Protein interactions related to biological ice nucleation, allergies, and inflammation

Anna Theresa Kunert

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Mainz, 3 March 2020, Anna Theresa Kunert

Der größte Feind der Qualität ist die Eile.

(Henry Ford)

Abstract

Proteins are involved in many environmental and physiological processes that are relevant for climate and public health, including ice nucleation, allergies, and inflammation. Upon exposure to reactive oxygen and nitrogen species, proteins can undergo chemical modifications influencing their physical, chemical, and biological properties. This dissertation addresses two aspects of current atmospheric and biomedical research related to protein interactions: (i) the ice nucleation activity of proteins and characterization of biological ice nuclei (IN), and (ii) the chemical reactivity and inflammatory potential of proteins acting as allergens or damage-associated molecular patterns (DAMPs).

In the first part of the dissertation, a fully-automated high-throughput Twin-plate Ice Nucleation Assay (TINA) was developed for efficient analysis and characterization of biological IN in laboratory and field samples. The instrument was used to investigate bacterial, fungal, and chemically modified IN as well as air particulate matter. Experiments with fungal ice nuclei from *Fusarium* revealed that the cell-free IN are smaller than 100 kDa and that molecular aggregates can be formed in aqueous solution. The IN activity was not affected by long-term storage, freeze-thaw cycles, and exposure to atmospherically relevant concentrations of ozone and nitrogen dioxide, but it was strongly reduced by heat treatment confirming that the *Fusarium* IN are proteinaceous. Moreover, TINA was used in related further studies investigating the effects of antifreeze proteins, salts, pH changes, and electrostatic interactions on the activity of bacterial IN from *Pseudomonas syringae*. The results provide new insights and help to unravel the molecular mechanisms of biological ice nucleation.

In the second part of the dissertation, the influence of chemical modifications on the allergenic and inflammatory potential of proteins was investigated, building on a review of air pollution effects on allergies. For the grass pollen allergen Phl p 5, the products and kinetics of reactions with O_3/NO_2 and $ONOO^-$ were characterized over a wide range of experimental conditions. The degrees of nitration and oligomerization were higher for $ONOO^-$ than O_3/NO_2, extending the mechanistic insights gained in related studies with other allergenic proteins and reference substances. Furthermore, chemical modification of the proteinous DAMPs α-Synuclein, heat shock protein 60, and high-mobility-group box 1 protein by $ONOO^-$ was found to enhance innate immune responses mediated by pattern recognition receptors, pro-inflammatory transcription factors, and cytokines (TLR4, NF-κB, TNF-α, IL-1β, IL-8). The mechanistic insights gained in these studies contribute to a molecular understanding of adverse health effects caused by oxidative stress and environmental change in the Anthropocene.

Zusammenfassung

Proteine sind in vielen Umwelt- und physiologischen Prozessen involviert einschließlich Eiskeimbildung, Allergien und Entzündungen, die für Klima und öffentliche Gesundheit relevant sind. Durch Wechselwirkungen mit reaktiven Sauerstoff- und Stickstoffspezies können Proteine chemisch modifiziert werden, was ihre physikalischen, chemischen und biologischen Eigenschaften verändert. Diese Dissertation befasst sich mit zwei Aspekten der aktuellen Atmosphären- und biomedizinischen Forschung in Bezug auf Proteinwechselwirkungen: (i) der Eisaktivität von Proteinen und der Charakterisierung von biologischen Eiskeimen, sowie (ii) der chemischen Reaktivität und dem Entzündungspotential von Proteinen, die als Allergene oder Schadens-assoziierte molekulare Muster (*engl.: damage-associated molecular patterns*, DAMPs) wirken.

Im ersten Teil der Dissertation wurde ein vollautomatischer Hochdurchsatz-Gefrierassay (*Twin-plate Ice Nucleation Assay*, TINA) für die effiziente Analyse und Charakterisierung von biologischen Eiskeimen in Labor- und Feldproben entwickelt. Das Instrument wurde für die Untersuchung von bakteriellen, pilzlichen und chemisch modifizierten Eiskeimen sowie von Luftstaub eingesetzt. Experimente mit pilzlichen Eiskeimen von *Fusarium* zeigten, dass die zellfreien Eiskeime kleiner als 100 kDa sind und dass in wässriger Lösung molekulare Aggregate geformt werden können. Die Eisaktivität wurde durch Langzeitlagerung, Gefrier-Tau-Zyklen und atmosphärisch relevanten Konzentrationen von Ozon und Stickstoffdioxid nicht beeinträchtigt, jedoch wurde sie durch Hitzebehandlungen stark reduziert, was bestätigt, dass Eiskeime von *Fusarium* Proteine enthalten. Darüber hinaus wurde TINA in ähnlichen weiteren Studien genutzt um die Effekte von Antigefrierproteinen, Salzen, pH-Veränderungen und elektrostatischen Wechselwirkungen auf die Aktivität der bakteriellen Eiskeime von *Pseudomonas syringae* zu untersuchen. Die Ergebnisse liefern neue Erkenntnisse und helfen die molekularen Mechanismen der biologischen Eiskeimbildung aufzuklären.

Im zweiten Teil der Dissertation wurde, aufbauend auf einem Review über die Auswirkungen von Luftverschmutzung auf Allergien, der Einfluss chemischer Modifikationen auf das allergene und entzündliche Potenzial von Proteinen untersucht. Für das Graspollenallergen Phl p 5 wurden die Produkte und Kinetiken der Reaktion mit O_3/NO_2 and $ONOO^-$ unter verschiedenen experimentellen Bedingungen charakterisiert. Die Nitrierungs- und Oligomerisierungsgrade waren für $ONOO^-$ höher als für O_3/NO_2, was die mechanistischen Erkenntnisse erweitert, die in verwandten Studien mit anderen allergenen Proteinen und Referenzsubstanzen gewonnen wurden. Des weiteren wurde herausgefunden, dass die chemische Modifizierung von proteinösen DAMPs (α-Synuklein, Hitzeschockprotein 60, High-Mobility-Group-Protein B1) durch $ONOO^-$ die angeborene Immunantwort verstärkt, die durch Mustererkennungsrezeptoren, entzündungsfördernden Transkriptionsfaktoren und Zytokine (TLR4, NF-κB, TNF-α, IL-1β, IL-8) vermittelt wird. Die in diesen Studien gewonnenen mechanistischen Erkenntnisse tragen zu einem besseren molekularen Verständnis der gesundheitsschädlichen Auswirkungen bei, die durch oxidativen Stress und Umweltveränderungen im Anthropozän verursacht werden.

Contents

1 Introduction

1.1 Atmospheric Aerosols and Biological Ice Nucleation

Atmospheric aerosols play important roles in climate, atmospheric chemistry, and public health (Andreae and Rosenfeld, 2008; Pöschl, 2005; Pöschl and Shiraiwa, 2015; Shiraiwa et al., 2017). Primary biological aerosol particles, in short bioaerosols, comprise living or dead organisms, reproductive units, and fragments or excretions from plants and animals (e.g., algae, bacteria, fungal spores, plant pollen, proteins and other biopolymers) (Després et al., 2012; Fröhlich-Nowoisky et al., 2016), spanning a size range from nanometers up to about a tenth of a millimeter with average atmospheric residence times between minutes and weeks (Burrows et al., 2009; Pöschl and Shiraiwa, 2015). They are directly released from the biosphere into the atmosphere, and in the course of atmospheric transport, airborne particles can undergo physical and chemical transformation, stress, and biological aging before they are deposited back to the ground (Fig. 1) (Fröhlich-Nowoisky et al., 2016; Pöschl et al., 2005; Pöschl and Shiraiwa, 2015).

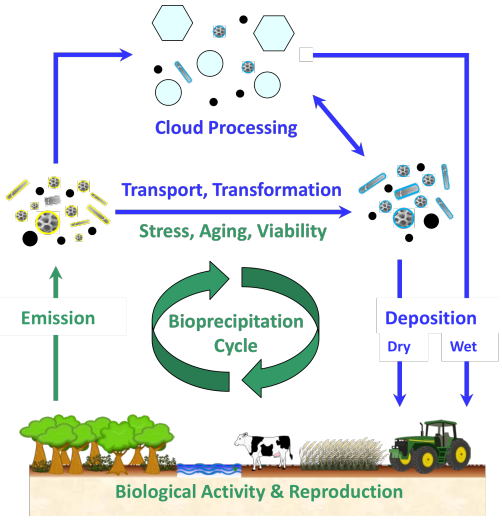

Figure 1: Bioaerosol cycling in the Earth system. Bioaerosols are directly released from the biosphere into the atmosphere, where they can be involved in cloud formation and precipitation and undergo physical and chemical transformation, stress, and biological aging before they are deposited back to the ground. Viable bioparticles can contribute to biological reproduction and further emission, which is particularly efficient when coupled to the water cycle (bioprecipitation). Figure adapted from Fröhlich-Nowoisky et al. (2016).

Some bioaerosols such as certain bacteria or plant pollen can act as ice nuclei (IN), but the impact of biological IN on cloud glaciation and the formation of precipitation is still poorly understood, and aerosol-cloud interactions are among the largest uncertainties in the assessment and understanding of climate change, the Earth system, and the Anthropocene (Boucher et al., 2013; Pöschl and Shiraiwa, 2015). Several studies suggest a triggering effect of biological IN for cloud formation and precipitation (Creamean et al., 2013; DeMott and Prenni, 2010; Failor et al., 2017; Hanlon et al., 2017; Joly et al., 2014; Petters and Wright, 2015; Pratt et al., 2009; Stopelli et al., 2015, 2017), and former studies have shown that biological particles are more efficient than mineral IN (Després et al., 2012; Hill et al., 2014; Hoose and Möhler, 2012; Huffman et al., 2013; Möhler et al., 2007; Pratt et al., 2009). Thus, biological IN are assumed to play an important role for clouds in the temperature range from -15 to 0 °C (DeMott and Prenni, 2010; Morris et al., 2014; Murray et al., 2012).

The best characterized biological IN are common plant-associated bacteria of the genera *Pseudomonas*, *Pantoea*, and *Xanthomonas* (Garnham et al., 2011; Govindarajan and Lindow, 1988; Graether and Jia, 2001; Green and Warren, 1985; Hill et al., 2014; Kim et al., 1987; Ling et al., 2018; Šantl-Temkiv et al., 2015; Schmid et al., 1997; Wolber et al., 1986), and, recently, an ice-nucleation-active (IN-active) *Lysinibacillus* was found (Failor et al., 2017). The first identified IN-active fungi were strains of the genus *Fusarium* (Hasegawa et al., 1994; Pouleur et al., 1992; Richard et al., 1996; Tsumuki et al., 1992). To date, a few more fungal genera with varying initial freezing temperatures such as *Isaria farinosa* ($\sim$ -4 °C), *Mortierella alpina* ($\sim$ -5 °C), *Puccinia* species (-4 to -8 °C), and *Sarocladium* (formerly named *Acremonium*) *implicatum* ($\sim$ -9 °C) have been identified as IN-active (Fröhlich-Nowoisky et al., 2015; Huffman et al., 2013; Morris et al., 2013; Richard et al., 1996). Although IN activity in fungi is known for decades, the IN could not be identified yet. Several studies hypothesize that a proteinaceous compound has to be involved, but the molecular structure remains unknown (Fröhlich-Nowoisky et al., 2015; Hasegawa et al., 1994; Kunert et al., 2019; Pouleur et al., 1992; Pummer et al., 2015; Tsumuki and Konno, 1994).

A wide range of different instruments has been developed and applied for the analysis of IN in immersion freezing experiments, including cloud chambers (e.g., Bundke et al., 2008; Möhler et al., 2003; Stratmann et al., 2004), continuous flow diffusion chambers (e.g., Kanji and Abbatt, 2009; Rogers et al., 2001; Salam et al., 2006), and droplet freezing assays (e.g., Budke and Koop, 2015; Stopelli et al., 2015; Whale et al., 2015). The latter type of instrument is the only one, which is suitable to measure very small IN concentrations in environmental samples that are active at temperatures above -10 °C (Stopelli et al., 2015). The basic operating principle of a droplet freezing assay is the simultaneous cooling of a defined number of aqueous droplets with equal volume in picoliter to milliliter range. Experiments can be performed using a stepped temperature profile or a constant cooling rate, which is similar to those in slowly ascending clouds ($< 1\,\mathrm{m\,s^{-1}}$) where precipitation is initiated by the formation of ice crystals ($< 1\,^{\circ}\mathrm{C\,min^{-1}}$) (Stopelli et al., 2015). The droplet freezing

is detected by either digital cameras based on the reduction of light transmission upon freezing of a liquid sample (e.g., Budke and Koop, 2015; Stopelli et al., 2015; Whale et al., 2015) or infrared cameras based on the latent heat release upon phase change of liquid water to ice (Harrison et al., 2018; Kunert et al., 2018; Zaragotas et al., 2016). The determination of frozen droplets at a given temperature or after a certain time interval enables the quantitative assessment of IN, which was established by Gabor Vali in 1971.

1.2 Protein Modification, Allergies, and Inflammation

Air pollutants can chemically modify proteins either directly in the environment or indirectly in the human body by inducing oxidative stress and inflammation (Reinmuth-Selzle et al., 2017). Responsible for these modifications are reactive oxygen or nitrogen species (ROS/RNS), such as ozone (O_3), hydroxyl radicals, hydrogen peroxide, superoxide (O_2^-), nitrogen oxide (NO), nitrogen dioxide (NO_2) or peroxynitrite ($ONOO^-$), which react with oxidation-sensitive amino acids like cysteine, histidine, methionine, phenylalanine, tryptophan, and tyrosine (Tyr), as well as aliphatic side chains and the peptide backbone (Bachi et al., 2013; Mudd et al., 1969; Sharma and Graham, 2010). Among them, the aromatic amino acid tyrosine is favored in the reaction of proteins with ROS/RNS resulting in the formation of 3-nitrotyrosine (NTyr) and dityrosine cross-links (Kampf et al., 2015; Reinmuth-Selzle et al., 2014; Walcher et al., 2003). Nitrotyrosine can be linked to many severe acute and chronic diseases, such as sepsis, cancer, cardiovascular and neurodegenerative diseases, and it is an important biomarker for inflammation and oxidative stress (Ischiropoulos et al., 1992; Ischiropoulos, 2009; Greenacre and Ischiropoulos, 2001; Hodara et al., 2004; Turko and Murad, 2002). Also protein dimerization and oligomerization are supposed to have a strong influence on the immunogenicity of proteins (Reinmuth-Selzle et al., 2017; Ziegler et al., 2020).

In the atmosphere, allergenic proteins can react with O_3 and NO_2 resulting in oxidized and nitrated proteins as well as protein oligomerization and degradation (Franze et al., 2005; Shiraiwa et al., 2012). The reaction requires a two-step mechanism, in which the protein reacts first with ozone, forming long-lived reactive oxygen intermediates (ROIs), such as tyrosyl radicals (Fig. 2). In a second step, the ROI reacts either with nitrogen dioxide resulting in the formation of 3-nitrotyrosine, combines with another ROI forming dityrosine cross-links, or undergo further oxidation reactions (Kampf et al., 2015; Liu et al., 2017; Shiraiwa et al., 2011).

A similar two-step mechanism is known for protein modification inside the human body. During oxidative stress and inflammation, NO and O_2^- are formed that can react in a diffusion-controlled reaction to the strong oxidizing and nitrating intermediate $ONOO^-$ (Beckman et al., 1990). Under physiological conditions, an equilibrium exists between $ONOO^-$ and its protonated form, peroxyni-

trous acid (ONOOH), that depends on the local pH (80 % $ONOO^-$ at pH 7.4) (Radi et al., 2001). The anion can react with carbon dioxide (CO_2) forming nitrosoperoxycarbonate ($ONOOCO_2^-$), which in turn decomposes to NO_2 and a carbonate radical (CO_3^-) (Gunaydin and Houk, 2009). These one-electron oxidants can attack the aromatic ring of a tyrosine residue, leading to the formation of a tyrosyl radical (Fig. 2). In a second step, the ROI either combines with NO_2 to yield 3-nitrotyrosine or reacts with another ROI forming dityrosine cross-links, similar to the reaction with O_3 and NO_2 (Gunaydin and Houk, 2009; Pfeiffer et al., 2000). Notably, the reactions involving free radicals are complex, depend on the pH, and yield a mixture of hydroxylated, nitrated, cross-linked, and degraded proteins (Davies and Delsignore, 1987; Gunaydin and Houk, 2009).

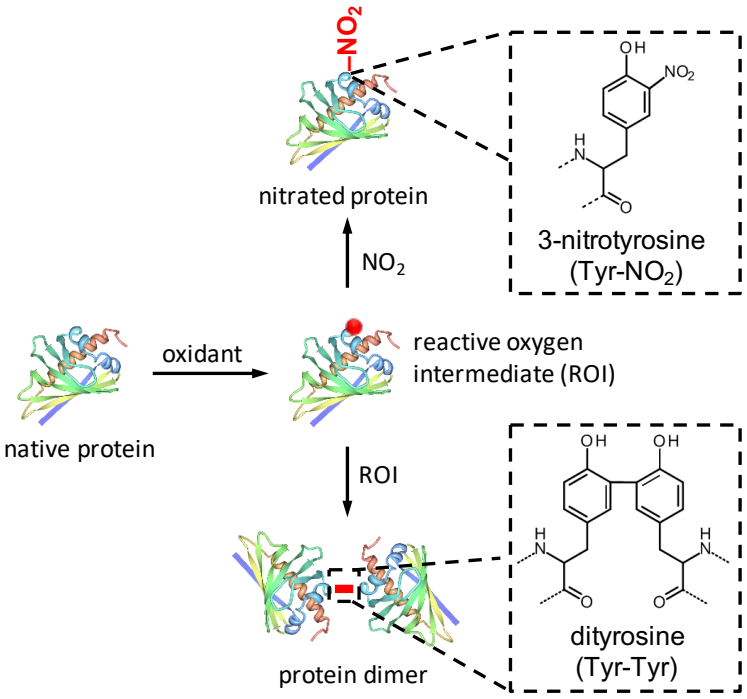

Figure 2: Two-step mechanism for the posttranslational modification of proteins. In the first step, a one-electron oxidant (O_3, NO_2, CO_3^-) reacts with the aromatic ring of a tyrosine residue forming a reactive oxygen intermediate (ROI), such as a tyrosyl radical (red dot). In a second step, the ROI either combines with NO_2 to yield 3-nitrotyrosine or reacts with another ROI, forming dityrosine cross-links (red bar) and thereby protein dimers and higher oligomers. Figure adapted from Reinmuth-Selzle et al. (2017).

Posttranslational modifications can change the protein structure and stability, affect hydrophobicity and acidity of binding sites, and thereby alter the protein function (Abello et al., 2009; Ackaert et al., 2014; Greenacre and Ischiropoulos, 2001; Ischiropoulos, 2009; Gruijthuijsen et al., 2006; Karle et al., 2012). For example, the addition of a sterically demanding NO_2 group at the ortho position of the aromatic ring causes a significant shift in the pKa value of the hydroxyl group from $\sim$10 to $\sim$7, thereby affecting the protein conformation (Bachi et al., 2013; Reinmuth-Selzle et al., 2017; Turko

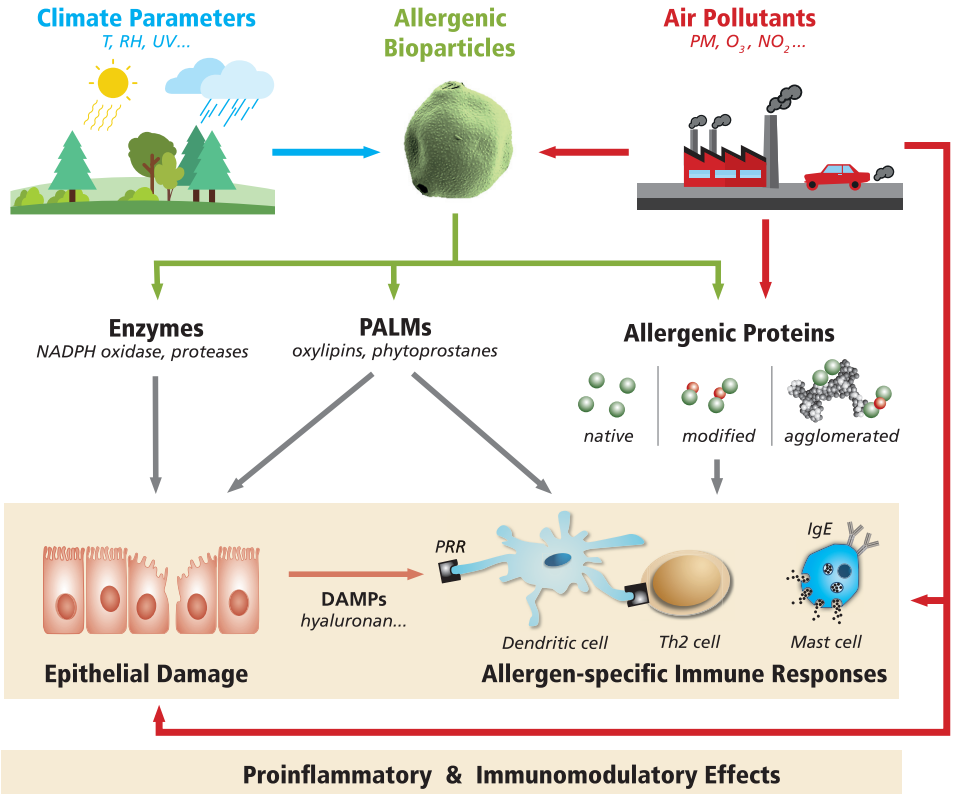

Figure 3: Pathways through which climate parameters and air pollutants can influence the release, potency, and effects of allergens and adjuvants: temperature (T), relative humidity (RH), ultraviolet (UV) radiation, particulate matter (PM), ozone and nitrogen oxides (O_3, NO_x), reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, pollen-associated lipid mediators (PALMs), damage-associated molecular patterns (DAMPs), pattern recognition receptors (PRR), type 2 T helper (Th2) cells, immunoglobulin E (IgE), allergenic proteins (green dots), and chemical modifications (red dots). Figure adapted from (Reinmuth-Selzle et al., 2017).

and Murad, 2002). A chemically modified protein with altered chemical and structural properties can act as adjuvant inducing or potentiating immunogenic reactions and inflammation (Fig. 3). Climate parameters and air pollution can further promote pro-inflammatory and immunomodulatory effects, as both factors can influence the release and thus the environmental abundance of allergenic proteins and biogenic adjuvants (Reinmuth-Selzle et al., 2017). For example, pollen grains can rupture under humid conditions or upon exposure to O_3 or NO_2, leading to subsequent release of cytoplasmic material into the atmosphere (Behrendt and Becker, 2001; Motta et al., 2006; Ouyang et al., 2016; Steiner et al., 2015; Taylor et al., 2004). Besides allergenic proteins, pollen and fungal spores also release other compounds that can act as adjuvants. In particular, the release of nonallergenic, bioactive, pollen-associated lipid mediators (PALMs) with pro-inflammatory and immunomodulatory effects can trigger and enhance allergies (Fig. 3) (Reinmuth-Selzle et al., 2017).

During inflammation, damage-associated molecular patterns (DAMPs) can be actively secreted by epithelial cells, monocytes, macrophages, and other cells, or passively released by damaged and dying cells (Fig. 3). Extracellularly, they can stimulate pattern recognition receptors (PRRs) such as the Toll-like receptor 4 (TLR4). TLR4 signaling leads to the activation of transcription factors like the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB), which ia a key activator of inflammatory cascades (Liu et al., 2014; Takeda and Akira, 2004). NF-κB induces the expression and modulates the secretion of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-8, which can stimulate the corresponding cytokine receptors leading to further activation of NF-κB and other signaling pathways (Liu et al., 2014, 2016; Varfolomeev and Vucic, 2018; Weber et al., 2010). This positive feedback can amplify and propagate inflammatory processes in autocrine or paracrine fashion (Gan et al., 2014; Lucas and Maes, 2013; Ziegler et al., 2020).

1.3 Research Objectives

This dissertation addresses two aspects of current atmospheric and biomedical research related to proteins: (i) Quantification and characterization of biological ice nuclei in laboratory and field samples, and (ii) studies on the chemical modification of allergenic and pro-inflammatory proteins. The specific objectives can be outlined as follows:

1. Development of a droplet freezing assay for efficient analysis and characterization of biological ice nuclei in laboratory and field samples.
2. Investigation of bacterial and fungal ice nuclei, their stability under atmospherically relevant conditions, and related protein interactions.
3. Elucidation of the reaction kinetics and products of allergenic proteins interacting with ozone, nitrogen dioxide, and peroxynitrite.
4. Investigation of the inflammatory potential of peroxynitrite-modified proteins acting as damage-associated molecular patterns.

2 Results and Conclusions

2.1 Overview

In the course of my dissertation, I studied protein interactions involved in environmental and physiological processes. The results obtained are described in a total of 16 manuscripts for publication in peer-reviewed scientific journals. The main results and conclusions are summarized below.

A droplet freezing assay was developed, which was applied in several biological IN studies with laboratory and field samples, leading to nine manuscripts including two first-author and three second-author manuscripts. Two of the papers have already been published in and two more are submitted to internationally leading journals of atmospheric science and physical chemistry and are attached in Appendix C.

In the field of allergenic and inflammatory processes, I performed two studies on the chemical modification of different proteins, one as first-author and the other one as co-first-author. I contributed to several other studies related to chemically modified proteins and their effects on human health, leading to five additional manuscripts including one second-author manuscript. Three of the papers have already been published in and one more is submitted to internationally leading journals of environmental science and biology and are attached in Appendix C.

2.2 Biological Ice Nucleation

2.2.1 Twin-plate Ice Nucleation Assay (TINA)

A new high-throughput droplet freezing instrument was developed for efficient analysis and characterization of biological IN in laboratory and field samples under immersion freezing conditions. The Twin-plate Ice Nucleation Assay (TINA) is a fully-automated setup to study ice nucleation and freezing events simultaneously in hundreds of microliter-range droplets using infrared detectors. The instrument was tested and characterized in experiments with bacterial and fungal IN from *Pseudomonas syringae* and *Mortierella alpina*, and it was applied to investigate the influence of chemical processing on the activity of biological IN, in particular the effects of oxidation and nitration reactions. To show

its applicability for field samples, TINA was used to study aqueous extracts of atmospheric aerosols, simultaneously analyzing a multitude of differently pre-treated samples to distinguish several types of IN. The results confirmed that TINA is suitable for high-throughput experiments and efficient analysis of biological IN in laboratory and field samples. For details see Appendix C.1, Kunert *et al.*, Atmos. Meas. Tech., 2018.

2.2.2 Macromolecular Fungal Ice Nuclei in *Fusarium*

Ice nucleation activity in fungi was first discovered in the cosmopolitan genus *Fusarium*, which can be regarded as the best studied IN-active fungus today, but the frequency and distribution of IN activity within *Fusarium* are still insufficiently investigated. More than 100 strains from 65 different *Fusarium* species were tested for IN activity, and in total, ∼11 % of all tested species included IN-active strains, and ∼16 % of all tested strains showed IN activity above 12 °C. Besides species with known IN activity, *F. armeniacum*, *F. begoniae*, *F. concentricum*, and *F. langsethiae* were newly identified as IN-active. Filtration experiments indicate that cell-free ice-nucleating macromolecules (INMs) from *Fusarium* are smaller than 100 kDa and that molecular aggregates can be formed in solution. Long-term storage and freeze-thaw cycle experiments as well as exposure to atmospherically relevant concentrations of ozone and nitrogen dioxide did not affect the activity of *Fusarium* IN. Heat treatments, however, strongly reduced the IN activity, confirming earlier hypotheses that the INM in *Fusarium* largely consists of a proteinaceous compound. The frequency and the wide distribution of IN activity within the genus *Fusarium*, combined with the stability of the IN under atmospherically relevant conditions, suggest that fungal IN may have a stronger influence on the Earth's water cycle and climate than previously assumed. Additional research is necessary to characterize the INMs in *Fusarium* and processes that can result in their agglomeration to larger protein complexes. To evaluate the implication of these IN on Earth's climate, additional work is required to study the abundance of *Fusarium* IN in environmental samples on a global scale. For details see Appendix C.2, Kunert *et al.*, Biogeosciences, 2019.

2.2.3 Related Studies

TINA was applied to related further IN studies investigating the interactions of bacterial IN from *Pseudomonas syringae* with antifreeze proteins (AFPs), salts, pH changes, and electrostatic interactions.

AFPs have been reported to be able to inhibit the activity of IN, but the generality of this effect is not understood, and for the few known examples of IN inhibition by AFPs, the molecular mechanisms remain unclear. A comprehensive evaluation of the effects of all major classes of AFPs on the activity of bacterial IN was performed. Certain AFPs inhibited the activity of bacterial IN, while other AFPs

showed no effect. Thus, the ability to inhibit the IN activity is not an intrinsic property of AFPs, and the interactions of IN and different AFPs proceed through protein-specific rather than universal molecular mechanisms. For details see Appendix C.3, Schwidetzky *et al.*, submitted to J. Phys. Chem. Lett., 2020a.

The activity of bacterial IN is also reduced by lowering the pH to acidic values, whereas a pH change to alkaline values does not affect the IN activity. The molecular origin for this pH sensitivity is unknown, but a better understanding would provide necessary insights into the driving forces of functional IN aggregation, which is required for the high activity of bacterial IN. Electrostatic interactions are important for the IN activity of *Pseudomonas syringae* as the net charge strongly correlates with the IN activity of the larger IN aggregates. In contrast, the activity of IN monomers is less affected by pH changes. Thus, electrostatic interactions play an essential role in the formation of the highly efficient IN aggregates. For details see Appendix C.4, Lukas *et al.*, submitted to J. Am. Chem. Soc., 2019.

Furthermore, the interactions of different salts with bacterial IN from *Pseudomonas syringae* was investigated. The IN activity was influenced by different salts following the Hofmeister series. Weakly hydrated ions like thiocyanate strongly inhibit bacterial ice nucleation, whereas enhancement was observed for strongly hydrated ions like sulfate. Ongoing experiments with surface-specific sum-frequency generation spectroscopy and molecular dynamic simulations investigate the ability of the different salts to interact with the IN proteins (Schwidetzky *et al.*, in preparation, 2020b).

2.3 Chemical Modification and Inflammatory Potential of Proteins

2.3.1 Timothy Grass Pollen Allergen Phl p 5

The allergenic potential of proteins may be enhanced via posttranslational modifications, but the molecular mechanisms and kinetics of the chemical modifications by O_3/NO_2 and $ONOO^-$ are still not fully understood. Tyrosine nitration and oligomerization of the grass pollen allergen Phl p 5 was investigated upon simultaneous exposure of O_3 and NO_2 and reaction with $ONOO^-$. The nitration degree and the oligomer formation was determined by reversed-phase and size-exclusion liquid chromatography. Both, exposure to O_3/NO_2 and reaction with $ONOO^-$, led to nitration and oligomerization of the allergen. The nitration degree and oligomer formation were dependent on the nitrating agent and the reaction conditions (concentration, exposure time), and they were higher for $ONOO^-$ than for O_3/NO_2. For the reaction with $ONOO^-$, a maximum nitration degree of 25 % was found, which corresponds to maximal ~ 6 nitrated tyrosine residues per Phl p 5 monomer, whereas exposure to O_3/NO_2 resulted in a maximum nitration degree of 8 % corresponding to ~ 1 nitrotyrosine per Phl p 5 monomer. The obtained results were similar to earlier studies investigating other

allergenic and reference proteins (Bet v 1, BSA). The data will be further interpreted with regard to protein structure, and kinetic modeling will be included in the future. For details see Appendix C.5, Kunert *et al.*, in preparation, 2020.

2.3.2 Pro-inflammatory Proteins α-Syn, HSP60, HMGB1

In the course of inflammatory processes, reactive oxygen and nitrogen species such as peroxynitrite can chemically modify proteins, but the effects of such modifications on human health are not well understood. It was investigated how the innate immune responses via TLR4 and NF-κB and the pro-inflammatory potential of the proteinous DAMPs α-Syn, HSP60, and HMGB1 changes upon chemical modification with peroxynitrite. For the peroxynitrite-modified proteins, a strongly enhanced activation of TLR4 and NF-κB was found as well as increased expression and secretion of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-8. The results show that peroxynitrite-modified DAMPs more potently amplify inflammation via TLR4 activation than the native DAMPs, and provide first evidence that such modifications can directly enhance innate immune responses via a defined receptor. For details see Appendix C.6, Ziegler *et al.*, submitted to Redox Biol., 2020.

2.3.3 Related Studies

Air pollution and climate change are potential drivers for the increasing burden of allergic diseases as the allergenic potential of airborne proteins may be enhanced via posttranslational modification induced by air pollutants like O_3 and NO_2. The molecular mechanisms by which air pollutants and climate parameters may influence allergic diseases, however, are complex and elusive.

The review article provides an overview of physical, chemical, and biological interactions between air pollution, climate change, allergens, adjuvants, and the immune system, addressing how these interactions may promote the development of allergies. Key findings from atmospheric, climate, and biomedical research were reviewed and synthesized. The current state of knowledge, open questions, and future research perspectives are outlined and discussed. For details see Appendix C.7, Reinmuth-Selzle *et al.*, Environ. Sci. Technol., 2017.

In another study, protein tyrosine nitration and oligomerization upon exposure to O_3 and NO_2 were studied, using bovine serum albumin as a model protein. Generally, more tyrosine residues were found to react via the nitration pathway than via the oligomerization pathway, and the experimental results were well reproduced by the kinetic multilayer model of aerosol surface and bulk chemistry. The extent of nitration and oligomerization strongly depends on relative humidity due to moisture-induced phase transition of proteins, highlighting the importance of cloud processing conditions for accelerated protein chemistry. For details see Appendix C.7, Liu *et al.*, Faraday Discuss., 2017.

2.4 Summary and Outlook

In the first part of this dissertation, a high-throughput droplet freezing assay was developed for efficient analysis and characterization of biological ice nuclei in laboratory and field samples. It was used to investigate bacterial, fungal, and chemically modified IN as well as air particulate matter. We found that proteinaceous, nanometer-sized ice nuclei in *Fusarium* are highly stable under atmospherically relevant conditions, suggesting that fungal IN may have a stronger influence on the Earth's water cycle and climate than previously assumed. Further studies revealed how the activity of bacterial IN from *Pseudomonas syringae* is affected by interactions with antifreeze proteins, salts, air pollutants, pH changes, and electric charges.

In the second part, the chemical reactivity and inflammatory potential of proteins acting as allergens or DAMPs was investigated. Chemical modification of the timothy grass pollen allergen Phl p 5 by O_3/NO_2 and $ONOO^-$ led to nitrated and oligomerized products, whereby higher reactivity was observed for the physiological reactant $ONOO^-$ than for the air pollutant mixture of O_3/NO_2. The results extend the mechanistic insights gained in related studies with other allergenic proteins and reference substances (Bet v 1, BSA). The chemical modification of the proteinous DAMPs α-Syn, HSP60, and HMGB1 enhanced innate immune responses via TLR4 and NF-κB. The results show that peroxynitrite-modified DAMPs more potently amplify inflammation via TLR4 activation than the native DAMPs, and provide first evidence that such modifications can directly enhance innate immune responses via a defined receptor.

The instrument developed and the measurement results obtained in this dissertation provide new insights and a basis for efficient further investigations of protein interactions relevant for climate and public health. Future challenges in these areas of research comprise the full elucidation of molecular mechanisms of biological ice nucleation as well as identifying and quantifying the immunochemical reactions and underlying mechanisms leading to allergies and inflammation in response to environmental pollution. A comprehensive understanding of the protein interactions and their effects in environmental and physiological processes will help to assess and mitigate the consequences of the steeply increasing and globally pervasive human influence on air quality, climate, and public health in the Anthropocene (Pöschl and Shiraiwa, 2015; Reinmuth-Selzle et al., 2017; Ziegler et al., 2020).

3 Bibliography

Abello, N., Kerstjens, H. A. M., Postma, D. S., and Bischoff, R. 2009. Protein Tyrosine Nitration: Selectivity, Physicochemical and Biological Consequences, Denitration, and Proteomics Methods for the Identification of Tyrosine-Nitrated Proteins. *J. Proteome Res.*, 8(7):3222–3238.

Ackaert, C., Kofler, S., Horejs-Hoeck, J., Zulehner, N., Asam, C., von Grafenstein, S., Fuchs, J. E., Briza, P., Liedl, K. R., Bohle, B., Ferreira, F., Brandstetter, H., Oostingh, G. J., and Duschl, A. 2014. The Impact of Nitration on the Structure and Immunogenicity of the Major Birch Pollen Allergen Bet v 1.0101. *PLoS One*, 9(8):e104520.

Andreae, M. and Rosenfeld, D. 2008. Aerosol–cloud–precipitation interactions. Part 1. The nature and sources of cloud-active aerosols. *Earth-Science Rev.*, 89(1-2):13–41.

Bachi, A., Dalle-Donne, I., and Scaloni, A. 2013. Redox Proteomics: Chemical Principles, Methodological Approaches and Biological/Biomedical Promises. *Chem. Rev.*, 113(1):596–698.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci.*, 87(4):1620–1624.

Behrendt, H. and Becker, W.-M. 2001. Localization, release and bioavailability of pollen allergens: the influence of environmental factors. *Curr. Opin. Immunol.*, 13(6):709–715.

Boucher, O., Randall, D., Artaxo, P., Bretherton, C., Feingold, G., Forster, P., Kerminen, V. M., Kondo, Y., Liao, H., Lohmann, U., Rasch, P., Satheesh, S. K., Sherwood, S., Stevens, B., and Zhang, X. Y. 2013. Clouds and Aerosols. In Stocker, T., Qin, D., Plattner, G.-K., Tignor, M., Allen, S., Boschung, J., Nauels, A., Xia, Y., And, V. B., and Midgley, P., editors, *Clim. Chang. 2013 Phys. Sci. Basis. Contrib. Work. Gr. I to Fifth Assess. Rep. Intergov. Panel Clim. Chang.*, pages 571–658. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Budke, C. and Koop, T. 2015. BINARY: an optical freezing array for assessing temperature and time dependence of heterogeneous ice nucleation. *Atmos. Meas. Tech.*, 8(2):689–703.

Bundke, U., Nillius, B., Jaenicke, R., Wetter, T., Klein, H., and Bingemer, H. 2008. The fast Ice Nucleus chamber FINCH. *Atmos. Res.*, 90(2-4):180–186.

Burrows, S. M., Butler, T., Jöckel, P., Tost, H., Kerkweg, A., Pöschl, U., and Lawrence, M. G. 2009. Bacteria in the global atmosphere – Part 2: Modeling of emissions and transport between different ecosystems. *Atmos. Chem. Phys.*, 9(23):9281–9297.

Creamean, J. M., Suski, K. J., Rosenfeld, D., Cazorla, A., DeMott, P. J., Sullivan, R. C., White, A. B., Ralph, F. M., Minnis, P., Comstock, J. M., Tomlinson, J. M., and Prather, K. A. 2013. Dust and Biological Aerosols from the Sahara and Asia Influence Precipitation in the Western U.S. *Science (80-.).*, 339(6127):1572–1578.

Davies, K. J. and Delsignore, M. E. 1987. Protein damage and degradation by oxygen radicals. III. Modification of secondary and tertiary structure. *J. Biol. Chem.*, 262(20):9908–13.

DeMott, P. J. and Prenni, A. J. 2010. New Directions: Need for defining the numbers and sources of biological aerosols acting as ice nuclei. *Atmos. Environ.*, 44(15):1944–1945.

Després, V. R., Huffman, J. A., Burrows, S. M., Hoose, C., Safatov, A. S., Buryak, G., Fröhlich-Nowoisky, J., Elbert, W., Andreae, M. O., Pöschl, U., and Jaenicke, R. 2012. Primary biological aerosol particles in the atmosphere: a review. *Tellus B Chem. Phys. Meteorol.*, 64(1):15598.

Failor, K. C., Schmale, D. G., Vinatzer, B. A., and Monteil, C. L. 2017. Ice nucleation active bacteria in precipitation are genetically diverse and nucleate ice by employing different mechanisms. *ISME J.*, 11(12):2740–2753.

Franze, T., Weller, M. G., Niessner, R., and Pöschl, U. 2005. Protein Nitration by Polluted Air. *Environ. Sci. Technol.*, 39(6):1673–1678.

Fröhlich-Nowoisky, J., Hill, T. C. J., Pummer, B. G., Yordanova, P., Franc, G. D., and Pöschl, U. 2015. Ice nucleation activity in the widespread soil fungus *Mortierella alpina*. *Biogeosciences*, 12(4):1057–1071.

Fröhlich-Nowoisky, J., Kampf, C. J., Weber, B., Huffman, J. A., Pöhlker, C., Andreae, M. O., Lang-Yona, N., Burrows, S. M., Gunthe, S. S., Elbert, W., Su, H., Hoor, P., Thines, E., Hoffmann, T., Després, V. R., and Pöschl, U. 2016. Bioaerosols in the Earth system: Climate, health, and ecosystem interactions. *Atmos. Res.*, 182:346–376.

Gan, L. T., Van Rooyen, D. M., Koina, M. E., McCuskey, R. S., Teoh, N. C., and Farrell, G. C. 2014. Hepatocyte free cholesterol lipotoxicity results from JNK1-mediated mitochondrial injury and is HMGB1 and TLR4-dependent. *J. Hepatol.*, 61(6):1376–1384.

Garnham, C. P., Campbell, R. L., Walker, V. K., and Davies, P. L. 2011. Novel dimeric β-helical model of an ice nucleation protein with bridged active sites. *BMC Struct. Biol.*, 11(1):36.

Govindarajan, A. G. and Lindow, S. E. 1988. Size of bacterial ice-nucleation sites measured *in situ* by radiation inactivation analysis. *Proc. Natl. Acad. Sci. U. S. A.*, 85(5):1334–1338.

Graether, S. P. and Jia, Z. 2001. Modeling *Pseudomonas syringae* Ice-Nucleation Protein as a β-Helical Protein. *Biophys. J.*, 80(3):1169–1173.

Green, R. L. and Warren, G. J. 1985. Physical and functional repetition in a bacterial ice nucleation gene. *Nature*, 317(6038):645–648.

Greenacre, S. A. and Ischiropoulos, H. 2001. Tyrosine nitration: Localisation, quantification, consequences for protein function and signal transduction. *Free Radic. Res.*, 34(6):541–581.

Gruijthuijsen, Y., Grieshuber, I., Stöcklinger, A., Tischler, U., Fehrenbach, T., Weller, M., Vogel, L., Vieths, S., Pöschl, U., and Duschl, A. 2006. Nitration Enhances the Allergenic Potential of Proteins. *Int. Arch. Allergy Immunol.*, 141(3):265–275.

Gunaydin, H. and Houk, K. N. 2009. Mechanisms of Peroxynitrite-Mediated Nitration of Tyrosine. *Chem. Res. Toxicol.*, 22(5):894–898.

Hanlon, R., Powers, C., Failor, K., Monteil, C. L., Vinatzer, B. A., and Schmale, D. G. 2017. Microbial ice nucleators scavenged from the atmosphere during simulated rain events. *Atmos. Environ.*, 163:182–189.

Harrison, A. D., Whale, T. F., Rutledge, R., Lamb, S., Tarn, M. D., Porter, G. C. E., Adams, M. P., McQuaid, J. B., Morris, G. J., and Murray, B. J. 2018. An instrument for quantifying heterogeneous ice nucleation in multiwell plates using infrared emissions to detect freezing. *Atmos. Meas. Tech.*, 11:5629–5641.

Hasegawa, Y., Ishihara, Y., and Tokuyama, T. 1994. Characteristics of Ice-nucleation Activity in *Fusarium avenaceum* IFO 7158. *Biosci. Biotechnol. Biochem.*, 58(12):2273–2274.

Hill, T. C. J., Moffett, B. F., DeMott, P. J., Georgakopoulos, D. G., Stump, W. L., and Franc, G. D. 2014. Measurement of Ice Nucleation-Active Bacteria on Plants and in Precipitation by Quantitative PCR. *Appl. Environ. Microbiol.*, 80(4):1256–1267.

Hodara, R., Norris, E. H., Giasson, B. I., Mishizen-Eberz, A. J., Lynch, D. R., Lee, V. M., and Ischiropoulos, H. 2004. Functional Consequences of α-Synuclein Tyrosine Nitration. *J. Biol. Chem.*, 279(46):47746–47753.

Hoose, C. and Möhler, O. 2012. Heterogeneous ice nucleation on atmospheric aerosols: a review of results from laboratory experiments. *Atmos. Chem. Phys.*, 12(20):9817–9854.

Huffman, J. A., Prenni, A. J., DeMott, P. J., Pöhlker, C., Mason, R. H., Robinson, N. H., Fröhlich-Nowoisky, J., Tobo, Y., Després, V. R., Garcia, E., Gochis, D. J., Harris, E., Müller-Germann, I.,

Ruzene, C., Schmer, B., Sinha, B., Day, D. A., Andreae, M. O., Jimenez, J. L., Gallagher, M., Kreidenweis, S. M., Bertram, A. K., and Pöschl, U. 2013. High concentrations of biological aerosol particles and ice nuclei during and after rain. *Atmos. Chem. Phys.*, 13(13):6151–6164.

Ischiropoulos, H. 2009. Protein tyrosine nitration—An update. *Arch. Biochem. Biophys.*, 484(2):117–121.

Ischiropoulos, H., Zhu, L., Chen, J., Tsai, M., Martin, J. C., Smith, C. D., and Beckman, J. S. 1992. Peroxynitrite-Mediated Tyrosine Nitration Catalyzed by Superoxide Dismutase. *Arch. Biochem. Biophys.*, 298(2):431–7.

Joly, M., Amato, P., Deguillaume, L., Monier, M., Hoose, C., and Delort, A.-M. 2014. Quantification of ice nuclei active at near 0 °C temperatures in low-altitude clouds at the Puy de Dôme atmospheric station. *Atmos. Chem. Phys.*, 14(15):8185–8195.

Kampf, C. J., Liu, F., Reinmuth-Selzle, K., Berkemeier, T., Meusel, H., Shiraiwa, M., and Pöschl, U. 2015. Protein Cross-Linking and Oligomerization through Dityrosine Formation upon Exposure to Ozone. *Environ. Sci. Technol.*, 49(18):10859–10866.

Kanji, Z. A. and Abbatt, J. P. D. 2009. The University of Toronto Continuous Flow Diffusion Chamber (UT-CFDC): A Simple Design for Ice Nucleation Studies. *Aerosol Sci. Technol.*, 43(7):730–738.

Karle, A. C., Oostingh, G. J., Mutschlechner, S., Ferreira, F., Lackner, P., Bohle, B., Fischer, G. F., Vogt, A. B., and Duschl, A. 2012. Nitration of the Pollen Allergen Bet v 1.0101 Enhances the Presentation of Bet v 1-Derived Peptides by HLA-DR on Human Dendritic Cells. *PLoS One*, 7(2):e31483.

Kim, H. K., Orser, C., Lindow, S. E., and Sands, D. C. 1987. *Xanthomonas campestris* pv. *translucens* Strains Active in Ice Nucleation. *Plant Dis.*, 71(11):994–996.

Kunert, A. T., Lamneck, M., Helleis, F., Pöhlker, M. L., Pöschl, U., and Fröhlich-Nowoisky, J. 2018. Twin-plate Ice Nucleation Assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples. *Atmos. Meas. Tech.*, 11(July):6327–6337.

Kunert, A. T., Pöhlker, M. L., Tang, K., Krevert, C. S., Wieder, C., Speth, K. R., Hanson, L. E., Morris, C. E., Schmale III, D. G., Pöschl, U., and Fröhlich-Nowoisky, J. 2019. Macromolecular fungal ice nuclei in *Fusarium*: effects of physical and chemical processing. *Biogeosciences*, 16(23):4647–4659.

Kunert, A. T., Reinmuth-Selzle, K., Ziegler, K., Krevert, C. S., Wieder, C., Weller, M. G., Lucas, K., Pöschl, U., and Fröhlich-Nowoisky, J. 2020. Reaction kinetics and products of nitration and oligomerization of the major grass pollen allergen Phl p 5 by peroxynitrite and nitrogen dioxide with ozone, *in preparation*.

Ling, M. L., Wex, H., Grawe, S., Jakobsson, J., Löndahl, J., Hartmann, S., Finster, K., Boesen, T., and Šantl-Temkiv, T. 2018. Effects of Ice Nucleation Protein Repeat Number and Oligomerization Level on Ice Nucleation Activity. *J. Geophys. Res. Atmos.*, 123(3):1802–1810.

Liu, F., Lakey, P. S. J., Berkemeier, T., Tong, H., Kunert, A. T., Meusel, H., Cheng, Y., Su, H., Fröhlich-Nowoisky, J., Lai, S., Weller, M. G., Shiraiwa, M., Pöschl, U., and Kampf, C. J. 2017. Atmospheric protein chemistry influenced by anthropogenic air pollutants: nitration and oligomerization upon exposure to ozone and nitrogen dioxide. *Faraday Discuss.*, 200:413–427.

Liu, Q., Li, A., Tian, Y., Wu, J. D., Liu, Y., Li, T., Chen, Y., Han, X., and Wu, K. 2016. The CXCL8-CXCR1/2 pathways in cancer. *Cytokine Growth Factor Rev.*, 31:61–71.

Liu, Y., Yin, H., Zhao, M., and Lu, Q. 2014. TLR2 and TLR4 in Autoimmune Diseases: a Comprehensive Review. *Clin. Rev. Allergy Immunol.*, 47(2):136–147.

Lucas, K. and Maes, M. 2013. Role of the Toll Like Receptor (TLR) Radical Cycle in Chronic Inflammation: Possible Treatments Targeting the TLR4 Pathway. *Mol. Neurobiol.*, 48(1):190–204.

Lukas, M., Schwidetzky, R., Kunert, A. T., Pöschl, U., Fröhlich-Nowoisky, J., Bonn, M., and Meister, K. 2019. Electrostatic Interactions Control the Functionality of Bacterial Ice Nucleators. *Submitt. to J. Am. Chem. Soc.*

Möhler, O., DeMott, P. J., Vali, G., and Levin, Z. 2007. Microbiology and atmospheric processes: the role of biological particles in cloud physics. *Biogeosciences*, 4(4):2559–2591.

Möhler, O., Stetzer, O., Schaefers, S., Linke, C., Schnaiter, M., Tiede, R., Saathoff, H., Krämer, M., Mangold, A., Budz, P., Zink, P., Schreiner, J., Mauersberger, K., Haag, W., Kärcher, B., and Schurath, U. 2003. Experimental investigation of homogeneous freezing of sulphuric acid particles in the aerosol chamber AIDA. *Atmos. Chem. Phys.*, 3(1):211–223.

Morris, C. E., Conen, F., Alex Huffman, J., Phillips, V., Pöschl, U., and Sands, D. C. 2014. Bioprecipitation: a feedback cycle linking Earth history, ecosystem dynamics and land use through biological ice nucleators in the atmosphere. *Glob. Chang. Biol.*, 20(2):341–351.

Morris, C. E., Sands, D. C., Glaux, C., Samsatly, J., Asaad, S., Moukahel, A. R., Gonçalves, F. L. T., and Bigg, E. K. 2013. Urediospores of rust fungi are ice nucleation active at $>-10\,^{\circ}\mathrm{C}$ and harbor ice nucleation active bacteria. *Atmos. Chem. Phys.*, 13(8):4223–4233.

Motta, A., Marliere, M., Peltre, G., Sterenberg, P., and Lacroix, G. 2006. Traffic-Related Air Pollutants Induce the Release of Allergen-Containing Cytoplasmic Granules from Grass Pollen. *Int. Arch. Allergy Immunol.*, 139(4):294–298.

Mudd, J. B., Leavitt, R., Ongun, A., and McManus, T. T. 1969. Reaction of ozone with amino acids and proteins. *Atmos. Environ.*, 3:669–682.

Murray, B. J., O'Sullivan, D., Atkinson, J. D., and Webb, M. E. 2012. Ice nucleation by particles immersed in supercooled cloud droplets. *Chem. Soc. Rev.*, 41(19):6519.

Ouyang, Y., Xu, Z., Fan, E., Li, Y., and Zhang, L. 2016. Effect of nitrogen dioxide and sulfur dioxide on viability and morphology of oak pollen. *Int. Forum Allergy Rhinol.*, 6(1):95–100.

Petters, M. D. and Wright, T. P. 2015. Revisiting ice nucleation from precipitation samples. *Geophys. Res. Lett.*, 42(20):8758–8766.

Pfeiffer, S., Schmidt, K., and Mayer, B. 2000. Dityrosine Formation Outcompetes Tyrosine Nitration at Low Steady-state Concentrations of Peroxynitrite. *J. Biol. Chem.*, 275(9):6346–6352.

Pöschl, U. 2005. Atmospheric Aerosols: Composition, Transformation, Climate and Health Effects. *Angew. Chemie Int. Ed.*, 44(46):7520–7540.

Pöschl, U., Rudich, Y., and Ammann, M. 2005. Kinetic model framework for aerosol and cloud surface chemistry and gas-particle interactions –Part 1: general equations, parameters, and terminology. *Atmos. Chem. Phys.*, 5(2):2111–2191.

Pöschl, U. and Shiraiwa, M. 2015. Multiphase Chemistry at the Atmosphere–Biosphere Interface Influencing Climate and Public Health in the Anthropocene. *Chem. Rev.*, 115(10):4440–4475.

Pouleur, S., Richard, C., Martin, J.-G., and Antoun, H. 1992. Ice Nucleation Activity in *Fusarium acuminatum* and *Fusarium avenaceum*. *Appl. Environ. Microbiol.*, 58(9):2960–2964.

Pratt, K. A., DeMott, P. J., French, J. R., Wang, Z., Westphal, D. L., Heymsfield, A. J., Twohy, C. H., Prenni, A. J., and Prather, K. A. 2009. *In situ* detection of biological particles in cloud ice-crystals. *Nat. Geosci.*, 2(6):398–401.

Pummer, B. G., Budke, C., Niedermeier, D., Felgitsch, L., Kampf, C. J., Huber, R. G., Liedl, K. R., Loerting, T., Moschen, T., Schauperl, M., Tollinger, M., Morris, C. E., Wex, H., Grothe, H., Pöschl, U., Koop, T., and Fröhlich-Nowoisky, J. 2015. Ice nucleation by water-soluble macromolecules. *Atmos. Chem. Phys.*, 15:4077–4091.

Radi, R., Peluffo, G., Alvarez, M. N., Naviliat, M., and Cayota, A. 2001. Unraveling peroxynitrite formation in biological systems. *Free Radic. Biol. Med.*, 30(5):463–488.

Reinmuth-Selzle, K., Ackaert, C., Kampf, C. J., Samonig, M., Shiraiwa, M., Kofler, S., Yang, H., Gadermaier, G., Brandstetter, H., Huber, C. G., Duschl, A., Oostingh, G. J., and Pösch 2014. Nitration of the Birch Pollen Allergen Bet v 1.0101: Efficiency and Site-Selectivity of Liquid and Gaseous Nitrating Agents. *J. Proteome Res.*, 13:1570–1577.

Reinmuth-Selzle, K., Kampf, C. J., Lucas, K., Lang-Yona, N., Fröhlich-Nowoisky, J., Shiraiwa, M., Lakey, P. S. J., Lai, S., Liu, F., Kunert, A. T., Ziegler, K., Shen, F., Sgarbanti, R., Weber, B., Bellinghausen, I., Saloga, J., Weller, M. G., Duschl, A., Schuppan, D., and Pöschl, U. 2017. Air Pollution and Climate Change Effects on Allergies in the Anthropocene: Abundance, Interaction, and Modification of Allergens and Adjuvants. *Environ. Sci. Technol.*, 51(8):4119–4141.

Richard, C., Martin, J. G., and Pouleur, S. 1996. Ice nucleation activity identified in some phytopathogenic *Fusarium* species. *Phytoprotection*, 77(April):83–92.

Rogers, D. C., DeMott, P. J., Kreidenweis, S. M., and Chen, Y. 2001. A Continuous-Flow Diffusion Chamber for Airborne Measurements of Ice Nuclei. *J. Atmos. Ocean. Technol.*, 18(5):725–741.

Salam, A., Lohmann, U., Crenna, B., Lesins, G., Klages, P., Rogers, D., Irani, R., MacGillivray, A., and Coffin, M. 2006. Ice Nucleation Studies of Mineral Dust Particles with a New Continuous Flow Diffusion Chamber. *Aerosol Sci. Technol.*, 40(2):134–143.

Šantl-Temkiv, T., Sahyoun, M., Finster, K., Hartmann, S., Augustin-Bauditz, S., Stratmann, F., Wex, H., Clauss, T., Woetmann Nielsen, N., Havskov Sorensen, J., Smith Korsholm, U., Wick, L. Y., and Gosewinkel Karlson, U. 2015. Characterization of airborne ice-nucleation-active bacteria and bacterial fragments. *Atmos. Environ.*, 109:105–117.

Schmid, D., Pridmore, D., Capitani, G., Battistutta, R., Neeser, J.-R., and Jann, A. 1997. Molecular organisation of the ice nucleation protein InaV from *Pseudomonas syringae*. *FEBS Lett.*, 414(3):590–594.

Schwidetzky, R., Kunert, A. T., Bonn, M., Pöschl, U., Ramlov, H., DeVries, A. L., Fröhlich-Nowoisky, J., and Meister, K. 2020a. To Freeze or Not to Freeze: Inhibition of Bacterial Ice Nucleators by Antifreeze Proteins. *Submitt. to J. Phys. Chem. Lett.*

Schwidetzky, R., Yar, A. Y., Lukas, M., Kunert, A. T., Pöschl, U., Koop, T., Bonn, M., Fröhlich-Nowoisky, J., Nagata, Y., and Meister, K. 2020b. Specific Ion Effects on Bacterial Ice Nucleation, *in preparation*.

Sharma, V. K. and Graham, N. J. D. 2010. Oxidation of Amino Acids, Peptides and Proteins by Ozone: A Review. *Ozone Sci. Eng.*, 32:81–90.

Shiraiwa, M., Selzle, K., Yang, H., Sosedova, Y., Ammann, M., and Pöschl, U. 2012. Multiphase chemical kinetics of the nitration of aerosolized protein by ozone and nitrogen dioxide. *Environ. Sci. Technol.*, 46(12):6672–6680.

Shiraiwa, M., Sosedova, Y., Rouvière, A., Hong, Y., Yingyi, Z., Abbatt, J. P. D., Ammann, M., and Pöschl, U. 2011. The role of long-lived reactive oxygen intermediates in the reaction of ozone with aerosol particles. *Nat. Chem.*, 3(4):291–295.

Shiraiwa, M., Ueda, K., Pozzer, A., Lammel, G., Kampf, C. J., Fushimi, A., Enami, S., Arangio, A. M., Fröhlich-Nowoisky, J., Fujitani, Y., Furuyama, A., Lakey, P. S., Lelieveld, J., Lucas, K., Morino, Y., Pöschl, U., Takahama, S., Takami, A., Tong, H., Weber, B., Yoshino, A., and Sato, K. 2017. Aerosol Health Effects from Molecular to Global Scales. *Environ. Sci. Technol.*, 51(23):13545–13567.

Steiner, A. L., Brooks, S. D., Deng, C., Thornton, D. C. O., Pendleton, M. W., and Bryant, V. 2015. Pollen as atmospheric cloud condensation nuclei. *Geophys. Res. Lett.*, 42:3596–3602.

Stopelli, E., Conen, F., Guilbaud, C., Zopfi, J., Alewell, C., and Morris, C. E. 2017. Ice nucleators, bacterial cells and *Pseudomonas syringae* in precipitation at Jungfraujoch. *Biogeosciences*, 14(5):1189–1196.

Stopelli, E., Conen, F., Morris, C. E., Herrmann, E., Bukowiecki, N., and Alewell, C. 2015. Ice nucleation active particles are efficiently removed by precipitating clouds. *Sci. Rep.*, 5:16433.

Stratmann, F., Kiselev, A., Wurzler, S., Wendisch, M., Heintzenberg, J., Charlson, R. J., Diehl, K., Wex, H., and Schmidt, S. 2004. Laboratory Studies and Numerical Simulations of Cloud Droplet Formation under Realistic Supersaturation Conditions. *J. Atmos. Ocean. Technol.*, 21(6):876–887.

Takeda, K. and Akira, S. 2004. TLR signaling pathways. *Semin. Immunol.*, 16(1):3–9.

Taylor, P. E., Flagan, R. C., Miguel, A. G., Valenta, R., and Glovsky, M. M. 2004. Birch pollen rupture and the release of aerosols of respirable allergens. *Clin. Exp. Allergy*, 34(10):1591–1596.

Tsumuki, H. and Konno, H. 1994. Ice Nuclei Produced by *Fusarium* sp. Isolated from the Gut of the Rice Stem Borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). *Biosci. Biotechnol. Biochem.*, 58(3):578–579.

Tsumuki, H., Konno, H., Maeda, T., and Okamoto, Y. 1992. An ice-nucleating active fungus isolated from the gut of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae). *J. Insect Physiol.*, 38(2):119–125.

Turko, I. V. and Murad, F. 2002. Protein Nitration in Cardiovascular Diseases. *Pharmacol. Rev.*, 54(4):619–634.

Vali, G. 1971. Quantitative Evaluation of Experimental Results an the Heterogeneous Freezing Nucleation of Supercooled Liquids. *J. Atmos. Sci.*, 28(3):402–409.

Varfolomeev, E. and Vucic, D. 2018. Intracellular regulation of TNF activity in health and disease. *Cytokine*, 101:26–32.

Walcher, W., Franze, T., Weller, M. G., Po, U., Huber, C. G., Mu, D., and Saarbru, D. 2003. Liquid- and Gas-Phase Nitration of Bovine Serum Albumin Studied by LC - MS and LC - MS / MS Using Monolithic Columns research articles. *J. Proteome Res.*, 2:534–542.

Weber, A., Wasiliew, P., and Kracht, M. 2010. Interleukin-1 (IL-1) Pathway. *Sci. Signal.*, 3(105):cm1.

Whale, T. F., Murray, B. J., O'Sullivan, D., Wilson, T. W., Umo, N. S., Baustian, K. J., Atkinson, J. D., Workneh, D. A., and Morris, G. J. 2015. A technique for quantifying heterogeneous ice nucleation in microlitre supercooled water droplets. *Atmos. Meas. Tech.*, 8(6):2437–2447.

Wolber, P. K., Deininger, C. A., Southworth, M. W., Vandekerckhovet, J., Van Montagut, M., and Warren, G. J. 1986. Identification and purification of a bacterial ice-nucleation protein. *Proc. Natl. Acad. Sci.*, 83:7256–7260.

Zaragotas, D., Liolios, N. T., and Anastassopoulos, E. 2016. Supercooling, ice nucleation and crystal growth: A systematic study in plant samples. *Cryobiology*, 72(3):239–243.

Ziegler, K., Kunert, A. T., Reinmuth-Selzle, K., Leifke, A. L., Widera, D., Weller, M. G., Schuppan, D., Fröhlich-Nowoisky, J., Lucas, K., and Pöschl, U. 2020. Chemical modification of pro-inflammatory proteins by peroxynitrite increases activation of TLR4 and NF-κB: Implications for the health effects of air pollution and oxidative stress. *Submitt. to Redox Biol.*

A List of Abbreviations

α-Syn	α-Synuclein
AFP	Antifreeze protein
BSA	Bovine serum albumin
DAMPs	Damage-associated molecular patterns
HSP60	Heat shock protein 60
HMGB1	High-mobility group box 1 protein
IN	Ice nuclei
INM	Ice-nucleating macromolecule
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
PRR	Pattern recognition receptor
RNS	Reactive nitrogen species
ROI	Reactive oxygen intermediate
ROS	Reactive oxygen species
TINA	Twin-plate Ice Nucleation Assay
TLR4	Toll-like receptor 4

B Personal List of Publications

Journal Articles

Published

1. Kunert, A. T., Pöhlker, M. L., Tang, K., Krevert, C. S., Wieder, C., Speth, K. R., Hanson, L. E., Morris, C. E., Schmale III, D. G., Pöschl, U., and Fröhlich-Nowoisky, J.: Macromolecular fungal ice nuclei in *Fusarium*: effects of physical and chemical processing, *Biogeosciences* **2019**, *16*, 4647–4659.

2. Kunert, A. T., Lamneck, M., Helleis, F., Pöschl, U., Pöhlker, M. L., Fröhlich-Nowoisky, J.: Twin-plate Ice Nucleation Assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples, *Atmos. Meas. Tech.* **2018**, *11*, 6327-6337.

3. Lang-Yona, N., Kunert, A. T., Vogel, L., Kampf, C. J., Bellinghausen, I., Saloga, J., Schink, A., Ziegler, K., Lucas, K., Pöschl, U., Weber, B., Fröhlich-Nowoisky, J.: Fresh water, marine and terrestrial cyanobacteria display distinct allergen characteristics, *Sci. Total Environ.* **2018**, *612*, 767-774.

4. Reinmuth-Selzle, K., Kampf, C. J., Lucas, K., Lang-Yona, N., Fröhlich-Nowoisky, J., Shiraiwa, M., Lakey, P. S. J., Lai, S., Liu, F., Kunert, A. T., Ziegler, K., Shen, F., Sgarbanti, R., Weber, B., Bellinghausen, I., Saloga, J., Weller, M. G., Duschl, A., Schuppan, D., Pöschl, U.: Air Pollution and Climate Change Effects on Allergies in the Anthropocene: Abundance, Interaction, and Modification of Allergens and Adjuvants, *Environ. Sci. Technol.* **2017**, *51*, 4119-4141.

5. Liu, F., Lakey, P. S. J., Berkemeier, T., Tong, H., Kunert, A. T., Meusel, H., Cheng, Y., Su, H., Fröhlich-Nowoisky, J., Lai, S., Weller, M. G., Shiraiwa, M., Pöschl, U., Kampf, C. J.: Atmospheric protein chemistry influenced by anthropogenic air pollutants: nitration and oligomerization upon exposure to ozone and nitrogen dioxide, *Faraday Discuss.* **2017**, *200*, 413-427.

Submitted

6. Ziegler, K., Kunert, A. T., Reinmuth-Selzle, K., Leifke, A. L., Widera, D., Weller, M. G., Schuppan, D., Fröhlich-Nowoisky, J., Lucas, K., Pöschl, U.: Chemical modification of pro-inflammatory proteins by peroxynitrite increases activation of TLR4 and NF-κB: Implications for the health effects of air pollution and oxidative stress, *submitted to Redox Biol.* **2020**.

7. Schwidetzky, R., Kunert, A. T., Bonn, M., Pöschl, U., Ramlov, H., DeVries, A. L., Fröhlich-Nowoisky, J., Meister, K.: To Freeze or Not to Freeze: Inhibition of Bacterial Ice Nucleators by Antifreeze Proteins, *submitted to J. Phys. Chem. Lett.* **2020**.

8. Lukas, M., Schwidetzky, R., Kunert, A. T., Pöschl, U., Fröhlich-Nowoisky, J., Bonn, M., and Meister, K.: Electrostatic Interactions Control the Functionality of Bacterial Ice Nucleators, *submitted to J. Am. Chem. Soc.* **2019**.

In Preparation

9. Kunert, A. T., Reinmuth-Selzle, K., Ziegler, K., Krevert, C. S., Wieder, C., Weller, M. G., Lucas, K., Pöschl, U., and Fröhlich-Nowoisky, J.: Reaction kinetics and products of nitration and oligomerization of the major grass pollen allergen Phl p 5 by peroxynitrite and nitrogen dioxide with ozone, *in preparation*.

10. Tang, K., Kunert, A. T., Sánchez-Parra, B., Barbosa, C., Campos, D., Kremper, L., Pickersgill, D. A., Prass, M., Artaxo, P., Pöhlker, C., Pöschl, U., Fröhlich-Nowoisky, J.: Characterization of ice nuclei in ATTO air filter samples, *in preparation*.

11. Lukas, M., Kunert, A. T., Kutus, B., Fröhlich-Nowoisky, J., Abdelmonem, A., Pöschl, U., Bonn, M., Backus, E. H. G.: Ice-nucleating bacteria and fungi: Increased order of water molecules at low temperatures, *in preparation*.

12. Sosso, G. C., Sudera, P., Kunert, A. T., Fröhlich-Nowoisky, J., Pöschl, U., Backus, E. H. G., Bonn, M., and Michaelides, A.: Same Chemistry, Different Structure: Understanding the Key Ingredients of Ice Formation, *in preparation*.

13. Reinmuth-Selzle, K., Tchipilov, T., Kunert, A. T., Ziegler, K., Lucas, K., Pöschl, U., Fröhlich-Nowoisky, J., Weller, M. G.: Quantitative protein analysis of challenging and modified protein samples, *in preparation*.

14. Schwidetzky, R., Yar, A. Y., Lukas, M., Kunert, A. T., Pöschl, U., Koop, T., Bonn, M., Fröhlich-Nowoisky, J., Nagata, Y., Meister, K.: Specific Ion Effects on Bacterial Ice Nucleation, *in preparation*.

15. Reinmuth-Selzle, K., Bellinghausen, I., Ziegler, K., Kunert, A. T., Leifke, A. L., Lucas, K., Fröhlich-Nowoisky, J., Pöschl, U.: Chemical modification of aeroallergens Bet v 1 and Phl p 5 by air pollutants and peroxynitrite affects serum IgE binding and TLR4 signaling, *in preparation*.

16. Tang, K., Yordanova, Y., Wehking, J., Sánchez-Parra, B., Pickersgill, D. A., Maier, S., Kunert, A. T., Sciare, J., Pöschl, U., Weber, B., Fröhlich-Nowoisky, J.: Bioaerosols in a mediterranean dryland: Community changes related to rainfall, *in preparation*.

Oral Presentations

1. Kunert, A. T., Ziegler, K., Krevert, C. S., Wieder, C., Reinmuth-Selzle, K., Lucas, K., Pöschl, U., Fröhlich-Nowoisky, J.: Nitration and oligomerization of allergens: efficiency and effects, Lindau Alumni Retreat, Heidelberg, Germany (19^{th} - 22^{nd} September 2019).

2. Kunert, A. T., Lamneck, M., Helleis, F., Pöschl, U., Pöhlker, M. L., Fröhlich-Nowoisky, J.: Twin-plate Ice Nucleation Assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples, European Aerosol Conference, Goteborg, Sweden (25^{th} - 30^{th} August 2019).

3. Kunert, A. T., Lamneck, M., Helleis, F., Pöschl, U., Pöhlker, M. L., Fröhlich-Nowoisky, J.: Twin-plate Ice Nucleation Assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples, International Conference on Carbonaceous Particles in the Atmosphere, Vienna, Austria (3^{rd} - 6^{th} April 2019).

4. Kunert, A. T., Ziegler, K., Krevert, C. S., Wieder, C., Reinmuth-Selzle, K., Lucas, K., Pöschl, U., Fröhlich-Nowoisky, J.: Nitration and Oligomerization of Allergens upon Exposure to Ozone and Nitrogen Dioxide as well as Peroxynitrite Treatment, 31^{st} Mainzer Allergy Workshop, Mainz, Germany (29^{th} - 30^{th} March 2019).

5. Kunert, A. T., Lamneck, M., Helleis, F., Pöschl, U., Pöhlker, M. L., Fröhlich-Nowoisky, J.: High-Throughput Measurements of Biological Ice Nuclei, Lindau Alumni Retreat, Frankfurt, Germany (17^{th} - 18^{th} November 2018).

6. Liu, F., Lakey, P. S. J., Berkemeier, T., Tong, H., Kunert, A. T., Meusel, H., Cheng, Y., Su, H., Fröhlich-Nowoisky, J., Lai, S., Weller, M. G., Shiraiwa, M., Pöschl, U., Kampf, C. J.: Atmospheric protein chemistry influenced by anthropogenic air pollutants: nitration and oligomerization upon exposure to ozone and nitrogen dioxide, 30^{th} Mainzer Allergy Workshop, Mainz, Germany (22^{nd} - 23^{rd} March 2018).

7. Kunert, A. T., Krevert, C. S., Speth, K. R., Scheel, J. F., Kopper, G. M., Kampf, C. J., Liu, F., Hanson, L. E., Franc, G. D., Pöschl, U., Morris, C., Schmale, D. G., Fröhlich-Nowoisky, J.:

Highly active, stable, and cell–free fungal ice nuclei are widespread among *Fusarium* spp, INUIT Final Conference and Second Atmospheric Ice Nucleation Conference, Grasellenbach, Germany (26th February - 1st March 2018).

8. Kunert, A. T., Lamneck, M., Gurk, C., Helleis, F., Klimach, T., Scheel, J., Pöschl, U., Fröhlich-Nowoisky, J.: TINA, a new fully automated high-performance droplet freezing assay coupled to a customized infrared detection system, 5th Workshop - Microphysics of Ice Clouds, Vienna, Austria (22nd - 23rd April 2017).

Poster Presentations

1. Kunert, A. T., Lamneck, M., Helleis, F., Pöschl, U., Pöhlker, M. L., Fröhlich-Nowoisky, J.: Twin-plate Ice Nucleation Assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples, General Assembly of the European Geoscience Union, Vienna, Austria (7th - 12nd April 2019).

2. Kunert, A. T., Lamneck, M., Helleis, F., Scheel, J., Pöschl, U., Fröhlich-Nowoisky, J.: TINA: Twin-plate ice nucleation assay with infrared detection for high-throughput droplet freezing experiments, INUIT Final Conference and Second Atmospheric Ice Nucleation Conference, Grasellenbach, Germany (26th February - 1st March 2018).

3. Kunert, A. T., Lamneck, M., Gurk, C., Helleis, F., Klimach, T., Scheel, J., Pöschl, U., Fröhlich-Nowoisky, J.: TINA, a new fully automated high-performance droplet freezing assay coupled to a customized infrared detection system, General Assembly of the European Geoscience Union, Vienna, Austria (23rd - 28th April 2017).

4. Kunert, A. T., Maurus, I., Liu, F., Reinmuth-Selzle, K., Kampf, C. J., Lang-Yona, N., Ziegler, K., Lucas, K., Fröhlich-Nowoisky, J., Pöschl, U.: Chemical Modification of Proteins by Anthropogenic Air Pollutants, WE-Heraeus-Seminar on Aerosol, Climate and Health, Bad Honnef, Germany (27th - 31st March 2017).

5. Kunert, A. T., Lamneck, M., Gurk, C., Helleis, F., Klimach, T., Scheel, J., Pöschl, U., Fröhlich-Nowoisky, J.: TINA: A New High-Performance Droplet Freezing Assay for the Analysis of Ice Nuclei with Complex Composition, General Assembly of the European Geoscience Union, Vienna, Austria (17th - 22nd April 2016).

6. Kunert, A. T., Lamneck, M., Gurk, C., Helleis, F., Klimach, T., Scheel, J., Pöschl, U., Fröhlich-Nowoisky, J.: TINA: A New High-Performance Droplet Freezing Assay for the Analysis of Ice Nuclei with Complex Composition, Spring Symposium of the Youth Organization of the German Chemical Society, Kiel, Germany (16th - 19th March 2016).

C Selected Publications

1. Kunert, A. T., Lamneck, M., Helleis, F., Pöschl, U., Pöhlker, M. L., Fröhlich-Nowoisky, J.: Twin-plate Ice Nucleation Assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples, *Atmos. Meas. Tech.* **2018**, *11*, 6327-6337.

2. Kunert, A. T., Pöhlker, M. L., Tang, K., Krevert, C. S., Wieder, C., Speth, K. R., Hanson, L. E., Morris, C. E., Schmale III, D. G., Pöschl, U., and Fröhlich-Nowoisky, J.: Macromolecular fungal ice nuclei in *Fusarium*: effects of physical and chemical processing, *Biogeosciences* **2019**, *16*, 4647–4659.

3. Schwidetzky, R., Kunert, A. T., Bonn, M., Pöschl, U., Ramlov, H., DeVries, A. L., Fröhlich-Nowoisky, J., Meister, K.: To Freeze or Not to Freeze: Inhibition of Bacterial Ice Nucleators by Antifreeze Proteins, *submitted to J. Phys. Chem. Lett.* **2020**.

4. Lukas, M., Schwidetzky, R., Kunert, A. T., Pöschl, U., Fröhlich-Nowoisky, J., Bonn, M., and Meister, K.: Electrostatic Interactions Control the Functionality of Bacterial Ice Nucleators, *submitted to J. Am. Chem. Soc.* **2019**.

5. Kunert, A. T., Reinmuth-Selzle, K., Ziegler, K., Krevert, C. S., Wieder, C., Weller, M. G., Lucas, K., Pöschl, U., and Fröhlich-Nowoisky, J.: Reaction kinetics and products of nitration and oligomerization of the major grass pollen allergen Phl p 5 by peroxynitrite and nitrogen dioxide with ozone, *in preparation*.

6. Ziegler, K., Kunert, A. T., Reinmuth-Selzle, K., Leifke, A. L., Widera, D., Weller, M. G., Schuppan, D., Fröhlich-Nowoisky, J., Lucas, K., Pöschl, U.: Chemical modification of pro-inflammatory proteins by peroxynitrite increases activation of TLR4 and NF-κB: Implications for the health effects of air pollution and oxidative stress, *submitted to Redox Biol.* **2020**.

7. Reinmuth-Selzle, K., Kampf, C. J., Lucas, K., Lang-Yona, N., Fröhlich-Nowoisky, J., Shiraiwa, M., Lakey, P. S. J., Lai, S., Liu, F., Kunert, A. T., Ziegler, K., Shen, F., Sgarbanti, R., Weber, B., Bellinghausen, I., Saloga, J., Weller, M. G., Duschl, A., Schuppan, D., Pöschl, U.: Air Pollution and Climate Change Effects on Allergies in the Anthropocene: Abundance, Interaction, and Modification of Allergens and Adjuvants, *Environ. Sci. Technol.* **2017**, *51*, 4119-4141.

8. Liu, F., Lakey, P. S. J., Berkemeier, T., Tong, H., Kunert, A. T., Meusel, H., Cheng, Y., Su, H., Fröhlich-Nowoisky, J., Lai, S., Weller, M. G., Shiraiwa, M., Pöschl, U., Kampf, C. J.: Atmospheric protein chemistry influenced by anthropogenic air pollutants: nitration and oligomerization upon exposure to ozone and nitrogen dioxide, *Faraday Discuss.* **2017**, *200*, 413-427.

C.1 Kunert *et al.*, Atmos. Meas. Tech., 2018

Twin-plate Ice Nucleation Assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples

Anna T. Kunert[1], Mark Lamneck[2], Frank Helleis[2], Ulrich Pöschl[1], Mira L. Pöhlker[1], Janine Fröhlich-Nowoisky[1]

[1]Multiphase Chemistry Department, Max Planck Institute for Chemistry, 55128 Mainz, Germany; [2]Instrument Development and Electronics, Max Planck Institute for Chemistry, 55128 Mainz, Germany

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Author contributions

ATK, ML, and FH developed the instrument. ATK, UP, JFN conceived and designed the experiments. ATK performed the experiments. MLP wrote the code to process the data and did the error calculation. All authors discussed the data and contributed to the writing of the manuscript.

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Twin-plate Ice Nucleation Assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples

Anna T. Kunert[1], **Mark Lamneck**[2], **Frank Helleis**[2], **Ulrich Pöschl**[1], **Mira L. Pöhlker**[1], **and Janine Fröhlich-Nowoisky**[1]

[1]Multiphase Chemistry Department, Max Planck Institute for Chemistry, 55128 Mainz, Germany
[2]Instrument Development and Electronics, Max Planck Institute for Chemistry, 55128 Mainz, Germany

Correspondence: Janine Fröhlich-Nowoisky (j.frohlich@mpic.de) and Mira L. Pöhlker (m.pohlker@mpic.de)

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Abstract. For efficient analysis and characterization of biological ice nuclei under immersion freezing conditions, we developed the Twin-plate Ice Nucleation Assay (TINA) for high-throughput droplet freezing experiments, in which the temperature profile and freezing of each droplet is tracked by an infrared detector. In the fully automated setup, a couple of independently cooled aluminum blocks carrying two 96-well plates and two 384-well plates, respectively, are available to study ice nucleation and freezing events simultaneously in hundreds of microliter-range droplets (0.1–40 µL). A cooling system with two refrigerant circulation loops is used for high-precision temperature control (uncertainty < 0.2 K), enabling measurements over a wide range of temperatures (~ 272–233 K) at variable cooling rates (up to 10 K min^{-1}).

The TINA instrument was tested and characterized in experiments with bacterial and fungal ice nuclei (IN) from *Pseudomonas syringae* (Snomax®) and *Mortierella alpina*, exhibiting freezing curves in good agreement with literature data. Moreover, TINA was applied to investigate the influence of chemical processing on the activity of biological IN, in particular the effects of oxidation and nitration reactions. Upon exposure of Snomax® to O_3 and NO_2, the cumulative number of IN active at 270–266 K decreased by more than 1 order of magnitude. Furthermore, TINA was used to study aqueous extracts of atmospheric aerosols, simultaneously investigating a multitude of samples that were pre-treated in different ways to distinguish different kinds of IN. For example, heat treatment and filtration indicated that most biological IN were larger than 5 µm. The results confirm that TINA is suitable for high-throughput experiments and efficient analysis of biological IN in laboratory and field samples.

1 Introduction

Clouds and aerosols still contribute the largest uncertainty to the evaluation of the Earth's changing energy budget (Boucher et al., 2013). Thus, the understanding of the contribution of atmospheric aerosols in cloud processes is of fundamental importance. Atmospheric ice nucleation is essential for cloud glaciation and precipitation, thereby influencing the hydrological cycle and climate. Ice can be formed via homogeneous nucleation in liquid water droplets or heterogeneous nucleation triggered by particles serving as atmospheric ice nuclei (IN) (Pruppacher and Klett, 1997).

A wide range of droplet freezing assays and instruments have been developed and applied for the analysis of IN in immersion freezing experiments (e.g., Budke and Koop, 2015; Fröhlich-Nowoisky et al., 2015; Häusler et al., 2018; Murray et al., 2010; O'Sullivan et al., 2014; Stopelli et al., 2014; Tobo, 2016; Vali, 1971b; Whale et al., 2015; Wright and Petters, 2013; Zaragotas et al., 2016). Most of the available assays and instruments, however, are limited to the investigation of small droplet numbers and use optical detection systems in the UV–Vis wavelength range.

Infrared (IR) detectors enable efficient detection of droplet freezing (Harrison et al., 2018; Zaragotas et al., 2016). Upon the phase change of water from liquid to solid, latent heat is released resulting in a sudden temperature change of the

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droplet, which can be detected by IR video thermography. In 1995, Ceccardi et al. (1995) used IR video thermography as a new technique to non-destructively study ice formation on plants by visualizing the changes in surface temperature. Wisniewski et al. (1997) evaluated the IR video thermography under controlled conditions and determined it as an excellent method for directly observing ice nucleation and propagation in plants. Since then, IR video thermography has been used in a range of studies investigating freezing in plants (e.g., Ball et al., 2002; Carter et al., 1999; Charrier et al., 2017; Fuller and Wisniewski, 1998; Hacker and Neuner, 2007; Pearce and Fuller, 2001; Sekozawa et al., 2004; Stier et al., 2003; Wisniewski et al., 2008; Workmaster, 1999). Further applications of IR video thermography are investigations of cold thermal stress in insects (Gallego et al., 2016), monitoring of freeze-drying processes (Emteborg et al., 2014), as well as detection of ice in wind turbine blades (Gómez Muñoz et al., 2016) and helicopter rotor blades (Hansman and Dershowitz, 1994). Freezing of single water droplets in an acoustic levitator has also been successfully observed by IR video thermography (Bauerecker et al., 2008).

Here, we introduce the Twin-plate Ice Nucleation Assay (TINA) for high-throughput droplet freezing experiments, in which the temperature profile and freezing of each droplet is tracked by an infrared detector. In the fully automated setup, a couple of independently cooled aluminum blocks are available to study ice nucleation and freezing events in nearly 1000 microliter-range droplets simultaneously. The instrument was developed in the course of the INUIT project over the last three years, in which it has been presented and discussed at several conferences and workshops (Kunert et al., 2016a,b, 2017a,b, 2018). We use the bacterial IN Snomax® and the IN-active fungus *Mortierella alpina* as biological test substances to investigate heterogeneous ice nucleation. Moreover, TINA is applied to investigate the effect of O_3 and NO_2 exposure on the IN activity of Snomax®. Furthermore, aqueous extracts of atmospheric aerosols are treated in different ways and are analyzed for different kinds of IN.

2 Experimental setup

2.1 Technical details

The core of TINA is composed of two independently cooled, customized sample holder aluminum blocks, which have been shaped for multiwell plates with 96 and 384 wells, respectively. In each cooling block, two multiwell plates can be analyzed simultaneously. The maximal droplet volume in the 96-well block is 250 µL, and the minimal droplet volume is 0.1 µL, which is the limit of our liquid handling station (epMotion ep5073, Eppendorf, Hamburg, Germany). For each experiment, new sterile multiwell plates are used (96-well: Axon Labortechnik, Kaiserslautern, Germany, 384-well: Eppendorf, Hamburg, Germany). As shown in Fig. 1, the design

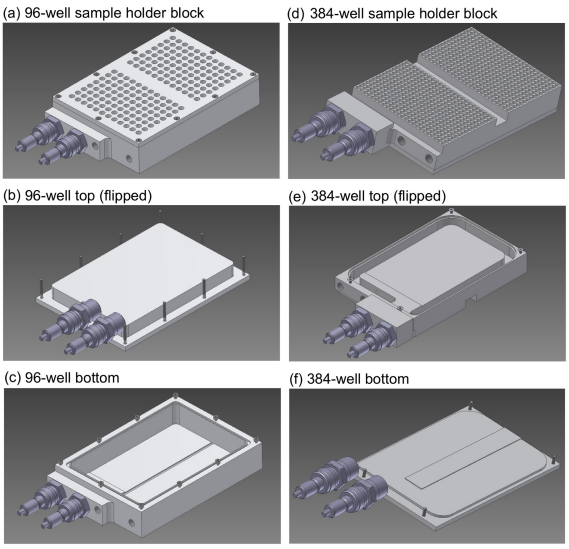

Figure 1. Sample holder and cooling blocks of the Twin-plate Ice Nucleation Assay (TINA) with **(a–c)** 96-well plates and **(d–f)** 384-well plates (CAD drawings).

of the two sample holder blocks is basically identical, but the detailed construction varies slightly. Both blocks consist of two parts, a trough and a cap, which are screwed together and sealed with an O-ring. But, for the 96-well block (Fig. 1a), the cap is at the top (Fig. 1b), and the trough is at the bottom (Fig. 1c), whereas, for the 384-well block (Fig. 1d), the trough is at the top (Fig. 1e) and the cap is at the bottom (Fig. 1f). Two openings with Swagelok® adapters for cooling liquid are placed next to each other, and the cooling liquid flows in a small passage around an elevation in the middle of the trough.

The customized sample holder blocks are cooled with a silicon-based cooling liquid (SilOil M80.055.03, Peter Huber Kältemaschinenbau AG, Offenburg, Germany) tempered by an external high-performance refrigeration bath circulator (CC-508 with Pilot ONE, Peter Huber Kältemaschinenbau AG), which can supply temperatures down to 218 K (−55 °C). Both sample holder blocks can be operated in parallel and independently of each other by use of two self-developed mixing valves and cooling loops (Fig. 2). This allows either the cooling of two different droplet freezing assays at the same time or the observation of 960 droplets in one experiment. The mixing of a cold and a warm loop of cooling liquid for each block enables a fast and precise adjustment of the sample holder block temperatures without being dependent on the cooling rate of the refrigeration bath circulator itself. In each experiment, the refrigeration bath circulator is cooled down 5 K below the coldest temperature, which is projected for the experiment, while no mixing of warm and cold cooling liquid occurs. By changing the posi-

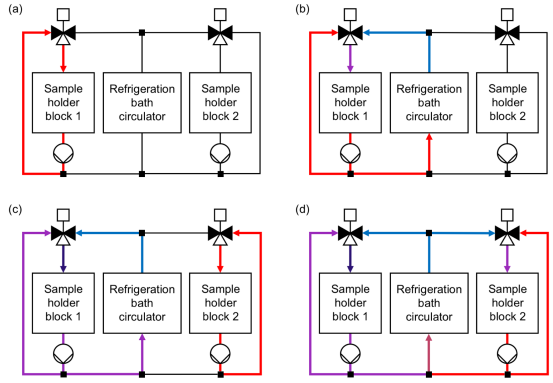

Figure 2. Cooling system layout and operating principle of the Twin-plate Ice Nucleation Assay (TINA). **(a)** Cooling liquid is pumped in warm cooling loop of sample holder block 1 without connection to colder cooling liquid provided by refrigeration bath circulator. **(b)** Mixing valve is opened for both warm cooling liquid of warm cooling loop and cold cooling liquid of refrigeration bath circulator. Position of mixing valve defines temperature within sample holder block 1. **(c)** Sample holder block 1 is cooled further down, while cooling liquid is pumped in warm cooling loop of sample holder block 2. **(d)** Sample holder block 2 can be run in parallel independently of the temperature in sample holder block 1.

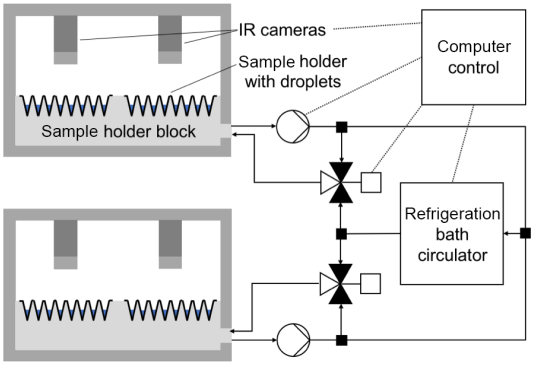

Figure 3. Schematic illustration of the overall setup: sample holder blocks, sample holders with droplets, IR cameras, cooling system with refrigeration bath circulator, pumps and mixing valves, computer control.

tion of the mixing valves for a defined period of time, cold and warm cooling liquids are mixed together so that the desired temperatures within the two blocks are reached. Two pumps (VPP-655 PWM Single Version, Alphacool International GmbH, Braunschweig, Germany) ensure the continuous circulation of cooling liquid through each block independently of the position of the mixing valves. Figure 3 is a schematic illustration of the overall setup of TINA.

2.2 Temperature control and calibration

Within each sample holder block, the temperature is measured with two temperature sensors, an NTC thermistor in the cooling liquid stream (TH-44033, resistance: 2255 Ω/298 K, interchangeability: ±0.1 K, Omega Engineering GmbH, Deckenpfronn, Germany) and a customized sensor with an NTC thermistor (10K3MRBD1, resistance: 10 000 Ω/298 K, interchangeability: ±0.2 K, TE Connectivity Company, Galway, Ireland) and a thermocouple (K type, 0.08 mm diameter, Omega), which were glued together in a 1/8 in. Swagelok® pipe, placed inside the elevated central part of the block. With further thermocouples connected to this reference, this offers the possibility of measuring temperature differences between the NTC thermistor and arbitrary points simultaneously. Another NTC thermistor (10K3MRBD1, resistance: 10 000 Ω/298 K, interchangeability: ±0.2 K, TE Connectivity Company) monitors the temperature behind each mixing valve. Temperature control within the entire system is achieved by a self-developed microcontroller-based electronic system. The analog input unit is equipped with a low-noise, 24 bit ADC (ADS1256, Texas Instruments Incorporated, Dallas, TX, USA), which assures the required accuracy to process the resolution of the used thermistors. All thermistors had been calibrated with a reference thermometer (2180A, Fluke Deutschland GmbH, Glottertal, Germany; 0.01 K resolution, system uncertainty δ_{Fluke}: ±0.08 K at 223 K and ±0.07 K at 273 K). Therefore, all thermistors were bound together with a PT100 sensor of the reference thermometer, and the bundle was placed inside a brass cylinder filled with cooling liquid. The cylinder was placed inside the cooling bath of the refrigeration bath circulator. The temperature within the bath was cooled down from 303.2 to 218.2 K (30.0 to −55.0 °C) in 5 K steps, warmed to 220.7 K (−52.5 °C), and raised again from 220.7 to 300.7 K (−52.5 to 27.5 °C) in 5 K steps. Each step was kept for 30 min to equilibrate the temperature, while the resistance of all thermistors and the temperature measured by the reference thermometer were monitored. For the conversion of the measured resistance of the thermistors into temperature, cubic spline interpolation was used ($\delta_{\text{Ipol}} < 0.01$ K). We obtained the thermistor calibration uncertainty $\delta_{\text{Thermistor}} < 0.09$ K ($\delta_{\text{Thermistor}} = \delta_{\text{Fluke}} + \delta_{\text{Ipol}}$).

To determine a potential temperature gradient of the sample holder blocks, two thermocouples (K type, 0.08 mm diameter, Omega) were positioned in various wells of multiwell plates (Fig. S1a and b in the Supplement), each filled with 30 µL pure water (see Sect. 3.1). These thermocouples were connected to the thermocouple in the elevation of each sample holder block, and the temperature offset between sample holder block and wells was measured for a continuous cooling rate of 1 K min^{-1} (Fig. S1c). Below −2 °C, the temperature offset between sample holder block and wells is nearly constant, in this example ∼ 0.16 and ∼ 0.19 K. The measurement was performed in duplicate for all observed

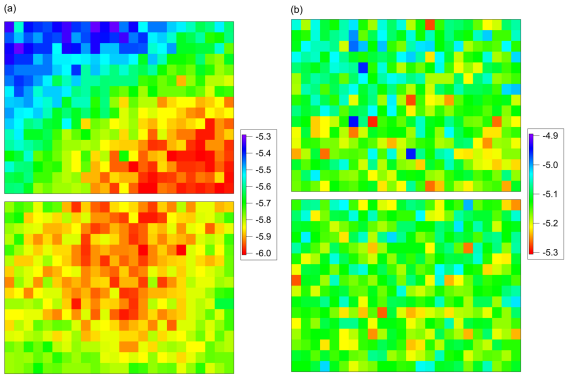

Figure 4. Measurement of temperature gradient of 384-well sample holder block using *Mortierella alpina* 13A as calibration substance. A correction matrix was calculated to compensate for temperature gradient and offset. **(a)** Data before correction. **(b)** Data after correction.

wells. Figure S2 shows the temperature gradient exemplarily for the 384-well sample holder block in a 2-D interpolation based on all measurements.

To characterize the uncertainty of this measurement, the two thermocouples were placed in an ice water bath, and the sample holder block was cooled down to 2, 1, 0, −1, and −2 °C (T_{block}), while the difference between the ice water and the block temperature was monitored by the thermocouples (T_{diffTC}) (Fig. S3). From these experiments, we obtained thermocouple uncertainties $\delta_{\text{TC}} < 0.05\,\text{K}$ ($\delta_{\text{TC}} = T_{\text{block}} + T_{\text{diffTC}}$).

Additionally, we used undiluted IN filtrate of *Mortierella alpina* 13A (see Sect. 3.2) as calibration substance, and a freezing experiment was performed as described for the biological reference materials (see Sect. 3.2). These results were used to compensate for the temperature gradient, and the thermocouple measurements were used to correct the temperature offset between gradient-corrected wells and thermistors. A correction matrix was calculated, and this matrix was used to correct subsequent freezing experiments. Figure 4 shows the results of the fungal IN filtrate measurement (a) before and (b) after correction. After correction, all fungal IN filtrate measurements showed a standard deviation of $< 0.06\,\text{K}$ (δ_{Morti}). From the calibration measurements, we obtained a total uncertainty estimate of $\delta_{\text{total}} < 0.2\,\text{K}$ ($\delta_{\text{total}} = \delta_{\text{Thermistor}} + \delta_{\text{TC}} + \delta_{\text{Morti}}$).

2.3 Infrared video thermography

Droplet freezing is determined by a distinct detection system, where the temperature profile of each single droplet is tracked by infrared cameras (Seek Thermal Compact XR, Seek Thermal Inc., Santa Barbara, CA, USA) coupled to a self-written software. The camera has a resolution of 206 × 156 pixels, and it takes 10 pictures per second. These pictures are averaged to one picture per second. The concept enables a doubtless determination of freezing events because freezing of supercooled liquid releases energy, which leads to an abrupt rise in the detected temperature of the observed droplet, as discussed earlier (Sect. 1). This detection system uses the IR video thermography only to determine freezing events, while the proper temperature is monitored by thermistors. Figure 5 is a sequence of infrared camera images showing 384 droplets during cooling and freezing (red circles). Software analysis uses a grid of 96 and 384 points, respectively, where the grid point is set to the center of each well enabling one to fit the dimensions of each plate under different perspective angles. The temperature is tracked for each well during the experiment. A self-written algorithm detects a local maximum shortly followed by a local minimum in the derivative of the temperature profile, which is caused

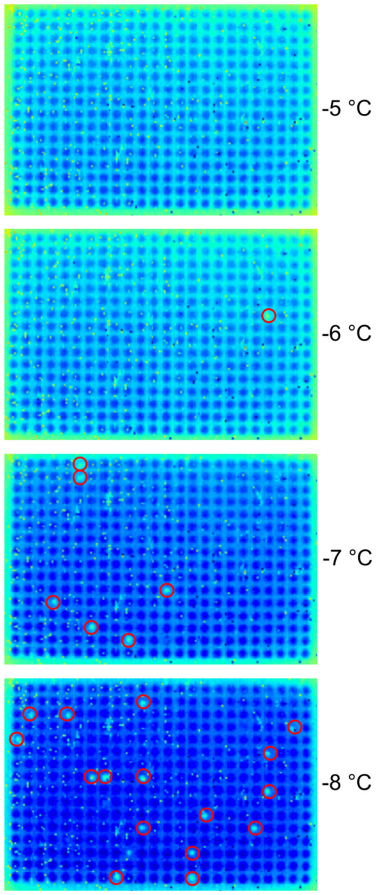

Figure 5. Sequence of infrared camera images showing 384 droplets during cooling. Red circles indicate freezing droplets.

by the release of latent heat during freezing. The software exports the data for each droplet in CSV format.

2.4 Data analysis

Assuming ice nucleation as a time-independent (singular) process, the number concentration of IN ($\frac{\Delta N_\mathrm{m}}{\Delta T}$) active at a certain temperature (T) per unit mass of material is given by Eq. (1) (Vali, 1971a).

$$\frac{\Delta N_\mathrm{m}}{\Delta T}(T) = -\ln\left(1 - \frac{s}{a - \sum_{i=0}^{j} s}\right) \cdot \frac{c}{\Delta T} \quad ; 0 \le j \le a \tag{1}$$

$$\text{with } c = \frac{V_\mathrm{wash}}{V_\mathrm{drop}} \cdot \frac{d}{m}, \tag{2}$$

where s is the number of freezing events in 0.1 K bins (ΔT), a is the number of all droplets, m is the mass of the particles in the initial suspension, V_wash is the volume of the initial suspension, V_drop is the droplet volume, and d is the dilution factor of the droplets relative to m. The measurement uncertainty ($\delta \frac{\Delta N_\mathrm{m}}{\Delta T}(T)$) was calculated using the counting error of s plus one digit and the Gaussian error propagation (Eq. 3).

$$\delta \frac{\Delta N_\mathrm{m}}{\Delta T}(T) = \sqrt{\left(\frac{1}{1 - \frac{s}{a - \sum_{i=0}^{j} s}} \cdot \frac{c}{\Delta T} \cdot \frac{\sqrt{s+1}}{a - \sum_{i=0}^{j} s}\right)^2 + \left(\frac{1}{1 - \frac{s}{a - \sum_{i=0}^{j} s}} \cdot \frac{c}{\Delta T} \cdot \frac{s \cdot \sqrt{\sum_{i=0}^{j} s + 1}}{\left(a - \sum_{i=0}^{j} s\right)^2}\right)^2} \tag{3}$$

The cumulative IN number concentration ($N_\mathrm{m}(T)$) is given by Eq. (4).

$$N_\mathrm{m}(T) = -\ln\left(1 - \frac{\sum_{i=0}^{j} s}{a}\right) \cdot c \quad ; 0 \le j \le a \tag{4}$$

The error of the cumulative IN number concentration ($\delta N_\mathrm{m}(T)$) was calculated using Eq. (5).

$$\delta N_\mathrm{m}(T) = \sqrt{\left(\frac{c}{1 - \frac{\sum_{i=0}^{j} s}{a}} \cdot \frac{\sqrt{\sum_{i=0}^{j} s + 1}}{a}\right)^2} \tag{5}$$

According to the above equations, the uncertainty is proportional to the number of frozen droplets per temperature bin. In the freezing experiments described below, the lowest number of freezing events and largest uncertainties were obtained at the lower and higher end of each dilution series (Poisson distribution). Data points with uncertainties $\ge 100\,\%$ were excluded (overall less than 6 % of the measurement data).

3 Freezing experiments

The fully automated TINA setup was tested and characterized for immersion freezing experiments with pure-water droplets, as well as Snomax® and IN filtrate of the fungus *Mortierella alpina* as biological reference substances. Moreover, TINA was used to study the effect of O_3 and NO_2 exposure on the IN activity of Snomax®. Furthermore, TINA was applied to atmospheric aerosol samples.

3.1 Pure water

Pure water was obtained from a Barnstead™ GenPure™ xCAD Plus water purification system (Thermo Scientific, Braunschweig, Germany). The water was autoclaved at 394 K (121 °C) for 20 min, filtered three times through a sterile 0.1 µm pore diameter sterile polyethersulfone (PES) vacuum filter unit (VWR International, Radnor, PA, USA), and autoclaved again.

For background measurements, 3 µL aliquots of autoclaved and filtered pure water were pipetted into new sterile multiwell plates by a liquid handling station. Therefore, four (96-well plate) and eight (384-well plate) different water samples were pipetted column-wise distributed into the plates. In total, six columns per sample were apportioned over the two twin plates, i.e., 48 droplets per sample in 96-well plates, and 96 droplets per sample in 384-well plates. The plates were placed in the sample holder blocks and were cooled down quickly to 273 K (0 °C) and, as soon as the temperature was stable for 1 min, in a continuous cooling rate of 1 K min^{-1} further down to 238 K (−35 °C).

As the phase transition from liquid water to ice is kinetically hindered, supercooled water can stay liquid at temperatures down to 235 K (−38 °C), where homogeneous ice nucleation takes place. This is only true for nanometer-sized droplets because the freezing temperature is dependent on droplet volume and cooling rate, and the classical nucleation theory predicts a homogeneous freezing temperature of about 240 K (−33 °C) for microliter-volume droplets using a cooling rate of 1 K min^{-1} (Fornea et al., 2009; Murray et al., 2010; Pruppacher and Klett, 1997; Tobo, 2016). However, several studies reported average freezing temperatures for microliter-volume droplets of pure water at significantly higher temperatures because of possible artifacts (e.g., Conen et al., 2011; Fröhlich-Nowoisky et al., 2015; Hill et al., 2016; Whale et al., 2015). To our knowledge, only two studies reported an average homogeneous freezing temperature of 240 K (−33 °C) for microliter-volume droplets and a cooling rate of 1 K min^{-1}, using hydrophobic surfaces as a contact area for the droplets (Fornea et al., 2009; Tobo, 2016). Providing microliter droplets free of suspended IN and surfaces free of contaminants is difficult, so the temperature limit below which freezing cannot be traced back to heterogeneous IN needs to be determined individually for each setup.

Our results showed that most pure-water droplets froze around 248 K (−25 °C) in 96-well plates (Fig. 6a) and around 245 K (−28 °C) in 384-well plates (Fig. 6b). The 96-well plates were obtained from a different manufacturer than the 384-well plates. All in all, these freezing temperatures are

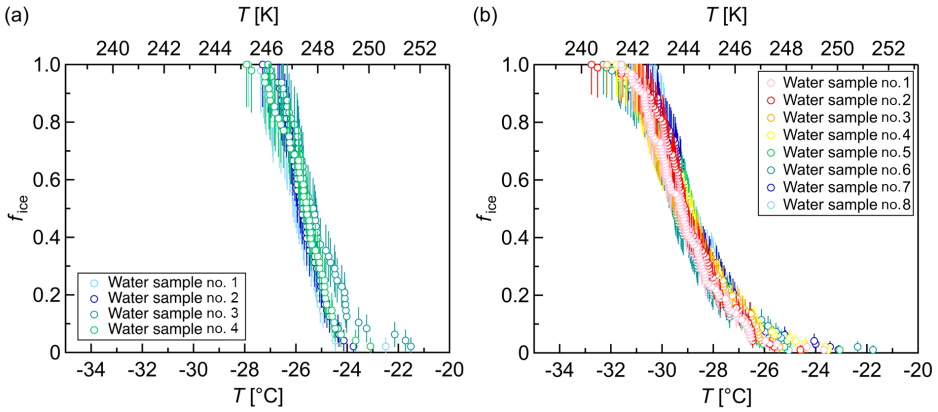

Figure 6. Freezing experiments with pure-water droplets. Fraction of frozen droplets (f_{ice}) vs. temperature (T) obtained with a continuous cooling rate of 1 K min^{-1} and a droplet volume of 3 µL. **(a)** Four different samples with 48 droplets each apportioned over two 96-well plates. **(b)** Eight different samples with 96 droplets each apportioned over two 384-well plates. The error bars were calculated using the counting error and the Gaussian error propagation. The temperature error is 0.2 K.

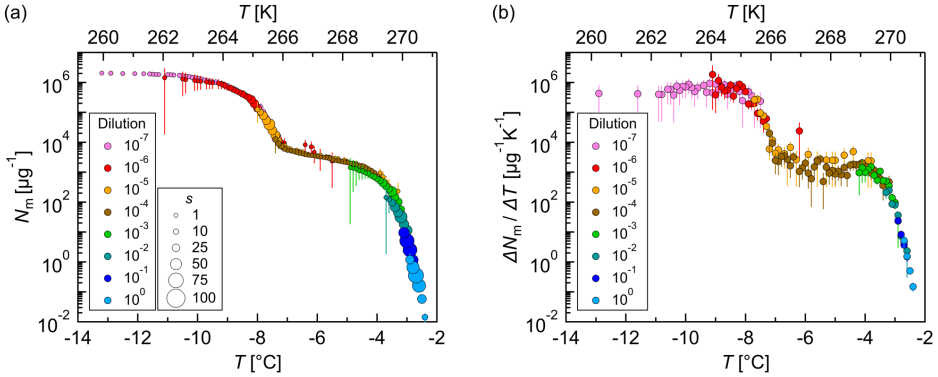

Figure 7. Measurements of dilution series of bacterial IN (Snomax®). **(a)** Cumulative number of IN (N_m) and **(b)** differential number of IN ($\Delta N_m/\Delta T$) per unit mass of Snomax® vs. temperature (T). Droplets of the same dilution of three independent measurements were added to a total droplet number of 288 (3 × 96 droplets). Symbol colors indicate different dilutions; symbol size indicates the number of frozen droplets per 0.1 K bin (s). The error bars were calculated using the counting error and the Gaussian error propagation. The temperature error is 0.2 K.

substantially above the expected temperatures for homogeneous nucleation of microliter droplets, but they are in accord with the results of Whale et al. (2015).

3.2 Biological reference materials

The performance of TINA was further assessed using Snomax® as a bacterial IN-active reference substance (e.g., Budke and Koop, 2015; Hartmann et al., 2013; Möhler et al., 2008; Turner et al., 1990; Ward and DeMott, 1989) and IN filtrate of the well-studied IN fungus *Mortierella alpina* (Fröhlich-Nowoisky et al., 2015; Pummer et al., 2015).

Snomax® was obtained from SMI Snow Makers AG (Thun, Switzerland), and a stock solution was prepared in pure water with an initial mass concentration of 1 mg mL^{-1}. This suspension was then serially diluted 10-fold with pure water by the liquid handling station. The resulting Snomax® concentrations varied between 1 mg mL^{-1} and 0.1 ng mL^{-1}, equivalent to a total mass of Snomax® between 3 µg and 0.3 pg, respectively, per 3 µL droplet.

Each dilution was pipetted column-wise distributed over the twin plates as described before in 96 droplets into 384-well plates by the liquid handling station. Two plates at a time were placed inside the 384-well sample holder block, and the plates were cooled down quickly to 273 K (0 °C) and, as

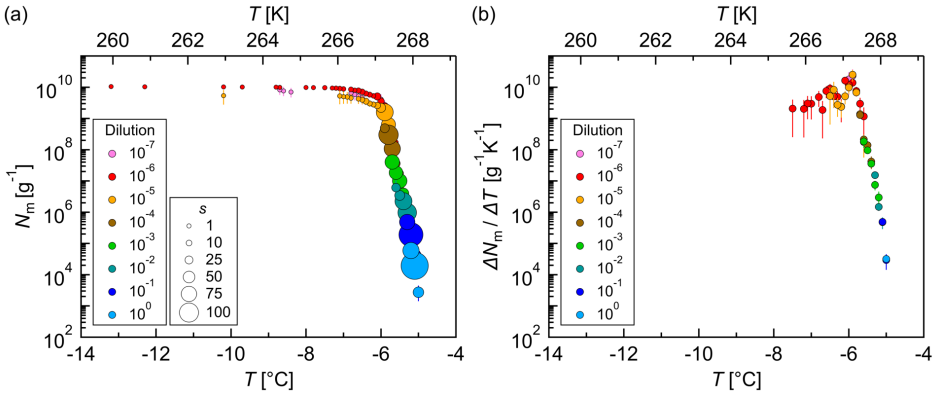

Figure 8. Measurements of dilution series of fungal IN (*Mortierella alpina* 13A). **(a)** Cumulative number of IN (N_m) and **(b)** differential number of IN ($\Delta N_m/\Delta T$) per unit mass of mycelium vs. temperature (T). Droplets of the same dilution of three independent measurements were added to a total droplet number of 288 (3×96 droplets). Symbol colors indicate different dilutions; symbol size indicates the number of frozen droplets per 0.1 K bin (s). The error bars were calculated using the counting error and the Gaussian error propagation. The temperature error is 0.2 K.

soon as the temperature was stable for 1 min, in a continuous cooling rate of 1 K min^{-1} further down to 253 K (−20 °C).

Three independent experiments with Snomax® showed reproducible results (Fig. S4), and, therefore, droplets of the same dilution were added to a total droplet number of 288. The obtained results were plotted in a cumulative and a differential IN spectrum (Fig. 7). The cumulative IN number concentration represents the total number of IN active above a certain temperature. The cumulative IN spectrum showed two strong increases: around 270 K (−3 °C) and around 265 K (−8 °C). These findings are in good agreement with the results of Budke and Koop (2015). The differential IN number concentration was calculated according to Vali (1971a), and it represents the number of IN active in a particular temperature interval. The differential IN spectrum showed a similar shape as the cumulative IN spectrum with a distinct plateau between 268 and 266 K (−5 and −7 °C) and two slight maxima, around 269 K (−4 °C) and around 264 K (−9 °C). This indicates the presence of highly efficient IN, active at a temperature of approximately 269 K (−4 °C), and less-efficient IN, active around 264 K (−9 °C). The fact that the less-efficient IN appeared in higher dilutions implies that they occur in higher concentrations than the highly efficient IN. The presence of further IN with lower freezing temperatures and low concentrations cannot be excluded.

The analysis of different IN active within a wide temperature range was only possible with the measurement of a dilution series. TINA enables the simultaneous measurement of such a dilution series with high statistics in a short period of time.

Mortierella alpina 13A was grown on full-strength PDA (VWR International GmbH, Darmstadt, Germany) at 277 K (4 °C) for 7 months. Fungal IN filtrate was prepared as described previously (Fröhlich-Nowoisky et al., 2015; Pummer et al., 2015) and contained IN from spores and mycelial surfaces. It was serially diluted 10-fold with pure water by the liquid handling station. The experiment was performed as described above.

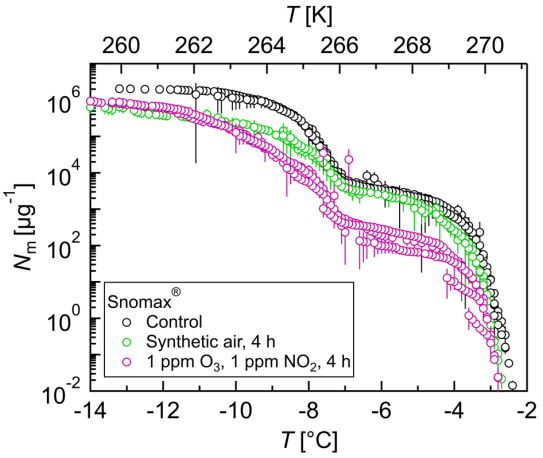

Figure 9. Freezing experiments with ozonized and nitrated bacterial IN. Cumulative number of IN (N_m) per unit mass of Snomax® vs. temperature (T). Droplets of the same dilution of three independent measurements were added to a total droplet number of 288 (3×96 droplets). Symbol colors indicate different exposure conditions. The error bars were calculated using the counting error and the Gaussian error propagation. The temperature error is 0.2 K.

For test measurements with fungal IN, IN filtrate of three different culture plates from *Mortierella alpina* 13A was

measured, and the results were reproducible (Fig. S5). The cumulative number of IN per gram mycelium only varied by 1 order of magnitude, which is a good achievement for a biological sample, and droplets of the same dilutions were added to a total droplet number of 288. A cumulative IN spectrum (Fig. 8a) and a differential IN spectrum (Fig. 8b) were plotted. The cumulative number of IN and the initial freezing temperature of 268 K (−5 °C) are in good agreement with the literature (Fröhlich-Nowoisky et al., 2015; Pummer et al., 2015). The cumulative and the differential IN spectra showed similar shapes with one maximum around 267 K (−6 °C), indicating the presence of one type of IN, which is highly efficient.

3.3 Ozonized and nitrated samples

To study the effect of O_3 and NO_2 exposure on the IN activity of Snomax®, an aliquot of 1 mL of a 1 mg mL^{-1} suspension of Snomax® in pure water was exposed in liquid phase to gases with or without O_3 and NO_2 as described in Liu et al. (2017).

Briefly, O_3 was produced by exposing synthetic air to UV light (L.O.T.-Oriel GmbH & Co. KG, Darmstadt, Germany), and the O_3 concentration was adjusted by tuning the amount of UV light. The gas flow was ∼ 1.9 L min^{-1}, and it was mixed with N_2 containing ∼ 5 ppmV NO_2 (AIR LIQUIDE Deutschland GmbH, Düsseldorf, Germany). The NO_2 concentration was regulated by the addition of the amount of the ∼ 5 ppmV NO_2 gas. The O_3 and NO_2 concentrations were monitored with commercial monitoring instruments (ozone analyzer: 49i, Thermo Scientific, Braunschweig, Germany; NO_x analyzer: 42i-TL, Thermo Scientific). The gas mixture was directly bubbled through 1 mL of the Snomax® solution at a flow rate of 60 mL min^{-1} using a Teflon tube (ID: 1.59 mm). The Snomax® solution was exposed to a mixture of 1 ppm O_3 and 1 ppm NO_2 for 4 h, representing the exposure to an atmospherically relevant amount of about 200 ppb each for about 20 h. The exposure experiments were performed in triplicate. After exposure, the treated samples were serially diluted and the IN activity was measured as described for the Snomax® reference measurements.

The results showed that gas exposure affected the IN activity of Snomax® (Fig. 9). High concentrations of O_3 and NO_2 reduced the cumulative number of IN from Snomax® between 1 and 2 orders of magnitude, while exposure to synthetic air showed smaller effects.

Snomax® contains IN proteins of the bacterium *Pseudomonas syringae*. Attard et al. (2012) found no significant or only weak effects of exposure to ∼ 100 ppb O_3 and ∼ 100 ppb NO_2 on the IN activity of two strains of *P. syringae*, and a variable response of a third strain, suggesting a strain-specific response.

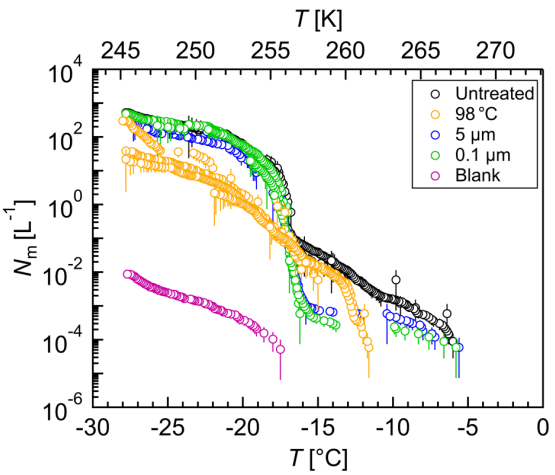

Figure 10. Freezing experiments with aqueous extracts of atmospheric aerosols. Cumulative number of IN per liter air (N_m) vs. temperature (T) for untreated (black), heated (yellow), 5 µm filtered (blue), 0.1 µm filtered (green), and blank (magenta) filter extracts. Droplets of the same dilution of two aliquots were added to a total droplet number of 192 (2 × 96 droplets). The error bars were calculated using the counting error and the Gaussian error propagation. The temperature error is 0.2 K.

3.4 Air filter samples

Total suspended particle samples were collected onto 150 mm glass fiber filters (Type MN 85/90, Macherey-Nagel GmbH, Düren, Germany) using a high-volume sampler (DHA-80, Digitel Elektronik AG, Hegnau, Switzerland) operated at 1000 L min^{-1}, which was placed on the roof of the Max Planck Institute for Chemistry (Mainz, Germany). There, a mix of urban and rural continental boundary layer air can be sampled in central Europe. The filter was taken in April 2018, and the sampling period was seven days, corresponding to a total air volume of approximately 10 000 m^3. Filters were pre-baked at 603 K (330 °C) for 10 h to remove any biological material, and blank samples were taken to detect possible contaminations. All filters were packed in pre-baked aluminum bags, and loaded filters were stored at 193 K (−80 °C) until analysis.

An aerosol and a blank filter were cut with a sterilized scissor into aliquots (∼ 1/16), and the exact percentage was determined gravimetrically. For reproducibility, two filter sample aliquots of each filter were extracted. Each filter sample aliquot was transferred into a sterile 50 mL tube (Greiner Bio-One, Kremsmünster, Austria), and 10 mL of pure water was added. The tubes were shaken horizontally at 200 rpm for 15 min. Afterwards, the filter was removed, and the aqueous extract was tested for IN activity. To further characterize the IN, the effects of filtration and heat treatment were investigated. Therefore, aliquots of the extract were treated as

follows: (i) 1 h at 371 K (98 °C), (ii) filtration through a 5 µm pore diameter filter (Acrodisc®, PES, Pall GmbH, Dreieich, Germany), (iii) filtration through a 5 and a 0.1 µm pore diameter filter (Acrodisc®).

Each solution (96 aliquots of 3 µL) was pipetted column-wise into 384-well plates by the liquid handling station. The plates were cooled down quickly to 273 K (0 °C) and, as soon as the temperature was stable for 1 min, at a continuous cooling rate of 1 K min^{-1} further down to 243 K (−30 °C).

Each solution of the two aliquots of each filter was measured separately, and droplets of the same solution were added to a total droplet number of 192 (2 × 96 droplets) (Figs. 10 and S6). All IN concentrations were calculated per liter air.

The untreated filter extract showed IN activity at relatively high temperatures with an initial freezing temperature of 267 K (−6 °C). The concentration of IN active at temperatures above 263 K (−10 °C) was about 0.001 L^{-1}, but heat treatment led to a loss of IN activity above 263 K (−10 °C). Because the activity of known biological IN results from proteins or proteinaceous compounds (Green and Warren, 1985; Kieft and Ruscetti, 1990; Pouleur et al., 1992; Tsumuki and Konno, 1994) and proteins are known to be heat-sensitive, the results suggest the presence of biological IN. The concentration of IN between 263 K (−10 °C) and 257 K (−16 °C) increased about 2 orders of magnitude and in a sudden increase another 2 orders between 257 K (−16 °C) and 256 K (−17 °C). The IN concentration below 256 K (−17 °C) increased continuously up to about 500 L^{-1}, but heat treatment reduced the IN concentration of up to 1 order of magnitude below 256 K (−17 °C). Filtration experiments did not affect the initial freezing temperature, but the concentration of biological IN decreased significantly. The results suggest the presence of many biological IN or agglomerates larger than 5 µm and of a few biological IN smaller than 0.1 µm. The cumulative number of IN active between 263 K (−10 °C) and 257 K (−16 °C) decreased up to 2 orders of magnitude upon filtration, but the IN concentration below 256 K (−17 °C) was not affected. The findings show that many IN active between 263 K (−10 °C) and 257 K (−16 °C) were larger than 5 µm, whereas IN active below 256 K (−17 °C) were smaller than 0.1 µm.

4 Conclusions

The new high-throughput droplet freezing assay TINA was introduced to study heterogeneous ice nucleation of microliter-range droplets in the immersion mode. TINA provides the analysis of 960 droplets simultaneously or 192 and 768 droplets in two independent experiments at the same time, enabling the analysis of many samples with high statistics in a short period of time. Moreover, an infrared camera-based detection system allows the reliable determination of droplet freezing. The setup was tested with Snomax® as bacterial IN and IN filtrate of *Mortierella alpina* as fungal IN. For these reference materials, both the initial freezing temperature and the cumulative number of IN per gram unit mass were in good agreement with the literature, which demonstrates the functionality of the new setup.

TINA was applied to study the effect of O_3 and NO_2 exposure on the IN activity of Snomax®, where high concentrations of O_3 and NO_2 reduced the IN activity significantly. Atmospheric aerosol samples from Mainz (Germany) were analyzed for IN activity to show the applicability of TINA for field samples. Here, the results suggest that most of the biological IN were larger than 5 µm. Moreover, many IN active between 263 K (−10 °C) and 257 K (−16 °C) were larger than 5 µm, whereas IN active below 256 K (−17 °C) were smaller than 0.1 µm. The results confirm that TINA is suitable for high-throughput experiments and efficient analysis of biological IN in laboratory and field samples.

Data availability. All data are available from the corresponding authors upon request.

Supplement. The supplement related to this article is available online at: https://doi.org/10.5194/amt-11-6327-2018-supplement.

Author contributions. ATK, ML, and FH developed the instrument. ATK, UP, JFN conceived and designed the experiments. ATK performed the experiments. MLP wrote the code to process the data and did the error calculation. All authors discussed the data and contributed to the writing of the manuscript.

Competing interests. The authors declare that they have no conflict of interest.

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References

Attard, E., Yang, H., Delort, A.-M., Amato, P., Pöschl, U., Glaux, C., Koop, T., and Morris, C. E.: Effects of atmospheric conditions on ice nucleation activity of Pseudomonas, Atmos. Chem. Phys., 12, 10667–10677, https://doi.org/10.5194/acp-12-10667-2012, 2012.

Ball, M. C., Wolfe, J., Canny, M., Hofmann, M., Nicotra, A. B., and Hughes, D.: Space and time dependence of temperature and freezing in evergreen leaves, Funct. Plant. Biol., 29, 1259–1272, 2002.

Bauerecker, S., Ulbig, P., Buch, V., Vrbka, L., and Jungwirth, P.: Monitoring ice nucleation in pure and salty water via high-speed imaging and computer simulations, J. Phys. Chem. C, 112, 7631–7636, https://doi.org/10.1021/jp711507f, 2008.

Boucher, O., Randall, D., Artaxo, P., Bretherton, C., Feingold, G., Forster, P., Kerminen, V.-M., Kondo, Y., Liao, H., Lohmann, U., Rasch, P., Satheesh, S. K., Sherwood, S., Stevens, B., and Zhang, X. Y.: Clouds and Aerosols, in: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, edited by: Stocker, T. F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S. K., Boschung, J., Nauels, A., Xia, Y., And, V. B., and Midgley, P. M., 571–657, Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 2013.

Budke, C. and Koop, T.: BINARY: an optical freezing array for assessing temperature and time dependence of heterogeneous ice nucleation, Atmos. Meas. Tech., 8, 689–703, https://doi.org/10.5194/amt-8-689-2015, 2015.

Carter, J., Brennan, R., and Wisniewski, M.: Low-temperature tolerance of blackcurrant flowers, HortScience, 34, 855–859, 1999.

Ceccardi, T. L., Heath, R. L., and Ting, I. P.: Low-temperature exotherm measurement using infrared thermography, HortScience, 30, 140–142, 1995.

Charrier, G., Nolf, M., Leitinger, G., Charra-Vaskou, K., Losso, A., Tappeiner, U., Améglio, T., and Mayr, S.: Monitoring of Freezing Dynamics in Trees: A Simple Phase Shift Causes Complexity, Plant Physiol., 173, 2196–2207, https://doi.org/10.1104/pp.16.01815, 2017.

Conen, F., Morris, C. E., Leifeld, J., Yakutin, M. V., and Alewell, C.: Biological residues define the ice nucleation properties of soil dust, Atmos. Chem. Phys., 11, 9643–9648, https://doi.org/10.5194/acp-11-9643-2011, 2011.

Emteborg, H., Zeleny, R., Charoud-Got, J., Martos, G., Lüddeke, J., Schellin, H., and Teipel, K.: Infrared thermography for monitoring of freeze-drying processes: Instrumental developments and preliminary results, J. Pharm. Sci., 103, 2088–2097, https://doi.org/10.1002/jps.24017, 2014.

Fornea, A. P., Brooks, S. D., Dooley, J. B., and Saha, A.: Heterogeneous freezing of ice on atmospheric aerosols containing ash, soot, and soil, J. Geophys. Res.-Atmos., 114, 1–12, https://doi.org/10.1029/2009JD011958, 2009.

Fröhlich-Nowoisky, J., Hill, T. C. J., Pummer, B. G., Yordanova, P., Franc, G. D., and Pöschl, U.: Ice nucleation activity in the widespread soil fungus Mortierella alpina, Biogeosciences, 12, 1057–1071, https://doi.org/10.5194/bg-12-1057-2015, 2015.

Fuller, M. and Wisniewski, M.: The use of infrared thermal imaging in the study of ice nucleation and freezing of plants, J. Therm. Biol., 23, 81–89, https://doi.org/10.1016/S0306-4565(98)00013-8, 1998.

Gallego, B., Verdú, J. R., Carrascal, L. M., and Lobo, J. M.: A protocol for analysing thermal stress in insects using infrared thermography, J. Therm. Biol., 56, 113–121, https://doi.org/10.1016/j.jtherbio.2015.12.006, 2016.

Gómez Muñoz, C. Q., García Márquez, F. P., and Sánchez Tomás, J. M.: Ice detection using thermal infrared radiometry on wind turbine blades, Measurement, 93, 157–163, https://doi.org/10.1016/j.measurement.2016.06.064, 2016.

Green, R. L. and Warren, G. J.: Physical and functional repetition in a bacterial ice nucleation gene, Nature, 317, 645–648, https://doi.org/10.1038/317645a0, 1985.

Hacker, J. and Neuner, G.: Ice propagation in plants visualized at the tissue level by infrared differential thermal analysis (IDTA), Tree Physiol., 27, 1661–70, https://doi.org/10.1093/treephys/27.12.1661, 2007.

Hansman, R. J. and Dershowitz, A. L.: Method of and apparatus for detection of ice accretion, United States Patent, Massachusetts Institute of Technology, Patent Number: 5 313 202, 1994.

Harrison, A. D., Whale, T. F., Rutledge, R., Lamb, S., Tarn, M. D., Porter, G. C. E., Adams, M. P., McQuaid, J. B., Morris, G. J., and Murray, B. J.: An instrument for quantifying heterogeneous ice nucleation in multiwell plates using infrared emissions to detect freezing, Atmos. Meas. Tech., 11, 5629–5641, https://doi.org/10.5194/amt-11-5629-2018, 2018.

Hartmann, S., Augustin, S., Clauss, T., Wex, H., Šantl-Temkiv, T., Voigtländer, J., Niedermeier, D., and Stratmann, F.: Immersion freezing of ice nucleation active protein complexes, Atmos. Chem. Phys., 13, 5751–5766, https://doi.org/10.5194/acp-13-5751-2013, 2013.

Häusler, T., Witek, L., Felgitsch, L., Hitzenberger, R., and Grothe, H.: Freezing on a Chip – A New Approach to Determine Heterogeneous Ice Nucleation of Micrometer-Sized Water Droplets, Atmosphere-Basel, 9, 140, https://doi.org/10.3390/atmos9040140, 2018.

Hill, T. C. J., DeMott, P. J., Tobo, Y., Fröhlich-Nowoisky, J., Moffett, B. F., Franc, G. D., and Kreidenweis, S. M.: Sources of organic ice nucleating particles in soils, Atmos. Chem. Phys., 16, 7195–7211, https://doi.org/10.5194/acp-16-7195-2016, 2016.

Kieft, T. L. and Ruscetti, T.: Characterization of Biological Ice Nuclei from a Lichen, J. Bacteriol., 172, 3519–3523, 1990.

Kunert, A. T., Scheel, J. F., Helleis, F., Klimach, T., Pöschl, U., and Fröhlich-Nowoisky, J.: New High-Performance Droplet Freezing Assay (HP-DFA) for the Analysis of Ice Nuclei with Complex Composition, in EGU General Assembly Conference Abstracts, vol. 18, EPSC2016-6293, 2016a.

Kunert, A. T., Scheel, J. F., Helleis, F., Klimach, T., Pöschl, U., and Fröhlich-Nowoisky, J.: TINA: A New High-Performance Droplet Freezing Assay for the Analysis of Ice Nuclei with Complex Composition, in: 4th Workshop – Microphysics of Ice Clouds, 2016b.

Kunert, A. T., Lamneck, M., Gurk, C., Helleis, F., Klimach, T., Scheel, J. F., Pöschl, U., and Fröhlich-Nowoisky, J.: TINA, a new fully automated high-performance droplet freezing assay cou-

pled to a customized infrared detection system, in: EGU General Assembly Conference Abstracts, vol. 19, p. 13571, 2017a.

Kunert, A. T., Lamneck, M., Gurk, C., Helleis, F., Klimach, T., Scheel, J. F., Pöschl, U., and Fröhlich-Nowoisky, J.: TINA, a new fully automated high-performance droplet freezing assay coupled to a customized infrared detection system, in: 5th Workshop – Microphysics of Ice Clouds, 2017b.

Kunert, A. T., Lamneck, M., Helleis, F., Scheel, J. F., Pöschl, U., and Fröhlich-Nowoisky, J.: TINA: Twin-plate ice nucleation assay with infrared detection for high-throughput droplet freezing experiments, in: INUIT Final Conference and 2nd Atmospheric Ice Nucleation Conference, 2018.

Liu, F., Lakey, P., Berkemeier, T., Tong, H., Kunert, A. T., Meusel, H., Su, H., Cheng, Y., Fröhlich-Nowoisky, J., Lai, S., Weller, M. G., Shiraiwa, M., Pöschl, U., and Kampf, C. J.: Atmospheric protein chemistry influenced by anthropogenic air pollutants: nitration and oligomerization upon exposure to ozone and nitrogen dioxide, Faraday Discuss., 200, 413–427, https://doi.org/10.1039/C7FD00005G, 2017.

Möhler, O., Georgakopoulos, D. G., Morris, C. E., Benz, S., Ebert, V., Hunsmann, S., Saathoff, H., Schnaiter, M., and Wagner, R.: Heterogeneous ice nucleation activity of bacteria: new laboratory experiments at simulated cloud conditions, Biogeosciences, 5, 1425–1435, https://doi.org/10.5194/bg-5-1425-2008, 2008.

Murray, B. J., Broadley, S. L., Wilson, T. W., Bull, S. J., Wills, R. H., Christenson, H. K., and Murray, E. J.: Kinetics of the homogeneous freezing of water, Phys. Chem. Chem. Phys., 12, 10380–10387, https://doi.org/10.1039/c003297b, 2010.

O'Sullivan, D., Murray, B. J., Malkin, T. L., Whale, T. F., Umo, N. S., Atkinson, J. D., Price, H. C., Baustian, K. J., Browse, J., and Webb, M. E.: Ice nucleation by fertile soil dusts: relative importance of mineral and biogenic components, Atmos. Chem. Phys., 14, 1853–1867, https://doi.org/10.5194/acp-14-1853-2014, 2014.

Pearce, R. S. and Fuller, M. P.: Freezing of Barley Studied by Infrared Video Thermography, Plant Physiol., 125, 227–240, 2001.

Pouleur, S., Richard, C., Martin, J. G., and Antoun, H.: Ice Nucleation Activity in Fusarium acuminatum and Fusarium avenaceum, Appl. Environ. Microbiol., 58, 2960–2964, 1992.

Pruppacher, H. R. and Klett, J. D.: Microphysics of Clouds and Precipitation, 2nd edn., Springer Netherlands, Dordrecht, 1997.

Pummer, B. G., Budke, C., Augustin-Bauditz, S., Niedermeier, D., Felgitsch, L., Kampf, C. J., Huber, R. G., Liedl, K. R., Loerting, T., Moschen, T., Schauperl, M., Tollinger, M., Morris, C. E., Wex, H., Grothe, H., Pöschl, U., Koop, T., and Fröhlich-Nowoisky, J.: Ice nucleation by water-soluble macromolecules, Atmos. Chem. Phys., 15, 4077–4091, https://doi.org/10.5194/acp-15-4077-2015, 2015.

Sekozawa, Y., Sugaya, S., and Gemma, H.: Observations of Ice Nucleation and Propagation in Flowers of Japanese Pear (Pyrus pyrifolia Nakai) using Infrared Video Thermography, J. Japan. Soc. Hort. Sci., 73, 1–6, https://doi.org/10.1248/cpb.37.3229, 2004.

Stier, J. C., Filiault, D. L., Wisniewski, M., and Palta, J. P.: Visualization of freezing progression in turfgrasses using infrared video thermography, Crop. Sci., 43, 415–420, 2003.

Stopelli, E., Conen, F., Zimmermann, L., Alewell, C., and Morris, C. E.: Freezing nucleation apparatus puts new slant on study of biological ice nucleators in precipitation, Atmos. Meas. Tech., 7, 129–134, https://doi.org/10.5194/amt-7-129-2014, 2014.

Tobo, Y.: An improved approach for measuring immersion freezing in large droplets over a wide temperature range, Sci. Rep., 6, 32930, https://doi.org/10.1038/srep32930, 2016.

Tsumuki, H. and Konno, H.: Ice Nuclei Produced by Fusarium sp. Isolated from the Gut of the Rice Stem Borer, Chilo suppressalis Walker (Lepidoptera: Pyralidae), Biosci. Biotechnol. Biochem., 58, 578–579, 1994.

Turner, M. A., Arellano, F., and Kozloff, L. M.: Three separate classes of bacterial ice nucleation structures, J. Bacteriol., 172, 2521–2526, 1990.

Vali, G.: Quantitative Evaluation of Experimental Results an the Heterogeneous Freezing Nucleation of Supercooled Liquids, J. Atmos. Sci., 28, 402–409, https://doi.org/10.1175/1520-0469(1971)028<0402:QEOERA>2.0.CO;2, 1971a.

Vali, G.: Supercooling of Water and Nucleation of Ice (Drop Freezer), Am. J. Phys., 39, 1125, https://doi.org/10.1119/1.1976585, 1971b.

Ward, P. J. and DeMott, P. J.: Preliminary experimental evaluation of Snomax snow inducer, Pseudomonas syringae, as an artificial ice nucleus for weather modification, J. Weather Modif., 21, 9–13, 1989.

Whale, T. F., Murray, B. J., O'Sullivan, D., Wilson, T. W., Umo, N. S., Baustian, K. J., Atkinson, J. D., Workneh, D. A., and Morris, G. J.: A technique for quantifying heterogeneous ice nucleation in microlitre supercooled water droplets, Atmos. Meas. Tech., 8, 2437–2447, https://doi.org/10.5194/amt-8-2437-2015, 2015.

Wisniewski, M., Lindow, S. E., and Ashworth, E. N.: Observations of Ice Nucleation and Propagation in Plants Using Infrared Video Thermography, Plant Physiol., 113, 327–334, 1997.

Wisniewski, M., Glenn, D. M., Gusta, L., and Fuller, M. P.: Using Infrared Thermography to Study Freezing in Plants, HortScience, 43, 1648–1651, 2008.

Workmaster, B.: Ice nucleation and propagation in cranberry uprights and fruit using infrared video thermography, J. Amer. Soc. Hort. Sci., 124, 619–625, 1999.

Wright, T. P. and Petters, M. D.: The role of time in heterogeneous freezing nucleation, J. Geophys. Res.-Atmos., 118, 3731–3743, https://doi.org/10.1002/jgrd.50365, 2013.

Zaragotas, D., Liolios, N. T., and Anastassopoulos, E.: Supercooling, ice nucleation and crystal growth: A systematic study in plant samples, Cryobiology, 72, 239–243, https://doi.org/10.1016/j.cryobiol.2016.03.012, 2016.

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Macromolecular fungal ice nuclei in *Fusarium*: effects of physical and chemical processing

Anna T. Kunert[1], Mira L. Pöhlker[1], Kai Tang[1], Carola S. Krevert[1], Carsten Wieder[1], Kai R. Speth[1], Linda E. Hanson[2], Cindy E. Morris[3], David G. Schmale III[4], Ulrich Pöschl[1], Janine Fröhlich-Nowoisky[1]

[1]Multiphase Chemistry Department, Max Planck Institute for Chemistry, 55128 Mainz, Germany; [2]USDA-ARS and Department of Plant, Soil and Microbial Science, Michigan State University, East Lansing, MI 48824, USA; [3]Plant Pathology Research Unit, INRA, 84143 Montfavet, France; [4]School of Plant and Environmental Sciences, Virginia Tech, Blacksburg, VA 24061, USA

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Author contributions

CEM, JFN, and UP designed the experiments. DGS III and LEH provided fungal cultures. CEM, DGS III, and JFN performed the initial screenings. ATK, KT, CSK, CW, and KRS performed the experiments. ATK, JFN, MLP, and UP discussed the results. ATK and JFN wrote the manuscript with contributions of all coauthors.

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Anna T. Kunert[1], Mira L. Pöhlker[1], Kai Tang[1], Carola S. Krevert[1], Carsten Wieder[1], Kai R. Speth[1], Linda E. Hanson[2], Cindy E. Morris[3], David G. Schmale III[4], Ulrich Pöschl[1], and Janine Fröhlich-Nowoisky[1]

[1]Multiphase Chemistry Department, Max Planck Institute for Chemistry, 55128 Mainz, Germany
[2]USDA-ARS and Department of Plant, Soil and Microbial Science, Michigan State University, East Lansing, MI 48824, USA
[3]Plant Pathology Research Unit, INRA, 84143 Montfavet, France
[4]School of Plant and Environmental Sciences, Virginia Tech, Blacksburg, VA 24061, USA

Correspondence: Janine Fröhlich-Nowoisky (j.frohlich@mpic.de)

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Abstract. Some biological particles and macromolecules are particularly efficient ice nuclei (IN), triggering ice formation at temperatures close to 0 °C. The impact of biological particles on cloud glaciation and the formation of precipitation is still poorly understood and constitutes a large gap in the scientific understanding of the interactions and coevolution of life and climate. Ice nucleation activity in fungi was first discovered in the cosmopolitan genus *Fusarium*, which is widespread in soil and plants, has been found in atmospheric aerosol and cloud water samples, and can be regarded as the best studied ice-nucleation-active (IN-active) fungus. The frequency and distribution of ice nucleation activity within *Fusarium*, however, remains elusive. Here, we tested more than 100 strains from 65 different *Fusarium* species for ice nucleation activity. In total, ∼ 11 % of all tested species included IN-active strains, and ∼ 16 % of all tested strains showed ice nucleation activity above −12 °C. Besides *Fusarium* species with known ice nucleation activity, *F. armeniacum*, *F. begoniae*, *F. concentricum*, and *F. langsethiae* were newly identified as IN-active. The cumulative number of IN per gram of mycelium for all tested *Fusarium* species was comparable to other biological IN like *Sarocladium implicatum*, *Mortierella alpina*, and Snomax®. Filtration experiments indicate that cell-free ice-nucleating macromolecules (INMs) from *Fusarium* are smaller than 100 kDa and that molecular aggregates can be formed in solution. Long-term storage and freeze–thaw cycle experiments revealed that the fungal IN in aqueous solution remain active over several months and in the course of repeated freezing and thawing. Exposure to ozone and nitrogen dioxide at atmospherically relevant concentration levels also did not affect the ice nucleation activity. Heat treatments at 40 to 98 °C, however, strongly reduced the observed IN concentrations, confirming earlier hypotheses that the INM in *Fusarium* largely consists of a proteinaceous compound. The frequency and the wide distribution of ice nucleation activity within the genus *Fusarium*, combined with the stability of the IN under atmospherically relevant conditions, suggest a larger implication of fungal IN on Earth's water cycle and climate than previously assumed.

1 Introduction

Ice particles in the atmosphere are formed either by homogeneous nucleation at temperatures below −38 °C or by heterogeneous nucleation catalyzed by particles or macromolecules serving as ice nuclei (IN) at warmer temperatures (Pruppacher and Klett, 1997; reviewed in detail in Fröhlich-Nowoisky et al., 2016 and Knopf et al., 2018). Biological particles in particular are expected to play an important role as IN in the temperature range from −15 to 0 °C, but the impact of biological particles on cloud glaciation and the formation of precipitation is still poorly understood (Coluzza et al., 2017). Several studies suggest a triggering effect of biological IN for cloud glaciation and formation of precipitation (Creamean et al., 2013; DeMott and Prenni, 2010; Failor et al., 2017; Hanlon et al., 2017; Joly et al., 2014;

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Petters and Wright, 2015; Pratt et al., 2009; Stopelli et al., 2015, 2017), and former studies have shown that biological particles are more efficient than mineral IN (DeMott and Prenni, 2010; Després et al., 2012; Hill et al., 2014; Hoose and Möhler, 2012; Huffman et al., 2013; Möhler et al., 2007; Morris et al., 2014; Murray et al., 2012; Pratt et al., 2009).

The best characterized biological IN are common plant-associated bacteria of the genera *Pseudomonas*, *Pantoea*, and *Xanthomonas* (Garnham et al., 2011; Govindarajan and Lindow, 1988; Graether and Jia, 2001; Green and Warren, 1985; Hill et al., 2014; Kim et al., 1987; Ling et al., 2018; Šantl-Temkiv et al., 2015; Schmid et al., 1997; Wolber et al., 1986), and, recently, an ice-nucleation-active (IN-active) *Lysinibacillus* was found (Failor et al., 2017). The first identified IN-active fungi were strains of the genus *Fusarium* (Hasegawa et al., 1994; Pouleur et al., 1992; Richard et al., 1996; Tsumuki et al., 1992). To date, a few more fungal genera with varying initial freezing temperatures such as *Isaria farinosa* ($\sim -4\,^{\circ}C$), *Mortierella alpina* ($\sim -5\,^{\circ}C$), *Puccinia* species (-4 to $-8\,^{\circ}C$), and *Sarocladium* (formerly named *Acremonium*) *implicatum* ($\sim -9\,^{\circ}C$) have been identified as IN-active (Fröhlich-Nowoisky et al., 2015; Huffman et al., 2013; Morris et al., 2013; Richard et al., 1996).

The genus *Fusarium* is cosmopolitan and includes saprophytes and pathogens of plants and animals (Leslie and Summerell, 2006; Nelson et al., 1994). Although they are considered to be primarily soilborne fungi, many species of *Fusarium* are airborne (Prussin et al., 2014; Schmale et al., 2012; Schmale and Ross, 2015), and they were found in atmospheric and cloud water samples (e.g., Amato et al., 2007; Fröhlich-Nowoisky et al., 2009; Fulton, 1966). Some species can cause wilts, blights, root rots, and cankers in agriculturally important crops worldwide (e.g., Schmale and Gordon, 2003; Wang and Jeffers, 2000). Other species can produce secondary metabolites known as mycotoxins that can cause a variety of acute and chronic health effects in humans and animals (e.g., Bush et al., 2004; Ichinoe et al., 1983).

While the factors for a positive selective pressure for ice nucleation activity in *Fusarium* and other fungi have not been directly identified, an ecological advantage of initiating ice formation is easily conceivable. Indeed, most IN-active bacteria and fungi are isolated from regions with seasonal temperatures below $0\,^{\circ}C$ (Diehl et al., 2002; Schnell and Vali, 1972). Ice nucleation activity at temperatures close to $0\,^{\circ}C$ could be beneficial for pathogens or might provide an ecological advantage for saprophytic *Fusarium* species by facilitating in the acquisition of nutrients liberated during cell rupture of the host (Lindow et al., 1982). Furthermore, IN on the surface of the mycelium could avoid physical damage of the fungus by protective extracellular freezing (Fröhlich-Nowoisky et al., 2015; Zachariassen and Kristiansen, 2000) or by binding moisture as ice in cold and dry seasons (Pouleur et al., 1992). With increasing temperatures, the retained water can be of advantage in early vegetative periods and for bacterial movement on the mycelial water film known as the fungal highway (Kohlmeier et al., 2005; Warmink et al., 2011). Moreover, ice nucleation activity might be beneficial for airborne *Fusarium* and for their return to Earth's surface under advantageous conditions in a feedback cycle known as bioprecipitation (Després et al., 2012; Morris et al., 2013, 2014; Sands et al., 1982). In addition, once the IN are released into the environment, they can adsorb to clay and might also be available in the atmosphere associated with soil dust particles (Conen et al., 2011; Fröhlich-Nowoisky et al., 2015, 2016; Hill et al., 2016; O'Sullivan et al., 2014, 2015, 2016; Sing and Sing, 2010).

The sources, abundance, and identity of biological IN are not well characterized (Coluzza et al., 2017), and it has been proposed that systematic surveys will likely increase the number of IN-active fungal species discovered (Fröhlich-Nowoisky et al., 2015). *Fusarium* is the best-known IN-active fungus, but the frequency and distribution of ice nucleation activity within *Fusarium* is not well known. In this study, more than 100 strains from 65 different *Fusarium* species were tested for ice nucleation activity in three laboratories with different freezing methods. A high-throughput droplet freezing assay was used to quantify the IN of selected *Fusarium* species, and filtration experiments were performed to estimate the size of the *Fusarium* IN. Furthermore, the stability of *Fusarium* IN upon exposure to ozone and nitrogen dioxide, under high and low or quickly changing temperatures, and after short- and long-term storage under various conditions was investigated.

2 Materials and methods

2.1 Origin and growth conditions of fungal cultures

Thirty *Fusarium* strains from USDA-ARS, Michigan State University (Linda E. Hanson, East Lansing, MI, USA), 13 strains from the Schmale Laboratory at Virginia Tech (David G. Schmale, Blacksburg, VA, USA), and 69 strains from the Kansas State University Teaching Collection (John F. Leslie, Manhattan, KS, USA) were screened for ice nucleation activity (Table S1 in the Supplement).

The strains from the USDA-ARS, Michigan State University, were collected from crop tissue (sugar beet). All isolates were from field-grown beets and were obtained by hyphal tip transfer. The strains from the Schmale Laboratory at Virginia Tech were collected with unmanned aircraft systems (UASs or drones) equipped with remotely operated sampling devices containing a *Fusarium* selective medium (e.g., Lin et al., 2013, 2014). All of the Schmale Laboratory strains were collected 100 m above ground level at the Kentland Farm in Blacksburg, Virginia, USA. Detailed information is not available for the sources of the strains for the Kansas State University Teaching collection. However, some of these strains are holotype strains referenced in Leslie and Summerell (2006).

The strains from the USDA-ARS, Michigan State University, were cultivated on dextrose peptone yeast extract agar, containing 10 g L^{-1} dextrose (VWR, Radnor, PA, USA), 3 g L^{-1} peptone (Difco Proteose Peptone No. 3, Becton, Dickinson and Company, Franklin Lakes, NY, USA), and 0.3 g L^{-1} yeast extract (Merck, Kenilworth, NJ, USA), and were filtered through a 0.2 µm pore diameter filter (PES disposable filter units, Life Science Products, Frederick, CO, USA). After filtration, 12 g L^{-1} agarose (Certified Molecular Biology Agarose, Bio-Rad, Hercules, CA, USA) was added, and the medium was sterilized by autoclaving at 121 °C for 20 min. The colonies were grown at 22 to 24 °C for 7 to 19 d. The strains from the Schmale Laboratory at Virginia Tech and the Kansas State University Teaching Collection were maintained in cryogenic storage at −80 °C and were grown on quarter-strength potato dextrose agar (Difco Laboratories, Detroit, USA) on 100 mm petri plates at ambient room temperature for 4 d prior to ice nucleation assays.

For quantitative analysis, exposure experiments, heat treatments, freeze–thaw cycles, and short- and long-term storage tests, a selection of IN-active tested strains was grown on full-strength potato dextrose agar (VWR International GmbH, Darmstadt, Germany) first at room temperature for 4 to 6 d and then at 6 °C for about 4 weeks. For filtration experiments, the fungal cultures were grown at 6 °C for up to 6 months.

2.2 Preparation and treatments of aqueous extracts

For LED-based Ice Nucleation Detection Apparatus (LINDA) (Stopelli et al., 2014) experiments (see Sect. 2.3), 4 mL of sterile 0.9 % NaCl was added to each of eight petri plates, and the fungal cultures were scraped with the flat end of a sterile bamboo skewer. The resulting suspension of mycelium and spores was filtered through a 100 µm filter (Corning Life Sciences, Reims, France).

For Twin-plate Ice Nucleation Assay (TINA) (Kunert et al., 2018) experiments (see Sect. 2.3) the fungal mycelium was scraped off the agar plate and transferred into a 15 mL tube (Greiner Bio One, Kremsmünster, Austria). The fresh weight of the mycelium was determined gravimetrically. Pure water was prepared as described in Kunert et al. (2018). Aliquots of 10 mL pure water were added before vortexing three times at 2700 rpm for 30 s (Vortex-Genie 2, Scientific Industries, Inc., Bohemia, NY, USA) and centrifugation at 4500 g for 10 min (Heraeus Megafuge 40, Thermo Scientific, Braunschweig, Germany). For all experiments, the aqueous extract were filtered successively through a 5 and a 0.1 µm PES syringe filter (Acrodisc®, Sigma-Aldrich, Taufkirchen, Germany), and the aqueous extracts contained IN from spores and mycelial surfaces.

For filtration experiments, the 0.1 µm filtrate was further filtered successively through 300 000 and 100 000 MWCO PES ultrafiltration units (Vivaspin®, Sartorius AG, Göttingen, Germany). After each filtration step, the IN concentration was determined using TINA.

For exposure experiments, aqueous extracts of *F. acuminatum* 3–68 and *F. avenaceum* 2–106 were exposed to high concentrations of O_3 and NO_2 as described in Liu et al. (2017). Briefly, a mixture of 1 ppm O_3 and 1 ppm NO_2 was bubbled through 1 mL aliquots of aqueous extract for 4 h, and the IN concentration was determined using TINA.

For heat treatment experiments, aliquots of aqueous extracts were incubated at 40, 70, and 98 °C for 1 h for each of *F. acuminatum* 3–68, *F. armeniacum* 20 970, *F. avenaceum* 2–106, and *F. langsethiae* 19 084. The IN concentration was determined using TINA.

For freeze–thaw cycles, the ice nucleation activity of *F. acuminatum* 3–68 was determined shortly after preparation of the aqueous extract and after storage at 6 °C for 24 h using TINA. Then, the aqueous extract was stored at −20 °C for 24 h and thawed again. The ice nucleation activity was tested before storage at −20 °C for an additional 24 h. After thawing, the ice nucleation activity was determined again.

For long-term storage experiments, the aqueous extracts of various *Fusarium* species were stored at 6 °C for about 4 months or at −20 °C for about 8 months, and the ice nucleation activity was determined using TINA.

2.3 Ice nucleation assays

Two independent droplet freezing assays conducted in two laboratories were used to investigate the distribution of ice nucleation activity within *Fusarium* in an initial screening.

First, a thermal cycler (PTC200, MJ Research, Hercules, CA, USA) was used as described in Fröhlich-Nowoisky et al. (2015) to screen 30 *Fusarium* strains from seven species from USDA-ARS, Michigan State University, in the temperature range from −2 to −9 °C. Mycelium was picked with sterile pipette tips (Eppendorf, Westbury, NY, USA) into 80 µL aliquots of 0.2 µm pore diameter filtered dextrose peptone yeast (DPY) broth in sterile 96-well polypropylene PCR plates (VWR International, LLC, Radnor, PA, USA). Up to seven droplets were measured for each sample, and the mean freezing temperature was calculated. Aliquots of uninoculated DPY broth were used as negative controls, which did not freeze in the investigated temperature interval.

Second, the LED-based Ice Nucleation Detection Apparatus (LINDA) was used as described by Stopelli et al. (2014) to screen 13 strains from the Schmale Laboratory at Virginia Tech and 69 strains from the Kansas State University Teaching Collection. Aliquots of 200 µL of each aqueous extract were transferred to three separate 500 µL tubes and placed on ice for 1 h prior to the LINDA experiments. LINDA was run from −1 to −20 °C, and images of the samples were recorded every 6 s. The mean freezing temperature for three droplets was calculated. Note that the aqueous extracts were prepared in 0.9 % NaCl solution, which could reduce the freezing temperatures by 0.5 °C based on theoret-

ical calculations. We cannot exclude, however, that the high concentration of IN compensates for the effect of NaCl on the freezing temperature. This is supported by the investigations of Stopelli et al. (2014), who did not find a systematic suppression of freezing at this salt concentration in LINDA experiments. As a negative control, a 0.9 % NaCl solution was added to three uninoculated agar plates, and the freezing started below −14 °C. As positive control, aqueous suspensions of *Pseudomonas syringae* CC94 from the collection of INRA (Avignon, France) (Berge et al., 2014), with a final OD_{580} of 0.5 to 0.7, i.e. $\sim 10^9$ bacteria mL^{-1}, were used for each experiment. The bacteria were grown on King's medium B (King et al., 1954) at 22 to 25 °C for 48 h, and aqueous suspensions were equilibrated at 4 °C for 1 to 4 h before LINDA experiments. The freezing temperatures of *P. syringae* CC94 ranged from −3.5 to −4.6 °C.

Ice nuclei of selected *Fusarium* species, which were long known for ice nucleation activity (*F. acuminatum*, *F. avenaceum*), as well as all the newly identified species, were further analyzed in immersion freezing mode using the high-throughput Twin-plate Ice Nucleation Assay (TINA) (Kunert et al., 2018). The aqueous extracts were serially diluted 10-fold with pure water by a liquid handling station (epMotion ep5073, Eppendorf, Hamburg, Germany) to a dilution at which droplets remained liquid in the investigated temperature interval. Of each dilution, 96 droplets (3 µL) were tested with a continuous cooling rate of 1 °C min^{-1} from 0 to −20 °C. Pure water samples (0.1 µm filtrated) served as a negative control for each experiment. These did not freeze in the observed temperature interval. The temperature was measured with an accuracy of 0.2 K (Kunert et al., 2018). The obtained fraction of frozen droplets (f_{ice}) and the counting error were used to calculate the cumulative number of IN (N_m) with the associated error using the Vali formula and the Gaussian error propagation (Kunert et al., 2018; Vali, 1971). For each experiment, the cumulative number of IN was averaged over all dilutions. If the experiment was repeated, the cumulative number of IN was averaged over all experiments, and the standard error was calculated. Three independent experiments with aqueous extracts from three individual fungal culture plates of the same strain showed similar results with only slight variation. An example of results is presented for *F. armeniacum* 20 970 (Fig. S1 in the Supplement).

Table 1. Ice-nucleation-active *Fusarium* strains with corresponding mean freezing temperatures of the initial screening. The newly identified IN-active *Fusarium* species are marked with an asterisk (*).

Species	Strain	T (°C)
F. acuminatum	1–3	−5.6
F. acuminatum	1–4	−5.0
F. acuminatum	1–5	−5.6
F. acuminatum	1–24	−3.5
F. acuminatum	2–38	−5.0
F. acuminatum	2–48	−5.6
F. acuminatum	2–109	−5.6
F. acuminatum	3–48	−5.0
F. acuminatum	3–68	−3.5
F. acuminatum	20 964	−6.2
*F. armeniacum**	20 970	−5.3
F. avenaceum	2–106	−5.0
F. avenaceum	11 440	−7.6
*F. begoniae**	10 767	−11.2
*F. concentricum**	10 765	−4.6
*F. langsethiae**	19 084	−9.4
F. tricinictum	20 990	−7.3

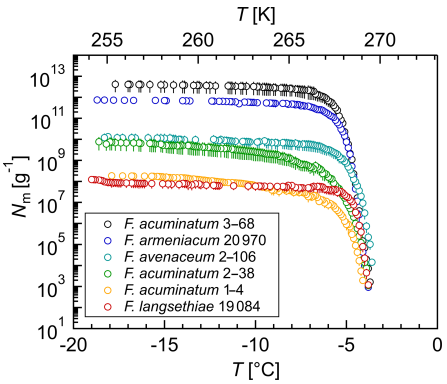

Figure 1. Overview of ice nucleation activity for selected *Fusarium* species and strains: cumulative number of IN (N_m) per gram of mycelium plotted against the temperature (T); arithmetic mean values and standard error of two independent experiments with aqueous extracts from two individual fungal culture plates of the same species.

3 Results and discussion

3.1 IN-active *Fusarium* species

Although several IN-active *Fusarium* species are known, the frequency and distribution of ice nucleation activity within the fungal genus *Fusarium* are still not well studied (Hasegawa et al., 1994; Humphreys et al., 2001; Pouleur et al., 1992; Richard et al., 1996; Tsumuki and Konno, 1994). Two initial screenings in the temperature range from −1 to −20 °C were performed to better evaluate the frequency of ice nucleation activity within *Fusarium*. A strain was defined as IN-active, when it initiated ice formation above −9 °C (thermal cycler) and −12 °C (LINDA), respectively.

In total, ∼ 16 % (18/112) of the tested strains showed ice nucleation activity with mean freezing temperatures of −3.5 to −11.2 °C (Table 1) in the typical range known for *Fusarium* (−1 and −9 °C) (Hasegawa et al., 1994; Humphreys et al., 2001; Pouleur et al., 1992; Richard et al., 1996; Tsumuki et al., 1992; Tsumuki and Konno, 1994). Most

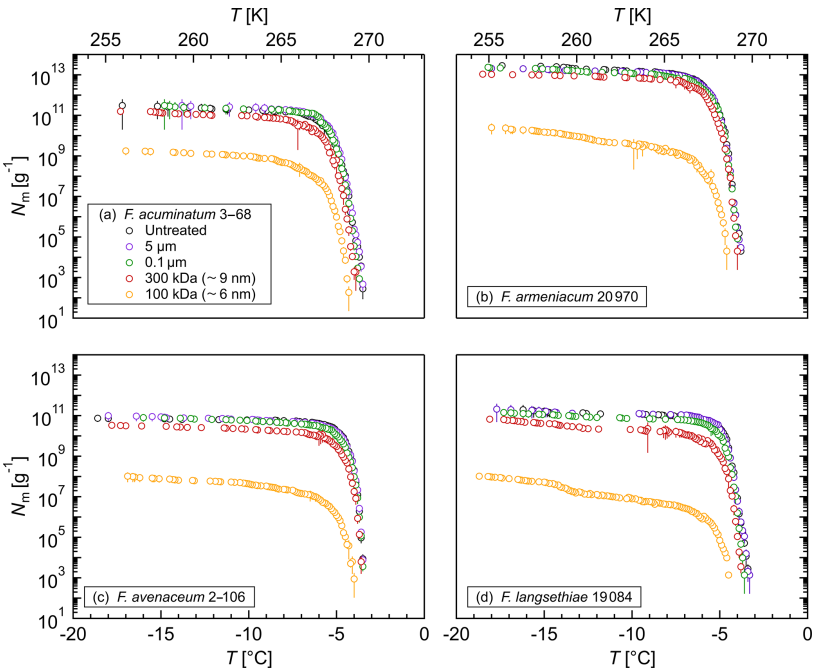

Figure 2. Size determination of the *Fusarium* IN upon filtration: cumulative number of IN (N_m) per gram of mycelium plotted against the temperature (T) for **(a)** *F. acuminatum* 3–68, **(b)** *F. armeniacum* 20 970, **(c)** *F. avenaceum* 2–106, and **(d)** *F. langsethiae* 19 084. The error bars were calculated using the counting error and the Gaussian error propagation.

formerly reported initial freezing temperatures were obtained with different *Fusarium* strains, growth conditions, and freezing assays, which might explain differences compared to our results. The high proportion of IN-active strains within *F. acuminatum* is consistent with previous reports (Pouleur et al., 1992; Tsumuki et al., 1995). Overall, ∼ 11 % (7/65) of the tested species included IN-active strains. In addition to strains from *Fusarium* species with known ice nucleation activity, four *Fusarium* species were newly identified as IN-active: *F. armeniacum*, *F. begoniae*, *F. concentricum*, and *F. langsethiae*. In further experiments, the ice nucleation activity of *F. begoniae* and *F. concentricum* could not be verified.

The newly identified IN-active species are cosmopolitan. *Fusarium armeniacum* is a toxigenic saprophyte (Burgess et al., 1993), causing seed and root rot on soybeans (Ellis et al., 2012). The geographical distribution has been reported as tropical and subtropical (Leslie and Summerell, 2006), but it was also found in Minnesota, USA (Kommedahl et al., 1979), and Australia (Burgess et al., 1993). *Fusarium begoniae* is a plant pathogen of Begonia found in Germany with a potential wider distribution (Nirenberg and O'Donnell, 1998). *Fusarium concentricum* is a plant pathogen, which is frequently found in Central America and isolated from bananas (Aoki et al., 2001; Leslie and Summerell, 2006), and *F. langsethiae* is a broadly distributed cereal pathogen (Torp and Nirenberg, 2004). Some strains of these newly identified IN-active species are known to produce mycotoxins, which can threaten the health of humans and animals (Fotso et al., 2012; Kokkonen et al., 2012; Wing et al., 1993a, b).

The results suggest that the ice nucleation activity within *Fusarium* is more widespread than previously known. Not all *Fusarium* species include IN-active strains and not all strains within one species show ice nucleation activity. Earlier studies including experiments suggested that *Fusarium* IN are proteins or at least contain a proteinaceous compound (Hasegawa et al., 1994; Pouleur et al., 1992; Tsumuki and Konno, 1994). Their production requires energy, and we might assume that this trait would not be expressed or maintained unless there was an ecological advantage. It is known that *Fusarium* can regulate the gene expression for IN production, depending on environmental conditions such as nutrient availability (Richard et al., 1996), and some *Fusarium* species reduce or lose their ice nucleation activity after several subcultures (Pummer et al., 2013; Tsumuki et al., 1995). Thus, we cannot exclude that all *Fusarium* strains have the ability to produce IN. From the phylogenetic distribution of ice nucleation activity across the genus *Fusarium*, we can speculate that ice nucleation activity is a very old trait, but either the gene expression requires a trigger, which is not yet identified, or the trait might be in the process of being lost. It is unlikely, however, that the age of the genetic determi-

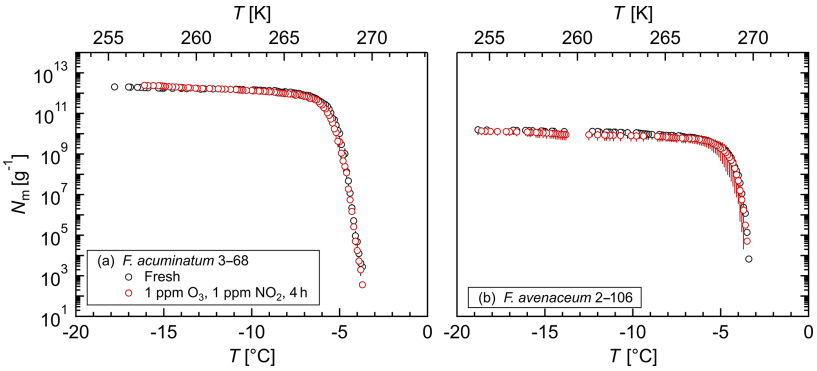

Figure 3. Exposure of aqueous extract from *Fusarium* to ozone and nitrogen dioxide: cumulative number of IN (N_m) per mass of mycelium plotted against the temperature (T) for **(a)** *F. acuminatum* 3–68 and **(b)** *F. avenaceum* 2–106; arithmetic mean values and standard error of two independent experiments with aqueous extracts from two individual fungal culture plates of the same species.

nants of fungal ice nucleation activity is older than that in bacteria, since fungi diverged well after the age that has been attributed to the bacterial IN gene (Morris et al., 2014), and the genetic determinants are not the same as those in bacteria.

3.2 Quantification and size determination of IN from selected *Fusarium* species

A selection of IN-active *Fusarium* species was further investigated by extensive droplet freezing assay analysis using TINA. All tested *Fusarium* strains initiated ice nucleation between −3 and −4 °C (Fig. 1). Differences in the freezing temperatures between the initial screening and the quantitative analysis can be due to different growth conditions and freezing assays. The cumulative number of IN (N_m) per gram of mycelium was in the range between 10^8 g^{-1} and 10^{13} g^{-1}. *Fusarium acuminatum* 3–68 showed the highest ice nucleation activity and *F. langsethiae* the lowest per gram of mycelium. The results are comparable to other IN-active microorganisms like *Sarocladium implicatum* (10^8 g^{-1}, Pummer et al., 2015), *Mortierella alpina* (10^9 g^{-1}, Fröhlich-Nowoisky et al., 2015; 10^{10} g^{-1}, Kunert et al., 2018), and the bacterial IN-active substance Snomax® containing *Pseudomonas syringae* (10^{12} g^{-1}, Budke and Koop, 2015; Kunert et al., 2018).

The size of the *Fusarium* IN was investigated by filtration experiments. Filtration through a 5 and a 0.1 µm filter did not affect the ice nucleation activity (Fig. 2), revealing that *Fusarium* IN are smaller than 100 nm, cell-free, easily removed from the fungus, and stay active in solution. This is in agreement with other *Fusarium* studies (O'Sullivan et al., 2015; Pouleur et al., 1992; Tsumuki and Konno, 1994). Moreover, biological ice-nucleating macromolecules (INMs) smaller than 200 nm were also found in various organisms, e.g., other fungi (Fröhlich-Nowoisky et al., 2015; Pummer et al., 2015); leaves, bark, and pollen from birch trees (*Betula* spp.) (Felgitsch et al., 2018; Pummer et al., 2012); leaf litter (Schnell and Vali, 1973); some microalgae (Tesson and Šantl-Temkiv, 2018); strains of *Lysinibacillus* (Failor et al., 2017); and biological particles in the sea surface microlayer (Irish et al., 2019; Wilson et al., 2015). Filtration through a 300 000 MWCO filter unit decreased the cumulative number of IN per gram of mycelium by about 50 % to 75 % depending on the *Fusarium* species, but a tremendous number of IN (10^{10}–10^{13} g^{-1}) still passed through the filter. The initial freezing temperature was slightly shifted towards lower temperatures. Further filtration through a 100 000 MWCO filter unit reduced the IN number to 10^8–10^{10} g^{-1}, which is less than 1 % of the initial IN concentration. Additionally, the initial freezing temperature was shifted about 1 °C towards lower temperatures.

As ice nucleation activity was found in all filtrates, the aqueous extract of *Fusarium* consists of a mixture of IN-active proteins with different sizes. We hypothesize that *Fusarium* IN are macromolecules (INMs) smaller than 100 kDa, which agglomerate to large protein complexes in solution. Some of these complexes fall apart upon filtration, so that the INMs can pass through the filter. The small shift in the initial freezing temperature suggests that these INMs reassemble again to aggregates after filtration, as larger IN nucleate at warmer temperatures (Govindarajan and Lindow, 1988; Pummer et al., 2015). Erickson (2009) determined the size of proteins based on theoretical calculations. As the interior of proteins is closely packed with no substantial holes and almost no water molecules inside, proteins are rigid structures with approximately the same density (~ 1.37 g cm^{-1}). Assuming the protein as a smooth spherical particle, the minimum diameter of the INM would be smaller than 6.1 nm. Our results are in accordance with Lagzian et al. (2014), who cloned and expressed a 49 kDa IN-active protein from *F. acuminatum*.

As *Fusarium* IN are cell-free and can easily be washed off the fungal surface, they can be released in high num-

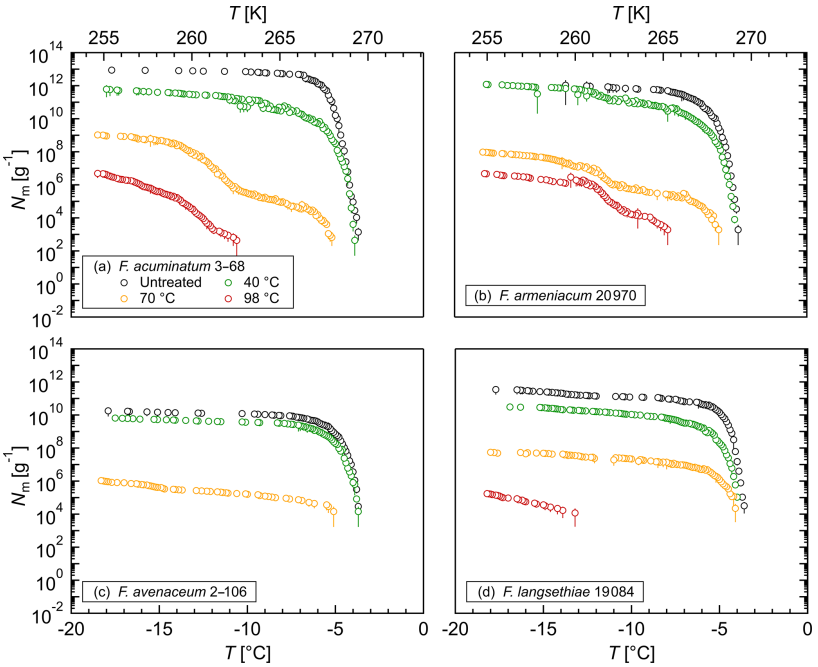

Figure 4. Effects of high temperatures on the ice nucleation activity of *Fusarium*: cumulative number of IN (N_m) per gram of mycelium plotted against the temperature (T) for **(a)** *F. acuminatum* 3–68, **(b)** *F. armeniacum* 20 970, **(c)** *F. avenaceum* 2–106, and **(d)** *F. langsethiae* 19 084. The error bars were calculated using the counting error and the Gaussian error propagation.

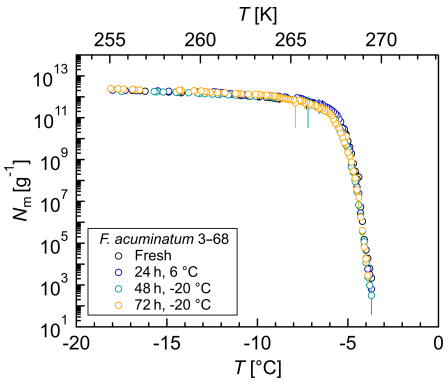

Figure 5. Effects of short-term storage and freeze–thaw cycles on the ice nucleation activity of *Fusarium acuminatum* 3–68: cumulative number of IN (N_m) per gram of mycelium plotted against the temperature (T). The same aqueous extract was measured immediately after preparation (black), after storage at 6 °C for 24 h (blue), after another 24 h stored at −20 °C (total 48 h; turquoise), and after another 24 h stored at −20 °C (total 72 h; yellow). The error bars were calculated using the counting error and the Gaussian error propagation.

bers into the environment. If they are not degraded by microorganisms before, the IN can adsorb to soil dust and be aerosolized while attached to these particles (Conen et al., 2011; Fröhlich-Nowoisky et al., 2015; Hill et al., 2016; O'Sullivan et al., 2014, 2015, 2016; Sing and Sing, 2010). This is in good agreement with Pruppacher and Klett (1997), who found a positive correlation between IN number concentration and particles in the coarse mode. Other releasing processes cannot be excluded; however, it is unlikely that the INMs are present in the atmosphere as individual aerosol particles. Individual proteins with a diameter of ∼ 6 nm, which may enter the atmosphere, would be in the nucleation mode size range, where particles tend to uptake gaseous compounds and grow to Aitken mode particles, which themselves tend to coagulate to larger agglomerates (Seinfeld and Pandis, 1998).

3.3 Stability of *Fusarium* IN

In the atmosphere, IN can interact with other aerosol particles or gases. They can be exposed to chemically modifying agents like ozone and nitrogen dioxide, as well as physical stressors like high and low or quickly changing temperatures. To investigate the stability of *Fusarium* IN, we performed exposure experiments, heat treatments, freeze–thaw cycles, and long-term storage tests.

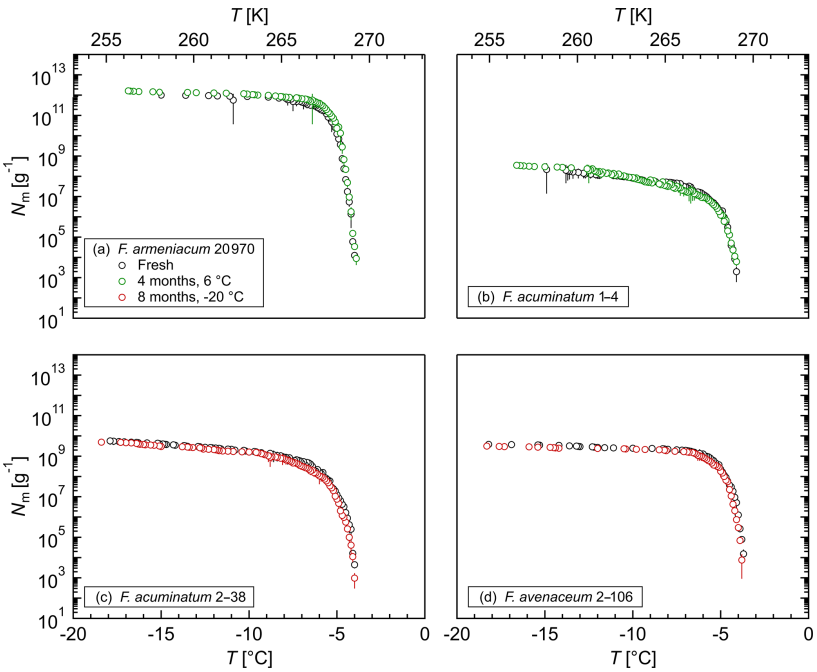

Figure 6. Effect of long-term storage on the ice nucleation activity of **(a)** *F. armeniacum* 20 970, **(b)** *F. acuminatum* 1–4, **(c)** *F. acuminatum* 2–38, and **(d)** *F. avenaceum* 2–106: cumulative number of IN (N_m) per gram of mycelium plotted against the temperature (T). The error bars were calculated using the counting error and the Gaussian error propagation.

The influence of chemical processing on the *Fusarium* IN, in particular oxidation and nitration reactions as occurring during atmospheric aging, was investigated by exposing aqueous extracts from *F. acuminatum* 3–68 and *F. avenaceum* 2–106 to high concentrations of ozone and nitrogen dioxide in liquid phase. Figure 3 shows that for both species neither the initial freezing temperature nor the cumulative number of IN per gram of mycelium was affected by exposure. These results demonstrate a high stability of *Fusarium* IN under oxidizing and nitrating conditions. This is in contrast to other biological IN, e.g., bacterial IN (Snomax®) (Kunert et al., 2018), birch and alder pollen (Gute and Abbatt, 2018), and dissolved organic matter (Borduas-Dedekind et al., 2019), where exposure to oxidizing agents reduced the IN activity.

The stability of the INM in *Fusarium* was investigated in heat treatment experiments. The ice nucleation activity was reduced significantly at a 40 °C treatment (Fig. 4). Between 40 % and 90 % of IN were lost at this temperature depending on the species, which supports the hypothesis that the INM in *Fusarium* consists of a proteinaceous compound. A heat treatment at 70 °C reduced the ice nucleation activity to less than 0.01 % compared to the initial level. Moreover, the initial freezing temperature was shifted to lower temperatures, indicating a breakdown of the large protein aggregates. After a 98 °C treatment, we still found ice nucleation activity for all investigated species except for *F. avenaceum* 2–106. The results are in agreement with previous studies, which also reported a reduction in ice nucleation activity with increasing temperature in heat treatment experiments (Hasegawa et al., 1994; Pouleur et al., 1992; Tsumuki and Konno, 1994). The remaining activity after the 98 °C treatment, however, could indicate that posttranslational modifications like glycosylation and therefore polysaccharides could play a role in the ice nucleation activity of *Fusarium*. Further systematic studies including chemical analyses are needed for elucidation.

To study the effects of short-term storage and freeze–thaw cycles on the ice nucleation activity of *F. acuminatum* 3–68, IN measurements of the same aqueous extract were performed at different time points (Fig. 5). The results of freshly prepared aqueous extract revealed that the highest activity of fungal IN was already developed during preparation of the filtrate and no time for equilibration was required. Storage of aqueous extract at 6 °C for 24 h did not affect the ice nucleation activity. Also, further storage at −20 °C for another 24 h and repeated freeze–thaw cycles had no impact on the ice nucleation activity. This means that, once in the atmosphere, the IN can undergo several freeze–thaw cycles without losing their activity and are still able to influence cloud glaciation and the formation of precipitation. This could be an explanation for why not all fungi are always IN-active as their IN are highly stable and quasi-recyclable. Ice nuclei might influence the availability of moisture over long time

periods, and if enough moisture is available in the environment, the necessity of IN production would be omitted, and the fungus could save energy.

In addition, the stability of the INM in *Fusarium* was studied in long-term storage tests, where aqueous extracts of various *Fusarium* species were stored at different temperatures for a long period of time. Figure 6 shows that storage at 6 °C for 4 months and at −20 °C for 8 months did not influence the ice nucleation activity of *F. armeniacum* 20 970, *F. acuminatum* 1–4, *F. avenaceum* 2–106, or *F. acuminatum* 2–38. The results demonstrate the high stability of the INMs in *Fusarium* in liquid and frozen solutions over long time periods, which makes *Fusarium* well applicable for laboratory IN studies. Moreover, the high stability is likely an advantage for these fungi to be linked to atmospheric processes.

4 Conclusions

The frequency and distribution of ice nucleation activity within the fungal genus *Fusarium* was investigated in a screening of more than 100 strains from 65 different *Fusarium* species. In total, ∼ 11 % (7/65) of all tested species included IN-active strains, and ∼ 16 % (18/112) of all tested strains showed ice nucleation activity, demonstrating the wide distribution of ice nucleation activity within *Fusarium*. Filtration experiments suggest that *Fusarium* IN form aggregates consisting of INMs smaller than 100 kDa (∼ 6 nm). Exposure experiments, freeze–thaw cycles, and long-term storage tests revealed a high stability of the INMs in *Fusarium*, demonstrating the suitability of *Fusarium* in laboratory IN studies. Heat treatments at 40 to 98 °C reduced the IN concentration significantly, supporting the hypothesis that the INM in *Fusarium* largely consists of a proteinaceous compound. An involvement of polysaccharides, however, cannot be excluded. The wide distribution of ice nucleation activity within the genus *Fusarium*, together with the stability of the INM in *Fusarium* under atmospherically relevant conditions, suggests that the implication of these IN on Earth's water cycle and climate might be more significant than previously assumed. Additional research is necessary to characterize the INMs in *Fusarium* and processes which can result in their agglomeration to larger protein complexes. To evaluate the implication of these IN on Earth's climate, additional work is required to study the abundance of *Fusarium* IN in environmental samples on a global scale.

Data availability. All data are available from the corresponding author upon request.

Supplement. The supplement related to this article is available online at: https://doi.org/10.5194/bg-16-4647-2019-supplement.

Author contributions. CEM, JFN, and UP designed the experiments. DGS III and LEH provided fungal cultures. CEM, DGS III, and JFN performed the initial screenings. ATK, KT, CSK, CW, and KRS performed the experiments. ATK, JFN, MLP, and UP discussed the results. ATK and JFN wrote the manuscript with contributions of all coauthors.

Competing interests. The authors declare that they have no conflict of interest.

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References

Amato, P., Parazols, M., Sancelme, M., Laj, P., Mailhot, G., and Delort, A. M.: Microorganisms isolated from the water phase of tropospheric clouds at the Puy de Dôme: Major groups and growth abilities at low temperatures, FEMS Microbiol. Ecol., 59, 242–254, https://doi.org/10.1111/j.1574-6941.2006.00199.x, 2007.

Aoki, T., O'Donnell, K., and Ichikawa, K.: *Fusarium fractiflexum* sp. nov. and two other species within the *Gibberella fujikuroi* species complex recently discovered in Japan that form aerial conidia in false heads, Mycoscience, 42, 461–478, https://doi.org/10.1007/BF02464343, 2001.

Berge, O., Monteil, C. L., Bartoli, C., Chandeysson, C., Guilbaud, C., Sands, D. C., and Morris, C. E.: A User's Guide to a Data Base of the Diversity of *Pseudomonas syringae* and Its Application to Classifying Strains in This Phylogenetic Complex, PLoS One, 9, e105547, https://doi.org/10.1371/journal.pone.0105547, 2014.

Borduas-Dedekind, N., Ossola, R., David, R. O., Boynton, L. S., Weichlinger, V., Kanji, Z. A., and McNeill, K.: Photomineralization mechanism changes the ability of dissolved organic matter to activate cloud droplets and to nucleate ice crystals, Atmos. Chem. Phys., 19, 12397–12412, https://doi.org/10.5194/acp-19-12397-2019, 2019.

Budke, C. and Koop, T.: BINARY: an optical freezing array for assessing temperature and time dependence of het-

erogeneous ice nucleation, Atmos. Meas. Tech., 8, 689–703, https://doi.org/10.5194/amt-8-689-2015, 2015.

Burgess, L. W., Forbes, G. A., Windels, C., Nelson, P. E., Marasas, W. F. O., and Gott, K. P.: Characterization and distribution of *Fusarium acuminatum* subsp. *armeniacum* subsp. nov., Mycologia, 85, 119–124, 1993.

Bush, B. J., Carson, M. L., Cubeta, M. A., Hagler, W. M., and Payne, G. A.: Infection and Fumonisin Production by *Fusarium verticillioides* in Developing Maize Kernels, Phytopathology, 94, 88–93, https://doi.org/10.1094/PHYTO.2004.94.1.88, 2004.

Coluzza, I., Creamean, J., Rossi, M. J., Wex, H., Alpert, P. A., Bianco, V., Boose, Y., Dellago, C., Felgitsch, L., Fröhlich-Nowoisky, J., Herrmann, H., Jungblut, S., Kanji, Z. A., Menzl, G., Moffett, B., Moritz, C., Mutzel, A., Pöschl, U., Schauperl, M., Scheel, J., Stopelli, E., Stratmann, F., Grothe, H., and Schmale III, D. G.: Perspectives on the Future of Ice Nucleation Research: Research Needs and Unanswered Questions Identified from Two International Workshops, Atmosphere-Basel, 8, 138, https://doi.org/10.3390/atmos8080138, 2017.

Conen, F., Morris, C. E., Leifeld, J., Yakutin, M. V., and Alewell, C.: Biological residues define the ice nucleation properties of soil dust, Atmos. Chem. Phys., 11, 9643–9648, https://doi.org/10.5194/acp-11-9643-2011, 2011.

Creamean, J. M., Suski, K. J., Rosenfeld, D., Cazorla, A., DeMott, P. J., Sullivan, R. C., White, A. B., Ralph, F. M., Minnis, P., Comstock, J. M., Tomlinson, J. M., and Prather, K. A.: Dust and Biological Aerosols from the Sahara and Asia Influence Precipitation in the Western U.S., Science, 339, 1572–1578, https://doi.org/10.1126/science.1227279, 2013.

DeMott, P. J. and Prenni, A. J.: New Directions: Need for defining the numbers and sources of biological aerosols acting as ice nuclei, Atmos. Environ., 44, 1944–1945, https://doi.org/10.1016/j.atmosenv.2010.02.032, 2010.

Després, V. R., Huffman, J. A., Burrows, S. M., Hoose, C., Safatov, A. S., Buryak, G., Fröhlich-Nowoisky, J., Elbert, W., Andreae, M. O., Pöschl, U., and Jaenicke, R.: Primary biological aerosol particles in the atmosphere: a review, Tellus B, 64, 15598, https://doi.org/10.3402/tellusb.v64i0.15598, 2012.

Diehl, K., Matthias-Maser, S., Jaenicke, R., and Mitra, S. K.: The ice nucleating ability of pollen: Part II. Laboratory studies in immersion and contact freezing modes, Atmos. Res., 61, 125–133, https://doi.org/10.1016/j.atmosres.2005.03.008, 2002.

Ellis, M. L., Diaz Arias, M. M., Leandro, L. F., and Mungvold, G. P.: First report of *Fusarium armeniacum* causing seed rot and root rot on soybean (Glycine max) in the United States, Plant Dis., 96, 1693, https://doi.org/10.1094/PDIS-05-12-0429-PDN, 2012.

Erickson, H. P.: Size and Shape of Protein Molecules at the Nanometer Level Determined by Sedimentation, Gel Filtration, and Electron Microscopy, Biol. Proced. Online, 11, 32–51, https://doi.org/10.1007/s12575-009-9008-x, 2009.

Failor, K. C., Schmale, D. G., Vinatzer, B. A., and Monteil, C. L.: Ice nucleation active bacteria in precipitation are genetically diverse and nucleate ice by employing different mechanisms, ISME J., 11, 2740–2753, https://doi.org/10.1038/ismej.2017.124, 2017.

Felgitsch, L., Baloh, P., Burkart, J., Mayr, M., Momken, M. E., Seifried, T. M., Winkler, P., Schmale III, D. G., and Grothe, H.: Birch leaves and branches as a source of ice-nucleating macromolecules, Atmos. Chem. Phys., 18, 16063–16079, https://doi.org/10.5194/acp-18-16063-2018, 2018.

Fotso, J., Leslie, J. F., and Smith, J. S.: Production of Beauvericin, Moniliformin, Fusaproliferin, and Fumonisins B_1, B_2, and B_3 by Fifteen Ex-Type Strains of *Fusarium* Species, Appl. Environ. Microb., 68, 5195–5197, https://doi.org/10.1128/AEM.68.10.5195-5197.2002, 2012.

Fröhlich-Nowoisky, J., Pickersgill, D. A., Després, V. R., and Pöschl, U.: High diversity of fungi in air particulate matter, P. Natl. Acad. Sci. USA, 106, 12814–12819, https://doi.org/10.1073/pnas.0811003106, 2009.

Fröhlich-Nowoisky, J., Hill, T. C. J., Pummer, B. G., Yordanova, P., Franc, G. D., and Pöschl, U.: Ice nucleation activity in the widespread soil fungus Mortierella alpina, Biogeosciences, 12, 1057–1071, https://doi.org/10.5194/bg-12-1057-2015, 2015.

Fröhlich-Nowoisky, J., Kampf, C. J., Weber, B., Huffman, J. A., Pöhlker, C., Andreae, M. O., Lang-Yona, N., Burrows, S. M., Gunthe, S. S., Elbert, W., Su, H., Hoor, P., Thines, E., Hoffmann, T., Després, V. R., and Pöschl, U.: Bioaerosols in the Earth system: Climate, health, and ecosystem interactions, Atmos. Res., 182, 346–376, https://doi.org/10.1016/j.atmosres.2016.07.018, 2016.

Fulton, J. D.: Microorganisms of the Upper Atmosphere: IV. Microorganisms of a Land Air Mass as it Traverses an Ocean, Appl. Envrion. Microb., 14, 241–244, 1966.

Garnham, C. P., Campbell, R. L., Walker, V. K., and Davies, P. L.: Novel dimeric β-helical model of an ice nucleation protein with bridged active sites, BMC Struct. Biol., 11, 36, https://doi.org/10.1186/1472-6807-11-36, 2011.

Govindarajan, A. G. and Lindow, S. E.: Size of bacterial ice-nucleation sites measured *in situ* by radiation inactivation analysis, P. Natl. Acad. Sci. USA, 85, 1334–1338, https://doi.org/10.1073/pnas.85.5.1334, 1988.

Graether, S. P. and Jia, Z.: Modeling *Pseudomonas syringae* Ice-Nucleation Protein as a β-Helical Protein, Biophys. J., 80, 1169–1173, https://doi.org/10.1016/S0006-3495(01)76093-6, 2001.

Green, R. L. and Warren, G. J.: Physical and functional repetition in a bacterial ice nucleation gene, Nature, 317, 645–648, https://doi.org/10.1038/317645a0, 1985.

Gute, E. and Abbatt, J. P. D.: Oxidative Processing Lowers the Ice Nucleation Activity of Birch and Alder Pollen, Geophys. Res. Lett., 45, 1647–1653, https://doi.org/10.1002/2017GL076357, 2018.

Hanlon, R., Powers, C., Failor, K., Monteil, C. L., Vinatzer, B. A., and Schmale, D. G.: Microbial ice nucleators scavenged from the atmosphere during simulated rain events, Atmos. Environ., 163, 182–189, https://doi.org/10.1016/j.atmosenv.2017.05.030, 2017.

Hasegawa, Y., Ishihara, Y., and Tokuyama, T.: Characteristics of Ice-nucleation Activity in *Fusarium avenaceum* IFO 7158, Biosci. Biotech. Bioch., 58, 2273–2274, https://doi.org/10.1271/bbb.58.2273, 1994.

Hill, T. C. J., Moffett, B. F., DeMott, P. J., Georgakopoulos, D. G., Stump, W. L., and Franc, G. D.: Measurement of Ice Nucleation-Active Bacteria on Plants and in Precipitation by Quantitative PCR, Appl. Environ. Microb., 80, 1256–1267, https://doi.org/10.1128/AEM.02967-13, 2014.

Hill, T. C. J., DeMott, P. J., Tobo, Y., Fröhlich-Nowoisky, J., Moffett, B. F., Franc, G. D., and Kreidenweis, S. M.: Sources of or-

ganic ice nucleating particles in soils, Atmos. Chem. Phys., 16, 7195–7211, https://doi.org/10.5194/acp-16-7195-2016, 2016.

Hoose, C. and Möhler, O.: Heterogeneous ice nucleation on atmospheric aerosols: a review of results from laboratory experiments, Atmos. Chem. Phys., 12, 9817–9854, https://doi.org/10.5194/acp-12-9817-2012, 2012.

Huffman, J. A., Prenni, A. J., DeMott, P. J., Pöhlker, C., Mason, R. H., Robinson, N. H., Fröhlich-Nowoisky, J., Tobo, Y., Després, V. R., Garcia, E., Gochis, D. J., Harris, E., Müller-Germann, I., Ruzene, C., Schmer, B., Sinha, B., Day, D. A., Andreae, M. O., Jimenez, J. L., Gallagher, M., Kreidenweis, S. M., Bertram, A. K., and Pöschl, U.: High concentrations of biological aerosol particles and ice nuclei during and after rain, Atmos. Chem. Phys., 13, 6151–6164, https://doi.org/10.5194/acp-13-6151-2013, 2013.

Humphreys, T. L., Castrillo, L. A., and Lee, M. R.: Sensitivity of Partially Purified Ice Nucleation Activity of *Fusarium acuminatum* SRSF 616, Curr. Microbiol., 42, 330–338, https://doi.org/10.1007/s002840010225, 2001.

Ichinoe, M., Kurata, H., Sugiura, Y., and Ueno, Y.: Chemotaxonomy of *Gibberella zeae* with Special Reference to Production of Trichothecenes and Zearalenone, Appl. Environ. Microb., 46, 1364–1369, 1983.

Irish, V. E., Hanna, S. J., Xi, Y., Boyer, M., Polishchuk, E., Ahmed, M., Chen, J., Abbatt, J. P. D., Gosselin, M., Chang, R., Miller, L. A., and Bertram, A. K.: Revisiting properties and concentrations of ice-nucleating particles in the sea surface microlayer and bulk seawater in the Canadian Arctic during summer, Atmos. Chem. Phys., 19, 7775–7787, https://doi.org/10.5194/acp-19-7775-2019, 2019.

Joly, M., Amato, P., Deguillaume, L., Monier, M., Hoose, C., and Delort, A.-M.: Quantification of ice nuclei active at near 0 °C temperatures in low-altitude clouds at the Puy de Dôme atmospheric station, Atmos. Chem. Phys., 14, 8185–8195, https://doi.org/10.5194/acp-14-8185-2014, 2014.

Kim, H. K., Orser, C., Lindow, S. E., and Sands, D. C.: *Xanthomonas campestris* pv. *translucens* Strains Active in Ice Nucleation, Plant Dis., 71, 994–996, https://doi.org/10.1094/PD-71-0994, 1987.

King, E. O., Ward, M. K., and Raney, D. E.: Two simple media for the demonstration of pyocyanin and fluorescin, Transl. Res., 44, 301–307, 1954.

Knopf, D. A., Alpert, P. A., and Wang, B.: The Role of Organic Aerosol in Atmospheric Ice Nucleation: A Review, ACS Earth Sp. Chem., 2, 168–202, https://doi.org/10.1021/acsearthspacechem.7b00120, 2018.

Kohlmeier, S., Smits, T. H. M., Ford, R. M., Keel, C., Harms, H., and Wick, L. Y.: Taking the Fungal Highway: Mobilization of Pollutant-Degrading Bacteria by Fungi, Environ. Sci. Technol., 39, 4640–4646, https://doi.org/10.1021/es047979z, 2005.

Kokkonen, M., Jestoi, M., and Laitila, A.: Mycotoxin production of *Fusarium langsethiae* and *Fusarium sporotrichioides* on cereal-based substrates, Mycotoxin Res., 28, 25–35, https://doi.org/10.1007/s12550-011-0113-8, 2012.

Kommedahl, T., Windels, C. E., and Stucker, R. E.: Occurrence of *Fusarium* Species in Roots and Stalks of Symptomless Corn Plants During the Growing Season, Phytopathology, 69, 961–966, 1979.

Kunert, A. T., Lamneck, M., Helleis, F., Pöschl, U., Pöhlker, M. L., and Fröhlich-Nowoisky, J.: Twin-plate Ice Nucleation Assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples, Atmos. Meas. Tech., 11, 6327–6337, https://doi.org/10.5194/amt-11-6327-2018, 2018.

Lagzian, M., Latifi, A. M., Bassami, M. R., and Mirzaei, M.: An ice nucleation protein from *Fusarium acuminatum*: cloning, expression, biochemical characterization and computational modeling, Biotechnol. Lett., 36, 2043–2051, https://doi.org/10.1007/s10529-014-1568-4, 2014.

Leslie, J. F. and Summerell, B. A.: The Fusarium Laboratory Manual, Blackwell Publishing, Ames, Iowa 50014, USA, 2006.

Lin, B., Bozorgmagham, A., Ross, S. D., and Schmale III, D. G.: Small fluctuations in the recovery of fusaria across consecutive sampling intervals with unmanned aircraft 100 m above ground level, Aerobiologia (Bologna), 29, 45–54, https://doi.org/10.1007/s10453-012-9261-3, 2013.

Lin, B., Ross, S. D., Prussin, A. J., and Schmale, D. G.: Seasonal associations and atmospheric transport distances of fungi in the genus *Fusarium* collected with unmanned aerial vehicles and ground-based sampling devices, Atmos. Environ., 94, 385–391, https://doi.org/10.1016/j.atmosenv.2014.05.043, 2014.

Lindow, S. E., Hirano, S. S., Barchet, W. R., Arny, D. C., and Upper, C. D.: Relationship between Ice Nucleation Frequency of Bacteria and Frost Injury, Plant Physiol., 70, 1090–1093, 1982.

Ling, M. L., Wex, H., Grawe, S., Jakobsson, J., Löndahl, J., Hartmann, S., Finster, K., Boesen, T., and Šantl-Temkiv, T.: Effects of Ice Nucleation Protein Repeat Number and Oligomerization Level on Ice Nucleation Activity, J. Geophys. Res.-Atmos., 123, 1802–1810, https://doi.org/10.1002/2017JD027307, 2018.

Liu, F., Lakey, P., Berkemeier, T., Tong, H., Kunert, A. T., Meusel, H., Su, H., Cheng, Y., Fröhlich-Nowoisky, J., Lai, S., Weller, M. G., Shiraiwa, M., Pöschl, U., and Kampf, C. J.: Atmospheric protein chemistry influenced by anthropogenic air pollutants: nitration and oligomerization upon exposure to ozone and nitrogen dioxide, Faraday Discuss., 200, 413–427, https://doi.org/10.1039/C7FD00005G, 2017.

Möhler, O., DeMott, P. J., Vali, G., and Levin, Z.: Microbiology and atmospheric processes: the role of biological particles in cloud physics, Biogeosciences, 4, 1059–1071, https://doi.org/10.5194/bg-4-1059-2007, 2007.

Morris, C. E., Sands, D. C., Glaux, C., Samsatly, J., Asaad, S., Moukahel, A. R., Gonçalves, F. L. T., and Bigg, E. K.: Urediospores of rust fungi are ice nucleation active at $> -10\,^{\circ}C$ and harbor ice nucleation active bacteria, Atmos. Chem. Phys., 13, 4223–4233, https://doi.org/10.5194/acp-13-4223-2013, 2013.

Morris, C. E., Conen, F., Alex Huffman, J., Phillips, V., Pöschl, U., and Sands, D. C.: Bioprecipitation: a feedback cycle linking Earth history, ecosystem dynamics and land use through biological ice nucleators in the atmosphere, Glob. Change Biol., 20, 341–351, https://doi.org/10.1111/gcb.12447, 2014.

Murray, B. J., O'Sullivan, D., Atkinson, J. D., and Webb, M. E.: Ice nucleation by particles immersed in supercooled cloud droplets, Chem. Soc. Rev., 41, 6519, https://doi.org/10.1039/c2cs35200a, 2012.

Nelson, P. E., Dignani, M. C., and Anaissie, E. J.: Taxonomy, biology, and clinical aspects of *Fusarium* species, Clin. Microbiol. Rev., 7, 479–504, https://doi.org/10.1128/CMR.7.4.479, 1994.

Nirenberg, H. I. and O'Donnell, K.: New *Fusarium* species and combinations within the *Gibberella fujikuroi* species complex, Mycologia, 90, 434–458, 1998.

O'Sullivan, D., Murray, B. J., Malkin, T. L., Whale, T. F., Umo, N. S., Atkinson, J. D., Price, H. C., Baustian, K. J., Browse, J., and Webb, M. E.: Ice nucleation by fertile soil dusts: relative importance of mineral and biogenic components, Atmos. Chem. Phys., 14, 1853–1867, https://doi.org/10.5194/acp-14-1853-2014, 2014.

O'Sullivan, D., Murray, B. J., Ross, J. F., Whale, T. F., Price, H. C., Atkinson, J. D., Umo, N. S., and Webb, M. E.: The relevance of nanoscale biological fragments for ice nucleation in clouds, Sci. Rep.-UK, 5, 8082, https://doi.org/10.1038/srep08082, 2015.

O'Sullivan, D., Murray, B. J., Ross, J. F., and Webb, M. E.: The adsorption of fungal ice-nucleating proteins on mineral dusts: a terrestrial reservoir of atmospheric ice-nucleating particles, Atmos. Chem. Phys., 16, 7879–7887, https://doi.org/10.5194/acp-16-7879-2016, 2016.

Petters, M. D. and Wright, T. P.: Revisiting ice nucleation from precipitation samples, Geophys. Res. Lett., 42, 8758–8766, https://doi.org/10.1002/2015GL065733, 2015.

Pouleur, S., Richard, C., Martin, J.-G., and Antoun, H.: Ice Nucleation Activity in *Fusarium acuminatum* and *Fusarium avenaceum*, Appl. Environ. Microb., 58, 2960–2964, 1992.

Pratt, K. A., DeMott, P. J., French, J. R., Wang, Z., Westphal, D. L., Heymsfield, A. J., Twohy, C. H., Prenni, A. J., and Prather, K. A.: *In situ* detection of biological particles in cloud ice-crystals, Nat. Geosci., 2, 398–401, https://doi.org/10.1038/ngeo521, 2009.

Pruppacher, H. R. and Klett, J. D.: Microphysics of Clouds and Precipitation, Springer Netherlands, Dordrecht, 2nd Edn., https://doi.org/10.1007/978-0-306-48100-0, 1997.

Prussin, A. J., Li, Q., Malla, R., Ross, S. D., and Schmale, D. G.: Monitoring the Long-Distance Transport of *Fusarium graminearum* from Field-Scale Sources of Inoculum, Plant Dis., 98, 504–511, https://doi.org/10.1094/PDIS-06-13-0664-RE, 2014.

Pummer, B. G., Bauer, H., Bernardi, J., Bleicher, S., and Grothe, H.: Suspendable macromolecules are responsible for ice nucleation activity of birch and conifer pollen, Atmos. Chem. Phys., 12, 2541–2550, https://doi.org/10.5194/acp-12-2541-2012, 2012.

Pummer, B. G., Atanasova, L., Bauer, H., Bernardi, J., Druzhinina, I. S., Fröhlich-Nowoisky, J., and Grothe, H.: Spores of many common airborne fungi reveal no ice nucleation activity in oil immersion freezing experiments, Biogeosciences, 10, 8083–8091, https://doi.org/10.5194/bg-10-8083-2013, 2013.

Pummer, B. G., Budke, C., Augustin-Bauditz, S., Niedermeier, D., Felgitsch, L., Kampf, C. J., Huber, R. G., Liedl, K. R., Loerting, T., Moschen, T., Schauperl, M., Tollinger, M., Morris, C. E., Wex, H., Grothe, H., Pöschl, U., Koop, T., and Fröhlich-Nowoisky, J.: Ice nucleation by water-soluble macromolecules, Atmos. Chem. Phys., 15, 4077–4091, https://doi.org/10.5194/acp-15-4077-2015, 2015.

Richard, C., Martin, J. G., and Pouleur, S.: Ice nucleation activity identified in some phytopathogenic *Fusarium* species, Phytoprotection, 77, 83–92, https://doi.org/10.7202/706104ar, 1996.

Sands, D. C., Langhans, V. E., Scharen, A. L., and de Smet, G.: The association between bacteria and rain and possible resultant meteorological implications, J. Hungarian Meteorol. Serv., 86, 148–152, 1982.

Šantl-Temkiv, T., Sahyoun, M., Finster, K., Hartmann, S., Augustin-Bauditz, S., Stratmann, F., Wex, H., Clauss, T., Woetmann Nielsen, N., Havskov Sorensen, J., Smith Korsholm, U., Wick, L. Y., and Gosewinkel Karlson, U.: Characterization of airborne ice-nucleation-active bacteria and bacterial fragments, Atmos. Environ., 109, 105–117, https://doi.org/10.1016/j.atmosenv.2015.02.060, 2015.

Schmale, D. G. and Gordon, T. R.: Variation in susceptibility to pitch canker disease, caused by *Fusarium circinatum*, in native stands of *Pinus muricata*, Plant Pathol., 52, 720–725, https://doi.org/10.1111/j.1365-3059.2003.00925.x, 2003.

Schmale, D. G. and Ross, S. D.: Highways in the Sky: Scales of Atmospheric Transport of Plant Pathogens, Annu. Rev. Phytopathol., 53, 591–611, https://doi.org/10.1146/annurev-phyto-080614-115942, 2015.

Schmale, D. G., Ross, S. D., Fetters, T. L., Tallapragada, P., Wood-Jones, A. K., and Dingus, B.: Isolates of *Fusarium graminearum* collected 40–320 meters above ground level cause Fusarium head blight in wheat and produce trichothecene mycotoxins, Aerobiologia (Bologna), 28, 1–11, https://doi.org/10.1007/s10453-011-9206-2, 2012.

Schmid, D., Pridmore, D., Capitani, G., Battistutta, R., Neeser, J.-R., and Jann, A.: Molecular organisation of the ice nucleation protein InaV from *Pseudomonas syringae*, FEBS Lett., 414, 590–594, https://doi.org/10.1016/S0014-5793(97)01079-X, 1997.

Schnell, R. C. and Vali, G.: Atmospheric Ice Nuclei from Decomposing Vegetation, Nature, 236, 163–165, https://doi.org/10.1038/236163a0, 1972.

Schnell, R. C. and Vali, G.: World-wide Source of Leaf-derived Freezing Nuclei, Nature, 246, 212–213, 1973.

Seinfeld, J. H. and Pandis, S. N.: Atmospheric chemistry and physics – from air pollution to climate change, John Wiley & Sons, New York, 1998.

Sing, D. and Sing, C. F.: Impact of Direct Soil Exposures from Airborne Dust and Geophagy on Human Health, Int. J. Environ. Res. Pu., 7, 1205–1223, https://doi.org/10.3390/ijerph7031205, 2010.

Stopelli, E., Conen, F., Zimmermann, L., Alewell, C., and Morris, C. E.: Freezing nucleation apparatus puts new slant on study of biological ice nucleators in precipitation, Atmos. Meas. Tech., 7, 129–134, https://doi.org/10.5194/amt-7-129-2014, 2014.

Stopelli, E., Conen, F., Morris, C. E., Herrmann, E., Bukowiecki, N., and Alewell, C.: Ice nucleation active particles are efficiently removed by precipitating clouds, Sci. Rep.-UK, 5, 16433, https://doi.org/10.1038/srep16433, 2015.

Stopelli, E., Conen, F., Guilbaud, C., Zopfi, J., Alewell, C., and Morris, C. E.: Ice nucleators, bacterial cells and Pseudomonas syringae in precipitation at Jungfraujoch, Biogeosciences, 14, 1189–1196, https://doi.org/10.5194/bg-14-1189-2017, 2017.

Tesson, S. V. M. and Šantl-Temkiv, T.: Ice Nucleation Activity and Aeolian Dispersal Success in Airborne and Aquatic Microalgae, Front. Microbiol., 9, 2681, https://doi.org/10.3389/fmicb.2018.02681, 2018.

Torp, M. and Nirenberg, H. I.: *Fusarium langsethiae* sp. nov. on cereals in Europe, Int. J. Food Microbiol., 95, 247–256, https://doi.org/10.1016/j.ijfoodmicro.2003.12.014, 2004.

Tsumuki, H. and Konno, H.: Ice Nuclei Produced by *Fusarium* sp. Isolated from the Gut of the Rice Stem Borer, *Chilo suppres-*

salis Walker (Lepidoptera: Pyralidae), Biosci. Biotech. Bioch., 58, 578–579, 1994.

Tsumuki, H., Konno, H., Maeda, T., and Okamoto, Y.: An ice-nucleating active fungus isolated from the gut of the rice stem borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae), J. Insect. Physiol., 38, 119–125, https://doi.org/10.1016/0022-1910(92)90040-K, 1992.

Tsumuki, H., Yanai, H., and Aoki, T.: Identification of Ice-nucleating Active Fungus Isolated from the Gut of the Rice Stem Borer, *Chilo suppressalis* Walker (Lepidoptera: Pyralidae) and a Search for Ice-nucleating Active *Fusarium* Species, Ann. Phytopathol. Soc. Japan, 61, 334–339, https://doi.org/10.3186/jjphytopath.61.334, 1995.

Vali, G.: Quantitative Evaluation of Experimental Results an the Heterogeneous Freezing Nucleation of Supercooled Liquids, J. Atmos. Sci., 28, 402–409, https://doi.org/10.1175/1520-0469(1971)028<0402:QEOERA>2.0.CO;2, 1971.

Wang, B. and Jeffers, S. N.: Fusarium Root and Crown Rot: A Disease of Container-Grown Hostas, Plant Dis., 84, 980–988, https://doi.org/10.1094/PDIS.2000.84.9.980, 2000.

Warmink, J., Nazir, R., Corten, B., and van Elsas, J.: Hitchhikers on the fungal highway: The helper effect for bacterial migration via fungal hyphae, Soil Biol. Biochem., 43, 760–765, https://doi.org/10.1016/j.soilbio.2010.12.009, 2011.

Wilson, T. W., Ladino, L. A., Alpert, P. A., Breckels, M. N., Brooks, I. M., Browse, J., Burrows, S. M., Carslaw, K. S., Huffman, J. A., Judd, C., Kilthau, W. P., Mason, R. H., McFiggans, G., Miller, L. A., Nájera, J. J., Polishchuk, E., Rae, S., Schiller, C. L., Si, M., Temprado, J. V., Whale, T. F., Wong, J. P. S., Wurl, O., Yakobi-Hancock, J. D., Abbatt, J. P. D., Aller, J. Y., Bertram, A. K., Knopf, D. A., and Murray, B. J.: A marine biogenic source of atmospheric ice-nucleating particles, Nature, 525, 234–238, https://doi.org/10.1038/nature14986, 2015.

Wing, N., Bryden, W., Lauren, D., and Burgess, L.: Toxigenicity of *Fusarium* species and subspecies in section Gibbosum from different regions of Australia, Mycol. Res., 97, 1441–1446, https://doi.org/10.1016/S0953-7562(09)80214-1, 1993a.

Wing, N., Lauren, D. R., Bryden, W. L., and Burgess, L. W.: Toxicity and Trichothecene Production by *Fusarium acuminatum* subsp. *acuminatum* and *Fusarium acuminatum* subsp. *armeniacum*, Nat. Toxins, 1, 229–234, 1993b.

Wolber, P. K., Deininger, C. A., Southworth, M. W., Vandekerckhovet, J., Van Montagut, M., and Warren, G. J.: Identification and purification of a bacterial ice-nucleation protein, P. Natl. Acad. Sci. USA, 83, 7256–7260, 1986.

Zachariassen, K. E. and Kristiansen, E.: Ice Nucleation and Antinucleation in Nature, Cryobiology, 41, 257–279, https://doi.org/10.1006/cryo.2000.2289, 2000.

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To Freeze or Not to Freeze: Inhibition of Bacterial Ice Nucleators by Antifreeze Proteins

Ralph Schwidetzky[1], Anna T. Kunert[2], Mischa Bonn[1], Ulrich Pöschl[2], Hans Ramlov[3], Arthur L. DeVries[4], Janine Fröhlich-Nowoisky[2], Konrad Meister[1,5]

[1]Max Planck Institute for Polymer Science, 55128 Mainz, Germany; [2]Max Planck Institute for Chemistry, 55128 Mainz, Germany; [3]Roskilde University, 4000 Roskilde, Denmark; [4]University of Illinois at Urbana–Champaign, Urbana, IL, 61801, USA; [5]University of Alaskas Southeast, Juneau, AK, 99801, USA

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Authors: Ralph Schwidetzky[1], Anna T. Kunert[2], Mischa Bonn[1], Ulrich Pöschl[2], Hans Ramløv[3], Arthur L. DeVries[4], Janine Fröhlich-Nowoisky[2] and Konrad Meister*[1,5]

Affiliations:

[1]Max Planck Institute for Polymer Science, 55128 Mainz, Germany

[2]Max Planck Institute for Chemistry, 55128 Mainz, Germany

[3]Roskilde University, 4000 Roskilde, Denmark

[4]University of Illinois at Urbana–Champaign, Urbana, IL, 61801, USA

[5]University of Alaskas Southeast, Juneau, AK, 99801, USA

*Correspondence to: meisterk@mpip-mainz.mpg.de

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Antifreeze Proteins, Ice-Nucleating Proteins, Protein Interactions, Heterogeneous Ice Nucleation, Snomax®

Abstract:

Cold-adapted organisms use antifreeze proteins (AFPs) or ice-nucleating proteins (INPs) for the survival in freezing habitats. AFPs have been reported to be able to inhibit the activity of INPs, a property that would be of great physiological relevance. The generality of this effect is not understood, and for the few known examples of INP inhibition by AFPs, the molecular mechanisms remain unclear. Here, we report a comprehensive evaluation of the effects of all major classes of AFPs on the activity of bacterial ice nucleators using a high-throughput ice nucleation assay. We find that bacterial INPs are inhibited by certain AFPs, while others show no effect. Thus, the ability to inhibit the activity of INPs is not an intrinsic property of AFPs, and the interactions of INPs and different AFPs proceed through protein-specific rather than universal molecular mechanisms.

Ice formation is the most important liquid-to-solid phase transition on earth and is critical for fields as diverse as cryobiology, geology, and climate science [1-2]. The crystallization of water into ice and the process of ice recrystallization upon thawing are further major contributors to cell death and lethal to most organisms [3]. Ice nucleation-active bacteria are a primary cause of frost damage to plants, and in the earth's atmosphere, they can contribute to the formation of snow and rain [4-6]. The formation of ice is thermodynamically favored in water at temperatures below 0 ˚C, but the initial crystallization is kinetically hindered [7]. As a result, pure water can be supercooled to temperatures as low as -46 ˚C, below which homogenous ice nucleation occurs [8]. Freezing of water in biological systems is a heterogeneous process, facilitated by the presence of ice-nucleating agents of biological and abiotic origins [9-11]. The most efficient biological ice nucleators are ice-nucleating proteins (INPs) from bacteria such as *Pseudomonas syringae* and *Erwinia herbicola,* which can initiate ice formation at temperatures close to 0 ˚C [12-13].

Most known bacterial ice nucleators consist of large proteins with an estimated weight of 150-180 kDa that are anchored in the outer cell membranes. INPs are typically present as monomers but have repeatedly been shown to aggregate in the bacterial outer membranes [14-17]. The ice nucleation induced by bacteria generally occurs in the ranges of -2 ˚C to -4 ˚C, -5 ˚C to -7 ˚C and below -7 ˚C and is associated with INP subpopulations of different sizes with the monomers being the least and the multimer aggregates being the most efficient [18-19]. Based on their activity in droplet freezing experiments, the bacterial INP aggregates are usually grouped into classes A, B, and C [19]. Class A represents large protein oligomers of up to a hundred INPs [14] and class C consists of a few single proteins [14, 20]. However, up to now, the details of the structure and functionality of the INP aggregates remain the object of active research. On the molecular scale, the INPs are believed to function by organizing water into ice-like patterns, which increase in size as the temperature decreases until they are large enough to form a stable embryonic crystal, which leads to ice growth [21-22]. Potent biological ice nucleators have also been identified in freeze-tolerant organisms, i.e., insects that survive the freezing of a fraction of their body fluids, pollen, and fungi, as integral parts of their freeze-tolerance strategy in nature [11, 23-26].

Freeze-avoiding organisms have evolved an opposite approach to ensure survival in subzero environments. They produce antifreeze proteins (AFPs) or antifreeze glycoproteins (AFGPs) that are able to bind to embryonic ice crystals and arrest their macroscopic growth [11, 27-28]. The success of AF(G)Ps as efficient protection against freezing can be witnessed by their wide distribution among organisms of different kingdoms, phyla, and species [11]. The AF(G)Ps found

in different organisms show a remarkable diversity in structures [29], but share the same capability of binding to ice and the lowering of the temperatures of ice growth [28]. Despite having diametrically opposite functions, the INPs resemble AF(G)Ps in many respects. Both classes of proteins can interact with ice, and molecular simulations suggest that the active ice-binding surfaces and adjacent interfacial water of INPs are similar to AF(G)Ps, just on a much larger scale [20, 30]. In fact, there is increasing experimental evidence that AF(G)Ps have ice-nucleating activity [31-33], but at temperatures much lower than those of INPs.

Interestingly, AF(G)Ps have frequently been reported to also inhibit the activity of biological ice nucleators [34-35]. Such an effect would be of physiological importance and could explain why some cold-adapted organisms produce both AFPs and INPs [11, 23]. However, the mechanism of the inhibition of INPs by AFPs remains largely unknown and is highly debated [34-36]. For instance, Parody-Morreale *et al*. reported that antifreeze glycoproteins (AFGPs) from *Dissostichus mawsoni* inhibited the ice-nucleating activity of the bacteria *E. herbicola* and *P. syringae* [37], whereas Holt *et al.* found that in the presence of AFGPs the ice-nucleating activity of *P. syringae* was slightly enhanced [36]. Olsen and Duman reported that AFPs from the beetle *Dendroides canadensis* inhibited the activity of the bacteria *Pseudomonas fluorescens*, but not the INPs from the crane fly *Tipula trivittata* [38]. While the above studies clearly demonstrate the importance of the subject, the reported experiments focused on a limited number of antifreeze proteins and left many open questions. Here, we investigate the effect of all major classes of AFPs and the non-AFP bovine serum albumin (BSA) on bacterial ice nucleators using the recently developed high-throughput twin-plate ice nucleation assay (TINA) [39]. TINA enables the simultaneous measurement of a complete dilution series with high statistics, enabling the analysis and characterization of the efficiency of biological ice nuclei and their inhibitors with high accuracy [39].

Figure 1 shows the results of TINA measurements of a dilution series of bacterial ice nucleators from *P. syringae* (Snomax®). Snomax® is widely used as a model and reference system for biological and atmospheric ice nucleation studies [39-41] and contains a preparation of freeze-dried irradiated bacteria cells of *P. syringae*. The initial mass concentration was 0.1 mg/mL and was then serially diluted 10-fold, over six orders of magnitude, with pure water using an automated liquid handling station. The resulting ice nucleator concentration ranged from 0.1 mg/mL to 0.1 ng/mL, per 3 µL droplet. The cumulative ice nucleator number concentration (N_m) was calculated using Vali's formula and represents the total number of ice nucleators that are active above a certain temperature [42].

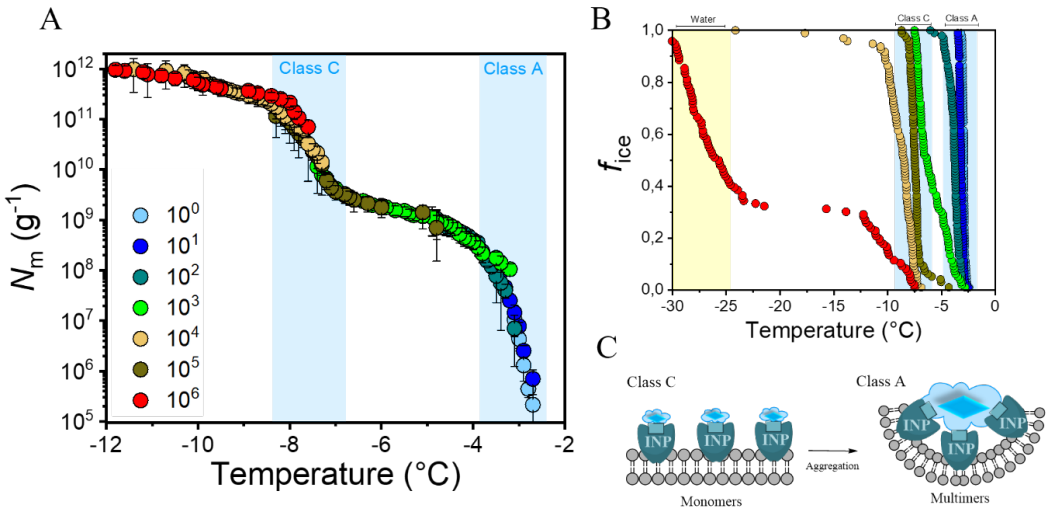

Figure 1. Freezing experiments of aqueous solutions of Snomax® containing bacterial ice nucleators from *P. syringae*. **A)** Cumulative number of ice nucleators (N_m) per unit mass of *P. syringae* vs. temperature for various dilutions indicated in the legend, starting with 0.1 mg/mL. The error bars were calculated using the counting error and the Gaussian error propagation. **B)** Fraction of frozen droplets (f_{ice}) for different *P. syringae* dilutions. Symbol colors indicate data from droplets with different concentrations and are identical to A. The temperature ranges for class A and C nucleators according to the definition by Turner *et al.* are shaded in light blue [19]. The yellow shaded region presents the temperature range in which pure water freezes in our system [39]. **C)** Schematic structure of class C and A nucleators. Class C consists of monomeric INPs, which aggregate to form the highly-efficient class A. The INPs are shown with their active ice-binding site and preordered water patterns in their vicinity.

For the pure bacterial ice nucleator solution, the spectrum shows two strong increases in $N_m(T)$ around ~-2.9 ˚C and ~-7.5 ˚C with distinct plateaus between ~-4.5 ˚C and ~-7 ˚C and above ~-9.5 ˚C (Figure 1). These values are reproducible (Supporting Figure 1), and consistent with previous studies [39-41]. The two distinct rises in the spectrum indicate that the ice nucleation activity of *P. syringae* stems from two distinct subpopulations of ice nucleators with different activation temperatures. These subpopulations have previously been assigned to isolated (~-7.5˚C) and aggregated (~-2.5˚C) INPs [19]. Aggregation of INPs occurs in the cell membrane of *P. syringae* under conditions of stress as shown in Figure 1C [13]. The aggregation of the INPs effectively increases the size of the ice-binding surface, which increases the ice nucleation activity though cooperative effects [43]. The plateaus at temperatures below the two increases of $N_m(T)$ indicate that there are no or few additional ice nucleators active at different temperatures in the investigated droplets [41]. The presence of two predominant ice nucleators becomes apparent when comparing the droplet freezing statistics of the different dilutions as shown in

Figure 1B. The two main bacterial ice nucleators with activities around ~-2.5 ˚C and ~-7.5 ˚C are apparent, as well as a third rise at ~-25 ˚C. We assign the observed rise at ~-2.5 ˚C to the highly efficient nucleators of class A and the less-efficient ice nucleators that cause nucleation around ~-7.5 ˚C to class C [19]. Protein complexes of class B were not clearly observed in our measurements which is in agreement with others [39, 41]. The third rise at ~-25 ˚C corresponds to the freezing point of pure water in our system [39]. At high Snomax® concentrations, intermolecular interactions between INPs and different bacterial fragments of Snomax® (*P. syringae*) occur, which results in aggregation and the formation of class A. Diluting the samples reduces the probability of such interactions and hence the formation of larger aggregates (Supporting Figure 2). At very high dilution, there are no more ice nucleators present and eventually, the curve resembles that of pure water.

Figure 2 shows the effect of BSA, the fish antifreeze proteins type 1 (AFP-1), type 3(AFP-3), the insect antifreeze protein from *Rhagium mordax* (*Rm*AFP), and the small antifreeze glycoproteins ($AFGP_{7-8}$) and larger $AFGP_{1-5}$ isoforms on the ice-nucleating activity of *P. syringae*. The investigated proteins vary significantly in terms of three-dimensional structures, putative ice-binding planes, and antifreeze activities [29]. The freezing assay experiments were performed at fixed AF(G)P concentration of 0.1 mg/mL, and the same dilution range for INP as that shown in Figure 1, which results in varying AF(G)P: INP ratios spanning from 1:1 (weight %) to a vast excess of AF(G)Ps.

The most efficient bacterial class A ice nucleators are predominately present in concentrated Snomax® (*P. syringae*) solutions. The addition of different proteins to the concentrated *P. syringae* solutions gives rise to three types of effect: (i) the addition of $AFGP_{1-5}$ has no effect on the freezing curve, and the freezing statistics are indistinguishable from that of Snomax® (*P. syringae*) in water. $AFGP_{1-5}$ is known to stabilize membranes [44-45], so that the aggregation of proteins in membrane structures (Figure 1B) is preserved and IN activity retained; (ii) AFP-3, $AFGP_{7-8}$, *Rm*AFP, and BSA have similar and rather small effects on the class A ice nucleators: The resulting response is comparable to that of the pure bacteria, only shifted by ~0.7 ˚C to lower temperatures (Figure 2A). This minor inhibition of the class A nucleators is statistically insignificant, but it should be noted that the f_{ice} at 0.5 was found to always be at lower temperatures compared to Snomax®. This could indicate a slight inhibition that originates from non-specific interactions of the different proteins with the outer cell membrane of *P. syringae*. Such interactions would perturb the formation of the precisely aligned INP aggregates that give rise to the high freezing temperatures, and, as a consequence, freezing occurs at lower

temperatures. This inhibition mechanism is independent of antifreeze activities, explaining why AFPs and non-AFPs show similar activities. (iii) The addition of AFP-1 shifts the freezing point by ~3.5 ˚C to lower temperatures. The resulting freezing temperature around ~-7 ˚C closely resembles that of class C nucleators. AFP-1 is known to insert and disturb model cell membranes [45-47] and evidently has the ability to prevent the formation of highly efficient class A aggregates, reducing the activity of class A to that of class C.

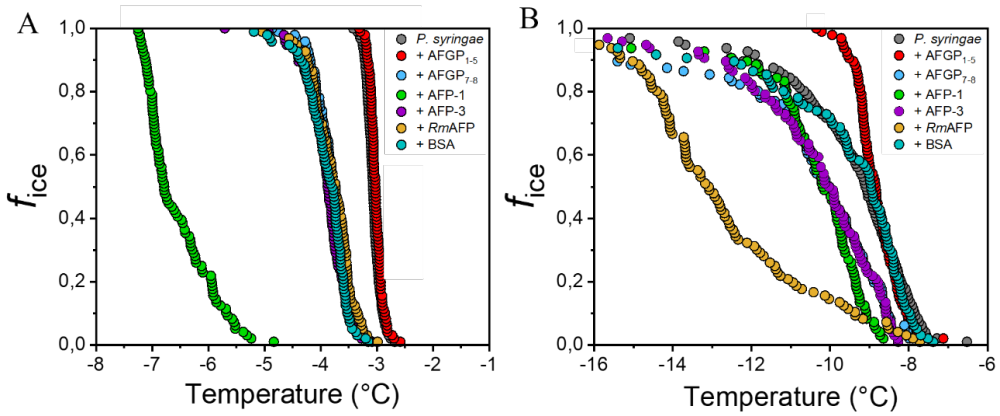

Figure 2: Freezing experiments of aqueous solutions of Snomax® containing ice nucleators from *P. syringae* in the presence of a variety of AF(G)Ps. Fraction of frozen droplets (f_{ice}) for concentrated (0.1 mg/mL, A) and diluted (1 ng/mL, B) Snomax® (*P. syringae*) solutions in the presence of different AF(G)Ps at fixed 0.1 mg/mL concentration. The high concentration Snomax® (*P. syringae*) solutions predominately contain INP aggregates (class A), while lower concentration solutions are mostly monomeric INPs (class C).

Additionally, we examined the effect of the AF(G)Ps on the class C ice nucleators of *P. syringae*, where the INPs are predominately present as smaller aggregates and monomers. We find that some AF(G)Ps inhibit ice nucleation activity and shift the freezing point to lower temperatures. Interestingly, the degree of the inhibition is markedly different from that observed for class A. For class C, *Rm*AFP is the most efficient inhibitor and AFP-1, the most efficient class A inhibitor, only shows moderate counter-activity. We further find that BSA shows no effect on class C, which is in line with previous studies that observed no inhibitory effect of non-AFPs on class C nucleators [34].

Interestingly, we find that the larger isoform $AFGP_{1-5}$ shows no inhibition but a moderate enhancement of the activity of class C nucleators, while the smaller isoform $AFGP_{7-8}$ inhibits the INPs. The enhancement of ice nucleation by the addition of $AFGP_{1-5}$ is interesting, since bacterial nucleators are very potent ice nucleators on their own. We rank the inhibition

efficiency in the order *Rm*AFP>>>>AFP-1=AFGP$_{7-8}$=AFP-3=BSA>AFGP$_{1-5}$. This ranking does not reflect the efficiency of the antifreeze activity of the proteins, except for *Rm*AFP, which is the most active AFP known.

To further explore the relationship between the inhibition of the INPs and antifreeze activity, we studied inactive mutants of AFP-3 and AFGP$_{1-5}$. In the AFP-3* variant, the threonine 18 residue was replaced by asparagine, which results in the elimination of antifreeze activity [29]. In the AFGP$_{1-5}$* variant, we oxidized the C-6 hydroxyls of the galactose moieties to carboxylic acids, which results in the elimination of >80 % of the antifreeze activity [48]. In Figure 3, we compare the true supercooling of all the AFPs and different variants at high and low Snomax® (*P. syringae*) concentrations. Supercooling values ΔT represent the temperature difference at $f_{ice} = 0.5$ between Snomax® (*P. syringae*) with and without the added protein at high (left panel) and low (right panel) Snomax® (*P. syringae*) concentrations. All proteins were measured at least three times. For both AFP-3s, we find no significant difference between active and inactive variants on the class A or C ice nucleators. For the modified AFGP-Carboxyl variant, we likewise observe no significant change relative to the natural AFGP$_{1-5}$. AFP-1 and *Rm*AFP both inhibit ice nucleation, yet each with marked and opposite efficiencies for class A and class C INPs.

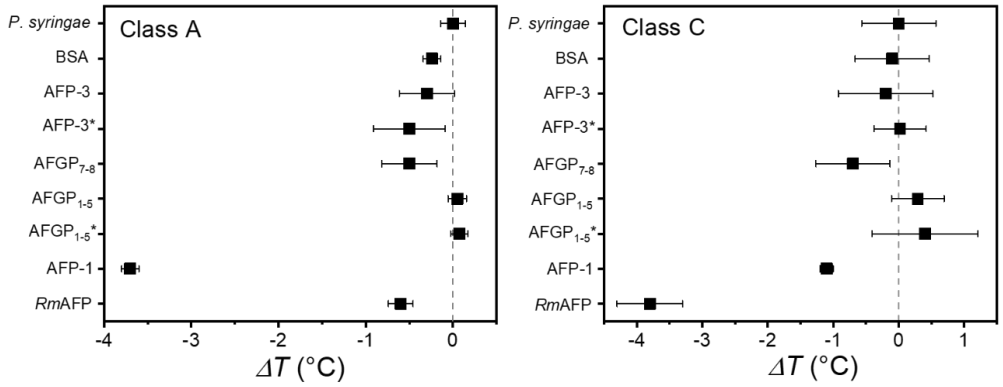

Figure 3: Ice nucleation inhibition efficiency of AF(G)Ps and non-AFP on class A (aggregates) and C (monomer) bacterial ice nucleators solutions. Shifts represent the difference of f_{ice} at 0.5 between *P. syringae* and added AF(G))P. Each experiment was performed at least three times, and the error bars represent the standard deviation between the individual measurements.

Freeze-avoiding organisms extend their supercooling abilities by the accumulation of colligative solutes, by the removal of ice nucleators, by inhibiting the activity of ice nucleators present, or alternately by inactivating embryonic ice crystals [11]. The importance of biological macromolecules that inhibit ice nucleators has been recognized, but there have been no systematic studies with sufficiently robust analytical methods [49]. AF(G)Ps were among the most studied systems due to their known properties to recognize and bind ice crystals inhibiting their growth [34-35]. However, the generality of such inhibition of INPs by AF(G)P is not well supported, and for the few reported examples of inhibition by AF(G)Ps, the mechanism is unknown. Our results provide clear evidence that some AF(G)Ps can inhibit the activity of bacterial INPs, while others show no effect. Bacterial INPs are known to form preordered "ice-like" interfacial water domains at their putative ice-binding sites [22]. Likewise, AFPs like *Rm*AFP are known to use preordered "ice-like" interfacial water domains for the recognition and subsequent attachment to ice [32, 50-53]. We speculate that the preordered water domains of the AFPs will have a high affinity for similar "ice-like" water domains and, therefore, for the preordered water of the INPs [50]. Consequently, upon diffusion of AFPs into the vicinity of INPs, they will remain longer in the hydration shell of INPs and thereby disturb the interfacial water arrangement required for a nucleation event. This form of inhibition would be dependent on the similarity of the structure and hydration motifs of AFPs and INPs and on the antifreeze activity of the AFPs, which has been related to the extent of their preordered interfacial water domains [52]. Bacterial INPs from *P. syringae* and hyperactive insect AFPs like *Rm*AFP are proposed to bind ice though a flat array of TxT repeats, where T is threonine and x a non-conserved amino acid [30]. Hence, the hyperactive *Rm*AFP shows strong inhibition. Non-AFPs and inactive AFP variants will also randomly diffuse through the preordered water domains of INPs, but lack the affinity for preordered interfacial water and therefore fail to mask the INPs. This hypothetical mechanism is schematically shown in Figure 4. AFGPs are unique because of their highly flexible solution structure, multiple ice-binding sites, and capability to stabilize membranes [44, 54-55]. Our observations that the larger $AFGP_{1-5}$ isoforms do not inhibit bacterial INPs but rather show no effect or even a slight enhancement are in agreement with Holt *et al.* [36]. We speculate that larger AFGPs can stabilize INPs in the membranes and potentially link them to form larger aggregates that nucleate at higher temperatures. Our results further demonstrate that the highly efficient class A nucleators are more vulnerable to inhibition. For the most active inhibitor AFP-1, we propose a mechanism in which the proteins interact with the cell membrane of the bacteria to prevent the aggregation of the INP monomers, thereby preventing the formation of the highly efficient class A nucleators. This mechanism is in line with findings that AFP-1 directly inserts

into model cell membranes, whereas AFGPs and other AFPs interact weaker and show less effects on the membrane [45-47]. The different mechanisms underlying the inhibition of bacterial INPs by AF(G)Ps are likely highly specific. Thus, we conclude that no universal INP inhibition mechanism through AFPs exists. This is further supported by the findings that AFP-1, the best inhibitor of bacterial INPs, had no effect on fungal INPs (Supporting Figure 3). Ice nucleation can be promoted by a range of properties, including charge [56], lattice matching [57], hydrophobicity [58] or morphology [59]. In such cases, it is not apparent that AF(G)Ps will show inhibitory activity.

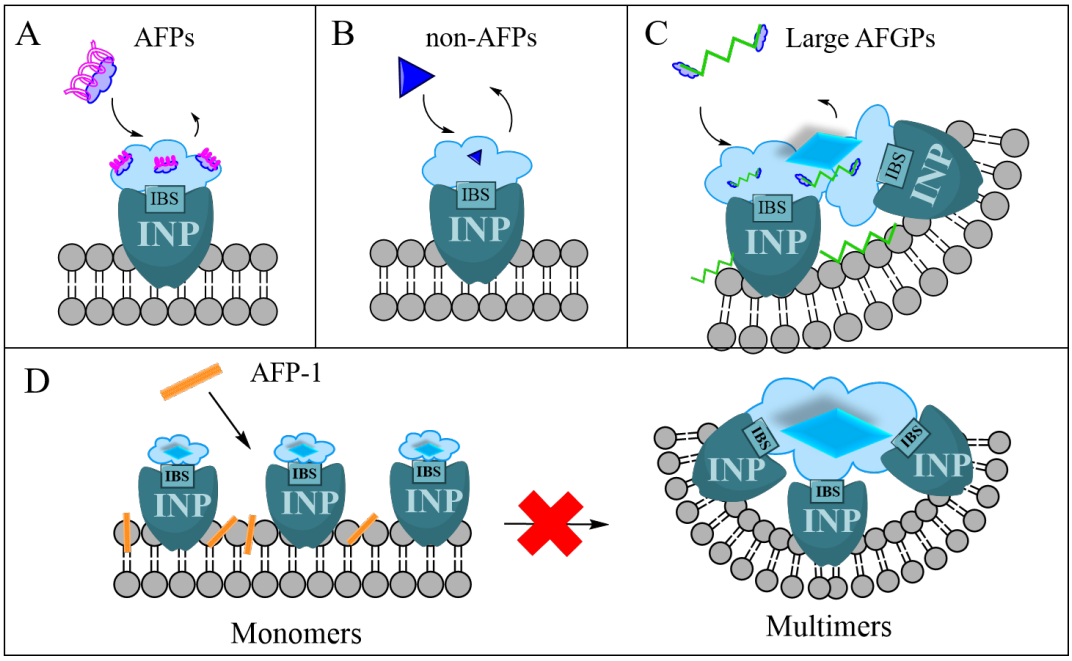

Figure 4: Proposed mechanism of the inhibition of ice nucleators from *P. syringae* by AF(G)Ps. INPs are shown with their active ice-binding sites (IBS) and preordered ice-like water patterns in their vicinity (blue clouds). **A)** Hyperactive AFPs like *Rm*AFP use preordered water domains to bind to ice. They also have structural similarity to the INPs of *P. syringae* [30]. Hence, preordered water domains of *Rm*AFP will have an affinity for the preordered interfacial water domains of the bacterial INPs. As a result, *Rm*AFP will disturb the preordering of the interfacial water that is needed for ice nucleation and masks the bacterial ice-nucleating sites. **B)** Non-AFPs have no preordered water domains (no blue cloud) and therefore no affinity for the interfacial water domains of INPs. **C)** Large AFGP isoforms can stabilize and link INPs to form larger aggregates that nucleate at higher temperatures. **D)** AFP-1 interacts with the cell membranes of the bacteria to prevent the functional aggregation of the INP monomers, which then give rise to the highly efficient class A nucleators.

Associated Content

Experimental details of the setups and the measurement procedure, Supporting Figures 1-3 (Ice nucleation measurements of Snomax®; Dynamic Light Scattering measurements; Ice nucleation measurements of fungal ice nucleators.)

Corresponding Author

Email: meisterk@mpip-mainz.mpg.de

Competing interests

The authors declare no competing financial interests.

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References

1. Vergara-Temprado, J.; Miltenberger, A. K.; Furtado, K.; Grosvenor, D. P.; Shipway, B. J.; Hill, A. A.; Wilkinson, J. M.; Field, P. R.; Murray, B. J.; Carslaw, K. S. Strong control of Southern Ocean cloud reflectivity by ice-nucleating particles. *Proceedings of the National Academy of Sciences* **2018,** *115* (11), 2687-2692.
2. Mazur, P. Cryobiology: the freezing of biological systems. *Science (New York, N.Y.)* **1970,** *168* (3934), 939-49.
3. Ramlov, H. Aspects of natural cold tolerance in ectothermic animals. *Hum Reprod* **2000,** *15 Suppl 5*, 26-46.
4. Morris, C. E.; Conen, F.; Alex Huffman, J.; Phillips, V.; Poschl, U.; Sands, D. C. Bioprecipitation: a feedback cycle linking earth history, ecosystem dynamics and land use through biological ice nucleators in the atmosphere. *Global change biology* **2014,** *20* (2), 341-51.
5. Failor, K. C.; Schmale, D. G.; Vinatzer, B. A.; Monteil, C. L. Ice nucleation active bacteria in precipitation are genetically diverse and nucleate ice by employing different mechanisms. *The ISME Journal* **2017,** *11* (12), 2740-2753.
6. Joly, M.; Amato, P.; Deguillaume, L.; Monier, M.; Hoose, C.; Delort, A. Direct quantification of total and biological ice nuclei in cloud water. *Atmos. Chem. Phys.* **2014,** *14*.
7. Murray, B. J.; Broadley, S. L.; Wilson, T. W.; Bull, S. J.; Wills, R. H.; Christenson, H. K.; Murray, E. J. Kinetics of the homogeneous freezing of water. *Physical Chemistry Chemical Physics* **2010,** *12* (35), 10380-10387.
8. Kim, K. H.; Späh, A.; Pathak, H.; Perakis, F.; Mariedahl, D.; Amann-Winkel, K.; Sellberg, J. A.; Lee, J. H.; Kim, S.; Park, J.; Nam, K. H.; Katayama, T.; Nilsson, A. Maxima in the thermodynamic response and correlation functions of deeply supercooled water. *Science (New York, N.Y.)* **2017,** *358* (6370), 1589-1593.

9. Zachariassen, K. E.; Kristiansen, E.; Pedersen, S. A.; Hammel, H. T. Ice nucleation in solutions and freeze-avoiding insects—homogeneous or heterogeneous? *Cryobiology* **2004,** *48* (3), 309-321.
10. Fröhlich-Nowoisky, J.; Kampf, C. J.; Weber, B.; Huffman, J. A.; Pöhlker, C.; Andreae, M. O.; Lang-Yona, N.; Burrows, S. M.; Gunthe, S. S.; Elbert, W.; Su, H.; Hoor, P.; Thines, E.; Hoffmann, T.; Després, V. R.; Pöschl, U. Bioaerosols in the Earth system: Climate, health, and ecosystem interactions. *Atmospheric Research* **2016,** *182*, 346-376.
11. Duman, J. G. Antifreeze and ice nucleator proteins in terrestrial arthropods. *Annual review of physiology* **2001,** *63*, 327-57.
12. Kozloff, L. M.; Schofield, M. A.; Lute, M. Ice nucleating activity of Pseudomonas syringae and Erwinia herbicola. *J Bacteriol* **1983,** *153* (1), 222-231.
13. Maki, L. R.; Galyan, E. L.; Chang-Chien, M. M.; Caldwell, D. R. Ice nucleation induced by pseudomonas syringae. *Applied microbiology* **1974,** *28* (3), 456-459.
14. Govindarajan, A. G.; Lindow, S. E. Size of bacterial ice-nucleation sites measured *in situ* by radiation inactivation analysis. *Proceedings of the National Academy of Sciences* **1988,** *85* (5), 1334-1338.
15. Southworth, M. W.; Wolber, P. K.; Warren, G. J. Nonlinear relationship between concentration and activity of a bacterial ice nucleation protein. *Journal of Biological Chemistry* **1988,** *263* (29), 15211-15216.
16. Mueller, G. M.; Wolber, P. K.; Warren, G. J. J. C. Clustering of ice nucleation protein correlates with ice nucleation activity. *Cryobiology* **1990,** *27* (4), 416-422.
17. Schmid, D.; Pridmore, D.; Capitani, G.; Battistutta, R.; Neeser, J.-R.; Jann, A. J. F. l. Molecular organisation of the ice nucleation protein InaV from Pseudomonas syringae. *FEBS Letters* **1997,** *414* (3), 590-594.
18. Yankofsky, S. A.; Levin, Z.; Bertold, T.; Sandlerman, N. Some Basic Characteristics of Bacterial Freezing Nuclei. *Journal of Applied Meteorology* **1981,** *20* (9), 1013-1019.
19. Turner, M. A.; Arellano, F.; Kozloff, L. M. Three separate classes of bacterial ice nucleation structures. *Journal of Bacteriology* **1990,** *172* (5), 2521-2526.
20. Garnham, C. P.; Campbell, R. L.; Walker, V. K.; Davies, P. L. Novel dimeric beta-helical model of an ice nucleation protein with bridged active sites. *BMC structural biology* **2011,** *11*, 36.
21. Knight, C. A. The Freezing of Supercooled Liquids. *American Journal of Physics* **1968,** *36* (5), 466-467.
22. Hudait, A.; Moberg, D. R.; Qiu, Y.; Odendahl, N.; Paesani, F.; Molinero, V. Preordering of water is not needed for ice recognition by hyperactive antifreeze proteins. *Proceedings of the National Academy of Sciences* **2018,** *115* (33), 8266-8271.
23. Dreischmeier, K.; Budke, C.; Wiehemeier, L.; Kottke, T.; Koop, T. Boreal pollen contain ice-nucleating as well as ice-binding 'antifreeze' polysaccharides. *Sci Rep* **2017,** *7*, 41890-41890.
24. Fröhlich-Nowoisky, J.; Hill, T. C. J.; Pummer, B. G.; Yordanova, P.; Franc, G. D.; Pöschl, U. Ice nucleation activity in the widespread soil fungus *Mortierella alpina*. *Biogeosciences* **2015,** *12* (4), 1057-1071.
25. Pummer, B. G.; Budke, C.; Augustin-Bauditz, S.; Niedermeier, D.; Felgitsch, L.; Kampf, C. J.; Huber, R. G.; Liedl, K. R.; Loerting, T.; Moschen, T.; Schauperl, M.; Tollinger, M.; Morris, C. E.; Wex, H.; Grothe, H.; Pöschl, U.; Koop, T.; Fröhlich-Nowoisky, J. Ice nucleation by water-soluble macromolecules. *Atmos. Chem. Phys.* **2015,** *15* (8), 4077-4091.
26. Kunert, A. T.; Pöhlker, M. L.; Krevert, C. S.; Wieder, C.; Speth, K. R.; Hanson, L. E.; Morris, C. E.; Schmale Iii, D. G.; Pöschl, U.; Fröhlich-Nowoisky, J. Highly active and stable fungal ice nuclei are widespread among Fusarium species. *Biogeosciences Discuss.* **2019,** *2019*, 1-19.
27. DeVries, A. L. Glycoproteins as biological antifreeze agents in antarctic fishes. *Science (New York, N.Y.)* **1971,** *172* (3988), 1152-5.
28. Raymond, J. A.; DeVries, A. L. Adsorption inhibition as a mechanism of freezing resistance in polar fishes. *Proceedings of the National Academy of Sciences* **1977,** *74* (6), 2589-2593.

29. Olijve, L. L. C.; Meister, K.; DeVries, A. L.; Duman, J. G.; Guo, S.; Bakker, H. J.; Voets, I. K. Blocking rapid ice crystal growth through nonbasal plane adsorption of antifreeze proteins. *Proceedings of the National Academy of Sciences* **2016,** *113* (14), 3740-3745.
30. Hudait, A.; Odendahl, N.; Qiu, Y.; Paesani, F.; Molinero, V. Ice-Nucleating and Antifreeze Proteins Recognize Ice through a Diversity of Anchored Clathrate and Ice-like Motifs. *Journal of the American Chemical Society* **2018,** *140* (14), 4905-4912.
31. Eickhoff, L.; Dreischmeier, K.; Zipori, A.; Sirotinskaya, V.; Adar, C.; Reicher, N.; Braslavsky, I.; Rudich, Y.; Koop, T. Contrasting Behavior of Antifreeze Proteins: Ice Growth Inhibitors and Ice Nucleation Promoters. *The Journal of Physical Chemistry Letters* **2019**, 966-972.
32. Liu, K.; Wang, C.; Ma, J.; Shi, G.; Yao, X.; Fang, H.; Song, Y.; Wang, J. Janus effect of antifreeze proteins on ice nucleation. *Proceedings of the National Academy of Sciences* **2016,** *113* (51), 14739-14744.
33. Wilson, P. W.; Osterday, K. E.; Heneghan, A. F.; Haymet, A. D. J. Type I Antifreeze Proteins Enhance Ice Nucleation above Certain Concentrations. *Journal of Biological Chemistry* **2010,** *285* (45), 34741-34745.
34. Tomalty, H. E.; Walker, V. K. Perturbation of bacterial ice nucleation activity by a grass antifreeze protein. *Biochemical and biophysical research communications* **2014,** *452* (3), 636-41.
35. Wilson, P. W.; Leader, J. P. Stabilization of supercooled fluids by thermal hysteresis proteins. *Biophysical Journal* **1995,** *68* (5), 2098-2107.
36. Holt, C. B. The effect of antifreeze proteins and poly(vinyl alcohol) on the nucleation of ice: A preliminary study. *Cryoletters* **2003,** *24* (5), 323-330.
37. Parody-Morreale, A.; Murphy, K. P.; Cera, E. D.; Fall, R.; DeVries, A. L.; Gill, S. J. Inhibition of bacterial ice nucleators by fish antifreeze glycoproteins. *Nature* **1988,** *333* (6175), 782-783.
38. Olsen, T. M.; Duman, J. G. Maintenance of the supercooled state in the gut fluid of overwintering pyrochroid beetle larvae, Dendroides canadensis : role of ice nucleators and antifreeze proteins. *Journal of Comparative Physiology B* **1997,** *167* (2), 114-122.
39. Kunert, A. T.; Lamneck, M.; Helleis, F.; Pöschl, U.; Pöhlker, M. L.; Fröhlich-Nowoisky, J. Twin-plate Ice Nucleation Assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples. *Atmos. Meas. Tech.* **2018,** *11* (11), 6327-6337.
40. Wex, H.; Augustin-Bauditz, S.; Boose, Y.; Budke, C.; Curtius, J.; Diehl, K.; Dreyer, A.; Frank, F.; Hartmann, S.; Hiranuma, N.; Jantsch, E.; Kanji, Z. A.; Kiselev, A.; Koop, T.; Möhler, O.; Niedermeier, D.; Nillius, B.; Rösch, M.; Rose, D.; Schmidt, C.; Steinke, I.; Stratmann, F. Intercomparing different devices for the investigation of ice nucleating particles using Snomax® as test substance. *Atmos. Chem. Phys.* **2015,** *15* (3), 1463-1485.
41. Budke, C.; Koop, T. BINARY: an optical freezing array for assessing temperature and time dependence of heterogeneous ice nucleation. *Atmos. Meas. Tech.* **2015,** *8* (2), 689-703.
42. Vali, G. Quantitative Evaluation of Experimental Results an the Heterogeneous Freezing Nucleation of Supercooled Liquids. *Journal of the Atmospheric Sciences* **1971,** *28* (3), 402-409.
43. Qiu, Y.; Hudait, A.; Molinero, V. How Size and Aggregation of Ice-Binding Proteins Control Their Ice Nucleation Efficiency. *Journal of the American Chemical Society* **2019,** *141* (18), 7439-7452.
44. Huelsz-Prince, G.; DeVries, A. L.; Bakker, H. J.; van Zon, J. S.; Meister, K. Effect of Antifreeze Glycoproteins on Organoid Survival during and after Hypothermic Storage. *Biomolecules* **2019,** *9* (3).
45. Tomczak, M. M.; Hincha, D. K.; Estrada, S. D.; Wolkers, W. F.; Crowe, L. M.; Feeney, R. E.; Tablin, F.; Crowe, J. H. A mechanism for stabilization of membranes at low temperatures by an antifreeze protein. *Biophys J* **2002,** *82* (2), 874-81.
46. Garner, J.; Inglis, S. R.; Hook, J.; Separovic, F.; Harding, M. M. A solid-state NMR study of the interaction of fish antifreeze proteins with phospholipid membranes. *European biophysics journal : EBJ* **2008,** *37* (6), 1031-8.
47. Kar, R. K.; Mroue, K. H.; Kumar, D.; Tejo, B. A.; Bhunia, A. Structure and Dynamics of Antifreeze Protein–Model Membrane Interactions: A Combined Spectroscopic and Molecular Dynamics Study. *The Journal of Physical Chemistry B* **2016,** *120* (5), 902-914.

48. Shier, W. T.; Lin, Y.; De Vries, A. L. Structure and mode of action of glycoproteins from an antarctic fish. *Biochimica et Biophysica Acta (BBA) - Protein Structure* **1972,** *263* (2), 406-413.
49. Fujikawa, S.; Kuwabara, C.; Kasuga, J.; Arakawa, K. Supercooling-Promoting (Anti-ice Nucleation) Substances. *Adv Exp Med Biol* **2018,** *1081*, 289-320.
50. Meister, K.; Strazdaite, S.; DeVries, A. L.; Lotze, S.; Olijve, L. L. C.; Voets, I. K.; Bakker, H. J. Observation of ice-like water layers at an aqueous protein surface. *Proceedings of the National Academy of Sciences* **2014,** *111* (50), 17732-17736.
51. Kozuch, D. J.; Stillinger, F. H.; Debenedetti, P. G. Combined molecular dynamics and neural network method for predicting protein antifreeze activity. *Proceedings of the National Academy of Sciences* **2018,** *115* (52), 13252-13257.
52. Meister, K.; Ebbinghaus, S.; Xu, Y.; Duman, J. G.; DeVries, A.; Gruebele, M.; Leitner, D. M.; Havenith, M. Long-range protein–water dynamics in hyperactive insect antifreeze proteins. *Proceedings of the National Academy of Sciences* **2013,** *110* (5), 1617-1622.
53. Meister, K.; Moll, C. J.; Chakraborty, S.; Jana, B.; DeVries, A. L.; Ramløv, H.; Bakker, H. J. Molecular structure of a hyperactive antifreeze protein adsorbed to ice. *The Journal of Chemical Physics* **2019,** *150* (13), 131101.
54. Giubertoni, G.; Meister, K.; DeVries, A. L.; Bakker, H. J. Determination of the Solution Structure of Antifreeze Glycoproteins Using Two-Dimensional Infrared Spectroscopy. *The Journal of Physical Chemistry Letters* **2019,** *10* (3), 352-357.
55. Hays, L. M.; Feeney, R. E.; Crowe, L. M.; Crowe, J. H.; Oliver, A. E. Antifreeze glycoproteins inhibit leakage from liposomes during thermotropic phase transitions. *Proceedings of the National Academy of Sciences* **1996,** *93* (13), 6835-40.
56. Hudait, A.; Molinero, V. Ice Crystallization in Ultrafine Water–Salt Aerosols: Nucleation, Ice-Solution Equilibrium, and Internal Structure. *Journal of the American Chemical Society* **2014,** *136* (22), 8081-8093.
57. Pruppacher, H. R. On the growth of ice crystals in supercooled water and aqueous solution drops. *Pure and Applied geophysics* **1967,** *68* (1), 186-195.
58. Li, K.; Xu, S.; Chen, J.; Zhang, Q.; Zhang, Y.; Cui, D.; Zhou, X.; Wang, J.; Song, Y. Viscosity of interfacial water regulates ice nucleation. *Applied Physics Letters* **2014,** *104* (10), 101605.
59. Zhang, Z.; Liu, X.-Y. Control of ice nucleation: freezing and antifreeze strategies. *Chemical Society Reviews* **2018,** *47* (18), 7116-7139.

Supplementary Material

To Freeze or Not to Freeze: Inhibition of Bacterial Ice Nucleators by Antifreeze Proteins

Ralph Schwidetzky[1], Anna T. Kunert[2], Mischa Bonn[1], Ulrich Pöschl[2], Hans Ramlov[3], Arthur L. DeVries[4], Janine Fröhlich-Nowoisky[2], Konrad Meister[1,5]

[1]Max Planck Institute for Polymer Science, 55128 Mainz, Germany; [2]Max Planck Institute for Chemistry, 55128 Mainz, Germany; [3]Roskilde University, 4000 Roskilde, Denmark; [4]University of Illinois at Urbana–Champaign, Urbana, IL, 61801, USA; [5]University of Alaskas Southeast, Juneau, AK, 99801, USA

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Supporting Information:

To Freeze or Not to Freeze: Inhibition of Bacterial Ice Nucleators by Antifreeze Proteins

Authors: Ralph Schwidetzky[1], Anna T. Kunert[2], Mischa Bonn[1], Ulrich Pöschl[2], Hans Ramløv[3], Arthur L. DeVries[4], Janine Fröhlich-Nowoisky[2] and Konrad Meister*[1,5]

Affiliations:

[1]Max Planck Institute for Polymer Science, 55128 Mainz, Germany

[2]Max Planck Institute for Chemistry, 55128 Mainz, Germany

[3]Roskilde University, 4000 Roskilde, Denmark

[4]University of Illinois at Urbana–Champaign, Urbana, IL, 61801, USA

[5]University of Alaska Southeast, Juneau, AK, 99801, USA

*Correspondence to: meisterk@mpip-mainz.mpg.de

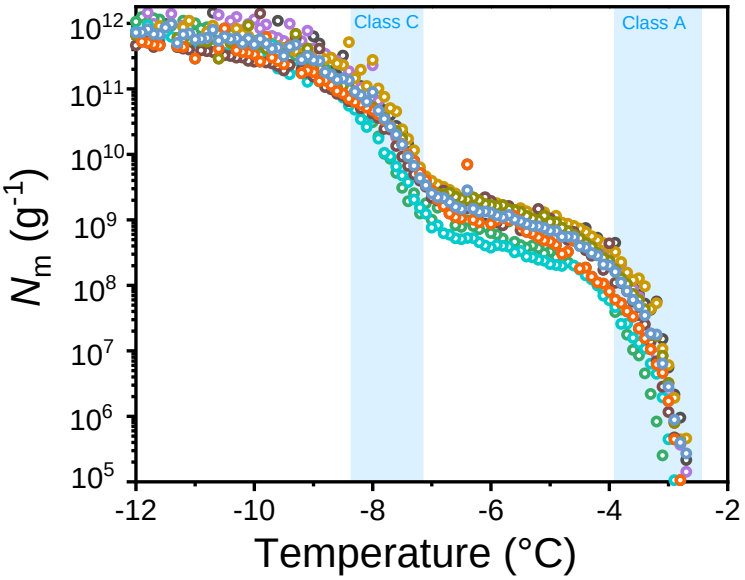
Experimental Methods

Protein Samples. Snomax® was obtained from SMI Snow Makers AG (Thun, Switzerland) and contains a preparation of fragmented freeze-dried, irradiated bacteria cells of *P syringae*. AFP-1 was purified from winter flounder *Pseudopleuronectes americanus*[1]. $AFGP_{1-5}$ and $AFGP_{7-8}$ were purified from the Antarctic toothfish *Dissostichus mawsoni*[2]. *Rm*AFP was obtained by recombinant protein expression as described elsewhere[3]. We investigated AFP-3 purified from the Antarctic eelpout (*Lycodichthys dearborni*) and obtained by recombinant protein expression, including the mutant T18N[4]. In this mutant, the threonine residue at position 18 is replaced by asparagine which causes the complete loss of antifreeze activity[4]. For the $AFGP_{1-5}$ variant, we oxidized the C-6 hydroxyl groups of the galactose moieties to carboxylic acids are described previously [5]. Only AF(G)P samples with proven antifreeze activity were used, and the activity was determined prior to the experiments using nanoliter cryoscopy. BSA was obtained from Sigma Aldrich and was used without further purification. Pure water was prepared as described elsewhere [6].

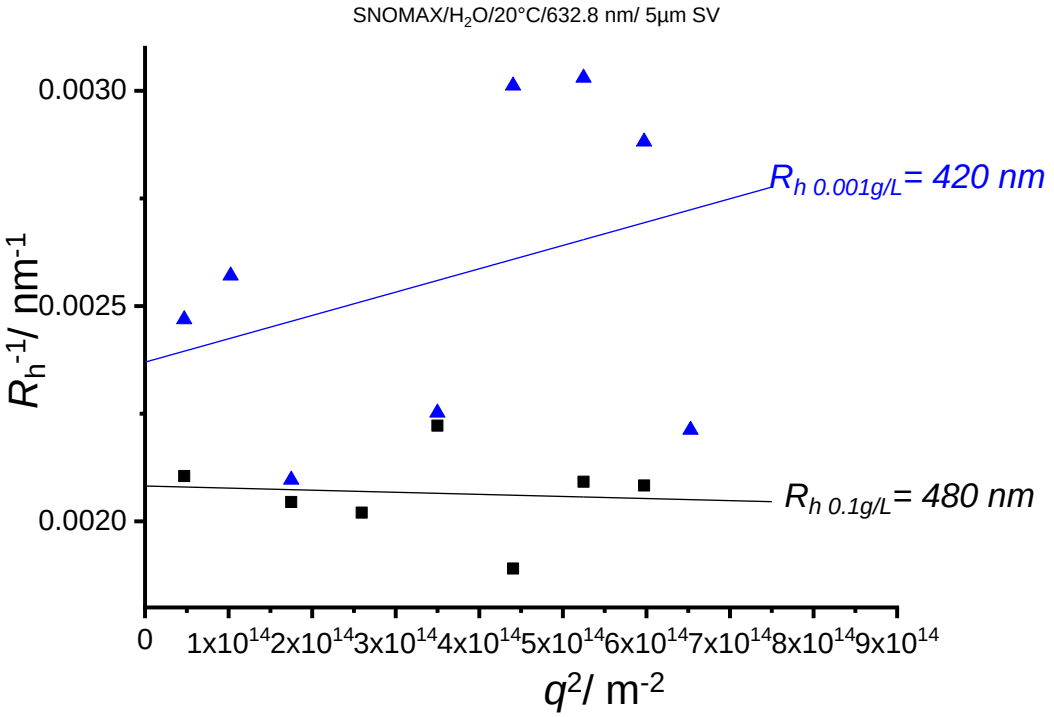
Fusarium acuminatum from USDA-ARS, Michigan State University (Linda E. Hanson, East Lansing, MI, USA) was cultivated on full-strength potato dextrose agar (VWR International GmbH, Darmstadt, Germany) first at room temperature for one week and then at 6 °C for about 4 weeks. Aqueous extract containing proteinaceous IN from spores and mycelial surfaces was prepared as described elsewhere [7].

TINA Experiments. Ice nucleation experiments were performed using a high-throughput droplet freezing assay. The details of the instrument have been described previously [6]. In a typical experiment, a 0.1 mg/mL solution of Snomax® (*P. syringae)* in pure water was serially diluted ten-fold by a liquid handling station (epMotion ep5073, Eppendorf, Hamburg, Germany) with either a solution of AF(G)Ps (0.1 mg/mL) or pure water. For each dilution, 96 droplets (3 µL) per dilution were placed on two 384-well-plates and tested with a continuous cooling-rate of 1 °C/min from 0 °C to -30 °C. The freezing of droplets was determined by two infrared cameras (Seek Therman Compact XR, Seek Thermal Inc., Santa Barbara, CA, USA). The uncertainty in the temperature of the setup was ±0.2 °C. The obtained fraction of frozen droplets and the counting error were used to calculate the cumulative number of ice nucleators with the associated error using the Vali formula and the Gaussian error propagation [6, 8]. The pH values of all samples were controlled before TINA measurements and were ~6-7. Samples were measured at least three times in independent experiments except for the variants which were measured twice.

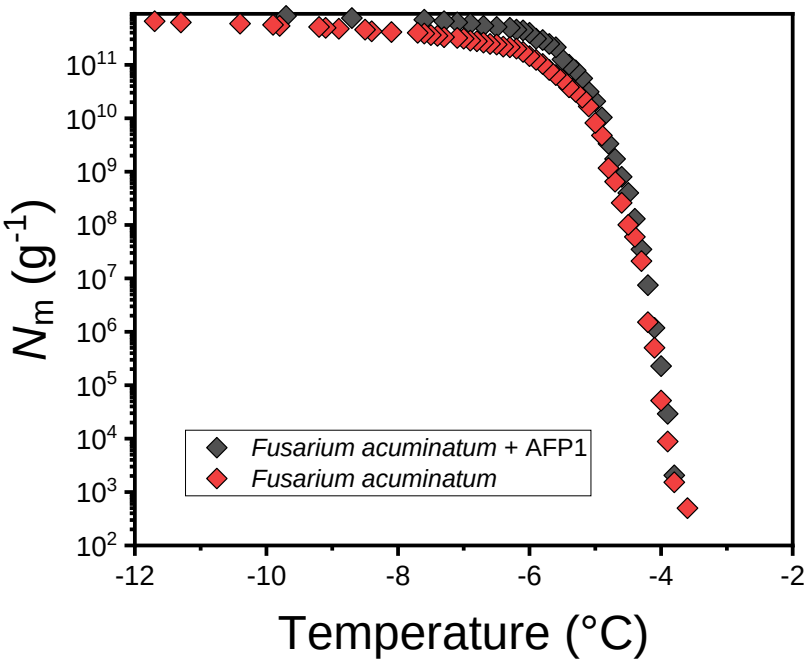
DLS measurements. The hydrodynamic radii (Rh) of 0.1 g/L and 0.001 g/L Snomax® in ultrapure water were determined using dynamic light scattering (DLS). Light scattering measurements were performed on an ALV spectrometer consisting of a goniometer and an ALV-5004 multiple-tau full-digital correlator (320 channels), which allows measurements over an angular range from 30° to 150°. A He-Ne laser (wavelength of 632.8 nm) was used as light source. Measurements were performed at 20 °C at 9 angles ranging from 30° to 150°.



Supporting Figure 1: Freezing experiments of aqueous solutions of Snomax® containing bacterial ice nucleators from *P. syringae*. Shown are the cumulative number of ice nucleators per unit mass of *P. syringae* vs. temperature for eleven independent experiments. The temperature ranges for class A and class C INPs according to the definition by Turner *et al.* are shaded in light blue[9].



Supporting Figure 2: Hydrodynamic radii of Snomax® containing bacterial ice nucleators from *P. syringae* at 0.1 g/L and at 0.001 g/L. The radii at higher concentration are larger, which suggests that larger aggregates are present in the solution.



Supporting Figure 3: Freezing experiments of aqueous extracts from *Fusarium acuminatum* containing fungal ice nucleating proteins and in the presence of AFP type 1. Shown is the cumulative number of ice nucleators (N_m) per gram of mycelium plotted against the temperature (T).

References:

1. Duman, J. G.; DeVries, A. L. Isolation, characterization, and physical properties of protein antifreezes from the winter flounder, Pseudopleuronectes americanus. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry* **1976,** *54* (3), 375-380.

2. Evans, C. W.; Gubala, V.; Nooney, R.; Williams, D. E.; Brimble, M. A.; Devries, A. L. How do Antarctic notothenioid fishes cope with internal ice? A novel function for antifreeze glycoproteins. *Antarctic Science* **2011,** *23* (1), 57-64.

3. Friis, D. S.; Kristiansen, E.; von Solms, N.; Ramløv, H. Antifreeze activity enhancement by site directed mutagenesis on an antifreeze protein from the beetle Rhagium mordax. *FEBS Letters* **2014,** *588* (9), 1767-1772.

4. Meister, K.; Strazdaite, S.; DeVries, A. L.; Lotze, S.; Olijve, L. L. C.; Voets, I. K.; Bakker, H. J. Observation of ice-like water layers at an aqueous protein surface. *Proceedings of the National Academy of Sciences* **2014,** *111* (50), 17732-17736.

5. Shier, W. T.; Lin, Y.; De Vries, A. L. Structure and mode of action of glycoproteins from an antarctic fish. *Biochimica et Biophysica Acta (BBA) - Protein Structure* **1972,** *263* (2), 406-413.

6. Kunert, A. T.; Lamneck, M.; Helleis, F.; Pöschl, U.; Pöhlker, M. L.; Fröhlich-Nowoisky, J. Twin-plate Ice Nucleation Assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples. *Atmos. Meas. Tech.* **2018,** *11* (11), 6327-6337.

7. Kunert, A. T.; Pöhlker, M. L.; Krevert, C. S.; Wieder, C.; Speth, K. R.; Hanson, L. E.; Morris, C. E.; Schmale Iii, D. G.; Pöschl, U.; Fröhlich-Nowoisky, J. Highly active and stable fungal ice nuclei are widespread among Fusarium species. *Biogeosciences Discuss.* **2019,** *2019*, 1-19

8. Vali, G. Quantitative Evaluation of Experimental Results an the Heterogeneous Freezing Nucleation of Supercooled Liquids. *Journal of the Atmospheric Sciences* **1971,** *28* (3), 402-409.

9. Turner, M. A.; Arellano, F.; Kozloff, L. M. Three separate classes of bacterial ice nucleation structures. *J Bacteriol* **1990,** *172* (5), 2521-2526.

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Electrostatic Interactions Control the Functionality of Bacterial Ice Nucleators

Max Lucas[1], Ralph Schwidetzky[1], Anna T. Kunert[2], Ulrich Pöschl[2], Janine Fröhlich-Nowoisky[2], Mischa Bonn[1], Konrad Meister[1]

[1]Max Planck Institute for Polymer Science, 55128 Mainz, Germany; [2]Max Planck Institute for Chemistry, 55128 Mainz, Germany

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Contribution

Aided in freezing experiments, data analysis, and writing of the manuscript.

Title: Electrostatic Interactions Control the Functionality of Bacterial Ice Nucleators

Authors: M. Lukas[1], R. Schwidetzky[1], A. T. Kunert[2], U. Pöschl[2], J. Fröhlich-Nowoisky[2], M. Bonn[1] and K. Meister[1]

Affiliations:

[1]Max Planck Institute for Polymer Research, 55128 Mainz, Germany

[2]Max Planck Institute for Chemistry, 55128 Mainz, Germany

Abstract:

Bacterial ice-nucleating proteins (INPs) promote heterogeneous ice nucleation more efficiently than any other known material. The details of their working mechanism remain elusive, but their high activity has been shown to involve the formation of functional INP aggregates. Here we reveal the importance of electrostatic interactions for the activity of INPs from the bacterium *Pseudomonas syringae* by combining a high-throughput ice nucleation assay with surface-specific sum-frequency generation spectroscopy. We determined the charge state of non-viable *P. syringae* as a function of pH by monitoring the degree of alignment of the interfacial water molecules and the corresponding ice nucleation activity. The net charge correlates with the ice nucleation activity of the INP aggregates, which is minimal at the isoelectric point. In contrast, the activity of INP monomers is less affected by pH changes. We conclude that electrostatic interactions play an essential role in the formation of the highly-efficient functionally aligned INP aggregates, providing a mechanism for promoting aggregation under conditions of stress that prompt the bacteria to nucleate ice.

Ice formation is the most important liquid-to-solid phase transition on earth and is strongly affected by the presence of nucleators that initiate heterogeneous ice nucleation at temperatures above -40 ˚C. There is a large variety of compounds of biological and abiotic origin that can act as ice nucleators, and their efficiency strongly differs[1-3]. The most efficient ice nucleators are bacteria from *Pseudomonas syringae,* which can initiate the crystallization of water at temperatures as high as −2 ˚C[4-5]. The ability of bacteria to nucleate ice is caused by specialized ice-nucleating proteins (INPs) that are exposed on the bacterial surface and are anchored in the outer membrane on the bacterial cell wall[6]. Bacterial INPs contain a large central-repeat domain that has been proposed to be the active site and which is responsible for ice nucleation through a mechanism that likely involves the preordering of water molecules [7,8]. Apart from the specific ice-binding site, the high ice nucleation activity of INPs has been shown to crucially depend on the ability to aggregate into larger functional protein clusters[9-11]. Based on their activity in droplet freezing experiments, the bacterial INP aggregates are typically divided into classes as shown in Figure 1[10]. Class A consists of large aggregates (>50 INPs) that are responsible for freezing at high subzero temperatures (−2 to −4 ˚C)[10]. Class B consists of smaller aggregates that induce freezing at −5 ˚C to −6.5 ˚C, and class C consists of mostly monomeric INPs that induce ice formation between −7 ˚C to −12 ˚C[10]. In nature, the aggregation of INPs occurs under conditions of stress, which require the bacteria to nucleate ice[5]. The INP aggregation mechanism and whether INP aggregation in cell membranes is promoted by a change in chemistry is unknown. Notably, lowering the pH to acidic values has repeatedly been shown to reduce the ice nucleation activity of bacterial INPs[10, 12-13]. In contrast, changing the pH to alkaline values did not affect the nucleation activity[10]. The molecular origin for this pH sensitivity is unknown, but a better understanding would provide needed insights into the driving forces of INP aggregation. Moreover, it would have direct implications for understanding biological ice nucleation in the atmosphere, where the pH levels are oftentimes acidic due to anthropogenic activities[12].

Figure 1 shows the results of ice nucleation measurements of the bacterial ice nucleator Snomax® at three pH values. Snomax® is a commonly used model system for biological and atmospheric ice nucleation studies[14-16], and it consists of a preparation of inactivated bacteria cells of *P. syringae*. The initial Snomax® solutions in water had a concentration of 0.1 mg/mL and a pH value of ~6.2. The samples were then serially diluted, resulting in concentrations from 1 ng/mL to 1 mg/mL. The cumulative ice nucleator number concentration (N_m) was

calculated using Vali's formula, and it represents the number of ice nucleators per unit weight that are active above a certain temperature[17].

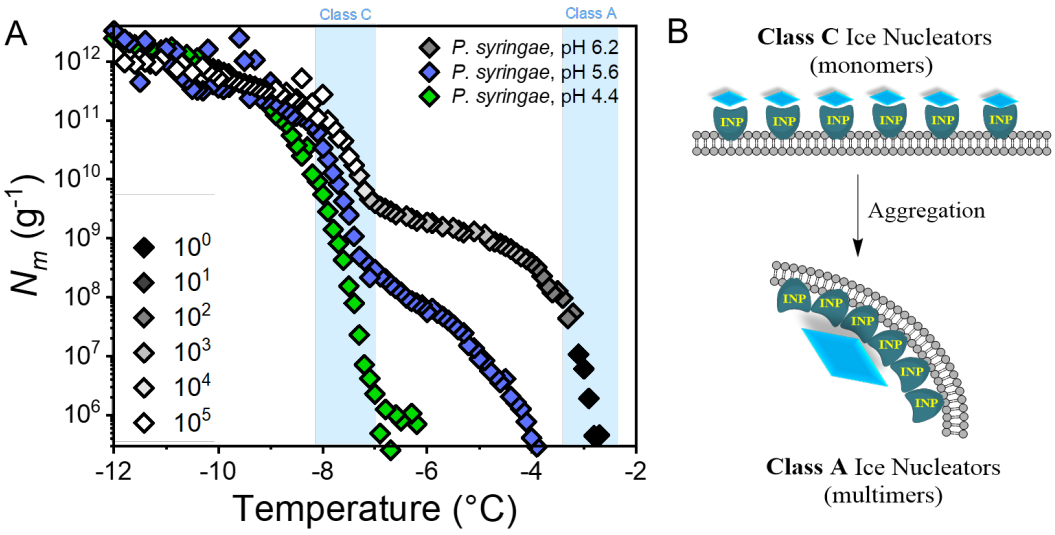

Figure 1: Freezing experiments of aqueous solutions of Snomax® containing bacterial ice nucleators from *P. syringae* in water and at different pH values. A) Shown is the cumulative number of ice nucleators (N_m) per unit mass of Snomax® vs. temperature. Numbers in the legend denote dilution factors. The temperature ranges for class A and C bacterial ice nucleators are shaded in blue[10]. B) Schematic structure of class A and C nucleators. Class C refers to monomeric INPs, which can aggregate to form the highly efficient class A nucleators.

For the bacterial ice nucleator solution in water (pH ~ 6.2), the spectrum shows two strong increases in $N_m(T)$ around ~ −2.9 °C and ~ −7.5 °C with plateaus between ~ −4.5 °C and ~ −7 °C and above ~ −9.5 °C. The two rises in the spectrum reveal that the ice nucleation activity of *P. syringae* stems from two classes of ice nucleators with different activation temperatures. The plateaus at temperatures T below each increase of $N_m(T)$ arise when fewer ice nucleators at these temperatures are present[15]. We attribute the observed rises at ~ −2.9°C and ~ −7.5°C to class A and C ice nucleators, respectively.

For lower pH solutions, the trend looks markedly different. At pH 5.5 the rise at ~ −2.9 °C is absent; instead, we observe a rise at ~ −4.5 °C. Further, the second rise at ~ −7.5 °C is slightly shifted by ~ −0.5 °C. Evidently, lowering the pH influences the ability of the ice-nucleating proteins to form the more efficient class A aggregates. Further lowering the pH fortifies this effect and at a pH of ~ 4.4, the class C nucleators have disappeared; apparently, class A

nucleators were converted into class C. While class A nucleators are very pH-sensitive, class C nucleators are only weakly affected by pH variations.

To obtain a more complete picture of the effect of the pH on the ice nucleation activity of the class A bacterial INPs we conducted a comprehensive evaluation of pH values from 2–10.5 as shown in Figure 2. We find that the change of pH gives rise to different types of effects as shown in Figure 2B: (i) Lowering the pH to acidic values shifts the freezing point of class A to lower temperatures. This trend increases until pH ~ 4, where the resulting freezing temperature ~ −7 ˚C closely resembles that of class C nucleators. The more acidic conditions clearly prevent the formation of highly efficient class A aggregates, reducing the activity of class A to that of class C. (ii) Raising the pH to basic conditions has a small effect on the class A ice nucleators and the resulting response looks similar to that of bacteria in water, only shifted by ~ 0.6 ˚C to lower temperatures (Figure S1, Figure 2B). (iii) Raising the pH to extreme basic conditions shifts the freezing point by ~ 3.5 ˚C to lower temperatures. The resulting freezing temperature ~ −7 ˚C resembles that of class C nucleators.

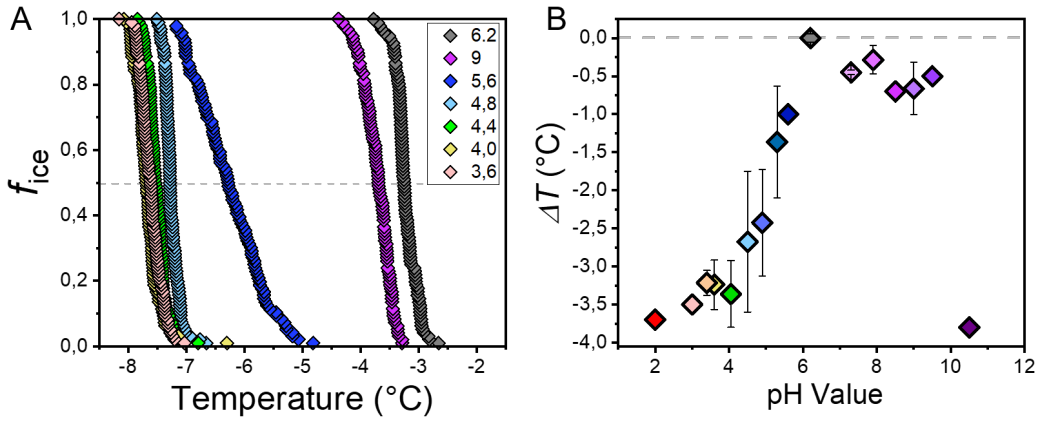

Figure 2: Freezing experiments of aqueous solutions of Snomax® containing bacterial ice nucleators from *P. syringae* at different pH values. A) Fraction of frozen droplets (f_{ice}) for highly concentrated (0.1 mg/mL) Snomax® solutions. B) Temperature shifts ΔT induced by different pH values. Shifts represent the difference at $f_{ice} = 0.5$ between Snomax® in water (pH 6.2) and at different pH values. Error bars represent the standard deviation for multiple independent measurements.

To investigate the molecular origin of the strong pH dependence of bacterial INP's ice nucleation efficiency, we conducted sum-frequency generation (SFG) spectroscopic experiments of Snomax® adsorbed to the air-water interface at different pH values. SFG is a

surface-specific method that can be used to probe the interfacial water of biomolecules[18-19]. In this technique, an infrared light pulse and a visible pulse are combined at a surface to generate light at the sum-frequency of the two incident fields. The technique is bulk-forbidden in isotropic media, and only ensembles of molecules with a net orientation, e.g. at an interface, can generate a detectable signal. The SFG signal intensity depends on the number of aligned molecules at the interface.

At charged surfaces, the surface field can align the water dipoles. Such charge-induced enhanced ordering of the interfacial water molecules causes the signal intensity in the O-H stretching region (~ 3150–3600 cm^{-1}) to increase, and, inversely, the SFG signal intensity can be used to quantify the amount of charge at the electrified surface. This concept has previously been applied to determine the isoelectric point (IEP) of proteins[20-23]. Figure 3A shows pH-dependent SFG spectra of aqueous solutions of 0.1 mg/mL Snomax® adsorbed to the air-water interface. In the frequency region from 2800–3100 cm^{-1}, the SFG spectra show strong signals from C-H stretching vibrations. We assign these bands to the methyl symmetric stretch (2880 cm^{-1}), a Fermi resonance (2940 cm^{-1}), and aromatic C-H (3050 cm^{-1}) vibrations. At frequencies above 3100 cm^{-1}, the spectrum shows a broad response from the O-H stretching band of interfacial water molecules. The SFG intensity of the CH and OH groups shows substantial changes when the solution pH is altered. We find that at pH values ~ 4.2 the intensity of the O-H stretching signals is close to zero, whereas, at values below and above, the intensity of the O-H bands increases markedly and dominates the SFG spectrum of *P. syringae*. The observed changes in the C-H region can be explained with interferences with the O-H resonances[21-22]. To more accurately determine the IEP of *P. syringae,* we integrated the SFG signal in the frequency region of ~ 3100–3600 cm^{-1}. Figure 3B shows the pH dependence of the integrated intensity of the O-H stretching region. The lowest value for *P. syringae* was found at pH ~ 4.2, which corresponds to the point of no net charge or IEP of the bacteria. This value is similar to the bulk IEP of 4.0 that was previously reported for *P. syringae*[24].

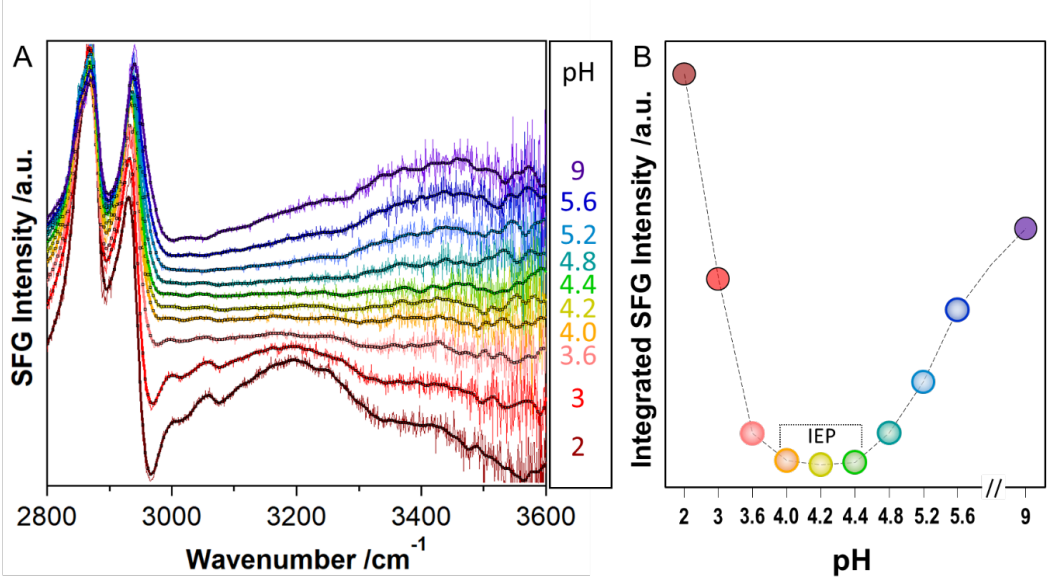

Figure 3: A) SFG spectra of aqueous solutions of Snomax® containing bacterial ice nucleators from *P. syringae* at the air-water interface as a function of bulk pH. The bulk concentration of Snomax® was 0.1 mg/mL. B) Integrated SFG intensity of the frequency region from 3100–3600 cm^{-1} for Snomax® (*P. syringae)* at different bulk pH values.

Ice nucleation activities of bacteria are being studied extensively due to their important roles in precipitation and frost injury of plants[25]. Elucidating the impact of environmental factors such as the pH is essential for understanding not only atmospheric ice nucleation processes, but also the functionality of INP aggregates. Electrostatic properties are governed by the distribution and ratio of charged and polar residues within protein structures and are among the most important factors that determine the functionality, stability, and interactions of proteins. We demonstrate that acidic pH values reduce the ice-nucleating activity of non-viable *P. syringae* bacteria and that the effect arises from the inactivation of the highly efficient class A aggregates active at high subzero temperatures. In contrast, we find no significant influence of basic pH values on the ice nucleation activity. The effect of acidic pH on the ice nucleation activity of *P. syringae* has previously been observed and those results are consistent with our findings[4, 10, 12-13]. Turner *et al.* proposed that the acidic pH denatures the larger class A INP complexes and that this process is irreversible[10]. Unlike *Turner et al.,* we observe that the ice nucleation activity of the class A INP aggregates can be partially recovered upon raising the pH back to neutral values (Figure S2-S4). Combined, the pH-dependent SFG and droplet freezing experiments revealed that eliminating the net negative charge of *P. syringae* correlates with the decrease of the ice nucleation activity from the large class A INP aggregates. We explain these

observations with the formation of misfolded INP aggregates as a result of the decreased charge repulsion. Upon lowering the pH towards pH 4.2, negatively charged amino acids are protonated, and the net charge is reduced. The large numbers of glutamic acid residues contained in INPs of *P. syringae* are likely candidates for protonation[8]. As a result of the protonation, the charge repulsion between INP monomers is absent, and hydrophobic interactions cause the INPs to misfold. These newly formed INP aggregates differ substantially from the precisely aligned functional class A INP aggregates and lack the ice nucleation activity at high subzero temperatures. Consequently, only single INPs or smaller aggregates, i.e. class C nucleators remain active at acidic pH, which is in line with our experimental observations. The finding that the ice nucleation activity can be recovered by going back to neutral pH, further provides evidence that no irreversible denaturation of INPs occurs. In nature, the aggregation of INPs occurs in the cell membrane of ice-nucleating bacteria under conditions of stress that require them to nucleate ice[5]. The alteration of the pH in the system would provide a means to trigger INP aggregation in the cell membrane.

Associated Content

Experimental setups, experimental methods, effect of alkaline pH on ice nucleation activity, effect of reversibility of acidic pH change, Supporting Figures S1-S4

Corresponding Author

meisterk@mpip-mainz.mpg.de

Notes

The authors declare no competing financial interests.

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References

1. Murray, B. J.; O'Sullivan, D.; Atkinson, J. D.; Webb, M. E., Ice nucleation by particles immersed in supercooled cloud droplets. *Chemical Society Reviews* **2012,** *41* (19), 6519-6554.
2. Pummer, B. G.; Budke, C.; Augustin-Bauditz, S.; Niedermeier, D.; Felgitsch, L.; Kampf, C. J.; Huber, R. G.; Liedl, K. R.; Loerting, T.; Moschen, T.; Schauperl, M.; Tollinger, M.; Morris, C. E.; Wex, H.; Grothe, H.; Pöschl, U.; Koop, T.; Fröhlich-Nowoisky, J., Ice nucleation by water-soluble macromolecules. *Atmos. Chem. Phys.* **2015,** *15* (8), 4077-4091.
3. Kunert, A. T.; Pöhlker, M. L.; Krevert, C. S.; Wieder, C.; Speth, K. R.; Hanson, L. E.; Morris, C. E.; Schmale Iii, D. G.; Pöschl, U.; Fröhlich-Nowoisky, J., Highly active and stable fungal ice nuclei are widespread among Fusarium species. *Biogeosciences Discuss.* **2019,** *2019*, 1-19.
4. Kozloff, L. M.; Schofield, M. A.; Lute, M., Ice nucleating activity of Pseudomonas syringae and Erwinia herbicola. *J Bacteriol* **1983,** *153* (1), 222-231.

5. Maki, L. R.; Galyan, E. L.; Chang-Chien, M. M.; Caldwell, D. R., Ice nucleation induced by pseudomonas syringae. *Applied microbiology* **1974,** *28* (3), 456-459.
6. Govindarajan, A. G.; Lindow, S. E., Size of bacterial ice-nucleation sites measured in situ by radiation inactivation analysis. *Proceedings of the National Academy of Sciences of the United States of America* **1988,** *85* (5), 1334-8.
7. Garnham, C. P.; Campbell, R. L.; Walker, V. K.; Davies, P. L., Novel dimeric beta-helical model of an ice nucleation protein with bridged active sites. *BMC structural biology* **2011,** *11*, 36.
8. Pandey, R.; Usui, K.; Livingstone, R. A.; Fischer, S. A.; Pfaendtner, J.; Backus, E. H. G.; Nagata, Y.; Fröhlich-Nowoisky, J.; Schmüser, L.; Mauri, S.; Scheel, J. F.; Knopf, D. A.; Pöschl, U.; Bonn, M.; Weidner, T., Ice-nucleating bacteria control the order and dynamics of interfacial water. **2016,** *2* (4), e1501630.
9. Gurian-Sherman, D.; Lindow, S. E., Bacterial ice nucleation: significance and molecular basis. *Faseb j* **1993,** *7* (14), 1338-43.
10. Turner, M. A.; Arellano, F.; Kozloff, L. M., Three separate classes of bacterial ice nucleation structures. *J Bacteriol* **1990,** *172* (5), 2521-2526.
11. Qiu, Y.; Hudait, A.; Molinero, V., How Size and Aggregation of Ice-Binding Proteins Control Their Ice Nucleation Efficiency. *Journal of the American Chemical Society* **2019,** *141* (18), 7439-7452.
12. Attard, E.; Yang, H.; Delort, A. M.; Amato, P.; Pöschl, U.; Glaux, C.; Koop, T.; Morris, C. E., Effects of atmospheric conditions on ice nucleation activity of Pseudomonas. *Atmos. Chem. Phys.* **2012,** *12* (22), 10667-10677.
13. Kawahara, H.; Tanaka, Y.; Obata, H., Isolation and Characterization of a Novel Ice-nucleating Bacterium, Pseudomonas sp. KUIN-4, Which Has Stable Activity in Acidic Solution. *Bioscience, Biotechnology, and Biochemistry* **1995,** *59* (8), 1528-1532.
14. Wex, H.; Augustin-Bauditz, S.; Boose, Y.; Budke, C.; Curtius, J.; Diehl, K.; Dreyer, A.; Frank, F.; Hartmann, S.; Hiranuma, N.; Jantsch, E.; Kanji, Z. A.; Kiselev, A.; Koop, T.; Möhler, O.; Niedermeier, D.; Nillius, B.; Rösch, M.; Rose, D.; Schmidt, C.; Steinke, I.; Stratmann, F., Intercomparing different devices for the investigation of ice nucleating particles using Snomax® as test substance. *Atmos. Chem. Phys.* **2015,** *15* (3), 1463-1485.
15. Budke, C.; Koop, T., BINARY: an optical freezing array for assessing temperature and time dependence of heterogeneous ice nucleation. *Atmos. Meas. Tech.* **2015,** *8* (2), 689-703.
16. Kunert, A. T.; Lamneck, M.; Helleis, F.; Pöschl, U.; Pöhlker, M. L.; Fröhlich-Nowoisky, J., Twin-plate Ice Nucleation Assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples. *Atmos. Meas. Tech.* **2018,** *11* (11), 6327-6337.
17. Vali, G., Quantitative Evaluation of Experimental Results an the Heterogeneous Freezing Nucleation of Supercooled Liquids. *Journal of the Atmospheric Sciences* **1971,** *28* (3), 402-409.
18. Meister, K.; Strazdaite, S.; DeVries, A. L.; Lotze, S.; Olijve, L. L. C.; Voets, I. K.; Bakker, H. J., Observation of ice-like water layers at an aqueous protein surface. *Proceedings of the National Academy of Sciences* **2014,** *111* (50), 17732-17736.
19. Meister, K.; Paananen, A.; Speet, B.; Lienemann, M.; Bakker, H. J., Molecular Structure of Hydrophobins Studied with Site-Directed Mutagenesis and Vibrational Sum-Frequency Generation Spectroscopy. *The journal of physical chemistry. B* **2017,** *121* (40), 9398-9402.
20. Devineau, S.; Inoue, K.-i.; Kusaka, R.; Urashima, S.-h.; Nihonyanagi, S.; Baigl, D.; Tsuneshige, A.; Tahara, T., Change of the isoelectric point of hemoglobin at the air/water interface probed by the orientational flip-flop of water molecules. *Physical Chemistry Chemical Physics* **2017,** *19* (16), 10292-10300.
21. Engelhardt, K.; Peukert, W.; Braunschweig, B., Vibrational sum-frequency generation at protein modified air–water interfaces: Effects of molecular structure and surface charging. *Current Opinion in Colloid & Interface Science* **2014,** *19* (3), 207-215.
22. Strazdaite, S.; Meister, K.; Bakker, H. J., Orientation of polar molecules near charged protein interfaces. *Physical Chemistry Chemical Physics* **2016,** *18* (10), 7414-7418.

23. Guckeisen, T.; Hosseinpour, S.; Peukert, W., Isoelectric Points of Proteins at the Air/Liquid Interface and in Solution. *Langmuir* **2019,** *35* (14), 5004-5012.
24. Horká, M.; Horký, J.; Matoušková, H.; Šlais, K., Free flow and capillary isoelectric focusing of bacteria from the tomatoes plant tissues. *Journal of Chromatography A* **2009,** *1216* (6), 1019-1024.
25. Morris, C. E.; Conen, F.; Alex Huffman, J.; Phillips, V.; Poschl, U.; Sands, D. C., Bioprecipitation: a feedback cycle linking earth history, ecosystem dynamics and land use through biological ice nucleators in the atmosphere. *Global change biology* **2014,** *20* (2), 341-51.

Supplementary Material

Electrostatic Interactions Control the Functionality of Bacterial Ice Nucleators

Max Lucas[1], Ralph Schwidetzky[1], Anna T. Kunert[2], Ulrich Pöschl[2], Janine Fröhlich-Nowoisky[2], Mischa Bonn[1], Konrad Meister[1]

[1]Max Planck Institute for Polymer Science, 55128 Mainz, Germany; [2]Max Planck Institute for Chemistry, 55128 Mainz, Germany

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Supporting Information:

Electrostatic Interactions Control the Functionality of Bacterial Ice Nucleators

Authors: M. Lukas[1], R. Schwidetzky[1], A.T. Kunert[2], U. Pöschl[2], J. Fröhlich-Nowoisky[2], M. Bonn[1], and K. Meister[1]

Affiliations:

[1]Max Planck Institute for Polymer Research, 55128 Mainz, Germany

[2]Max Planck Institute for Chemistry, 55128 Mainz, Germany

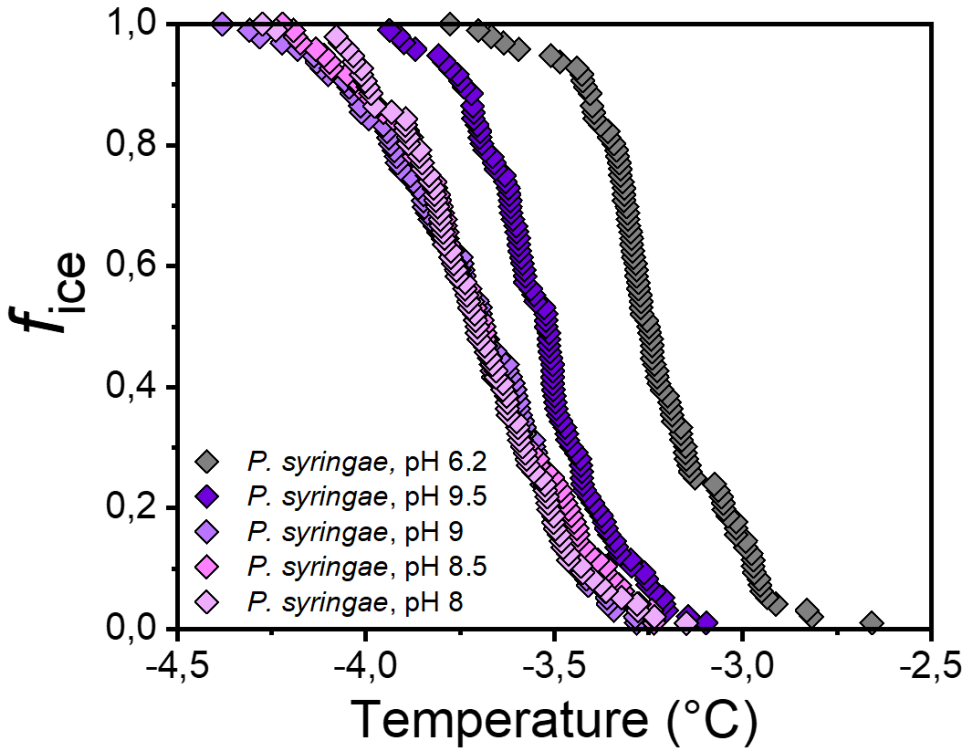

Figure S1: Freezing experiments of aqueous solutions of Snomax® containing bacterial ice nucleators from *P. syringae* at alkaline pH values. Fraction of frozen droplets (f_{ice}) for highly concentrated (0.1 mg/mL samples) Snomax® (*P. syringae*) solutions.

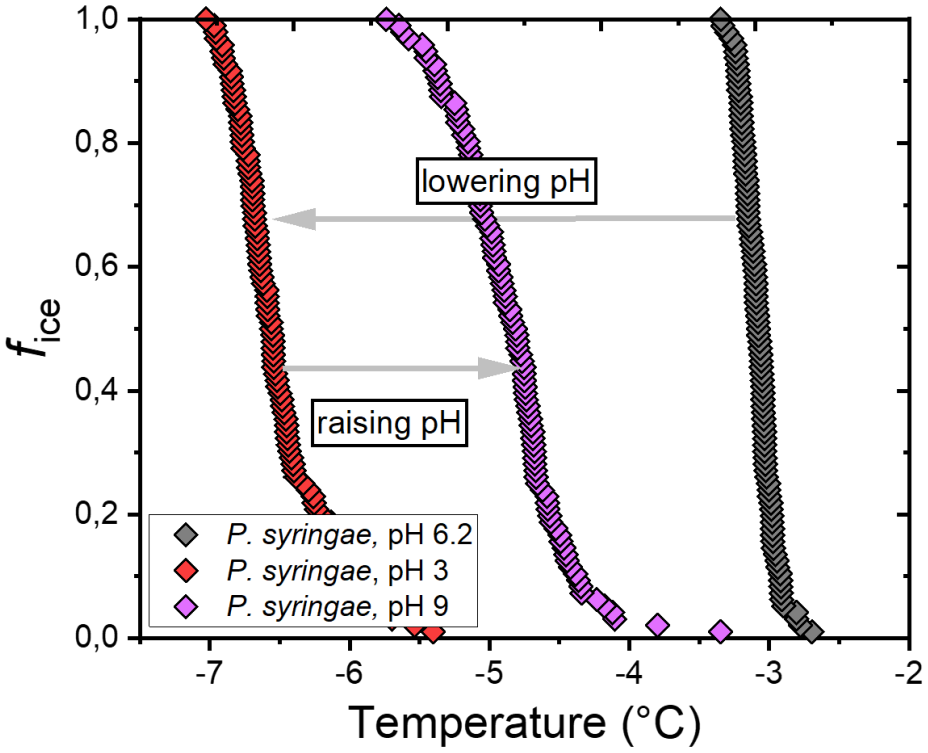

Figure S2: Freezing experiments of aqueous solutions of Snomax® containing bacterial ice nucleators from *P. syringae* as a function of pH values. Fraction of frozen droplets (f_{ice}) for highly concentrated (0.1 mg/mL) Snomax® (*P. syringae)* solutions, in which the pH was first decreased using HCl and then increased using NaOH.

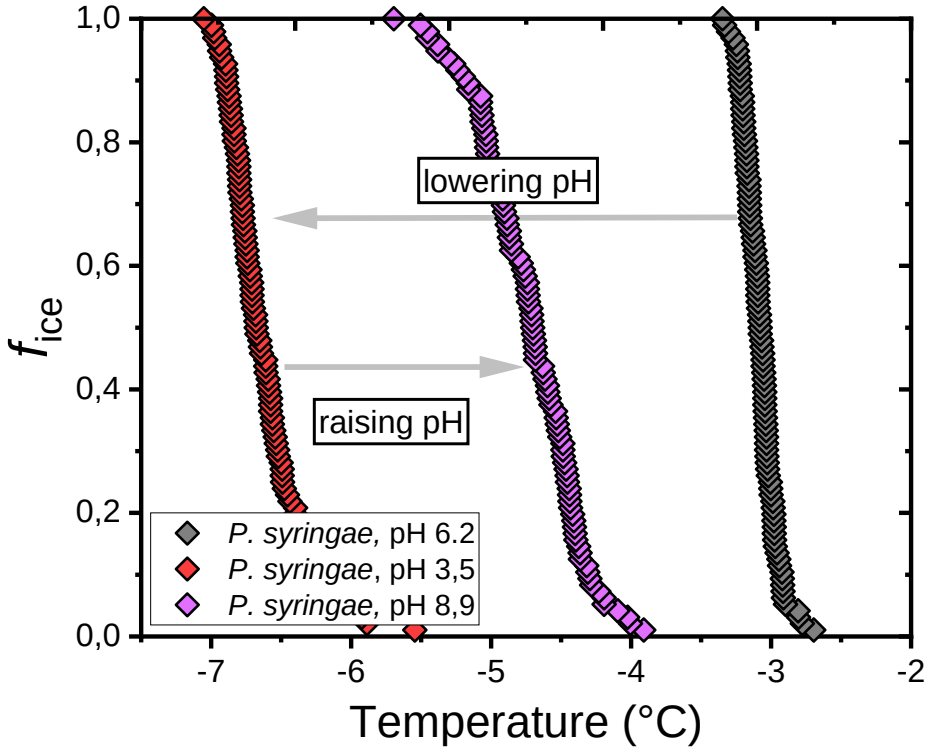

Figure S3: Freezing experiments of aqueous solutions of Snomax® containing bacterial ice nucleators from *P. syringae* as a function of pH values. Fraction of frozen droplets (f_{ice}) for highly concentrated (0.1 mg/mL) Snomax® (*P. syringae)* solutions, in which the pH was first decreased using HCl and then increased using NaOH.

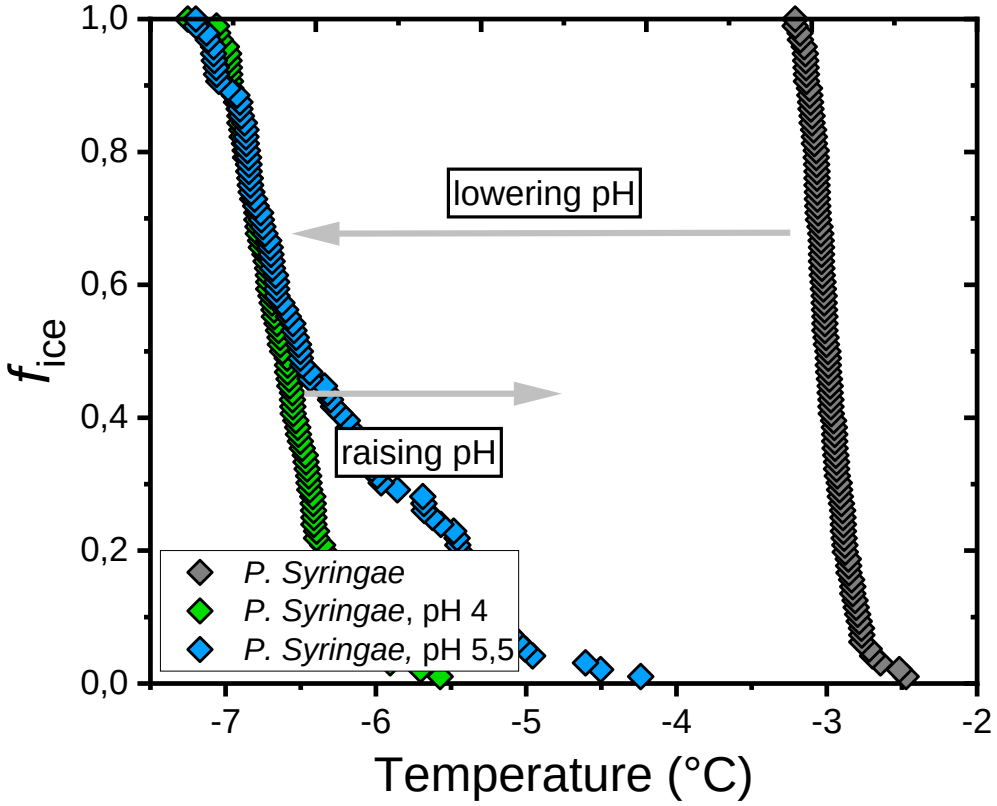

Figure S4: Freezing experiments of aqueous solutions of Snomax® containing bacterial ice nucleators from *P. syringae* as a function of pH values. Fraction of frozen droplets (f_{ice}) for highly concentrated (0.1 mg/mL) Snomax® (*P. syringae)* solutions, in which the pH was first decreased and then increased using acetate buffer.

Materials and Methods

Samples: Pure water was prepared as described elsewhere[1]. Snomax® was obtained from SMI Snow Makers AG (Thun, Switzerland) and contains a preparation of freeze-dried, irradiated bacteria cells of *Pseudomonas syringae*. Buffer materials (Tris, PBS, acetate) as well as NaOH and HCl were obtained from Sigma. The concentration of Snomax® was 0.1 mg/mL, and the pH value in pure water was 6.2 +/- 0.2. The SFG experiments and TINA experiments were performed in either pure water or in 0.1 M buffer solution (acetate, PBS, Tris,) of the respected pH. The ionic strength of the solutions was 0.1 M and adjusted by adding NaCl. The pH values of all samples were controlled before each measurement.

TINA Experiments. Ice nucleation experiments were performed using a high-throughput droplet freezing assay. The details of the instrument have been described recently[1]. In a typical experiment, the investigated ice nucleator sample was serially diluted 10-fold by a liquid handling station (epMotion ep5073, Eppendorf, Hamburg, Germany). 96 droplets (3 µL) per dilution were placed on two 384-well-plates and tested with a continuous cooling-rate of 1 °C/min from 0 °C to -20 °C. The temperature of droplet-freezing was determined by two infrared cameras (Seek Therman Compact XR, Seek Thermal Inc., Santa Barbara, CA, USA). The uncertainty in the temperature of the setup was ±0.2°C. The obtained fraction of frozen droplets were used to calculate the cumulative number of ice nucleators using the Vali formula. Experiments were performed 3 - 6 times on independent samples.

Sum-Frequency Generation Spectroscopy Experiments:

The details of the experimental setup have been described previously[2]. For the SFG intensity spectra, we used a conventional SFG setup in reflection geometry. A broadband IR (~ 5 µJ, full width half maximum (FWHM) of ~ 450 cm^{-1}) and a narrowband VIS (~ 13 µJ, centered at ~ 800 nm, FWHM of ~ 15 cm^{-1}) beam were focused and spatially and temporally overlapped on the sample surface, with incident angles of 36° (VIS) and 41° (IR) with respect to the surface normal. The spectra presented in this study were simultaneously recorded in the C-H and O-H-stretch region. The spectral resolution was limited by the bandwidth of the VIS. The generated SFG signal was collimated by a lens, directed and focused onto a spectrograph (*Acton SP 300i, Princeton Instruments*) and detected by a camera (*Newton 970, Andor Instruments*). All spectra were obtained in the ssp-polarization combination (s-polarized SFG, s-polarized VIS, p-polarized IR). Background spectra where taken with a blocked IR beam and

all spectra were normalized to reference spectra from z-cut quartz. The SFG measurements were performed in a custom-made Teflon trough at room temperature. The Snomax® (*P. syringae*) solutions were measured at a concentration of 0.1 g/L. Samples were measured in allowed to equilibrate for two hours before measurements. The equilibration of the samples at the interface was complete, when the surface pressure was constant and the SFG spectra did not change within one hour.

Colligative effects

We exclude significant contributions of colligative effects on our results, since we performed measurements in buffer systems or by adjusting the pH value using NaOH and HCl. The ionic strength was kept constant at 0.1 M for all solutions and adjusted by adding NaCl. The maximum theoretical shift due to colligative effects in the TINA measurements would be ~ 0.4 ˚C which is less than the observed shifts.

1. Kunert, A. T.; Lamneck, M.; Helleis, F.; Pöschl, U.; Pöhlker, M. L.; Fröhlich-Nowoisky, J., Twin-plate Ice Nucleation Assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples. *Atmos. Meas. Tech.* **2018,** *11* (11), 6327-6337.
2. Dreier, L. B.; Wolde-Kidan, A.; Bonthuis, D. J.; Netz, R. R.; Backus, E. H. G.; Bonn, M., Unraveling the Origin of the Apparent Charge of Zwitterionic Lipid Layers. *The Journal of Physical Chemistry Letters* **2019,** *10* (20), 6355-6359.

C.5 Kunert *et al.*, in preparation, 2020

Reaction kinetics and products of nitration and oligomerization of the major grass pollen allergen Phl p 5 by peroxynitrite and nitrogen dioxide with ozone

Anna T. Kunert[1], Kathrin Reinmuth-Selzle[1], Kira Ziegler[1], Carola S. Krevert[1], Carsten Wieder[1], Michael G. Weller[2], Kurt Lucas[1], Ulrich Pöschl[1], Janine Fröhlich-Nowoisky[1]

[1]Max Planck Institute for Chemistry, Multiphase Chemistry Department, 55128 Mainz, Germany; [2]Division 1.5 Protein Analysis, Federal Institute for Materials Research and Testing (BAM), 12489 Berlin, Germany

Contribution

KL, UP, and JFN designed the study. ATK, KRS, KZ, CSK, and CW performed the experiments. ATK, KRS, KZ,KL, UP, JF analyzed, interpreted and discussed the results. CSK, CW, and MGW contributed to the discussion. ATK and JFN wrote the manuscript.

Reaction kinetics and products of nitration and oligomerization of the major grass pollen allergen Phl p 5 by peroxynitrite and nitrogen dioxide with ozone

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[1]Multiphase Chemistry Department, Max Planck Institute for Chemistry, 55128 Mainz, Germany
[1]Division 1.5 Protein Analysis, Federal Institute for Materials Research and Testing (BAM), 12489 Berlin, Germany

Correspondence: Janine Fröhlich-Nowoisky (j.frohlich@mpic.de)

Abstract. Air pollution is associated with the increasing burden of allergenic diseases. The allergenic potential of proteins may be enhanced via posttranslational modifications induced by anthropogenic air pollutants like ozone (O_3) and nitrogen dioxide (NO_2). Furthermore, air pollution causes severe lung diseases involving inflammations, and inhaled allergens can be chemically modified by peroxynitrite ($ONOO^-$), which is released during inflammations in the human body. The molecular mechanisms and kinetics of the chemical modifications that enhance the allergenicity of proteins, however, are still not fully understood. We studied protein tyrosine nitration and oligomerization of the major timothy grass pollen allergen Phl p 5 upon simultaneous exposure of O_3 and NO_2 and reaction with $ONOO^-$. The nitration degree and oligomer formation were analyzed by reversed-phase and size-exclusion liquid chromatography. Both, exposure to O_3/NO_2 and reaction with $ONOO^-$, led to nitration and oligomerization of the allergen. The nitration degree and oligomer formation were dependent on the nitrating agent and the reaction conditions (concentration, exposure time), and they were higher for $ONOO^-$ than for O_3/NO_2. For the reaction with $ONOO^-$, we found a maximum nitration degree of 25 %, which corresponds to maximal $\sim$6 nitrated tyrosine residues per Phl p 5 monomer, whereas exposure to O_3/NO_2 resulted in a maximum nitration degree of 8 % corresponding to $\sim$1 nitrotyrosine per Phl p 5 monomer. The obtained results were similar to earlier studies investigating other allergenic proteins and reference substances (Bet v 1, BSA). The data will be further interpreted with regard to protein structure, and kinetic modeling will be included in the future.

1 Introduction

The prevalence of allergic diseases and asthma are increasing worldwide, and especially the complexity and severity of allergic diseases in children and young adults continue to rise (Asher et al., 2006; Pawankar et al., 2008, 2013; Pawankar, 2014). The rapid increase in the prevalence, however, especially in industrialized countries, cannot be explained by genetic factors alone, and environmental factors need to be included (Pawankar et al., 2008, 2013; Reinmuth-Selzle et al., 2017; Traidl-Hoffmann et al., 2009). Indeed, several studies have suggested that allergic diseases are enhanced by traffic-related air pollution with high concentrations of the reactive trace gases ozone (O_3) and nitrogen dioxide (NO_2) (D'Amato et al., 2017; Shiraiwa et al.,

2012a), and it has been shown that birch and ragweed pollen from urban areas had a higher allergenic potential than pollen from rural areas (Bryce et al., 2010; Ghiani et al., 2012). Pollen can rupture upon exposure to O_3 or NO_2 or under humid conditions and release cytoplasmic material including allergenic proteins and biogenic adjuvants into the atmosphere (Motta et al., 2006; Ouyang et al., 2016; Taylor et al., 2002, 2004). The released proteins can interact with gaseous and particulate air pollutants leading to the formation of modified proteins. Moreover, allergenic proteins can be chemically modified in the human body during oxidative stress and inflammation induced upon exposure to air pollutants (Reinmuth-Selzle et al., 2017). These chemical posttranslational modifications can change the protein structure and stability, affect hydrophobicity and acidity of binding sites, and thereby alter the immunogenicity of the proteins (Abello et al., 2009; Ackaert et al., 2014; Greenacre and Ischiropoulos, 2001; Ischiropoulos, 2009; Gruijthuijsen et al., 2006; Karle et al., 2012).

In the atmosphere, allergenic proteins can react with O_3 and NO_2 resulting in oxidized and nitrated proteins as well as protein oligomerization and degradation (Franze et al., 2005; Shiraiwa et al., 2012b). The reaction requires a two-step mechanism, in which the protein reacts first with ozone, forming long-lived reactive oxygen intermediates (ROIs) such as tyrosyl radicals. In a second step, the ROI reacts either with nitrogen dioxide resulting in the formation of 3-nitrotyrosine (NTyr) residues, combines with another ROI forming dityrosine crosslinks, or undergo further oxidation reactions (Kampf et al., 2015; Liu et al., 2017a; Shiraiwa et al., 2011).

A similar two-step mechanism is known for protein modification inside the human body. During oxidative stress and inflammation, nitrogen oxide radicals (NO) and superoxide anions (O_2^-) are formed, which in turn rapidly react to the strong oxidizing and nitrating peroxynitrite ($ONOO^-$) (Beckman et al., 1990). This intermediate can react with carbon dioxide (CO_2) forming nitrosoperoxycarbonate ($ONOOCO_2^-$), which in turn decomposes to a nitrogen dioxide radical (NO_2) and a carbonate radical (CO_3^-) (Gunaydin and Houk, 2009). These one-electron oxidants attack the aromatic ring of a tyrosine residue (Tyr), leading to the formation of a tyrosyl radical. In a second step, the ROI either combines with NO_2 to yield 3-nitrotyrosine or reacts with another ROI forming dityrosine crosslinks, similar to the reaction with O_3 and NO_2 (Gunaydin and Houk, 2009; Pfeiffer et al., 2000).

In this study, we investigated the nitration and oligomerization of the major timothy grass pollen allergen Phl p 5.0101, simply termed Phl p 5 hereafter, by O_3 and NO_2 mimicking air pollution effects and by $ONOO^-$ mimicking endogenous protein modification during oxidative stress. The recombinant Phl p 5 has a molecular mass of 28.6 kDa and consists of 278 amino acids including 12 tyrosine residues. The exposure experiments were performed in aqueous phase at low (50 ppb/50 ppb) or high (200 ppb/200 ppb) O_3 and NO_2 concentrations for 0.5-10 h. The endogenous protein modification was performed with 0.07-0.4 mM $ONOO^-$ concentrations. Reversed-phase high-performance liquid chromatography coupled to diode array detection analysis was used to determine the total tyrosine nitration degree for the native and modified proteins. Additionally, we used size exclusion chromatography to investigate protein oligomer formation and to determine the individual tyrosine nitration degrees for the different oligomer fractions.

2 Materials and methods

2.1 Chemicals

Pure water was taken from a Barnstead™ GenPure™ xCAD plus water purification system (Thermo Scientific, Braunschweig, Germany). The water was autoclaved at 121 °C for 20 min and filtered three times through a sterile 0.1 µm pore diameter sterile polyethersulfone (PES) vacuum filter unit (VWR International, Radnor, PA, USA). Timothy grass (*Phleum pratense*) pollen allergen 5 (Phl p 5.0101) was obtained from Biomay AG (Vienna, Austria), and a stock solution (1 mg mL^{-1}) was prepared in pure water prior to each experiment. The solution was incubated at room temperature for at least 30 min to ensure complete reconstitution of the protein. Sodium peroxynitrite (160-200 mM) was purchased from Merck (Darmstadt, Germany), and ammonium bicarbonate (NH_4HCO_3) was from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Water with 0.1 % trifluoroacetic acid (TFA) was purchased from VWR International GmbH (Darmstadt, Germany), and acetonitril (ACN) and sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$) were purchased from Carl Roth GmbH & Co. KG. A 150 mM $NaH_2PO_4 \cdot H_2O$ buffer was prepared, and the pH was adjusted to 7 by the addition of sodium hydroxide (NaOH), which was obtained from VWR.

2.2 Protein modification by O_3/NO_2

The experimental setup was described previously (Kampf et al., 2015; Kunert et al., 2018; Liu et al., 2017a; Reinmuth-Selzle et al., 2014), and it was extended by incorporating an oven (Heratherm IGS60, Thermo Scientific) to maintain a constant temperature during the exposure (Fig. S1). Briefly, O_3 was generated by synthetic air passing through a UV lamp (L.O.T.-Oriel GmbH & Co. KG, Darmstadt, Germany) at ∼1.9 L min^{-1}, and the O_3 concentration was adjusted by tuning the amount of UV light. The gas flow was humidified by passing through a Nafion® gas humidifier (MH-110-12F-4, PermaPure, Lakewood, NJ, USA) operated with pure water. The gas flow was mixed with N_2 containing ∼5 ppmV NO_2 (Air Liquide Deutschland GmbH, Düsseldorf, Germany), and the NO_2 concentration was regulated by the amount of the added ∼5 ppmV NO_2 gas. After mixing, the gas flow was split, one flow was equipped with a humidity sensor (FHA 646-E1C with Alemo 2590-3 instrument, Ahlborn, Mess- und Regelungstechnik, Germany) and used to react with the samples, and the other one was used to determine the O_3 and NO_2 concentrations. The O_3 and NO_2 concentrations were monitored using commercial monitoring instruments (ozone analyzer: 49i, Thermo Scientific, Braunschweig, Germany; NOx analyzer: 42i-TL, Thermo Scientific). The O_3/NO_2 gas mixtures were directly bubbled through 1.5 mL of 0.13 mg mL^{-1} Phl p 5 aqueous solutions at a flow rate of 60 mL min^{-1} using a Teflon tube (ID: 1.59 mm). The experiments were performed in duplicates.

2.3 Protein modification by $ONOO^-$

For each reaction, 300 µL protein stock solution (1 mg mL^{-1}) was transferred into a brown reaction tube (Eppendorf, Hamburg, Germany), and 7.7 µL 2 M NH_4HCO_3 buffer was added to yield a final buffer concentration of 0.05 M. After being thawed on ice, an aliquot of peroxynitrite was diluted with 0.3 M NaOH in the ratio 1/1 to reduce the pipetting error for small volumes.

The diluted peroxynitrite solution was added to the protein sample to yield $ONOO^-$/Tyr molar ratios of 0.1/1 as well as 0.5/1, and the original peroxynitrite solution was added to yield $ONOO^-$/Tyr molar ratios of 1/1, 3/1, and 5/1. The reaction was performed on ice for 110 min. Afterwards, the sample was pipetted into a 10 kDa centrifugal filter (Amicon® Ultra; Merck) and centrifuged at 14 000 g for 2 min (5427 R, Eppendorf). Then, 200 µL pure water was added, and the procedure was repeated four times. For sample recovery, the filter was turned upside down into a clean micro centrifuge tube and centrifuged at 1 000 g for 2 min. To remove possible sample residues, the filter was washed with 200 µL pure water and centrifuged again upside down into the concentrated protein sample. The experiments were performed in duplicates.

2.4 HPLC-DAD analysis

The total nitration degrees were determined as described previously (Selzle et al., 2013) using an high-performance liquid chromatography coupled to diode array detection (HPLC-DAD; Agilent Technologies 1260 Infinity series, Waldbronn, Germany). The HPLC system consisted of a quaternary pump (G1311B), an autosampler (G7129A), a column thermostat (G1316C), and a photodiode array detector (G1315C). ChemStation software (Rev. C.01.07, Agilent) was used for system control and data analysis. A monomerically bound C_{18} column (Vydac 238TP, 250 mm x 2.1 mm i.d., 5 µm, Hichrom, Berkshire, UK) was used for chromatographic separation. Gradient elution was performed at a flow rate of 0.2 mL min^{-1} with 0.1 % (v/v) TFA in water and ACN. The sample injection volume was 10 µL, and each chromatographic run was performed in duplicate.

The protein oligomer mass fractions and the individual nitration degrees were determined simultaneously as described in Liu et al. (2017b) using an HPLC-DAD system (Agilent Technologies 1260 Infinity II series), which consisted of a binary pump (G7112B), a multisampler (G7167A), a column thermostat (G7116A), and a photodiode array detector (DAD, G7115A). ChemStation software (Rev. C.01.08, Agilent) was used for system control and data analysis. Molecular weight separation by size exclusion chromatography (SEC) was carried out using a SEC column (PSS Proteema BioInert Micro 300 Å, 250 mm x 4.6 mm inner diameter, 3 µm particle size; PSS Polymer Standards Service GmbH, Mainz, Germany). The sample injection volume was 30 µL, and each chromatographic run was performed in duplicate.

3 Results and discussion

The nitration and oligomerization of recombinant Phl p 5 modified in aqueous solution by O_3/NO_2 and $ONOO^-$, respectively, was studied in a total of 34 experiments. We determined the total nitration degree averaged over all tyrosine residues in the protein and the oligomerization combined with individual nitration degrees for the different protein oligomer mass fractions using reversed-phase and size-exclusion chromatography.

3.1 Protein modification by O_3/NO_2

3.1.1 Total nitration degree

Figure 1 shows the effect of low (50 ppb/50 ppb) and high (200 ppb/200 ppb) concentrations of O_3 and NO_2 on the nitration of recombinant Phl p 5. Exposure experiments were performed in aqueous phase and at varying exposure times between 0.5 h and 10 h. The total nitration degree (ND_{tot}) increased over the course of reaction time and with O_3/NO_2 concentrations. The maximum total nitration degree was up to 8 ± 1 % after 10 h of exposure at high O_3/NO_2 concentrations, reaching a plateau after 4 h. This plateau corresponds to ~ 1.1 nitrated Tyr residue per Phl p 5 monomer. No plateau is reached for the exposure at low concentrations, and a linear increase is found with the highest value of 4 ± 1 % after 10 h. We assume that a similar plateau will be reached for the exposure at low concentrations for longer exposure times. Reinmuth-Selzle et al. (2014) investigated the chemical modification of the major birch pollen allergen Bet v 1.0101 by O_3/NO_2 in aqueous phase and found a nitration degree of $\sim 22 \pm 7$ % after 17 h of exposure at 120 ppb O_3 and 120 ppb NO_2. As these reaction conditions are comparable to the ones used in this study (longer exposure times but lower O_3/NO_2 concentrations for Reinmuth-Selzle et al. (2014)), these results suggest that the allergen Bet v 1 is more easily nitrated than Phl p 5. This would mean that the Tyr residues of Bet v 1 are easier accessible for the air pollutants than the ones in the grass pollen allergen. Considering that the Bet v 1 molecule contains in total 7 Tyr residues, the nitration degree would corresponds to ~ 1.5 nitrated Tyr residues per Bet v 1 monomer, which is similar to the results obtained in this study.

3.1.2 Oligomerization and individual nitration degree

To investigate the temporal evolution of protein dimers and higher oligomers and to determine their individual nitration degrees, the Phl p 5 samples exposed to O_3 and NO_2 were further analyzed by size-exclusion chromatography. Figure 2 shows the effect of low (50 ppb/50 ppb) and high (200 ppb/200 ppb) concentrations of O_3 and NO_2 on the protein oligomerization of recombinant Phl p 5. Generally, the mass fractions of Phl p 5 dimers and higher oligomers increased over the course of reaction time while the mass fraction of Phl p 5 monomers decreased. We found up to 17 ± 2 % dimers at low O_3/NO_2 concentrations and up to 21 ± 3 % at high concentrations after 10 h of exposure (Fig. 2b). This is not a big difference, but the temporal evolution of the dimers differed strongly for the two conditions. At low O_3/NO_2 concentrations, the dimer fraction increased only slightly in the beginning and became stronger after 4 h. In contrast, at high concentrations, the dimer fraction increased rapidly within the first 4 h of exposure reaching a plateau for longer exposure times. A stronger effect of the different O_3/NO_2 concentrations on the oligomerization was found for higher oligomers. Only 7 ± 1 % of higher oligomers ($n \geq 3$) were formed upon exposure to low O_3/NO_2 concentrations, whereas up to 30 ± 5 % were formed at high concentrations after 10 h of exposure (Fig. 2c). The minimum mass fraction of Phl p 5 monomers correspondingly was 76 ± 2 % for low O_3/NO_2 concentrations and 49 ± 2 % for high concentrations (Fig. 2a). Our results are in good agreement with Liu et al. (2017a), who investigated the nitration and oligomerization of the model protein bovine serum albumin (BSA) upon exposure to different O_3/NO_2 concentrations in aqueous phase. They found up to ~ 20 % dimers and up to ~ 5 % of higher oligomers after 10 h of exposure at 50 ppb O_3 and 50 ppb NO_2.

Additionally, we determined the individual nitration degrees for the different Phl p 5 oligomer mass fractions (Fig. 3). The individual nitration degrees for the Phl p 5 monomers and dimers increased over the course of reaction time. The maximum nitration degree for the Phl p 5 monomer mass fraction was 3 ± 1 % for low O_3/NO_2 concentrations and 8 ± 2 % for high concentrations after 10 h of exposure, reaching a plateau corresponding to ~ 1 nitrated Tyr residue per Phl p 5 monomer (NTyr/Monomer) after 4 h of exposure at high O_3/NO_2 concentrations (Fig. 3a), which is in good agreement with the results obtained by reversed-phase chromatography. The maximum nitration degree for the Phl p 5 dimer mass fraction was 8 ± 1 % for low O_3/NO_2 concentrations and 12 ± 2 % for high concentrations after 10 h of exposure (Fig. 3b). A plateau was reached already after 2 h of exposure for both concentrations, corresponding to ~ 2.2 and ~ 3.4 nitrated Tyr residues per Phl p 5 dimer (NTyr/Dimer), respectively. The results for the higher oligomer mass fraction ($n \geq 3$) show a different trend independently of the exposure time and O_3/NO_2 concentrations 3c). The individual nitration degree for this fraction is on average 14 ± 4 %. As we found higher nitration degrees for the dimer than for the monomer mass fraction, our results are in contrast to the results of Liu et al. (2017a), who found nitration degrees of ~ 6 % for the monomer and ~ 4 % for the dimer mass fraction after 10 h of exposure at 50 ppb O_3 and 50 ppb NO_2.

The observed time and concentration dependence for the nitration and oligomerization of the major grass pollen allergen Phl p 5 confirms earlier studies with the model protein BSA (Liu et al., 2017a; Shiraiwa et al., 2012b), who discovered that the first and rate limiting step is the reaction with O_3 forming tyrosyl radicals as long-lived reactive oxygen intermediates (ROIs). The second step is then the fast reaction of the ROIs with NO_2 leading to the formation of 3-nitrotyrosine or the reaction with another ROI forming dityrosine cross-links and thereby protein dimers and higher oligomers.

3.2 Protein modification by $ONOO^-$

3.2.1 Total nitration degree

For the investigation of endogenous protein modifications, e.g., during oxidative stress, recombinant Phl p 5 was treated with different concentrations of $ONOO^-$. Experiments were performed in aqueous phase with $ONOO^-$/Tyr molar ratios of 0.1/1, 0.5/1, 1/1, 3/1, and 5/1. Figure 4 shows the effect of different $ONOO^-$ concentrations on the nitration of recombinant Phl p 5. The total nitration degree (ND_{tot}) increased with increasing $ONOO^-$ concentration. The maximum total nitration degree was up to 25 ± 3 % for a $ONOO^-$/Tyr molar ratio of 5/1. For a molar ratio larger than 1/1, the total nitration degree leveled to a plateau between 20 and 25 %, which corresponds to $\sim 3-3.5$ nitrated Tyr residues per Phl p 5 monomer. These results are in good agreement with Reinmuth-Selzle et al. (2014), who investigated the nitration of the major birch pollen allergen Bet v 1.0101 by $ONOO^-$. At a $ONOO^-$/Tyr molar ratio of 5/1, they found a total nitration degree of $\sim 24 \pm 2$ %.

3.2.2 Oligomerization and individual nitration degree

The temporal evolution of protein dimers and higher oligomers and their individual nitration degrees were analyzed by size-exclusion chromatorgraphy. Figure 5 shows the effect of different concentrations of $ONOO^-$ on the oligomerization of recombinant Phl p 5. The highest oligomer formation was found for the $ONOO^-$/Tyr molar ratios of 0.5/1 and 1/1, reaching up to

26 ± 1 % for dimers and 18 ± 3 % for higher oligomers ($n \geq 3$). The minimum mass fraction of monomers correspondingly was 56 ± 5 %. For $ONOO^-$/Tyr molar ratios of 3/1 and 5/1, however, lower oligomer formation was found.

Additionally, we determined the individual nitration degrees for the different oligomer mass fractinos. Figure 6 shows that the individual nitration degrees for the different Phl p 5 mass fractions increased with increasing concentration of $ONOO^-$. The maximum nitration degree for the Phl p 5 monomer mass fraction was 29 ± 1 %, which corresponds to ~ 4 nitrated Tyr residues per Phl p 5 monomer. The maximum nitration degree for the Phl p 5 dimer mass fraction was 37 ± 3 %, which corresponds to ~ 10 nitrated Tyr residues per Phl p 5 dimer. The maximum nitration degree for the Phl p 5 mass fraction of higher oligomers ($n \geq 3$) was 37 ± 4 %.

Our results show that the formation of oligomers predominates at low $ONOO^-$ concentrations, whereas the nitration of Tyr residues mainly takes place at high concentrations. This is in good agreement with Pfeiffer et al. (2000), who found that the dimerization of Tyr radicals outcompeted the nitration reaction at low $ONOO^-$ concentrations.

4 Conclusions

In this study, we analyzed the reaction products of the timothy grass pollen allergen Phl p 5 exposed to O_3 and NO_2 mimicking air pollution and $ONOO^-$ mimicking oxidative stress and inflammation. The results show that the tyrosine nitration degree and the formation of protein dimers and higher oligomers depend on the nitrating agent and the reaction conditions (concentration, exposure time). Both, the nitration degree and the oligomer formation, were higher for the reaction with $ONOO^-$. The maximum nitration degree of 25% was achieved by reaction with $ONOO^-$, while exposure to O_3/NO_2 yielded ND values of up to 8%. Nitrated oligomers were formed upon exposure to O_3/NO_2 and reaction with $ONOO^-$. We observed that the tyrosine cross-linking predominates at low $ONOO^-$ concentrations, whereas nitrotyrosines are mainly formed at higher concentrations. Our results are in accordance with earlier studies investigating the chemical modification of other proteins by O_3/NO_2 and $ONOO^-$. The data will be further interpreted with regard to the protein structure, and kinetic modeling will be included in the future. The various reaction products including nitrated and cross-linked proteins might have different allergenic potential. Thus, related further studies are required to investigate the immunogenicity of the different nitrated and cross-linked variants separately. The efficient nitration and oligomerization of allergenic proteins by atmospheric air pollutants and the enhanced allergenic and immunostimulatory potential of chemically modified proteins call for action to improve air quality and public health in the Anthropocene.

Data availability. All data are available from the corresponding authors upon request.

Author contributions. KL, UP, and JFN designed the study. ATK, KRS, KZ, CSK, and CW performed the experiments. ATK, KRS, KZ, KL, UP, JF analyzed, interpreted and discussed the results. CSK, CW, and MGW contributed to the discussion. ATK and JFN wrote the manuscript.

Competing interests. The authors declare that they have no conflict of interest.

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References

Abello, N., Kerstjens, H. A. M., Postma, D. S., and Bischoff, R.: Protein Tyrosine Nitration: Selectivity, Physicochemical and Biological Consequences, Denitration, and Proteomics Methods for the Identification of Tyrosine-Nitrated Proteins, J. Proteome Res., 8, 3222–3238, https://doi.org/10.1021/pr900039c, https://pubs.acs.org/doi/abs/10.1021/pr900039c, 2009.

Ackaert, C., Kofler, S., Horejs-Hoeck, J., Zulehner, N., Asam, C., von Grafenstein, S., Fuchs, J. E., Briza, P., Liedl, K. R., Bohle, B., Ferreira, F., Brandstetter, H., Oostingh, G. J., and Duschl, A.: The Impact of Nitration on the Structure and Immunogenicity of the Major Birch Pollen Allergen Bet v 1.0101, PLoS One, 9, e104 520, https://doi.org/10.1371/journal.pone.0104520, https://dx.plos.org/10.1371/journal.pone.0104520, 2014.

Asher, M. I., Montefort, S., Björkstén, B., Lai, C. K., Strachan, D. P., Weiland, S. K., and Williams, H.: Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys, Lancet, 368, 733–743, https://doi.org/10.1016/S0140-6736(06)69283-0, https://linkinghub.elsevier.com/retrieve/pii/S0140673606692830, 2006.

Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A.: Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide., Proc. Natl. Acad. Sci., 87, 1620–1624, https://doi.org/10.1073/pnas.87.4.1620, http://www.pnas.org/cgi/doi/10.1073/pnas.87.4.1620, 1990.

Bryce, M., Drews, O., Schenk, M., Menzel, A., Estrella, N., Weichenmeier, I., Smulders, M., Buters, J., Ring, J., Görg, A., Behrendt, H., and Traidl-Hoffmann, C.: Impact of Urbanization on the Proteome of Birch Pollen and Its Chemotactic Activity on Human Granulocytes, Int. Arch. Allergy Immunol., 151, 46–55, https://doi.org/10.1159/000232570, https://www.karger.com/Article/FullText/232570, 2010.

D'Amato, G., Vitale, C., Rosario, N., Neto, H. J. C., Chong-Silva, D. C., Mendonça, F., Perini, J., Landgraf, L., Solé, D., Sánchez-Borges, M., Ansotegui, I., and D'Amato, M.: Climate change, allergy and asthma, and the role of tropical forests, World Allergy Organ. J., 10, 11, https://doi.org/10.1186/s40413-017-0142-7, http://dx.doi.org/10.1186/s40413-017-0142-7https://linkinghub.elsevier.com/retrieve/pii/S1939455119300110, 2017.

Franze, T., Weller, M. G., Niessner, R., and Pöschl, U.: Protein Nitration by Polluted Air, Environ. Sci. Technol., 39, 1673–1678, https://doi.org/10.1021/es0488737, http://www.ncbi.nlm.nih.gov/pubmed/15819224http://pubs.acs.org/doi/abs/10.1021/es0488737, 2005.

Ghiani, A., Aina, R., Asero, R., Bellotto, E., and Citterio, S.: Ragweed pollen collected along high-traffic roads shows a higher allergenicity than pollen sampled in vegetated areas, Allergy, 67, 887–894, https://doi.org/10.1111/j.1398-9995.2012.02846.x, http://doi.wiley.com/10.1111/j.1398-9995.2012.02846.x, 2012.

Greenacre, S. A. and Ischiropoulos, H.: Tyrosine nitration: Localisation, quantification, consequences for protein function and signal transduction, Free Radic. Res., 34, 541–581, https://doi.org/10.1080/10715760100300471, http://www.tandfonline.com/doi/full/10.1080/10715760100300471, 2001.

Gruijthuijsen, Y., Grieshuber, I., Stöcklinger, A., Tischler, U., Fehrenbach, T., Weller, M., Vogel, L., Vieths, S., Pöschl, U., and Duschl, A.: Nitration Enhances the Allergenic Potential of Proteins, Int. Arch. Allergy Immunol., 141, 265–275, https://doi.org/10.1159/000095296, https://www.karger.com/Article/FullText/95296, 2006.

Gunaydin, H. and Houk, K. N.: Mechanisms of Peroxynitrite-Mediated Nitration of Tyrosine, Chem. Res. Toxicol., 22, 894–898, https://doi.org/10.1021/tx800463y, https://pubs.acs.org/doi/10.1021/tx800463y, 2009.

Ischiropoulos, H.: Protein tyrosine nitration—An update, Arch. Biochem. Biophys., 484, 117–121, https://doi.org/10.1016/j.abb.2008.10.034, http://dx.doi.org/10.1016/j.abb.2008.10.034https://linkinghub.elsevier.com/retrieve/pii/S0003986108005031, 2009.

Kampf, C. J., Liu, F., Reinmuth-Selzle, K., Berkemeier, T., Meusel, H., Shiraiwa, M., and Pöschl, U.: Protein Cross-Linking and Oligomerization through Dityrosine Formation upon Exposure to Ozone, Environ. Sci. Technol., 49, 10 859–10 866, https://doi.org/10.1021/acs.est.5b02902, 2015.

Karle, A. C., Oostingh, G. J., Mutschlechner, S., Ferreira, F., Lackner, P., Bohle, B., Fischer, G. F., Vogt, A. B., and Duschl, A.: Nitration of the Pollen Allergen Bet v 1.0101 Enhances the Presentation of Bet v 1-Derived Peptides by HLA-DR on Human Dendritic Cells, PLoS One, 7, e31 483, https://doi.org/10.1371/journal.pone.0031483, https://dx.plos.org/10.1371/journal.pone.0031483, 2012.

Kunert, A. T., Lamneck, M., Helleis, F., Pöhlker, M. L., Pöschl, U., and Fröhlich-Nowoisky, J.: Twin-plate Ice Nucleation Assay (TINA) with infrared detection for high-throughput droplet freezing experiments with biological ice nuclei in laboratory and field samples, Atmos. Meas. Tech., 11, 6327–6337, https://doi.org/10.5194/amt-2018-230, https://doi.org/10.5194/amt-2018-230, 2018.

Liu, F., Lakey, P. S. J., Berkemeier, T., Tong, H., Kunert, A. T., Meusel, H., Cheng, Y., Su, H., Fröhlich-Nowoisky, J., Lai, S., Weller, M. G., Shiraiwa, M., Pöschl, U., and Kampf, C. J.: Atmospheric protein chemistry influenced by anthropogenic air pollutants: nitration and oligomerization upon exposure to ozone and nitrogen dioxide, Faraday Discuss., 200, 413–427, https://doi.org/10.1039/C7FD00005G, http://pubs.rsc.org/en/Content/ArticleLanding/2017/FD/C7FD00005Ghttp://xlink.rsc.org/?DOI=C7FD00005G, 2017a.

Liu, F., Reinmuth-Selzle, K., Lai, S., Weller, M. G., Pöschl, U., and Kampf, C. J.: Simultaneous determination of nitrated and oligomerized proteins by size exclusion high-performance liquid chromatography coupled to photodiode array detection, J. Chromatogr. A, 1495, 76–82, https://doi.org/10.1016/j.chroma.2017.03.015, http://dx.doi.org/10.1016/j.chroma.2017.03.015https://linkinghub.elsevier.com/retrieve/pii/S0021967317303795, 2017b.

Motta, A., Marliere, M., Peltre, G., Sterenberg, P., and Lacroix, G.: Traffic-Related Air Pollutants Induce the Release of Allergen-Containing Cytoplasmic Granules from Grass Pollen, Int. Arch. Allergy Immunol., 139, 294–298, https://doi.org/10.1159/000091600, https://www.karger.com/Article/FullText/91600, 2006.

Ouyang, Y., Xu, Z., Fan, E., Li, Y., and Zhang, L.: Effect of nitrogen dioxide and sulfur dioxide on viability and morphology of oak pollen, Int. Forum Allergy Rhinol., 6, 95–100, https://doi.org/10.1002/alr.21632, http://doi.wiley.com/10.1002/alr.21632, 2016.

Pawankar, R.: Allergic diseases and asthma: a global public health concern and a call to action, World Allergy Organ. J., 7, 12, https://doi.org/10.1186/1939-4551-7-12, http://dx.doi.org/10.1186/1939-4551-7-12https://linkinghub.elsevier.com/retrieve/pii/S193945511930242X, 2014.

Pawankar, R., Baena-Cagnani, C. E., Bousquet, J., Canonica, G. W., Cruz, A. A., Kaliner, M. A., and Lanier, B. Q.: State of World Allergy Report 2008: Allergy and Chronic Respiratory Diseases, World Allergy Organ. J., 1, S4–S17, https://doi.org/10.1186/1939-4551-1-S1-S4, http://dx.doi.org/10.1186/1939-4551-1-S1-S4https://linkinghub.elsevier.com/retrieve/pii/S1939455119306040, 2008.

Pawankar, R., Canonica, G. W., Holgate, S. T., Lockey, Richard, F., and Blaiss, M. S., eds.: White Book on Allergy: Update 2013, World Allergy Organization (WAO), Milwaukee, Wisconsin, 2013.

Pfeiffer, S., Schmidt, K., and Mayer, B.: Dityrosine Formation Outcompetes Tyrosine Nitration at Low Steady-state Concentrations of Peroxynitrite, J. Biol. Chem., 275, 6346–6352, https://doi.org/10.1074/jbc.275.9.6346, http://www.jbc.org/lookup/doi/10.1074/jbc.275.9.6346, 2000.

Reinmuth-Selzle, K., Ackaert, C., Kampf, C. J., Samonig, M., Shiraiwa, M., Kofler, S., Yang, H., Gadermaier, G., Brandstetter, H., Huber, C. G., Duschl, A., Oostingh, G. J., and Pösch: Nitration of the Birch Pollen Allergen Bet v 1.0101: Efficiency and Site-Selectivity of Liquid and Gaseous Nitrating Agents, J. Proteome Res., 13, 1570–1577, https://doi.org/10.1021/pr401078h, 2014.

Reinmuth-Selzle, K., Kampf, C. J., Lucas, K., Lang-Yona, N., Fröhlich-Nowoisky, J., Shiraiwa, M., Lakey, P. S. J., Lai, S., Liu, F., Kunert, A. T., Ziegler, K., Shen, F., Sgarbanti, R., Weber, B., Bellinghausen, I., Saloga, J., Weller, M. G., Duschl, A., Schuppan, D., and Pöschl, U.: Air Pollution and Climate Change Effects on Allergies in the Anthropocene: Abundance, Interaction, and Modification of Allergens and Adjuvants, Environ. Sci. Technol., 51, 4119–4141, https://doi.org/10.1021/acs.est.6b04908, http://pubs.acs.org/doi/abs/10.1021/acs.est.6b04908https://pubs.acs.org/doi/10.1021/acs.est.6b04908, 2017.

Selzle, K., Ackaert, C., Kampf, C. J., Kunert, A. T., Duschl, A., Oostingh, G. J., and Pöschl, U.: Determination of nitration degrees for the birch pollen allergen Bet v 1, Anal. Bioanal. Chem., 405, 8945–8949, https://doi.org/10.1007/s00216-013-7324-0, 2013.

Shiraiwa, M., Ammann, M., Koop, T., and Pöschl, U.: Gas uptake and chemical aging of semisolid organic aerosol particles, Proc. Natl. Acad. Sci. U. S. A., 108, 11 003–11 008, https://doi.org/10.1073/pnas.1103045108, http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3131339&tool=pmcentrez&rendertype=abstract, 2011.

Shiraiwa, M., Selzle, K., and Pöschl, U.: Hazardous components and health effects of atmospheric aerosol particles: reactive oxygen species, soot, polycyclic aromatic compounds and allergenic proteins, Free Radic. Res., pp. 1–13, https://doi.org/10.3109/10715762.2012.663084, http://www.ncbi.nlm.nih.gov/pubmed/22300277, 2012a.

Shiraiwa, M., Selzle, K., Yang, H., Sosedova, Y., Ammann, M., and Pöschl, U.: Multiphase chemical kinetics of the nitration of aerosolized protein by ozone and nitrogen dioxide, Environ. Sci. Technol., 46, 6672–6680, https://doi.org/10.1021/es300871b, 2012b.

Taylor, P. E., Flagan, R. C., Valenta, R., and Glovsky, M.: Release of allergens as respirable aerosols: A link between grass pollen and asthma, J. Allergy Clin. Immunol., 109, 51–56, https://doi.org/10.1067/mai.2002.120759, https://linkinghub.elsevier.com/retrieve/pii/S0091674902503754, 2002.

Taylor, P. E., Flagan, R. C., Miguel, A. G., Valenta, R., and Glovsky, M. M.: Birch pollen rupture and the release of aerosols of respirable allergens, Clin. Exp. Allergy, 34, 1591–1596, https://doi.org/10.1111/j.1365-2222.2004.02078.x, http://doi.wiley.com/10.1111/j.1365-2222.2004.02078.x, 2004.

Traidl-Hoffmann, C., Jakob, T., and Behrendt, H.: Determinants of allergenicity, J. Allergy Clin. Immunol., 123, 558–566, https://doi.org/10.1016/j.jaci.2008.12.003, http://dx.doi.org/10.1016/j.jaci.2008.12.003https://linkinghub.elsevier.com/retrieve/pii/S0091674908023233, 2009.

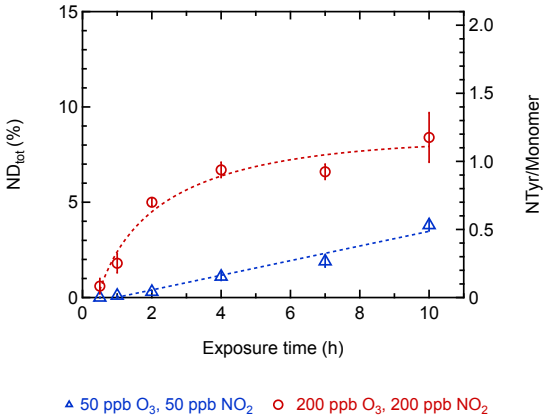

Figure 1. Total nitration degree (ND_{tot}) of Phl p 5 exposed to low and high concentrations of O_3 and NO_2 (primary y-axis) and number of nitrotyrosines (NTyr) per Phl p 5 monomer (secondary y-axis) for different exposure times. The data points and error bars represent the arithmetic mean values and standard deviations of duplicate experiments. Dashed lines represent preliminary polynomial fits for better visualization.

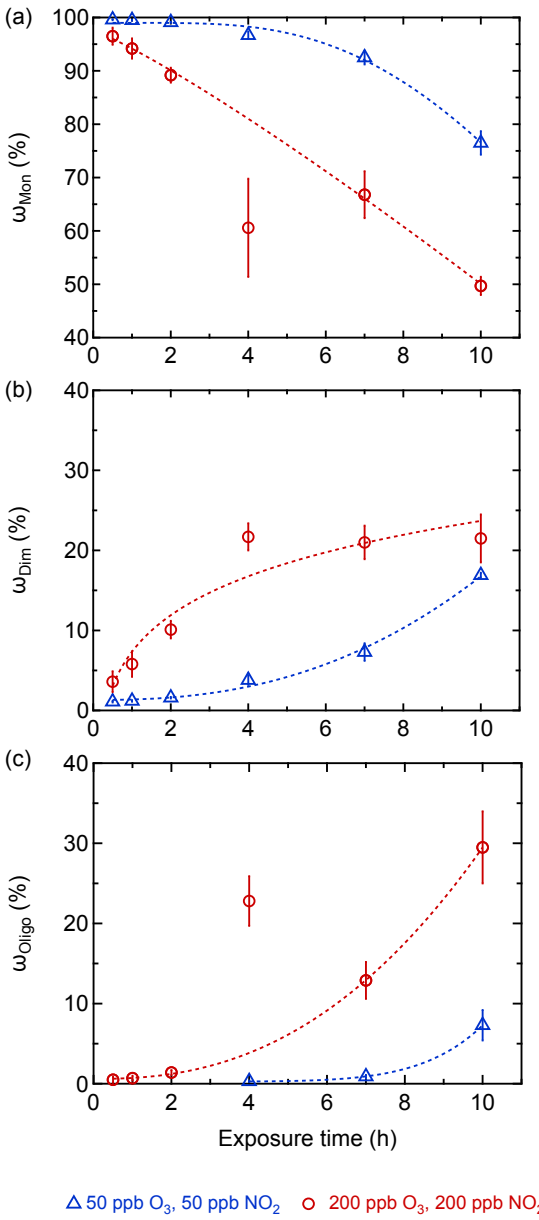

Figure 2. Temporal evolution of protein oligomer mass fractions of Phl p 5 exposed to low and high concentrations of O_3 and NO_2 for different exposure times: (a) monomers (ω_{Mon}), (b) dimers (ω_{Dim}), and (c) higher oligomers ($n \geq 3$) (ω_{Oligo}). The data points and error bars represent the arithmetic mean values and standard deviations of duplicate experiments. Dashed lines represent preliminary polynomial fits for better visualization.

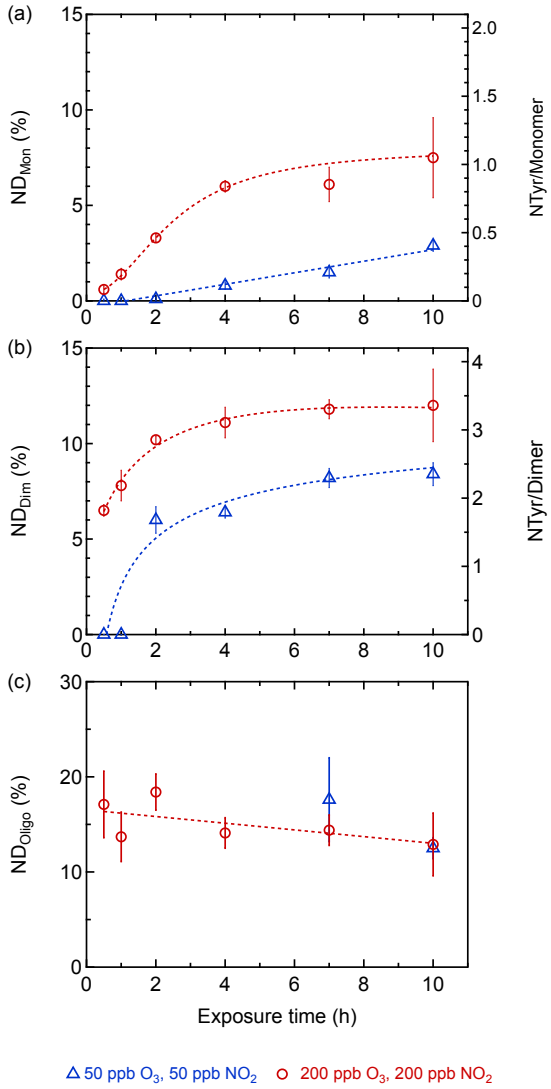

Figure 3. Individual nitration degrees of Phl p 5 oligomer mass fractions (primary y-axis) and number of nitrotyrosines (NTyr) per Phl p 5 monomer and dimer, respectivly, (secondary y-axis) for different exposure times: (a) monomers (ND_{Mon}), (b) dimers (ND_{Dim}), and (c) higher oligomers ($n \geq 3$) (ND_{Oligo}). The data points and error bars represent the arithmetic mean values and standard deviations of duplicate experiments. Dashed lines represent preliminary polynomial fits for better visualization.

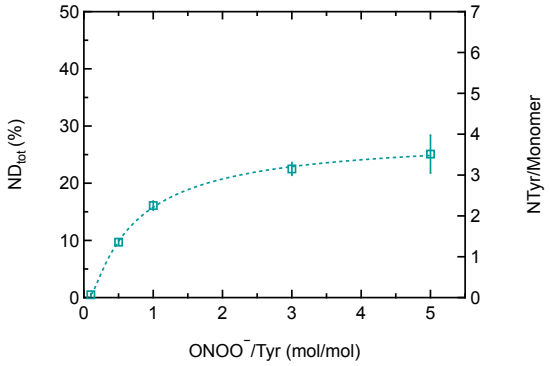

Figure 4. Total nitration degree (ND_{tot}) of Phl p 5 (primary y-axis) and number of nitrotyrosines (NTyr) per Phl p 5 monomer (secondary y-axis) for different $ONOO^-$ concentrations. The data points and error bars represent the arithmetic mean values and standard deviations of duplicate experiments. Dashed lines represent preliminary polynomial fits for better visualization.

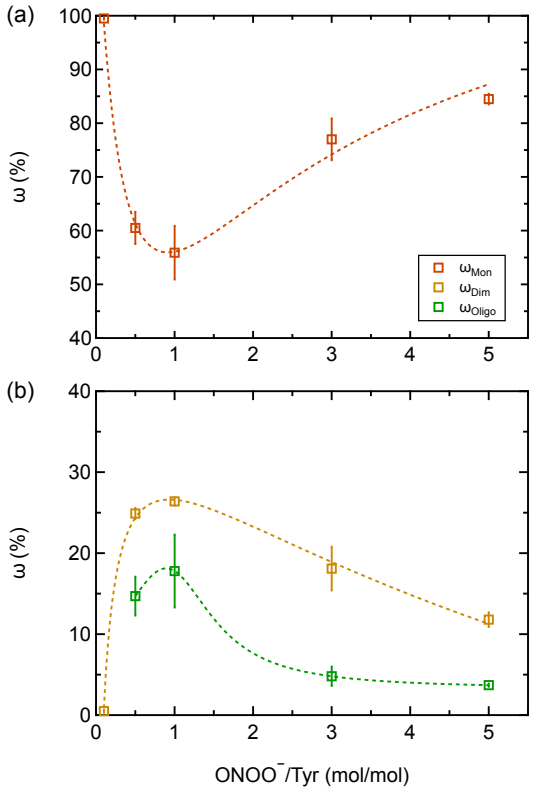

Figure 5. Formation of protein oligomer mass fractions of Phl p 5 treated with different $ONOO^-$ concentrations: (a) monomers (ω_{Mon}), (b) dimers (ω_{Dim}) and higher oligomers ($n \geq 3$) (ω_{Oligo}). The data points and error bars represent the arithmetic mean values and standard deviations of duplicate experiments. Dashed lines represent preliminary polynomial fits for better visualization.

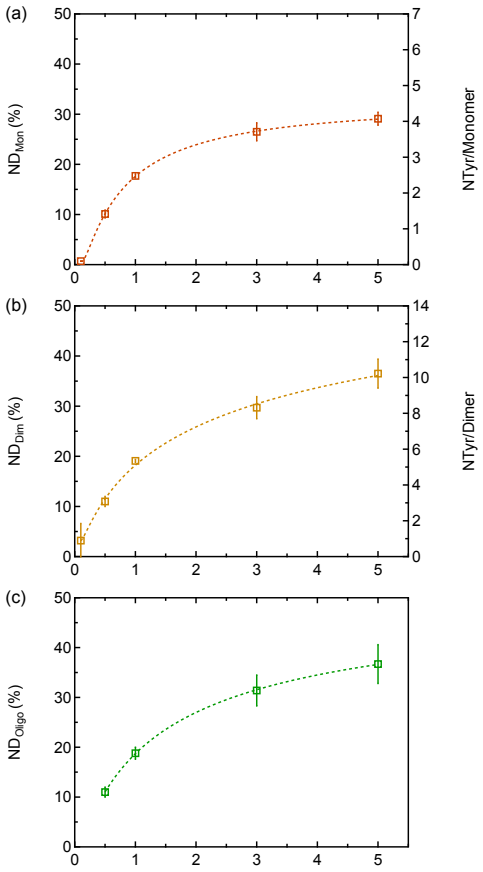

Figure 6. Individual nitration degrees of Phl p 5 oligomer mass fractions (primary y-axis) and number of nitrotyrosines (NTyr) per Phl p 5 monomer and dimer, respectivly, (secondary y-axis) for different $ONOO^-$ concentrations: (a) monomers (ND_{Mon}), (b) dimers (ND_{Dim}), and (c) higher oligomers ($n \geq 3$) (ND_{Oligo}). The data points and error bars represent the arithmetic mean values and standard deviations of duplicate experiments. Dashed lines represent preliminary polynomial fits for better visualization.

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Chemical modification of pro-inflammatory proteins by peroxynitrite increases activation of TLR4 and NF-κB: Implications for the health effects of air pollution and oxidative stress

Kira Ziegler[1], Anna T. Kunert[1], Kathrin Reinmuth-Selzle[1], Anna Lena Leifke[1], Darius Widera[2], Michael G. Weller[3], Detlef Schuppan[4,5], Janine Fröhlich-Nowoisky[1], Kurt Lucas[1], Ulrich Pöschl[1]

[1]Max Planck Institute for Chemistry, Multiphase Chemistry Department, 55128 Mainz, Germany; [2]Stem Cell Biology and Regenerative Medicine Group, School of Pharmacy, University of Reading, RG6 6AP Reading, UK; [3]Federal Institute for Materials Research and Testing (BAM), Berlin, Germany; [4]Institute of Translational Immunology, University Medical Center of the Johannes Gutenberg University, 55131 Mainz, Germany; [5]Division of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, MA 02215, USA

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Author contributions

KL and UP conceived and directed the study. KZ, ATK, KRS, ALL, and KL designed and performed the experiments. KZ, ATK, KRS, MGW, JFN, KL, and UP analyzed, interpreted and discussed the data. DW and DS contributed to the design, interpretation, and discussion. All authors contributed to the preparation and editing of the manuscript. KZ and ATK contributed equally.

Chemical modification of pro-inflammatory proteins by peroxynitrite increases activation of TLR4 and NF-κB: Implications for the health effects of air pollution and oxidative stress

Kira Ziegler[a,1], Anna T. Kunert[a,1], Kathrin Reinmuth-Selzle[a], Anna Lena Leifke[a], Darius Widera[b], Michael G. Weller[c], Detlef Schuppan[d,e], Janine Fröhlich-Nowoisky[a], Kurt Lucas[a,**], and Ulrich Pöschl[a,*]

[a]Max Planck Institute for Chemistry, Multiphase Chemistry Department, 55128 Mainz, Germany

[b]Stem Cell Biology and Regenerative Medicine Group, School of Pharmacy, University of Reading, RG6 6AP Reading, UK

[c]Federal Institute for Materials Research and Testing (BAM), Berlin, Germany

[d]Institute of Translational Immunology, University Medical Center of the Johannes Gutenberg University, 55131 Mainz, Germany

[e]Division of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, MA 02215, USA

[1]equal co-author contributions

*corresponding author: u.poschl@mpic.de

**corresponding author: k.lucas@mpic.de

Keywords

Protein modification

TLR4

Peroxynitrite

α-Synuclein

HSP60

HMGB1

Abbreviations

α-Syn - α-Synuclein

HSP60 - heat shock protein 60

HMGB1 - high-mobility-group box 1 protein

OVA - ovalbumin

TLR4 - Toll-like receptor 4

NF-κB - nuclear factor 'kappa-light-chain-enhancer' of activated B-cells

DAMP - damage-associated molecular patterns

IL - interleukin

Abstract

Environmental pollutants like fine particulate matter can cause adverse health effects through oxidative stress and inflammation. Reactive oxygen and nitrogen species (ROS/RNS) such as peroxynitrite can chemically modify proteins, but the effects of such modifications on the immune system and human health are not well understood. In the course of inflammatory processes, the Toll-like receptor 4 (TLR4) can sense damage-associated molecular patterns (DAMPs). Here, we investigate how the TLR4 response and pro-inflammatory potential of the proteinous DAMPs α-Synuclein (α-Syn), heat shock protein 60 (HSP60), and high-mobility-group box 1 protein (HMGB1), which are relevant in neurodegenerative and cardiovascular diseases, changes upon chemical modification with peroxynitrite.

For the peroxynitrite-modified proteins, we found a strongly enhanced activation of TLR4 and of the pro-inflammatory transcription factor NF-κB in stable reporter cell lines as well as increased mRNA expression and secretion of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-8 in human monocytes (THP-1). This enhanced activation of innate immunity via TLR4 is mediated by covalent chemical modifications of the studied DAMPs.

Our results show that proteinous DAMPs modified by peroxynitrite more potently amplify inflammation via TLR4 activation than the native DAMPs, and provide first evidence that such modifications can directly enhance innate immune responses via a defined receptor. These findings suggest that environmental pollutants and related ROS/RNS may play a role in promoting acute and chronic inflammatory disorders by structurally modifying the body's own DAMPs. This may have important consequences for chronic neurodegenerative, cardiovascular or gastrointestinal diseases that are prevalent in modern societies, and calls for action, to improve air quality and climate in the Anthropocene.

1. Introduction

Reactive oxygen and nitrogen species (ROS/RNS) like peroxynitrite play important roles in oxidative stress and adverse health effects induced upon exposure to environmental pollutants and in the course of inflammatory diseases [1–8]. Peroxynitrite (ONO_2^-) is generated upon reaction of superoxide (O_2^-) and nitric oxide (NO) [9]. It can react with amino acids like tyrosine, leading to the formation of nitrotyrosine, dityrosine, and protein oligomers [10–15]. Nitrotyrosine and dityrosine are known as markers of inflammation and oxidative stress, which can influence the chemical and physiological properties of proteins [16–19]. For example, nitration can change the binding of proteins to certain receptors and thus modulate downstream signaling cascades [12,14,20–22]. Notably, preferential recognition of nitrotyrosine epitopes by immunoglobulins was reported for several inflammatory diseases [23–25]. Dityrosine crosslinks can be intra- or intermolecular, altering protein structures, causing protein aggregation/oligomerization, and affecting protein function [13,26,27].

During inflammation, damage-associated molecular patterns (DAMPs) can be actively secreted by epithelial cells, monocytes, macrophages, and other cells, or passively released by damaged and dying cells [28]. Proteinous DAMPs can have various intracellular functions, e.g., as chaperones, and when released as extracellular proteins, they can stimulate pattern recognition receptors (PRR) such as the Toll-like receptor 4 (TLR4) [29]. TLR4 signaling leads to the activation of transcription factors like the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) and the interferon regulatory transcription factor 3 (IRF3), which are key activators of inflammatory cascades [30,31]. NF-κB induces the expression and modulates the secretion of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-8, which can stimulate the corresponding cytokine receptors leading to further activation of NF-κB and other signaling pathways [30,32–34]. This positive feedback can amplify and propagate inflammatory processes in autocrine or paracrine fashion [35,36].

In this study, we investigate if and how peroxynitrite can enhance the activation of TLR4 and NF-κB by chemical modification of disease-related proteins acting as DAMPs (Table S1): α-Synuclein, heat shock protein 60, and high-mobility-group box 1 protein (HMGB1).

α-Synuclein (α-Syn) is a neuronal protein that regulates exocytosis and endocytosis of synaptic vesicles as well as ATP synthase [37–40]. Oxidative and nitrating conditions can lead to the formation of nitrated, misfolded or aggregated α-Syn, which is linked to neurodegenerative disorders such as Parkinson’s disease [39,41–46]. Oligomeric and misfolded α-Syn can be released from neuronal cells by exocytosis and stimulate TLR4 and other receptors [43,47–49].

Heat shock protein 60 (HSP60) is a mitochondrial chaperone that assists correct folding of imported mitochondrial proteins [50,51]. HSP60 can be released by stressed or necrotic cells, and modulate immune response by stimulating TLR4 and other receptors [52–58]. It contributes to the pathogenesis of chronic inflammatory diseases such as Crohn´s disease, diabetes, and atherosclerosis [59–63].

High-mobility-group box 1 protein (HMGB1) is a ubiquitously expressed nuclear protein involved in DNA replication, recombination, transcription, and repair [29,64–66]. HMGB1 can be released by activated or damaged cells and secreted by immune cells in response to microbial or pro-inflammatory stimuli [66–69]. HMGB1 can stimulate TLR4, the advanced glycosylation end product-specific receptor (RAGE), and other receptors [28,65,70–72]. It is involved in severe acute and chronic diseases, such as sepsis, cancer, cardiovascular and neurodegenerative diseases [65,66,69,73,74].

The investigated proteins were exposed to peroxynitrite, and covalent chemical modifications were analyzed by liquid chromatography (HPLC), gel electrophoresis (SDS-PAGE), and western blots (anti-nitrotyrosine, anti-dityrosine). TLR4 and NF-κB activation were determined in stable reporter cell lines using bioluminescence detection (HeLa TLR4, THP-1 NF-κB). Moreover, mRNA expression and secretion of various cytokines were measured in human monocytes (THP-1) as detailed below.

2. Materials and Methods

2.1 Protein modification and analysis

2.1.1 Peroxynitrite modification

Protein stock solutions (1 mg mL^{-1}) of α-Syn (Merck Millipore, Darmstadt, Germany), HSP60 (Abcam, Cambridge, UK), HMGB1 (Sigma Aldrich, Taufkirchen, Germany), and Ovalbumin (InvivoGen, Toulouse, France) were prepared in PBS (Thermo Fisher Scientific, Darmstadt, Germany). Ammonium bicarbonate (≥ 98 %, Ph. Eur., BP, Carl Roth, Karlsruhe, Germany) was dissolved in water to yield a final buffer concentration of 2 M, and the pH was adjusted to 7.8 by the addition of 1 M hydrogen chloride (37 % stock solution, Merck Millipore). For each reaction, 300-500 µL protein solution was transferred into a brown reaction tube (Eppendorf, Hamburg, Germany), and 7.5-12.5 µL ammonium bicarbonate buffer (2 M) was added to yield a final buffer concentration of 50 mM. After being thawed on ice, 1-8 µL sodium peroxynitrite solution (160-200 mM, Merck Millipore) was added to yield a molar ratio of peroxynitrite to tyrosine residues of 5:1, and the reaction was performed on ice for 110 min. Thereafter, the

sample was pipetted into a 10 kDa centrifugal filter (Amicon®, Merck Millipore) and centrifuged at 14,000 x g for 2 min (5427 R, Eppendorf). The sample was washed five times with 200 µL PBS and centrifugation at 14,000 x g for 2 min. For sample recovery, the filter was turned upside down, transferred into a clean microcentrifuge tube, and centrifuged at 1,000 x g for 2 min. To recover possible sample residues, the filter was washed with 200 µL fresh PBS and centrifuged upside down at 1,000 x g for 2 min into the concentrated protein sample. Ovalbumin (OVA), which is not a TLR4 agonist, was treated the same way and served as negative control in the cell culture experiments described below. For mock samples, 500 µL pure PBS was mixed with 12.5 µL ammonium bicarbonate buffer (2 M) and 8 µL peroxynitrite and treated as described above.

Protein concentrations were determined using a Synergy Neo plate reader (BioTek, Bad Friedrichshall, Germany) measuring the absorbance at 260 nm / 280 nm. For each protein, a dilution series of the native protein (25-1000 µg mL^{-1}) was used for calibration. Three microliters of modified protein solution were transferred into a micro-volume plate in triplicates (Take3 trio, BioTek), and the absorbance was measured. The protein concentrations were confirmed by SDS-PAGE and silver stain (see section 2.1.3).

For each of the investigated proteins, multiple samples of protein solution were chemically modified as outlined above, and selected samples were characterized by the analytical methods described below. An overview of the analytical results is given in Table S2 (tyrosine nitration degree, dimer/oligomer fraction).

2.1.2 HPLC-DAD analysis

For the investigated proteins, tyrosine nitration degrees were determined as described in Selzle et al. [75]. Briefly, an HPLC−DAD system (Agilent Technologies 1260 Infinity series, Waldbronn, Germany) equipped with a monomerically bound C_{18} column (Vydac 238TP, 250 mm x 2.1 mm i.d., 5 µm, Hichrom, Berkshire, UK) was used for chromatographic separation. Gradient elution was performed with 0.1 % (v/v) trifluoroacetic acid in water (VWR International GmbH, Darmstadt, Germany) and acetonitrile (Carl Roth), and absorbance was measured at wavelengths of 280 nm and 357 nm. Injection volume was 10 µL, and each chromatographic run was performed in duplicates. For system control and data analysis, ChemStation Software was used (Rev. C.01.07, Agilent). Nitration was observed in the modified samples of all proteins, and tyrosine nitration degrees were quantified in two independent experiments as specified in Table S2. The tyrosine nitration degree is defined as the concentration of nitrotyrosine divided by the sum of the concentrations of nitrotyrosine and tyrosine [75].

2.1.3 SDS-PAGE and silver stain

Protein oligomerization was visualized and quantified by silver stained SDS-PAGE (Thermo Fisher Scientific). Protein samples dissolved in PBS were mixed with an equivalent volume of 2x Laemmli buffer, containing 65.8 mM Tris-HCl (pH 6.8, Carl Roth), 26.3 % glycerol (v/v, Carl Roth), 2.1 % SDS (Carl Roth), 0.02 % bromophenol blue (Sigma Aldrich) and 5.0 % 2-mercaptoethanol (Sigma Aldrich), and heated at 95 °C for 5 min. The samples (50 ng in 10 µL) were loaded onto PROTEAN Precast gels (4–20 %, Bio-Rad, Munich, Germany) together with 5 µL Color Prestained Protein Standard, Broad Range (11–245 kDa, New-England Biolabs, Frankfurt, Germany) and stained with a silver stain kit (Thermo Fisher Scientific) following manufacturer's instructions. For image acquisition and quantification of protein monomers, dimers, and higher oligomers, a ChemiDoc system (Bio-Rad) with Image Lab software 5.2.1 (Bio-Rad) was used. Protein dimers occurred in the modified samples of all proteins (Fig. S1, Table S2), and signals of higher oligomers were observed but exceeded the analytical detection limit (3 %) only for modified HSP60 (23 ± 13 %).

2.1.4 Western blot analysis

The presence of nitrotyrosine and dityrosine residues was investigated by SDS-PAGE and subsequent western blot analysis. The native and modified protein samples were prepared in Laemmli-buffer as described in section 2.1.3. As sensitivities for the antibodies varied, different amounts of protein per lane were applied for nitrotyrosine staining (2 µg α-Syn, 5 µg HSP60, and 10 µg HMGB1) and dityrosine staining (5 µg α-Syn, 5 µg HSP60, and 5 µg HMGB1). For nitrotyrosine staining, α-Syn, HSP60, and HMGB1 were separated by a 12 %, 8 %, and 10 % SDS polyacrylamide gel, respectively, and by a Mini-PROTEAN Precast gel (4-20 %, Bio-Rad) for dityrosine staining. Thereafter, the gels were transferred onto 0.45 µm PVDF membranes (Merck Millipore) using a semi-dry transfer unit (Hoefer, Holliston, MA, USA). The membranes were blocked with 5 % fat-free milk powder (Cell Signaling Technology, Leiden, Netherlands) in Tris-buffered saline with 0.1 % Tween-20 (TBS-T) at RT for 2 h, followed by an overnight incubation at 4 °C with nitrotyrosine antibody (mouse monoclonal [clone HM11], 1:1000, Cat # 321900, Thermo Fisher Scientific) and dityrosine antibody (mouse monoclonal [clone 7D4], 1:10,000, Cat # NBP2-59360, Novus Biologicals, Centennial, CO, USA), respectively, diluted in 5 % fat-free milk powder in TBS-T. All membranes were washed four times with TBS-T for 5 min each and incubated with the horseradish peroxidase coupled secondary antibody (goat anti-mouse, polyclonal, 1:10,000, Cat # 115-035-062, Jackson Immuno Research Europe Ltd, Ely, UK) at RT for 1 h, diluted in TBS-T. For detection, the membranes were washed four times with TBS-T for 5 min, and protein bands were developed using the chemiluminescence system ECL-femto (Thermo Fisher Scientific) for nitrotyrosine and ECL-plus (Thermo Fisher Scientific) for dityrosine according to the manufacturer's protocol. The bands were detected in a ChemiDoc system, and images were processed using Image Lab software 5.2.1.

Nitrotyrosine and dityrosine were detected in peroxynitrite-modified samples of all investigated proteins. For the native proteins, neither nitrotyrosine nor dityrosine were detectable in the western blots (Fig. S1).

2.1.5 Endotoxin quantification

The amount of endotoxin in native and modified protein samples was quantified by a Pierce™ LAL Chromogenic Endotoxin Quantitation Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. For this purpose, protein stock solutions were diluted to 1 µg mL^{-1} with endotoxin-free water. The endotoxin level in all protein samples was less than 1 EU per µg of protein.

2.2 Cell culture experiments

All cell experiments were performed with the same final concentrations of native and modified protein samples (α-Syn: 50.0 µg mL^{-1}, HSP60: 14.3 µg mL^{-1}, HMGB1: 3.6 µg mL^{-1}, OVA: 50.0 µg mL^{-1}) except for mRNA expression of α-Syn (35.7 µg mL^{-1}). LPS from *E. coli* O111:B4, 25 ng mL^{-1}, InvivoGen) was used as positive control (LPS-EB for HeLa TLR4 experiments; LPS-EB ultrapure for THP-1-Lucia™ and THP-1 experiments), and medium and mock served as negative controls. Protein solutions were diluted in the respective cell culture medium.

2.2.1 HeLa TLR4 dual reporter cells

Using a HeLa TLR4 dual luciferase reporter cell line (Novus Biologicals), we simultaneously determined TLR4 activation and cell viability [15]. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Thermo Fisher Scientific) containing 25 mM D-glucose and 1 mM sodium pyruvate supplemented with 10 % heat-inactivated fetal bovine serum (FBS superior, Cat # S0615, Lot #0973F, Biochrom, Berlin, Germany), 1 % penicillin/streptomycin (Thermo Fisher Scientific) and 140 µg mL^{-1} hygromycin B (InvivoGen) in a humidified atmosphere of 5 % CO_2 at 37 °C. For each sample and replicate, 20,000 HeLa TLR4 dual reporter cells in 100 µL medium per well were seeded in a flat-bottom 96-well plate (Greiner, Frickenhausen, Germany). After 24 h, the cells were incubated in triplicates with 50 µL of the respective protein or control sample for 7 h. Thereafter, cells were washed with 200 µL PBS and lysed using 15 µL passive lysis buffer (Promega, Mannheim, Germany) at -80 °C overnight. The read out was performed using the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's protocol. Both luminescence signals were measured in a Synergy Neo plate reader. To calculate the normalized TLR4 activity, TLR4-driven *Renilla* luciferase signal was divided by the constitutive, CMV-driven firefly luciferase signal that served as a surrogate marker for cell viability. For each experiment, LPS-treated cells were used as a positive

control, and the arithmetic mean was set to 100 %. This value was used to normalize the measurement results of all protein and control samples. Arithmetic mean values and standard deviations were calculated from the normalized values of three (HSP60) or four (α-Syn, HMGB1, OVA) independent experiments performed in triplicates.

2.2.2 THP-1 NF-κB reporter cells and receptor antagonists

To assess the NF-κB activity of cells treated with different native and modified proteins, THP-1-Lucia™ NF-κB cells (InvivoGen) were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific) containing 25 mM D-glucose and 1 mM sodium pyruvate supplemented with 10 % heat-inactivated FBS, 100 µg mL^{-1} Zeocin™ (InvivoGen), and 1 % penicillin/streptomycin in a humidified atmosphere of 5 % CO_2 at 37 °C. For each sample and replicate, 100,000 cells in 50 µL medium per well were seeded in a flat-bottom 96-well plate. To inhibit TLR4 or RAGE signaling, cells were pre-incubated with 50 µL of the TLR4 antagonist TAK242 or the RAGE antagonist FPS-ZM1 for 4 h. TAK242 (25 mM, Merck Millipore) was provided in dimethyl sulfoxide (DMSO) and further diluted with medium to a final concentration of 0.36 µg mL^{-1}. FPS-ZM1 (25 mg, Merck Millipore) was dissolved in 250 µL DMSO and further diluted with medium to a final concentration of 3.3 µg mL^{-1}. If no antagonist was applied, 50 µL medium was added to the cells. Subsequently, cells were incubated in triplicates with 50 µL of the respective protein or control samples for 24 h. Cells treated with DMSO in medium (4.4 µg mL^{-1}) showed no NF-κB activation or toxic effects. Activity of NF-κB was measured by QUANTI-Luc™ reagent (InvivoGen) according to manufacturer's instructions. Briefly, 10 µL of cell culture supernatant was transferred into a white plate (LUMITRAC™, Greiner), and mixed with 50 µL of QUANTI-Luc™ reagent. The luminescence was detected in a Synergy Neo plate reader. For each experiment, LPS-treated cells were used as positive control, and the arithmetic mean was set to 100 %. This value was used to normalize the measurement results of all protein and control samples. Arithmetic mean values and standard deviation were calculated from the normalized values of three (α-Syn) or four (HSP60, HMGB1, OVA) independent experiments performed in triplicates. Assessment of cell viability was performed in triplicates using the alamarBlue™ cell viability reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. Excitation was performed at 560 nm, and emission was measured at 590 nm in a Synergy Neo plate reader.

2.2.3 Cytokine immunoassay

Human THP-1 monocyte cells (ATCC, Manassas, VA, USA) were grown in RPMI 1640 medium supplemented with 10 % heat-inactivated FBS, 0.05 mM 2-mercaptoethanol (Sigma Aldrich), and 1 % penicillin/streptomycin. Quantification of cytokine secretion was performed using a multiplex immunoassay. For each sample and replicate, 100,000 human THP-1

monocytes in 100 µL medium per well were seeded in a V-shaped 96-well plate (Greiner) and incubated with 50 µL of the respective protein or control samples over 24 h. Thereafter, cells were centrifuged at 200 x g for 5 min (5427 R, Eppendorf), and the supernatant was analyzed in duplicates for TNF-α, IL-1β, and IL-8 by a multiplex assay kit (R&D systems Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. The read-out was performed on a MAGPIX device (Luminex, Austin, TX, USA), and arithmetic mean values and standard deviation of the duplicates were calculated. Three independent experiments were performed showing similar trends. Assessment of cell viability was performed as described in section 2.2.2.

2.2.4 mRNA extraction and qPCR analysis

Quantification of mRNA expression of TNF-α, IL-1β, IL-8, and CXCL-10 was performed using real-time quantitative PCR (qPCR). For each sample and replicate, 400,000 human THP-1 monocyte cells in 1 mL medium per well were seeded in a 6-well cell culture plate (Greiner). On the next day, cells were incubated with 1 mL of the respective protein or control samples for 4 h. For qPCR analysis, cells were harvested by centrifugation at 500 x g for 5 min. Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Hilden, Germany) following the spin technology protocol. Total RNA yield was determined using a Take3 trio micro-volume plate in a Synergy Neo plate reader. Using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), 500 ng of total RNA per sample were transcribed into cDNA. Afterwards, qPCR was performed in duplicates using 10 ng of cDNA mixed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) to a final concentration of 333 nM for each primer, which were designed using PrimerBlast (NCBI) and the respective template genes (Table S3). As reference genes served Peptidylprolyl isomerase A (PPIA) and TATA-binding protein (TBP). Reactions were performed at 98 °C for 30 s, followed by 37 cycles of 98 °C for 10 s, and 60 °C for 25 s. Gene expression was calculated according to the $2^{-\Delta\Delta CT}$ method using the Bio-Rad CFX Manager Software 3.1. Arithmetic mean values and standard deviation of the duplicates were calculated. Three independent experiments were performed showing similar trends.

2.2.5 Statistical analyses

GraphPad Prism version 6.07 (GraphPad, San Diego, CA, USA) was used for statistical analysis. Unpaired t-tests were performed to observe differences between the native and peroxynitrite-modified proteins. The results were considered as significant when $*p < 0.05$, $**p < 0.01$, $***p < 0.005$.

3. Results

For the investigated proteins in native and peroxynitrite-modified form, TLR4 and NF-κB activation were determined in stable reporter cell lines (HeLa TLR4, THP-1 NF-κB). Additionally, mRNA expression and secretion of various cytokines induced by NF-κB were measured in human monocytes (THP-1).

As shown in Figures 1 and 2, chemical modification by peroxynitrite significantly increased activation of TLR4 and NF-κB for all three investigated proteins. Relative to the native protein, the TLR4 activity increased by a factor of ~1.4 for α-Syn and HSP60 and by a factor of ~2.2 for HMGB1 (Fig. 1, Table S4), while the NF-κB activity increased by a factor of ~1.6 for α-Syn and HSP60 and by a factor of ~4.2 for HMGB1 (Fig. 2, Table S4). Inhibition of the TLR4 receptor by the antagonist TAK242 reduced the NF-κB response to both, the modified and native protein by more than 80 % for α-Syn, by more than 90 % for HSP60, and by 30-40 % for HMGB1, while the RAGE inhibitor FPS-ZM1 had no substantial effect (Figs. 2). These inhibition experiments indicate that the enhancement of NF-κB activation by peroxynitrite-modification is predominantly mediated by TLR4 for α-Syn and HSP60, while for HMGB1 also other receptors such as RAGE and TLR2 may be involved [70,71].

Figure 3 shows that the chemically modified proteins also enhanced the secretion of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-8. Relative to the native protein, the secretion increased 1.2-2.0 fold for α-Syn, 1.2-8.8 fold for HSP60, and 2.1-16.7 fold for HMGB1 (Table S5). The mRNA expression of the investigated cytokines showed the same trend for modified HSP60 and HMGB1, but not for modified α-Syn (Fig. S2, Table S6). The mRNA results obtained for CXCL-10 indicate that the chemically modified proteins increased not only the activation of NF-κB (MyD88 pathway), but also the activation of the IRF3 (TRIF/TRAF) pathway of TLR4 signaling [76,77]. The negative controls of native and peroxynitrite-modified OVA exhibited no substantial TLR4 and NF-κB activity, cytokine secretion, or mRNA expression (Figs. 1-3, Fig. S2), and the applied protein concentrations did not affect the viability of the investigated cells (Figs. S3-S5), suggesting that the induced protein modifications were only relevant in the context of the TLR4-activating proteins.

Overall, our experimental results demonstrate that peroxynitrite treatment enhances the inflammatory potential of α-Syn, HSP60, and HMGB1 *in vitro*. This can be explained by changes in protein-receptor interactions related to chemical modifications like nitrotyrosine, intramolecular dityrosine crosslinks, protein dimers and higher oligomers as detected in the peroxynitrite-modified samples of the investigated proteins.

4. Discussion

Nitrotyrosine was detected in all chemically modified protein samples (Fig. S1, Table S2), and the introduction of a nitro group can strongly affect the chemical and physiological properties of a protein [78]. Notably, nitrotyrosine is more acidic than tyrosine, which can affect the isoelectric point of a protein, change the binding to receptors and ligands, and modulate downstream signaling cascades [12,14,20–22]. Nitration tends to enhance the immunogenicity of proteins, and nitrated proteins are thus used as model antigens or allergens [2,24,25,79–83]. The reason for this striking immunological property of nitrated proteins, which has also been linked to autoimmunity, is not completely clear [16,84]. The generation of neo-epitopes seems to be an important factor in adaptive immune responses [22,24], and nitrotyrosine was found to be associated with increased TLR4 signaling and innate immune responses related to chronic inflammatory diseases [15,18,78,85–88]. However, we here show for the first time that there is a direct enhancement of TLR4 activation by the peroxynitrite-induced modification of natural (human) DAMPs that play a role as innate immune activators in acute and chronic inflammation.

Besides nitrotyrosine, also dityrosine was detected in all chemically modified protein samples (Fig. S1). The detection of dityrosine in the monomeric form of the modified proteins implies that intramolecular crosslinks were formed and may have induced conformational changes affecting protein-receptor interactions. For HMGB1, it is well known that TLR4 binding depends on the oxidation state of the protein, and that an intramolecular disulfide bridge is crucial for TLR4 dimerization and activation [89,90]. By analogy, intramolecular dityrosine crosslinks among the tyrosine residues in HMGB1 might also induce or stabilize conformational arrangements that are relevant for protein-receptor interactions and TLR4 activation. Further investigations will be required to clarify if and how intramolecular dityrosine crosslinks may contribute to the enhancement of TLR4 activity observed for peroxynitrite-modified HMGB1 and other proteins.

Protein dimers and higher oligomers were detected in all chemically modified protein samples (Fig. S1, Table S2). They comprise more than one receptor interaction domain, can act as multivalent ligands, and may thus more efficiently promote TLR4 dimerization and signaling [15,91–93]. Such multivalency effects are common in biological systems, where higher functional affinities are observed for dimeric and oligomeric agonists, leading to enhanced receptor clustering and signal transduction [94–97]. Thus, the formation of dimers and oligomers by intermolecular dityrosine crosslinking may well explain the enhanced TLR4 and NF-κB activity observed upon stimulation with peroxynitrite-modified samples compared to the native form of the investigated proteins. For α-Syn, this is consistent with earlier studies

reporting that oligomeric α-Syn can enhance TLR4 signaling and pro-inflammatory cytokine production in microglia and astrocytes, which may contribute to development and progression of Parkinson's disease [43,98]. Similar effects have been reported for the interaction of amyloid beta in Alzheimer's disease, where aggregates trigger TLR2 and TLR4 [99,100], and nitration critically enhances amyloid beta aggregation and plaque formation [101]. For all receptors of the TLR family [102,103] as well as RAGE [104–106] and other receptors [107,108], activation and signal transduction requires dimerization of the receptor molecules, which may be promoted by dimerized or oligomeric ligands. Thus, we suggest that the peroxynitrite-induced dimerization or oligomerization of HSP60 and HMGB1 may also play a critical role for PRR signaling in inflammatory diseases.

As outlined above, the proteins α-Syn, HSP60, and HMGB1 are involved in many severe diseases. If *in vivo* studies confirm that nitrotyrosine residues or dityrosine crosslinks in proteinous DAMPs play an important role in amplifying inflammatory processes through enhanced TLR4 signaling, the chemically modified proteins may serve as useful clinical markers providing mechanistic insight. Such markers and insight may also help to advance medical treatment options that involve scavenging of peroxynitrite [109–111] or the inhibition of peroxynitrite formation [112,113].

5. Conclusions

In this study, we have shown that peroxynitrite can induce chemical modifications that enhance the TLR4 activity and inflammatory potential of proteinous DAMPs like α-Syn, HSP60, and HMGB1. Besides peroxynitrite, a wide range of other endogenous or exogenous ROS/RNS can also modify the chemical structure, properties, and effects of proteins [13]. In particular, air pollutants such as fine particulate matter, nitrogen oxides, and ozone can trigger or enhance oxidative stress, nitration and oligomerization of proteinous DAMPs and allergens, immune reactions, and feedback cycles of inflammation [1,2,13,15,36,79,114–116].

Figure 4 illustrates how chemically modified DAMPs may amplify oxidative stress and innate immune responses through a positive feedback loop of pro-inflammatory signaling via TLR4 or other PRR. Such feedback and self-amplification provide a potential mechanistic rationale for the development of inflammatory disorders related to environmental pollution. In particular, it may help to explain the massive burden of disease attributable to air pollution, where the underlying chemical and physiological mechanisms are not yet well understood [4–6]. Environmental pollutants may generate exogenous ROS/RNS and oxidative stress, triggering inflammatory processes that lead to the formation of endogenous ROS/RNS and release of DAMPs. The DAMPs can activate PRR (TLR, RAGE, etc.) that induce further pro-

inflammatory signaling and responses via transcription factors (NF-κB, IRF3, etc.), cytokines and cytokine receptors (IL-1, IL-8, etc.). This positive feedback can be additionally enhanced if the DAMPs undergo chemical modification by ROS/RNS, and if the modified DAMPs lead to stronger activation of PRR than the native DAMPs, as observed in this study. Such effects may have important consequences for chronic neurodegenerative, cardiovascular or gastrointestinal diseases and allergies that are prevalent in modern societies. We suggest to consider and further elucidate these processes, the role of environmental pollutants, and potential needs and perspectives for societal action with regard to the steeply increasing and globally pervasive human influence on air quality, climate, and public health in the Anthropocene [2,117].

Conflict of interest

The authors declare no conflict of interest.

Author contributions

KL and UP conceived and directed the study. KZ, ATK, KRS, ALL, and KL designed and performed the experiments. KZ, ATK, KRS, MGW, JFN, KL, and UP analyzed, interpreted and discussed the data. DW and DS contributed to the design, interpretation, and discussion. All authors contributed to the preparation and editing of the manuscript. KZ and ATK contributed equally.

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6. Literature

[1] M. Shiraiwa, K. Ueda, A. Pozzer, G. Lammel, C.J. Kampf, A. Fushimi, S. Enami, A.M. Arangio, J. Fröhlich-Nowoisky, Y. Fujitani, A. Furuyama, P.S.J. Lakey, J. Lelieveld, K. Lucas, Y. Morino, U. Pöschl, S. Takahama, A. Takami, H. Tong, B. Weber, A. Yoshino, K. Sato, Aerosol Health Effects from Molecular to Global Scales, Environ. Sci. Technol. 51 (2017) 13545–13567. https://doi.org/10.1021/acs.est.7b04417.

[2] K. Reinmuth-Selzle, C.J. Kampf, K. Lucas, N. Lang-Yona, J. Fröhlich-Nowoisky, M. Shiraiwa, P.S.J. Lakey, S. Lai, F. Liu, A.T. Kunert, K. Ziegler, F. Shen, R. Sgarbanti, B. Weber, I. Bellinghausen, J. Saloga, M.G. Weller, A. Duschl, D. Schuppan, U. Pöschl, Air Pollution and Climate Change Effects on Allergies in the Anthropocene: Abundance, Interaction, and Modification of Allergens and Adjuvants, Environ. Sci. Technol. 51 (2017) 4119–4141. https://doi.org/10.1021/acs.est.6b04908.

[3] T. Münzel, A. Daiber, Environmental Stressors and Their Impact on Health and Disease with Focus on Oxidative Stress, Antioxid. Redox Signal. 28 (2018) 735–740. https://doi.org/10.1089/ars.2017.7488.

[4] A.J. Cohen, M. Brauer, R. Burnett, H.R. Anderson, J. Frostad, K. Estep, K. Balakrishnan, B. Brunekreef, L. Dandona, R. Dandona, V. Feigin, G. Freedman, B. Hubbell, A. Jobling, H. Kan, L. Knibbs, Y. Liu, R. Martin, L. Morawska, C.A. Pope, H. Shin, K. Straif, G. Shaddick, M. Thomas, R. van Dingenen, A. van Donkelaar, T. Vos, C.J.L. Murray, M.H. Forouzanfar, Estimates and 25-year trends of the global burden of disease attributable to ambient air pollution: an analysis of data from the Global Burden of Diseases Study 2015, Lancet. 389 (2017) 1907–1918. https://doi.org/10.1016/S0140-6736(17)30505-6.

[5] J. Lelieveld, U. Pöschl, Chemists can help to solve the air-pollution health crisis, Nature. 551 (2017) 291–293. https://doi.org/10.1038/d41586-017-05906-9.

[6] J. Lelieveld, K. Klingmüller, A. Pozzer, U. Pöschl, M. Fnais, A. Daiber, T. Münzel, Cardiovascular disease burden from ambient air pollution in Europe reassessed using novel hazard ratio functions, Eur. Heart J. 40 (2019) 1590–1596. https://doi.org/10.1093/eurheartj/ehz135.

[7] H. Sies, Oxidative stress: oxidants and antioxidants, Exp. Physiol. 82 (1997) 291–295. https://doi.org/10.1113/expphysiol.1997.sp004024.

[8] F.J. Kelly, Oxidative stress: its role in air pollution and adverse health effects, Occup. Environ. Med. 60 (2003) 612–616. https://doi.org/10.1136/oem.60.8.612.

[9] H. Sies, C. Berndt, D.P. Jones, Oxidative Stress, Annu. Rev. Biochem. 86 (2017) 715–748. https://doi.org/10.1146/annurev-biochem-061516-045037.

[10] A. van der Vliet, J.P. Eiserich, C.A. Oneill, B. Halliwell, C.E. Cross, Tyrosine Modification by Reactive Nitrogen Species: A Closer Look, Arch. Biochem. Biophys. 319 (1995) 341–349. https://doi.org/10.1006/abbi.1995.1303.

[11] C. Quijano, B. Alvarez, R.M. Gatti, O. Augusto, R. Radi, Pathways of peroxynitrite oxidation of thiol groups, Biochem. J. 322 (1997) 167–173. https://doi.org/10.1042/bj3220167.

[12] K. Reinmuth-Selzle, C. Ackaert, C.J. Kampf, M. Samonig, M. Shiraiwa, S. Kofler, H. Yang, G. Gadermaier, H. Brandstetter, C.G. Huber, A. Duschl, G.J. Oostingh, Pösch, Nitration of the Birch Pollen Allergen Bet v 1.0101: Efficiency and Site-Selectivity of Liquid and Gaseous Nitrating Agents, J. Proteome Res. 13 (2014) 1570–1577. https://doi.org/10.1021/pr401078h.

[13] A. Bachi, I. Dalle-Donne, A. Scaloni, Redox Proteomics: Chemical Principles, Methodological Approaches and Biological/Biomedical Promises, Chem. Rev. 113 (2013) 596–698. https://doi.org/10.1021/cr300073p.

[14] C. Ackaert, S. Kofler, J. Horejs-Hoeck, N. Zulehner, C. Asam, S. von Grafenstein, J.E. Fuchs, P. Briza, K.R. Liedl, B. Bohle, F. Ferreira, H. Brandstetter, G.J. Oostingh, A. Duschl, The Impact of Nitration on the Structure and Immunogenicity of the Major Birch Pollen Allergen Bet v 1.0101, PLoS One. 9 (2014) e104520. https://doi.org/10.1371/journal.pone.0104520.

[15] K. Ziegler, J. Neumann, F. Liu, J. Fröhlich-Nowoisky, C. Cremer, J. Saloga, K. Reinmuth-Selzle, U. Pöschl, D. Schuppan, I. Bellinghausen, K. Lucas, Nitration of wheat amylase trypsin inhibitors increases their innate and adaptive immunostimulatory potential in vitro, Front. Immunol. 10 (2019) 1–10. https://doi.org/10.3389/fimmu.2018.03174.

[16] H. Ischiropoulos, Protein tyrosine nitration—An update, Arch. Biochem. Biophys. 484 (2009) 117–121. https://doi.org/10.1016/j.abb.2008.10.034.

[17] T. DiMarco, C. Giulivi, Current analytical methods for the detection of dityrosine, a biomarker of oxidative stress, in biological samples, Mass Spectrom. Rev. 26 (2007) 108–120. https://doi.org/10.1002/mas.20109.

[18] S.A.B. Greenacre, H. Ischiropoulos, Tyrosine nitration: Localisation, quantification, consequences for protein function and signal transduction, Free Radic. Res. 34

(2001) 541–581. https://doi.org/10.1080/10715760100300471.

[19] M. del Carmen Baez, M. de La Paz Scribano, M. Tarán, I. Fonseca, A. Balceda, S. Blencio, M. Moya, Nitrotyrosine as a biomarker: recovery of histopathological atherogenic lesions in rats treated with ascorbic acid, Trends Med. 18 (2018) 1–5. https://doi.org/10.15761/TiM.1000152.

[20] I. V. Turko, F. Murad, Protein Nitration in Cardiovascular Diseases, Pharmacol. Rev. 54 (2002) 619–634. https://doi.org/10.1124/pr.54.4.619.

[21] V. De Filippis, R. Frasson, A. Fontana, 3-Nitrotyrosine as a spectroscopic probe for investigating protein protein interactions., Protein Sci. 15 (2006) 976–986. https://doi.org/10.1110/ps.051957006.

[22] G. Ferrer-Sueta, N. Campolo, M. Trujillo, S. Bartesaghi, S. Carballal, N. Romero, B. Alvarez, R. Radi, Biochemistry of Peroxynitrite and Protein Tyrosine Nitration, Chem. Rev. 118 (2018) 1338–1408. https://doi.org/10.1021/acs.chemrev.7b00568.

[23] F. Khan, A.A. Siddiqui, Prevalence of anti-3-nitrotyrosine antibodies in the joint synovial fluid of patients with rheumatoid arthritis, osteoarthritis and systemic lupus erythematosus, Clin. Chim. Acta. 370 (2006) 100–107. https://doi.org/10.1016/j.cca.2006.01.020.

[24] Y.K. Gruijthuijsen, I. Grieshuber, A. Stöcklinger, U. Tischler, T. Fehrenbach, M.G. Weller, L. Vogel, S. Vieths, U. Pöschl, A. Duschl, Nitration Enhances the Allergenic Potential of Proteins, Int. Arch. Allergy Immunol. 141 (2006) 265–275. https://doi.org/10.1159/000095296.

[25] L. Thomson, M. Tenopoulou, R. Lightfoot, E. Tsika, I. Parastatidis, M. Martinez, T.M. Greco, P.-T. Doulias, Y. Wu, W.H.W. Tang, S.L. Hazen, H. Ischiropoulos, Immunoglobulins Against Tyrosine-Nitrated Epitopes in Coronary Artery Disease, Circulation. 126 (2012) 2392–2401. https://doi.org/10.1161/CIRCULATIONAHA.112.103796.

[26] D. Balasubramanian, R. Kanwar, Molecular pathology of dityrosine cross-links in proteins: Structural and functional analysis of four proteins, Mol. Cell. Biochem. 234–235 (2002) 27–38. https://doi.org/10.1023/A:1015927907418.

[27] S. Mukherjee, E.A. Kapp, A. Lothian, A.M. Roberts, Y. V. Vasil'ev, B.A. Boughton, K.J. Barnham, W.M. Kok, C.A. Hutton, C.L. Masters, A.I. Bush, J.S. Beckman, S.G. Dey, B.R. Roberts, Characterization and Identification of Dityrosine Cross-Linked Peptides Using Tandem Mass Spectrometry, Anal. Chem. 89 (2017) 6136–6145.

https://doi.org/10.1021/acs.analchem.7b00941.

[28] J.S. Roh, D.H. Sohn, Damage-Associated Molecular Patterns in Inflammatory Diseases, Immune Netw. 18 (2018) 1–14. https://doi.org/10.4110/in.2018.18.e27.

[29] E. Vénéreau, C. Ceriotti, M.E. Bianchi, DAMPs from Cell Death to New Life, Front. Immunol. 6 (2015) 1–11. https://doi.org/10.3389/fimmu.2015.00422.

[30] Y. Liu, H. Yin, M. Zhao, Q. Lu, TLR2 and TLR4 in Autoimmune Diseases: a Comprehensive Review, Clin. Rev. Allergy Immunol. 47 (2014) 136–147. https://doi.org/10.1007/s12016-013-8402-y.

[31] K. Takeda, S. Akira, TLR signaling pathways, Semin. Immunol. 16 (2004) 3–9. https://doi.org/10.1016/j.smim.2003.10.003.

[32] E. Varfolomeev, D. Vucic, Intracellular regulation of TNF activity in health and disease, Cytokine. 101 (2018) 26–32. https://doi.org/10.1016/j.cyto.2016.08.035.

[33] A. Weber, P. Wasiliew, M. Kracht, Interleukin-1 (IL-1) Pathway, Sci. Signal. 3 (2010) cm1. https://doi.org/10.1126/scisignal.3105cm1.

[34] Q. Liu, A. Li, Y. Tian, J.D. Wu, Y. Liu, T. Li, Y. Chen, X. Han, K. Wu, The CXCL8-CXCR1/2 pathways in cancer, Cytokine Growth Factor Rev. 31 (2016) 61–71. https://doi.org/10.1016/j.cytogfr.2016.08.002.

[35] L.T. Gan, D.M. Van Rooyen, M.E. Koina, R.S. McCuskey, N.C. Teoh, G.C. Farrell, Hepatocyte free cholesterol lipotoxicity results from JNK1-mediated mitochondrial injury and is HMGB1 and TLR4-dependent, J. Hepatol. 61 (2014) 1376–1384. https://doi.org/10.1016/j.jhep.2014.07.024.

[36] K. Lucas, M. Maes, Role of the Toll Like Receptor (TLR) Radical Cycle in Chronic Inflammation: Possible Treatments Targeting the TLR4 Pathway, Mol. Neurobiol. 48 (2013) 190–204. https://doi.org/10.1007/s12035-013-8425-7.

[37] C.-C. Huang, T.-Y. Chiu, T.-Y. Lee, H.-J. Hsieh, C.-C. Lin, L.-S. Kao, Soluble α-synuclein facilitates priming and fusion by releasing Ca2+ from the thapsigargin-sensitive Ca2+ pool in PC12 cells, J. Cell Sci. 131 (2018) jcs213017. https://doi.org/10.1242/jcs.213017.

[38] T. Logan, J. Bendor, C. Toupin, K. Thorn, R.H. Edwards, α-Synuclein promotes dilation of the exocytotic fusion pore, Nat. Neurosci. 20 (2017) 681–689. https://doi.org/10.1038/nn.4529.

[39] J. Burré, The Synaptic Function of α-Synuclein, J. Parkinsons. Dis. 5 (2015) 699–713.

https://doi.org/10.3233/JPD-150642.

[40] M.H.R. Ludtmann, P.R. Angelova, N.N. Ninkina, S. Gandhi, V.L. Buchman, A.Y. Abramov, Monomeric Alpha-Synuclein Exerts a Physiological Role on Brain ATP Synthase, J. Neurosci. 36 (2016) 10510–10521. https://doi.org/10.1523/JNEUROSCI.1659-16.2016.

[41] Y. He, Z. Yu, S. Chen, Alpha-Synuclein Nitration and Its Implications in Parkinson's Disease, ACS Chem. Neurosci. 10 (2019) 777–782. https://doi.org/10.1021/acschemneuro.8b00288.

[42] Y.K. Al-Hilaly, L. Biasetti, B.J.F. Blakeman, S.J. Pollack, S. Zibaee, A. Abdul-Sada, J.R. Thorpe, W.-F. Xue, L.C. Serpell, The involvement of dityrosine crosslinking in α-synuclein assembly and deposition in Lewy Bodies in Parkinson's disease, Sci. Rep. 6 (2016) 39171. https://doi.org/10.1038/srep39171.

[43] C.D. Hughes, M.L. Choi, M. Ryten, L. Hopkins, A. Drews, J.A. Botía, M. Iljina, M. Rodrigues, S.A. Gagliano, S. Gandhi, C. Bryant, D. Klenerman, Picomolar concentrations of oligomeric alpha-synuclein sensitizes TLR4 to play an initiating role in Parkinson's disease pathogenesis, Acta Neuropathol. 137 (2019) 103–120. https://doi.org/10.1007/s00401-018-1907-y.

[44] S. Schildknecht, H.R. Gerding, C. Karreman, M. Drescher, H.A. Lashuel, T.F. Outeiro, D.A. Di Monte, M. Leist, Oxidative and nitrative alpha-synuclein modifications and proteostatic stress: implications for disease mechanisms and interventions in synucleinopathies, J. Neurochem. 125 (2013) 491–511. https://doi.org/10.1111/jnc.12226.

[45] R. Hodara, E.H. Norris, B.I. Giasson, A.J. Mishizen-Eberz, D.R. Lynch, V.M.Y. Lee, H. Ischiropoulos, Functional Consequences of α-Synuclein Tyrosine Nitration, J. Biol. Chem. 279 (2004) 47746–47753. https://doi.org/10.1074/jbc.M408906200.

[46] A. Villar-Piqué, T. Lopes da Fonseca, R. Sant'Anna, É.M. Szegö, L. Fonseca-Ornelas, R. Pinho, A. Carija, E. Gerhardt, C. Masaracchia, E. Abad Gonzalez, G. Rossetti, P. Carloni, C.O. Fernández, D. Foguel, I. Milosevic, M. Zweckstetter, S. Ventura, T.F. Outeiro, Environmental and genetic factors support the dissociation between α-synuclein aggregation and toxicity, Proc. Natl. Acad. Sci. 113 (2016) E6506–E6515. https://doi.org/10.1073/pnas.1606791113.

[47] C. Kim, D.-H. Ho, J.-E. Suk, S. You, S. Michael, J. Kang, S. Joong Lee, E. Masliah, D. Hwang, H.-J. Lee, S.-J. Lee, Neuron-released oligomeric α-synuclein is an

endogenous agonist of TLR2 for paracrine activation of microglia, Nat. Commun. 4 (2013) 1562. https://doi.org/10.1038/ncomms2534.

[48] A. Jang, H.-J. Lee, J.-E. Suk, J.-W. Jung, K.-P. Kim, S.-J. Lee, Non-classical exocytosis of α-synuclein is sensitive to folding states and promoted under stress conditions, J. Neurochem. 113 (2010) 1263–1274. https://doi.org/10.1111/j.1471-4159.2010.06695.x.

[49] H.-J. Lee, S. Patel, S.-J. Lee, Intravesicular Localization and Exocytosis of α-Synuclein and its Aggregates, J. Neurosci. 25 (2005) 6016–6024. https://doi.org/10.1523/JNEUROSCI.0692-05.2005.

[50] G. Levy-Rimler, P. Viitanen, C. Weiss, R. Sharkia, A. Greenberg, A. Niv, A. Lustig, Y. Delarea, A. Azem, The effect of nucleotides and mitochondrial chaperonin 10 on the structure and chaperone activity of mitochondrial chaperonin 60, Eur. J. Biochem. 268 (2001) 3465–3472. https://doi.org/10.1046/j.1432-1327.2001.02243.x.

[51] S. Vilasi, R. Carrotta, M.R. Mangione, C. Campanella, F. Librizzi, L. Randazzo, V. Martorana, A. Marino Gammazza, M.G. Ortore, A. Vilasi, G. Pocsfalvi, G. Burgio, D. Corona, A. Palumbo Piccionello, G. Zummo, D. Bulone, E. Conway de Macario, A.J.L. Macario, P.L. San Biagio, F. Cappello, Human Hsp60 with Its Mitochondrial Import Signal Occurs in Solution as Heptamers and Tetradecamers Remarkably Stable over a Wide Range of Concentrations, PLoS One. 9 (2014) e97657. https://doi.org/10.1371/journal.pone.0097657.

[52] R.M. Vabulas, P. Ahmad-Nejad, C. da Costa, T. Miethke, C.J. Kirschning, H. Häcker, H. Wagner, Endocytosed HSP60s Use Toll-like Receptor 2 (TLR2) and TLR4 to Activate the Toll/Interleukin-1 Receptor Signaling Pathway in Innate Immune Cells, J. Biol. Chem. 276 (2001) 31332–31339. https://doi.org/10.1074/jbc.M103217200.

[53] S. Gupta, A.A. Knowlton, HSP60 trafficking in adult cardiac myocytes: role of the exosomal pathway, Am. J. Physiol. Circ. Physiol. 292 (2007) H3052–H3056. https://doi.org/10.1152/ajpheart.01355.2006.

[54] F.J. Quintana, I.R. Cohen, The HSP60 immune system network, Trends Immunol. 32 (2011) 89–95. https://doi.org/10.1016/j.it.2010.11.001.

[55] T. Zininga, L. Ramatsui, A. Shonhai, Heat Shock Proteins as Immunomodulants, Molecules. 23 (2018) 2846. https://doi.org/10.3390/molecules23112846.

[56] L. Stefano, G. Racchetti, F. Bianco, N. Passini, R.S. Gupta, P.P. Bordignon, J. Meldolesi, The surface-exposed chaperone, Hsp60, is an agonist of the microglial

TREM2 receptor, J. Neurochem. 110 (2009) 284–294. https://doi.org/10.1111/j.1471-4159.2009.06130.x.

[57] M. Cohen-Sfady, G. Nussbaum, M. Pevsner-Fischer, F. Mor, P. Carmi, A. Zanin-Zhorov, O. Lider, I.R. Cohen, Heat Shock Protein 60 Activates B Cells via the TLR4-MyD88 Pathway, J. Immunol. 175 (2005) 3594–3602. https://doi.org/10.4049/jimmunol.175.6.3594.

[58] J. Juwono, R.D. Martinus, Does Hsp60 Provide a Link between Mitochondrial Stress and Inflammation in Diabetes Mellitus?, J. Diabetes Res. 2016 (2016) 1–6. https://doi.org/10.1155/2016/8017571.

[59] T. Koeck, J.A. Corbett, J.W. Crabb, D.J. Stuehr, K.S. Aulak, Glucose-modulated tyrosine nitration in beta cells: Targets and consequences, Arch. Biochem. Biophys. 484 (2009) 221–231. https://doi.org/10.1016/j.abb.2009.01.021.

[60] M.-F. Tsan, B. Gao, Cytokine function of heat shock proteins, Am. J. Physiol. Physiol. 286 (2004) C739–C744. https://doi.org/10.1152/ajpcell.00364.2003.

[61] Q. Xiao, K. Mandal, G. Schett, M. Mayr, G. Wick, F. Oberhollenzer, J. Willeit, S. Kiechl, Q. Xu, Association of Serum-Soluble Heat Shock Protein 60 With Carotid Atherosclerosis, Stroke. 36 (2005) 2571–2576. https://doi.org/10.1161/01.STR.0000189632.98944.ab.

[62] M. Rizzo, A. J.L. Macario, E. Conway de Macario, I. Gouni-Berthold, H. K. Berthold, G. Battista Rini, G. Zummo, F. Cappello, Heat Shock Protein-60 and Risk for Cardiovascular Disease, Curr. Pharm. Des. 17 (2011) 3662–3668. https://doi.org/10.2174/138161211798220981.

[63] V. Rodolico, G. Tomasello, M. Zerilli, A. Martorana, A. Pitruzzella, A. Marino Gammazza, S. David, G. Zummo, P. Damiani, S. Accomando, E. Conway de Macario, A.J.L. Macario, F. Cappello, Hsp60 and Hsp10 increase in colon mucosa of Crohn's disease and ulcerative colitis, Cell Stress Chaperones. 15 (2010) 877–884. https://doi.org/10.1007/s12192-010-0196-8.

[64] P. Mandke, K.M. Vasquez, Interactions of high mobility group box protein 1 (HMGB1) with nucleic acids: Implications in DNA repair and immune responses, DNA Repair (Amst). 83 (2019) 102701. https://doi.org/10.1016/j.dnarep.2019.102701.

[65] J.R. Klune, R. Dhupar, J. Cardinal, T.R. Billiar, A. Tsung, HMGB1: Endogenous Danger Signaling, Mol. Med. 14 (2008) 476–484. https://doi.org/10.2119/2008-00034.Klune.

[66] A. Raucci, S. Di Maggio, F. Scavello, A. D'Ambrosio, M.E. Bianchi, M.C. Capogrossi, The Janus face of HMGB1 in heart disease: a necessary update, Cell. Mol. Life Sci. 76 (2019) 211–229. https://doi.org/10.1007/s00018-018-2930-9.

[67] T. Bonaldi, Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion, EMBO J. 22 (2003) 5551–5560. https://doi.org/10.1093/emboj/cdg516.

[68] S. Gardella, C. Andrei, D. Ferrera, L. V. Lotti, M.R. Torrisi, M.E. Bianchi, A. Rubartelli, The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway, EMBO Rep. 3 (2002) 995–1001. https://doi.org/10.1093/embo-reports/kvf198.

[69] I.E. Dumitriu, P. Baruah, B. Valentinis, R.E. Voll, M. Herrmann, P.P. Nawroth, B. Arnold, M.E. Bianchi, A.A. Manfredi, P. Rovere-Querini, Release of High Mobility Group Box 1 by Dendritic Cells Controls T Cell Activation via the Receptor for Advanced Glycation End Products, J. Immunol. 174 (2005) 7506–7515. https://doi.org/10.4049/jimmunol.174.12.7506.

[70] V. Chandrashekaran, R.K. Seth, D. Dattaroy, F. Alhasson, J. Ziolenka, J. Carson, F.G. Berger, B. Kalyanaraman, A.M. Diehl, S. Chatterjee, HMGB1-RAGE pathway drives peroxynitrite signaling-induced IBD-like inflammation in murine nonalcoholic fatty liver disease, Redox Biol. 13 (2017) 8–19. https://doi.org/10.1016/j.redox.2017.05.005.

[71] M. Yu, H. Wang, A. Ding, D.T. Golenbock, E. Latz, C.J. Czura, M.J. Fenton, K.J. Tracey, H. Yang, HMGB1 signals through toll-like receptor (TLR) 4 and TLR2, Shock. 26 (2006) 174–179. https://doi.org/10.1097/01.shk.0000225404.51320.82.

[72] W.L. Anggayasti, R.L. Mancera, S. Bottomley, E. Helmerhorst, The self-association of HMGB1 and its possible role in the binding to DNA and cell membrane receptors, FEBS Lett. 591 (2017) 282–294. https://doi.org/10.1002/1873-3468.12545.

[73] P. Fang, M. Schachner, Y.-Q. Shen, HMGB1 in Development and Diseases of the Central Nervous System, Mol. Neurobiol. 45 (2012) 499–506. https://doi.org/10.1007/s12035-012-8264-y.

[74] R. Kang, Q. Zhang, H.J. Zeh, M.T. Lotze, D. Tang, HMGB1 in Cancer: Good, Bad, or Both?, Clin. Cancer Res. 19 (2013) 4046–4057. https://doi.org/10.1158/1078-0432.CCR-13-0495.

[75] K. Selzle, C. Ackaert, C.J. Kampf, A.T. Kunert, A. Duschl, G.J. Oostingh, U. Pöschl,

Determination of nitration degrees for the birch pollen allergen Bet v 1, Anal. Bioanal. Chem. 405 (2013) 8945–8949. https://doi.org/10.1007/s00216-013-7324-0.

[76] H. Yanai, S. Chiba, S. Hangai, K. Kometani, A. Inoue, Y. Kimura, T. Abe, H. Kiyonari, J. Nishio, N. Taguchi-Atarashi, Y. Mizushima, H. Negishi, R. Grosschedl, T. Taniguchi, Revisiting the role of IRF3 in inflammation and immunity by conditional and specifically targeted gene ablation in mice, Proc. Natl. Acad. Sci. 115 (2018) 5253–5258. https://doi.org/10.1073/pnas.1803936115.

[77] M.T. Zeuner, C.L. Krüger, K. Volk, K. Bieback, G.S. Cottrell, M. Heilemann, D. Widera, Biased signalling is an essential feature of TLR4 in glioma cells, Biochim. Biophys. Acta - Mol. Cell Res. 1863 (2016) 3084–3095. https://doi.org/10.1016/j.bbamcr.2016.09.016.

[78] N. Abello, H.A.M. Kerstjens, D.S. Postma, R. Bischoff, Protein Tyrosine Nitration: Selectivity, Physicochemical and Biological Consequences, Denitration, and Proteomics Methods for the Identification of Tyrosine-Nitrated Proteins, J. Proteome Res. 8 (2009) 3222–3238. https://doi.org/10.1021/pr900039c.

[79] T. Franze, M.G. Weller, R. Niessner, U. Pöschl, Protein Nitration by Polluted Air, Environ. Sci. Technol. 39 (2005) 1673–1678. https://doi.org/10.1021/es0488737.

[80] A.C. Karle, G.J. Oostingh, S. Mutschlechner, F. Ferreira, P. Lackner, B. Bohle, G.F. Fischer, A.B. Vogt, A. Duschl, Nitration of the Pollen Allergen Bet v 1.0101 Enhances the Presentation of Bet v 1-Derived Peptides by HLA-DR on Human Dendritic Cells, PLoS One. 7 (2012) e31483. https://doi.org/10.1371/journal.pone.0031483.

[81] L.L. Hardy, D.A. Wick, J.R. Webb, Conversion of Tyrosine to the Inflammation-Associated Analog 3′-Nitrotyrosine at Either TCR- or MHC-Contact Positions Can Profoundly Affect Recognition of the MHC Class I-Restricted Epitope of Lymphocytic Choriomeningitis Virus Glycoprotein 33 by CD8 T Ce, J. Immunol. 180 (2008) 5956–5962. https://doi.org/10.4049/jimmunol.180.9.5956.

[82] E. Untersmayr, S.C. Diesner, G.J. Oostingh, K. Selzle, T. Pfaller, C. Schultz, Y. Zhang, D. Krishnamurthy, P. Starkl, R. Knittelfelder, E. Förster-Waldl, A. Pollak, O. Scheiner, U. Pöschl, E. Jensen-Jarolim, A. Duschl, Nitration of the Egg-Allergen Ovalbumin Enhances Protein Allergenicity but Reduces the Risk for Oral Sensitization in a Murine Model of Food Allergy, PLoS One. 5 (2010) e14210. https://doi.org/10.1371/journal.pone.0014210.

[83] A.S. Ondracek, D. Heiden, G.J. Oostingh, E. Fuerst, J. Fazekas-Singer, C. Bergmayr,

J. Rohrhofer, E. Jensen-Jarolim, A. Duschl, E. Untersmayr, Immune Effects of the Nitrated Food Allergen Beta-Lactoglobulin in an Experimental Food Allergy Model, Nutrients. 11 (2019) 2463. https://doi.org/10.3390/nu11102463.

[84] H.C. Birnboim, A.-M. Lemay, D.K.Y. Lam, R. Goldstein, J.R. Webb, Cutting Edge: MHC Class II-Restricted Peptides Containing the Inflammation-Associated Marker 3-Nitrotyrosine Evade Central Tolerance and Elicit a Robust Cell-Mediated Immune Response, J. Immunol. 171 (2003) 528–532. https://doi.org/10.4049/jimmunol.171.2.528.

[85] J.M. Souza, G. Peluffo, R. Radi, Free Radical Biology & Medicine Protein tyrosine nitration — Functional alteration or just a biomarker ?, 45 (2008) 357–366. https://doi.org/10.1016/j.freeradbiomed.2008.04.010.

[86] X. Tun, K. Yasukawa, K. Yamada, Nitric Oxide Is Involved in Activation of Toll-Like Receptor 4 Signaling through Tyrosine Nitration of Src Homology Protein Tyrosine Phosphatase 2 in Murine Dextran Sulfate-Induced Colitis, Biol. Pharm. Bull. 41 (2018) 1843–1852. https://doi.org/10.1248/bpb.b18-00558.

[87] P.F. Good, A. Hsu, P. Werner, D.P. Perl, C.W. Olanow, Protein Nitration in Parkinson's Disease, J. Neuropathol. Exp. Neurol. 57 (1998) 338–342. https://doi.org/10.1097/00005072-199804000-00006.

[88] C.A. Sacksteder, W.-J. Qian, T. V. Knyushko, H. Wang, M.H. Chin, G. Lacan, W.P. Melega, D.G. Camp, R.D. Smith, D.J. Smith, T.C. Squier, D.J. Bigelow, Endogenously Nitrated Proteins in Mouse Brain: Links to Neurodegenerative Disease, Biochemistry. 45 (2006) 8009–8022. https://doi.org/10.1021/bi060474w.

[89] C. Janko, M. Filipović, L.E. Munoz, C. Schorn, G. Schett, I. Ivanović-Burmazović, M. Herrmann, Redox Modulation of HMGB1-Related Signaling, Antioxid. Redox Signal. 20 (2014) 1075–1085. https://doi.org/10.1089/ars.2013.5179.

[90] S. Sun, M. He, S. VanPatten, Y. Al-Abed, Mechanistic insights into high mobility group box-1 (HMGb1)-induced Toll-like receptor 4 (TLR4) dimer formation, J. Biomol. Struct. Dyn. 37 (2019) 3721–3730. https://doi.org/10.1080/07391102.2018.1526712.

[91] C.L. Krüger, M.-T. Zeuner, G.S. Cottrell, D. Widera, M. Heilemann, Quantitative single-molecule imaging of TLR4 reveals ligand-specific receptor dimerization, Sci. Signal. 10 (2017) eaan1308. https://doi.org/10.1126/scisignal.aan1308.

[92] S.L. Latty, J. Sakai, L. Hopkins, B. Verstak, T. Paramo, N.A. Berglund, E. Cammarota, P. Cicuta, N.J. Gay, P.J. Bond, D. Klenerman, C.E. Bryant, Activation of Toll-like

receptors nucleates assembly of the MyDDosome signaling hub, Elife. 7 (2018) 1–15. https://doi.org/10.7554/eLife.31377.

[93] J. Neumann, K. Ziegler, M. Gelléri, J. Fröhlich-Nowoisky, F. Liu, I. Bellinghausen, D. Schuppan, U. Birk, U. Pöschl, C. Cremer, K. Lucas, Nanoscale distribution of TLR4 on primary human macrophages stimulated with LPS and ATI, Nanoscale. 11 (2019) 9769–9779. https://doi.org/10.1039/C9NR00943D.

[94] K.W. Foreman, A general model for predicting the binding affinity of reversibly and irreversibly dimerized ligands, PLoS One. 12 (2017) e0188134. https://doi.org/10.1371/journal.pone.0188134.

[95] J.E. Gestwicki, C.W. Cairo, L.E. Strong, K.A. Oetjen, L.L. Kiessling, Influencing Receptor−Ligand Binding Mechanisms with Multivalent Ligand Architecture, J. Am. Chem. Soc. 124 (2002) 14922–14933. https://doi.org/10.1021/ja027184x.

[96] L.L. Kiessling, A.C. Lamanna, Multivalency in Biological Systems, in: 2003: pp. 345–357. https://doi.org/10.1007/978-94-007-0958-4_26.

[97] L.L. Kiessling, J.E. Gestwicki, L.E. Strong, Synthetic Multivalent Ligands as Probes of Signal Transduction, Angew. Chemie Int. Ed. 45 (2006) 2348–2368. https://doi.org/10.1002/anie.200502794.

[98] L. Fellner, R. Irschick, K. Schanda, M. Reindl, L. Klimaschewski, W. Poewe, G.K. Wenning, N. Stefanova, Toll-like receptor 4 is required for α-synuclein dependent activation of microglia and astroglia, Glia. 61 (2013) 349–360. https://doi.org/10.1002/glia.22437.

[99] M. Jana, C.A. Palencia, K. Pahan, Fibrillar Amyloid-β Peptides Activate Microglia via TLR2: Implications for Alzheimer's Disease, J. Immunol. 181 (2008) 7254–7262. https://doi.org/10.4049/jimmunol.181.10.7254.

[100] M.L.D. Udan, D. Ajit, N.R. Crouse, M.R. Nichols, Toll-like receptors 2 and 4 mediate Aβ(1–42) activation of the innate immune response in a human monocytic cell line, J. Neurochem. 104 (2008) 524–533. https://doi.org/10.1111/j.1471-4159.2007.05001.x.

[101] M.P. Kummer, M. Hermes, A. Delekarte, T. Hammerschmidt, S. Kumar, D. Terwel, J. Walter, H.-C. Pape, S. König, S. Roeber, F. Jessen, T. Klockgether, M. Korte, M.T. Heneka, Nitration of Tyrosine 10 Critically Enhances Amyloid β Aggregation and Plaque Formation, Neuron. 71 (2011) 833–844. https://doi.org/10.1016/j.neuron.2011.07.001.

[102] B. Beutler, Inferences, questions and possibilities in Toll-like receptor signalling, Nature. 430 (2004) 257–263. https://doi.org/10.1038/nature02761.

[103] J.Y. Kang, J.-O. Lee, Structural Biology of the Toll-Like Receptor Family, Annu. Rev. Biochem. 80 (2011) 917–941. https://doi.org/10.1146/annurev-biochem-052909-141507.

[104] J. Xue, M. Manigrasso, M. Scalabrin, V. Rai, S. Reverdatto, D.S. Burz, D. Fabris, A.M. Schmidt, A. Shekhtman, Change in the Molecular Dimension of a RAGE-Ligand Complex Triggers RAGE Signaling, Structure. 24 (2016) 1509–1522. https://doi.org/10.1016/j.str.2016.06.021.

[105] W. Wei, L. Lampe, S. Park, B.S. Vangara, G.S. Waldo, S. Cabantous, S.S. Subaran, D. Yang, E.G. Lakatta, L. Lin, Disulfide Bonds within the C2 Domain of RAGE Play Key Roles in Its Dimerization and Biogenesis, PLoS One. 7 (2012) e50736. https://doi.org/10.1371/journal.pone.0050736.

[106] H. Zong, A. Madden, M. Ward, M.H. Mooney, C.T. Elliott, A.W. Stitt, Homodimerization Is Essential for the Receptor for Advanced Glycation End Products (RAGE)-mediated Signal Transduction, J. Biol. Chem. 285 (2010) 23137–23146. https://doi.org/10.1074/jbc.M110.133827.

[107] M.R. Walter, The molecular basis of IL-10 function: From receptor structure to the onset of signaling, in: S. Fillatreau, A. O'Garra (Eds.), Curr. Top. Microbiol. Immunol., Springer, Berlin, Heidelberg, 2014: pp. 191–212. https://doi.org/10.1007/978-3-662-43492-5_9.

[108] M. Simons, E. Gordon, L. Claesson-Welsh, Mechanisms and regulation of endothelial VEGF receptor signalling, Nat. Rev. Mol. Cell Biol. 17 (2016) 611–625. https://doi.org/10.1038/nrm.2016.87.

[109] A. Daiber, S. Daub, M. Bachschmid, S. Schildknecht, M. Oelze, S. Steven, P. Schmidt, A. Megner, M. Wada, T. Tanabe, T. Münzel, S. Bottari, V. Ullrich, Protein Tyrosine Nitration and Thiol Oxidation by Peroxynitrite—Strategies to Prevent These Oxidative Modifications, Int. J. Mol. Sci. 14 (2013) 7542–7570. https://doi.org/10.3390/ijms14047542.

[110] P. Pacher, J. Beckman, L. Liaudet, Nitric Oxide and Peroxynitrite, Physiol. Rev. 87 (2007) 315–424. https://doi.org/10.1152/physrev.00029.2006.

[111] S. Das, F. Alhasson, D. Dattaroy, S. Pourhoseini, R.K. Seth, M. Nagarkatti, P.S. Nagarkatti, G.A. Michelotti, A.M. Diehl, B. Kalyanaraman, S. Chatterjee, NADPH

Oxidase-Derived Peroxynitrite Drives Inflammation in Mice and Human Nonalcoholic Steatohepatitis via TLR4-Lipid Raft Recruitment, Am. J. Pathol. 185 (2015) 1944–1957. https://doi.org/10.1016/j.ajpath.2015.03.024.

[112] H. Zhang, J. Joseph, J. Feix, N. Hogg, B. Kalyanaraman, Nitration and Oxidation of a Hydrophobic Tyrosine Probe by Peroxynitrite in Membranes: Comparison with Nitration and Oxidation of Tyrosine by Peroxynitrite in Aqueous Solution, Biochemistry. 40 (2001) 7675–7686. https://doi.org/10.1021/bi002958c.

[113] T.L. Poulos, H. Li, Nitric oxide synthase and structure-based inhibitor design, Nitric Oxide. 63 (2017) 68–77. https://doi.org/10.1016/j.niox.2016.11.004.

[114] C.J. Kampf, F. Liu, K. Reinmuth-Selzle, T. Berkemeier, H. Meusel, M. Shiraiwa, U. Pöschl, Protein Cross-Linking and Oligomerization through Dityrosine Formation upon Exposure to Ozone, Environ. Sci. Technol. 49 (2015) 10859–10866. https://doi.org/10.1021/acs.est.5b02902.

[115] P.S.J. Lakey, T. Berkemeier, H. Tong, A.M. Arangio, K. Lucas, U. Pöschl, M. Shiraiwa, Chemical exposure-response relationship between air pollutants and reactive oxygen species in the human respiratory tract, Sci. Rep. 6 (2016) 32916. https://doi.org/10.1038/srep32916.

[116] M. Shiraiwa, K. Selzle, U. Pöschl, Hazardous components and health effects of atmospheric aerosol particles: reactive oxygen species, soot, polycyclic aromatic compounds and allergenic proteins, Free Radic. Res. (2012) 1–13. https://doi.org/10.3109/10715762.2012.663084.

[117] U. Pöschl, M. Shiraiwa, Multiphase Chemistry at the Atmosphere–Biosphere Interface Influencing Climate and Public Health in the Anthropocene, Chem. Rev. 115 (2015) 4440–4475. https://doi.org/10.1021/cr500487s.

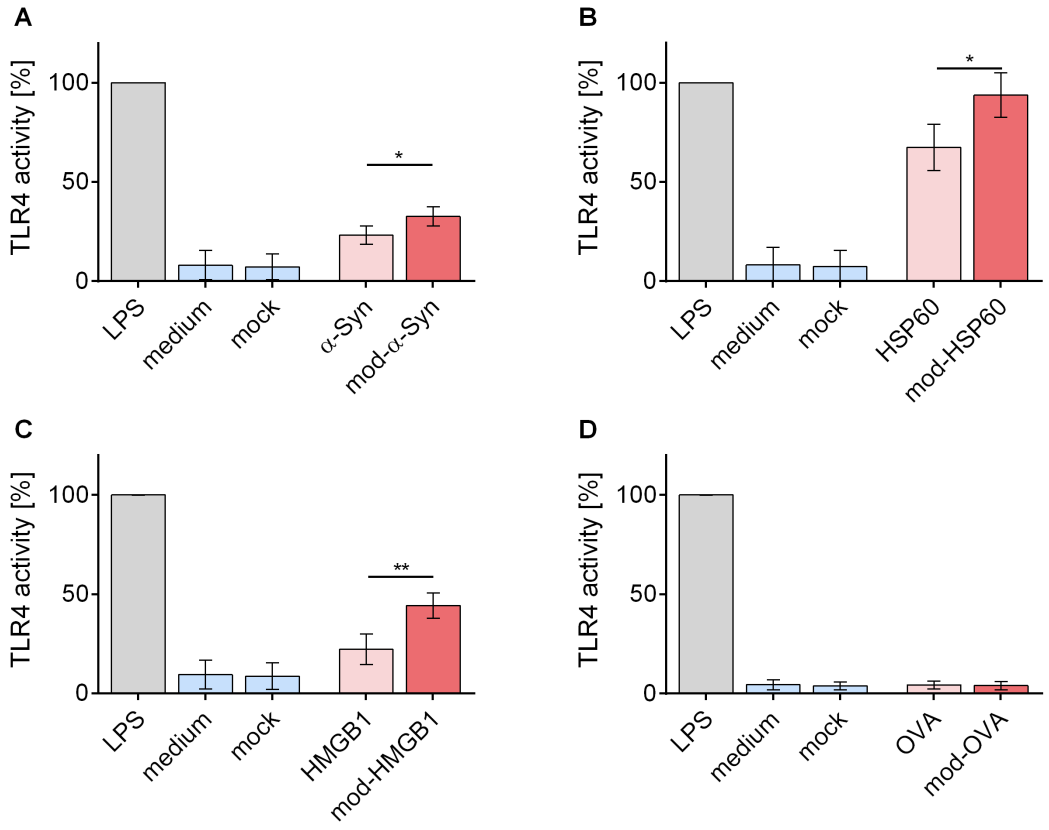

Figure 1: TLR4 activation by native and peroxynitrite-modified proteins. TLR4 activity in HeLa TLR4 dual luciferase reporter cells determined for α-Syn (A), HSP60 (B), HMGB1 (C), and OVA (D) normalized to LPS. Arithmetic mean values and standard deviations of three to four independent experiments performed in triplicates (*$p < 0.05$, **$p < 0.01$).

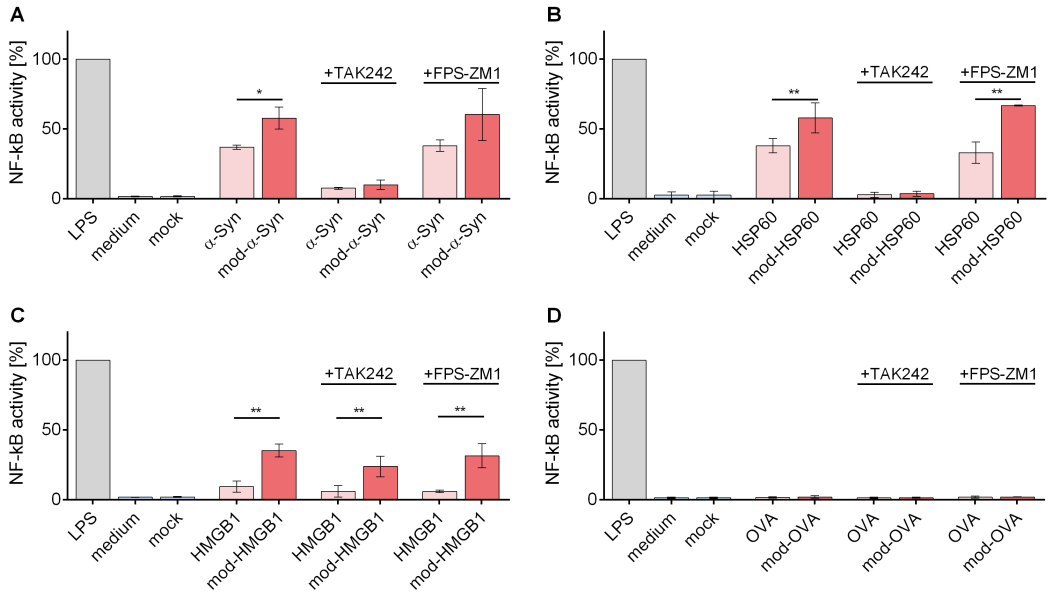

Figure 2: NF-κB activation by native and peroxynitrite-modified proteins. NF-κB activity in THP-1-Lucia™ NF-κB cells determined for α-Syn (A), HSP60 (B), HMGB1 (C), and OVA (D) normalized to LPS. Inhibition experiments with TLR4 antagonist TAK242 and RAGE antagonist FPS-ZM1. Arithmetic mean values and standard deviations of three to four independent experiments performed in triplicates (*p < 0.05, **p < 0.01).

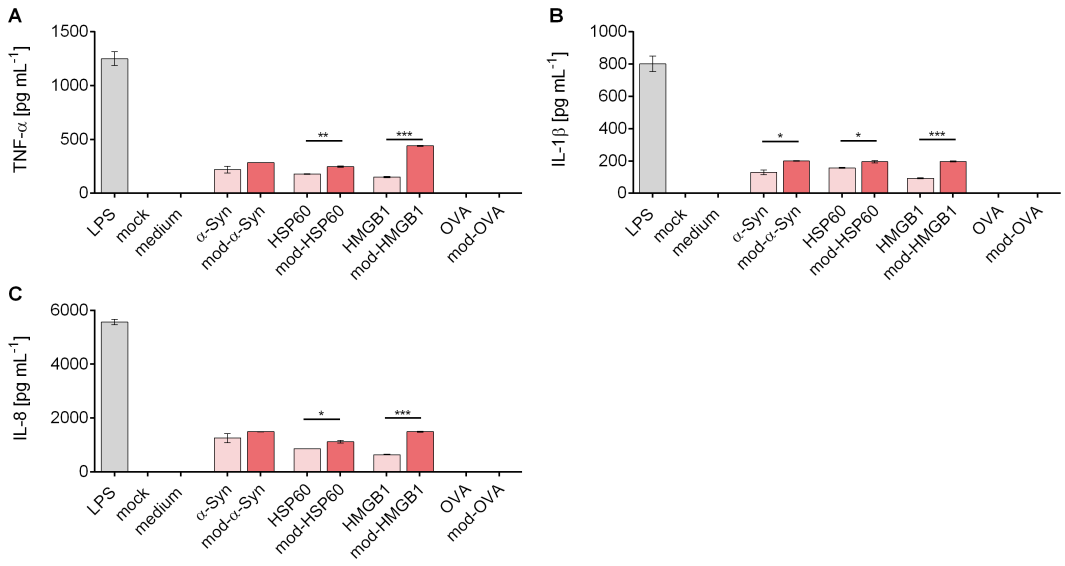

Figure 3: Pro-inflammatory cytokine secretion in response to native and peroxynitrite-modified proteins. Release of TNF-α (A), IL-1β (B), and IL-8 (C) determined for THP-1 monocytes after incubation over 24 h. Data for mock, medium, and OVA/mod-OVA are near or below the relevant limits of detection (~2-20 pg mL^{-1}). Arithmetic mean values and standard deviations of a representative experiment performed in duplicates (*p < 0.05, **p < 0.01, ***p < 0.005).

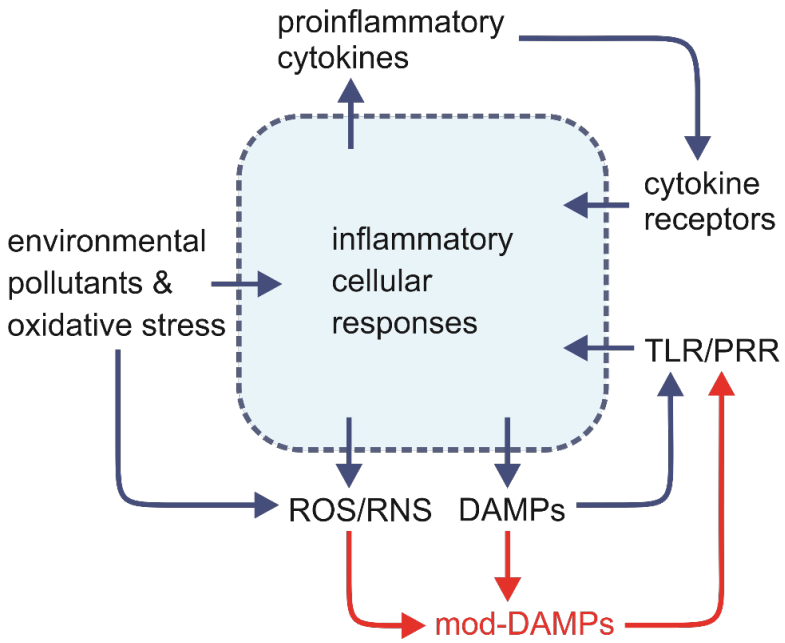

Figure 4: Amplification of inflammatory processes and innate immune responses through chemically modified DAMPs. Environmental pollutants and oxidative stress can induce an increase of reactive oxygen and nitrogen species (ROS/RNS), the formation of chemically modified damage-associated molecular patterns (mod-DAMPs), an increase of pro-inflammatory signaling via Toll-like receptors and other pattern recognition receptors (TLR/PRR), an increase of proinflammatory cytokines, and further inflammatory cellular responses.

Supplementary Material

Chemical modification of pro-inflammatory proteins by peroxynitrite increases activation of TLR4 and NF-κB: Implications for the health effects of air pollution and oxidative stress

Kira Ziegler[1], Anna T. Kunert[1], Kathrin Reinmuth-Selzle[1], Anna Lena Leifke[1], Darius Widera[2], Michael G. Weller[3], Detlef Schuppan[4,5], Janine Fröhlich-Nowoisky[1], Kurt Lucas[1], Ulrich Pöschl[1]

[1]Max Planck Institute for Chemistry, Multiphase Chemistry Department, 55128 Mainz, Germany; [2]Stem Cell Biology and Regenerative Medicine Group, School of Pharmacy, University of Reading, RG6 6AP Reading, UK; [3]Federal Institute for Materials Research and Testing (BAM), Berlin, Germany; [4]Institute of Translational Immunology, University Medical Center of the Johannes Gutenberg University, 55131 Mainz, Germany; [5]Division of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, MA 02215, USA

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Supplement to

Chemical modification of pro-inflammatory proteins by peroxynitrite increases activation of TLR4 and NF-κB: Implications for the health effects of air pollution and oxidative stress

Kira Ziegler[a,1], Anna T. Kunert[a,1], Kathrin Reinmuth-Selzle[a], Anna Lena Leifke[a], Darius Widera[b], Michael G. Weller[c], Detlef Schuppan[d,e], Janine Fröhlich-Nowoisky[a], Kurt Lucas[a,**], and Ulrich Pöschl[a,*]

[a]Max Planck Institute for Chemistry, Multiphase Chemistry Department, 55128 Mainz, Germany

[b]Stem Cell Biology and Regenerative Medicine Group, School of Pharmacy, University of Reading, RG6 6AP Reading, UK

[c]Federal Institute for Materials Research and Testing (BAM), Berlin, Germany

[d]Institute of Translational Immunology, University Medical Center of the Johannes Gutenberg University, 55131 Mainz, Germany

[e]Division of Gastroenterology, Beth Israel Deaconess Medical Center, Harvard Medical School, MA 02215, USA

[1]equal co-author contributions

*corresponding author: u.poschl@mpic.de

**corresponding author: k.lucas@mpic.de

Table S1: Overview of investigated proteins: molecular mass, chain length (number of amino acids), number of tyrosine residues, occurrence, and characteristic features.

Protein	Mass [kDa]	Length	Tyrosine residues	Occurrence	Characteristic features
α-Synuclein (α-Syn)	19.5	140	4	Neurons	TLR4-activating DAMP associated with Parkinson's disease
Heat shock protein 60 (HSP60)	60.0	573	5	Most human cell types	Mitochondrial chaperone and TLR4-activating DAMP, many diseases
High-mobility-group box 1 (HMGB1)	25.0	215	7	Most human cell types	Nuclear protein and TLR4-activating DAMP, many diseases
Ovalbumin (OVA)	42.8	386	10	Egg white	Negative control without TLR4 activation

Table S2: Tyrosine nitration degree and relative fractions of monomers and dimers in native and peroxynitrite-modified proteins (arithmetic mean values and standard deviations). Nitration degrees were determined by HPLC for two independently prepared samples measured in duplicates; monomer/dimer fractions were determined by SDS-PAGE for three to four independently prepared samples.

Protein	Nitration degree [%]	Monomer fraction [%]	Dimer fraction [%]
α-Syn	< 1	97 ± 4	< 3
mod-α-Syn	39 ± 6	81 ± 11	16 ± 7
HSP60	4 ± 3	98 ± 1	< 3
mod-HSP60	68 ± 16	55 ± 7	23 ± 8
HMGB1	2 ± 0.2	92 ± 9	12 ± 7
mod-HMGB1	13 ± 8	87 ± 11	17 ± 9
OVA	< 1	84 ± 4	16 ± 4
mod-OVA	13 ± 2	83 ± 8	15 ± 5

Table S3: Target genes and sequences of real-time quantitative PCR (qPCR) primers for mRNA expression of cytokines and reference genes (PPIA, TBP).

Gene	Accession number	Sequence 5´→ 3´
TNF-α	NM_000594.4	fw GCCCAGGCAGTCAGATCATCTT
		rev CCTCAGCTTGAGGGTTTGCTACA
IL-1β	NM_000576.2	fw GCCCTAAACAGATGAAGTGCTC
		rev GAACCAGCATCTTCCTCAG
IL-8	NM_000584.3	fw AGTCCTTGTTCCACTGTGCCTTGG
		rev TGCTTCCACATGTCCTCACAACATC
CXCL-10	NM_001565.4	fw CTGTACGCTGTACCTGCATCAGCA
		rev ACACGTGGACAAAATTGGCTTGC
PPIA	NM_021130.4	fw TCTGCACTGCCAAGACTGAG
		rev TGGTCTTGCCATTCCTGGAC
TBP	NM_001172085.1	fw TGAGCCAGAGTTATTTCCTGGT
		rev AATTTCTGCTCTGACTTTAGCACC

Table S4: Enhancement factors of TLR4 and NF-κB activity for peroxynitrite-modified protein relative to the native protein. Arithmetic mean values and standard errors for three to four independent experiments performed in triplicates.

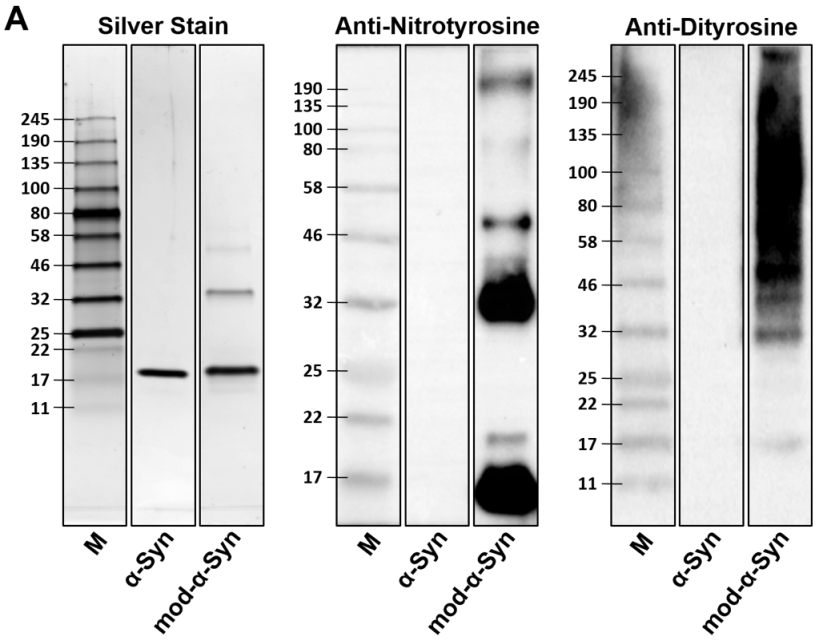
	α-Syn	**HSP60**	**HMGB1**
TLR4	1.4 ± 0.2	1.4 ± 0.1	2.2 ± 0.4
NF-κB	1.6 ± 0.1	1.6 ± 0.2	4.2 ± 0.8

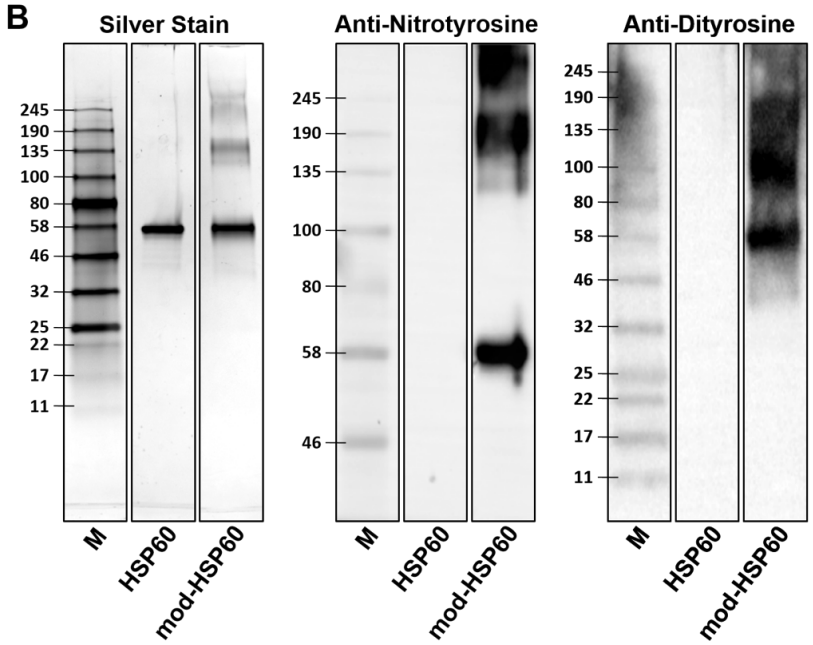
Table S5: Enhancement factors of pro-inflammatory cytokine secretion for peroxynitrite-modified protein relative to the native protein. Arithmetic mean values and standard errors for independent experiments performed in duplicates.

	α-Syn	**HSP60**	**HMGB1**
TNF-α	1.3 ± 0.1	1.4 ± 0.1	3.0 ± 0.2
	1.3 ± 0.2	1.4 ± 0.04	2.9 ± 0.1
	2.0 ± 0.2	8.8 ± 0.5	16.7 ± 2.5
IL-1β	1.5 ± 0.1	1.3 ± 0.1	2.2 ± 0.2
	1.5 ± 0.2	1.2 ± 0.1	2.1 ± 0.1
	1.7 ± 0.1	4.2 ± 0.1	6.8 ± 0.9
IL-8	1.2 ± 0.1	1.3 ± 0.1	2.3 ± 0.2
	1.2 ± 0.2	1.3 ± 0.1	2.3 ± 0.1
	1.6 ± 0.2	8.8 ± 0.5	8.6 ± 1.0

Table S6: Enhancement factors of pro-inflammatory cytokine mRNA expression for peroxynitrite-modified protein relative to the native protein. Arithmetic mean values and standard errors for three to four experiments performed in duplicates.

	α-Syn	**HSP60**	**HMGB1**
TNF-α	2.2 ± 0.1	-	4.4 ± 0.1
	1.0 ± 0.1	14.7 ± 0.4	1.9 ± 0.3
	1.0 ± 0.03	1.3 ± 0.1	2.4 ± 0.1
	1.0 ± 0.1	1.2 ± 0.1	-
IL-1 β	2.1 ± 0.1	-	4.3 ± 0.1
	0.9 ± 0.1	56.3 ± 3.1	3.3 ± 0.5
	0.9 ± 0.03	1.4 ± 0.04	3.0 ± 0.2
	1.1 ± 0.1	1.4 ± 0.1	-
IL-8	1.2 ± 0.1	-	3.0 ± 0.1
	0.8 ± 0.1	12.9 ± 0.9	1.7 ± 0.2
	0.8 ± 0.03	1.3 ± 0.1	2.3 ± 0.2
	1.0 ± 0.1	1.3 ± 0.02	-
CXCL-10	1.1 ± 0.03	-	8.4 ± 0.5
	1.1 ± 0.1	8.7 ± 0.6	1.9 ± 0.2
	0.9 ± 0.1	1.3 ± 0.1	2.4 ± 0.1
	1.1 ± 0.1	1.0 ± 0.03	-


A
Silver Stain
Anti-Nitrotyrosine
Anti-Dityrosine
245
190
135
100
80
58
46
32
25
22
17
11
M
α-Syn
mod-α-Syn


B
Silver Stain
Anti-Nitrotyrosine
Anti-Dityrosine
245
190
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58
46
32
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22
17
11
M
HSP60
mod-HSP60

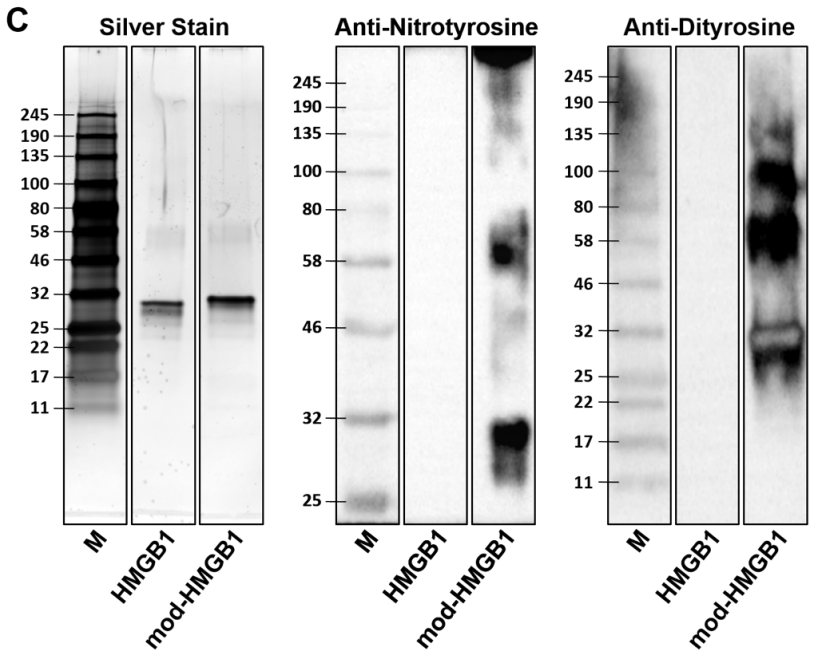

Figure S1: SDS-PAGE with silver stain and western blots against nitro- and dityrosine for native and peroxynitrite-modified proteins. Expected molecular masses of monomers: α-Syn, 19.5 kDa (A), HSP60, 60.0 kDa (B), and HMGB1, 25.0 kDa (C). Silver stain and marker (M) of western blots were taken in bright field and merged with the chemiluminescence western blot pictures against nitro- and dityrosine of native and peroxynitrite-modified proteins.

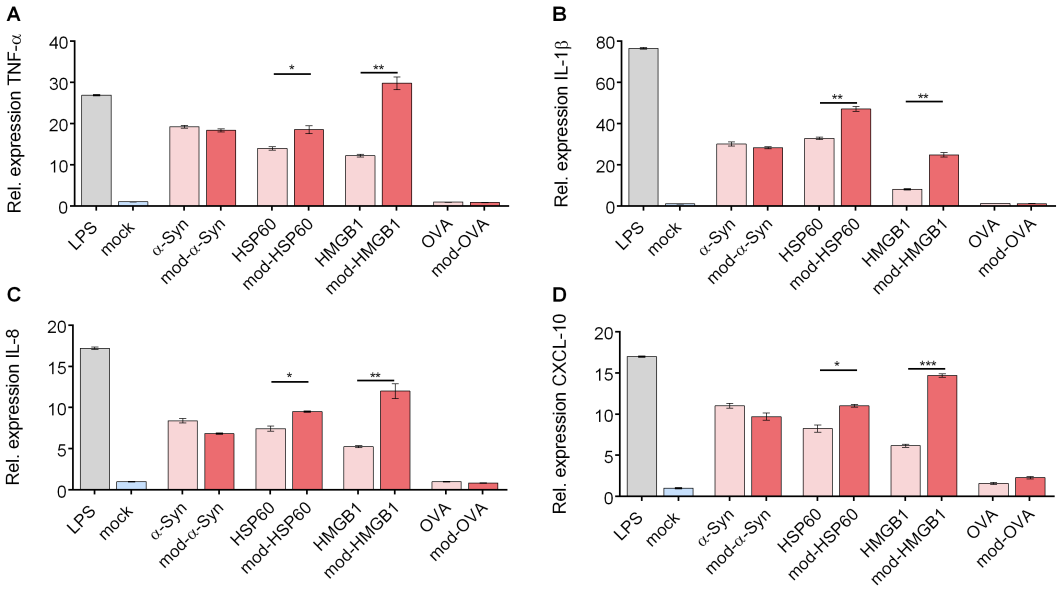

Figure S2: Expression of pro-inflammatory cytokines in response to native and peroxynitrite-modified proteins. mRNA quantification of gene expression of TNF-α (A), IL-1β (B), IL-8 (C), and CXCL-10 (D) determined for THP-1 monocytes after incubation over 4 h, corrected against two reference genes (PPIA, TBP) and normalized to mock. Arithmetic mean values and standard deviation of a representative experiment performed in duplicates (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$).

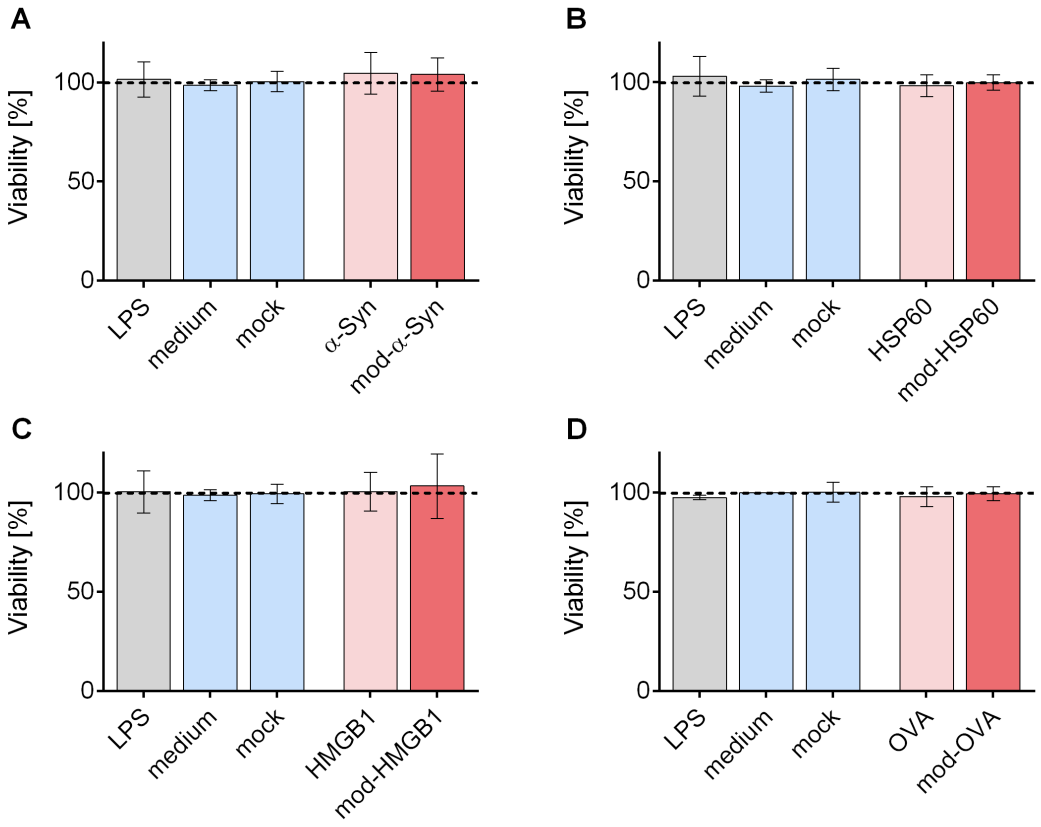

Figure S3: Viability of HeLa TLR4 dual reporter cells treated with native and peroxynitrite-modified proteins. Cell viability assessed for α-Syn (A), HSP60 (B), HMGB1 (C), and OVA (D) using the firefly signal normalized to medium-treated cells. Arithmetic mean values and standard deviations of three to four independent experiments performed in triplicates.

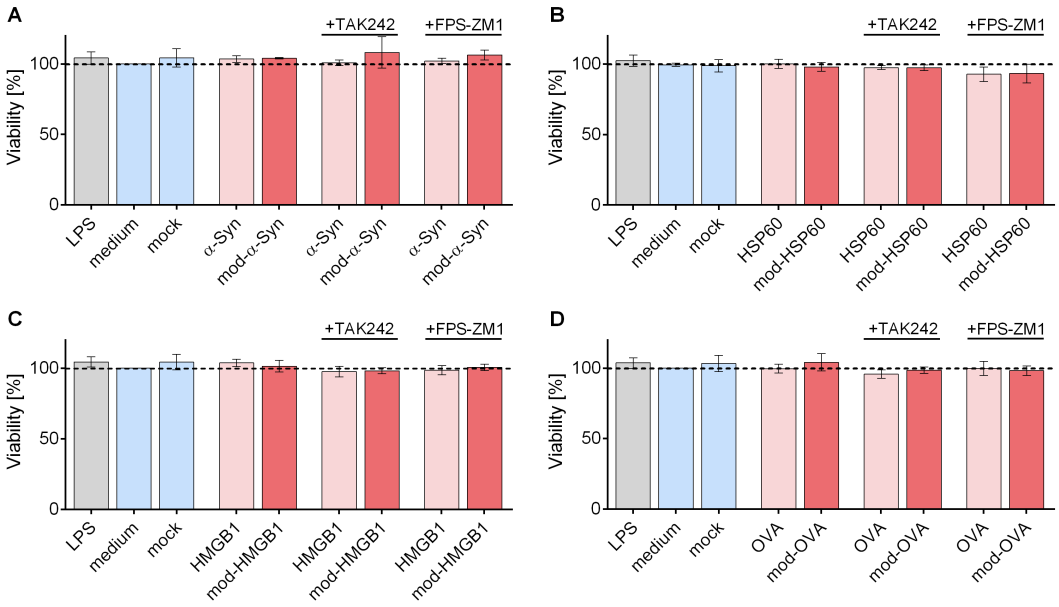

Figure S4: Viability of THP-1-Lucia™ NF-κB reporter cells treated with native and peroxynitrite-modified proteins. Cell viability assessed for α-Syn (A), HSP60 (B), HMGB1 (C), and OVA (D) with and without TLR4 or RAGE antagonist using the alamarBlue™ cell viability reagent normalized to medium-treated cells. Arithmetic mean values and standard deviations of three to four independent experiments performed in triplicates.

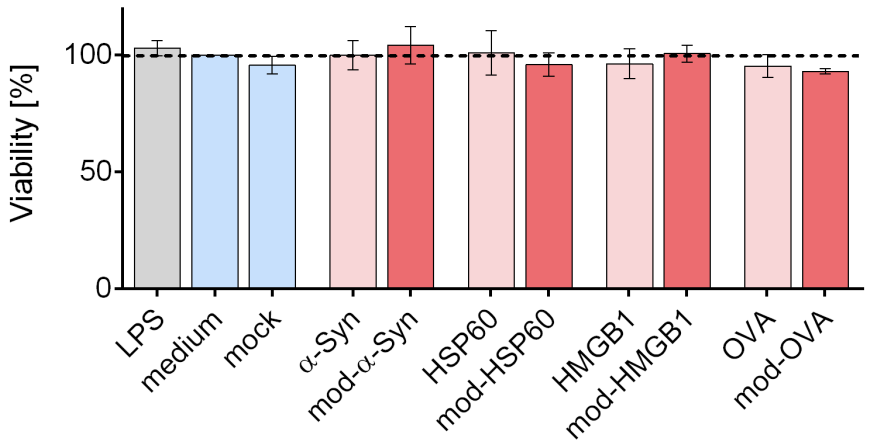

Figure S5: Viability of human THP-1 monocytes treated with native and peroxynitrite-modified proteins. Cell viability assessed for α-Syn, HSP60, HMGB1, and OVA using the alamarBlue™ cell viability reagent normalized to medium-treated cells. Arithmetic mean values and standard deviations of three independent experiments performed in triplicates.

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Air Pollution and Climate Change Effects on Allergies in the Anthropocene: Abundance, Interaction, and Modification of Allergens and Adjunvants

Kathrin Reinmuth-Selzle[1], Christopher J. Kampf[1,2], Kurt Lucas[1], Naama Lang-Yona[1], Janine Fröhlich-Nowoisky[1], Manabu Shiraiwa[1,3], Pascale S. J. Lakey[1], Senchao Lai[1,4], Fobang Liu[1], Anna T. Kunert[1], Kira Ziegler[1], Fangxia Shen[1], Rossella Sgarbanti[1], Bettina Weber[1], Iris Bellinghausen[5], Joachim Saloga[5], Michael G. Weller[6], Albert Duschl[7], Detlef Schuppan[8,9], Ulrich Pöschl[1]

[1]Multiphase Chemistry Department, Max Planck Institute for Chemistry, Mainz, 55128, Germany; [2]Institute of Inorganic and Analytical Chemistry, Johannes Gutenberg University, Mainz, 55128, Germany; [3]Department of Chemistry, University of California, Irvine, California 92697-2025, United States; [4]South China University of Technology, School of Environment and Energy, Guangzhou, 510006, China; [5]Department of Dermatology, University Medical Center, Johannes Gutenberg University, Mainz, 55131, Germany; [6]Division 1.5 Protein Analysis, Federal Institute for Materials Research and Testing (BAM), Berlin, 12489, Germany; [7]Department of Molecular Biology, University of Salzburg, 5020 Salzburg, Austria; [8]Institute of Translational Immunology and Research Center for Immunotherapy, Institute of Translational Immunology, University Medical Center, Johannes Gutenberg University, Mainz, 55131 Germany; [9]Division of Gastroenterology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215, United States

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Contribution

Aided in writing the manuscript.

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Air Pollution and Climate Change Effects on Allergies in the Anthropocene: Abundance, Interaction, and Modification of Allergens and Adjuvants

Kathrin Reinmuth-Selzle,[*,†,○] Christopher J. Kampf,[*,†,‡,○] Kurt Lucas,[†] Naama Lang-Yona,[†] Janine Fröhlich-Nowoisky,[†] Manabu Shiraiwa,[†,§] Pascale S. J. Lakey,[†] Senchao Lai,[†,∥] Fobang Liu,[†] Anna T. Kunert,[†] Kira Ziegler,[†] Fangxia Shen,[†] Rossella Sgarbanti,[†] Bettina Weber,[†] Iris Bellinghausen,[⊥] Joachim Saloga,[⊥] Michael G. Weller,[#] Albert Duschl,[¶] Detlef Schuppan,[■,△] and Ulrich Pöschl[*,†]

[†]Multiphase Chemistry Department, Max Planck Institute for Chemistry, Mainz, 55128, Germany

[‡]Institute of Inorganic and Analytical Chemistry, Johannes Gutenberg University, Mainz, 55128, Germany

[§]Department of Chemistry, University of California, Irvine, California 92697-2025, United States

[∥]South China University of Technology, School of Environment and Energy, Guangzhou, 510006, China

[⊥]Department of Dermatology, University Medical Center, Johannes Gutenberg University, Mainz, 55131, Germany

[#]Division 1.5 Protein Analysis, Federal Institute for Materials Research and Testing (BAM), Berlin, 12489, Germany

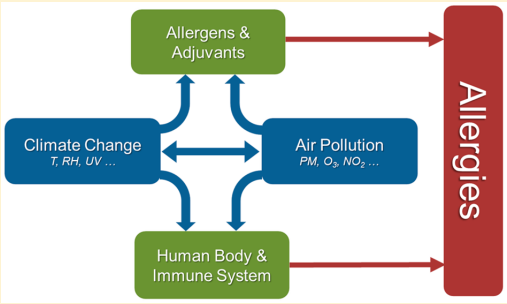
[¶]Department of Molecular Biology, University of Salzburg, 5020 Salzburg, Austria

[■]Institute of Translational Immunology and Research Center for Immunotherapy, Institute of Translational Immunology, University Medical Center, Johannes Gutenberg University, Mainz, 55131 Germany

[△]Division of Gastroenterology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215, United States

Supporting Information

ABSTRACT: Air pollution and climate change are potential drivers for the increasing burden of allergic diseases. The molecular mechanisms by which air pollutants and climate parameters may influence allergic diseases, however, are complex and elusive. This article provides an overview of physical, chemical and biological interactions between air pollution, climate change, allergens, adjuvants and the immune system, addressing how these interactions may promote the development of allergies. We reviewed and synthesized key findings from atmospheric, climate, and biomedical research. The current state of knowledge, open questions, and future research perspectives are outlined and discussed. The Anthropocene, as the present era of globally pervasive anthropogenic influence on planet Earth and, thus, on the human environment, is characterized by a strong increase of carbon dioxide, ozone, nitrogen oxides, and combustion- or traffic-related particulate matter in the atmosphere. These environmental factors can enhance the abundance and induce chemical modifications of allergens, increase oxidative stress in the human body, and skew the immune system toward allergic reactions. In particular, air pollutants can act as adjuvants and alter the immunogenicity of allergenic proteins, while climate change affects the atmospheric abundance and human exposure to bioaerosols and aeroallergens. To fully understand and effectively mitigate the adverse effects of air pollution and climate change on allergic diseases, several challenges remain to be resolved. Among these are the identification and quantification of immunochemical reaction pathways involving allergens and adjuvants under relevant environmental and physiological conditions.



1. INTRODUCTION AND MOTIVATION

Allergies are hypersensitivities initiated by specific immunologic mechanisms (abnormal adaptive immune responses).[1−3] They constitute a major health issue in most modern societies, and related diseases, such as allergic rhinitis, atopic asthma, eczema (atopic dermatitis), and food allergies, have strongly increased during the past decades.[4−12] While some of the perceived rise in allergies may be due to improved diagnosis, the prevalence of allergic diseases has genuinely increased with industrialization

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and with the adoption of a "Western" lifestyle.[13] The development of allergies is a complex multifactorial process that involves various factors influencing the body's predisposition and immune response, and the manifestation of allergic diseases depends on exposure to allergens, adjuvants and other environmental and lifestyle factors (Figure S1 and section S1).[3,4,14−16] Among the risk factors for allergic diseases are the genetic predisposition of the individual (referred to as atopy), reduced childhood exposure to pathogens and parasites ("hygiene hypothesis"), diet/nutrition, psychological/social stress, and environmental pollution, including outdoor and indoor air pollutants (ozone, nitrogen oxides, diesel exhaust particles, tobacco smoke, etc.).[4,12,17−35] As outlined in Figure 1,

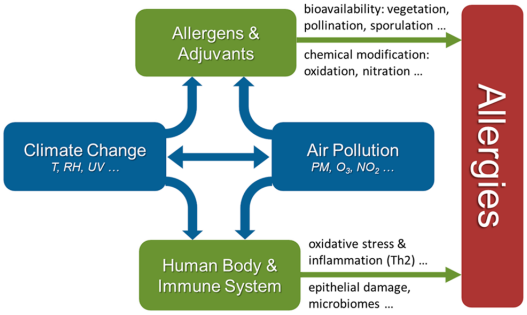

Figure 1. Interplay of air pollution and climate change can promote allergies by influencing the human body and immune system, as well as the abundance and potency of environmental allergens and adjuvants.

climate change and air pollution can influence the bioavailability and potency of allergens and adjuvants in multiple ways, including changes in vegetation cover, pollination and sporulation periods, and chemical modifications. Moreover, climatic conditions and air pollutants may skew physiological processes and the immune system toward the development of allergies, for example, by oxidative stress and inflammation, disruption of protective epithelial barriers, and disturbance of related microbial communities (microbiomes).[4,8,35−38]

The term Anthropocene describes the present era of globally pervasive and steeply increasing anthropogenic/human influence on planet Earth, including the land surface, biosphere and atmosphere.[38−44] Human activities have become a driving force that changes many characteristics of our environment such as biodiversity and air quality on local, regional, and global scales, for example, through land use change, agriculture, fossil fuel burning, traffic emissions, and the release of industrial products.[38,39,41,43,45−49] While the basic concept of the Anthropocene, as introduced by Nobel laureate Paul J. Crutzen and colleagues,[39,44,50] is widely accepted and increasingly used across the sciences and humanities, the actual beginning of the Anthropocene as a new geological epoch is still under investigation and discussion.[38,45−47,51−64] The proposed dates range from early human history via the 19th century (industrialization) to the 1960s (nuclear weapon testing and "Great Acceleration").[45−47,58−64] Since the industrialization of the 19th century and especially during the "Great Acceleration" of the 20th century, the primary emission, secondary formation, and concentration of air pollutants like ozone, nitrogen, and sulfur oxides, soot, and a wide range of other reactive trace gases and aerosols have greatly increased relative to preindustrial times, especially in densely populated and industrialized areas but also in agricultural environments and around the globe.[38,47,65−69] For example, the average mixing ratios of ozone in continental background air have increased by factors of 2−4 from around 10−20 ppb from the beginning of the 19th century to 30−40 ppb in the 21st century, and the number and mass concentrations of aerosol particles in polluted urban air are typically by 1−2 orders of magnitude higher than in pristine air of remote continental regions ($\sim 10^2$−10^3 cm^{-3} and ∼1−10 $\mu g\ m^{-3}$ vs $\sim 10^3$−10^5 cm^{-3} and ∼10−100 $\mu g\ m^{-3}$).[38,70]

Numerous studies indicate that ozone and air particulate matter have strong effects on human health and mortality as well as on agricultural crop yields.[71−80] In view of these findings, it appears unlikely that the strong environmental changes of the Anthropocene would have no effect on the interaction of the human immune system with environmental stimuli, including allergens and adjuvants. Indeed, it seems necessary to address the question whether human activities are creating a hazardous atmosphere that may severely affect public health.[35,37,38,81,82] Figure 2 illustrates how climate parameters and air pollutants can exert proinflammatory and immunomodulatory effects.[8] As detailed in the following sections, both air pollutants and climate parameters can influence the environmental abundance of allergenic bioparticles and the release of allergenic proteins and biogenic adjuvants. Moreover, air pollutants can chemically modify and agglomerate allergenic proteins, and they can act as adjuvants inducing epithelial damage and inflammation.

Several reviews have addressed the general determinants of allergenicity[3−8,83−85] and various environmental risk factors for allergic diseases.[4,9,12,34,36,86−101] In this Critical Review, we attempt to summarize, update, and synthesize the different perspectives and most relevant findings reported in earlier reviews and recent research articles addressing the effects of air pollutants and climate parameters on allergies. A central aim of this article is to review and outline both proven and potential effects of the globally pervasive environmental changes that are characteristic for the Anthropocene; a holistic view of environmentally caused changes in the abundance, interaction, and modification of allergens and related substances is provided. Our target audience comprises physical, chemical, and biomedical scientists interested in environmental effects on public health. Sections 2−4 deal with specific environmental processes and air pollutants that are likely to affect the development of allergies in the Anthropocene, that is, in an environment strongly influenced by human activity. Section 5 provides an outlook identifying key questions and promising directions of future research. For orientation of readers not familiar with the basics of allergic sensitization and response, section S1 outlines key features of the immunochemical interactions involved in IgE-mediated allergies (type I hypersensitivities)[3−5,14−16,84,102−136] on which this article is mainly focused and which usually involve Th2 cell-mediated inflammation[137,138] (Figure S2).

2. ABUNDANCE AND RELEASE OF ALLERGENS AND ADJUVANTS

Environmental allergens are mostly proteins derived from plants, animals, and fungi that can trigger chemical and biological reaction cascades in the immune system leading to allergic sensitization and formation of IgE antibodies (section S1).[8,84,103,105−109] Prominent examples are major allergens of

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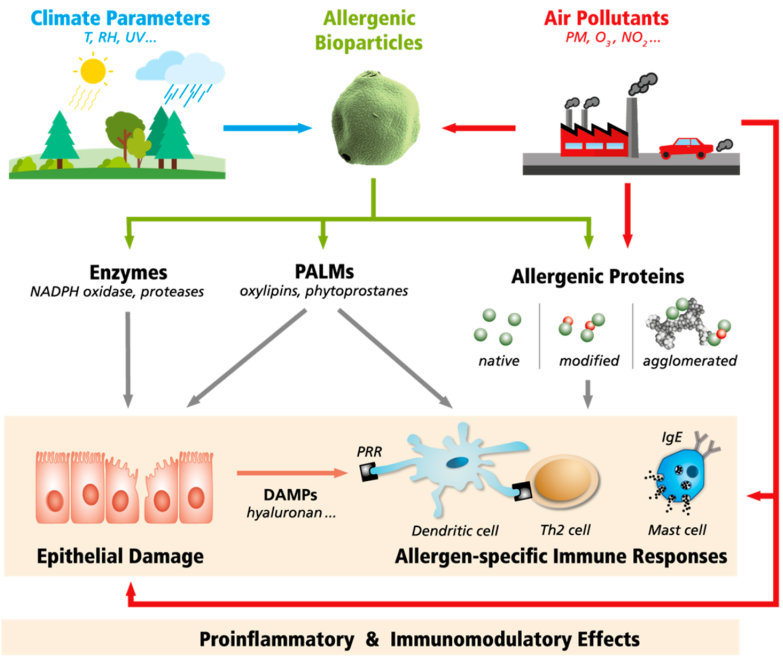

Figure 2. Pathways through which climate parameters and air pollutants can influence the release, potency, and effects of allergens and adjuvants: temperature (T), relative humidity (RH), ultraviolet (UV) radiation, particulate matter (PM), ozone and nitrogen oxides (O_3, NO_x), reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, pollen-associated lipid mediators (PALMs), damage-associated molecular patterns (DAMPs), pattern recognition receptors (PRR), type 2 T helper (Th2) cells, immunoglobulin E (IgE), allergenic proteins (green dots), and chemical modifications (red dots).

birch pollen (Bet v 1), timothy grass pollen (Phl p 1), ragweed (*Ambrosia*, Amb a 1), molds (*Alternaria alternata*, Alt a 1, *Cladosporium herbarum*, Cla h 1, *Aspergillus fumigatus*, Asp f 1), and dust mites (Der p 1).[4,139,140] Besides allergens, also adjuvants and their interaction with the immune system play a critical role in the development of allergies. Here, we use the term adjuvant generically for substances that are promoting pro-allergic immune responses. Adjuvants can trigger the immune system by inducing tissue damage and subsequent enhanced uptake of allergens, by inducing oxidative stress and activation of immune cells, by coexposure with the allergen favoring Th2 responses, or by modification of allergens enhancing their allergic potential. An overview of biogenic and anthropogenic adjuvants, including particulate matter as well as trace gases, and their effects on the immune system is given in Table 1.

Climate change is influencing vegetation patterns and plant physiology through spatial and temporal changes in temperature and humidity (Figure 1),[141−143] and increasing atmospheric carbon dioxide (CO_2) affects plant biology by supplying more carbon for photosynthesis, biomass production, and growth (CO_2 fertilization).[144,145] These factors can influence the spread of invasive plants, the beginning, duration, and intensity of pollination, the fruiting patterns and sporulation of fungi, as well as the allergen content and allergenicity of pollen grains, fungal spores, and other biological aerosol particles (Figure 2).[12,90,93,96−98,145−162] Specific examples of climate change effects on allergenic plants and fungi are outlined in Table 2. Climate and land use change are also expected to influence the composition and spread of microbial surface communities (cryptogamic covers), from which allergenic cyanobacteria and other microbial allergens or adjuvants can be emitted to the atmosphere.[163−174] Moreover, the frequency and intensity of dust storms are expected to increase,[141,175−179] and dust particles are known to carry biological and organic components with pathogenic, allergenic, and adjuvant activity.[152,154,180−187] Dust storms have been shown to cause and aggravate respiratory disorders including atopic asthma and allergic rhinitis.[181,188−191] So-called "thunderstorm asthma" is characterized by acute asthma exacerbations possibly caused by the dispersion of inhalable allergenic particles derived from plant pollen and fungal spores by osmotic rupture.[145,192] On the other hand, climate change-related regional enhancements of outdoor humidity and indoor home dampness may also lead to an increase of respiratory symptoms and atopic asthma induced by allergenic and adjuvant substances from fungi, other microbes, and mite.[12,193−196]

Pollen grains generally belong to the coarse fraction of air particulate matter (particle diameters >10 μm), but fungal spores and pollen fragments are also found in fine particulate matter (<2.5 μm; PM2.5), which can penetrate deep into the human respiratory tract and alveolar regions of the lung.[152,153,197−203] Allergenic proteins can be released from pollen and spores after cell damage or under humid conditions.[204] In particular, pollen rupture due to an osmotic shock during rain can lead to outbreaks of thunderstorm asthma.[145,192,205,206] Furthermore, peaks of high concentrations of pollen, fungal spores, and other primary biological aerosol (PBA) particles have been observed at the onset of heavy rain

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Table 1. Biogenic and Anthropogenic Adjuvants with Reported Pro-allergic Effects: (I) Pollen-Associated and Microbial Compounds, Such as Pollen-Associated Lipid Mediators (PALMs), Bacterial Lipopolysaccharides (LPS), and Fungal β-Glucans and (II) Anthropogenic Pollutants and Chemicals Including Air Particulate Matter, Gaseous Oxidants, and Organic Compounds

substances	effects
(I) Pollen-Associated and Microbial Compounds	
proteases	disrupt intracellular adhesion; stimulate protease activated receptors (PAR) inducing inflammation and enhanced IgE production[204,447,448]
	fungal proteases activate TLR4[449]
leukotrien-like PALMs	attract and activate innate cells like neutrophils and eosinophils[450]
phytoprostane PALMs	inhibit IL12 production and enhance IgE production[107]
NADPH oxidase	ROS production and inflammation[451]
adenosine	Th2 cytokine profile and inflammation[452]
flavonoids	modulate immune responses as ligands of allergenic proteins, e.g., a natural ligand of Bet v 1 is a quercetin and binds to the C-terminal helix[372,453]
	the pollen-derived flavonoid isorhamnetin modulates the immunological barrier function of the epithelium[454]
bacterial LPS	trigger TLR4 in dose dependent manner, induce a Th2 bias and allergic inflammation[455]
gram-positive bacteria	induce DC maturation by upregulation of CD80, CD83, and CD86[445]
fungal β-glucans	activate C-type lectin receptor[105]
fungal VOC	stimulate inflammatory response[456]
(II) Anthropogenic Pollutants and Chemicals	
air particulate matter (PM)	diesel exhaust particles (DEP) increase Th2 sensitization to coinhaled allergens (IgE isotype switching and production, mast cell and basophil degranulation, cytokine production (e.g., IL-4); exaberates allergic airway responses[86,457−462]
	PM and DEP induce ROS production and inflammation[86,463−465]
	DEP suppress alveolar macrophage function[466,467]
	DEP and cigartette smoke can increase thymic stromal lymphopoietin (TSLP) expression in epithelial cells[468,469]
	DEP induce permeability of epithelial cells; disrupt tight junctions by a ROS-mediated pathway[470,471]
	PM increase the expression of costimulatory molecules on DCs (MHC class II, CD40, CD80, CD86)[86,469]
	ultrafine particles (UFP < 100 nm) and DEP alter soluble protein levels (e.g., surfactant protein D, complement protein C3), increase levels of e.g., glycerin-aldehyde-3-phosphate-dehydrogenase (GADPH), manganese superoxide dismutase (MnSOD), or mitochondrial heat shock protein (Hsp 90)[472,473]
	PM2.5 and DEP activate complement proteins (C3)[474,475]
	black carbon (BC) and DEP induce epigenetic effects: DNA methylation in genes associated with Th2 polarization[476−478]
	DEP and cigarette smoke induce epithelial damage, oxidatitive stress, and inflammation[460]
	prenatal and postnatal exposure to environmental tobacco smoke (EST) is associated with asthma and wheezing[34,479,480]
	transition metals and other redox-active compounds (organic peroxides, quinones) induce ROS production and inflammation via Fenton-like reactions[38,129,309,311,481−483]
	colocalization of allergens on gold nanoparticles can facilitate IgE-receptor cross-linking[244]
ozone (O_3)	cause oxidative stress, airway inflammation, increased airway permeability[329,362,368,484]
	formation of protein ROI (reactive oxygen intermediates) and protein dimers[329,362] elevated levels of complement protein C3a[485]
	degradation of high molecular weight to low molecular weight hyaluronan, which is a DAMP that activates the TLR4 pathway[407,486]
nitrogen oxides (NO_x = NO + NO_2)	nitration of allergens[328,329]
	skew towards Th2 response,[487] increase eosinophilic inflammation,[488] and enhance airway permeability[484]
volatile, semivolatile and low-volatile organic compounds (VOC, SVOC, LVOC)	significant positive association between formaldehyde exposure and childhood asthma[272]
	antimicrobial endocrine disrupting compounds such as parabens and triclosan are associated with allergic sensitization[489,490] Bisphenol A can increase Il-4 and IgE levels[491]
	dermal and pulmonary exposure to indoor VOC elicit irritant and allergic responses[270,271]

and moist weather conditions;[200,207,208] and increased concentrations of free allergen molecules in fine air particulate matter have been observed after rainfall.[209] Prominent airborne fungi, such as *Cladosporium herbarum*, *Alternaria alternata*, and *Aspergillus fumigatus*, have been found to release higher amounts of allergens after germination under humid conditions,[210] and certain allergens are expressed only following germination.[210,211] Air pollutants, such as ozone, nitrogen oxides, and acids, can also interact with PBA particles, damage their envelope, and facilitate the release of allergenic substances, such as cytoplasmic granules from pollen (Figure S3).[205,212,213]

Besides allergenic proteins, pollen and fungal spores also release other compounds that can act as adjuvants (Table 1). In particular, the release of nonallergenic, bioactive, pollen-associated lipid mediators (PALMs) with pro-inflammatory and immunomodulatory effects can trigger and enhance allergies (Figure 2).[8,109,214−217] For example, skin prick tests of pollen allergens elicited larger wheals when tested together with low molecular weight compounds extracted from pollen.[218] The release of these substances can be influenced by climatic conditions and air pollution, and significantly higher levels were found for pollen collected near roads with heavy traffic.[205] Leukotriene-like PALMs (oxylipins) have the potential to attract and activate innate immune cells like neutrophils and eosinophils.[214,217] Other PALMs such as phytoprostanes (lipophilic counterparts of prostaglandins) are water-soluble and can inhibit the production of interleukin 12 (IL-12) by dendritic cells in the lower respiratory tract, thus favoring an allergenic Th2 T cell response.[8,215] A recent study showed that the low-molecular-weight fraction of phytopros-

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Table 2. Climate Change Effects on the Abundance and Properties as Reported for Selected Plants and Fungi Emitting Aeroallergens

allergenic species	effect of increasing temperature and CO_2 concentration
Ambrosia artemisiifolia (ragweed)	increased pollen and allergen production, plant migration and spreading[157,492−495]
	changes in pollen transcriptome, changes in allergenic potential, increase in flavonoid metabolites[158]
Betula spp. (birch)	earlier pollination start, increased pollen production[161,267,496]
Phleum pratense L. (timothy grass)	increased pollen production[159]
Alternaria spp. (mold)	increased spore numbers, decreased allergen per spore[146,156,160]
Aspergillus fumigatus (mold)	modified allergenicity and Asp f 1 content, increased spore numbers[146,155,497]
Cladosporium spp. (mold)	increased spore numbers[146]
Penicillium spp. (mold)	increased spore numbers[146]

tane E1 (PPE1) in ragweed pollen extract specifically enhanced IgE production in Th2 primed B cells. It was suggested that pollen-derived nonallergenic substances might be responsible for aggravation of IgE-mediated allergies.[219]

Fine aerosol particles and a wide range of inorganic, organic and biological substances from both natural and anthropogenic sources (e.g., secondary organic material, sulfuric and nitric acid, microbial compounds) can agglomerate and accumulate on the surface of pollen, fungal spores, and other PBA particles as illustrated in Figure S3.[152,205,220−223] An overview of reported air pollutant effects on the allergenic potential of plant pollen and fungal spores is given in Table S1.[38,205,221,224−240] Moreover, free allergens and adjuvants can bind to particulate pollutants, such as dust, soot, black/elemental carbon (BC/EC), and diesel exhaust particles (DEP) carrying the allergens and adjuvants into peripheral and deep airways.[241−243] The colocalization of allergens and adjuvants on particle surfaces (sorption layers, protein coronas) might also promote allergic sensitization and response by providing multiple/multivalent epitopes that facilitate receptor cross-linking (similar to parasitic organisms, against which IgE is naturally deployed).[244,245]

During recent years, great progress has been made in the development and application of efficient sampling and measurement methods for bioaerosol particles and components, including microscopic, spectroscopic, mass spectrometric, genomic, and proteomic analyses.[152,246−253] These and related advances in measurement and modeling techniques for health and climate relevant air contaminants (aerosols and trace gases) are expected to enable comprehensive characterization and forecasting of allergenic and adjuvant substances, as well as their mixing state in outdoor and indoor air.[38,70,254−268] Note that indoor air quality is usually influenced by both outdoor air pollutants (O_3, NO_x, PM2.5, etc.) and additional pollutants from indoor sources (e.g., formaldehyde and other organic compounds).[35,37,265,269−274] The data from ambient and individual monitoring and modeling of aeroallergen and adjuvant exposure can then be applied in epidemiological studies to better understand the risk factors of allergic sensitization and disease.[74−76,275−280]

Several epidemiological studies and meta-analyses reported that respiratory allergies and atopic dermatitis are associated with exposure to traffic-related air pollution (TRAP), but different results were obtained for different diseases and locations/studies.[281−293] TRAP is a complex mixture comprising variable proportions of particulate matter and gaseous pollutants from traffic-related primary emissions, as well as secondary pollutants formed by chemical reactions in the atmosphere.[283] Among the pollutants from primary emissions (combustion and noncombustion sources) are road dust, tire and break wear, soot/DEP, BC/EC, metals, polycyclic aromatic

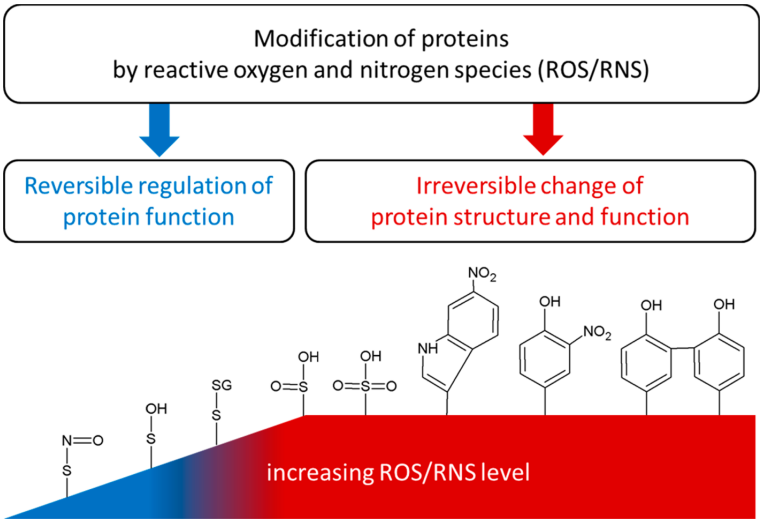

Figure 3. Upon interaction with reactive oxygen and nitrogen species (ROS/RNS), proteins can undergo a wide range of reversible and irreversible chemical modifications. Among the most commonly formed functional groups and products are *S*-nitrosothiol (SNO), sulfenic acid (SOH), disulfides with protein thiols or low molecular mass thiols (e.g., with glutathione, SSG), sulfinic acid (SO_2H), sulfonic acid (SO_3H), nitrotryptophan, nitrotyrosine, and dityrosine. Adapted from ref 317. Copyright 2013 American Chemical Society.

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hydrocarbons (PAH), and nitrogen oxides (NO_x); among the secondary pollutants are ozone, nitrates, and secondary organic aerosols (SOA).[38,70,273,283] A recent review concluded that epidemiological studies were restricted by imprecise methods of assessing both TRAP exposure and related health effects.[283] Accordingly, several studies called for more comprehensive investigations of TRAP markers, personal exposure, and lifetime outcomes.[281,294,295] The application of improved measurement and modeling techniques as outlined above should enable refined epidemiological studies and more targeted testing of hypotheses by resolving different types of TRAP (e.g., freshly emitted DEP vs resuspended road dust; soot and polycyclic hydrocarbons vs trace metals; ozone vs nitrogen oxides; etc.).

3. CHEMICAL MODIFICATION OF PROTEINS AND AMINO ACIDS

Chemical modification by air pollutants can lead to changes in the structure of protein macromolecules (amino acid oxidation, peptide backbone cleavage, conformational changes, cross-linking, and oligomerization), and affect protein stability and other properties, such as hydrophobicity and acidity of binding sites.[296−303] These and other posttranslational protein modifications may induce multiple effects in the molecular interaction of allergens with the immune system: (1) stability effects influencing the accumulation and degradation of allergenic proteins, the duration of exposure times to cellular receptors, and the process of antigen presentation via major histocompatibility complex (MHC) class II;[304,305] (2) epitope effects, that is, generation of new epitopes or modification of existing epitopes, changing the binding properties of antibodies and receptors, by direct chemical modification or as a result of conformational changes; (3) adjuvant effects, that is, generation of new adjuvant functions or modification of existing adjuvant functions such as lipid-binding capacities due to modified ligand binding sites; and (4) agglomeration effects, that is, multiplication or shielding of epitopes or adjuvant functions by cross-linking (oligomerization) of allergenic proteins, which may enhance the cross-linking of effector cell receptors (FcεRI) or sterically hinder molecular and cellular interactions.[307,308306229]

In the atmosphere, reactive oxygen and nitrogen species (ROS/RNS) are generated via photochemistry and gas-phase, heterogeneous, and multiphase reactions involving atmospheric oxidants and aerosol particles. In the human body, ROS/RNS can be formed upon exposure to air pollutants[38,309−312] or radiation (UV, X-rays, γ-rays),[313] and by regular physiological reactions.[314] For example, ROS/RNS are generated during oxidative metabolism as well as in cellular responses to foreign or danger signals (cytokines, xenobiotics, bacterial invasion).[315] Low amounts of ROS/RNS are involved in intra- and intercellular redox signaling processes, for example, oxidizing low molecular mass thiols and protein thiols (Figure 3).[316,317] An imbalance between oxidants and antioxidants in favor of oxidants (e.g., induced by air pollutants) can lead to irreversible damage of cellular lipids, proteins, nucleic acids, and carbohydrates, eventually resulting in cell death.[38,317,318] Rising levels of atmospheric oxidants and air particulate matter may lead to protein modifications in the atmosphere, as well as in the human body because of elevated oxidative stress levels, especially in the epithelial lining fluid (section 4).[38] Moreover, air pollutants and climatic stress factors, such as UV radiation, drought, salinity, and temperature extremes, can also induce higher ROS/RNS levels inside plants, which may lead to chemical modification of plant proteins, including allergens.[38,142,143] In the course of the Anthropocene, the ambient concentrations of many ROS/RNS have strongly increased because of emissions from traffic and combustion sources, as well as other industrial and agricultural activities like nitrogen fertilization of soils.[37,38,82,319,320]

In the following, we focus on irreversible modifications of allergenic proteins, such as oxidation, nitration, and cross-linking (Figure 3) by endogenous and exogenous ROS and RNS, like ozone (O_3), hydroxyl radicals (OH), hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), nitric oxide (NO), nitrogen dioxide (NO_2), nitrous acid (HONO), nitric acid (HNO_3), peroxyacetylnitrate (PAN), peroxynitrite ($ONOO^-$), and nitrate radicals (NO_3). ROS and RNS can react with oxidation-sensitive amino acids, such as cysteine (Cys), methionine (Met), tryptophan (Trp), tyrosine (Tyr), phenylalanine (Phe), and histidine (His), as well as with aliphatic side chains and the peptide backbone.[317,321−324] For example, OH radicals can cause backbone cleavage by abstracting hydrogen atoms from the α-carbon of any amino acid in the polypeptide backbone. Subsequent reactions lead to oxidative degradation of the protein and the formation of amide and carbonyl groups.[321,325,326] Oxidation reactions can result in aggregation, fragmentation, and denaturation of proteins.[327−329] While oxidative degradation appears likely to reduce the recognition of allergenic proteins, other chemical modifications, such as nitration or cross-linking may enhance the potency of allergens.[8,229,306−308,328,330−332]

The reaction of proteins with nitrating agents leads mainly to the nitration of the aromatic amino acid tyrosine forming 3-nitrotyrosine (NTyr).[333] The addition of the rather bulky NO_2 group at the ortho position of the aromatic ring induces a significant shift in the pK_a value of the tyrosine residue (Tyr) from ∼10 to ∼7, thus increasing the acidity of the hydroxyl group. These structural and chemical changes of the amino acid can affect the conformation and function of proteins.[334,335] For example, the modification of tyrosine residues can influence cell signaling through the important role of receptor tyrosine kinases, which are key regulators of cellular processes.[336] Moreover, nitrotyrosine has been reported as a biomarker for oxidative stress, inflammation, and a wide range of diseases.[296,301,337,338]

Early immunological studies already suggested that dinitrophenyl derivatives of proteins and peptides evade immune tolerance and boost immune responses.[339,340] As early as 1934, the allergic reaction to dinitrophenol was described,[341] and dinitrophenyl haptens became very popular reagents for the experimental induction of allergies.[342−344] Thus, nitrated aromatics and especially nitrophenols can be considered corner stones in the field of allergy research, suggesting that protein nitration by air pollutants might play a role in the development of allergies.[330]

Indeed, several studies showed enhanced allergenic potentials for nitrated pollen allergens,[229,305,306] nitrated fungal allergens,[237] and nitrated food allergens.[304,345] For example, the most efficiently nitrated tyrosine residue in the food allergen ovalbumin (OVA) is part of human and murine IgE epitopes and also belongs to a human T cell epitope.[304] Recent studies suggest that nitration may also affect the allergenic potential and adjuvant activity of α-amylase/trypsin inhibitors (ATIs) from wheat and other gluten-containing grains, which act as aeroallergens in baker's asthma and are involved in hyper-

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sensitivities and chronic inflammation of the gastrointestinal tract.[346−351] Nitrated variants of the major birch pollen allergen Bet v 1 induced enhanced levels of specific IgE in murine models, possibly because of the formation of neo-epitopes.[229] Nitration of Bet v 1 also increased the presentation of allergen-derived peptides by antigen presenting cells (APC).[305] Moreover, increased proteolytic stability, up-regulation of CCL17 (Th2-associated chemokine secreted by dendritic cells, DC), and alterations of T cell proliferation and stimulatory capacities have been observed for nitrated Bet v 1.[306] Nitrated proteins also have been observed to modulate the antioxidant levels in murine pneumocytes.[352] In a recent study, in vivo fumigation of ragweed pollen with NO_2 resulted in an altered proteomic pattern including nitrosylation products and the treated pollen showed higher IgE recognition in immunoblots.[239] Enhanced allergenic potential was also observed for *Betula pendula*, *Ostrya carpinifolia*, and *Carpinus betulus* pollen after NO_2 exposure (Table S1).[236]

Reaction product studies and kinetic experiments have shown that environmentally relevant O_3 and NO_2 concentrations can induce protein nitration on tyrosine residues.[237,328−330,333,353−355] This is in line with earlier observations that atmospheric oxidation and nitration processes leads to the formation of nitrophenols and dinitrophenols,[356] and that nitration is an important reaction pathway particularly in the atmospheric aqueous phase.[357,358] Especially, aromatic amino acids like tyrosine and tryptophan can react with atmospheric nitrating agents, such as ozone/NO_2 mixtures or peroxyacetylnitrate (PAN).[330,359] Under photochemical smog conditions in polluted urban environments (high O_3 and NO_2 concentrations), proteins on the surface of aerosol particles can be efficiently nitrated within minutes to hours.[328,330] The reaction kinetics also depends strongly on ambient relative humidity: At high relative humidity and especially during aqueous phase processing (when aerosol particles are activated as cloud or fog droplets), nitration may proceed efficiently also within the particle bulk.[328,360,361]

Mechanistically, the reaction between O_3/NO_2 and tyrosine involves the formation of long-lived reactive oxygen intermediates (ROI), likely via hydrogen abstraction from the phenolic OH group, yielding tyrosyl radicals (phenoxy radical derivatives of tyrosine) that can further react with NO_2 to form nitrotyrosine residues as shown in Figure 4.[329,362,363] The two-step protein nitration by air pollutants is similar to the endogenous nitration of proteins by peroxynitrite ($ONOO^-$)[298,328,364] formed from nitrous oxide (NO) and superoxide anions (O_2^-).[301,365,366] For endogeneous protein nitration by $ONOO^-$, both radical and electron transfer reaction pathways have been proposed.[367] Besides nitration, tyrosyl radicals can also undergo hydroxylation or self-reaction (cross-linking) to form dityrosine derivatives (Figure 4).[368]

The site selectivity of protein nitration is influenced by the molecular structure of the protein, the nitrating agent, and the reaction conditions. For example, different preferred reaction sites were observed for the birch pollen allergen Bet v 1, the egg allergen ovalbumin, and bovine serum albumin.[304,328,333,354] Upon exposure of Bet v 1 to atmospherically relevant concentrations of O_3/NO_2 and physiologically relevant concentrations of $ONOO^-$, the preferred sites of nitration were tyrosine residues with high solvent accessibility and within a hydrophobic environment. Accordingly, nitrated tyrosine residues occurred mainly in the C-terminal helix and in the hydrophobic cavity (Figure S4).[328] Both are key positions for

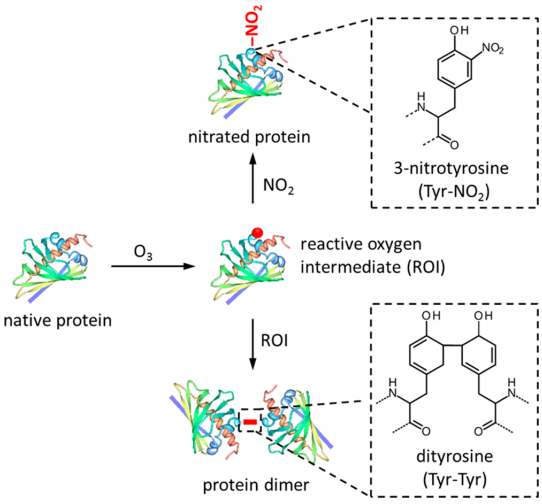

Figure 4. Posttranslational modification of proteins exposed to ozone (O_3) and nitrogen dioxide (NO_2). The initial reaction with O_3 leads to the formation of reactive oxygen intermediates (ROI, tyrosyl radicals), which can further react with each other to form cross-linked proteins (dityrosine) or with NO_2 to form nitrated proteins (nitrotyrosine). The shown protein is Bet v 1.0101 (PDB accession code 4A88,[370] created with the PDB protein workshop 3.9[498]), for which nitration and cross-linking were found to influence the immunogenicity and allergenic potential.[229,305,306,328] Red dot indicates a tyrosyl radical; red bar indicates dityrosine cross-link.

the binding of specific IgE,[369] as well as ligands like fatty acids, cytokines, and flavonoids.[370−372] The binding of such ligands may be involved in allergic and inflammatory immune responses by stabilizing Bet v 1 against endo/lysosomal degradation.[373] Moreover, nitration-related changes in ligand-binding capacity might influence the interaction of allergenic proteins like Bet v 1 with adjuvant substances like lipopolysaccharide (LPS) and induce a shift from Th1 to Th2 responses, thus resulting in increased allergenicity.[306]

Dimerization and oligomerization are supposed to have a strong influence on the immunogenicity of allergenic proteins and are common features of major allergens like Bet v 1.[307,308] The cross-linking of IgE receptors (FcεRI) on effector cells is a key element of allergic reactions and requires IgE antibody clustering on the cell surface,[374,375] which may be facilitated by multivalent allergens, such as oligomers of allergenic proteins providing multiple epitopes of the same kind.[122,376] Moreover, cross-linking can make proteins less susceptible to enzymatic proteolysis and influence immune responses.[313,373,377] Indeed, immune responses to oligomers and aggregates of certain allergenic proteins were found to be enhanced compared to the monomeric form of the allergenic protein.[307,308,378−380] The clustering of allergenic proteins on nanoparticle surfaces (protein coronas) can also modulate allergic respones depending on protein and particle properties.[244] Accordingly, the investigation and effects of allergen colocalization on the surface of inhalable ambient particles, such as pollen fragments or soot (DEP), are potentially important research perspectives.

Oxidative protein cross-linking can occur upon (a) tyrosyl radical coupling through dityrosine cross-links, (b) Schiff-base coupling of oxidation-derived protein carbonyl groups with the ε-amino groups of lysine residues, and (c) intermolecular

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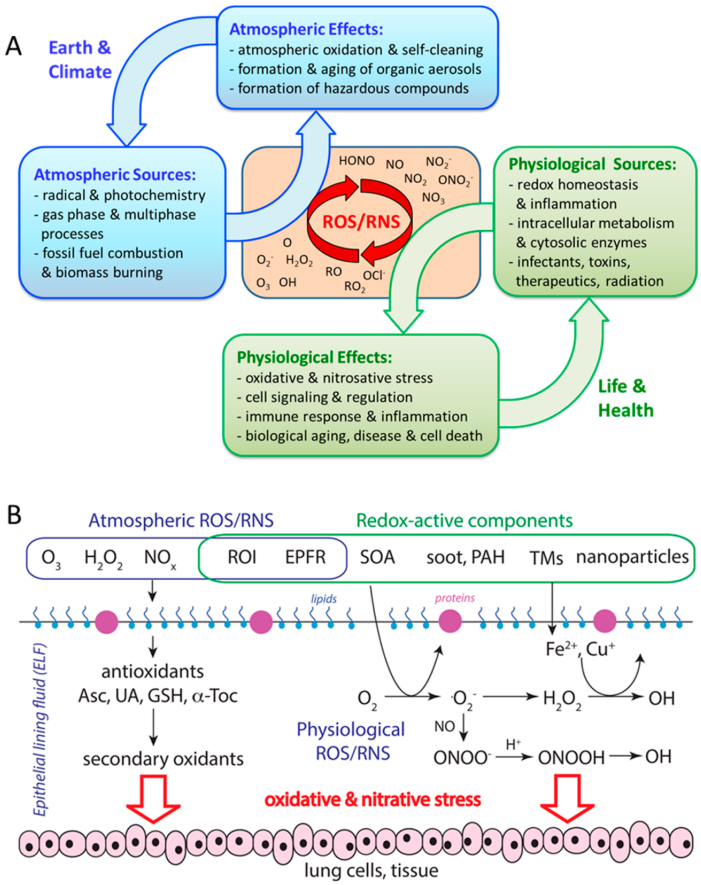

Figure 5. (A) Sources, effects, and interactions at the interface of atmospheric and physiological chemistry with feedback loops involving Earth System, climate, life, and health. (B) Interactions of atmospheric and physiological ROS/RNS with antioxidants (ascorbate, uric acid, reduced glutathione, α-tocopherol) in the epithelial lining fluid (ELF) of the human respiratory tract. Redox-active components, including reactive oxygen intermediates (ROI), soot, quinones and transition metals can induce ROS formation in vivo, leading to oxidative stress and biological aging. Adapted from ref 38. Copyright 2015 American Chemical Society.

disulfide coupling.[381] Recently, protein cross-linking and oligomerization upon exposure to atmospherically relevant concentrations of O_3 have been shown to proceed via the formation dityrosine cross-links as outlined in Figure 4, yielding up to ~10% of dimers, trimers, and higher oligomers of a model protein within minutes to hours of exposure under summer smog conditions.[368] Similar reaction mechanisms involving reactive oxygen intermediates may also be responsible for the protein cross-linking observed upon reaction with physiological and synthetic nitrating agents like $ONOO^-$ and tetranitromethane, respectively.[306,313,382,383] Cross-linking upon reaction with tetranitromethane was suggested to alter the immunogenicity and enhance the allergenicity of Bet v 1 through decreased endolysosomal degradation leading to extended MHC class II antigen presentation.[306] On the other hand, oligomerization of allergens induced by modification with glutaraldehyde, that is, formation of glutaraldehyde bridges between nucleophilic amino acid residues (in particular lysine), was suggested to reduce immunogenicity and allergenicity due to delayed allergen uptake and presentation by dendritic cells.[384,385]

As illustrated in Figure S2, the processes of allergic sensitization and response involve a wide range of interactions between protein molecules dissolved in liquids (blood, lymph, etc.) and embedded in semisolid structures (membranes, cells, tissues), which can be regarded as protein multiphase chemistry.[38] Protein reactions with ROS/RNS are generally pH-dependent and yield a mixture of hydroxylated, nitrated, cross-linked, aggregated or degraded products.[386−391] To assess immune responses to specific posttranslational modifications of proteins, it is necessary to carefully characterize the investigated samples and avoid artifacts or misinterpretations that might arise from interferences between different reaction products and pathways, for example, nitration vs dimerization or oligomerization of proteins exposed to oxidizing and nitrating agents (Figure 4).

4. EPITHELIAL SURFACE INTERACTIONS

The deposition of particles in the respiratory tract is size-dependent and deposited particles are removed by a number of physical, chemical, and biological clearance processes, including

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mucociliary movement, endo- and phagocytosis, dissolution, leaching, and protein binding.[201] Thus, the first step of an inhaled allergen-carrying particle is evading the mechanical defenses of the respiratory tract and passing, for example, alveolar macrophages, which prevent inappropriate immune activation by removing inhaled allergens via phagocytosis.[392−394] The epithelial surface is a protective barrier, which protects the underlying tissue from many inhaled substances. The epithelial cells are covered by a viscous mucosal lining rich in immune cells and soluble components, such as antioxidants, complement proteins, and surfactant proteins.[201,395,396] As the epithelium is more than a passive protective barrier, it recruits and activates more specialized immune cells and promotes inflammatory responses,[397] allergy is also discussed to be an epithelial barrier disease.[15,131,398−400] For example, nasal epithelium is clearly different between healthy and allergic subjects and only in allergic subjects the transport of Bet v 1 is caveolar-mediated.[401]

Air pollutants interacting with epithelial surfaces can act as adjuvants promoting pro-allergic innate and adaptive immune reactions as outlined in Table 2 and section S1. For example, they can induce inflammation and disrupt epithelial barriers, facilitating the access of allergens to immunogenic effector cells.[8,86] In particular, air particulate matter can trigger ROS production through Fenton-like reactions and the activation of macrophages, mitochondria and enzymes related to the oxidant/antioxidant balance (e.g., NADPH oxidase, glutathione peroxidase).[309,310,402−405] Additionally, pollution-derived ROS can induce proinflammatory responses by the production of damage associated molecular patterns (DAMPs oxidized phospholipids, hyaluronic acid, etc.) and trigger immune reactions leading to acute or chronic inflammation,[29,406] for example, through feedback cycles involving Toll-like receptors (TLR) and other pattern recognition receptors (PRR) (Figure S5).[407] Ozone and particulate matter can prime the airways for pro-allergic responses, and TLR signaling plays an important role in pollutant-induced inflammation.[408,409] During inflammation, inducible nitric oxide synthase (iNOS) that is mainly expressed in innate immune cells (monocytes, macrophages, dendritic cells) provides high amounts of nitrogen oxide (NO), which can react with superoxide radicals to form peroxynitrite ($ONOO^-$), a central endogenous nitrating agent for proteins.[301] In addition, particulate and gaseous pollutants may also drive pro-allergic inflammation through the generation of oxidative stress involving elevated levels of $ONOO^-$.[410]

As illustrated in Figure 5A, epithelial surfaces are interfaces coupling the atmospheric and the physiological production, cycling, and effects of ROS/RNS.[38] Specific interactions of atmospheric ROS/RNS with antioxidants in the epithelial lining fluid are shown in Figure 5B. An increase of ozone from typical background concentration levels (∼30 ppb) to summer smog conditions (>100 ppb) reduces the chemical half-life of antioxidants from days to hours,[309] which may be comparable or shorter than the physiological replenishment rates.[411] Furthermore, the adjuvant effect of ambient ultrafine particles was correlated with their oxidant potential.[412] Major contributors to the redox properties of ambient particles are transition metals, polycyclic aromatic hydrocarbons, and derivatives (PAH, nitro/oxy-PAH), and semiquinones.[38,312,412−415] In addition, the deposition of acidic particles may reduce the pH of the epithelial lining fluid (ELF). For healthy people the mean pH is ∼7.4, while in people with diseases (e.g., asthma, acid reflux) it can be as low as ∼4.[416,417] Oxidant exposure and changes of pH can alter reaction pathways of antioxidants[418] and also decrease the activities of antioxidant-related enzymes in the ELF, which are also reduced in smokers and people suffering from lung diseases.[419−421]

Recent studies yielded chemical exposure-response relations between ambient concentrations of air pollutants and the production rates and concentrations of ROS in the ELF of the human respiratory tract.[309] As illustrated in Figure 6, the total

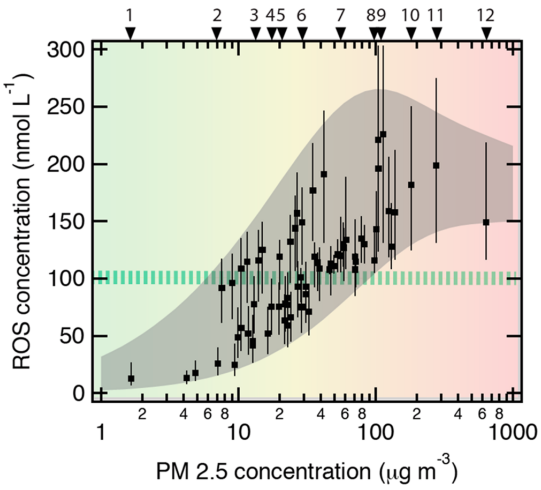

Figure 6. Chemical exposure-response relations between ambient concentrations of fine particulate matter (PM2.5) and the concentration of reactive oxygen species (ROS) in the epithelial lining fluid (ELF) of the human respiratory tract. The green-striped horizontal bar indicates the ROS level characteristic for healthy humans (∼100 nmol L^{-1}). The gray envelope represents the range of aerosol-induced ROS concentrations obtained with approximate upper and lower limit mass fractions of redox-active components observed in ambient PM2.5. The data points represent various geographic locations for which measured or estimated mass fractions are available, including (1) Amazon, Brazil (pristine rainforest air); (2) Edinburgh, UK; (3) Toronto, Canada; (4) Tokyo, Japan; (5) Budapest, Hungary; (6) Hong Kong, China; (7) Milan, Italy; (8) Guangzhou, China; (9) Pune, India; (10) Beijing, China; (11) New Delhi, India; (12) Sumatra, Indonesia (biomass burning/peat fire smoke). Adapted from Lakey, S. J. P.; Berkemeier, T.; Tong, H.; Arangio, A. M.; Lucas, K.; Pöschl, U.; Shiraiwa, M. Chemical exposure-response relationship between air pollutants and reactive oxygen species in the human respiratory tract. *Sci. Rep.* **2016**, *6*, 32916. DOI: 10.1038/srep32916.[309] Copyright 2016 Lakey et al.

concentration of ROS generated by redox-active substances contained in fine particulate matter (PM2.5) deposited in the ELF ranges from ∼10 nmol L^{-1} under clean conditions up to almost ∼250 nmol L^{-1} under highly polluted conditions. Thus, the inhalation of PM2.5 can increase ROS concentrations in the ELF to levels that exceed physiological background levels (50−200 nmol L^{-1}) and are characteristic for respiratory diseases.[309,422] In addition to the effects of PM2.5, ambient ozone readily saturates the ELF and can enhance oxidative stress by depleting antioxidants and surfactants.[309] Ozone also reacts with skin lipids (e.g., squalene) and generates organic compounds (e.g., mono- and dicarbonyls) that can act as irritants.[269] These and related organic compounds were found to act as adjuvants in the development of respiratory allergies as well as atopic dermatitis.[270,271,423,424] Some air pollutants and

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chemical reaction products formed at epithelial interfaces are sufficiently long-lived and mobile to diffuse through membranes and interact with the neural, cardiovascular, and immune system networks of the human body.[314,425−429] Through these and related physiological interactions involving DAMPs, inflammatory mediators, cytokines, leukocytes etc., oxidative stress and inflammation caused by air pollutants may propagate from the respiratory tract and skin to other parts of the human organism and exert systemic influence on the development of allergies, reaching also the gastrointestinal tract.[38,429]

A wide variety of commensal, symbiotic, and pathogenic microorganisms are found on the epithelial surfaces of the human body, such as the skin, lungs, and the gastrointestinal tract. Recent research suggests that the human microbiome is important to maintain physiological functions and to induce immune regulation by balancing the activities of Th1 and Th2 cells.[430−433] Normal microbial colonization in early life can promote tolerance to aeroallergens via induced regulatory T cells.[434] The development and composition of the human microbiome are influenced by many factors such as diet, infections, medical treatment, and also environmental factors.[435] For example, air pollutants and climatic stress factors may disturb microbial communities through oxidative stress, inflammation, and changes in environmental biodiversity.[4,36] Modifications in the composition of the gastrointestinal and lung microbiome can in turn affect the development of allergies in accordance with the "hygiene hypothesis"[36,436−440] and may also promote pathogenic species that can contribute to these diseases.[4,441−443] Recent studies revealed differences in the structure and composition of microbiota in the lower airways of healthy and asthmatic people: *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* are the most common phyla found in airways of healthy subjects, whereas increased concentrations of pathogenic Proteobacteria, such as *Haemophilus*, *Moraxella*, and *Neisseria* spp., were found in asthma patients.[442,443] Moreover, viral infections can exacerbate allergies.[31] It is still unclear, however, if these changes are a cause or a consequence of the disease. Moreover, it has been suggested that air pollutants, especially air particulate matter, ingested together with food can trigger and accelerate the development of gastrointestinal inflammatory diseases by altering the gastrointestinal microbiome and immune functions.[444] Besides the human microbiome, also microbes associated with allergenic pollen (pollen microbiome) and other aeroallergens may act as adjuvants when deposited on epithelial surfaces.[235,445]

5. CONCLUSIONS AND OUTLOOK

As the globally pervasive anthropogenic influence continues to shape planet Earth and the human environment in the Anthropocene, it becomes increasingly important to understand and assess the potential effects of environmental change on human health. The widespread increase of allergies and their complex dependence on multiple influencing factors, including environmental pollution, indicate that allergic diseases are a major challenge with regard to maintaining and improving public health.

Anthropogenic emissions of atmospheric trace substances are affecting air quality and climate on local, regional, and global scales. Changes in atmospheric aerosol composition, oxidant concentrations, and climate parameters can induce chemical modifications of allergens, increase oxidative stress in the human body, and skew the immune system toward allergic reactions. In particular, air pollutants can act as adjuvants and alter the immunogenicity of allergenic proteins, while climate change affects the abundance and properties of bioaerosols as carriers of aeroallergens. The production, release and properties of allergens and adjuvants are subject to various human interferences with the biosphere and climate system, including air pollutant interactions with natural and agricultural vegetation, fertilization and land-use change, as well as plant breeding and genetic engineering.

The following key questions remain to be resolved to understand and mitigate potential effects of air pollution and climate change on the observed increase and future development of allergies:

(Q1) Which air pollutant and climate change effects have the largest potential to influence on the abundance and potency of allergens and adjuvants in the human environment (indoor and outdoor)?

(Q2) Which elements and reaction pathways of the immune system are particularly susceptible to disturbance by air pollutants, and what are the most relevant chemical and physiological mechanisms (adjuvant activity vs allergen modification)?

(Q3) Which environmental and physiological parameters are needed and best suited to account for and assess air pollutant and climate change effects in epidemiological studies of allergic diseases (attribution and prediction of environmental risk factors)?

(Q4) How important are air pollutant and climate change effects relative to other environmental, lifestyle, genetic and epigenetic risk factors for allergic diseases?

Recommendations on how to address these key questions in future research are listed in Table S2, building on and extending suggestions given in related review and perspective articles (e.g., refs 8, 12, 93, and 280). Beyond addressing the above questions, it appears worthwhile to explore which components of the immune system could be modulated to prevent adverse effects of air pollution, for example, whether therapeutic monoclonal antibodies against relevant cytokines (e.g., IL-4, IL-5, IL-13) or IgE antibodies could make a difference. Further information about ongoing efforts and future perspectives of mitigating the health effects of climate change and air pollution is available from various national and international government agencies, medical institutions and related organizations (e.g., refs 4, 37, and 446). For efficient scientific progress, it will be important to combine and optimize state-of-the-art methods and results of environmental, immunological and epidemiological studies, tightly coupling physical, chemical, biological, and medical techniques and knowledge. One of the challenges consists in identifying and quantifying the mechanisms and feedback loops of immunochemical reactions in response to environmental influencing factors, including chemical modifications and interactions of allergens and adjuvants under realistic environmental and physiological conditions. For this purpose, the results of laboratory experiments and monitoring networks with improved detection methods for allergens, adjuvants and reactive intermediates should be used to design and inform epidemiological studies targeting the effects of different types and combinations of air pollutants and climate parameters.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b04908.

Allergic sensitization and response, air pollution effects on the allergenic potential of plant pollen and fungal spores, research activities proposed to address the key questions, essential steps and influencing factors in the development of IgE-mediated allergies, simplified scheme of major processes involved in allergic sensitization and response, scanning electron micrographs of oak and birch pollen, 3D-structure of the major birch pollen allergen Bet v 1.0101, and TLR4-radical cycle of inflammation (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*Tel: +49 6131 305 7007. E-mail: k.selzle@mpic.de.
*Tel: +49 6131 392 2417. E-mail: kampfc@uni-mainz.de.
*Tel: +49 6131 305 7001. E-mail: u.poschl@mpic.de.

ORCID

Kathrin Reinmuth-Selzle: 0000-0001-7274-3848
Senchao Lai: 0000-0002-4990-3679
Albert Duschl: 0000-0002-7034-9860

Author Contributions

○K.R.-S. and C.J.K. contributed equally.

Notes

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■ REFERENCES

(1) Johansson, S. G. O.; Bieber, T.; Dahl, R. Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. *J. Allergy Clin. Immunol.* **2004**, *113* (5), 832−836.
(2) Tanno, L. K.; Calderon, M. A.; Smith, H. E.; Sanchez-Borges, M.; Sheikh, A.; Demoly, P. Dissemination of definitions and concepts of allergic and hypersensitivity conditions. *World Allergy Organ. J.* **2016**, *9* (1), 24.
(3) Galli, S. J.; Tsai, M.; Piliponsky, A. M. The development of allergic inflammation. *Nature* **2008**, *454* (7203), 445−454.
(4) Adkinson, N. F., Jr.; Bochner, B. S.; Burks, A. W.; Busse, W. W.; Holgate, S. T.; Lemanske, R. F., Jr.; O'Hehir, R. E. *Middleton's Allergy Principles and Practice*, 8th ed.; Elsevier: 2014; Vol. *1 and 2*, pp 1896.
(5) Huby, R. D. J.; Dearman, R. J.; Kimber, I. Why are some proteins allergens? *Toxicol. Sci.* **2000**, *55* (2), 235−246.
(6) Palm, N. W.; Rosenstein, R. K.; Medzhitov, R. Allergic host defences. *Nature* **2012**, *484* (7395), 465−472.
(7) Shakib, F.; Ghaemmaghami, A. M.; Sewell, H. F. The molecular basis of allergenicity. *Trends Immunol.* **2008**, *29* (12), 633−642.
(8) Traidl-Hoffmann, C.; Jakob, T.; Behrendt, H. Determinants of allergenicity. *J. Allergy Clin. Immunol.* **2009**, *123* (3), 558−566.
(9) Ring, J.; Eberlein-Koenig, B.; Behrendt, H. Environmental pollution and allergy. *Ann. Allergy, Asthma, Immunol.* **2001**, *87* (6), 2−6.
(10) Pawankar, R.; Baena-Cagnani, C.; Bousquet, J.; Walter Canonica, G.; Cruz, A.; Kaliner, M.; Lanier, B.; Henley, K. State of World Allergy Report 2008: Allergy and Chronic Respiratory Diseases. *World Allergy Organ. J.* **2008**, *1* (Suppl 1), S4−S17.
(11) Langen, U.; Schmitz, R.; Steppuhn, H. Prevalence of allergic diseases in Germany. Results of the German Health Interview and Examination Survey for Adults (DEGS1). *Bundesgesundheitsblatt-Gesundheitsforschung-Gesundheitsschutz* **2013**, *56* (5−6), 698−706.
(12) D'Amato, G.; Holgate, S. T.; Pawankar, R. Meteorological conditions, climate change, new emerging factors, and asthma and related allergic disorders. A statement of the World Allergy Organization. *World Allergy Organ. J.* **2015**, *8*, 25.
(13) Graham-Rowe, D. Lifestyle: When allergies go west. *Nature* **2011**, *479* (7374), S2−S4.
(14) Valenta, R.; Hochwallner, H.; Linhart, B.; Pahr, S. Food Allergies: The Basics. *Gastroenterology* **2015**, *148* (6), 1120−1131.
(15) Papazian, D.; Hansen, S.; Wurtzen, P. A. Airway responses towards allergens - from the airway epithelium to T cells. *Clin. Exp. Allergy* **2015**, *45* (8), 1268−1287.
(16) Lambrecht, B. N.; Hammad, H. The airway epithelium in asthma. *Nat. Med.* **2012**, *18* (5), 684−692.
(17) Evans, H.; Mitre, E. Worms as therapeutic agents for allergy and asthma: Understanding why benefits in animal studies have not translated into clinical success. *J. Allergy Clin. Immunol.* **2015**, *135* (2), 343−353.
(18) Ring, J.; Kramer, U.; Schafer, T.; Behrendt, H. Why are allergies increasing? *Curr. Opin. Immunol.* **2001**, *13* (6), 701−708.
(19) Heinrich, J.; Popescu, M. A.; Wjst, M.; Goldstein, I. F.; Wichmann, H. E. Atopy in children and parental social class. *Am. J. Public Health* **1998**, *88* (9), 1319−1324.
(20) Larrick, J. W.; Buckley, C. E., III; Machamer, C. E.; Schlagel, G. D.; Yost, J. A.; Blessingmoore, J.; Levy, D. Does hyperimmunoglobulinemia-E protect tropical populations from allergic disease? *J. Allergy Clin. Immunol.* **1983**, *71* (2), 184−188.
(21) Olesen, A. B.; Juul, S.; Birkebaek, N.; Thestrup-Pedersen, K. Association between atopic dermatitis and insulin-dependent diabetes mellitus: a case-control study. *Lancet* **2001**, *357* (9270), 1749−1752.
(22) Coca, A. F.; Cooke, R. A. On the classification of the phenomena of hypersensitiveness. *J. Immunol.* **1923**, *8* (3), 163−182.
(23) Holt, P. G.; Thomas, W. R. Sensitization to airborne environmental allergens: unresolved issues. *Nat. Immunol.* **2005**, *6* (10), 957−960.
(24) Bégin, P.; Nadeau, K. C. Epigenetic regulation of asthma and allergic disease. *Allergy, Asthma, Clin. Immunol.* **2014**, *10* (1), 27.
(25) Ring, J.; Akdis, C.; Lauener, R.; et al. Global Allergy Forum and Second Davos Declaration 2013 Allergy: Barriers to cure - challenges and actions to be taken. *Allergy* **2014**, *69* (8), 978−982.
(26) Portelli, M. A.; Hodge, E.; Sayers, I. Genetic risk factors for the development of allergic disease identified by genome-wide association. *Clin. Exp. Allergy* **2015**, *45* (1), 21−31.
(27) Kramer, U.; Koch, T.; Ranft, U.; Ring, J.; Behrendt, H. Traffic-related air pollution is associated with atopy in children living in urban areas. *Epidemiology (Cambridge, Mass.)* **2000**, *11* (1), 64−70.

DOI: 10.1021/acs.est.6b04908
Environ. Sci. Technol. 2017, 51, 4119−4141

(28) Martino, D. J.; Prescott, S. L. Progress in understanding the epigenetic basis for immune development, immune function, and the rising incidence of allergic disease. *Curr. Allergy Asthma Rep.* **2013**, *13* (1), 85−92.
(29) Peden, D. B. Does air pollution really cause allergy? *Clin. Exp. Allergy* **2015**, *45* (1), 3−5.
(30) Miller, R. L.; Peden, D. B. Environmental Impacts on Immune Responses in Atopy and Asthma. *J. Allergy Clin. Immunol.* **2014**, *134* (5), 1001−1008.
(31) Gaffin, J. M.; Kanchongkittiphon, W.; Phipatanakul, W. Perinatal and early childhood environmental factors influencing allergic asthma immunopathogenesis. *Int. Immunopharmacol.* **2014**, *22* (1), 21−30.
(32) Wahn, U. What drives the allergic march? *Allergy* **2000**, *55* (7), 591−599.
(33) Krämer, U.; Behrendt, H.; Dolgner, R.; Ranft, U.; Ring, J.; Willer, H.; Schlipkoter, H. W. Airway diseases and allergies in East and West German children during the first 5 years after reunification: time trends and the impact of sulphur dioxide and total suspended particles. *Int. J. Epidemiol.* **1999**, *28* (5), 865−873.
(34) Castro-Rodriguez, J. A.; Forno, E.; Rodriguez-Martinez, C. E.; Celedon, J. C. Risk and Protective Factors for Childhood Asthma: What Is the Evidence? *J. Allergy Clin. Immunol.-Pract.* **2016**, *4* (6), 1111−1122.
(35) Bernstein, J. A.; Alexis, N.; Barnes, C.; et al. Health effects of air pollution. *J. Allergy Clin. Immunol.* **2004**, *114* (5), 1116−1123.
(36) Kim, B.-J.; Lee, S.-Y.; Kim, H.-B.; Lee, E.; Hong, S.-J. Environmental Changes, Microbiota, and Allergic Diseases. *Allergy, Asthma Immunol. Res.* **2014**, *6* (5), 389−400.
(37) Brunekreef, B.; Holgate, S. T. Air pollution and health. *Lancet* **2002**, *360* (9341), 1233−1242.
(38) Pöschl, U.; Shiraiwa, M. Multiphase Chemistry at the Atmosphere−Biosphere Interface Influencing Climate and Public Health in the Anthropocene. *Chem. Rev.* **2015**, *115* (10), 4440−4475.
(39) Crutzen, P. J. Geology of mankind. *Nature* **2002**, *415* (6867), 23−23.
(40) Steffen, W.; Crutzen, P. J.; McNeill, J. R. The Anthropocene: Are humans now overwhelming the great forces of nature. *Ambio* **2007**, *36* (8), 614−621.
(41) Zalasiewicz, J.; Williams, M.; Steffen, W.; Crutzen, P. The New World of the Anthropocene. *Environ. Sci. Technol.* **2010**, *44* (7), 2228−2231.
(42) Zalasiewicz, J.; Crutzen, P. J.; Steffen, W., The Anthropocene. In *Geologic Time Scale 2012, Vols 1 & 2*, Gradstein, F. M., Ogg, J. G., Mark Schmitz, M., Ogg, G., Eds.; Elsevier, 2012; 1033−1040.
(43) Steffen, W.; Grinevald, J.; Crutzen, P.; McNeill, J. The Anthropocene: conceptual and historical perspectives. *Philos. Trans. R. Soc., A* **2011**, *369* (1938), 842−867.
(44) Crutzen, P. J. Anthropocene man. *Nature* **2010**, *467* (7317), S10.
(45) Foley, S. F.; Gronenborn, D.; Andreae, M. O.; et al. The Palaeoanthropocene−The beginnings of anthropogenic environmental change. *Anthropocene* **2013**, *3*, 83−88.
(46) Lewis, S. L.; Maslin, M. A. Defining the Anthropocene. *Nature* **2015**, *519* (7542), 171−180.
(47) Waters, C. N.; Zalasiewicz, J.; Summerhayes, C. The Anthropocene is functionally and stratigraphically distinct from the Holocene. *Science* **2016**, *351* (6269), aad2622.
(48) Canfield, D. E.; Glazer, A. N.; Falkowski, P. G. The Evolution and Future of Earth's Nitrogen Cycle. *Science* **2010**, *330* (6001), 192−196.
(49) Heald, C. L.; Spracklen, D. V. Land Use Change Impacts on Air Quality and Climate. *Chem. Rev.* **2015**, *115* (10), 4476−4496.
(50) Crutzen, P. J.; Stoermer, E. F. The "Anthropocene". *Global Change Newsletter* **2000**, *41*, 17.
(51) Crutzen, P. J. The effects of industrial and agricultural practices on atmospheric chemistry and climate during the anthropocene. *J. Environ. Sci. Health, Part A: Toxic/Hazard. Subst. Environ. Eng.* **2002**, *37* (4), 423−424.
(52) Crutzen, P. J. Atmospheric Chemistry in the "Anthropocene". In *Challenges of a Changing Earth*, Proceedings of the Global Change Open Science Conference, Amsterdam, The Netherlands, 10−13 July 2001; Steffen, W.; Jäger, J.; Carson, D. J.; Bradshaw, C., Eds.; Springer Berlin Heidelberg: Berlin, Heidelberg, 2002; 45−48.
(53) Williams, J.; Crutzen, P. J. Perspectives on our planet in the Anthropocene. *Environ. Chem.* **2013**, *10* (4), 269−280.
(54) Suni, T.; Guenther, A.; Hansson, H. C.; et al. The significance of land-atmosphere interactions in the Earth system—iLEAPS achievements and perspectives. *Anthropocene* **2015**, *12*, 69−84.
(55) Schäfer, S.; Stelzer, H.; Maas, A.; Lawrence, M. G. Earth's future in the Anthropocene: Technological interventions between piecemeal and utopian social engineering. *Earth's Future* **2014**, *2* (4), 239−243.
(56) Brondizio, E. S.; O'Brien, K.; Bai, X.; et al. Re-conceptualizing the Anthropocene: A call for collaboration. *Glob. Environ. Change* **2016**, *39*, 318−327.
(57) Lawrence, M. G.; Crutzen, P. J. Was breaking the taboo on research on climate engineering via albedo modification a moral hazard or a moral imperative? *Earth's Future* **2017**, *5*, 136.
(58) Zalasiewicz, J.; Waters, C. N.; Williams, M.; et al. When did the Anthropocene begin? A mid-twentieth century boundary level is stratigraphically optimal. *Quat. Int.* **2015**, *383*, 196−203.
(59) Waters, C. N.; Zalasiewicz, J. A.; Williams, M.; Ellis, M. A.; Snelling, A. M. A stratigraphical basis for the Anthropocene? *Geol. Soc. Spec. Publ.* **2014**, *395*, 1−21.
(60) Zalasiewicz, J.; Williams, M.; Waters, C. N., Can an Anthropocene Series be defined and recognized? In *Stratigraphical Basis for the Anthropocene*; Waters, C. N., Zalasiewicz, J. A., Williams, M., Ellis, M., Snelling, A. M., Eds.; Geological Society: London, 2014; Vol. *395*, 39−53.
(61) Zalasiewicz, J.; Williams, M.; Haywood, A.; Ellis, M. The Anthropocene: a new epoch of geological time? *Philos. Trans. R. Soc., A* **2011**, *369* (1938), 835−841.
(62) Zalasiewicz, J.; Williams, M.; Steffen, W.; Crutzen, P. Response to "The Anthropocene forces us to reconsider adaptationist models of human-environment interactions". *Environ. Sci. Technol.* **2010**, *44* (16), 6008−6008.
(63) Steffen, W.; Leinfelder, R.; Zalasiewicz, J.; et al. Stratigraphic and Earth System approaches to defining the Anthropocene. *Earth's Future* **2016**, *4* (8), 324−345.
(64) Williams, M.; Zalasiewicz, J.; Waters, C. N.; et al. The Anthropocene: a conspicuous stratigraphical signal of anthropogenic changes in production and consumption across the biosphere. *Earth's Future* **2016**, *4* (3), 34−53.
(65) Cooper, O. R.; Parrish, D. D.; Ziemke, J. Global distribution and trends of tropospheric ozone: An observation-based review. *Elementa-Science of the Anthropocene* **2014**, *2*, No. 000029.
(66) Monks, P. S.; Archibald, A. T.; Colette, A.; et al. Tropospheric ozone and its precursors from the urban to the global scale from air quality to short-lived climate forcer. *Atmos. Chem. Phys.* **2015**, *15* (15), 8889−8973.
(67) Monks, P. S.; Granier, C.; Fuzzi, S.; et al. Atmospheric composition change - global and regional air quality. *Atmos. Environ.* **2009**, *43* (33), 5268−5350.
(68) Pusede, S. E.; Steiner, A. L.; Cohen, R. C. Temperature and Recent Trends in the Chemistry of Continental Surface Ozone. *Chem. Rev.* **2015**, *115* (10), 3898−3918.
(69) Andreae, M. O. Aerosols before pollution. *Science* **2007**, *315* (5808), 50−51.
(70) Seinfeld, J. H.; Pandis, S. N. *Atmospheric Chemistry and Physics: From Air Pollution to Climate Change*, 3rd ed.; John Wiley & Sons, 2016; pp 1152.
(71) Fishman, J.; Creilson, J. K.; Parker, P. A.; Ainsworth, E. A.; Vining, G. G.; Szarka, J.; Booker, F. L.; Xu, X. An investigation of widespread ozone damage to the soybean crop in the upper Midwest determined from ground-based and satellite measurements. *Atmos. Environ.* **2010**, *44* (18), 2248−2256.

DOI: 10.1021/acs.est.6b04908
Environ. Sci. Technol. 2017, 51, 4119−4141

(72) Lelieveld, J.; Evans, J. S.; Fnais, M.; Giannadaki, D.; Pozzer, A. The contribution of outdoor air pollution sources to premature mortality on a global scale. *Nature* **2015**, *525* (7569), 367−371.
(73) Lelieveld, J.; Barlas, C.; Giannadaki, D.; Pozzer, A. Model calculated global, regional and megacity premature mortality due to air pollution. *Atmos. Chem. Phys.* **2013**, *13* (14), 7023−7037.
(74) Brauer, M.; Freedman, G.; Frostad, J.; et al. Ambient Air Pollution Exposure Estimation for the Global Burden of Disease 2013. *Environ. Sci. Technol.* **2016**, *50* (1), 79−88.
(75) Brauer, M.; Amann, M.; Burnett, R. T.; et al. Exposure Assessment for Estimation of the Global Burden of Disease Attributable to Outdoor Air Pollution. *Environ. Sci. Technol.* **2012**, *46* (2), 652−660.
(76) West, J. J.; Cohen, A.; Dentener, F.; et al. What We Breathe Impacts Our Health: Improving Understanding of the Link between Air Pollution and Health. *Environ. Sci. Technol.* **2016**, *50* (10), 4895−4904.
(77) Correia, A. W.; Pope, C. A., 3rd; Dockery, D. W.; Wang, Y.; Ezzati, M.; Dominici, F. Effect of air pollution control on life expectancy in the United States: an analysis of 545 U.S. counties for the period from 2000 to 2007. *Epidemiology (Cambridge, Mass.)* **2013**, *24* (1), 23−31.
(78) Pope, C. A., 3rd; Dockery, D. W. Health effects of fine particulate air pollution: lines that connect. *J. Air Waste Manage. Assoc.* **2006**, *56* (6), 709−742.
(79) Dockery, D. W.; Pope, C. A., 3rd; Xu, X.; Spengler, J. D.; Ware, J. H.; Fay, M. E.; Ferris, B. G., Jr.; Speizer, F. E. An association between air pollution and mortality in six U.S. cities. *N. Engl. J. Med.* **1993**, *329* (24), 1753−1759.
(80) Gao, M.; Guttikunda, S. K.; Carmichael, G. R.; Wang, Y. S.; Liu, Z. R.; Stanier, C. O.; Saide, P. E.; Yu, M. Health impacts and economic losses assessment of the 2013 severe haze event in Beijing area. *Sci. Total Environ.* **2015**, *511*, 553−561.
(81) Whitmee, S.; Haines, A.; Beyrer, C.; et al. Safeguarding human health in the Anthropocene epoch: report of The Rockefeller Foundation−Lancet Commission on planetary health. *Lancet* **2015**, *386* (10007), 1973−2028.
(82) Brunekreef, B.; Sunyer, J. Asthma, rhinitis and air pollution: is traffic to blame? *Eur. Respir. J.* **2003**, *21* (6), 913−915.
(83) Breiteneder, H.; Mills, E. N. C. Plant food allergens - structural and functional aspects of allergenicity. *Biotechnol. Adv.* **2005**, *23* (6), 395−399.
(84) Scheurer, S.; Toda, M.; Vieths, S. What makes an allergen? *Clin. Exp. Allergy* **2015**, *45*, 1150−1161.
(85) Thomas, W. R.; Hales, B. J.; Smith, W.-A. Structural biology of allergens. *Curr. Allergy Asthma Rep.* **2005**, *5* (5), 388−393.
(86) Saxon, A.; Diaz-Sanchez, D. Air pollution and allergy: you are what you breathe. *Nat. Immunol.* **2005**, *6* (3), 223−226.
(87) Kim, K. H.; Jahan, S. A.; Kabir, E. A review on human health perspective of air pollution with respect to allergies and asthma. *Environ. Int.* **2013**, *59*, 41−52.
(88) Bartra, J.; Mullol, J.; del Cuvillo, A.; Davila, I.; Ferrer, M.; Jauregui, I.; Montoro, J.; Sastre, J.; Valero, A. Air pollution and allergens. *J. Invest. Allergol. Clin. Immunol.* **2007**, *17*, 3−8.
(89) Blando, J.; Bielory, L.; Nguyen, V.; Diaz, R.; Jeng, H. A. Anthropogenic Climate Change and Allergic Diseases. *Atmosphere* **2012**, *3* (1), 200−212.
(90) D'Amato, G.; Baena-Cagnani, C. E.; Cecchi, L.; et al. Climate change, air pollution and extreme events leading to increasing prevalence of allergic respiratory diseases. *Multidscip. Respir. Med.* **2013**, *8*, 12.
(91) Shea, K. M.; Truckner, R. T.; Weber, R. W.; Peden, D. B. Climate change and allergic disease. *J. Allergy Clin. Immunol.* **2008**, *122* (3), 443−453.
(92) Beggs, P. J.; Bambrick, H. J. Is the global rise of asthma an early impact of anthropogenic climate change? *Environ. Health Perspect.* **2005**, *113* (8), 915−919.
(93) Reid, C. E.; Gamble, J. L. Aeroallergens, Allergic Disease, and Climate Change: Impacts and Adaptation. *EcoHealth* **2009**, *6* (3), 458−470.
(94) Shiraiwa, M.; Selzle, K.; Pöschl, U. Hazardous components and health effects of atmospheric aerosol particles: reactive oxygen species, soot, polycyclic aromatic compounds and allergenic proteins. *Free Radical Res.* **2012**, *46* (8), 927−939.
(95) Frank, U.; Ernst, D. Effects of NO_2 and ozone on pollen allergenicity. *Front. Plant Sci.* **2016**, *7*, 91 DOI: 10.3389/fpls.2016.00091.
(96) D'Amato, G.; Vitale, C.; De Martino, A.; et al. Effects on asthma and respiratory allergy of Climate change and air pollution. *Multidiscip. Respir. Med.* **2015**, *10*, 39−39.
(97) D'Amato, M.; Vitale, C.; Stanziola, A.; Molino, A.; Vatrella, A.; D'Amato, G. Update on Effects of Climate Changes on Respiratory Allergy. *Allergy, Asthma & Immunophysiology: From Genes to Clinical Management*, New York, NY, April 26−29, 2014; pp 45−52.
(98) Ziska, L. H.; Beggs, P. J. Anthropogenic climate change and allergen exposure: The role of plant biology. *J. Allergy Clin. Immunol.* **2012**, *129* (1), 27−32.
(99) Tibbetts, J. H. Air Quality and Climate Change: A Delicate Balance. *Environ. Health Perspect.* **2015**, *123* (6), A148−A153.
(100) Schiavoni, G.; D'Amato, G.; Afferni, C. The dangerous liaison between pollens and pollution in respiratory allergy. *Ann. Allergy, Asthma, Immunol.* **2017**, *118* (3), 269−275.
(101) Jenerowicz, D.; Silny, W.; Danczak-Pazdrowska, A.; Polanska, A.; Osmola-Mankowska, A.; Olek-Hrab, K. Environmental factors and allergic diseases. *Ann. Agric. Environ. Med.* **2012**, *19* (3), 475−481.
(102) Coombs, R. R. A.; Gell, P. G. H., The classification of allergic reactions underlying disease. In *Clinical Aspects of Immunology*; Gell PGH, C. R. e., Ed.; Blackwell Science, 1963.
(103) Radauer, C.; Bublin, M.; Wagner, S.; Mari, A.; Breiteneder, H. Allergens are distributed into few protein families and possess a restricted number of biochemical functions. *J. Allergy Clin. Immunol.* **2008**, *121* (4), 847−852.
(104) Lambrecht, B. N.; Hammad, H. The immunology of asthma. *Nat. Immunol.* **2015**, *16* (1), 45−56.
(105) Lambrecht, B. N.; Hammad, H. Allergens and the airway epithelium response: Gateway to allergic sensitization. *J. Allergy Clin. Immunol.* **2014**, *134* (3), 499−507.
(106) Iwasaki, A.; Medzhitov, R. Regulation of Adaptive Immunity by the Innate Immune System. *Science* **2010**, *327* (5963), 291−295.
(107) Deifl, S.; Bohle, B. Factors influencing the allergenicity and adjuvanticity of allergens. *Immunotherapy* **2011**, *3* (7), 881−893.
(108) Thomas, W. R. Innate affairs of allergens. *Clin. Exp. Allergy* **2013**, *43* (2), 152−163.
(109) Gómez-Casado, C.; Díaz-Perales, A. Allergen-Associated Immunomodulators: Modifying Allergy Outcome. *Arch. Immunol. Ther. Exp.* **2016**, *64* (5), 339−347.
(110) Neurath, M. F.; Finotto, S.; Glimcher, L. H. The role of Th1/Th2 polarization in mucosal immunity. *Nat. Med.* **2002**, *8* (6), 567−573.
(111) Berin, M. C.; Sampson, H. A. Mucosal immunology of food allergy. *Curr. Biol.* **2013**, *23* (9), R389−R400.
(112) Lombardi, V.; Singh, A. K.; Akbari, O. The Role of Costimulatory Molecules in Allergic Disease and Asthma. *Int. Arch. Allergy Immunol.* **2010**, *151* (3), 179−189.
(113) Wills-Karp, M.; Koehl, J. New insights into the role of the complement pathway in allergy and asthma. *Curr. Allergy Asthma Rep.* **2005**, *5* (5), 362−369.
(114) Zhang, X.; Köhl, J. A complex role for complement in allergic asthma. *Expert Rev. Clin. Immunol.* **2010**, *6* (2), 269−277.
(115) Tangye, S. G.; Ma, C. S.; Brink, R.; Deenick, E. K. The good, the bad and the ugly - T-FH cells in human health and disease. *Nat. Rev. Immunol.* **2013**, *13* (6), 412−426.
(116) Schmudde, I.; Laumonnier, Y.; Köhl, J. Anaphylatoxins coordinate innate and adaptive immune responses in allergic asthma. *Semin. Immunol.* **2013**, *25*, 2−11.

DOI: 10.1021/acs.est.6b04908
Environ. Sci. Technol. 2017, 51, 4119−4141

(117) Pandya, P. H.; Wilkes, D. S. Complement system in lung disease. *Am. J. Respir. Cell Mol. Biol.* **2014**, *51* (4), 467−473.
(118) Khan, M. A.; Assiri, A. M.; Broering, D. C. Complement mediators: key regulators of airway tissue remodeling in asthma. *J. Transl. Med.* **2015**, *13* (1), 272.
(119) Akdis, C. A. Therapies for allergic inflammation: refining strategies to induce tolerance. *Nat. Med.* **2012**, *18* (5), 736−749.
(120) Pellerin, L.; Jenks, J. A.; Begin, P.; Bacchetta, R.; Nadeau, K. C. Regulatory T cells and their roles in immune dysregulation and allergy. *Immunol. Res.* **2014**, *58* (2−3), 358−368.
(121) Garman, S. C.; Wurzburg, B. A.; Tarchevskaya, S. S.; Kinet, J. P.; Jardetzky, T. S. Structure of the Fc fragment of human IgE bound to its high-affinity receptor Fc epsilon RI alpha. *Nature* **2000**, *406* (6793), 259−266.
(122) Posner, R. G.; Savage, P. B.; Peters, A. S.; Macias, A.; DelGado, J.; Zwartz, G.; Sklar, L. A.; Hlavacek, W. S. A quantitative approach for studying IgE−FcεRI aggregation. *Mol. Immunol.* **2002**, *38* (16−18), 1221−1228.
(123) Skoner, D. R. Allergic rhinitis: Definition, epidemiology, detection, and pathophysiology, diagnosis. *J. Allergy Clin. Immunol.* **2001**, *108* (1), S2−S8.
(124) Greiner, A. N.; Hellings, P. W.; Rotiroti, G.; Scadding, G. K. Allergic rhinitis. *Lancet* **2011**, *378* (9809), 2112−2122.
(125) Bianchi, M. E. DAMPs, PAMPs and alarmins: all we need to know about danger. *J. Leukocyte Biol.* **2007**, *81* (1), 1−5.
(126) Thomas, W. R. Allergen Ligands in the Initiation of Allergic Sensitization. *Curr. Allergy Asthma Rep.* **2014**, *14* (5), 10.
(127) Trompette, A.; Divanovic, S.; Visintin, A.; et al. Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* **2009**, *457* (7229), 585−588.
(128) Karp, C. L. Guilt by intimate association: What makes an allergen an allergen? *J. Allergy Clin. Immunol.* **2010**, *125* (5), 955−960.
(129) Zuo, L.; Lucas, K.; Fortuna, C. A.; Chuang, C. C.; Best, T. M. Molecular Regulation of Toll-like Receptors in Asthma and COPD. *Front. Physiol.* **2015**, *6*, 312.
(130) Holgate, S. T. Innate and adaptive immune responses in asthma. *Nat. Med.* **2012**, *18* (5), 673−683.
(131) Holgate, S. The sentinel role of the airway epithelium in asthma pathogenesis. *Immunol. Rev.* **2011**, *242*, 205−219.
(132) Salimi, M.; Barlow, J. L.; Saunders, S. P.; et al. A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *J. Exp. Med.* **2013**, *210* (13), 2939−2950.
(133) Bartemes, K. R.; Kephart, G. M.; Fox, S. J.; Kita, H. Enhanced innate type 2 immune response in peripheral blood from patients with asthma. *J. Allergy Clin. Immunol.* **2014**, *134* (3), 671−678.e4.
(134) Bernink, J. H.; Germar, K.; Spits, H. The role of ILC2 in pathology of type 2 inflammatory diseases. *Curr. Opin. Immunol.* **2014**, *31*, 115−120.
(135) Ho, J.; Bailey, M.; Zaunders, J.; Mrad, N.; Sacks, R.; Sewell, W.; Harvey, R. J. Group 2 innate lymphoid cells (ILC2s) are increased in chronic rhinosinusitis with nasal polyps or eosinophilia. *Clin. Exp. Allergy* **2015**, *45* (2), 394−403.
(136) Scanlon, S. T.; McKenzie, A. N. The messenger between worlds: the regulation of innate and adaptive type-2 immunity by innate lymphoid cells. *Clin. Exp. Allergy* **2015**, *45* (1), 9−20.
(137) Wynn, T. A. Type 2 cytokines: mechanisms and therapeutic strategies. *Nat. Rev. Immunol.* **2015**, *15* (5), 271−282.
(138) Woodruff, P. G.; Modrek, B.; Choy, D. F.; Jia, G. Q.; Abbas, A. R.; Ellwanger, A.; Arron, J. R.; Koth, L. L.; Fahy, J. V. T-helper Type 2-driven Inflammation Defines Major Subphenotypes of Asthma. *Am. J. Respir. Crit. Care Med.* **2009**, *180* (5), 388−395.
(139) Twaroch, T. E.; Curin, M.; Valenta, R.; Swoboda, I. Mold Allergens in Respiratory Allergy: From Structure to Therapy. *Allergy, Asthma Immunol. Res.* **2015**, *7* (3), 205−220.
(140) Radauer, C.; Nandy, A.; Ferreira, F.; et al. Update of the WHO/IUIS Allergen Nomenclature Database based on analysis of allergen sequences. *Allergy* **2014**, *69* (4), 413−419.
(141) Stocker, T. F., Qin, D.; Plattner, G.-K.; Tignor, M.; Allen, S.K.; Boschung, J.; Nauels, A.; Xia, Y.; Bex, V.; Midgley, P.M. *The Physical Science Basis*, Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, IPCC, 2013: Climate Change 2013; Cambridge University Press: Cambridge, United Kingdom, 2013; p 1525.
(142) Gill, S. S.; Tuteja, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.* **2010**, *48* (12), 909−930.
(143) Apel, K.; Hirt, H. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* **2004**, *55*, 373−399.
(144) Ciais, P.; Sabine, C.; Bala, G. et al. Carbon and Other Biogeochemical Cycles. In *Climate Change 2013: The Physical Science Basis*, Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change; Stocker, T. F., Qin, D., Plattner, G.-K., Tignor, M., Allen, S.K., Boschung, J., Nauels, A., Xia, Y., Bex, V., Midgley, P.M., Ed.; Cambridge University Press: Cambridge, United Kingdom, 2013; pp 465−570.
(145) Cecchi, L.; D'Amato, G.; Ayres, J. G.; et al. Projections of the effects of climate change on allergic asthma: the contribution of aerobiology. *Allergy* **2010**, *65* (9), 1073−1081.
(146) Klironomos, J. N.; Rillig, M. C.; Allen, M. F.; Zak, D. R.; Pregitzer, K. S.; Kubiske, M. E. Increased levels of airborne fungal spores in response to Populus tremuloides grown under elevated atmospheric CO_2. *Can. J. Bot.* **1997**, *75* (10), 1670−1673.
(147) Ciappetta, S.; Ghiani, A.; Gilardelli, F.; Bonini, M.; Citterio, S.; Gentili, R. Invasion of Ambrosia artemisiifolia in Italy: Assessment via analysis of genetic variability and herbarium data. *Flora (Jena)* **2016**, *223*, 106−113.
(148) Ziska, L. H.; McConnell, L. L. Climate Change, Carbon Dioxide, and Pest Biology: Monitor, Mitigate, Manage. *J. Agric. Food Chem.* **2016**, *64* (1), 6−12.
(149) Ziska, L. H.; Tomecek, M. B.; Valerio, M.; Thompson, J. P. Evidence for recent evolution in an invasive species, Microstegium vimineum, Japanese stiltgrass. *Weed Res.* **2015**, *55* (3), 260−267.
(150) Ziska, L.; Knowlton, K.; Rogers, C.; et al. Recent warming by latitude associated with increased length of ragweed pollen season in central North America. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108* (10), 4248−4251.
(151) D'Amato, G. Effects of climatic changes and urban air pollution on the rising trends of respiratory allergy and asthma. *Multidiscip. Respir. Med.* **2011**, *6* (1), 28−37.
(152) Despres, V. R.; Huffman, J. A.; Burrows, S. M.; et al. Primary biological aerosol particles in the atmosphere: a review. *Tellus, Ser. B* **2012**, *64*, 15598.
(153) Despres, V. R.; Nowoisky, J. F.; Klose, M.; Conrad, R.; Andreae, M. O.; Pöschl, U. Characterization of primary biogenic aerosol particles in urban, rural, and high-alpine air by DNA sequence and restriction fragment analysis of ribosomal RNA genes. *Biogeosciences* **2007**, *4* (6), 1127−1141.
(154) Fröhlich-Nowoisky, J.; Kampf, C. J.; Weber, B.; et al. Bioaerosols in the Earth System: Climate, Health, and Ecosystem Interactions. *Atmos. Res.* **2016**, *182*, 346−376.
(155) Lang-Yona, N.; Levin, Y.; Dannemiller, K. C.; Yarden, O.; Peccia, J.; Rudich, Y. Changes in atmospheric CO2 influence the allergenicity of Aspergillus fumigatus. *Glob. Change Biol.* **2013**, *19* (8), 2381−2388.
(156) Wolf, J.; O'Neill, N. R.; Rogers, C. A.; Muilenberg, M. L.; Ziska, L. H. Elevated Atmospheric Carbon Dioxide Concentrations Amplify Alternaria alternata Sporulation and Total Antigen Production. *Environ. Health Perspect.* **2010**, *118* (9), 1223−1228.
(157) Vogel, H.; Pauling, A.; Vogel, B. Numerical simulation of birch pollen dispersion with an operational weather forecast system. *Int. J. Biometeorol.* **2008**, *52* (8), 805−814.
(158) El Kelish, A.; Zhao, F.; Heller, W.; et al. Ragweed (Ambrosia artemisiifolia) pollen allergenicity: SuperSAGE transcriptomic analysis upon elevated CO_2 and drought stress. *BMC Plant Biol.* **2014**, *14*, 176.
(159) Albertine, J. M.; Manning, W. J.; DaCosta, M.; Stinson, K. A.; Muilenberg, M. L.; Rogers, C. A. Projected Carbon Dioxide to

DOI: 10.1021/acs.est.6b04908
Environ. Sci. Technol. 2017, 51, 4119−4141

Increase Grass Pollen and Allergen Exposure Despite Higher Ozone Levels. *PLoS One* **2014**, *9* (11), e111712.
(160) Kasprzyk, I.; Rodinkova, V.; Šaulienė, I.; et al. Air pollution by allergenic spores of the genus Alternaria in the air of central and eastern Europe. *Environ. Sci. Pollut. Res.* **2015**, *22* (12), 9260−9274.
(161) Newnham, R. M.; Sparks, T. H.; Skjoth, C. A.; Head, K.; Adams-Groom, B.; Smith, M. Pollen season and climate: Is the timing of birch pollen release in the UK approaching its limit? *Int. J. Biometeorol.* **2013**, *57* (3), 391−400.
(162) Weber, R. W. Aerobiology of Outdoor Allergens A2—Adkinson, N. Franklin. In *Middleton's Allergy*, 8th ed.; Bochner, B. S., Burks, A. W., Busse, W. W., Holgate, S. T., Lemanske, R. F., O'Hehir, R. E., Eds.; Elsevier: London, 2014; 430−452.
(163) Cohen, S. G.; Reif, C. B. Cutaneous Sensitization to Blue-Green Algae. *J. Allergy* **1953**, *24* (5), 452−457.
(164) Stewart, I.; Webb, P. M.; Schluter, P. J.; Fleming, L. E.; Burns, J. W.; Gantar, M.; Backer, L. C.; Shaw, G. R. Epidemiology of recreational exposure to freshwater cyanobacteria - an international prospective cohort study. *BMC Public Health* **2006**, *6* (1), 9310.
(165) Genitsaris, S.; Kormas, K. A.; Moustaka-Gouni, M. Airborne algae and cyanobacteria: occurrence and related health effects. *Front. Biosci., Elite Ed.* **2011**, *3* (1), 772−787.
(166) Petrus, M.; Culerrier, R.; Campistron, M.; Barre, A.; Rouge, P. First case report of anaphylaxis to spirulin: identification of phycocyanin as responsible allergen. *Allergy* **2010**, *65* (7), 924−925.
(167) Geh, E. N.; Ghosh, D.; McKell, M.; de la Cruz, A. A.; Stelma, G.; Bernstein, J. A. Identification of Microcystis aeruginosa Peptides Responsible for Allergic Sensitization and Characterization of Functional Interactions between Cyanobacterial Toxins and Immunogenic Peptides. *Environ. Health Persp.* **2015**, *123* (11), 1159−1166.
(168) Garcia-Pichel, F.; Loza, V.; Marusenko, Y.; Mateo, P.; Potrafka, R. M. Temperature Drives the Continental-Scale Distribution of Key Microbes in Topsoil Communities. *Science* **2013**, *340* (6140), 1574−1577.
(169) Elbert, W.; Weber, B.; Burrows, S.; Steinkamp, J.; Buedel, B.; Andreae, M. O.; Pöschl, U. Contribution of cryptogamic covers to the global cycles of carbon and nitrogen. *Nat. Geosci.* **2012**, *5* (7), 459−462.
(170) Weber, B.; Büdel, B.; Belnap, J. *Biological Soil Crusts: An Organizing Principle in Drylands*; Springer International Publishing: Switzerland, 2016; Vol. *226*.
(171) Reed, S. C.; Coe, K. K.; Sparks, J. P.; Housman, D. C.; Zelikova, T. J.; Belnap, J. Changes to dryland rainfall result in rapid moss mortality and altered soil fertility. *Nat. Clim. Change* **2012**, *2* (10), 752−755.
(172) Escolar, C.; Martínez, I.; Bowker, M. A.; Maestre, F. T. Warming reduces the growth and diversity of biological soil crusts in a semi-arid environment: implications for ecosystem structure and functioning. *Philos. Trans. R. Soc., B* **2012**, *367* (1606), 3087−3099.
(173) Ferrenberg, S.; Reed, S. C.; Belnap, J. Climate change and physical disturbance cause similar community shifts in biological soil crusts. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112* (39), 12116−12121.
(174) Lang-Yona, N.; Kunert, A. T.; Vogel, L. et al. Fresh Water, Marine and Terrestrial Cyanobacteria Display Distinct Allergen Characteristics. **2017**, submitted for publication.
(175) Stanelle, T.; Bey, I.; Raddatz, T.; Reick, C.; Tegen, I. Anthropogenically induced changes in twentieth century mineral dust burden and the associated impact on radiative forcing. *J. Geophys. Res.-Atmos.* **2014**, *119* (23), 13526−13546.
(176) McLeman, R. A.; Dupre, J.; Berrang Ford, L.; Ford, J.; Gajewski, K.; Marchildon, G. What we learned from the Dust Bowl: lessons in science, policy, and adaptation. *Popul. Environ.* **2014**, *35* (4), 417−440.
(177) Neff, J. C.; Ballantyne, A. P.; Farmer, G. L.; et al. Increasing eolian dust deposition in the western United States linked to human activity. *Nat. Geosci.* **2008**, *1* (3), 189−195.
(178) Mahowald, N. M.; Kloster, S.; Engelstaedter, S.; et al. Observed 20th century desert dust variability: impact on climate and biogeochemistry. *Atmos. Chem. Phys.* **2010**, *10* (22), 10875−10893.
(179) Mulitza, S.; Heslop, D.; Pittauerova, D.; et al. Increase in African dust flux at the onset of commercial agriculture in the Sahel region. *Nature* **2010**, *466* (7303), 226−228.
(180) Esmaeil, N.; Gharagozloo, M.; Rezaei, A.; Grunig, G. Dust events, pulmonary diseases and immune system. *Am. J. Clin. Exp. Immunl.* **2014**, *3* (1), 20−29.
(181) Goudie, A. S. Desert dust and human health disorders. *Environ. Int.* **2014**, *63*, 101−113.
(182) Griffin, D. W. Atmospheric movement of microorganisms in clouds of desert dust and implications for human health. *Clin. Microbiol. Rev.* **2007**, *20* (3), 459−477.
(183) Kellogg, C. A.; Griffin, D. W. Aerobiology and the global transport of desert dust. *Trends Ecol. Evol.* **2006**, *21* (11), 638−644.
(184) Leski, T. A.; Malanoski, A. P.; Gregory, M. J.; Lin, B. C.; Stenger, D. A. Application of a Broad-Range Resequencing Array for Detection of Pathogens in Desert Dust Samples from Kuwait and Iraq. *Appl. Environ. Microb.* **2011**, *77* (13), 4285−4292.
(185) Ortiz-Martinez, M. G.; Rodriguez-Cotto, R. I.; Ortiz-Rivera, M. A.; Pluguez-Turull, C. W.; Jimenez-Velez, B. D. Linking Endotoxins, African Dust PM10 and Asthma in an Urban and Rural Environment of Puerto Rico. *Mediators Inflammation* **2015**, *2015*, 784212.
(186) Maki, T.; Susuki, S.; Kobayashi, F.; et al. Phylogenetic analysis of atmospheric halotolerant bacterial communities at high altitude in an Asian dust (KOSA) arrival region, Suzu City. *Sci. Total Environ.* **2010**, *408* (20), 4556−4562.
(187) Yamaguchi, N.; Ichijo, T.; Sakotani, A.; Baba, T.; Nasu, M. Global dispersion of bacterial cells on Asian dust. *Sci. Rep.* **2012**, *2*, 525.
(188) Watanabe, M.; Yamasaki, A.; Burioka, N.; et al. Correlation between Asian Dust Storms and Worsening Asthma in Western Japan. *Allergol. Int.* **2011**, *60*, 267−275.
(189) Kanatani, K. T.; Ito, I.; Al-Delaimy, W. K.; Adachi, Y.; Mathews, W. C.; Ramsdell, J. W. Desert Dust Exposure Is Associated with Increased Risk of Asthma Hospitalization in Children. *Am. J. Respir. Crit. Care Med.* **2010**, *182* (12), 1475−1481.
(190) Gyan, K.; Henry, W.; Lacaille, S.; Laloo, A.; Lamsee-Ebanks, C.; McKay, S.; Antoine, R. M.; Monteil, M. A. African dust clouds are associated with increased paediatric asthma accident and emergency admissions on the Caribbean island of Trinidad. *Int. J. Biometeorol.* **2005**, *49* (6), 371−376.
(191) Chang, C. C.; Lee, I. M.; Tsai, S. S.; Yang, C. Y. Correlation of Asian dust storm events with daily clinic visits for allergic rhinitis in Taipei, Taiwan. *J. Toxicol. Environ. Health, Part A* **2006**, *69* (3), 229−235.
(192) D'Amato, G.; Vitale, C.; D'Amato, M.; et al. Thunderstorm-related asthma: what happens and why. *Clin. Exp. Allergy* **2016**, *46* (3), 390−396.
(193) Mendell, M. J.; Mirer, A. G.; Cheung, K.; Tong, M.; Douwes, J. Respiratory and Allergic Health Effects of Dampness, Mold, and Dampness-Related Agents: A Review of the Epidemiologic Evidence. *Environ. Health Perspect.* **2011**, *119* (6), 748−756.
(194) Tischer, C. G.; Hohmann, C.; Thiering, E.; et al. Meta-analysis of mould and dampness exposure on asthma and allergy in eight European birth cohorts: an ENRIECO initiative. *Allergy* **2011**, *66* (12), 1570−1579.
(195) Dannemiller, K. C.; Gent, J. F.; Leaderer, B. P.; Peccia, J. Indoor microbial communities: Influence on asthma severity in atopic and nonatopic children. *J. Allergy Clin. Immunol.* **2016**, *138* (1), 76−83.
(196) Platts-Mills, T. A. E. Indoor Allergens A2—Adkinson, N. Franklin. In *Middleton's Allergy*, 8th ed.; Bochner, B. S., Burks, A. W., Busse, W. W., Holgate, S. T., Lemanske, R. F., O'Hehir, R. E., Eds. Elsevier: London, 2014; 453−469.
(197) Elbert, W.; Taylor, P. E.; Andreae, M. O.; Pöschl, U. Contribution of fungi to primary biogenic aerosols in the atmosphere: wet and dry discharged spores, carbohydrates, and inorganic ions. *Atmos. Chem. Phys.* **2007**, *7* (17), 4569−4588.
(198) Fröhlich-Nowoisky, J.; Burrows, S. M.; Xie, Z.; et al. Biogeography in the air: fungal diversity over land and oceans. *Biogeosciences* **2012**, *9* (3), 1125−1136.

DOI: 10.1021/acs.est.6b04908
Environ. Sci. Technol. 2017, 51, 4119−4141

(199) Fröhlich-Nowoisky, J.; Pickersgill, D. A.; Despres, V. R.; Pöschl, U. High diversity of fungi in air particulate matter. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106* (31), 12814−12819.
(200) Müller-Germann, I.; Vogel, B.; Vogel, H.; Pauling, A.; Fröhlich-Nowoisky, J.; Pöschl, U.; Despres, V. R. Quantitative DNA Analyses for Airborne Birch Pollen. *PLoS One* **2015**, *10* (10), e0140949.
(201) Oberdorster, G.; Oberdorster, E.; Oberdorster, J. Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. *Environ. Health Perspect.* **2005**, *113* (7), 823−839.
(202) Taylor, P. E.; Flagan, R. C.; Miguel, A. G.; Valenta, R.; Glovsky, M. M. Birch pollen rupture and the release of aerosols of respirable allergens. *Clin. Exp. Allergy* **2004**, *34* (10), 1591−1596.
(203) Taylor, P. E.; Flagan, R. C.; Valenta, R.; Glovsky, M. M. Release of allergens as respirable aerosols: A link between grass pollen and asthma. *J. Allergy Clin. Immunol.* **2002**, *109* (1), 51−56.
(204) Knutsen, A. P.; Bush, R. K.; Demain, J. G.; et al. Fungi and allergic lower respiratory tract diseases. *J. Allergy Clin. Immunol.* **2012**, *129* (2), 280−291.
(205) Behrendt, H.; Becker, W. M. Localization, release and bioavailability of pollen allergens: the influence of environmental factors. *Curr. Opin. Immunol.* **2001**, *13* (6), 709−715.
(206) Taylor, P. E.; Jonsson, H. Thunderstorm asthma. *Curr. Allergy Asthma Rep.* **2004**, *4* (5), 409−413.
(207) Taylor, P. E.; Jacobson, K. W.; House, J. M.; Glovsky, M. M. Links between Pollen, Atopy and the Asthma Epidemic. *Int. Arch. Allergy Immunol.* **2007**, *144* (2), 162−170.
(208) Huffman, J. A.; Prenni, A. J.; DeMott, P. J.; et al. High concentrations of biological aerosol particles and ice nuclei during and after rain. *Atmos. Chem. Phys.* **2013**, *13* (13), 6151−6164.
(209) Schäppi, G. F.; Suphioglu, C.; Taylor, P. E.; Knox, R. B. Concentrations of the major birch tree allergen Bet v 1 in pollen and respirable fine particles in the atmosphere. *J. Allergy Clin. Immunol.* **1997**, *100* (5), 656−661.
(210) Green, B. J.; Zinovia Mitakakis, T. Z.; Tovey, E. R. Allergen detection from 11 fungal species before and after germination. *J. Allergy Clin. Immunol.* **2003**, *111* (2), 285−289.
(211) Sporik, R. B.; Arruda, L. K.; Woodfolk, J.; Chapman, M. D.; Plattsmills, T. A. E. Environmental exposure to aspergillus-fumigatus allergen (asp-f-i). *Clin. Exp. Allergy* **1993**, *23* (4), 326−331.
(212) Motta, A. C.; Marliere, M.; Peltre, G.; Sterenberg, P. A.; Lacroix, G. Traffic-Related Air Pollutants Induce the Release of Allergen-Containing Cytoplasmic Granules from Grass Pollen. *Int. Arch. Allergy Immunol.* **2006**, *139* (4), 294−298.
(213) Ouyang, Y.; Xu, Z.; Fan, E.; Li, Y.; Zhang, L. Effect of nitrogen dioxide and sulfur dioxide on viability and morphology of oak pollen. *Int. Forum Allergy Rhinol.* **2016**, *6* (1), 95−100.
(214) Gilles, S.; Mariani, V.; Bryce, M.; Mueller, M. J.; Ring, J.; Behrendt, H.; Jakob, T.; Traidl-Hoffmann, C. Pollen allergens do not come alone: pollen associated lipid mediators (PALMS) shift the human immune systems towards a T(H)2-dominated response. *Allergy, Asthma, Clin. Immunol.* **2009**, *5* (1), 3.
(215) Gilles-Stein, S.; Traidl-Hoffmann, C. Pollen are more than allergen carriers. *Allergologie* **2016**, *39* (2), 69−76.
(216) Gilles, S.; Beck, I.; Lange, S.; Ring, J.; Behrendt, H.; Traidl-Hoffmann, C. Non-allergenic factors from pollen modulate T helper cell instructing notch ligands on dendritic cells. *World Allergy Organ. J.* **2015**, *8*, 2.
(217) Traidl-Hoffmann, C.; Kasche, A.; Jakob, T.; Huger, M.; Plotz, S.; Feussner, I.; Ring, J.; Behrendt, H. Lipid mediators from pollen act as chemoattractants and activators of polymorphonuclear granulocytes. *J. Allergy Clin. Immunol.* **2002**, *109* (5), 831−838.
(218) Gilles-Stein, S.; Beck, I.; Chaker, A.; et al. Pollen derived low molecular compounds enhance the human allergen specific immune response in vivo. *Clin. Exp. Allergy* **2016**, *46* (10), 1355−1365.
(219) Oeder, S.; Alessandrini, F.; Wirz, O. F.; et al. Pollen-derived nonallergenic substances enhance Th2-induced IgE production in B cells. *Allergy* **2015**, *70* (11), 1450−1460.
(220) Degobbi, C.; Lopes, F.; Carvalho-Oliveira, R.; Munoz, J. E.; Saldiva, P. H. N. Correlation of fungi and endotoxin with PM2.5 and meteorological parameters in atmosphere of Sao Paulo, Brazil. *Atmos. Environ.* **2011**, *45* (13), 2277−2283.
(221) Majd, A.; Chehregani, A.; Moin, M.; Gholami, M.; Kohno, S.; Nabe, T.; Shariatzade, M. A. The effects of air pollution on structures, proteins and allergenicity of pollen grains. *Aerobiologia* **2004**, *20* (2), 111−118.
(222) Pöschl, U.; Martin, S. T.; Sinha, B.; et al. Rainforest Aerosols as Biogenic Nuclei of Clouds and Precipitation in the Amazon. *Science* **2010**, *329* (5998), 1513−1516.
(223) Ring, J.; Buters, J.; Behrendt, H. Particulate and Pollen Interactions A2—Adkinson, N. Franklin. In *Middleton's Allergy*, 8th ed.; Bochner, B. S., Burks, A. W., Busse, W. W., Holgate, S. T., Lemanske, R. F., O'Hehir, R. E., Eds. Elsevier: London, 2014; 497−507.
(224) Beck, I.; Jochner, S.; Gilles, S.; et al. High Environmental Ozone Levels Lead to Enhanced Allergenicity of Birch Pollen. *PLoS One* **2013**, *8* (11), e80147.
(225) Bryce, M.; Drews, O.; Schenk, M. F.; et al. Impact of Urbanization on the Proteome of Birch Pollen and Its Chemotactic Activity on Human Granulocytes. *Int. Arch. Allergy Immunol.* **2010**, *151* (1), 46−55.
(226) Chehregani, A.; Majde, A.; Moin, M.; Gholami, M.; Ali Shariatzadeh, M.; Nassiri, H. Increasing allergy potency of Zinnia pollen grains in polluted areas. *Ecotoxicol. Environ. Saf.* **2004**, *58* (2), 267−272.
(227) Cortegano, I.; Civantos, E.; Aceituno, E.; del Moral, A.; Lopez, E.; Lombardero, M.; del Pozo, V.; Lahoz, C. Cloning and expression of a major allergen from Cupressus arizonica pollen, Cup a 3, a PR-5 protein expressed under polluted environment. *Allergy* **2004**, *59* (5), 485−490.
(228) Ghiani, A.; Aina, R.; Asero, R.; Bellotto, E.; Citterio, S. Ragweed pollen collected along high-traffic roads shows a higher allergenicity than pollen sampled in vegetated areas. *Allergy* **2012**, *67* (7), 887−894.
(229) Gruijthuijsen, Y. K.; Grieshuber, I.; Stocklinger, A.; et al. Nitration enhances the allergenic potential of proteins. *Int. Arch. Allergy Immunol.* **2006**, *141* (3), 265−275.
(230) Jin, H. J.; Choi, G. S.; Shin, Y. S.; Kim, J. H.; Kim, J. E.; Ye, Y. M.; Park, H. S. The Allergenic Potency of Japanese Hop Pollen Is Increasing With Environmental Changes in Korea. *Allergy, Asthma Immunol. Res.* **2013**, *5* (5), 309−314.
(231) Suarez-Cervera, M.; Castells, T.; Vega-Maray, A.; et al. Effects of air pollution on Cup a 3 allergen in Cupressus arizonica pollen grains. *Ann. Allergy, Asthma, Immunol.* **2008**, *101* (1), 57−66.
(232) Pöschl, U. Atmospheric aerosols: Composition, transformation, climate and health effects. *Angew. Chem., Int. Ed.* **2005**, *44* (46), 7520−7540.
(233) Kanter, U.; Heller, W.; Durner, J.; et al. Molecular and Immunological Characterization of Ragweed (Ambrosia artemisiifolia L.) Pollen after Exposure of the Plants to Elevated Ozone over a Whole Growing Season. *PLoS One* **2013**, *8* (4), e61518.
(234) Pasqualini, S.; Tedeschini, E.; Frenguelli, G.; Wopfner, N.; Ferreira, F.; D'Amato, G.; Ederli, L. Ozone affects pollen viability and NAD(P)H oxidase release from Ambrosia artemisiifolia pollen. *Environ. Pollut.* **2011**, *159* (10), 2823−2830.
(235) Obersteiner, A.; Gilles, S.; Frank, U. Pollen-Associated Microbiome Correlates with Pollution Parameters and the Allergenicity of Pollen. *PLoS One* **2016**, *11* (2), e0149545.
(236) Cuinica, L. G.; Abreu, I.; Esteves da Silva, J. E. Effect of air pollutant NO_2 on Betula pendula, Ostrya carpinifolia and Carpinus betulus pollen fertility and human allergenicity. *Environ. Pollut.* **2014**, *186*, 50−55.
(237) Lang-Yona, N.; Shuster-Meiseles, T.; Mazar, Y.; Yarden, O.; Rudich, Y. Impact of urban air pollution on the allergenicity of Aspergillus fumigatus conidia: Outdoor exposure study supported by laboratory experiments. *Sci. Total Environ.* **2016**, *541*, 365−371.
(238) Ribeiro, H.; Duque, L.; Sousa, R.; Cruz, A.; Gomes, C.; Esteves da Silva, J. E.; Abreu, I. Changes in the IgE-reacting protein profiles of Acer negundo, Platanus x acerifolia and Quercus robur pollen in

DOI: 10.1021/acs.est.6b04908
Environ. Sci. Technol. 2017, 51, 4119−4141

response to ozone treatment. *Int. J. Environ. Health Res.* **2014**, *24* (6), 515−527.
(239) Zhao, F.; Elkelish, A.; Durner, J.; et al. Common ragweed (Ambrosia artemisiifolia L.): allergenicity and molecular characterization of pollen after plant exposure to elevated NO_2. *Plant, Cell Environ.* **2016**, *39* (1), 147−164.
(240) Sénéchal, H.; Visez, N.; Charpin, D.; et al. A Review of the Effects of Major Atmospheric Pollutants on Pollen Grains, Pollen Content, and Allergenicity. *Sci. World J.* **2015**, *2015*, 940243.
(241) Knox, R. B.; Suphioglu, C.; Taylor, P.; Desai, R.; Watson, H. C.; Peng, J. L.; Bursill, L. A. Major grass pollen allergen Lol p 1 binds to diesel exhaust particles: Implications for asthma and air pollution. *Clin. Exp. Allergy* **1997**, *27* (3), 246−251.
(242) Ormstad, H. Suspended particulate matter in indoor air: adjuvants and allergen carriers. *Toxicology* **2000**, *152* (1−3), 53−68.
(243) Namork, E.; Johansen, B. V.; Lovik, M. Detection of allergens adsorbed to ambient air particles collected in four European cities. *Toxicol. Lett.* **2006**, *165* (1), 71−78.
(244) Radauer-Preiml, I.; Andosch, A.; Hawranek, T.; Luetz-Meindl, U.; Wiederstein, M.; Horejs-Hoeck, J.; Himly, M.; Boyles, M.; Duschl, A. Nanoparticle-allergen interactions mediate human allergic responses: protein corona characterization and cellular responses. *Part. Fibre Toxicol.* **2015**, *13*, 3−3.
(245) Sompayrac, L. *How the Immune System Works*, 3rd ed.; Blackwell Publishing, 2008.
(246) Buters, J.; Prank, M.; Sofiev, M.; et al. Variation of the group 5 grass pollen allergen content of airborne pollen in relation to geographic location and time in season. *J. Allergy Clin. Immunol.* **2015**, *136* (1), 87−U179.
(247) Buters, J. T. M.; Thibaudon, M.; Smith, M.; et al. Release of Bet v 1 from birch pollen from 5 European countries. Results from the HIALINE study. *Atmos. Environ.* **2012**, *55*, 496−505.
(248) Creer, S.; Deiner, K.; Frey, S.; Porazinska, D.; Taberlet, P.; Thomas, W. K.; Potter, C.; Bik, H. M. The ecologist's field guide to sequence-based identification of biodiversity. *Methods in Ecology and Evolution* **2016**, *7*, 1008−1018.
(249) Lang-Yona, N.; Dannemiller, K.; Yamamoto, N.; Burshtein, N.; Peccia, J.; Yarden, O.; Rudich, Y. Annual distribution of allergenic fungal spores in atmospheric particulate matter in the Eastern Mediterranean; a comparative study between ergosterol and quantitative PCR analysis. *Atmos. Chem. Phys.* **2012**, *12* (5), 2681−2690.
(250) Liu, F.; Lai, S.; Reinmuth-Selzle, K.; Scheel, J. F.; Fröhlich-Nowoisky, J.; Després, V. R.; Hoffmann, T.; Pöschl, U.; Kampf, C. J. Metaproteomic analysis of atmospheric aerosol samples. *Anal. Bioanal. Chem.* **2016**, *408* (23), 6337−6348.
(251) West, J. S.; Kimber, R. B. E. Innovations in air sampling to detect plant pathogens. *Ann. Appl. Biol.* **2015**, *166* (1), 4−17.
(252) Estillore, A. D.; Trueblood, J. V.; Grassian, V. H. Atmospheric chemistry of bioaerosols: heterogeneous and multiphase reactions with atmospheric oxidants and other trace gases. *Chem. Sci.* **2016**, *7*, 6604−6616.
(253) Pöhlker, C.; Huffman, J. A.; Pöschl, U. *Atmos. Meas. Tech.* **2012**, *5* (1), 37−71.
(254) Iglesias-Otero, M. A.; Fernandez-Gonzalez, M.; Rodriguez-Caride, D.; Astray, G.; Mejuto, J. C.; Rodriguez-Rajo, F. J. A model to forecast the risk periods of Plantago pollen allergy by using the ANN methodology. *Aerobiologia* **2015**, *31* (2), 201−211.
(255) Laskin, A.; Gilles, M. K.; Knopf, D. A.; Wang, B.; China, S. Progress in the Analysis of Complex Atmospheric Particles. *Annu. Rev. Anal. Chem.* **2016**, *9* (1), 117−143.
(256) Marecal, V.; Peuch, V. H.; Andersson, C.; et al. A regional air quality forecasting system over Europe: the MACC-II daily ensemble production. *Geosci. Model Dev.* **2015**, *8* (9), 2777−2813.
(257) Noziere, B.; Kaberer, M.; Claeys, M.; et al. The Molecular Identification of Organic Compounds in the Atmosphere: State of the Art and Challenges. *Chem. Rev.* **2015**, *115* (10), 3919−3983.
(258) Prank, M.; Chapman, D. S.; Bullock, J. M.; et al. An operational model for forecasting ragweed pollen release and dispersion in Europe. *Agric. For. Meteorol* **2013**, *182-183*, 43−53.
(259) Sujaritpong, S.; Dear, K.; Cope, M.; Walsh, S.; Kjellstrom, T. Quantifying the health impacts of air pollution under a changing climate—a review of approaches and methodology. *Int. J. Biometeorol.* **2014**, *58* (2), 149−160.
(260) Tesson, S. V. M; Skjoth, C. A.; Santl-Temkiv, T.; Londahl, J. Airborne Microalgae: Insights, Opportunities, and Challenges. *Appl. Environ. Microbiol.* **2016**, *82* (7), 1978−1991.
(261) Thompson, T. M.; Saari, R. K.; Selin, N. E. Air quality resolution for health impact assessment: influence of regional characteristics. *Atmos. Chem. Phys.* **2014**, *14* (2), 969−978.
(262) Tsigaridis, K.; Daskalakis, N.; Kanakidou, M.; et al. The AeroCom evaluation and intercomparison of organic aerosol in global models. *Atmos. Chem. Phys.* **2014**, *14* (19), 10845−10895.
(263) von Schneidemesser, E.; Monks, P. S.; Allan, J. D.; et al. Chemistry and the Linkages between Air Quality and Climate Change. *Chem. Rev.* **2015**, *115* (10), 3856−3897.
(264) Weschler, C. J. Roles of the human occupant in indoor chemistry. *Indoor Air* **2016**, *26* (1), 6−24.
(265) Weschler, C. Chemistry in indoor environments: 20 years of research. *Indoor Air* **2011**, *21* (3), 205−218.
(266) Zhang, R.; Wang, G.; Guo, S.; Zamora, M. L.; Ying, Q.; Lin, Y.; Wang, W.; Hu, M.; Wang, Y. Formation of Urban Fine Particulate Matter. *Chem. Rev.* **2015**, *115* (10), 3803−3855.
(267) Zhang, R.; Duhl, T.; Salam, M. T.; et al. Development of a regional-scale pollen emission and transport modeling framework for investigating the impact of climate change on allergic airway disease. *Biogeosciences* **2014**, *11* (6), 1461−1478.
(268) Fiore, A. M.; Dentener, F. J.; Wild, O.; et al. Multimodel estimates of intercontinental source-receptor relationships for ozone pollution. *J. Geophys. Res.* **2009**, *114* (D04), D04301.
(269) Lakey, P. S. J.; Wisthaler, A.; Berkemeier, T.; Mikoviny, T.; Pöschl, U.; Shiraiwa, M. Chemical kinetics of multiphase reactions between ozone and human skin lipids: Implications for indoor air quality and health effects. *Indoor Air* **2016**, DOI: 10.1111/ina.12360.
(270) Anderson, S. E.; Franko, J.; Jackson, L. G.; Wells, J. R.; Ham, J. E.; Meade, B. J. Irritancy and allergic responses induced by exposure to the indoor air chemical 4-oxopentanal. *Toxicol. Sci.* **2012**, *127* (2), 371−381.
(271) Anderson, S. E.; Wells, J. R.; Fedorowicz, A.; Butterworth, L. F.; Meade, B. J.; Munson, A. E. Evaluation of the Contact and Respiratory Sensitization Potential of Volatile Organic Compounds Generated by Simulated Indoor Air Chemistry. *Toxicol. Sci.* **2007**, *97* (2), 355−363.
(272) McGwin, G.; Lienert, J.; Kennedy, J. I. Formaldehyde Exposure and Asthma in Children: A Systematic Review. *Environ. Health Perspect.* **2010**, *118* (3), 313−317.
(273) Finlayson-Pitts, B. J.; Pitts, J. N. *Chemistry of the Upper and Lower Atmosphere*; Academic Press: San Diego, CA, 2000.
(274) Hernandez, M. L.; Peden, D. B. Air Pollution: Indoor and Outdoor A2—Adkinson, N. Franklin. In *Middleton's Allergy*, 8th ed.; Bochner, B. S., Burks, A. W., Busse, W. W., Holgate, S. T., Lemanske, R. F., O'Hehir, R. E., Eds.; Elsevier: London, 2014; 482−496.
(275) Berger, U.; Kmenta, M.; Bastl, K. Individual pollen exposure measurements: are they feasible? *Curr. Opin. Allergy Clin. Immunol.* **2014**, *14* (3), 200−205.
(276) Bastl, K.; Kmenta, M.; Pessi, A.-M.; et al. First comparison of symptom data with allergen content (Bet v 1 and Phl p 5 measurements) and pollen data from four European regions during 2009−2011. *Sci. Total Environ.* **2016**, *548-549*, 229−235.
(277) Caillaud, D.; Martin, S.; Segala, C.; Besancenot, J.-P.; Clot, B.; Thibaudon, M.; French Aerobiology Network. Effects of Airborne Birch Pollen Levels on Clinical Symptoms of Seasonal Allergic Rhinoconjunctivitis. *Int. Arch. Allergy Immunol.* **2014**, *163* (1), 43−50.
(278) Lim, S. S.; Vos, T.; Flaxman, A. D.; et al. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990−2010: a systematic

DOI: 10.1021/acs.est.6b04908
Environ. Sci. Technol. 2017, 51, 4119−4141

analysis for the Global Burden of Disease Study 2010. *Lancet* **2012**, *380* (9859), 2224−2260.
(279) Kmenta, M.; Bastl, K.; Jaeger, S.; Berger, U. Development of personal pollen information-the next generation of pollen information and a step forward for hay fever sufferers. *Int. J. Biometeorol.* **2014**, *58* (8), 1721−1726.
(280) Exley, K.; Robertson, S.; Pope, F. D.; Harrison, R. M.; Gant, T. W. Workshop on the sources, quantification and health implications of bioaerosols. *Am. J. Pharmacol. Toxicol.* **2014**, *9* (3), 189−199.
(281) Bowatte, G.; Lodge, C.; Lowe, A. J.; Erbas, B.; Perret, J.; Abramson, M. J.; Matheson, M.; Dharmage, S. C. The influence of childhood traffic-related air pollution exposure on asthma, allergy and sensitization: a systematic review and a meta-analysis of birth cohort studies. *Allergy* **2015**, *70* (3), 245−256.
(282) Gehring, U.; Wijga, A. H.; Brauer, M.; Fischer, P.; de Jongste, J. C.; Kerkhof, M.; Oldenwening, M.; Smit, H. A.; Brunekreef, B. Traffic-related Air Pollution and the Development of Asthma and Allergies during the First 8 Years of Life. *Am. J. Respir. Crit. Care Med.* **2010**, *181* (6), 596−603.
(283) Guarnieri, M.; Balmes, J. R. Outdoor air pollution and asthma. *Lancet* **2014**, *383* (9928), 1581−1592.
(284) McConnell, R.; Islam, T.; Shankardass, K.; et al. Childhood Incident Asthma and Traffic-Related Air Pollution at Home and School. *Environ. Health Perspect.* **2010**, *118* (7), 1021−1026.
(285) Morgenstern, V.; Zutavern, A.; Cyrys, J.; et al. Atopic diseases, allergic sensitization, and exposure to traffic-related air pollution in children. *Am. J. Respir. Crit. Care Med.* **2008**, *177* (12), 1331−1337.
(286) Anderson, H. R.; Favarato, G.; Atkinson, R. W. Long-term exposure to air pollution and the incidence of asthma: meta-analysis of cohort studies. *Air Qual., Atmos. Health* **2013**, *6* (1), 47−56.
(287) Esposito, S.; Galeone, C.; Lelii, M.; et al. Impact of air pollution on respiratory diseases in children with recurrent wheezing or asthma. *BMC Pulm. Med.* **2014**, *14*, 130.
(288) Meng, Y. Y.; Wilhelm, M.; Rull, R. P.; English, P.; Ritz, B. Traffic and outdoor air pollution levels near residences and poorly controlled asthma in adults. *Ann. Allergy, Asthma, Immunol.* **2007**, *98* (5), 455−463.
(289) Kim, J. J.; Smorodinsky, S.; Lipsett, M.; Singer, B. C.; Hodgson, A. T.; Ostro, B. Traffic-related air pollution near busy roads: the East Bay Children's Respiratory Health Study. *Am. J. Respir. Crit. Care Med.* **2004**, *170* (5), 520−526.
(290) Bowatte, G.; Lodge, C. J.; Knibbs, L. D.; et al. Traffic-related air pollution exposure is associated with allergic sensitization, asthma, and poor lung function in middle age. *J. Allergy Clin. Immunol.* **2017**, *139*, 122−129.e1.
(291) Krämer, U.; Sugiri, D.; Ranft, U.; et al. Eczema, respiratory allergies, and traffic-related air pollution in birth cohorts from small-town areas. *J. Dermatol. Sci.* **2009**, *56* (2), 99−105.
(292) Anderson, H. R.; Favarato, G.; Atkinson, R. W. Long-term exposure to outdoor air pollution and the prevalence of asthma: meta-analysis of multi-community prevalence studies. *Air Qual., Atmos. Health* **2013**, *6* (1), 57−68.
(293) Devereux, G.; Matsui, E. C.; Burney, P. G. J., Epidemiology of Asthma and Allergic Airway Diseases A2—Adkinson, N. Franklin. In *Middleton's Allergy*, 8th ed.; Bochner, B. S., Burks, A. W., Busse, W. W., Holgate, S. T., Lemanske, R. F., O'Hehir, R. E., Eds.; Elsevier: London, 2014; pp 754−789.
(294) Janssen, N. A. H.; Hoek, G.; Simic-Lawson, M.; et al. Black Carbon as an Additional Indicator of the Adverse Health Effects of Airborne Particles Compared with PM10 and PM2.5. *Environ. Health Perspect.* **2011**, *119* (12), 1691−1699.
(295) Brauer, M. How Much, How Long, What, and Where. *Proc. Am. Thorac. Soc.* **2010**, *7* (2), 111−115.
(296) Greenacre, S. A. B.; Ischiropoulos, H. Tyrosine nitration: Localisation, quantification, consequences for protein function and signal transduction. *Free Radical Res.* **2001**, *34* (6), 541−581.
(297) Ischiropoulos, H. Biological selectivity and functional aspects of protein tyrosine nitration. *Biochem. Biophys. Res. Commun.* **2003**, *305* (3), 776−783.
(298) Radi, R. Nitric oxide, oxidants, and protein tyrosine nitration. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101* (12), 4003−4008.
(299) Souza, J. M.; Peluffo, G.; Radi, R. Protein tyrosine nitration - Functional alteration or just a biomarker? *Free Radical Biol. Med.* **2008**, *45* (4), 357−366.
(300) Abello, N.; Kerstjens, H. A. M.; Postma, D. S.; Bischoff, R. Protein Tyrosine Nitration: Selectivity, Physicochemical and Biological Consequences, Denitration, and Proteomics Methods for the Identification of Tyrosine-Nitrated Proteins. *J. Proteome Res.* **2009**, *8* (7), 3222−3238.
(301) Ischiropoulos, H. Protein tyrosine nitration-An update. *Arch. Biochem. Biophys.* **2009**, *484* (2), 117−121.
(302) Jones, L. H. Chemistry and Biology of Biomolecule Nitration. *Chem. Biol.* **2012**, *19* (9), 1086−1092.
(303) Radi, R. Protein Tyrosine Nitration: Biochemical Mechanisms and Structural Basis of Functional Effects. *Acc. Chem. Res.* **2013**, *46* (2), 550−559.
(304) Untersmayr, E.; Diesner, S. C.; Oostingh, G. J.; et al. Nitration of the Egg-Allergen Ovalbumin Enhances Protein Allergenicity but Reduces the Risk for Oral Sensitization in a Murine Model of Food Allergy. *PLoS One* **2010**, *5* (12), e14210.
(305) Karle, A. C.; Oostingh, G. J.; Mutschlechner, S.; Ferreira, F.; Lackner, P.; Bohle, B.; Fischer, G. F.; Vogt, A. B.; Duschl, A. Nitration of the Pollen Allergen Bet v 1.0101 Enhances the Presentation of Bet v 1-Derived Peptides by HLA-DR on Human Dendritic Cells. *PLoS One* **2012**, *7* (2), e31483.
(306) Ackaert, C.; Kofler, S.; Horejs-Hoeck, J.; et al. The impact of nitration on the structure and immunogenicity of the major birch pollen allergen Bet v 1.0101. *PLoS One* **2014**, *9* (8), e104520.
(307) Schöll, I.; Kalkura, N.; Shedziankova, Y.; et al. Dimerization of the major birch pollen allergen Bet v 1 is important for its in vivo IgE-cross-linking potential in mice. *J. Immunol.* **2005**, *175* (10), 6645−6650.
(308) Rouvinen, J.; Janis, J.; Laukkanen, M. L. Transient Dimers of Allergens. *PLoS One* **2010**, *5* (2), e9037.
(309) Lakey, S. J. P.; Berkemeier, T.; Tong, H.; Arangio, A. M.; Lucas, K.; Pöschl, U.; Shiraiwa, M. Chemical exposure-response relationship between air pollutants and reactive oxygen species in the human respiratory tract. *Sci. Rep.* **2016**, *6*, 32916.
(310) Gurgueira, S. A.; Lawrence, J.; Coull, B.; Murthy, G. G. K.; Gonzalez-Flecha, B. Rapid increases in the steady-state concentration of reactive oxygen species in the lungs and heart after particulate air pollution inhalation. *Environ. Health Perspect.* **2002**, *110* (8), 749−755.
(311) Charrier, J. G.; McFall, A. S.; Richards-Henderson, N. K.; Anastasio, C. Hydrogen Peroxide Formation in a Surrogate Lung Fluid by Transition Metals and Quinones Present in Particulate Matter. *Environ. Sci. Technol.* **2014**, *48* (12), 7010−7017.
(312) Verma, V.; Fang, T.; Xu, L.; Peltier, R. E.; Russell, A. G.; Ng, N. L.; Weber, R. J. Organic Aerosols Associated with the Generation of Reactive Oxygen Species (ROS) by Water-Soluble PM2.5. *Environ. Sci. Technol.* **2015**, *49* (7), 4646−4656.
(313) Stadtman, E. R.; Levine, R. L. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. *Amino Acids* **2003**, *25* (3−4), 207−218.
(314) Winterbourn, C. C. Reconciling the chemistry and biology of reactive oxygen species. *Nat. Chem. Biol.* **2008**, *4* (5), 278−286.
(315) Ray, P. D.; Huang, B.-W.; Tsuji, Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell. Signalling* **2012**, *24* (5), 981−990.
(316) Zhang, Y.; Du, Y.; Le, W.; Wang, K.; Kieffer, N.; Zhang, J. Redox control of the survival of healthy and diseased cells. *Antioxid. Redox Signaling* **2011**, *15* (11), 2867−2908.
(317) Bachi, A.; Dalle-Donne, I.; Scaloni, A. Redox Proteomics: Chemical Principles, Methodological Approaches and Biological/Biomedical Promises. *Chem. Rev.* **2013**, *113* (1), 596−698.
(318) Halliwell, B. G. J. *Free Radicals in Biology and Medicine*; Oxford University Press: Oxford, U.K., 2007; pp 851.

DOI: 10.1021/acs.est.6b04908
Environ. Sci. Technol. 2017, 51, 4119−4141

(319) Oswald, R.; Behrendt, T.; Ermel, M.; et al. HONO Emissions from Soil Bacteria as a Major Source of Atmospheric Reactive Nitrogen. *Science* **2013**, *341* (6151), 1233−1235.
(320) Su, H.; Cheng, Y.; Oswald, R.; et al. Soil Nitrite as a Source of Atmospheric HONO and OH Radicals. *Science* **2011**, *333* (6049), 1616−1618.
(321) Roeser, J.; Bischoff, R.; Bruins, A. P.; Permentier, H. P. Oxidative protein labeling in mass-spectrometry-based proteomics. *Anal. Bioanal. Chem.* **2010**, *397* (8), 3441−3455.
(322) Mudd, J. B.; Leavitt, R.; Ongun, A.; McManus, T. T. Reaction of ozone with amino acids and proteins. *Atmos. Environ.* **1969**, *3* (6), 669−681.
(323) Pryor, W. A.; Giamalva, D. H.; Church, D. F. Kinetics of ozonation. 2. Amino-acids and model compounds in water and comparisons to rates in nonpolar-solvents. *J. Am. Chem. Soc.* **1984**, *106* (23), 7094−7100.
(324) Sharma, V. K.; Graham, N. J. D. Oxidation of Amino Acids, Peptides and Proteins by Ozone: A Review. *Ozone: Sci. Eng.* **2010**, *32* (2), 81−90.
(325) Garrison, W. M. Reaction mechanisms in the radiolysis of peptides, polypeptides, and proteins. *Chem. Rev.* **1987**, *87* (2), 381−398.
(326) Xu, G. H.; Chance, M. R. Hydroxyl radical-mediated modification of proteins as probes for structural proteomics. *Chem. Rev.* **2007**, *107* (8), 3514−3543.
(327) Davies, K. J. A. Protein damage and degradation by oxygen radicals 0.1. General-aspects. *J. Biol. Chem.* **1987**, *262* (20), 9895−9901.
(328) Reinmuth-Selzle, K.; Ackaert, C.; Kampf, C. J.; et al. Nitration of the Birch Pollen Allergen Bet v 1.0101: Efficiency and Site-Selectivity of Liquid and Gaseous Nitrating Agents. *J. Proteome Res.* **2014**, *13* (3), 1570−1577.
(329) Shiraiwa, M.; Selzle, K.; Yang, H.; Sosedova, Y.; Ammann, M.; Pöschl, U. Multiphase Chemical Kinetics of the Nitration of Aerosolized Protein by Ozone and Nitrogen Dioxide. *Environ. Sci. Technol.* **2012**, *46* (12), 6672−6680.
(330) Franze, T.; Weller, M. G.; Niessner, R.; Pöschl, U. Protein nitration by polluted air. *Environ. Sci. Technol.* **2005**, *39* (6), 1673−1678.
(331) Kofler, S.; Ackaert, C.; Samonig, M.; et al. Stabilization of the dimeric birch pollen allergen Bet v 1 impacts its immunological properties. *J. Biol. Chem.* **2014**, *289* (1), 540−551.
(332) Ghiani, A.; Bruschi, M.; Citterio, S.; Bolzacchini, E.; Ferrero, L.; Sangiorgi, G.; Asero, R.; Perrone, M. G. Nitration of pollen aeroallergens by nitrate ion in conditions simulating the liquid water phase of atmospheric particles. *Sci. Total Environ.* **2016**, *573*, 1589−1597.
(333) Walcher, W.; Franze, T.; Weller, M. G.; Pöschl, U.; Huber, C. G. Liquid- and gas-phase nitration of bovine serum albumin studied by LC-MS and LC-MS/MS using monolithic columns. *J. Proteome Res.* **2003**, *2* (5), 534−542.
(334) Hodara, R.; Norris, E. H.; Giasson, B. I.; Mishizen-Eberz, A. J.; Lynch, D. R.; Lee, V. M. Y.; Ischiropoulos, H. Functional consequences of alpha-synuclein tyrosine nitration - Diminished binding to lipid vesicles and increased fibril formation. *J. Biol. Chem.* **2004**, *279* (46), 47746−47753.
(335) Turko, I. V.; Murad, F. Protein nitration in cardiovascular diseases. *Pharmacol. Rev.* **2002**, *54* (4), 619−634.
(336) Lemmon, M. A.; Schlessinger, J. Cell Signaling by Receptor Tyrosine Kinases. *Cell* **2010**, *141* (7), 1117−1134.
(337) Ghosh, S.; Janocha, A. J.; Aronica, M. A.; et al. Nitrotyrosine proteome survey in asthma identifies oxidative mechanism of catalase inactivation. *J. Immunol.* **2006**, *176* (9), 5587−5597.
(338) Murata, M.; Kawanishi, S. Oxidative DNA damage induced by nitrotyrosine, a biomarker of inflammation. *Biochem. Biophys. Res. Commun.* **2004**, *316* (1), 123−128.
(339) Eisen, H. N.; Carsten, M. E.; Belman, S. Studies of hypersensitivity to low molecular weight substances 3. The 2,4 Dinitrophenyl group as a determinat in the precipitin reaction. *J. Immunol.* **1954**, *73* (5), 296−308.
(340) Ovary, Z.; Benacerraf, B. Immunological specificity of secondary response with dinitrophenylated proteins. *Exp. Biol. Med.* **1963**, *114* (1), 72−76.
(341) Frumess, G. M. Allergic reaction to dinitrophenol - Report of case. *J. Am. Med. Assoc* **1934**, *102*, 1219−1220.
(342) Parker, C. W.; Kern, M.; Eisen, H. N. Polyfunctional dinitrophenyl haptens as reagents for elicitation of immediate type allergic skin responses. *J. Exp. Med.* **1962**, *115* (4), 789−801.
(343) Ishida, M.; Amesara, R.; Ukai, K.; Sakakura, Y. Antigen (DNP-AS)-induced rhinitis model in guinea-pigs. *Ann. Allergy* **1994**, *72* (3), 240−244.
(344) Landsteiner, K.; Jacobs, J. Studies on the sensitization of animals with simple chemical compounds. *J. Exp. Med.* **1935**, *61* (5), 643−656.
(345) Diesner, S. C.; Schultz, C.; Ackaert, C.; et al. Nitration of β-Lactoglobulin but Not of Ovomucoid Enhances Anaphylactic Responses in Food Allergic Mice. *PLoS One* **2015**, *10* (5), e0126279.
(346) Pastorello, E. A.; Farioli, L.; Conti, A.; et al. Wheat IgE-mediated food allergy in European patients: alpha-amylase inhibitors, lipid transfer proteins and low-molecular-weight glutenins. Allergenic molecules recognized by double-blind, placebo-controlled food challenge. *Int. Arch. Allergy Immunol.* **2007**, *144* (1), 10−22.
(347) Junker, Y.; Zeissig, S.; Kim, S.-J.; et al. Wheat amylase trypsin inhibitors drive intestinal inflammation via activation of toll-like receptor 4. *J. Exp. Med.* **2012**, *209* (13), 2395−2408.
(348) Sander, I.; Rozynek, P.; Rihs, H. P.; et al. Multiple wheat flour allergens and cross-reactive carbohydrate determinants bind IgE in baker's asthma. *Allergy* **2011**, *66* (9), 1208−1215.
(349) Zevallos, V. F.; Raker, V.; Tenzer, S.; et al. Nutritional Wheat Amylase-Trypsin Inhibitors Promote Intestinal Inflammation via Activation of Myeloid Cells. *Gastroenterology* **2016**, DOI: 10.1053/j.gastro.2016.12.006.
(350) Becker, K.; Lucas, K.; Bockamp, E.; Zevallos, V. F.; Ashfaq-Khan, M.; Bellinghausen, I.; Saloga, J.; Schuppan, D.; Pöschl, U. 28. Mainzer Allergie-Workshop-Abstract: Modulation of innate immune reactions upon interaction of the Toll-like receptor 4 with chemically modified allergens. *Allergo Journal* **2016**, *25* (1), 36−36.
(351) Ziegler, K.; Lucas, K.; Bellinghausen, I.; Liu, F.; Ashfaq-Khan, M.; Saloga, J.; Schuppan, D.; Pöschl, U. 29. Mainzer Allergie-Workshop-Abstract: The effect of nitration on the allergenicity of wheat derived alpha amylase trypsin inhibitors. *Allergo J.* **2017**, *26* (1), 48−48.
(352) Hochscheid, R.; Schreiber, N.; Kotte, E.; Weber, P.; Cassel, W.; Yang, H.; Zhang, Y.; Pöschl, U.; Müller, B. Nitration of Protein Without Allergenic Potential Triggers Modulation of Antioxidant Response in Type II Pneumocytes. *J. Toxicol. Environ. Health, Part A* **2014**, *77* (12), 679−695.
(353) Yang, H.; Zhang, Y. Y.; Pöschl, U. Quantification of nitrotyrosine in nitrated proteins. *Anal. Bioanal. Chem.* **2010**, *397* (2), 879−886.
(354) Zhang, Y. Y.; Yang, H.; Pöschl, U. Analysis of nitrated proteins and tryptic peptides by HPLC-chip-MS/MS: site-specific quantification, nitration degree, and reactivity of tyrosine residues. *Anal. Bioanal. Chem.* **2011**, *399* (1), 459−471.
(355) Selzle, K.; Ackaert, C.; Kampf, C. J.; Kunert, A. T.; Duschl, A.; Oostingh, G. J.; Pöschl, U. Determination of nitration degrees for the birch pollen allergen Bet v 1. *Anal. Bioanal. Chem.* **2013**, *405* (27), 8945−8949.
(356) Nojima, K.; Fukaya, K.; Fukui, S.; Kanno, S. Studies on photochemistry of aromatic hydrocarbons II: The formation of nitrophenols and nitrobenzene by the photochemical reaction of benzene in the presence of nitrogen monoxide. *Chemosphere* **1975**, *4* (2), 77−82.
(357) Kohler, M.; Heeb, N. V. Determination of nitrated phenolic compounds in rain by liquid chromatography/atmospheric pressure chemical ionization mass spectrometry. *Anal. Chem.* **2003**, *75* (13), 3115−3121.

DOI: 10.1021/acs.est.6b04908
Environ. Sci. Technol. 2017, 51, 4119−4141

(358) Vione, D.; Maurino, V.; Minero, C.; Pelizzetti, E. Aqueous atmospheric chemistry: Formation of 2,4-dinitrophenol upon nitration of 2-nitrophenol and 4-nitrophenol in solution. *Environ. Sci. Technol.* **2005**, *39* (20), 7921−7931.
(359) Lin, J. K.; Chen, K. J.; Liu, G. Y.; Chu, Y. R.; Lin-Shiau, S. Y. Nitration and hydroxylation of aromatic amino acid and guanine by the air pollutant peroxyacetyl nitrate. *Chem.-Biol. Interact.* **2000**, *127* (3), 219−236.
(360) Mikhailov, E.; Vlasenko, S.; Martin, S. T.; Koop, T.; Pöschl, U. Amorphous and crystalline aerosol particles interacting with water vapor: conceptual framework and experimental evidence for restructuring, phase transitions and kinetic limitations. *Atmos. Chem. Phys.* **2009**, *9* (2), 9491−9522.
(361) Shiraiwa, M.; Ammann, M.; Koop, T.; Pöschl, U. Gas uptake and chemical aging of semisolid organic aerosol particles. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108* (27), 11003−11008.
(362) Shiraiwa, M.; Sosedova, Y.; Rouviere, A.; Yang, H.; Zhang, Y. Y.; Abbatt, J. P. D.; Ammann, M.; Pöschl, U. The role of long-lived reactive oxygen intermediates in the reaction of ozone with aerosol particles. *Nat. Chem.* **2011**, *3* (4), 291−295.
(363) Sandhiya, L.; Kolandaivel, P.; Senthilkumar, K. Oxidation and Nitration of Tyrosine by Ozone and Nitrogen Dioxide: Reaction Mechanisms and Biological and Atmospheric Implications. *J. Phys. Chem. B* **2014**, *118* (13), 3479−3490.
(364) Radi, R.; Peluffo, G.; Alvarez, M. N.; Naviliat, M.; Cayota, A. Unraveling peroxynitrite formation in biological systems. *Free Radical Biol. Med.* **2001**, *30* (5), 463−488.
(365) Beckman, J. S.; Beckman, T. W.; Chen, J.; Marshall, P. A.; Freeman, B. A. Apparent hydroxyl radical production by peroxynitrite-implications for endothelial injury from nitric-oxide and superoxide. *Proc. Natl. Acad. Sci. U. S. A.* **1990**, *87* (4), 1620−1624.
(366) Ischiropoulos, H.; Zhu, L.; Chen, J.; Tsai, M.; Martin, J. C.; Smith, C. D.; Beckman, J. S. Peroxynitrite-mediated tyrosine nitartion catalyzed by superoxide-dismutase. *Arch. Biochem. Biophys.* **1992**, *298* (2), 431−437.
(367) Grossi, L. Evidence of an electron-transfer mechanism in the peroxynitrite-mediated oxidation of 4-alkylphenols and tyrosine. *J. Org. Chem.* **2003**, *68* (16), 6349−6353.
(368) Kampf, C. J.; Liu, F.; Reinmuth-Selzle, K.; Berkemeier, T.; Meusel, H.; Shiraiwa, M.; Pöschl, U. Protein Cross-Linking and Oligomerization through Dityrosine Formation upon Exposure to Ozone. *Environ. Sci. Technol.* **2015**, *49* (18), 10859−10866.
(369) Hecker, J.; Diethers, A.; Schulz, D.; et al. An IgE epitope of Bet v 1 and fagales PR10 proteins as defined by a human monoclonal IgE. *Allergy* **2012**, *67* (12), 1530−1537.
(370) Kofler, S.; Asam, C.; Eckhard, U.; Wallner, M.; Ferreira, F.; Brandstetter, H. Crystallographically Mapped Ligand Binding Differs in High and Low IgE Binding Isoforms of Birch Pollen Allergen Bet v 1. *J. Mol. Biol.* **2012**, *422* (1), 109−123.
(371) Mogensen, J. E.; Wimmer, R.; Larsen, J. N.; Spangfort, M. D.; Otzen, D. E. The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. *J. Biol. Chem.* **2002**, *277* (26), 23684−23692.
(372) Seutter von Loetzen, C.; Hoffmann, T.; Hartl, M. J.; Schweimer, K.; Schwab, W.; Rosch, P.; Hartl-Spiegelhauer, O. Secret of the major birch pollen allergen Bet v 1: identification of the physiological ligand. *Biochem. J.* **2014**, *457* (3), 379−390.
(373) Asam, C.; Batista, A. L.; Moraes, A. H.; et al. Bet v 1-a Trojan horse for small ligands boosting allergic sensitization? *Clin. Exp. Allergy* **2014**, *44* (8), 1083−1093.
(374) Gould, H. J.; Sutton, B. J. IgE in allergy and asthma today. *Nat. Rev. Immunol.* **2008**, *8* (3), 205−217.
(375) Rosenwasser, L. J. Mechanisms of IgE Inflammation. *Curr. Allergy Asthma Rep.* **2011**, *11* (2), 178−183.
(376) Hlavacek, W. S.; Perelson, A. S.; Sulzer, B.; Bold, J.; Paar, J.; Gorman, W.; Posner, R. G. Quantifying aggregation of IgE-Fc epsilon RI by multivalent antigen. *Biophys. J.* **1999**, *76* (5), 2421−2431.
(377) Davies, K. J. A. Degradation of oxidized proteins by the 20S proteasome. *Biochimie* **2001**, *83* (3−4), 301−310.
(378) Rosenberg, A. S. Effects of protein aggregates: An immunologic perspective. *AAPS J.* **2006**, *8* (3), E501−E507.
(379) Bellinghausen, I.; Haeringer, B.; Lafargue, B.; Strand, D.; Koenig, B.; Decker, H.; Saloga, J. Allergological implication of the quaternary hexameric structure of the cockroach allergen Per a 3. *Clin. Exp. Allergy* **2008**, *38* (3), 539−548.
(380) Vrtala, S.; Fohr, M.; Campana, R.; Baumgartner, C.; Valent, P.; Valenta, R. Genetic engineering of trimers of hypoallergenic fragments of the major birch pollen allergen, Bet v 1, for allergy vaccination. *Vaccine* **2011**, *29* (11), 2140−2148.
(381) Stadtman, E. R. Protein oxidation and aging. *Free Radical Res.* **2006**, *40* (12), 1250−1258.
(382) Ahmad, P.; Moinuddin; Ali, A. Peroxynitrite induced structural changes result in the generation of neo-epitopes on human serum albumin. *Int. J. Biol. Macromol.* **2013**, *59* (0), 349−356.
(383) Pfeiffer, S.; Schmidt, K.; Mayer, B. Dityrosine formation outcompetes tyrosine nitration at low steady-state concentrations of peroxynitrite - Implications for tyrosine modification by nitric oxide/superoxide in vivo. *J. Biol. Chem.* **2000**, *275* (9), 6346−6352.
(384) Heydenreich, B.; Bellinghausen, I.; Lorenz, S.; Henmar, H.; Strand, D.; Wurtzen, P. A.; Saloga, J. Reduced in vitro T-cell responses induced by glutaraldehyde-modified allergen extracts are caused mainly by retarded internalization of dendritic cells. *Immunology* **2012**, *136* (2), 208−217.
(385) Lund, L.; Henmar, H.; Wurtzen, P. A.; Lund, G.; Hjortskov, N.; Larsen, J. N. Comparison of allergenicity and immunogenicity of an intact allergen vaccine and commercially available allergoid products for birch pollen immunotherapy. *Clin. Exp. Allergy* **2007**, *37* (4), 564−571.
(386) Beckman, J. S. Oxidative Damage and Tyrosine Nitration from Peroxynitrite. *Chem. Res. Toxicol.* **1996**, *9* (5), 836−844.
(387) Davies, K. J. A.; Lin, S. W.; Pacifici, R. E. PROTEIN damage and degradation by oxygen radicals 0.4. Degradation of denatured protein. *J. Biol. Chem.* **1987**, *262* (20), 9914−9920.
(388) Gunaydin, H.; Houk, K. N. Mechanisms of Peroxynitrite-Mediated Nitration of Tyrosine. *Chem. Res. Toxicol.* **2009**, *22* (5), 894−898.
(389) Davies, K. J. A.; Delsignore, M. E. Protein damage and degradation by oxygen radicals 0.3. Modification of secondary and tertiary structure. *J. Biol. Chem.* **1987**, *262* (20), 9908−9913.
(390) Prütz, W. A.; Mönig, H.; Butler, J.; Land, E. J. Reactions of nitrogen dioxide in aqueous model systems: Oxidation of tyrosine units in peptides and proteins. *Arch. Biochem. Biophys.* **1985**, *243* (1), 125−134.
(391) Dalsgaard, T. K.; Nielsen, J. H.; Brown, B. E.; Stadler, N.; Davies, M. J. Dityrosine, 3,4-Dihydroxyphenylalanine (DOPA), and Radical Formation from Tyrosine Residues on Milk Proteins with Globular and Flexible Structures as a Result of Riboflavin-Mediated Photo-oxidation. *J. Agric. Food Chem.* **2011**, *59* (14), 7939−7947.
(392) Bunn, H. J.; Hewitt, C. R. A.; Grigg, J. Suppression of autologous peripheral blood mononuclear cell proliferation by alveolar macrophages from young infants. *Clin. Exp. Immunol.* **2002**, *128* (2), 313−317.
(393) Rubins, J. B. Alveolar Macrophages. *Am. J. Respir. Crit. Care Med.* **2003**, *167* (2), 103−104.
(394) Hussell, T.; Bell, T. J. Alveolar macrophages: plasticity in a tissue-specific context. *Nat. Rev. Immunol.* **2014**, *14* (2), 81−93.
(395) Knowles, M. R.; Boucher, R. C. Mucus clearance as a primary innate defense mechanism for mammalian airways. *J. Clin. Invest.* **2002**, *109* (5), 571−577.
(396) Minnicozzi, M.; Sawyer, R. T.; Fenton, M. J. Innate immunity in allergic disease. *Immunol. Rev.* **2011**, *242*, 106−127.
(397) Golebski, K.; Roschmann, K. I. L.; Toppila-Salmi, S.; Hammad, H.; Lambrecht, B. N.; Renkonen, R.; Fokkens, W. J.; van Drunen, C. M. The multi-faceted role of allergen exposure to the local airway mucosa. *Allergy* **2013**, *68* (2), 152−160.
(398) Mattila, P.; Joenvaara, S.; Renkonen, J.; Toppila-Salmi, S.; Renkonen, R. Allergy as an epithelial barrier disease. *Clin. Transl. Allergy* **2011**, *1* (1), 5.

DOI: 10.1021/acs.est.6b04908
Environ. Sci. Technol. 2017, 51, 4119−4141

(399) Irvine, A. D.; McLean, W. H.; Leung, D. Y. Filaggrin mutations associated with skin and allergic diseases. *N. Engl. J. Med.* **2011**, *365* (14), 1315−1327.
(400) Gandhi, V. D.; Vliagoftis, H. Airway Epithelium Interactions with Aeroallergens: Role of Secreted Cytokines and Chemokines in Innate Immunity. *Front. Immunol.* **2015**, *6*, 147.
(401) Joenvaara, S.; Mattila, P.; Renkonen, J.; et al. Caveolar transport through allergen Bet v 1 in allergic nasal epithelium of birch pollen patients. *J. Allergy Clin. Immunol.* **2009**, *124* (1), 135−142.
(402) Borcherding, J.; Baltrusaitis, J.; Chen, H.; et al. Iron oxide nanoparticles induce Pseudomonas aeruginosa growth, induce biofilm formation, and inhibit antimicrobial peptide function. *Environ. Sci.: Nano* **2014**, *1* (2), 123−132.
(403) Stohs, S. J.; Bagchi, D. Oxidative mechanisms in the toxicity of metal ions. *Free Radical Biol. Med.* **1995**, *18* (2), 321−336.
(404) Becker, S.; Soukup, J. M.; Gilmour, M. I.; Devlin, R. B. Stimulation of human and rat alveolar macrophages by urban air particulates: effects on oxidant radical generation and cytokine production. *Toxicol. Appl. Pharmacol.* **1996**, *141* (2), 637−648.
(405) Zorov, D. B.; Juhaszova, M.; Sollott, S. J. Mitochondrial ROS-induced ROS release: An update and review. *Biochim. Biophys. Acta, Bioenerg.* **2006**, *1757* (5−6), 509−517.
(406) Auerbach, A.; Hernandez, M. L. The effect of environmental oxidative stress on airway inflammation. *Curr. Opin. Allergy Clin. Immunol.* **2012**, *12* (2), 133−139.
(407) Lucas, K.; Maes, M. Role of the Toll Like receptor (TLR) radical cycle in chronic inflammation: possible treatments targeting the TLR4 pathway. *Mol. Neurobiol.* **2013**, *48* (1), 190−204.
(408) Bauer, R. N.; Diaz-Sanchez, D.; Jaspers, I. Effects of air pollutants on innate immunity: the role of Toll-like receptors and nucleotide-binding oligomerization domain-like receptors. *J. Allergy Clin. Immunol.* **2012**, *129* (1), 14−24.
(409) Peden, D. B. The role of oxidative stress and innate immunity in O_3 and endotoxin-induced human allergic airway disease. *Immunol. Rev.* **2011**, *242* (1), 91−105.
(410) Manzo, N.; LaGier, A.; Slade, R.; Ledbetter, A.; Richards, J.; Dye, J. Nitric oxide and superoxide mediate diesel particle effects in cytokine-treated mice and murine lung epithelial cells– implications for susceptibility to traffic-related air pollution. *Part. Fibre Toxicol.* **2012**, *9* (1), 43.
(411) Ghio, A. J.; Turi, J. L.; Yang, F.; Garrick, L. M.; Garrick, M. D. Iron homeostasis in the lung. *Biol. Res.* **2006**, *39* (1), 67−77.
(412) Li, N.; Wang, M. Y.; Bramble, L. A.; Schmitz, D. A.; Schauer, J. J.; Sioutas, C.; Harkema, J. R.; Nel, A. E. The Adjuvant Effect of Ambient Particulate Matter Is Closely Reflected by the Particulate Oxidant Potential. *Environ. Health Perspect.* **2009**, *117* (7), 1116−1123.
(413) Verma, V.; Rico-Martinez, R.; Kotra, N.; King, L.; Liu, J. M.; Snell, T. W.; Weber, R. J. Contribution of Water-Soluble and Insoluble Components and Their Hydrophobic/Hydrophilic Subfractions to the Reactive Oxygen Species-Generating Potential of Fine Ambient Aerosols. *Environ. Sci. Technol.* **2012**, *46* (20), 11384−11392.
(414) Gehling, W.; Dellinger, B. Environmentally Persistent Free Radicals and Their Lifetimes in PM2.5. *Environ. Sci. Technol.* **2013**, *47* (15), 8172−8178.
(415) Antinolo, M.; Willis, M. D.; Zhou, S.; Abbatt, J. P. D. Connecting the oxidation of soot to its redox cycling abilities. *Nat. Commun.* **2015**, *6*, 6812.
(416) Paget-Brown, A. O.; Ngamtrakulpanit, L.; Smith, A.; Bunyan, D.; Hom, S.; Nguyen, A.; Hunt, J. F. Normative data for pH of exhaled breath condensate. *Chest* **2006**, *129* (2), 426−430.
(417) Ricciardolo, F. L. M.; Gaston, B.; Hunt, J. Acid stress in the pathology of asthma. *J. Allergy Clin. Immunol.* **2004**, *113* (4), 610−619.
(418) Enami, S.; Hoffmann, M. R.; Colussi, A. J. Acidity enhances the formation of a persistent ozonide at aqueous ascorbate/ozone gas interfaces. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105* (21), 7365−7369.
(419) Pannala, V. R.; Bazil, J. N.; Camara, A. K. S.; Dash, R. K. A mechanistic mathematical model for the catalytic action of glutathione peroxidase. *Free Radical Res.* **2014**, *48* (4), 487−502.
(420) Bentley, A. R.; Emrani, P.; Cassano, P. A. Genetic variation and gene expression in antioxidant-related enzymes and risk of chronic obstructive pulmonary disease: a systematic review. *Thorax* **2008**, *63* (11), 956−961.
(421) Avissar, N. E.; Reed, C. K.; Cox, C.; Frampton, M. W.; Finkelstein, J. N. Ozone, but not nitrogen dioxide, exposure decreases glutathione peroxidases in epithelial lining fluid of human lung. *Am. J. Respir. Crit. Care Med.* **2000**, *162* (4), 1342−1347.
(422) Corradi, M.; Pignatti, P.; Brunetti, G.; Goldoni, M.; Caglieri, A.; Nava, S.; Moscato, G.; Balbi, B. Comparison between exhaled and bronchoalveolar lavage levels of hydrogen peroxide in patients with diffuse interstitial lung diseases. *Acta. Biomed* **2008**, *79* (Suppl 1), 73−78.
(423) Ahn, K. The role of air pollutants in atopic dermatitis. *J. Allergy Clin. Immunol.* **2014**, *134* (5), 993−999.
(424) Kim, J.; Kim, E. H.; Oh, I.; Jung, K.; Han, Y.; Cheong, H. K.; Ahn, K. Symptoms of atopic dermatitis are influenced by outdoor air pollution. *J. Allergy Clin. Immunol.* **2013**, *132* (2), 495−498.
(425) Pacher, P.; Beckman, J. S.; Liaudet, L. Nitric Oxide and Peroxynitrite in Health and Disease. *Physiol. Rev.* **2007**, *87* (1), 315−424.
(426) Cole, T. B.; Coburn, J.; Dao, K.; Roque, P.; Chang, Y. C.; Kalia, V.; Guilarte, T. R.; Dziedzic, J.; Costa, L. G. Sex and genetic differences in the effects of acute diesel exhaust exposure on inflammation and oxidative stress in mouse brain. *Toxicology* **2016**, *374*, 1−9.
(427) Li, N.; Georas, S.; Alexis, N.; Fritz, P.; Xia, T.; Williams, M. A.; Horner, E.; Nel, A. A work group report on ultrafine particles (American Academy of Allergy, Asthma & Immunology): Why ambient ultrafine and engineered nanoparticles should receive special attention for possible adverse health outcomes in human subjects. *J. Allergy Clin. Immunol.* **2016**, *138* (2), 386−396.
(428) Roque, P. J.; Dao, K.; Costa, L. G. Microglia mediate diesel exhaust particle-induced cerebellar neuronal toxicity through neuro-inflammatory mechanisms. *NeuroToxicology* **2016**, *56*, 204−214.
(429) Togias, A. Systemic effects of local allergic disease. *J. Allergy Clin. Immunol.* **2004**, *113* (1), S8−S14.
(430) Round, J. L.; Mazmanian, S. K. The gut microbiota shapes intestinal immune responses during health and disease. *Nat. Rev. Immunol.* **2009**, *9* (5), 313−323.
(431) Hooper, L. V.; Littman, D. R.; Macpherson, A. J. Interactions between the microbiota and the immune system. *Science (Washington, DC, U. S.)* **2012**, *336* (6086), 1268−1273.
(432) Haahtela, T.; Holgate, S.; Pawankar, R.; et al. The biodiversity hypothesis and allergic disease: world allergy organization position statement. *World Allergy Organ. J.* **2013**, *6* (1), 3.
(433) Hanski, I.; von Hertzen, L.; Fyhrquist, N.; et al. Environmental biodiversity, human microbiota, and allergy are interrelated. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109* (21), 8334−8339.
(434) Gollwitzer, E. S.; Saglani, S.; Trompette, A.; Yadava, K.; Sherburn, R.; McCoy, K. D.; Nicod, L. P.; Lloyd, C. M.; Marsland, B. J. Lung microbiota promotes tolerance to allergens in neonates via PD-L1. *Nat. Med.* **2014**, *20* (6), 642−647.
(435) Shanahan, F. The gut microbiota-a clinical perspective on lessons learned. *Nat. Rev. Gastroenterol. Hepatol.* **2012**, *9* (10), 609−614.
(436) Legatzki, A.; Rosler, B.; von Mutius, E. Microbiome diversity and asthma and allergy risk. *Curr. Allergy Asthma Rep.* **2014**, *14* (10), 466.
(437) Blázquez, A. B.; Berin, M. C. Microbiome and food allergy. *Transl. Res.* **2017**, *179*, 199−203.
(438) Riiser, A. The human microbiome, asthma, and allergy. *Allergy, Asthma, Clin. Immunol.* **2015**, *11*, 35.
(439) McCoy, K. D.; Koeller, Y. New developments providing mechanistic insight into the impact of the microbiota on allergic disease. *Clin. Immunol.* **2015**, *159* (2), 170−176.
(440) Fujimura, K. E.; Lynch, S. V. Microbiota in Allergy and Asthma and the Emerging Relationship with the Gut Microbiome. *Cell Host Microbe* **2015**, *17* (5), 592−602.

DOI: 10.1021/acs.est.6b04908
Environ. Sci. Technol. 2017, 51, 4119−4141

(441) Edwards, M. R.; Bartlett, N. W.; Hussell, T.; Openshaw, P.; Johnston, S. L. The microbiology of asthma. *Nat. Rev. Microbiol.* **2012**, *10* (7), 459−471.
(442) Hilty, M.; Burke, C.; Pedro, H.; et al. Disordered Microbial Communities in Asthmatic Airways. *PLoS One* **2010**, *5* (1), e8578.
(443) Huang, Y. J.; Nelson, C. E.; Brodie, E. L.; et al. Airway microbiota and bronchial hyperresponsiveness in patients with suboptimally controlled asthma. *J. Allergy Clin. Immunol.* **2011**, *127* (2), 372−381.
(444) Salim, S. Y.; Kaplan, G. G.; Madsen, K. L. Air pollution effects on the gut microbiota: a link between exposure and inflammatory disease. *Gut microbes* **2014**, *5* (2), 215−219.
(445) Heydenreich, B.; Bellinghausen, I.; Koenig, B.; Becker, W. M.; Grabbe, S.; Petersen, A.; Saloga, J. Gram-positive bacteria on grass pollen exhibit adjuvant activity inducing inflammatory T cell responses. *Clin. Exp. Allergy* **2012**, *42* (1), 76−84.
(446) CDC Website: Climate and Health. http://www.cdc.gov/climateandhealth/BRACE.htm.
(447) Runswick, S.; Mitchell, T.; Davies, P.; Robinson, C.; Garrod, D. R. Pollen proteolytic enzymes degrade tight junctions. *Respirology* **2007**, *12* (6), 834−842.
(448) Reed, C. E.; Kita, H. The role of protease activation of inflammation in allergic respiratory diseases. *J. Allergy Clin. Immunol.* **2004**, *114* (5), 997−1008.
(449) Millien, V. O.; Lu, W.; Shaw, J.; et al. Cleavage of fibrinogen by proteinases elicits allergic responses through Toll-like receptor 4. *Science* **2013**, *341* (6147), 792−796.
(450) Gilles, S.; Mariani, V.; Bryce, M.; Mueller, M. J.; Ring, J.; Jakob, T.; Pastore, S.; Behrendt, H.; Traidl-Hoffmann, C. Pollen-derived E1-phytoprostanes signal via PPAR-gamma and NF-kappaB-dependent mechanisms. *J. Immunol.* **2009**, *182* (11), 6653−8.
(451) Boldogh, I.; Bacsi, A.; Choudhury, B. K.; Dharajiya, N.; Alam, R.; Hazra, T. K.; Mitra, S.; Goldblum, R. M.; Sur, S. ROS generated by pollen NADPH oxidase provide a signal that augments antigen-induced allergic airway inflammation. *J. Clin. Invest.* **2005**, *115* (8), 2169−2179.
(452) Wimmer, M.; Alessandrini, F.; Gilles, S.; et al. Pollen-derived adenosine is a necessary cofactor for ragweed allergy. *Allergy* **2015**, *70* (8), 944−954.
(453) Berrens, L.; de la Cuadra Lopez, B. Complement activating agents in allergenic extracts. *Inflammation Res.* **1997**, *46* (11), 455−460.
(454) Blume, C.; Swindle, E. J.; Gilles, S.; Traidl-Hoffmann, C.; Davies, D. E. Low molecular weight components of pollen alter bronchial epithelial barrier functions. *Tissue barriers* **2015**, *3* (3), e1062316.
(455) Eisenbarth, S. C.; Piggott, D. A.; Huleatt, J. W.; Visintin, I.; Herrick, C. A.; Bottomly, K. Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J. Exp. Med.* **2002**, *196* (12), 1645−1651.
(456) Inamdar, A. A.; Bennett, J. W. A common fungal volatile organic compound induces a nitric oxide mediated inflammatory response in Drosophila melanogaster. *Sci. Rep.* **2014**, *4*, 3833.
(457) Diaz-Sanchez, D.; Garcia, M. P.; Wang, M.; Jyrala, M.; Saxon, A. Nasal challenge with diesel exhaust particles can induce sensitization to a neoallergen in the human mucosa. *J. Allergy Clin. Immunol.* **1999**, *104* (6), 1183−1188.
(458) Riedl, M. A.; Landaw, E. M.; Saxon, A.; Diaz-Sanchez, D. Initial high-dose nasal allergen exposure prevents allergic sensitization to a neoantigen. *J. Immunol.* **2005**, *174* (11), 7440−7445.
(459) Pandya, R. J.; Solomon, G.; Kinner, A.; Balmes, J. R. Diesel exhaust and asthma: Hypotheses and molecular mechanisms of action. *Environ. Health Perspect.* **2002**, *110*, 103−112.
(460) Maes, T.; Provoost, S.; Lanckacker, E. A.; Cataldo, D. D.; Vanoirbeek, J. A.; Nemery, B.; Tournoy, K. G.; Joos, G. F. Mouse models to unravel the role of inhaled pollutants on allergic sensitization and airway inflammation. *Respir. Res.* **2010**, *11*, 7.
(461) Provoost, S.; Maes, T.; Joos, G. F.; Tournoy, K. G. Monocyte-derived dendritic cell recruitment and allergic T(H)2 responses after exposure to diesel particles are CCR2 dependent. *J. Allergy Clin. Immunol.* **2012**, *129* (2), 483−91.
(462) Devouassoux, G.; Saxon, A.; Metcalfe, D. D.; Prussin, C.; Colomb, M. G.; Brambilla, C.; Diaz-Sanchez, D. Chemical constituents of diesel exhaust particles induce IL-4 production and histamine release by human basophils. *J. Allergy Clin. Immunol.* **2002**, *109* (5), 847−853.
(463) Hiura, T. S.; Li, N.; Kaplan, R.; Horwitz, M.; Seagrave, J. C.; Nel, A. E. The role of a mitochondrial pathway in the induction of apoptosis by chemicals extracted from diesel exhaust particles. *J. Immunol.* **2000**, *165* (5), 2703−2711.
(464) Hiura, T. S.; Kaszubowski, M. P.; Li, N.; Nel, A. E. Chemicals in diesel exhaust particles generate reactive oxygen radicals and induce apoptosis in macrophages. *J. Immunol.* **1999**, *163* (10), 5582−5591.
(465) Dick, C. A.; Brown, D. M.; Donaldson, K.; Stone, V. The role of free radicals in the toxic and inflammatory effects of four different ultrafine particle types. *Inhalation Toxicol.* **2003**, *15* (1), 39−52.
(466) Siegel, P. D.; Saxena, R. K.; Saxena, Q. B.; Ma, J. K. H.; Ma, J. Y. C.; Yin, X. J.; Castranova, V.; Al-Humadi, N.; Lewis, D. M. Effect of diesel exhaust particulate (DEP) on immune responses: Contributions of particulate versus organic soluble components. *J. Toxicol. Environ. Health, Part A* **2004**, *67* (3), 221−231.
(467) Yang, H. M.; Antonini, J. M.; Barger, M. W.; Butterworth, L.; Roberts, J. R.; Ma, J. K. H.; Castranova, V.; Ma, J. Y. C. Diesel exhaust particles suppress macrophage function and slow the pulmonary clearance of Listeria monocytogenes in rats. *Environ. Health Perspect.* **2001**, *109* (5), 515−521.
(468) Bleck, B.; Tse, D. B.; Gordon, T.; Ahsan, M. R.; Reibman, J. Diesel Exhaust Particle-Treated Human Bronchial Epithelial Cells Upregulate Jagged-1 and OX40 Ligand in Myeloid Dendritic Cells via Thymic Stromal Lymphopoietin. *J. Immunol.* **2010**, *185* (11), 6636−6645.
(469) Li, N.; Buglak, N. Convergence of air pollutant-induced redox-sensitive signals in the dendritic cells contributes to asthma pathogenesis. *Toxicol. Lett.* **2015**, *237* (1), 55−60.
(470) Bayram, H.; Devalia, J. L.; Sapsford, R. J.; Ohtoshi, T.; Miyabara, Y.; Sagai, M.; Davies, R. J. The effect of diesel exhaust particles on cell function and release of inflammatory mediators from human bronchial epithelial cells in vitro. *Am. J. Respir. Cell Mol. Biol.* **1998**, *18* (3), 441−448.
(471) Fukuoka, A.; Matsushita, K.; Morikawa, T.; Takano, H.; Yoshimoto, T. Diesel exhaust particles exacerbate allergic rhinitis in mice by disrupting the nasal epithelial barrier. *Clin. Exp. Allergy* **2016**, *46* (1), 142−152.
(472) Kang, X. D.; Li, N.; Wang, M. Y.; Boontheung, P.; Sioutas, C.; Harkema, J. R.; Bramble, L. A.; Nel, A. E.; Loo, J. A. Adjuvant effects of ambient particulate matter monitored by proteomics of bronchoalveolar lavage fluid. *Proteomics* **2010**, *10* (3), 520−531.
(473) Xiao, G. G.; Nel, A. E.; Loo, J. A. Nitrotyrosine-modified proteins and oxidative stress induced by diesel exhaust particles. *Electrophoresis* **2005**, *26* (1), 280−292.
(474) Kanemitsu, H.; Nagasawa, S.; Sagai, M.; MORI, Y. Complement activation by diesel exhaust particles (DEP). *Biol. Pharm. Bull.* **1998**, *21* (2), 129−132.
(475) Walters, D. M.; Breysse, P. N.; Schofield, B.; Wills-Karp, M. Complement factor 3 mediates particulate matter−induced airway hyperresponsiveness. *Am. J. Respir. Cell Mol. Biol.* **2002**, *27* (4), 413−418.
(476) Liu, J.; Ballaney, M.; Al-alem, U.; Quan, C.; Jin, X.; Perera, F.; Chen, L. C.; Miller, R. L. Combined inhaled diesel exhaust particles and allergen exposure alter methylation of T helper genes and IgE production in vivo. *Toxicol. Sci.* **2008**, *102* (1), 76−81.
(477) Sofer, T.; Baccarelli, A.; Cantone, L.; Coull, B.; Maity, A.; Lin, X.; Schwartz, J. Exposure to airborne particulate matter is associated with methylation pattern in the asthma pathway. *Epigenomics* **2013**, *5* (2), 147−154.
(478) Tezza, G.; Mazzei, F.; Boner, A. Epigenetics of allergy. *Early Hum. Dev.* **2013**, *89*, S20−S21.

DOI: 10.1021/acs.est.6b04908
Environ. Sci. Technol. 2017, 51, 4119−4141

(479) Vork, K. L.; Broadwin, R. L.; Blaisdell, R. J. Developing asthma in childhood from exposure to secondhand tobacco smoke: Insights from a meta-regression. *Environ. Health Perspect.* **2007**, *115* (10), 1394−1400.
(480) Burke, H.; Leonardi-Bee, J.; Hashim, A.; Pine-Abata, H.; Chen, Y.; Cook, D. G.; Britton, J. R.; McKeever, T. M. Prenatal and passive smoke exposure and incidence of asthma and wheeze: systematic review and meta-analysis. *Pediatrics* **2012**, *129* (4), 735−744.
(481) Ni, L.; Chuang, C.-C.; Zuo, L. Fine particulate matter in acute exacerbation of COPD. *Front. Physiol.* **2015**, *6*, 294.
(482) Jiang, L.; Diaz, P. T.; Best, T. M.; Stimpfl, J. N.; He, F.; Zuo, L. Molecular characterization of redox mechanisms in allergic asthma. *Ann. Allergy, Asthma, Immunol.* **2014**, *113* (2), 137−142.
(483) Zuo, L.; Otenbaker, N. P.; Rose, B. A.; Salisbury, K. S. Molecular mechanisms of reactive oxygen species-related pulmonary inflammation and asthma. *Mol. Immunol.* **2013**, *56* (1−2), 57−63.
(484) Bayram, H.; Rusznak, C.; Khair, O. A.; Sapsford, R. J.; Abdelaziz, M. M. Effect of ozone and nitrogen dioxide on the permeability of bronchial epithelial cell cultures of non-asthmatic and asthmatic subjects. *Clin. Exp. Allergy* **2002**, *32* (9), 1285−1292.
(485) Park, J.-W.; Taube, C.; Joetham, A.; et al. Complement activation is critical to airway hyperresponsiveness after acute ozone exposure. *Am. J. Respir. Crit. Care Med.* **2004**, *169* (6), 726−732.
(486) Cyphert, J. M.; Trempus, C. S.; Garantziotis, S. Size Matters: Molecular Weight Specificity of Hyaluronan Effects in Cell Biology. *Int. J. Cell Biol.* **2015**, *2015*, 563818.
(487) Bevelander, M.; Mayette, J.; Whittaker, L. A.; et al. Nitrogen dioxide promotes allergic sensitization to inhaled antigen. *J. Immunol.* **2007**, *179* (6), 3680−3688.
(488) Ezratty, V.; Guillossou, G.; Neukirch, C.; et al. Repeated nitrogen dioxide exposures and eosinophilic airway inflammation in asthmatics: a randomized crossover study. *Environ. Health Perspect.* **2014**, *122* (8), 850−855.
(489) Savage, J. H.; Matsui, E. C.; Wood, R. A.; Keet, C. A. Urinary levels of triclosan and parabens are associated with aeroallergen and food sensitization. *J. Allergy Clin. Immunol.* **2012**, *130* (2), 453−460.
(490) Clayton, E. M. R.; Todd, M.; Dowd, J. B.; Aiello, A. E. The Impact of Bisphenol A and Triclosan on Immune Parameters in the U.S. Population, NHANES 2003−2006. *Environ. Health Perspect.* **2011**, *119* (3), 390−396.
(491) Lee, M. H.; Chung, S. W.; Kang, B. Y.; Park, J.; Lee, C. H.; Hwang, S. Y.; Kim, T. S. Enhanced interleukin-4 production in CD4+ T cells and elevated immunoglobulin E levels in antigen-primed mice by bisphenol A and nonylphenol, endocrine disruptors: involvement of nuclear factor-AT and Ca2+. *Immunology* **2003**, *109* (1), 76−86.
(492) Wayne, P.; Foster, S.; Connolly, J.; Bazzaz, F.; Epstein, P. Production of allergenic pollen by ragweed (Ambrosia artemisiifolia L.) is increased in CO2-enriched atmospheres. *Ann. Allergy, Asthma, Immunol.* **2002**, *88* (3), 279−282.
(493) Singer, B. D.; Ziska, L. H.; Frenz, D. A.; Gebhard, D. E.; Straka, J. G. Increasing Amb a 1 content in common ragweed (Ambrosia artemisiifolia) pollen as a function of rising atmospheric CO_2 concentration. *Funct. Plant Biol.* **2005**, *32* (7), 667−670.
(494) Ziska, L. H.; Gebhard, D. E.; Frenz, D. A.; Faulkner, S.; Singer, B. D.; Straka, J. G. Cities as harbingers of climate change: Common ragweed, urbanization, and public health. *J. Allergy Clin. Immunol.* **2003**, *111* (2), 290−295.
(495) Clot, B. Trends in airborne pollen: An overview of 21 years of data in Neuchatel (Switzerland). *Aerobiologia* **2003**, *19* (3−4), 227−234.
(496) Ahlholm, J. U.; Helander, M. L.; Savolainen, J. Genetic and environmental factors affecting the allergenicity of birch (Betula pubescens ssp. czerepanovii Orl. Hamet-Ahti) pollen. *Clin. Exp. Allergy* **1998**, *28* (11), 1384−1388.
(497) Low, S. Y.; Dannemiller, K.; Yao, M.; Yamamoto, N.; Peccia, J. The allergenicity of Aspergillus fumigatus conidia is influenced by growth temperature. *Fungal Biol.* **2011**, *115* (7), 625−632.
(498) Moreland, J. L.; Gramada, A.; Buzko, O. V.; Zhang, Q.; Bourne, P. E. The Molecular Biology Toolkit (MBT): a modular platform for developing molecular visualization applications. *BMC Bioinf.* **2005**, *6* (21), 21.

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Atmospheric protein chemistry influenced by anthropogenic air pollutants: nitration and oligomerization upon exposure to ozone and nitrogen dioxide

Fobang Liu[1], Pascale S. J. Lakey[1], Thomas Berkemeier[1,2], Haijie Tong[1], Anna T. Kunert[1], Hannah Meusel[1], Yafang Cheng[1], Hang Su[1], Janine Fröhlich-Nowoisky[1], Senchao Lai[3], Michael G. Weller[4], Manabu Shiraiwa[5], Ulrich Pöschl[1], Christopher J. Kampf[1,6]

[1]Multiphase Chemistry Department, Max Planck Institute for Chemistry, Hahn-Meitner-Weg 1, 55128 Mainz, Germany; [2]School of Chemical & Biomolecular Engineering, Georgia Tech, Atlanta, GA, USA; [3]School of Environment and Energy, South China University of Technology, Higher Education Mega Center, Guangzhou 510006, P. R. China; [4]Division 1.5 Protein Analysis, Federal Institute for Materials Research and Testing (BAM), Richard-Willstätter-Str. 11, 12489 Berlin, Germany; [5]Department of Chemistry, University of California, Irvine, CA, USA; [6]Institute for Organic Chemistry, Johannes Gutenberg University Mainz, Duesbergweg 10-14, 55128 Mainz, Germany.

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Atmospheric protein chemistry influenced by anthropogenic air pollutants: nitration and oligomerization upon exposure to ozone and nitrogen dioxide†

Fobang Liu,[a] Pascale S. J. Lakey,[a] Thomas Berkemeier,[ab] Haijie Tong,[a] Anna Theresa Kunert,[a] Hannah Meusel,[a] Yafang Cheng,[a] Hang Su,[a] Janine Fröhlich-Nowoisky,[a] Senchao Lai,[c] Michael G. Weller,[d] Manabu Shiraiwa,[e] Ulrich Pöschl[a] and Christopher J. Kampf *[af]

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The allergenic potential of airborne proteins may be enhanced *via* post-translational modification induced by air pollutants like ozone (O_3) and nitrogen dioxide (NO_2). The molecular mechanisms and kinetics of the chemical modifications that enhance the allergenicity of proteins, however, are still not fully understood. Here, protein tyrosine nitration and oligomerization upon simultaneous exposure of O_3 and NO_2 were studied in coated-wall flow-tube and bulk solution experiments under varying atmospherically relevant conditions (5–200 ppb O_3, 5–200 ppb NO_2, 45–96% RH), using bovine serum albumin as a model protein. Generally, more tyrosine residues were found to react *via* the nitration pathway than *via* the oligomerization pathway. Depending on reaction conditions, oligomer mass fractions and nitration degrees were in the ranges of 2.5–25% and 0.5–7%, respectively. The experimental results were well reproduced by the kinetic multilayer model of aerosol surface and bulk chemistry (KM-SUB). The extent of nitration and oligomerization strongly depends on relative humidity (RH) due to moisture-induced phase transition of proteins, highlighting the importance of cloud

[a]*Multiphase Chemistry Department, Max Planck Institute for Chemistry, Hahn-Meitner-Weg 1, 55128 Mainz, Germany*
[b]*School of Chemical & Biomolecular Engineering, Georgia Tech, Atlanta, GA, USA*
[c]*School of Environment and Energy, South China University of Technology, Higher Education Mega Center, Guangzhou 510006, P. R. China*
[d]*Division 1.5 Protein Analysis, Federal Institute for Materials Research and Testing (BAM), Richard-Willstätter-Str. 11, 12489 Berlin, Germany*
[e]*Department of Chemistry, University of California, Irvine, CA, USA*
[f]*Institute for Organic Chemistry, Johannes Gutenberg University Mainz, Duesbergweg 10-14, 55128 Mainz, Germany. E-mail: kampfc@uni-mainz.de*

† Electronic supplementary information (ESI) available: Chemical mechanism and corresponding parameters used in kinetic modelling (Table S1), schematic experimental setup (Fig. S1), and SEC calibration curve (Fig. S2). See DOI: 10.1039/c7fd00005g

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processing conditions for accelerated protein chemistry. Dimeric and nitrated species were major products in the liquid phase, while protein oligomerization was observed to a greater extent for the solid and semi-solid phase states of proteins. Our results show that the rate of both processes was sensitive towards ambient ozone concentration, but rather insensitive towards different NO_2 levels. An increase of tropospheric ozone concentrations in the Anthropocene may thus promote pro-allergic protein modifications and contribute to the observed increase of allergies over the past decades.

1. Introduction

Allergies represent an important issue for human health and the prevalence of allergic diseases has been increasing worldwide over the past decades.[1,2] Among other explanations, air pollution has been proposed as a potential driver for this increase.[3–6] It is well established that air pollutants, especially diesel exhaust particles (DEPs), can act as adjuvants and facilitate allergic sensitization in the human body.[7,8] Air pollutants like nitrogen dioxide (NO_2), sulfur dioxide (SO_2), and ozone (O_3) have been shown to interact with and modify allergen carriers like pollen grains and fungal spores, increasing the release of allergenic proteins.[8,9] Moreover, post-translational modifications (PTM) of allergenic proteins can be induced by reactive trace gases such as O_3 and NO_2 which modifies their structure and activity, thus altering the immunogenicity of the proteins.[10–13]

Airborne allergenic proteins (aeroallergens) are contained not only in coarse biological particles such as pollen grains,[14] but also in the fine fraction of air particulate matter (aerodynamic diameter < 2.5 μm).[15–17] The occurrence of allergenic proteins in fine particles can be explained by several processes, including the release of pollen cytoplasmic granules (PCGs) from the rupture of pollen grains,[9] fragmentation of airborne cellular material,[18] and contact transfer of allergenic proteins onto fine particles.[18,19] Therefore, aeroallergens can be directly exposed to ambient O_3 and NO_2, promoting chemical modifications like tyrosine (Tyr) nitration and oligomerization.

Laboratory and field investigations have shown that proteins can be oxidized, nitrated and/or oligomerized upon exposure to NO_2 and O_3 in synthetic gas mixtures or polluted urban air.[11,12,15,20] The mechanisms of protein nitration by O_3 and NO_2, and protein cross-linking (oligomerization) by O_3 both involve the formation of long-lived reactive oxygen intermediates (ROIs), which are most likely tyrosyl radicals, as proposed earlier.[12,20–24] The ROIs can subsequently react with each other forming dityrosine (DTyr) crosslinks with NO_2 to form nitrotyrosine (NTyr) residues, or undergo further oxidation reactions. Using quantum chemical methods, Sandhiya *et al.*[22] showed that six different intermediates can be formed through the initial oxidation of Tyr residues by O_3, out of which the tyrosyl radical is favorable due to a small energy barrier, particularly in the aqueous phase. In the absence of NO_2, tyrosyl radicals can undergo self-reaction to stabilize in the form of dimers. Under physiological conditions, Pfeiffer *et al.*[25] found that DTyr was a major product of Tyr modification caused by low steady-state concentrations of peroxynitrite, while high fluxes (>2 μM s^{-1}) of nitrogen oxide/superoxide anions (NO/O_2^-) are required to render peroxynitrite an efficient trigger of Tyr nitration. Thus, kinetic competition between Tyr nitration and dimerization (or oligomerization) upon protein exposure to O_3 and NO_2 can be

expected, which needs to be explored in detail to assess the relevant atmospheric conditions that favor potentially health relevant protein modifications.

In this study, we explored the oxidation, nitration, and oligomerization reactions of proteins induced by O_3 and NO_2, and their kinetics under different atmospherically relevant conditions using bovine serum albumin (BSA) as a model protein. Coated-wall flow-tube and bulk solution experiments were performed to study the kinetics of protein nitration and oligomerization at O_3 and NO_2 concentrations of 5–200 ppb, and relative humidities (RH) of 45% and 96%, utilizing a size exclusion chromatography/spectrophotometry method. Additionally, we used the kinetic multilayer model of aerosol surface and bulk chemistry (KM-SUB)[26] to investigate which chemical reactions and transport processes control the concentration and time dependence of protein oligomerization and nitration.

2. Experimental

2.1. Materials

Bovine serum albumin (BSA, A5611) and sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$, 71504), were purchased from Sigma Aldrich (Germany). Sodium hydroxide (NaOH, 0583) was purchased from VWR (Germany). 10× Tris/glycine/SDS (161-0732) was purchased from Bio-Rad Laboratories (USA). High purity water (18.2 MΩ cm) for chromatography was taken from a Milli-Q Integral 3 water purification system (Merck Millipore, USA). The high purity water (18.2 MΩ cm) was autoclaved before use if not specified otherwise.

2.2. Protein O_3/NO_2 exposure setup

Reactions of BSA with O_3/NO_2 mixtures were performed both homogeneously in aqueous solutions and heterogeneously *via* the exposure of BSA-coated glass tubes to gaseous O_3/NO_2 at different levels of relative humidity (RH). Before the exposure experiments, BSA solutions (0.6 mL, 0.33 mg mL^{-1}) were loaded into the glass tube and dried by passing a nitrogen (N_2, 99.999%) flow at ~1 L min^{-1} through a specific rotating device,[27] which is essential to ensure homogeneous coating and experiment reproducibility. The BSA-coated glass tube was then connected to the experimental setup. The experimental setup (Fig. S1, ESI†) was described previously,[20] and was extended by incorporating an additional flow of NO_2 after the humidifier.

Briefly, ozone was produced from synthetic air passed through a UV lamp (L.O.T.-Oriel GmbH & Co. KG, Germany) at ~1.9 L min^{-1}. The gas flow was then split and one flow was passed through a Nafion® gas humidifier (MH-110-12F-4, PermaPure, USA) operated with autoclaved high purity water, while the other flow remained dry. RH could be varied in a wide range by adjusting the ratio between the dry and humidified air flow. During the experiments, the standard deviation from the set RH values was <2% RH. The gas flow with a set O_3 concentration and RH was then mixed with a N_2 flow containing ~5 ppmV NO_2 (Air Liquide, Germany). The NO_2 concentrations were adjusted by varying the flow rate (20–80 mL min^{-1}) of the ~5 ppmV NO_2 flow. The combined gas flow was then directed through the BSA-coated glass tube. The concentrations of O_3 and NO_2 as well as RH were measured using commercial monitoring instruments (Ozone analyzer,

49i, Thermo Scientific, Germany; NO_x analyzer, 42i-TL, Thermo Scientific, Germany; RH sensor FHA 646-E1C with ALMEMO 2590-3 instrument, Ahlborn, Mess- und Regelungstechnik, Germany). After exposure, the proteins were extracted from the glass tube with 1.5 mL of 1× Tris/glycine/SDS (pH 8.3) buffer to avoid precipitation of protein oligomers in the extract solution.

For homogeneous bulk solution reactions, the O_3/NO_2 gas mixtures were directly bubbled through 1.5 mL of 0.13 mg mL^{-1} BSA aqueous solutions (pH 7.0 ± 0.2; measured by a pH meter, model WTW multi 350i, WTW, Germany) at a flow rate of 60 mL min^{-1} using a Teflon tube (ID: 1.59 mm). All heterogeneous and homogeneous exposure experiments were performed in duplicate.

2.3. SEC-HPLC-DAD analysis

Product analysis was performed using high-performance liquid chromatography coupled to diode array detection (HPLC-DAD, Agilent Technologies 1200 series). The HPLC-DAD system consisted of a binary pump (G1379B), an autosampler with a thermostat (G1330B), a column thermostat (G1316B), and a photodiode array detector (DAD, G1315C). ChemStation software (Rev. B.03.01, Agilent) was used for system control and data analysis. Molecular weight (MW) separation by size exclusion chromatography (SEC) was carried out using an AdvanceBio SEC column (Agilent, 300 Å, 300 × 4.6 mm, 2.7 μm). Isocratic separation at a flow rate of 0.35 mL min^{-1} was carried out using a mobile phase of 150 mM NaH_2PO_4 buffer (adjusted to pH 7 with 10 M NaOH (aq)) after injecting 40 μL of sample. The absorbance was monitored by the DAD at wavelengths of 220, 280 and 357 nm. Each chromatographic run was performed in duplicate.

A protein standard mix 15–600 kDa (69385, Sigma Aldrich, Germany) containing bovine thyroglobulin (MW = 670 kDa), γ-globulin from bovine blood (MW = 150 kDa), chicken egg albumin, grade VI (MW = 44.3 kDa), and ribonuclease A (MW = 13.7 kDa) was used for SEC column calibration (elution time *vs.* log MW). Details can be found in Fig. S2 in the ESI.† It should be noted that SEC separates molecules according to their hydrodynamic sizes, thus only approximate molecular masses can be obtained by this calibration method.

2.4. Determination of protein oligomer mass fractions and nitration degrees

A detailed description of the simultaneous determination of protein oligomer mass fractions and nitration degrees using the SEC-HPLC-DAD analysis described above can be found in Liu *et al.*[28] Briefly, we report the formation of BSA oligomers as the temporal evolution in the ratios of the respective oligomers (dimers, trimers, and oligomers with $n \geq 4$) to the sum of monomer and all oligomer peak areas at the absorption wavelength of 220 nm. Assuming that the molar extinction coefficients of the individual protein oligomer fractions are multiples of the monomer extinction coefficient, the calculated oligomer ratios correspond to the mass fractions (ω) of the individual oligomers. Nitration degrees (NDs), defined as the concentration of nitrotyrosine (NTyr) divided by the sum of the concentrations of NTyr and Tyr, were obtained for BSA monomers and dimers, using the respective peak areas of the monomer and dimer signals at wavelengths of 280 nm and 357 nm. For calculation of the total protein ND, the sum of the peak areas of all protein signals at wavelengths of 280 nm and 357 nm was used. Note that corresponding to the definition of the ND, the same number of nitrated Tyr

residues in a BSA monomer and dimer will lead to a factor of 2 difference in the individual NDs, because a BSA dimer contains twice the number of Tyr residues compared to the monomer. Further information on the calculation of NDs can be found in Liu *et al.*[28] The values and errors of the calculated NDs and oligomer mass fractions represent the arithmetic mean values and standard deviations of duplicate experiments.[29] The commercially available BSA also contains dimers and trimers of the protein as well as pre-nitrated monomers and dimers (~NDs 0.9%). Therefore, the reported values of oligomer mass fractions and NDs were corrected for these background signals.

3. Results and discussion

3.1. Protein oligomerization

Fig. 1 and 2 show the effects of varying NO_2 and O_3 concentrations on protein oligomerization for homogeneous bulk solution and coated-wall flow-tube experiments, respectively. Exposures were carried out at fixed NO_2

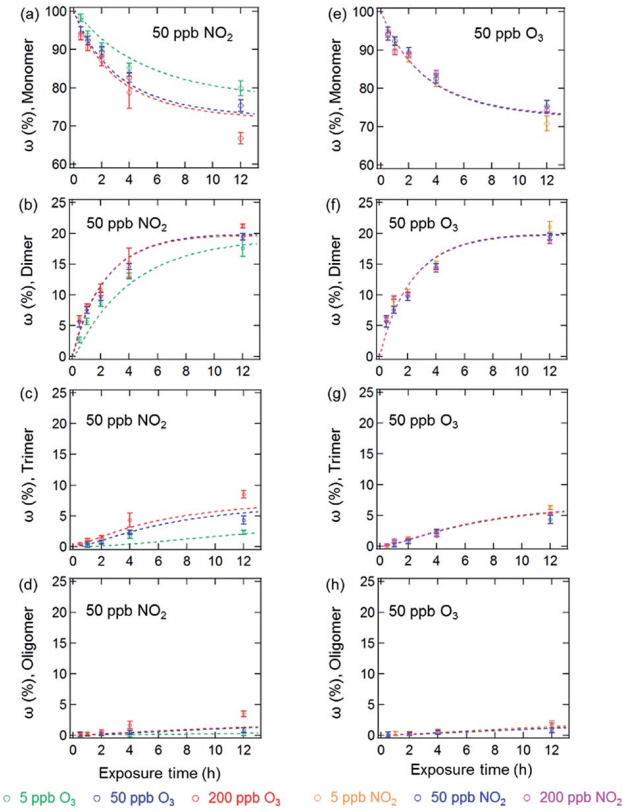

Fig. 1 Temporal evolution of protein oligomer mass fractions (ω (%), monomer, dimer, trimer and oligomer ($n \geq 4$)) in the aqueous phase reaction of BSA with O_3/NO_2: ((a)–(d)) at a fixed NO_2 concentration of 50 ppb with varied O_3 concentrations; ((e)–(h)) at a fixed O_3 concentration of 50 ppb with varied NO_2 concentrations. The data points and error bars represent the arithmetic mean values and standard deviations of duplicate experiments. The dashed lines are the results of the kinetic model.

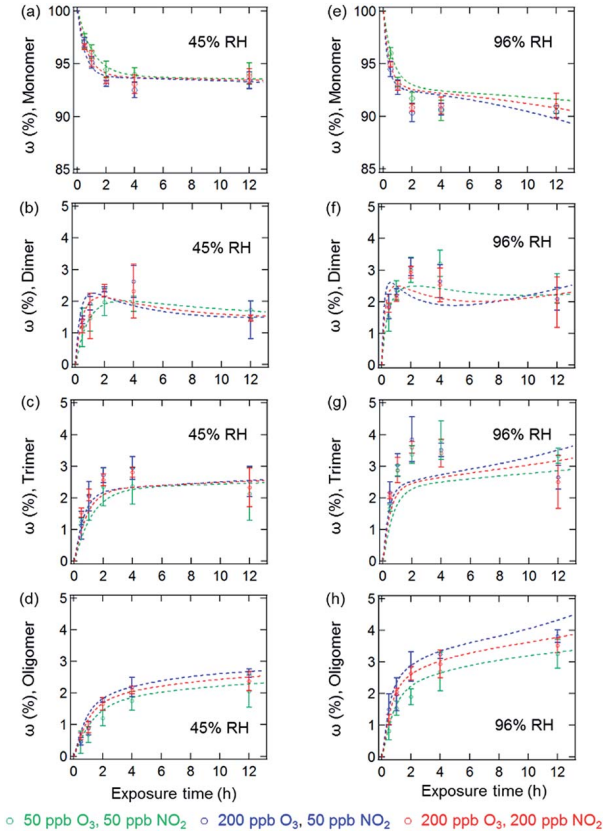

Fig. 2 Temporal evolution of protein oligomer mass fractions (ω (%), monomer, dimer, trimer and oligomer ($n \geq 4$)) upon exposure of BSA films (thickness 34 nm) to various O_3/NO_2 concentrations: ((a)–(d)) at 45% RH; ((e)–(h)) at 96% RH. The data points and error bars represent the arithmetic mean values and standard deviations of duplicate experiments. The dashed lines are the results of the kinetic model.

concentrations with varying O_3 concentrations and *vice versa*. The exposure time was varied from 0.5 up to 12 h. While in bulk solution experiments dimers were generally observed as the major reaction products of BSA with O_3 and NO_2, trimers or higher oligomers can be dominant products in the coated-wall flow-tube experiments at longer exposure times, depending on the experimental conditions.

The results of the bulk solution experiments on protein oligomerization are illustrated in Fig. 1. Generally, the mass fractions of dimers, trimers, and higher oligomers increase with increasing reaction times, reaching up to 21 $\pm$ 1% for dimers, 9 $\pm$ 1% for trimers, and 4 $\pm$ 1% for oligomers with $n \geq 4$ after 12 h of exposure. The minimum mass fraction of monomers correspondingly was found to be 66%. While varying the O_3 concentrations (Fig. 1a–d, fixed 50 ppb of NO_2) from 5 to 200 ppb significantly affected the temporal evolutions observed for the mass fractions of the different oligomers, changing the NO_2 concentration (Fig. 1e–h, fixed 50 ppb of O_3) in the same range did not result in significant changes in oligomer mass fractions. The solubility of O_3 and NO_2 in water is

$\sim 10^{-5}$ mol mL^{-1} (derived from their Henry's law constants: $K_{sol,cc,O_3} \approx K_{sol,cc,NO_2} \approx 10^{-2}$ M atm^{-1})[30] under our experimental conditions. Increasing O_3 and NO_2 gas concentrations between 5 to 200 ppb should result in concentrations of O_3 and NO_2 between 7×10^{-11} to 3×10^{-9} M in the aqueous phase. Thus, the insignificant change of oligomer mass fractions with varied NO_2 concentration should not be caused by a saturation of dissolved NO_2 in the concentration range investigated (5 to 200 ppb).

Mechanistically, the reactions between O_3/NO_2 and protein Tyr residues involve the formation of ROIs (tyrosyl radicals) resulting from the reaction of Tyr with O_3. These ROIs can then either react with NO_2 to form NTyr residues or cross-link due to intermolecular DTyr formation.[6,20] Ozonolysis of Tyr may also result in other oxidized products such as 3,4-dihydroxyphenylalanine (DOPA).[31] However, the reaction mechanism for the formation of these oxidized products is not the focus of this study and we only consider these modified Tyr derivatives in the proposed mechanism (Table S1†) as a portion of the oxidized amino acid residues. Regardless, an inhibition of intermolecular DTyr cross-linking would be expected with increasing NO_2 concentrations. However, no such behavior was observed. Furthermore, similar protein oligomer mass fractions were observed previously in the absence of NO_2 for BSA exposed to O_3 in bulk solution experiments with comparable levels of O_3 (50 and 200 ppb).[20] This observation may be due to the high number of accessible Tyr residues on the dissolved BSA molecules in solution, because after 12 h of exposure, 66% of BSA (Fig. 1a) is still present in monomeric form.

The results of the coated-wall flow-tube experiments on protein oligomerization are illustrated in Fig. 2. In these experiments, thin protein films were exposed to O_3/NO_2 mixtures. A film thickness of $\sim$34 nm, or roughly five layers of BSA, can be calculated assuming an even distribution of the BSA molecules on the inner surface of the glass tube.[20] The experiments were performed at 45% and 96% RH with O_3/NO_2 concentrations of 50/50, 200/50, and 50/200 ppb, respectively. Generally, the reactive sites accessible for oligomerization reactions of the proteins are limited here compared to aqueous solutions, leading to smaller mass fractions of protein oligomers. Furthermore, we observed a 30–40% reduction of the overall oligomer mass fraction (dimer, trimer, and oligomer $\geq$4) compared to similar flow-tube experiments in the absence of NO_2 for comparable RH and O_3 concentrations.[20] Apparently, the lower diffusivity of the proteins in this solid (45% RH) or semi-solid (96% RH) state induces a competition between DTyr and NTyr formation, also indicated by the observation of slower reaction rates for oligomerization in the bulk of the thin protein film compared to its surface.[20,23]

In contrast to the bulk solution experiments, which show a steady increase of the oligomer mass fractions with exposure time, dimer and trimer mass fractions in the flow tube experiments peaked at exposure times of 2–4 h, depending on RH and trace gas concentrations, while only higher oligomers steadily increased over the course of the reaction time (see Fig. 2). This result indicates that as the exposure proceeds, the formation of dimers and trimers becomes slower than their consumption converting them into higher oligomers. The characteristic residence times (lifetimes) of biological particles in the atmosphere can range from hours to weeks, depending on their sizes, aerodynamic, and hygroscopic properties.[32] Our observation indicates that the initial exposure (<2–4 h) of proteins to O_3 and NO_2 mainly leads to the formation of protein dimers and

trimers, and as the exposure proceeds, protein oligomers could be the dominant protein species, *e.g.*, on the surface of bioaerosol particles.

3.2. Protein nitration

Fig. 3 and 4 show the effects of varying NO_2 and O_3 concentrations on the nitration of protein monomers and dimers in homogeneous bulk solution and coated-wall flow-tube experiments, respectively. Exposures were carried out at fixed NO_2 concentrations with varying O_3 concentrations and *vice versa*. The exposure time was varied from 0.5 up to 12 h. While in previous studies total protein nitration degrees (NDs) were investigated upon exposure to O_3 and NO_2,[12,33] we explicitly explored and characterized the reaction kinetics of protein nitration, resolving the individual NDs of the protein monomer and its various oligomers over the course of reaction time.

The results of the bulk solution experiments on protein nitration are illustrated in Fig. 3. The maximum ND of protein monomers and dimers were found to be 7% and 5% after 12 h exposure to 200 ppb O_3 and 50 ppb NO_2, respectively. These results correspond to 1.4 NTyr residues per monomer molecule and 2 NTyr residues per dimer molecule (NTyr/monomer and NTyr/dimer are shown as secondary *y*-axes in Fig. 3 and 4). We found a positive relationship between the NDs and O_3 concentration (Fig. 3a and b), particularly that the increase of O_3 concentration by one order of magnitude from 5 to 50 ppb resulted in an increase of NDs from 4.2 ± 0.2% to 6.9 ± 0.2%, and 2.0 ± 0.3% to 4.5 ± 0.3% after 12 h exposure for the monomer and dimer, respectively. Also, for protein nitration, no significant difference was found when concentrations of NO_2 were varied from 5 to 200 ppb at a fixed O_3 concentration of 50 ppb, as shown in Fig. 3c and d. These results are in accordance with the observations made by Shiraiwa *et al.*[23] on the

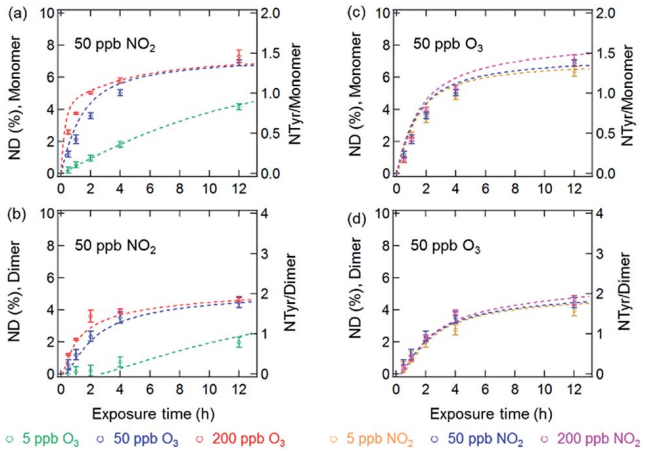

Fig. 3 NDs of protein monomer and dimer (primary *y*-axis), and NTyr number per monomer and dimer (secondary *y*-axis) plotted against reaction time in the aqueous phase reaction of BSA with O_3/NO_2: (a) and (b) at a fixed NO_2 concentration of 50 ppb with varied O_3 concentrations, and (c) and (d) at a fixed O_3 concentration of 50 ppb with varied NO_2 concentrations. The data points and error bars represent the arithmetic mean values and standard deviations of duplicate experiments. The dashed lines are the results of the kinetic model.

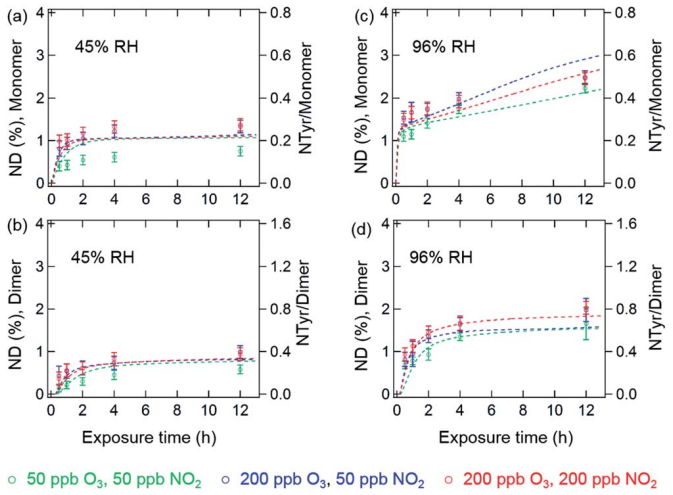

Fig. 4 NDs of protein monomer and dimer (primary *y*-axis), and NTyr number per monomer and dimer (secondary *y*-axis) plotted against exposure time upon the exposure of BSA films (thickness 34 nm) to various O_3/NO_2 concentrations: (a) and (b) at 45% RH, and (c) and (d) at 96% RH. The data points and error bars represent the arithmetic mean values and standard deviations of duplicate experiments. The dashed lines are the results of the kinetic model.

study of the reactive uptake of NO_2 by aerosolized proteins. They found that the uptake coefficient of NO_2 (γ_{NO_2}) increased with increasing O_3 concentrations while γ_{NO_2} decreased gradually with increasing NO_2 concentration. Thus, our results confirm that the protein reaction with O_3 and formation of ROI is the rate-limiting step for protein nitration.[21,23] Shiraiwa *et al.*[23] have excluded NO_3 or N_2O_5 (formed through NO_2 oxidation by O_3) as major contributors to protein nitration. Ghiani *et al.*[34] reported that nitration of proteins can also occur by nitrate ions in bulk solutions without UV irradiation under acidic conditions (pH < 3 for BSA). The reaction of NO_2 with water can form nitric acid and thereby nitrate ions might appear in the BSA solution. However, we found that the pH of the BSA solutions stayed relatively constant (pH 6.6 ± 0.2; pH meter model WTW multi 350i) for a reaction time of 12 h at 200 ppb NO_2 and 50 ppb O_3, indicating that nitration induced by nitrate ions is likely a minor or negligible pathway in this study. This hypothesis is consistent with the results in Fig. 3c and d that show only a slight increase in ND (monomer, 6.3 ± 0.3% to 6.9 ± 0.2%, and dimer, 4.0 ± 0.3% to 4.5 ± 0.3%, for 12 h reaction) for a one order of magnitude increase in the NO_2 concentration from 5 to 50 ppb.

The results of the temporal increase of NDs of monomer and dimer for reactions of the thin protein films with O_3 and NO_2 concentrations of 50 and 200 ppb at 45% and 96% RH are illustrated in Fig. 4. Here, the NDs for monomer and dimer at 45% RH were found to be around 1% and 0.8% for 12 h exposure, corresponding to 0.2 NTyr/monomer molecule and 0.3 NTyr/dimer molecule. Note that the protein coating consisted of ~5 layers. Therefore, the results likely indicate that on average one Tyr per BSA monomer molecule located on the surface of the protein film was efficiently nitrated, while the bulk nitration occurred at much slower rates. This highly reactive site could be the Tyr residue at

position 161 (Y161), as suggested by Zhang *et al.*[35] The maximum NDs for monomer and dimer reached up to 2.5 ± 0.1% and 2.0 ± 0.1% at 96% RH, respectively. However, both of the maximum NDs at 45% and 96% RH were much lower than those obtained for O_3/NO_2 exposure in aqueous solutions (200 ppb O_3 and 50 ppb NO_2). This discrepancy can be explained by a decrease in viscosity and an increase in diffusivity on going from an amorphous solid (45% RH) or semi-solid (96% RH) protein on a coated wall to an aqueous protein solution and was able to be reproduced using a model (see below).[36,37] Furthermore, the NDs of BSA in this study are comparable to those found by Yang *et al.*[33] using the same protein, whereas the nitration efficiency of BSA in general is found to be much lower than the one observed for the major birch pollen allergen Bet v 1 in similar exposure experiments,[12] indicating a strong influence of molecular structure and potentially the amino acid sequence of the reacting protein. From previous mass spectrometric analysis of the site selectivity of protein nitration by O_3/NO_2, it is known that only 3 out of 21 Tyr residues in BSA can be detected in the nitrated form, while in Bet v 1, 4 out of 7 Tyr residues can be nitrated.[12,35] Thus, besides the types of nitrating agents (*e.g.* $ONOO^-$ or O_3/NO_2) and reaction conditions (in aqueous solution or heterogeneous exposure), the nitration efficiency also depends on the fraction of reactive Tyr residues in the investigated protein.

3.3. Kinetics and mechanism of protein nitration and oligomerization by O_3/NO_2

The model results for the reactions of proteins with O_3/NO_2 under the various exposure conditions are shown as the dashed lines in Fig. 1–4. A chemical mechanism involving 19 reactions (see Table S1† for details) was applied in two kinetic models, *i.e.*, a box model for bulk solution experiments and a kinetic multilayer model for aerosol surface and bulk chemistry (KM-SUB)[26] for flow tube

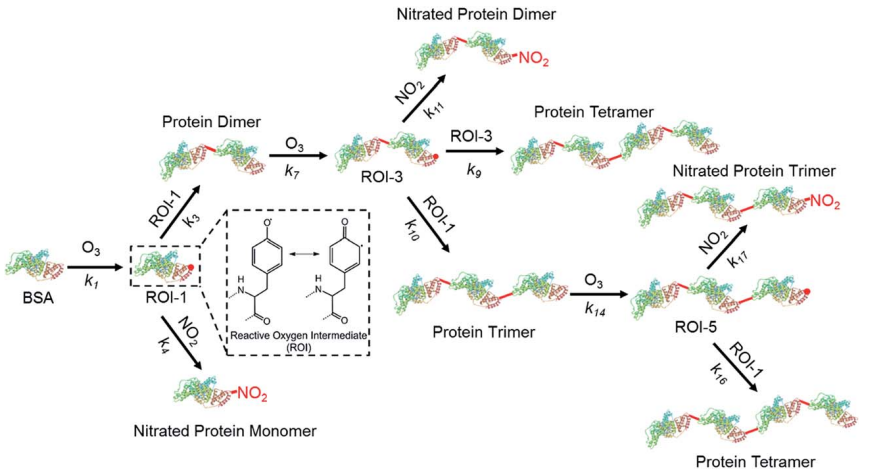

Fig. 5 Schematic overview of the most relevant reactions for protein nitration and oligomerization upon exposure to ozone and nitrogen dioxide. The reactions correspond to protein surface Tyr nitration and oligomerization in the mechanism presented in Table S1.† The molecular structure of the protein (BSA, PDB accession number 3V03) was created using RCSB PDB protein workshop (4.2.0) software.

experiments to fit the experimental data. The most relevant reactions for this mechanism are illustrated in Fig. 5. The first step in the mechanism is the reaction of a Tyr residue with O_3 forming tyrosyl radicals as long-lived reactive oxygen intermediates (ROIs). In the second step of the process, the ROIs can react with each other to form dimers, or with NO_2 to form nitrated monomers. Note that for simplification, each molecule only contains one reactive tyrosine residue, while nitrated and oxidized monomers, dimers and trimers are unable to take part in further reactions in the kinetic model. A dimer can react further with O_3 to form a dimeric ROI, which may react with NO_2 to form a nitrated protein dimer, with monomeric ROI to form a protein trimer or with another dimeric ROI to form a protein tetramer.

The following assumptions were made to enable modelling of the reaction system for bulk solution and coated-wall flow-tube experiments using the sets of physicochemical parameters shown in Table S1 (ESI†): BSA molecules have reactive amino acid residues on their surface (AA1) and in their bulk (AA2), both of them reactive towards O_3. While ROIs formed in the protein bulk can only react with NO_2 to form NTyr, they are unable to form intermolecular DTyr due to steric hindrance.[38] These assumptions were also applied to dimers and trimers. Besides Tyr, O_3 can also oxidize other amino acid residues, *i.e.*, cysteine (Cys), tryptophan (Trp), methionine (Met) and histidine (His).[31] Among them, only Cys is able to cross-link proteins directly upon O_3 exposure, yielding intermolecular disulfide bridges, as one free Cys is available in BSA.[39] This reversible cross-linking mechanism has been shown to be only a minor contributor to protein oligomerization upon O_3/NO_2 exposure previously.[20] The kinetic parameters were obtained using a global optimization method combining a uniformly-sampled Monte Carlo search with a genetic algorithm (MCGA method).[40,41] The genetic algorithm was terminated when the correlation between experimental data and model output reached an optimum. Concentrations of O_3 and NO_2 in the aqueous phase can be estimated using the published Henry's law constant of $K_{sol,cc,O_3} \approx K_{sol,cc,NO_2} \approx 10^{-2}$ M atm^{-1}, which were used as fixed values in the model.[30]

The temporal evolution of NDs and oligomer mass fractions in aqueous solution is well reproduced by the model (Fig. 1 and 3). For the heterogeneous reactions studied in the coated-wall flow-tube experiments at 45% RH and 96% RH, some substantial deviations between modelled and measured data can be observed, and the coefficient of determination (R^2 value) is approximately a factor of two lower than for the aqueous data. For example, the oligomer mass fractions ω at 45% RH in Fig. 2a–d are qualitatively captured fairly well, while the model fails to reproduce their evolution at higher RH, especially for the dimer and trimer (Fig. 2f and g). The observed reduction of dimers in flow tube experiments could be reproduced by the model including the reactions on the surface, where the rates are four orders of magnitude higher than that of bulk reactions. Despite simple model assumptions when describing the complex reaction system that was studied, the model reproduces the experimental data reasonably well with an overall R^2 value of 0.88 for Fig. 1–4. Most of the optimized parameters obtained for the flow tube experiments were close to or the same as those for aqueous solutions (for details see Table S1, ESI†). However, note that some of the rate coefficients would be expected to change as the liquid water content and viscosity varies. Water could actively take part in some of the reactions and its presence at different concentrations could lead to changes in experimental conditions, such

as pH, which would influence the rate of the reactions. As the viscosity increases it is also expected that some rate coefficients would decrease as they become diffusion-limited as per the Smoluchowski diffusion equation.[42,43]

The second-order rate coefficients obtained as model outputs and shown in Table S1† are mostly consistent with previous studies.[20,23] However, as the complex reaction mixture has been reflected in only 19 chemical reactions, the absolute numbers of the rate coefficients obtained for the individual reactions likely do not reflect reality, because further secondary chemistry of various kinds is not included explicitly. It should also be noted that different types of tyrosine residues have not been explicitly included within the model, although these can nitrate at different rates and have different surface accessibilities.[35,44] Nevertheless, qualitatively, the model results suggest that protein nitration occurs at faster rates than protein oligomerization. The observed and modelled preference of nitration over oligomerization can be rationalized by comparing the mass fraction of nitrated monomer (calculated by multiplying the mass fraction of monomer with NTyr/monomer) with the total oligomer mass fraction. Nitrated monomers were observed to have two times or higher mass fractions compared to all other oligomer mass fractions combined under all experimental conditions. This result indicates that Tyr nitration outcompetes the dimerization/oligomerization process.[22,45]

4. Implications and conclusions

In this study we investigated the kinetics and mechanism of the nitration and oligomerization of proteins induced by O_3 and NO_2 under different atmospherically relevant conditions. We showed the concentration and time dependence of the formation of dimers, trimers and higher protein oligomers as well as their individual nitration degrees. The temporal evolution of the concentrations of these multiple reaction products was well reproduced by a kinetic model with 19 chemical reactions. Protein nitration was found to be kinetically favored over protein oligomerization under the experimental conditions studied in this work. On the basis of the observation that nitrated oligomers formed upon exposure to O_3/NO_2, we suggest further investigation on the allergenic and immunogenic effects by nitrated protein oligomers. The nitrated oligomers were also found in the physiologically-relevant peroxynitrite-induced protein nitration and oligomerization,[46] for which the mechanism is similar to the one we reported here.[25,47]

The implications of protein chemistry with O_3 and NO_2 under atmospherically relevant conditions are illustrated in Fig. 6. The overall nitration and oligomerization rates were both almost one order of magnitude higher in the aqueous phase than for 45% RH, indicating an increased relevance of the processes under cloud-processing conditions. Also, the yields of protein nitration and oligomerization can be strongly influenced by changes in relative humidity leading to changes of phase states. The protein dimers can yield up to 20% (by mass) for 12 h exposure in the liquid phase and the NDs of monomers and dimers can be up to 7% and 5%, respectively. This result indicates that on average, 1.4 Tyr residues in each monomeric protein molecule and 2 Tyr residues in each dimeric molecule are present in their nitrated forms. For proteins in solid or semi-solid phase states, our measurement and model results suggest that higher protein oligomers are likely to be found at lower RH, *e.g.*, on the surface of bioaerosols, whereas the

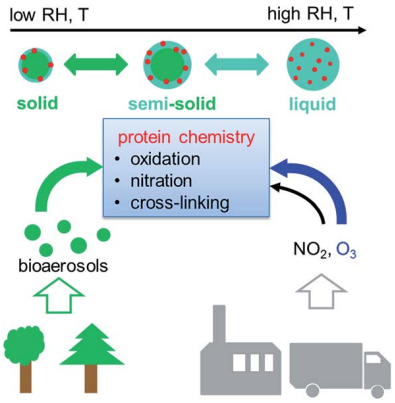

Fig. 6 Atmospheric protein chemistry by ozone (O_3) and nitrogen dioxide (NO_2). Reaction rates are limited by the phase state of proteins and hence the diffusivity of oxidants and protein molecules, which changes with relative humidity (RH) and temperature (*T*). Air pollutants such as NO_2 and O_3 can enhance allergen release from bioaerosols (*e.g.*, pollen) with O_3 being more important in triggering the nitration, cross-linking and oxidation of allergenic proteins in bioaerosols and other protein-containing particles (*e.g.*, Bet v 1 on urban road dust[15]).

NDs of monomers and dimers remain at ~1–2%. Using *ab initio* calculations, Sandhiya *et al.*[22] also showed that increased temperature can speed up the formation of tyrosyl radicals by ozonolysis. Thus, it is expected that nitrated and dimeric protein species could be important products of exposure to O_3 and NO_2 under tropical or summer smog conditions. These differences in reaction kinetics should be taken into account in studies on the physiological effects of proteins exposed to anthropogenic air pollutants, as the allergenic proteins in various oligomerization and nitration states might have a different immunogenic potential.

Both increasing levels of O_3 and NO_2 are able to damage pollen grains and facilitate the release of allergens in polluted environments.[3,8,9] However, our results show that the tyrosine nitration and cross-linking of proteins are sensitive towards an increase in O_3 concentration and rather insensitive towards changes in ambient NO_2 concentrations. Therefore, effective control of the enhanced allergenicity induced by air pollutant-modified aeroallergens should point towards the decrease of ambient ozone concentrations.

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Notes and references

1 M. I. Asher, S. Montefort, B. Björkstén, C. K. Lai, D. P. Strachan, S. K. Weiland, H. Williams and I. P. T. S. Group, *Lancet*, 2006, **368**, 733–743.

2 R. Pawankar, C. E. Baena-Cagnani, J. Bousquet, G. W. Canonica, A. A. Cruz, M. A. Kaliner and B. Q. Lanier, *World Allergy Organ. J.*, 2008, **1**, S4–S17.
3 U. Frank and D. Ernst, *Front. Plant Sci.*, 2016, **7**, 91.
4 K.-H. Kim, S. A. Jahan and E. Kabir, *Environ. Int.*, 2013, **59**, 41–52.
5 U. Pöschl and M. Shiraiwa, *Chem. Rev.*, 2015, **115**, 4440–4475.
6 K. Reinmuth-Selzle, C. J. Kampf, K. Lucas, N. Lang-Yona, J. Fröhlich-Nowoisky, M. Shiraiwa, P. S. J. Lakey, S. Lai, F. Liu, A. T. Kunert, K. Ziegler, F. Shen, R. Sgarbanti, B. Weber, M. G. Weller, I. Bellinghausen, J. Saloga, A. Duschl, D. Schuppan and U. Pöschl, *Environ. Sci. Technol.*, 2017, **51**, 4119–4141.
7 D. Diaz-Sanchez, M. P. Garcia, M. Wang, M. Jyrala and A. Saxon, *J. Allergy Clin. Immunol.*, 1999, **104**, 1183–1188.
8 I. Beck, S. Jochner, S. Gilles, M. McIntyre, J. T. Buters, C. Schmidt-Weber, H. Behrendt, J. Ring, A. Menzel and C. Traidl-Hoffmann, *PLoS One*, 2013, **8**, e80147.
9 Y. Ouyang, Z. Xu, E. Fan, Y. Li and L. Zhang, *Int. Forum. Allergy Rhinol.*, 2016, **6**, 95–100.
10 C. Ackaert, S. Kofler, J. Horejs-Hoeck, N. Zulehner, C. Asam, S. von Grafenstein, J. E. Fuchs, P. Briza, K. R. Liedl and B. Bohle, *PLoS one*, 2014, **9**, e104520.
11 N. Lang-Yona, T. Shuster-Meiseles, Y. Mazar, O. Yarden and Y. Rudich, *Sci. Total Environ.*, 2016, **541**, 365–371.
12 K. Reinmuth-Selzle, C. Ackaert, C. J. Kampf, M. Samonig, M. Shiraiwa, S. Kofler, H. Yang, G. Gadermaier, H. Brandstetter and C. G. Huber, *J. Proteome Res.*, 2014, **13**, 1570–1577.
13 A. D. Estillore, J. V. Trueblood and V. H. Grassian, *Chem. Sci.*, 2016, **7**, 6604–6616.
14 J. Fröhlich-Nowoisky, C. J. Kampf, B. Weber, J. A. Huffman, C. Pöhlker, M. O. Andreae, N. Lang-Yona, S. M. Burrows, S. S. Gunthe, W. Elbert, H. Su, P. Hoor, E. Thines, T. Hoffmann, V. R. Després and U. Pöschl, *Atmos. Res.*, 2016, **182**, 346–376.
15 T. Franze, M. G. Weller, R. Niessner and U. Pöschl, *Environ. Sci. Technol.*, 2005, **39**, 1673–1678.
16 J. T. Buters, A. Kasche, I. Weichenmeier, W. Schober, S. Klaus, C. Traidl-Hoffmann, A. Menzel, J. Huss-Marp, U. Krämer and H. Behrendt, *Int. Arch. Allergy Immunol.*, 2007, **145**, 122–130.
17 F. Liu, S. Lai, K. Reinmuth-Selzle, J. F. Scheel, J. Fröhlich-Nowoisky, V. R. Després, T. Hoffmann, U. Pöschl and C. J. Kampf, *Anal. Bioanal. Chem.*, 2016, **408**, 6337–6348.
18 P. Taylor, R. Flagan, A. Miguel, R. Valenta and M. Glovsky, *Clin. Exp. Allergy*, 2004, **34**, 1591–1596.
19 G. F. Schäppi, P. E. Taylor, I. A. Staff, C. Suphioglu and R. B. Knox, *Sex. Plant Reprod.*, 1997, **10**, 315–323.
20 C. J. Kampf, F. Liu, K. Reinmuth-Selzle, T. Berkemeier, H. Meusel, M. Shiraiwa and U. Pöschl, *Environ. Sci. Technol.*, 2015, **49**, 10859–10866.
21 M. Shiraiwa, Y. Sosedova, A. Rouvière, H. Yang, Y. Zhang, J. P. Abbatt, M. Ammann and U. Pöschl, *Nat. Chem.*, 2011, **3**, 291–295.
22 L. Sandhiya, P. Kolandaivel and K. Senthilkumar, *J. Phys. Chem. B*, 2014, **118**, 3479–3490.

23 M. Shiraiwa, K. Selzle, H. Yang, Y. Sosedova, M. Ammann and U. Pöschl, *Environ. Sci. Technol.*, 2012, **46**, 6672–6680.
24 M. Shiraiwa, K. Selzle and U. Pöschl, *Free Radical Res.*, 2012, **46**, 927–939.
25 S. Pfeiffer, K. Schmidt and B. Mayer, *J. Biol. Chem.*, 2000, **275**, 6346–6352.
26 M. Shiraiwa, C. Pfrang and U. Pöschl, *Atmos. Chem. Phys.*, 2010, **10**, 3673–3691.
27 G. Li, H. Su, X. Li, U. Kuhn, H. Meusel, T. Hoffmann, M. Ammann, U. Pöschl, M. Shao and Y. Cheng, *Atmos. Chem. Phys.*, 2016, **16**, 10299–10311.
28 F. Liu, K. Reinmuth-Selzle, S. Lai, M. G. Weller, U. Pöschl and C. J. Kampf, *J. Chromatogr. A*, 2017, **1495**, 76–82.
29 S. L. Ellison, M. Rosslein and A. Williams, in *Quantifying uncertainty in analytical measurement*, Eurachem, 2000.
30 R. Sander, *Atmos. Chem. Phys.*, 2015, **15**, 4399–4981.
31 V. K. Sharma and N. J. Graham, *Ozone: Sci. Eng.*, 2010, **32**, 81–90.
32 V. R. Després, J. A. Huffman, S. M. Burrows, C. Hoose, A. S. Safatov, G. Buryak, J. Fröhlich-Nowoisky, W. Elbert, M. O. Andreae, U. Pöschl and R. Jaenicke, *Tellus, Ser. B*, 2012, **64**, 1–58.
33 H. Yang, Y. Zhang and U. Pöschl, *Anal. Bioanal. Chem.*, 2010, **397**, 879–886.
34 A. Ghiani, M. Bruschi, S. Citterio, E. Bolzacchini, L. Ferrero, G. Sangiorgi, R. Asero and M. G. Perrone, *Sci. Total Environ.*, 2016, **573**, 1589–1597.
35 Y. Zhang, H. Yang and U. Pöschl, *Anal. Bioanal. Chem.*, 2011, **399**, 459–471.
36 M. Shiraiwa, M. Ammann, T. Koop and U. Pöschl, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 11003–11008.
37 E. Mikhailov, S. Vlasenko, S. Martin, T. Koop and U. Pöschl, *Atmos. Chem. Phys.*, 2009, **9**, 9491–9522.
38 W. H. Heijnis, H. L. Dekker, L. J. de Koning, P. A. Wierenga, A. H. Westphal, C. G. de Koster, H. Gruppen and W. J. van Berkel, *J. Agric. Food Chem.*, 2010, **59**, 444–449.
39 T. Ueki, Y. Hiragi, M. Kataoka, Y. Inoko, Y. Amemiya, Y. Izumi, H. Tagawa and Y. Muroga, *Biophys. Chem.*, 1985, **23**, 115–124.
40 T. Berkemeier, S. S. Steimer, U. K. Krieger, T. Peter, U. Pöschl, M. Ammann and M. Shiraiwa, *Phys. Chem. Chem. Phys.*, 2016, **18**, 12662–12674.
41 A. M. Arangio, J. H. Slade, T. Berkemeier, U. Pöschl, D. A. Knopf and M. Shiraiwa, *J. Phys. Chem. A*, 2015, **119**, 4533–4544.
42 W. Scheider, *J. Phys. Chem.*, 1972, **76**, 349–361.
43 L. J. Lapidus, W. A. Eaton and J. Hofrichter, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 7220–7225.
44 B. Petersen, T. N. Petersen, P. Andersen, M. Nielsen and C. Lundegaard, *BMC Struct. Biol.*, 2009, **9**, 1.
45 J. M. Souza, G. Peluffo and R. Radi, *Free Radical Biol. Med.*, 2008, **45**, 357–366.
46 Y. J. Zhang, Y. F. Xu, X. Q. Chen, X. C. Wang and J.-Z. Wang, *FEBS Lett.*, 2005, **579**, 2421–2427.
47 R. Radi, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 4003–4008.