



Soft matter characterization beyond resolution limit

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Abstract

Nature has designed complex machineries and structures that often rely on interactions between nanometer-sized objects. These objects have inspired scientists to rationally design nanomaterials (NMs). Interest in NMs has led to the development of highly impactful applications in fields ranging from biomedicine to coatings. However, their nanometric dimensionality, combined with resolution limits, complicates clear and immediate characterization. This challenge has led to the development of high-resolution microscopy tools and sophisticated analysis algorithms that work in synergy to overcome the limitations of physical resolution. Among these, two award-winning methodologies deserve particular mention: cryogenic electron microscopy (CryoEM) and super-resolution microscopy (SRM). In this thesis, I apply these advanced methods to investigate novel NMs, specifically amyloid peptide nanofibrils (PNFs) and polymeric nanoparticles (NPs).

To begin with, I will show the importance of atomistic investigation of amyloid PNFs. To do so, I combine CryoEM imaging with single particle analysis tools to unravel the structure derived from a novel short self-assembling peptide. The study discovered that, alongside the common cross- β motif- characteristic in amyloid fibrils- a novel trimeric junction motif is formed. Cooperation of these two motifs elegantly assembles with an hexagonal distribution, producing within the fibril a multi-compartment nanotube. This unexpected discovery underscores the importance of structural investigations in nanomaterials, highlighting the potential for tailoring specific applications based on their unique architectures.

The characterization analysis in the thesis then shifts to a different type of systems: multicomponent polymeric NPs. Atomistic analysis of these NPs is not yet possible, thus the focus shifted to morphological investigation. Specifically, SRM and electron energy loss spectroscopy were applied to detect the distribution of the distinct chemical moieties within single NPs. Notably, I not only present an overview of a showcase example but rather focus on (semi)-quantitative description. Specifically, I focus on describing composition, size and morphology of small multi-polymeric NPs. The performed image analysis allows critical assessment of the microscopy data removing user-biased image interpretation.

Finally, the concluding section of my thesis continues to address polymeric NPs. However, the characterization analysis shifts its focus to the NP surface. The objective of this investigation is to conduct a detailed analysis of the distribution of functional groups. The analysis is conducted from an ensemble average perspective, with the objective of focusing on the distribution of different types of functional groups: total, visible, and accessible. Furthermore, the accessible functional groups are investigated with high-

resolution microscopy in order to enable particle-to-particle differentiation.

In summary, my work presents a fundamental characterization approach for NMs, aiming to unravel fine details that can improve the understanding of their function.

Zusammenfassung

Die Natur hat komplexe geschaffen, die häufig auf der Interaktion zwischen Objekten im Nanometerbereich beruhen. Diese Objekte haben Wissenschaftler dazu inspiriert, Nanomaterialien (NM) zu entwerfen. Das Interesse an Nanomaterialien hat zur Entwicklung äußerst wirkungsvoller Anwendungen in Bereichen der Biomedizin bis hin zu Beschichtungen geführt. Ihre nanoskalige Dimension in Verbindung mit den Auflösungsgrenzen erschwert jedoch eine eindeutige und unmittelbare Charakterisierung. Diese Herausforderung hat zur Entwicklung von hochauflösenden Mikroskopietechniken und ausgefeilten Analysealgorithmen geführt, die in Synergie arbeiten, um die Grenzen der physikalischen Auflösung zu überwinden. Unter diesen sind zwei preisgekrönte Methoden besonders hervorzuheben: die kryogene Elektronenmikroskopie (CryoEM) und die Super-Resolution-Mikroskopie (SRM). In dieser Arbeit wende ich diese fortschrittlichen Methoden an, um neuartige NMs zu untersuchen, insbesondere Amyloid-Peptid-Nanofibrillen (PNFs) und polymere Nanopartikel (NPs).

Zunächst werde ich die Bedeutung der atomistischen Untersuchung von Amyloid-PNFs aufzeigen. Dafür kombiniere ich die CryoEM-Bildgebung mit Werkzeugen zur Einzelpartikelanalyse, um die Struktur eines neuartigen kurzen, sich selbst zusammensetzenden Peptids zu entschlüsseln. Die Studie ergab, dass neben dem üblichen Kreuz- β Motiv, das für Amyloidfibrillen charakteristisch ist, ein neuartiges trimeres Kreuzungs-Motiv gebildet wird. Das Zusammenwirken dieser beiden Motive führt zu einer eleganten hexagonalen Verteilung, wodurch innerhalb der Fibrille ein Nanoröhrchen mit mehreren Kompartimenten entsteht. Diese unerwartete Entdeckung unterstreicht die Bedeutung von Strukturuntersuchungen an Nanomaterialien und verdeutlicht das Potenzial für maßgeschneiderte Anwendungen auf der Grundlage ihrer einzigartigen Architekturen.

Die Charakterisierungsanalyse in dieser Arbeit verlagert sich dann auf eine andere Art von Systemen: mehrphasige polymere NPs. Eine atomistische Analyse dieser NPs ist noch nicht möglich, so dass sich der Schwerpunkt auf morphologische Untersuchungen verlagert. Konkret wurden SRM und Elektronenenergieverlustspektroskopie eingesetzt, um die Verteilung der verschiedenen Phasen innerhalb einzelner NPs zu ermitteln. Ich präsentiere nicht nur einen Überblick über ein Musterbeispiel, sondern konzentriere mich auf die (semiquantitative) Beschreibung. Insbesondere konzentriere ich mich auf die Beschreibung der Zusammensetzung, Größe und Morphologie von kleinen multipolymeren NPs. Die durchgeführte Bildanalyse ermöglicht eine kritische Bewertung der Mikroskopiedaten und beseitigt so eine voreingenommene Bildinterpretation durch den Benutzer.

Der abschließende Abschnitt meiner Arbeit befasst sich weiterhin mit polymeren

NPs. Allerdings verlagert sich der Schwerpunkt der Charakterisierungsanalyse auf die Oberfläche der NP. Ziel dieser Untersuchung ist es, eine detaillierte Analyse der Verteilung der funktionellen Gruppen durchzuführen. Die Analyse wird aus der Perspektive des Ensemble-Mittelwerts durchgeführt, wobei der Schwerpunkt auf der Verteilung der verschiedenen Arten von funktionellen Gruppen liegt: insgesamt, sichtbar und zugänglich. Darüber hinaus werden die zugänglichen funktionellen Gruppen mit hochauflösender Mikroskopie untersucht, um eine Differenzierung von Partikel zu Partikel zu ermöglichen.

Zusammenfassend lässt sich sagen, dass meine Arbeit einen grundlegenden Ansatz zur Charakterisierung von NMs darstellt, mit dem Ziel, feine Details zu entschlüsseln, die das Verständnis ihrer Funktion verbessern können.

Acknowledgments

This section might need to be longer than my whole thesis. Science is a team effort, and here I want to acknowledge all the people that have contributed to the work in the last 4 years.

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I now want to conclude this acknowledgment section by reporting a statement that reflects how I feel within science:

If an atom can be both a wave and a particle, then I chose to be simultaneously a genius and an idiot.

— FraaaMazzz

Table of Contents

Abstract	IV
Zusammenfassung	VI
Acknowledgments	VIII
1 Motivation and Introduction	3
1.1 Peptide Based Nanofibrils	4
1.2 Polymer Based Nanoparticles	7
1.3 Nanomaterial Characterization Techniques	9
1.4 Thesis Motivation and Outline	11
2 Insight of the Primary Techniques	15
2.1 TEM: Transmission Electron Microscopy	15
2.1.1 Image Formation in TEM	15
2.1.2 From TEM to CryoEM	18
2.1.3 Image Acquisition Setup and Requirements	19
2.2 SPA: Single Particle Analysis	21
2.2.1 SPA Theoretical Background	21
2.2.2 SPA Experimental Setup	21
2.3 EELS: Electron Energy Loss Spectroscopy	29
2.3.1 EELS Core Concepts	29
2.4 SRM: Super Resolution Microscopy	30
2.4.1 Optical Setup	31
2.4.2 Working Principle of SRM	31
2.4.3 Image Analysis Setup	32
2.4.4 dSTORM: direct STochastic Optical Reconstruction Microscopy .	33
2.4.5 DNA-PAINT: DNA Point Accumulation for Imaging in Na-noscale Topography	34
3 Unraveling Structural Characteristics of HONEY ASAP: Hexago- nally Ordered Nanofibrils Easily Yielded by Amphiphilic Self As- sembly Peptides	37
3.1 Introduction	39
3.2 Nanofibril Formation	40
3.3 Morphology Analysis	47
3.4 Atomic Modeling Reveals Key Interactions	50
3.5 The Leading Role of Junction Stabilization	54
3.6 Interpretation of DAP's Key Interactions	57

3.7	Summary and Outlook	60
3.8	Materials and Methods	61
4	Chemical Phase Distribution Within Polymeric Nanoparticles	69
4.1	Introduction	72
4.2	Polyurethane-Polyacrylate Nanoparticle Synthesis and Bulk Characterization.	74
4.3	Nanoparticle Characterization via Electron Microscopy.	76
4.4	Nanoparticle Polymer Composition Detection via dSTORM	78
4.5	Subpopulation Size Determination	81
4.6	Hybrid Morphology Analysis Based on Localization Overlap.	83
4.7	Morphology determination via Elemental Analysis	86
4.7.1	Overview of the Nanoparticle Fabrication	86
4.7.2	EELS Characterization	86
4.8	Summary and Outlook	90
4.9	Material and Methods	91
4.9.1	dSTORM Relevant Nanoparticles	91
4.9.2	EELS Relevant Nanoparticles	93
5	Functional Group Distribution within Nanoparticles	95
5.1	Introduction	97
5.2	Polymer and Nanoparticle Synthesis	99
5.3	Total Functional Group Detection	102
5.4	Visible Functional Group Detection	104
5.5	Accessible Functional Group Detection	105
5.6	Albumin Conjugation to COOH-Nanoparticles	109
5.7	DNA-PAINT Imaging	110
5.8	Summary and Outlook	113
5.9	Material and Methods	114
6	Summary	121
	Bibliography	121
	Abbreviations	143
	Materials	149
	Appendix	151
	Curriculum Vitae	164
	List of Publications	165

Chapter 1

Motivation and Introduction

Since the beginning of time, humans have been drawn to the observation and understanding of their surroundings, leading to the characterization of objects. Over time, this pursuit has evolved to encompass smaller and smaller objects, beyond reach of the naked eye. The invisibility of such structures is constrained by the resolution limit of human vision. With advances in science and technology, this limit has continually been pushed forward, notably with the invention of various types microscopes that use external sources-beyond our eyes-to illuminate and visualize these small objects. However, these microscopes have their own resolution limits. For optical microscopes, which use light as an illumination source, the resolution is dictated by Abbe’s law,¹ inversely proportional to the wavelength of light. Thus, the typical resolution is limited to around 250 nm.² Yet, recent breakthroughs have pushed this limit to sub-nanometer levels.³ Major advances were recognized in the 2014 Nobel Prize in Chemistry awarded to Eric Betzig, Stefan W. Hell, and William E. Moerner “*for the development of super resolution microscopy*”.⁴ Although limited by the Abbe law as well, electron microscopes are capable of achieving atomic resolution. Yet, electron microscopy faces practical limitations imposed by the materials under observation.⁵ Specifically, electron damage restricts the application of high-resolution analysis to beam-stable inorganic materials-dead matter.⁶ Nevertheless, scientific progress has overcome these barriers and was recognized with the 2017 Nobel Prize in Chemistry awarded to Jacques Dubochet, Joachim Frank, and Richard Henderson “*for developing cryo-electron microscopy for the high-resolution structure determination of biomolecules in solution*”.⁷

These groundbreaking advancements not only expand our ability to visualize the ‘invisible’ but also hold profound implications for various fields, from elucidating the structure of biomolecules in solution⁸ to revolutionizing our understanding of cellular processes.⁹ While these advancements have been developed for the biological field, they have also shown their potential in characterization of nanomaterials (NMs).^{10,11} NMs embody a diverse range of materials, characterized by their dimensions falling within the nanoscale- 100 nm.¹² In this thesis, I focus on 0D and 1D NMs¹³, respectively nanoparticles (NPs) and nanofibrils, each presenting unique properties and applications.

The chemical composition of NMs, in this thesis, is based on organic polymers that, together with their dimensionality, impart soft matter traits.¹⁴ These traits, characterized by their responsiveness to small perturbations,¹⁵ arise from intramolecular interaction energies comparable to thermal energy (kT), rendering them highly susceptible to

thermal stress.¹⁶ This deformability impart flexibility and adaptability, underpinning a myriad of natural and engineered systems. For instance, the self-assembly and hierarchical organization inherent to soft matter systems offer approaches for creating functional materials with tailored properties, such as photonic crystals¹⁷ and responsive gels.¹⁸

While soft matter enables exceptional features, it is essential to recognize that polymers can give rise to fundamental differences. For example, synthetic polymers, such as polystyrene, can assemble into amorphous yet highly stable systems, such as NPs.¹⁹ Conversely, bio-organic polymers, such as proteins or DNA, exhibit the ability to self-assemble into highly organized systems with precise spatial organization like peptide nanofibrils²⁰ or DNA-origami.²¹ Nature’s engineering of these systems provides them with unparalleled flexibility and adaptability. These inherent differences are not only of fundamental scientific interest, but also have implications in the way these materials can be characterized. While many techniques can be adapted for each of these systems, it is crucial to tailor the scientific approach to account for these variations. For instance, when employing microscopy imaging, certain polymers that produce highly ordered structures allow the use of image-averaging to boost signal-to-noise ratio (SNR) and increase the level of detail observed. Conversely, applying the same technique on polydisperse polymer structures will report a less detailed information. By acknowledging and understanding these distinctions, researchers can refine their scientific questions and unlock deeper insights into the behavior and properties of soft matter systems.

In this thesis I focus on soft matter NMs with the aim of understanding the interplay between structure and function, where structure is induced by precise polymer chemistry design, aiming to engineer NMs and tailor their function and application. Specifically, two distinct types of polymers are examined: a peptide-based biopolymer that self-assembles into highly organized nanofibrils, and synthetic polymers (blends and co-polymers) employed in the formation of NPs with spherical morphology. Through systematic characterization of these polymer-NM systems, this study aims to elucidate how specific chemical compositions and morphologies dictate the properties and behaviors in the resulting NMs. By doing so, I pave the way for the deliberate design of advanced materials with tailored functionalities.

1.1 Peptide Based Nanofibrils

I would like to dive into a background description of nanofibrils relevant to the work presented in this thesis. Nanofibrils are ubiquitous in nature: from nucleic acids to proteins and sugars.²² This thesis will focus on amyloids, a specific type of peptide nanofibrils (PNFs), while also briefly mentioning the relevance of nanotubes- defined as hollow nanofibrils.

Amyloid fibrils are proteinaceous fibrillar aggregates composed of amino acid-based polymers (proteins or peptides) that share a common architectural framework (Figure 1.1). Their core unit comprises β -strand secondary structures, which, through hydrogen bonding, undergo self-assembly to form tertiary structures characterized by β -sheets. These sheets, with an approximate distance of 4.8 Å between chains, further aggregate in a cross- β manner (two β -sheets facing each other). These cross- β structures are based on steric zippers motifs in which the polymer’s side chains interlock to promote optimal interaction.^{23–25} Such interaction can be driven both by hydrophobic residue interactions

or a polar zipper, reliant on hydrogen bonds (e.g., glutamine and asparagine).^{26,27} Amyloid fibrils can be categorized into three primary groups: pathological, functional, and artificial.²⁴ Despite their differing roles, all amyloid fibrils exhibit notable mechanical and chemical stability.^{24,28}

Pathological amyloids represent the earliest-discovered form of amyloid fibrils, notably observed within amyloid plaques associated with neurodegenerative diseases and systemic amyloidosis.²⁹ One of the most commonly addressed protein in this regards is Amyloid- β ($A\beta$) that produces amyloid plaques in Alzheimer disease patients.³⁰ These plaques primarily consist of misfolded proteins that aggregate into insoluble fibrillar structures.³¹ A defining trait of pathological amyloids is their ability to propagate, serving as seeds for further growth of amyloid fibrils.^{30,32} Given their involvement in the pathological field, understanding their structural characteristics has been of paramount importance in the investigation of their toxicity mechanism. The advancement of cryogenic electron microscopy (CryoEM) has facilitated a detailed atomistic exploration of these amyloids.³³ CryoEM analysis detected isomorphism, where morphological similarities occur across distinct proteins.³¹ These morphological similarities are observable at a micrometer level. Yet, at a sub-micrometer level, considerable structural differences can be found due to variations in the in-plane arrangement of the peptides.³¹ These differences manifest in diverse assembly patterns influenced by factors such as growth

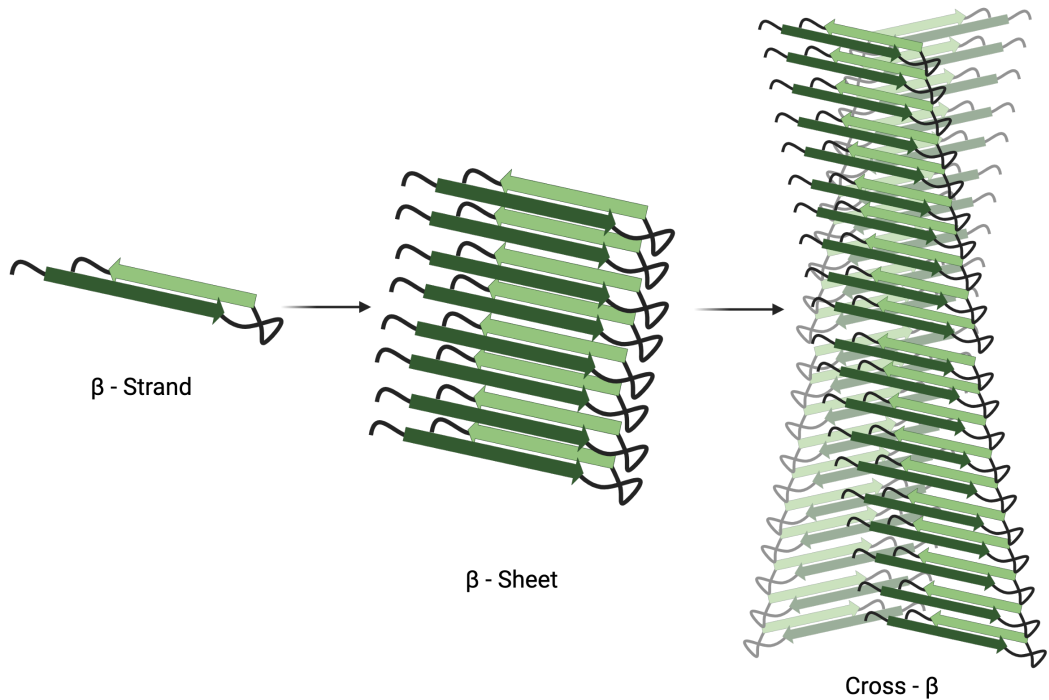


Figure 1.1: Amyloid structural features. Schematic representation of the amyloid formation. As a first step, the peptide’s primary structure produces β -strands. Multiple β -strands then interact along the fibril’s axis with identical copies to form β -sheet as a secondary structure. Lastly, sheets come together with cross- β interactions to form quaternary structures.

conditions (i.e. *in vivo* VS *in vitro*),³⁴ as well as variations in polymer sequence (proteolytic cleavage occurring at similar sites). Therefore, the diverse assembly of pathological amyloids can be interpreted as a machinery defect. Specifically, these sequences did not evolve to play a pathological role but, as commonly observed in disease, are an evolutionary error³⁵.

Contrarily, functional amyloids, are elegantly designed by nature to fulfill specific biological functions, ranging from structural support to memory storage²⁶ and antimicrobial defense.^{36,35} These amyloids typically exhibit minimal polymorphism, with single fibril morphologies commonly observed, and their production mechanisms appear tightly regulated by the organism.^{28,37} Notable examples include the Curli fibril in *E. coli*³⁸ and the Fap fibril in *Pseudomonas*.³⁹ These fibrils have been associated to biofilm formation that enhances bacterial resilience against environmental stressors (e.g. cell survival in drying conditions). Recent advancements have elucidated the atomic-scale structures of additional functional amyloids, such as the Orb2 amyloid in *Drosophila*, implicated in long-term memory formation.²⁶ This discovery contrasts starkly with the detrimental effects of pathological amyloids on memory function, as seen in Alzheimer's disease. Similarly, the Uperin 3.5 amyloid in *Uperoleia mjobergii* exhibits antimicrobial properties.³⁶ Notably, both Orb2 and Uperin 3.5 amyloids display trimeric interactions, distinct from typical pathological amyloid interactions (Figure 1.2). Collectively, these insights underscore the dynamic and diverse roles of functional amyloids in biological systems.

Scientists have drawn inspiration from the diverse functionalities of natural amyloid fibrils to engineer synthetic amyloids tailored for diverse applications. Drawing inspiration from the structure of SEVI fibrils-responsible for enhanced HIV infection,⁴¹ researchers developed amyloid-forming peptides enriched with positively charged amino acids (lysine and histidine), enhancing viral infectivity in gene therapy.⁴² Additionally, innovative approaches have utilized amyloids with amine groups to selectively interact with CO₂, offering potential solutions for carbon capture.⁴³ From an environmental point of view, Mezzenga and colleagues transformed industrial waste proteins into amyloid fibrils for water filtration, capitalizing on their selective ion interaction properties.^{44–46} Furthermore, synthetic amyloids have been designed to mimic enzyme-like

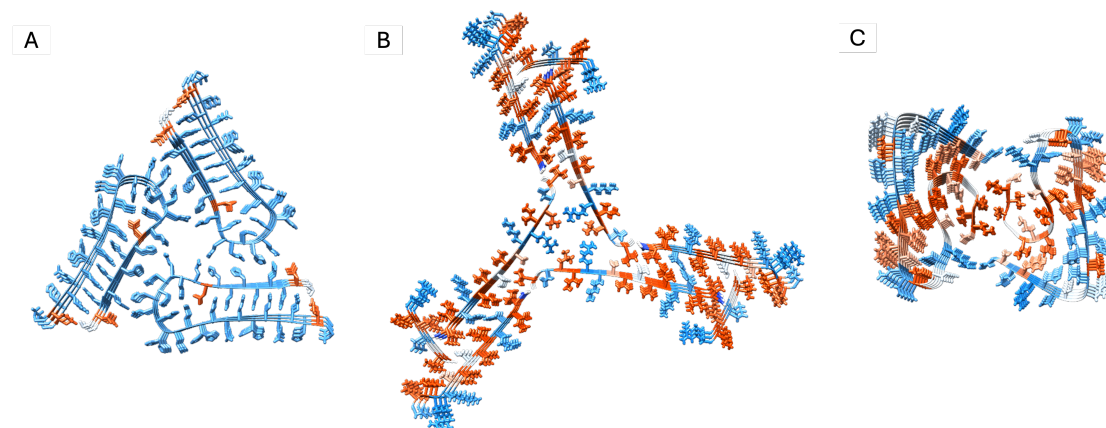


Figure 1.2: CryoEM atomic models of Orb2 (PDB: 6VPS²⁶), uperin 3.5 (PDB: 7QV5³⁶), and A β -42 (PDB: 5OQV⁴⁰)

activities, with peptides coordinating specific amino acids and metals to catalyze reactions.⁴⁷ Similarly, recent studies, inspired by pathological amyloids (e.g., A β -42) with enzyme-like activities,^{48,49} demonstrated the design of short peptides capable of catalytic activity.⁵⁰ Specifically, in their work, efforts are directed towards uncovering structural characteristics essential for elucidating and hypothesizing the catalytic mode of action. All the listed examples highlight the versatility and potential application of synthetic amyloids nanofibrils in nanotechnology.

While synthetic amyloids have received considerable attention in research, investigations into synthetic amyloid with nanotube characteristics (hollow nanofibrils) remain relatively limited.⁵¹ Instead, much attention has been directed towards exploring other types of protein and peptide nanotubes, often constructed using peptide macrocycles as building blocks.^{52,53} These macrocycles, characterized by closed structures where the N and C terminals are connected, facilitate the formation of inherent pores that contribute to the hollow nature of the final nanofibrils. Additionally, the peptide nature of the building block promotes hydrogen bonding between chains, facilitating self-assembly into nanotubes. However, the synthesis process for such building blocks can be more complex compared to linear peptide synthesis, posing challenges for their fabrication. Despite the absence of specific studies on amyloid-based nanotubes, these alternative nanotubes have attracted significant interest and investigation. Engineered to possess inherent pores and functionalities, they hold promise for various applications, including ion transport. Nonetheless, the complexity of the synthesis process underscores the need for further research and optimization in this area.^{52,53}

1.2 Polymer Based Nanoparticles

We now move onto the second type of polymers investigated in this thesis: synthetic polymers for the formation of NPs. Polymer-based NPs represent an important subset of NMs with 0D dimensionality. They offer unique properties and versatile applications across diverse scientific realms such as coatings,^{54–57} photocatalysis,^{58–60} and nanomedicine.^{61–63} In this section I provide, in this section, a brief overview of NPs in these fields to contextualize the significance of my research.

Coating applications exploit the versatility of soft matter to impart specialized functionalities to surfaces.⁶⁴ For instance, paints are often colloidal systems that, after application on a designated surface (e.g. a car), produce a film with multiple functionalities, including decoration (e.g., color) and protection (e.g. corrosion prevention).⁶⁴ Hence, these type of colloidal solutions require proper formulation of the primary materials (e.g., polymers) in order to produce a film with the desired properties (e.g., transparency).⁶⁵ One of the prominent systems in the field is water-based polyurethane (PU).⁶⁶ Such a system was introduced in response to environmental concerns surrounding volatile organic compounds emitted by traditional solvent-based coatings. Today, water-based PU systems have become a cornerstone of modern coating technology, steadily replacing conventional solvent-born systems.⁶⁷ This transition to water-based formulations necessitated the conversion of PU polymers dispersed in organic solvents into PU NPs in water, leveraging principles of colloidal science. Subsequent research has focused on enhancing the performance of these coatings by incorporating additives⁶⁸ such as acrylates.^{67,69–71} The optimization of synthesis protocols has been paramount in achieving films with

superior abrasion, corrosion, and water resistance, while simultaneously addressing cost-effectiveness and minimizing solvent pollution.⁷⁰ In the realm of PU-acrylate coatings, three major synthesis routes have emerged (Figure 1.3): 1) physically blending PU and polyacrylate (PA) NPs, 2) emulsion polymerization of acrylate monomers in a solution containing PU NPs covalently conjugated to acrylic reactive groups, and 3) interpenetrating polymer networks utilizing PU NPs as seeds for acrylate monomer polymerization. Methods 2 and 3 have demonstrated improved film performance thanks to homogeneous distribution of the two chemical moieties within the film.⁷⁰ Consequently, there is considerable interest in elucidating the nature of NP solutions and their correlation with the functional properties of the final materials (e.g. film homogeneity).⁷²

Moving from coatings to photocatalysis, photocatalytic-NPs are utilized to harness light energy for catalytic reactions. Specifically, light is utilized to activate catalytic units, promoting chemical reactions under mild conditions and overcoming high activation energy barriers.⁷³ While traditional homogeneous photocatalytic reactions yield high efficiencies, they often suffer from drawbacks such as high cost and intrinsic toxicity of the catalyst.⁷⁴ In response, researchers have developed systems that maintain catalytic activity in solution with improved cost-effectiveness and reduced toxicity.⁷⁵ A key strategy involves the use of highly dispersible porous NPs, which provide increased surface area, leading to more active sites,⁷⁶ enhanced light absorption through scattering,⁷⁷ and facilitated recovery of the material for re-use in subsequent reactions.⁵⁹ For example, silica NPs can be engineered to incorporate photocatalytically relevant moieties, demonstrating the potential of NPs to advance photocatalytic processes.^{78,79}

Lastly, in the field of nanomedicine, polymeric NPs have attracted significant in-

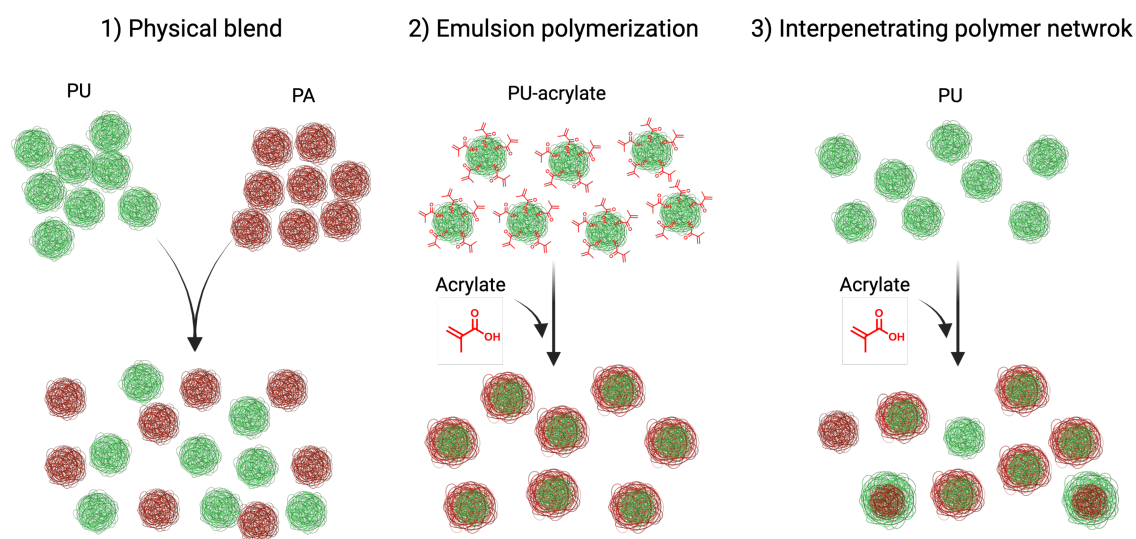


Figure 1.3: Schematic representation of the three major synthesis strategies to obtain PU-PA colloidal suspension. Method 1) shows the physical blending of separately obtained PU and PA NPs. Method 2) shows the emulsion polymerization of acrylate monomers on acrylate-functionalized PU NPs. Method 3) shows the interpenetrating polymer network approach where PU NPs are swollen with acrylate monomer and then polymerized. The final PU-PA system is believed to produce respectively: phase separated PU and PA NPs, core-shell PU-PA NPs, and hybrid PU-PA NPs.⁷⁰

terest from the scientific community due to their ability to encapsulate molecules of interest.^{63,80} These NPs can be utilized as protective carriers, shielding the encapsulated molecules from binding to plasma proteins, thus suppressing clearance rates and enhancing biodistribution and tissue uptake.⁶² To optimize NP delivery within the body, surface functionalization with specific molecules is often employed. For instance, the incorporation of antibodies can induce targeted interactions, guiding NPs to specific cell surfaces of interest.^{81–83} Similarly, conjugation with enzymes enables the creation of nanomotors capable of motion,^{84–86} which can enhance cargo delivery by overcoming Brownian motion. Additionally, surface functionalization with carbohydrates,^{87,88} peptides,^{89,90} or other small molecules⁹¹ further expands the versatility and functionality of NPs. Regardless of the molecule type, the introduction of these functional groups is crucial. Consequently, significant efforts are being directed towards optimizing synthetic strategies to achieve homogeneous and efficient materials.

1.3 Nanomaterial Characterization Techniques

The NMs discussed thus far are imperceptible to the naked eye, underscoring the importance of detailed characterization to discern their distinctive properties. A plethora of characterization techniques have emerged over the years, each tailored to address specific scientific questions about these materials. In this brief introduction, rather than listing all available techniques, the focus lies on describing the fundamental questions researchers seek to answer and the corresponding techniques employed to address them. Specifically, I will elucidate how to understand the structural and morphological characteristics of PNFs and polymeric NPs (Figure 1.4).

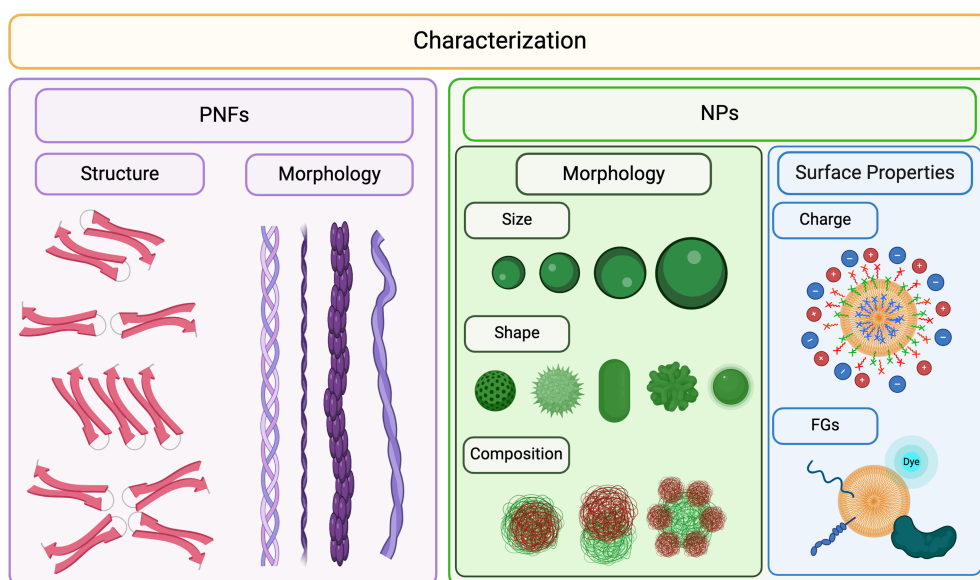


Figure 1.4: Characterization overview. Schematic representation of the characterization information investigated in this thesis. The structure is divided in PNFs and NPs characterization. PNFs are investigated to comprehend atomic structure of the peptide chains and morphological differences. NPs are characterized from a morphology point of view to comprehend size, shape, and composition. Surface properties of the NPs are also characterized to comprehend their charge and functional group (FG) distribution and composition.

When investigating PNFs, high resolution microscopy tools such as atomic force microscopy (AFM), transmission electron microscopy (TEM), or CryoEM provide images that elucidate morphology features.⁹² These techniques offer valuable insights into the overall architecture and organization of PNFs. Their use is crucial for visualization of the peptides self-assembly and for the detection of different assembly states due to varying experimental conditions.^{93,94} An example is the reversible formation of PNFs under acidic pH conditions.⁹⁴ Additionally to monitoring reversible assembly effect, such type of morphological analysis can be of the utmost importance in scanning the complex energy landscape associated to macro-molecular self-assembly.⁹⁵ While such tools largely improves the comprehension of PNFs, they are not able to detect differences at the sub-nanometer level.⁹⁶ In such cases a more in-depth analysis is required to visualize atomic variations, thus the shift to structural analysis.

In PNFs, primary structure is inherently dictated by the amino acid sequence. Thus, for short synthetic peptides it is dictated by the selected synthesis, typically with an automated solid-phase synthesis.⁵² In this technique a stepwise elongation of polypeptides is achieved by sequentially reacting each amino acid on a solid resin.⁹⁷ The synthesis is followed by a purification step (high performance liquid chromatography, HPLC).⁹⁷ Additionally, techniques such as mass spectrometry serve to validate the correct amino acid sequence by confirming the precise molecular weight.⁹⁷

Transitioning to secondary structure characterization, this aspect depends on the spatial arrangement of amino acids within the backbone of amyloid molecules. Circular dichroism (CD) or fourier-transform infrared (FTIR) spectroscopy are classical methods employed for characterizing secondary structures.⁹⁸ However, challenges may rise with short synthetic peptides, as these techniques have been calibrated on proteins rather than short peptides thus hampering detailed analysis.⁹²

Moving further, tertiary and quaternary structure characterization dives into the intricate three-dimensional interactions between individual chains within amyloid assemblies.⁹⁹ While obtaining a comprehensive understanding of tertiary structure remains complex, certain techniques provide partial insights. For instance, small dyes such as Thioflavin-T (ThT) produce intense fluorescence signals when interacting with amyloid fibrils, offering some information about 3-dimensional interaction formation.^{100,101} However, achieving a full reconstruction of protein structures is necessary to obtain atomistic understanding of the molecular disposition. Historically, for globular proteins, this was accomplished through protein crystallization followed by X-ray crystallography.¹⁰² Recent advancements in single particle analysis (SPA) coupled with CryoEM¹⁰³ have enabled structural determination of proteins in their native environment thanks to the an image-averaging approach (details in Section 2.2). Yet, this method is primarily applied to natural amyloids,^{27,104} particularly in pathological systems to elucidate underlying processes involved with the pathology. Contrarily, for synthetic amyloids, while some structures have been investigated,^{50,105} this atomistic analysis remains far from typical procedure.

Transitioning to polymeric NPs, structural and atomistic investigations pose a challenge due to heterogeneity arising from both the polymer and the NP itself, complicating the SPA approach. Consequently, NP characterization focuses on morphology (size and shape) and surface properties.¹⁰⁶ Size information is commonly assessed using scattering techniques such as dynamic light scattering (DLS).¹⁰⁷ While DLS offers rapid and con-

venient size distribution analysis, it has a bias towards larger particles and limitations in capturing the distribution of smaller NPs.¹⁰⁷ Advanced scattering techniques like small-angle X-ray scattering (SAXS), coupled with fitting methods, can provide additional shape information and are particularly useful for more complex structures like core-shell nanoparticles.¹⁰⁶ However, a more conventional approach to shape analysis involves high-resolution microscopy techniques such as AFM,¹⁰⁸ scanning electron microscopy (SEM),¹⁰⁹ or TEM.¹¹⁰ Despite their effectiveness, these techniques typically require samples to be in a dry state, which may introduce artifacts, especially for film-forming polymeric NPs prone to aggregation upon drying. To mitigate these challenges, shape investigations should ideally be conducted in the sample’s native environment, achieved through specialized methods like CryoEM or super-resolution microscopy (SRM).

When NPs are obtained by combining multiple chemical components, hybrid NPs, understanding how these different chemicals distribute at the single NP level is of great importance, as composition can significantly affect function.⁷⁰ Hence, in these systems, NP morphology can be influenced not only by size and shape but also by chemical composition distribution.^{111,112} To visualize this distribution, microscopy methods such as SEM or TEM are often coupled with elemental analysis tools. Examples include energy-dispersive X-ray spectroscopy (EDX)¹¹³ and electron energy-loss spectroscopy (EELS, detailed in Section 2.3).⁷⁸ While these techniques are well established for metallic-type NPs, their application in polymeric particles, especially smaller ones, remains an area of ongoing investigation. In exceptional cases, electron beam sensitivity can be used to distinguish phases. For instance, in the case of Janus NPs composed by a poly-styrene and poly-lactic acid where the beam-stable polymer (poly-styrene) is observed while the correspondent one (poly-lactic acid) is disintegrated generating distinct black and white particles.¹¹¹ Alternatively, selective fluorescent labels can be introduced for multi-color SRM, offering chemically specific visualization.^{114,115}

Lastly, surface properties, including charge density, significantly influence stability, dispersibility, and reactivity thus rendering this investigation crucial for optimal NP performance.¹⁰⁶ Zeta potential measurements offer insights into surface charge and its impact on particle stability,¹⁰⁷ although strong ionic strength in solution can neutralize charge stabilization, leading to particle aggregation.¹⁰⁶ Additionally, surface modification is often employed to tailor NPs for specific applications, utilizing functional groups as reactive handles for conjugating molecules of interest.^{81–86} Characterizing surface composition can be achieved through techniques such as potentiometric titration,¹¹⁶ which provides information on visible functional groups. An example is particle charge detection that quantifies charged groups.¹¹⁷ Accessible functional groups, crucial for conjugation strategies, are typically identified through fluorescent labeling approaches, enabling direct or indirect quantification.^{116,118,119}

1.4 Thesis Motivation and Outline

The previous sections aimed to elucidate the significance of amyloid PNFs and NPs in various applications and to underscore the necessity for meticulous characterization in order to comprehend their distinctive properties. Through such characterization, scientists can obtain valuable information regarding the behavior of these NMs. For instance, structural investigation of A β extracted from humans and mice has revealed

differences that suggest different formation mechanisms in the two species, which has led to the development of improved animal models.⁴⁰ For antibody-functionalized NPs, verifying the proper orientation of the biomolecule on the NP surface is of paramount importance for clinical translation.¹²⁰

These examples serve as the foundation for structure-function studies and represent the primary focus of my thesis. This correlation between structure and function is not only fundamental for optimizing existing NMs but also essential for driving the advancement of nanotechnology by engineering NMs with tailored properties. The advancement of this field is reliant on a profound comprehension and lucid representation of the materials that are being created. In other words, in order to advance the development of NMs with specific properties for various applications, it is essential to gain a comprehensive understanding of the structures and characteristics of these materials, as well as to be able to visualize them. The following chapters will therefore examine these concepts in greater detail.

Chapter 2, focuses on elucidating the core concepts of the primary techniques employed in this thesis. Such techniques are not a standard characterization approach; hence, a detailed description can facilitate a practical understanding of the research output. The objective of this chapter is to provide a foundation for critical analysis of the findings presented in subsequent chapters.

Chapter 3 moves onto the first investigated system: amyloid PNFs. These systems have been the subject of extensive investigation,⁹³ yet, there remains a notable gap in the literature with regard to our understanding of their atomic-level disposition. While these materials may serve their intended purposes, a more profound comprehension of their atomic arrangement could potentially enhance the efficacy of these systems or even inspire novel modifications. Consequently, the atomic structure of a novel fibril-based on a short peptide- was elucidated via CryoEM and SPA. The structure is termed HONEY ASAP, an acronym that describes the key features of the produced structure: Hexagonally Ordered Nanofibril Easily Yielded by Amphiphilic Self-Assembly Peptide. Unraveling the atomic details of this novel NM has detected a unprecedented structure based on a hexagonal disposition of the peptides that confers a nanotube characteristics to the fibril. The findings underscore the significance of elucidating the atomic structure of PNFs that leads to a detail comprehension of self-assembly motifs and can inspire the development of novel applications and new NMs.

Chapter 4 shifts the focus to a different system: multi-component NPs where the characterization is redirected to morphology analysis. Literature presents numerous studies that elucidate chemical distribution in larger systems, such as micrometer-sized particles.^{114,121} However, there is a lack of methods for detailed investigation of chemical composition distribution within polymeric NPs. The reduced dimensionality of the particles poses a challenge in characterization. Therefore, there is a necessity for high-resolution techniques and meticulous analysis, such as chromatic correction methods discussed in Section 2.4.1. For this reason, I introduce dual color dSTORM as a suitable technique for such analysis. Importantly, I propose a (semi)-quantitative characterization of composition, size and morphology on an industrially relevant NP system. Additionally, through EELS, I demonstrate the characterization of a beam-stable NP. The detected core-shell morphology improves the interpretation of the mechanism behind the photocatalytic activity of this NP.

Lastly, **Chapter 5** continues to address polymeric NPs by shifting the characterization to surface analysis. In this chapter, I diverge from the analysis of multi-component systems and instead focus on NPs derived from co-polymers. The objective is to conduct a comprehensive analysis of the distribution of functional groups. The analysis is performed from an ensemble averaged point of view to focus on different type of functional groups: total, visible and accessible. Furthermore, the accessible functional groups are investigated with high-resolution microscopy: DNA Point Accumulation for Imaging in Nanoscale Topography. This imaging enables particle-to-particle differentiation.

Overall, in this thesis I aim to transform high-resolution images into quantifiable data. This approach demonstrated that simply capturing images was insufficient for a comprehensive sample analysis. Contrarily, it was necessary to perform detailed image processing to translate visually appealing images into quantitative data. By applying detailed processing, my thesis serves as a fundamental characterization of PNFs and polymeric NPs. The finding can later on serve as the basis for improved understanding of the NMs function.

Chapter 2

Insight of the Primary Techniques

To comprehensively evaluate the materials discussed in this thesis, it is essential to have a thorough understanding of the two microscopy techniques upon which the work is based. Therefore, this chapter delves into the detailed description of both electron microscopy and fluorescence microscopy. The goal is to elucidate the fundamental principles underlying image formation in TEM and fluorescence microscopy. Additionally, the chapter briefly mentions electron energy loss spectroscopy (EELS) as a related technique to electron microscopy. The focus then shifts to the image analysis methodologies employed to enhance the resolution limits of these microscopy techniques: single particle analysis (SPA) and super resolution microscopy (SRM). SPA enables the restoration of Å-resolution information for biomaterials, while SRM elevates optical resolution from 250 nm to 20 nm. SRM necessitates specific imaging conditions: different experimental setups lead to distinct methodologies. The methods covered in this chapter include direct stochastic optical reconstruction microscopy (dSTORM) and point accumulation for imaging in nanoscale topography (PAINT).

2.1 TEM: Transmission Electron Microscopy

As first technique, electron microscopy is presented. More specifically, I elucidate in this section the basic aspects that allow the formation and analysis of TEM images. Thus in this section the microscope set up is first described in order to define the concept that allow image formation. The next step is to introduce core aspects of CryoEM and how it differentiates from conventional TEM. Lastly, to introduce SPA, the imaging set up is described.

2.1.1 Image Formation in TEM

The electron microscopes are composed of three principal components: electron source, lenses and detectors (Figure 2.1). An electron source, typically a field emission gun, emits electrons generating the electron beam. This beam is then focused by the condenser lens system before passing through the sample with the assistance of objective lenses, culminating in image formation. Subsequently, the produced signal is passed through the projection lenses to obtain optimal magnification. Finally the electrons are

visualized by the detector system.

To enhance image quality, an additional component can be added between the sample and detector: the energy filter. Its feature is to remove electrons of unwanted energy. To comprehend its mechanism it is necessary to further understand the image formation mechanism. The latter is based on the electron-sample interaction which can be either elastically or inelastically scattered thus generating respectively amplitude or phase contrast.

The **amplitude contrast** derives from the particle nature of the electron and its inelastic scattering resulting in energy loss. This contrast mechanism is based on the presence or absence of material: empty space allows the electron to pass freely (e.g. Figure 2.2 B), while material, such as a NP, induces electron interaction, hindering their clear transit to the detector (e.g. Figure 2.2 A). Consequently, amplitude contrast aids in detecting sample thickness: regions of greater thickness heightened scattering, yielding a darker signal, whereas thinner areas yield the opposite effect.

In thick samples, electrons may undergo multiple scattering to penetrate the entire object. Consequently, these electrons lose a significant amount of energy, which causes the magnetic fields of the objective and projector lenses to misproject the electrons onto the detector and thereby diminishing image quality. Implementing an energy filter, which only selects electrons close to zero-energy-loss (primarily scattered electrons), can enhance image quality by reducing the impact of multiple scattering.

The **phase contrast** on the other hand derives from the wave nature of electrons and their elastic scattering. When an electron wave reaches the sample, it interacts with each of the atoms. Such interaction causes a change in the amplitude and phase of the electron wave, which lead each atom to act as the source of a new wave (e.g. Figure 2.2 C). Consequently, multiple waves are generated within the sample due to the interaction with the electron beam. Thus, the sample acts as a new source of waves. Such waves then undergo constructive or destructive interference. Constructive interaction enhances information detection, while destructive interference leads to information attenuation in the final image. This interaction is visually depicted in the diffraction pattern of

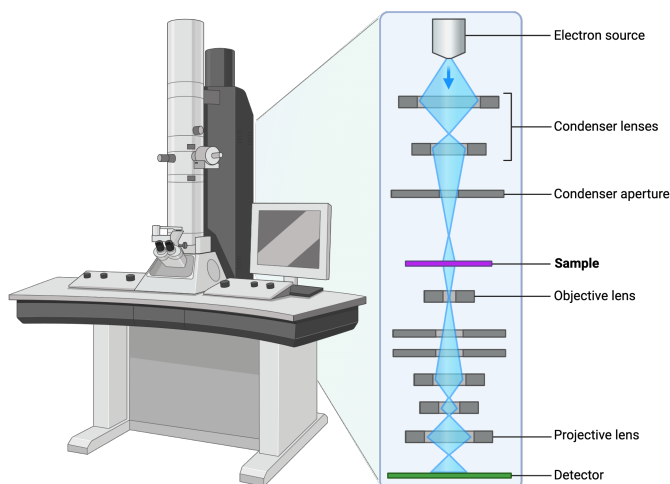


Figure 2.1: Schematic representation of the TEM microscope primary components.

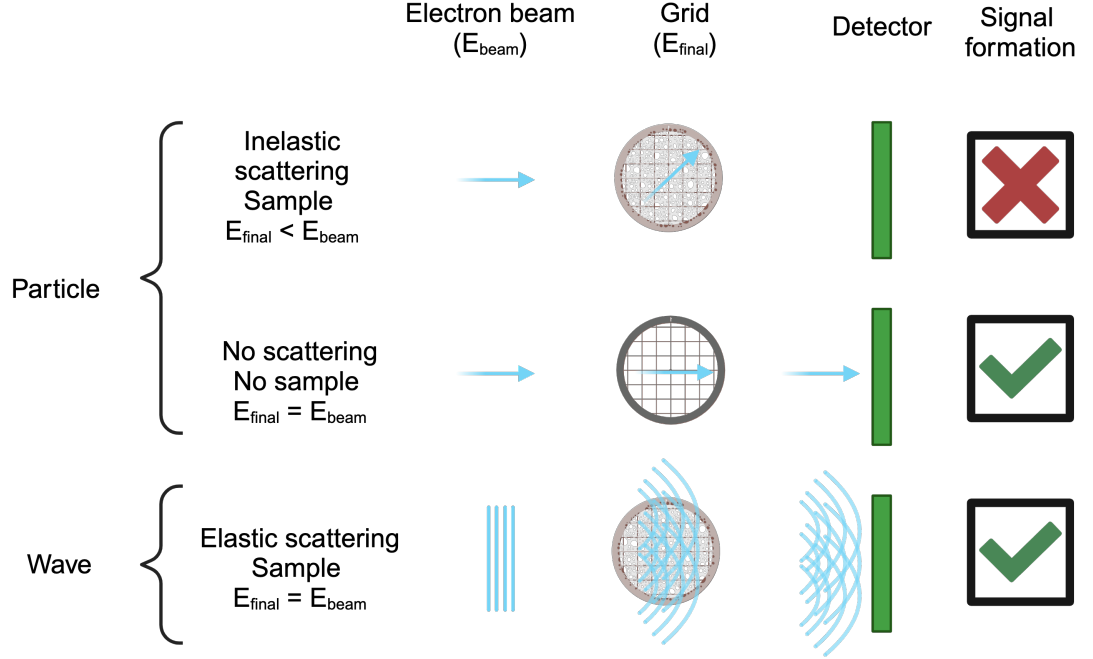


Figure 2.2: Beam-sample interaction scheme. (A) is inelastic scattering, no electrons reach the detector. (B) no interaction thus, together with case A, produces amplitude contrast. (C) elastic scattering (no energy loss) so the electrons reach the detector and produce the image, yet this happens based on wave interactions.

the image and is mathematically described by the contrast transfer function (CTF), represented by a sinusoidal function.

The CTF quantifies the extent to which phase contrast is conveyed in the final image. It is important to recognize that phase information is not directly represented in real space but rather in reciprocal space. Consequently, its domain is characterized by spatial frequencies (expressed in reciprocal angstroms, $1/\text{\AA}$), not physical distances ($\text{\AA}$). This implies that the CTF reports on how the spatial frequencies, which are details at different length scales, are recorded in the final images. Thus, it is acting as a band pass filter which selects the information to convey. Therefore, the oscillating nature of the CTF is critical; as the CTF approaches zero, a portion of the signal is lost, directly challenging the detectability of image features at specific spatial frequencies. For example, if the CTF equals zero at a spatial frequency of $1/100 \text{ \AA}$, details in real space (e.g., the final image) with dimensions of $100 \text{ \AA}$ become undetectable.

Furthermore, CTF depends on the defocus (z-height) at which the image is acquired. In perfect focus ($\Delta z = 0$), the CTF reaches zero at high spatial frequencies. Consequently, at lower frequency, including those representing interesting structural details (e.g. a characteristic pattern), the CTF exhibits diminished values, hindering the detection of these objects. Therefore, images are typically acquired in defocus condition to improve object detection. This concept is depicted in Figure 2.3.

In Panel A of Figure 2.3, the defocus value is close to zero ($\Delta z = 0.5 \mu\text{M}$). In this image (panel on the right), sample identification is hindered due to the stipes thickness being approximately $40 \text{ \AA}$. At the corresponding spatial frequency ($1/40 \text{ \AA}$), the CTF is dim. On the contrary, in Panel B of Figure 2.3, with higher defocus ($\Delta z = 1.77 \mu\text{M}$), the CTF exhibits a robust signal at the corresponding spatial frequency, resulting in clear depiction of the particle.

Understanding the relationship between the CTF and the information contained in the images is essential for SPA as it is used to extract high resolution information during the reconstruction process.

2.1.2 From TEM to CryoEM

Before diving into the description of image acquisition, it is important to highlight the important concepts which are the basis of CryoEM. The Nobel prize winning breakthrough in the field occurred when Dubochet and his team devised a method to produce TEM grids under cryogenic conditions without the formation of crystalline ice¹²³. Their protocol, based on plunge freezing, utilizes liquid ethane near its freezing temperature (90 K). Liquid ethane, unlike liquid nitrogen, lacks the Leidenfrost effect¹²⁴. This means that liquid ethane does not form an insulating gas layer above the solvent. Consequently,

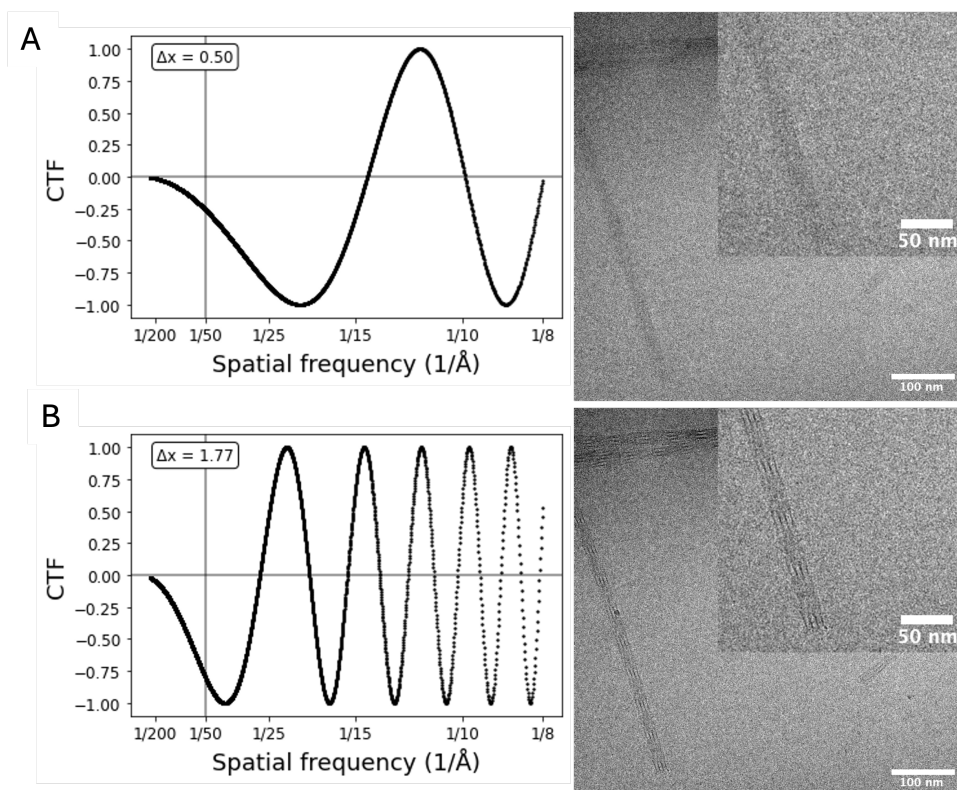


Figure 2.3: CTF example curves. Simulated CTF curves with respective images and a close up to the fibril features. The CTF was calculated using the equation reported by Thuman *et. al.*¹²². The values used are $Q = 0$, $C_s = 2.7 \text{ nm}$, $\lambda_e = 0.04 \text{ \AA}$ and Δz either $0.5 \mu\text{m}$ (A) or $1.77 \mu\text{m}$ (B).

when the sample is plunged, it makes direct contact with the freezing liquid without passing through a cold gas layer. As a result, liquid ethane presents a high cooling rate, enabling water in solution to solidify in its amorphous state rather than crystallizing. Hence, as water does not undergo expansion due to crystallization, the sample retains its original form.

The developed procedure was the following. Copper grids, pre-coated with a thin layer of carbon and featuring predefined pores, undergo plasma treatment. This step is crucial to minimize the hydrophobic effect, which otherwise causes aqueous samples to form rounded drops on the grid, resulting in non-homogeneous sample distribution. The treated grid is then transferred into a plunging system, consisting of a humidity chamber coupled with a liquid nitrogen holder to maintain the liquid ethane at 90 K. Once the grid is transferred in the humidity chamber, a small aliquot (e.g. 4 μ L) of sample are applied and the grid is blotted with specific force and a duration (e.g. 1 sec). Such step allows the removal of excess sample and the formation of a thin layer of aqueous solution. The grid is then immediately plunged in liquid ethane and is now ready to be imaged in the microscope.

All the above mentioned parameters need to be adapted for the sample in question. For instance, A β and insulin amyloid fibrils often pose challenges during grid deposition. Therefore, after deposition, a period of interaction is often required to ensure proper fibril deposition. In some cases, blotting the grid only from the back (the opposite side of the sample application) is necessary to prevent fibrils from adhering to the blotting paper. Additionally, fibrils may tend to cluster and form gel-like networks, leading to non-uniform distribution on the grid. The presence of large clusters results in greater sample thickness (approximately 100 nm) in specific areas, leading to ice contamination or increased electron scattering, ultimately diminishing image quality. Thus, adapting parameters and employing meticulous techniques are essential to overcome sample-specific challenges and ensure high-quality imaging outcomes in CryoEM.

2.1.3 Image Acquisition Setup and Requirements

In SPA, large data acquisition are fundamental, typically more than 1'000 images are required for optimal data analysis. Thus, software, such as SerialEM, have been developed to automate data acquisition.¹²⁵ The software involves imaging the sample with step approach. Initially, an overview of the grid, known as ATLAS, is acquired at very low magnification (135x). Subsequent stages involve employing a low dose acquisition composed of three imaging modes: search (low magnification, e.g. 3.6kx), view (medium magnification, e.g. 15kx) and record (high magnification, e.g. 81k). These imaging modes require optimization to regulate the pixel size and the sample's exposure to electrons, to reduce beam damage. Pixel size is crucial in determining the highest achievable resolution. The latter is governed by the Nyquist-Shannon theorem which, in CryoEM, states that the achievable resolution will be twice the pixel size. The pixel size sets the required magnification.¹²⁶ Sample stability is then assessed by analyzing the diffraction pattern in the protein area. Modification in the diffraction pattern can identify the onset of information loss. The modification is assessed by collecting movies rather than images. For example, instead of utilizing an exposure time of 2 sec, the data can be acquired by collecting 20 images with an exposure time of 0.1 sec. In such a way, the diffraction pattern of each frame can be analyzed to determine beam damage.

Additionally, by collecting movies rather than images it is possible to correct for small shifts in the sample during acquisition time that can reduce image quality. Hence, when acquiring data sets for image reconstruction it is invaluable to record movies.

Following the selection of crucial low dose parameters, a systematic approach, outlined in Figure 2.4, is performed. The ATLAS (Figure 2.4 A) overview enables the identification of areas of interest, such as those highlighted in green (Figure 2.4), for low or mid magnification mapping. Utilizing the search or view mode, maps of these areas are generated (Figure 2.4 B-E). This stage facilitates the detection of regions rich in sample (green), empty (red), or with ice contamination (orange arrows), enabling the appropriate selection of points for high magnification imaging.

Subsequently, data acquisition is automated through a script, capable of executing essential operations like auto-focusing. As discussed in detail previously, focusing is indispensable for accurate data analysis. Employing different defocus values generates CTF curves with high contrast transfer at various spatial frequencies. Thus, during SPA it is possible, by analyzing multiple CTFs, to recover most of the information of interest for high resolution reconstruction. Consequently, it is imperative for the script to acquire images at a range of defocus values.

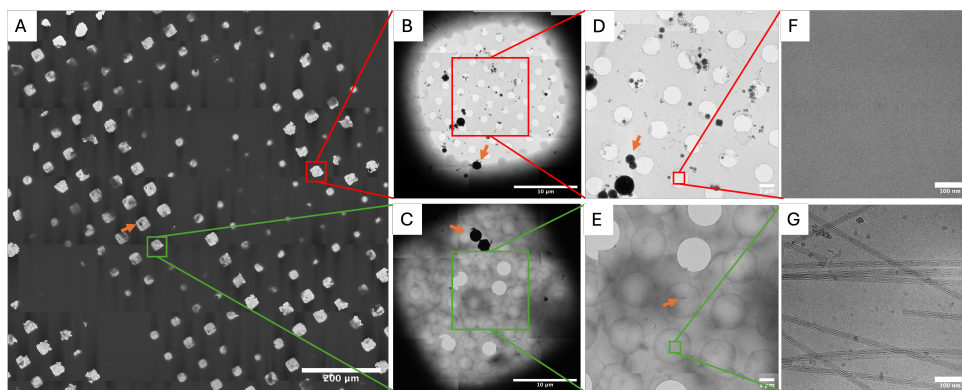


Figure 2.4: Step by step approach for large data collection. (A) Zoomed-in of an ATLAS. (B) and (C) are a low magnification map respectively in an bad (red) and good (green) area. (D) and (E) zoom in the low magnification map and highlight empty and filled holes. An example of these is reported respectively in (F) and (G). The orange arrows examples of highlight ice contamination.

2.2 SPA: Single Particle Analysis

Now that the fundamentals of image formation in TEM have been comprehended, it is possible to leverage this understanding to obtain high-resolution information. Such information is a product of the SPA method, which was pioneered by Nobel laureates Joachim Frank and Richard Henderson, alongside their respective teams. In this section, the core concepts of SPA are explored, elucidating the steps undertaken throughout this thesis. Particular emphasis will be placed on SPA for structures exhibiting helical symmetry, as these constitute the primary focus of my research.

2.2.1 SPA Theoretical Background

The core concept of SPA revolves around enhancing the signal-to-noise ratio (SNR) by averaging identical images, thereby amplifying the desired signal while minimizing noise. This principle is coupled with the necessity to capture multiple images from various angles, facilitating the detection of protein projections from different orientations. Subsequently, the improved SNR images from different angles are integrated into a three-dimensional space to generate the final image.

Mathematically, this process entails applying a fast Fourier transform (FFT) to the collected images, revealing their respective diffraction patterns. Conceptually, the key steps are presented sequentially, as depicted in 2.5. Initially, a first image is transformed into its diffraction pattern and positioned in a 3D-reciprocal space (Panel A, Figure 2.5). Subsequently, the same transformation is applied to a second image. Given that both images depict the same sample (e.g. protein) from different orientations, they exhibit common features. These features are represented in their FFT by the detection of a common line where their diffraction patterns are identical. This commonality allows for the overlaying of the two images (Panel B, Figure 2.5). However, to accurately determine the angular orientation, a third image is required. By comparing the FFT of the third image to both the first and second images, the angular constraints necessary to orient the figures in 3D space are introduced (Panel C, Figure 2.5). This iterative procedure is applied across the entire dataset which in turn produces a 3D diffraction pattern. Subsequently, by applying an inverse FFT, the reconstruction of the 3D structure becomes feasible.

2.2.2 SPA Experimental Setup

The fundamentals of SPA algorithms have now been described. Yet, from a user standpoint, the process can be summarized into five essential steps schematized in Figure 2.6:

1. **From movies to micrographs:** movies undergo a drift and gain correction with CTF determination
2. **From micrographs to particles:** within the micrographs a box size is selected for particle picking which are then classified in the 2D space
3. **From particles to volume:** an initial volume is determined to be used as a reference to align the particles and to obtain the final high resolution information

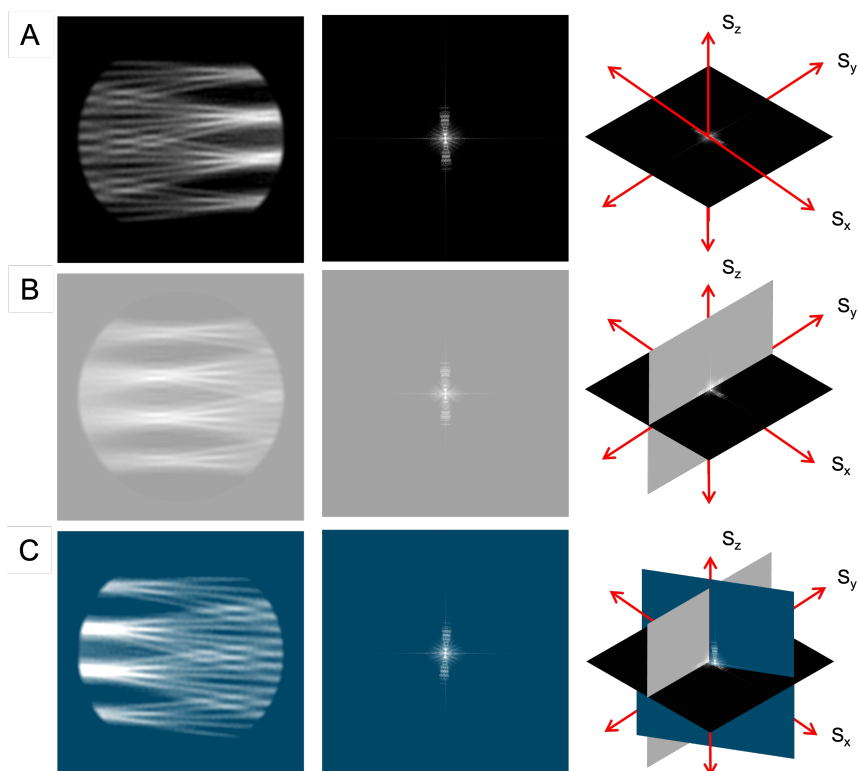


Figure 2.5: Schematic representation of the SPA steps. Image A, it's diffraction pattern and its orientation in the reciprocal space. Image B is then overlaid based on their common line. Finally image C is added to the mix.

4. **Atomic modeling:** the atomic resolution volume is used to fit the molecular model of the sample

These are executed in various software typically Relion,^{127,128} Cryosparc,¹²⁹ or Scipion.¹³⁰ Each software uses slight differences between the algorithms but the conceptual basics are the same and will be described in the following sections.

From Movies to Micrographs

The first step in SPA is to analyze the raw data (i.e. the movies) and to convert it into analyzable data (i.e. the micrographs). It is important to acquire movies with short exposure times to monitor sample progression during acquisition, enabling the identification of beam-induced shifts or damage. Drift correction algorithms are then employed to align movie frames, reducing blurring effects that could hamper subsequent analysis steps. Additionally, gain correction is necessary to address imperfections in the detection camera, which may distort the detected signal.

Subsequently, the diffraction pattern of each image is determined by applying an FFT. Proper modeling of the data involves fitting a correct CTF to determine the defocus value of the sample. Initially, this is performed as an average for the entire image. In the final stages of reconstruction, the CTF fitting is refined on a per-particle basis to optimize data alignment. This step is critical due to variations in sample thickness

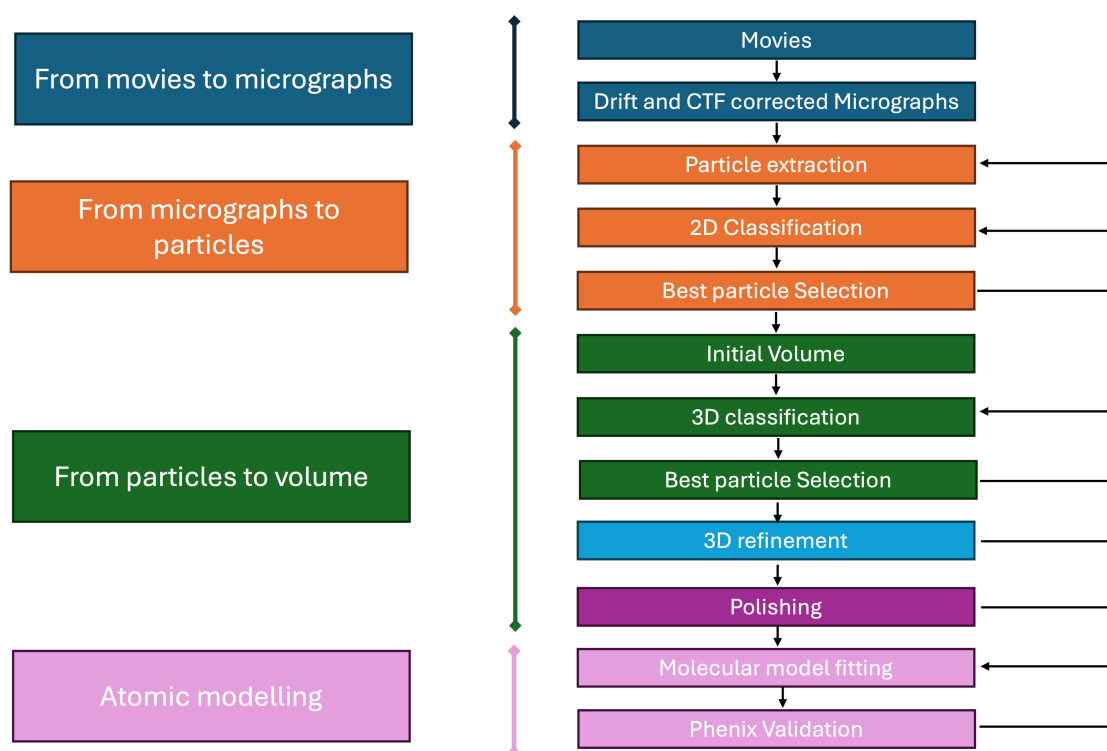


Figure 2.6: Schematic approach of SPA on a software. Each step is highlighted on the left with a specific color (blue, orange, green and pink). On the right further details of each steps are indicated. The arrows indicate possible next steps during the reconstruction process.

caused by various factors such as heterogeneous water distribution within grid holes and sample clustering. Thus, accurate determination of each particle's defocus is essential for enhancing high-resolution detection, as opposed to relying solely on the overall image defocus.

The importance of defocus determination is further highlighted by the necessity to discard highly defocused micrographs. These images exhibit high signal in the low-resolution regime and highly oscillating information in the high-resolution regime. Such contrasting behavior can lead to misinterpretation of high-resolution details, potentially compromising the quality of reconstruction. An example is depicted in Figure 2.7. Comparing defocus value of 0 μm and 3 μm highlights how at higher defocus the oscillating natures of the CTF hampers proper signal interpretation.

From Micrographs to Particles

Once the corrected micrographs are generated, individual proteins within each micrograph are identified. However, this task is extremely tedious, thus software, based on artificial intelligent and deep networks, have been develop to automate, speed up and improve final picking. These tools rely on preliminary data, such as pre-picked particles or initial projections which require some manual input to optimize their performance. While such software excels in handling large proteins with relatively simple projections (e.g., circles, squares, or triangles), their effectiveness diminishes when dealing with long fibers, particularly in cases of high fibril polymorphism. This limitation becomes espe-

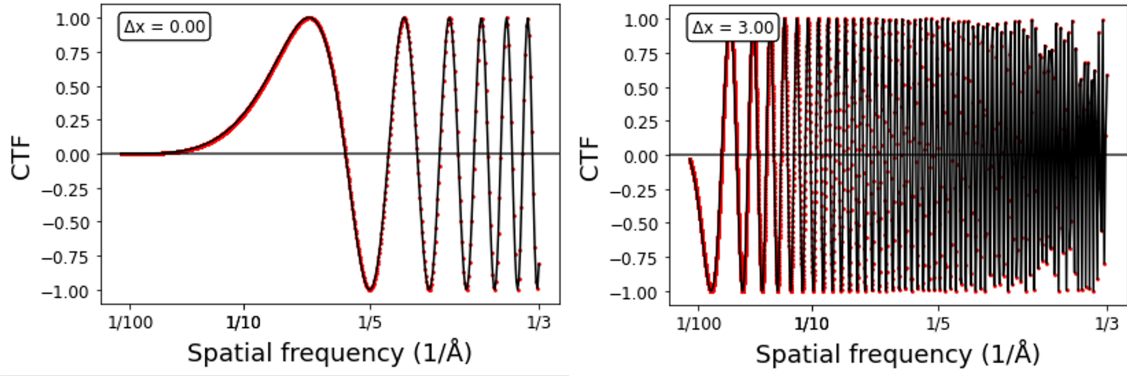


Figure 2.7: Examples of CTF curves. Simulated CTF curves using the equation reported by Thuman *et. al.*¹²². The values used are $Q = 0$, $C_s = 2.7$ nm, $\lambda_e = 0.04$ Å and Δz either 0 μ m or 3 μ m.

cially critical during 3D reconstruction, where a high degree of dataset homogeneity is crucial. Heterogeneity within the dataset introduces incorrect signals, thus amplifying the noise component and lowering the SNR. Such phenomena can not only reduce the quality of the final reconstruction but also hinder the detection of any useful information (e.g. detection of the protein backbone).

Once the picking is completed it is necessary to extract the particle by selecting a box size, which means subdividing the full micrographs into smaller sections which contain the protein. It should be kept in mind that while the protein in the image is of a certain size (e.g. 100 Å), the diffraction pattern is actually allocated into a larger region (e.g. 200 Å), thus the neighbouring section of the protein is of invaluable importance as it constrains information required during reconstruction. In standard SPA the rule of thumb is to use a box size of approximately two times the size of the protein e.g. 20 nm protein thus 40 nm box size. Yet, this is not applicable for helical structures and the box size determination requires a trial and error approach. Often it is necessary to re-extract particles with a different box size while using the information acquired in the previous steps to improve the final reconstruction.

The extracted particles undergo a 2D classification process where particles sharing the same orientation are grouped together. Within each cluster, the images are averaged to generate a 2D class average with an increased SNR. Therefore, it is imperative to obtain a large number of 2D classes, which depict the protein projection from various angles. This makes 2D classification a vital analysis step in standard SPA, as it can identify unwanted proteins. For instance, consider apoferritin, a standard protein used in SPA testing. Despite high sample purity, apoferritin may contain traces of iron in its core. Consequently, in a 2D classification, the apoferritin core may appear different from that of the correct protein (e.g. as a bright spot), enabling the removal of particles from these classes and allowing for the use of a more homogeneous dataset.

However, in the case of fibrils, a different approach is necessary. Unlike standard proteins, long fibrils typically exhibit a "twisting view" (e.g., the side view depicted in line with the micrograph, xz-plane), as they orient flat on the grid surface, making the "top view" (e.g., inside the fibril, xy-plane) rarely observable. Therefore, 2D classification in fibrils is utilized to characterize fundamental helical parameters, such as the cross-over

and rise values (further discussed in section 2.2.2).

From Particles to Volume

To establish a reliable correlation among the numerous high-quality particles extracted, it is indispensable to detect an initial object (initial volume) to serve as reference. The choice of initial volume can be performed in a variety of ways. For fibrils, a featureless cylinder often proves optimal. It's crucial to apply a low-pass filter to the initial volume to hide high-resolution details while retaining low-resolution information, as using a highly detailed reference volume can bias the alignment process. Typically, during the initial reconstruction steps a 60 Å low pass filter is used which means that none of the features below 60 Å are evident. This is a very strong filter and an example of how this filter plays a role on the removal of high resolution details is reported in Figure 2.8.

Once the initial volume is selected, the extracted particles undergo an iterative cleaning and refinement process to homogenize the dataset, enabling the generation of a high-resolution 3D volume for atomic modeling. At first, the particles are aligned to the initial volume and subjected to a 3D classification, typically with a small number of classes, often up to five, to identify major structural variances. This initial classification aims to provide an overview of the dataset and identify major structural variances.

Subsequently, the classification undergoes iterative refinement, where the number of classes is reduced to focus on distinguishing between particles with optimal or with less favorable alignment. This process aims to separate particles that contribute to higher

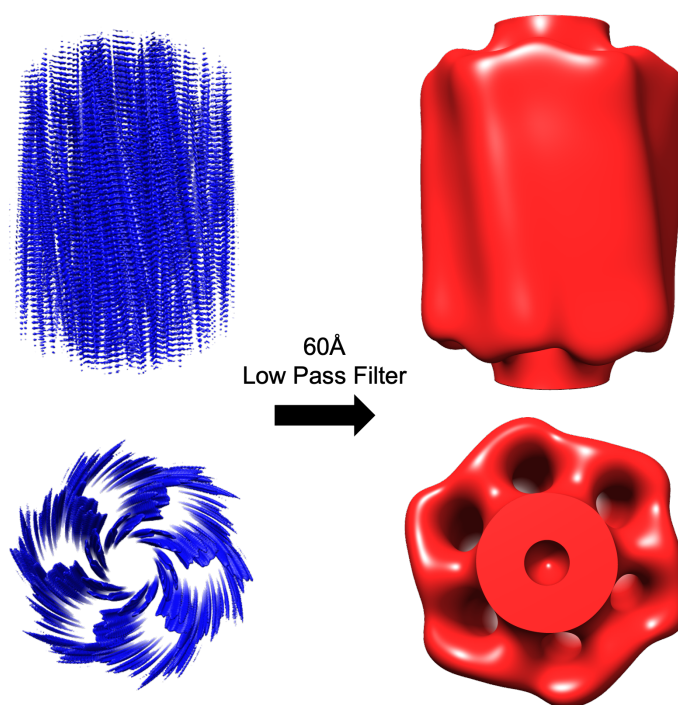


Figure 2.8: Low pass filter effect. Side and top view of a 3D volume before (blue) and after (red) the use of a 60 Å low pass filter.

resolution volumes from those that contribute to lower resolution ones. The iterative nature of this process involves using the product of novel alignments (e.g. a higher resolution volume) as references for further alignments, ensuring continuous improvement in dataset homogeneity and volume resolution.

Ultimately, this iterative classification process continues until the molecular structure can be reliably detected. Following this, a 3D refinement is conducted to precisely determine the resolution using the gold standard method. This refinement process entails splitting the dataset into two random subsets, aligning each independently, and quantifying the correlation between the two datasets using Fourier Shell Correlation (FSC) analysis.^{131,132} FSC is a cross-correlation function that measures the similarity between the FFT of the two objects at different spatial frequencies.¹³¹ An example is reported in Figure 2.9, at low spatial frequencies (representing low-resolution information), the correlation is typically high, while it diverges at higher spatial frequencies (representing high-resolution information). In SPA, it is common practice to determine the spatial frequency where FSC equals 0.143, as this frequency reflects the resolution of the reconstructed volume.¹³²

Once a high-resolution volume (above 4 Å) has been obtained, further polishing steps can be undertaken. These steps include optimizing the CTF fitting on a per-particle basis and employing Bayesian polishing to improve particle alignment. As these polishing steps require a higher computational cost compared to those performed in the initial stage (from movies to micrographs, section 2.2.2), they are applied only to the optimized dataset subsequent to the aforementioned cleaning steps.

Helical Symmetry Parameters for Volume Determination

Before diving into the atomic modeling, a brief insert is included to discuss 3D reconstruction of structures exhibiting helical symmetries, such as the ones investigated

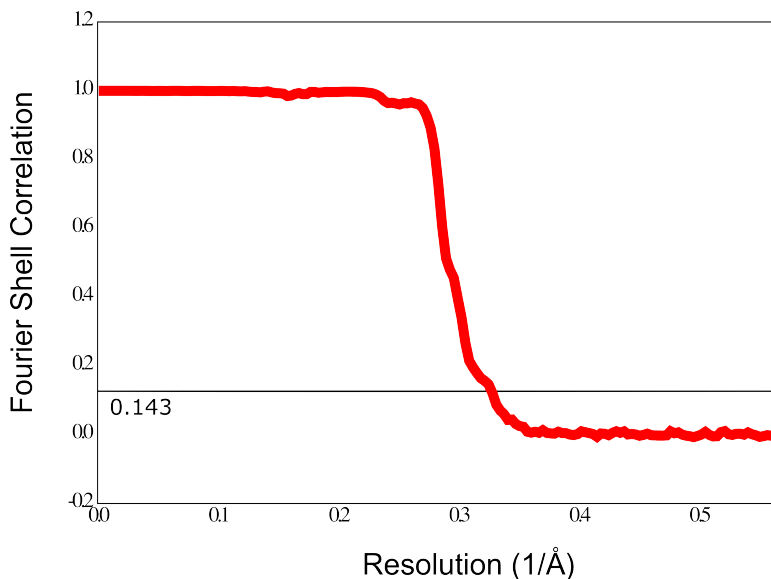


Figure 2.9: FSC curve. Example of a FSC curve highlighting the 0.143 value used to define the resolution of the reconstructed volume.

in this thesis.

To comprehend this, it is essential to outline the characteristics of helical symmetry. Helical symmetries are observed when an object can be depicted as a monomeric unit rotated by an angle $\Delta\theta$ (twist, $^\circ$) and translated by Δz (rise, $\text{\AA}$) along the helical axis (z-axis) in every xy-plane. Figure 2.10 provides a graphical representation of helical symmetry, highlighting another crucial parameter, p : the pitch. p denotes the length along the helical axis required for a monomer to complete a full 360° rotation. Thus, it enables the correlation between rise and twist, as expressed in the following equation:

$$\text{Rise}(\Delta z) : \text{Pitch}(p) = \text{Twist}(\Delta\theta) : 360 \quad (2.1)$$

In SPA for helical structures, the determination of rise and twist is crucial for successful volume determination. Therefore, during 3D classification or refinement, algorithms often integrate a local search for these values. Such search is computationally intensive, thus it is necessary to input preliminary values close to the real ones leading to the need to determine their potential values. The rise can be estimated in one of two ways. The first option is to observe the diffraction pattern of a micrograph as shown in Figure 2.11 A, the FFT clearly evidence an intense bright signal at a spatial frequency which corresponds to a $4.8 \text{ \AA}$ feature. The second option is reported in Figure 2.11 B. The 2D class, which thanks to the clustering has an increased SNR, presents an striped pattern, highlighted in the line profile below. By measuring the distance between each peak (example reported in the insert) the average rise can be determined.

Twist determination, on the other hand, relies on the fibril's pitch, as outlined in Equation 2.1. This feature manifests in micrographs as the cross-over distance, where the same fibril feature recurs as highlighted in Figure 2.12. It's important to note that additional rotational symmetry operations, such as C2, often coexist in the xy-plane. These operations imply that rotating the helix by $180^\circ (= 60^\circ \times 2)$ around the z-axis should yield the same feature. Consequently, the pitch is no longer equivalent to the cross-over but is instead $n \times$ cross-over, leading to a proportional error in the twist. Symmetry operations typically become apparent after several rounds of refinement, thereby introducing

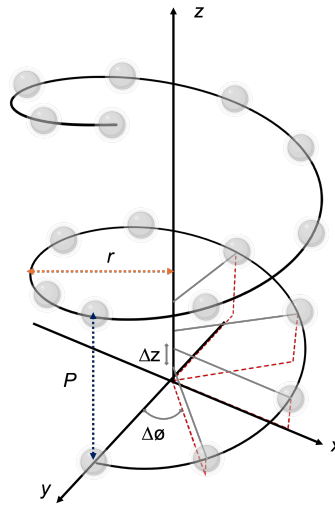


Figure 2.10: Helical representation. Schematic representation of an helix highlighting the key parameters that compose it: pitch (p), rise (Δz) and twist ($\Delta\theta$)

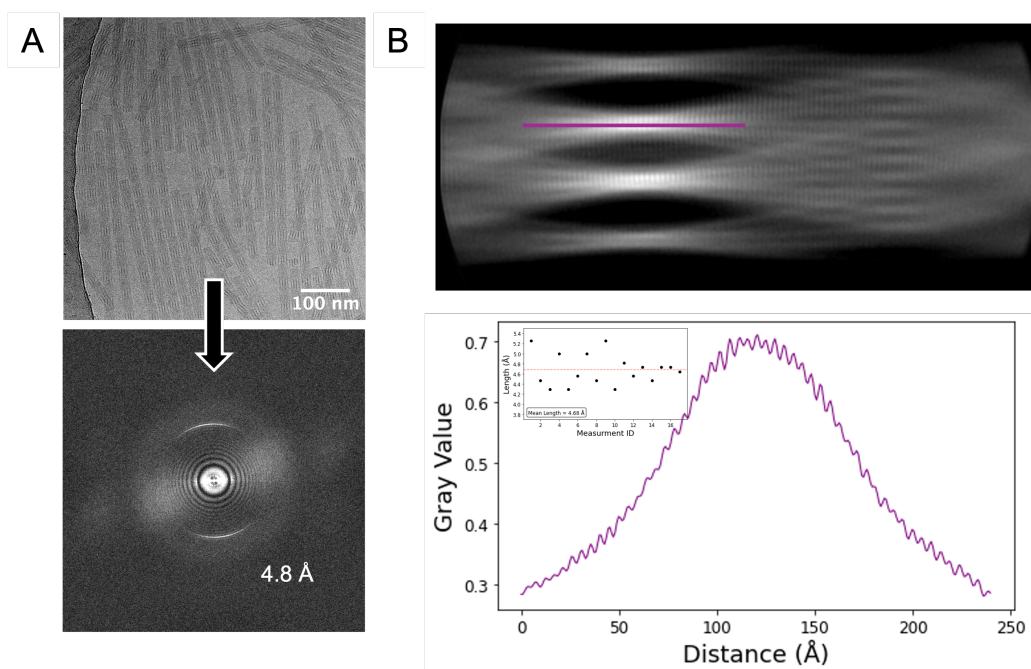


Figure 2.11: Examples of rise determination. Panel (A) reports a micrograph and its FFT (i.e. the diffraction pattern). Panel (B) shows a 2D class, a small section is highlighted (purple) and its line plot is reported below. The insert show the average (red) and specific distance between the peaks which report the average rise value.

an error in the alignment. Despite this, the formula serves as a fundamental starting point for fibril reconstruction, and in the event of symmetry detection, the values can be subsequently adjusted.



Figure 2.12: Cross-over feature. Example of cross-over detection on a fibril. Scale bar = 50 nm.

Atomic Modeling

The high-resolution volume obtained from all the above mentioned steps serves as the basis for fitting the atomic structure of the material. This fitting is performed utilizing various software. Specifically, in this thesis, UCSF Chimera,¹³³ COOT,¹³⁴ and Phenix¹³⁵ were employed. UCSF Chimera and COOT are visualization software where electronic densities and atomic structures can be compared. Additionally, these software enable calculations which aid the fitting of atoms into the electronic densities. The primary difference is that UCSF Chimera is preferred for visualization of the system while COOT for atomic fitting. Lastly, Phenix is a software for model building, in this work the MolProbity¹³⁶ plugin was used to interpret the quality of the fitting.

The typical atomic modeling procedure begins with producing the molecular model in Chimera, followed by manually overlaying the model onto the atomic density or using a rigid body fit. The resulting atomic model is saved in Chimera, with the volume coordinates serving as reference points, before being transferred to Phenix for model validation.

In Phenix, the quality of the fitting is assessed, with errors such as improper rotamers or atomic clashes (atom density which are overlaid) being identified and reported. Phenix offers direct visualization of these errors through its integration with Coot. Subsequently, automated fitting functions in Coot and/or Chimera, combined with manual adjustments to atom positions, are utilized to iteratively refine the atomic model. The final analysis, facilitated by the validation tool in Phenix, provides an assessment of the overall quality of the atomic model.

2.3 EELS: Electron Energy Loss Spectroscopy

While we have explored the benefits of utilizing TEM, it's important to acknowledge that this instrument offers versatility beyond imaging alone. In this thesis, we extend the capabilities of the TEM by leveraging its integration with a spectrometer for EELS. In the following section, we dive into the fundamental principles of EELS, highlighting its significance in elucidating nanomaterial characterization.

2.3.1 EELS Core Concepts

In Section 2.1.1, the interaction of electrons with the sample was discussed. In TEM imaging, both elastic and inelastic scattering are crucial for image formation. However, when transitioning to EELS, the interaction necessary to extract information relies on inelastic scattering. When a high-energy source, such as electron beam in an electron microscope, interacts with the sample, it can cause an electron in the inner-shell of the atom to be ejected. In these instances, the electron undergoes a loss of energy unique to the atomic structure of the sample. Consequently, analyzing this energy loss enables elemental analysis of the sample, facilitating investigation into its precise atomic composition.

Therefore, in EELS, it is necessary to detect energy differences in the electron beam after it interacts with the specimen. To achieve this, an additional component is integrated into the microscope setup: the spectrometer. This device, which acts as a magnetic prism, divides the electron beam based on its energy levels, resulting in the formation of a spectrum. On this spectrum, the x-axis denotes energy loss (eV), while the y-axis represents intensity, influenced by factors like elemental concentration. The spectrum typically comprises two main segments depicted in Figure 2.13: the low-loss and high-loss regions. The former predominantly consists of unscattered electrons, whereas the latter holds significant importance as it reveals the elemental composition of the sample.

EELS operates as a point analysis technique, focusing on individual measurement points rather than imaging entire fields like conventional TEM imaging does. Consequently, EELS requires the microscope to operate in STEM mode, which means to be

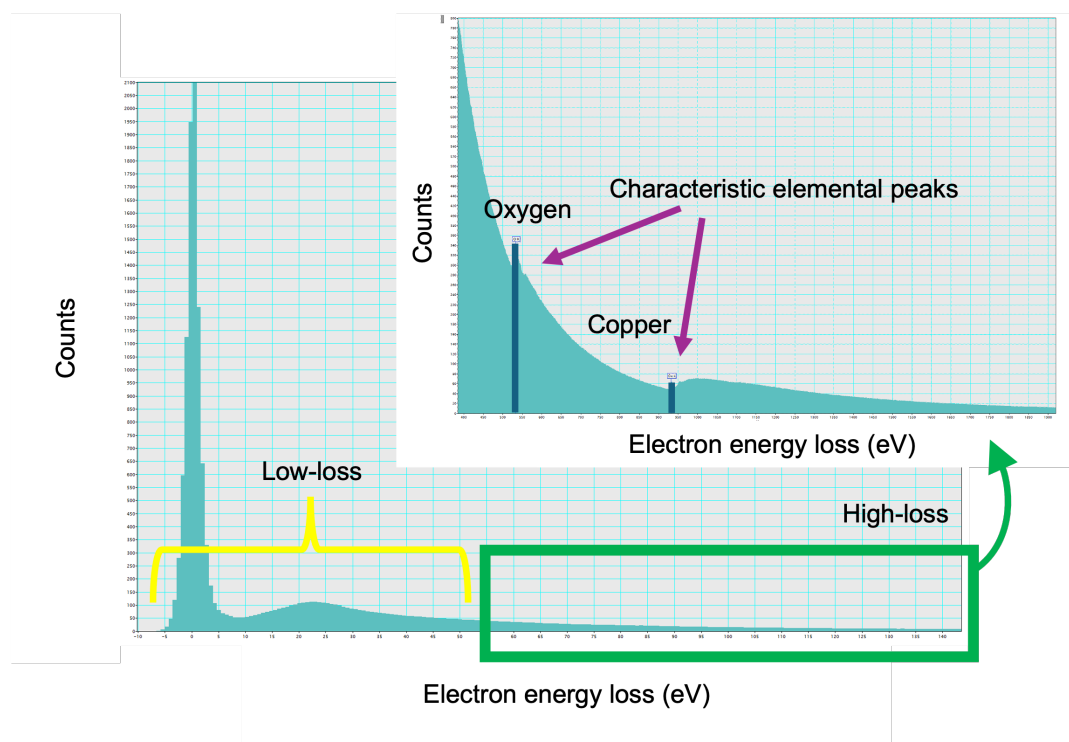


Figure 2.13: EELS spectra. Example of a EELS spectra highlighting the low-loss region (between 0 and 50 eV, yellow) and the high-loss region (above 50 eV, green). The insert zooms in the high-loss region and highlights characteristic elemental peaks (Oxygen and Copper). Data was produced from an in house measurement of an EELS map.

working in Scanning-TEM. This mode scans individual points and moves the electron beam accordingly to gather data from multiple locations. As a result, longer acquisition times are necessary in STEM mode compared to TEM. Although this scanning approach is primarily used for beam-stable materials, it allows elemental analysis at multiple points. Such elemental information can then be visualized as EELS maps, offering a clear representation of the sample's composition.

2.4 SRM: Super Resolution Microscopy

Continuing from the preceding discussion on various microscopy techniques, this section introduces the final technique utilized in this thesis: super resolution microscopy (SRM). The groundbreaking advancements in SRM were pioneered by Nobel laureates Eric Betzig, Stefan W. Hell, and William E. Moerner, along with their respective teams. Their work revolutionized fluorescence microscopy (FM), overcoming the theoretical limitations dictated by the Abbe diffraction law.¹³⁷ Within this section, I will outline the required microscope setup for performing FM, emphasizing key features essential for SRM. Subsequently, I will dive into the fundamental principles that enable the surpassing of resolution limits. Finally, I will discuss two specific types of SRM utilized in this thesis: dSTORM and DNA-PAINT.

2.4.1 Optical Setup

Before delving into the details of SRM, it is imperative to elucidate the microscope setup, schematically represented in Figure 2.14. For SRM, a conventional fluorescence microscope can be used. The key components of this microscope include the source, lenses, mirrors, objective, and detector. In FM, a laser serves as the source, its beam focused by a set of initial lenses onto a dichroic mirror, which directs the light into the objective lenses. The objective lenses, in direct contact with the sample, are responsible for magnification. Upon interaction with the sample, the light induces fluorescence, which is collected by the objective and directed to a second mirror. This mirror reflects the light to a dichroic mirror, which separates the light depending on its different wavelengths before transmitting it to the detector.

In SRM, an additional element is often required: a mirror within the objective lens capable of tilting the light source. While in standard FM, the light typically traverses the sample directly (epifluorescence), this method can lead to a decrease in SNR. Therefore, often in SRM, the light source must reach the sample, deposited on a glass coverslip, in a tilted manner with a specific angle to achieve total internal reflection fluorescence (TIRF). The differences between epifluorescence and TIRF are schematically depicted in the inset of Figure 2.14. In TIRF, due to reflection, the light source penetrates only a shallow depth, illuminating approximately 200 nm of the sample. Consequently, the signal originates solely from emitters localized within this specific region, minimizing background effects.

Subsequently, the image is generated when a dye at an xy location emits light, captured by the camera and recorded at its xy location. If an object is labeled with dyes of multiple color each emitting at different wavelengths, the camera records their positions at slightly different locations. This is due to chromatic aberration in the dichroic mirror. While such errors are minor and negligible (e.g., 1 pixel shift) in typical FM, in SRM, where pixel size is mathematically reduced, meticulous correction for chromatic aberrations is necessary.

2.4.2 Working Principle of SRM

The Nobel Prize-winning breakthrough that paved the way for SRM involved limiting dye emission through techniques such as STimulated Emission Depletion (STED) and single molecule microscopy. The steps involved in the latter are illustrated in Figure 2.15 and described below. When an object, such as a NP, is fully labeled with dyes, each dye can be activated sequentially, one at a time. The emitted light from each dye is then detected and analyzed, allowing for the localization of each element. This process is repeated for all dyes, enabling the rendering of higher-resolution images.

The detection of the signal on the camera follows a characteristic shape known as the point spread function (PSF). Typically described by a 2D Gaussian model,¹³⁸ understanding the PSF's shape allows for curve fitting and localization of its maxima. In single molecule localization microscopy (SMLM), the position of the emitter is reflected by these maxima, enabling nanometer-precision detection.

Therefore, SMLM necessitates an object with blinking capability, where dyes are turned on and off sequentially, and a movie capturing this blinking process is recorded,

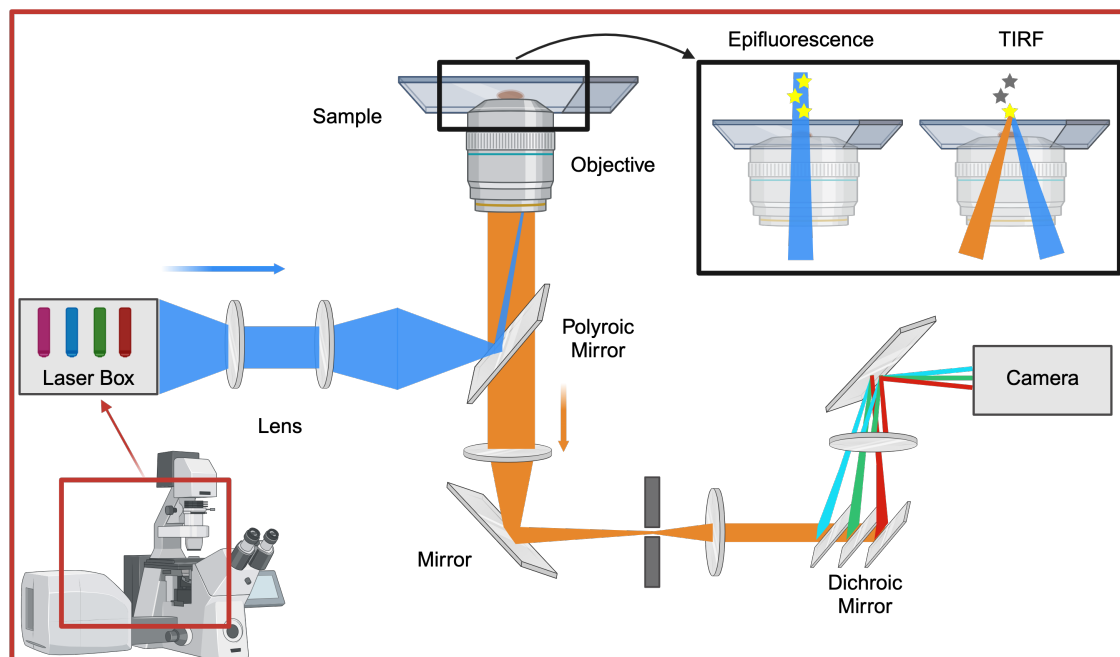


Figure 2.14: Schematic representation of a microscope for SRM. The core set up is the same as a standard fluorescence microscope. The insert highlight the properties of the objective which, thanks to a mirror can tilt the light thus adjusting the directionality and allowing the microscope to be used in TIRF mode. Figure created with BioRender.com

usually for about 10 minutes. The duration of blinking events depends on the underlying mechanism, typically ranging from tens to hundreds of milliseconds. Determining the appropriate acquisition time is crucial, as shorter exposure times yield more localizations, enhancing image quality but increasing file size and potentially reducing SNR. Thus, exposure time selection is strongly dependent on the experimental setup.

2.4.3 Image Analysis Setup

The movies collected are then processed with Fiji,¹³⁹ a freely available image analysis software. Specifically, Fiji provides the ThunderSTORM¹⁴⁰ plugin that is specifically designed to localize blinking events. An example of how to approach the analysis is reported in Figure 2.16. In each frame, the software applies Gaussian profiling to the point spread function (PSF) using pre-selected parameters (Figure 2.16 A). The fitting process identifies the localization in each frame, indicated by the red cross in Figure 2.16 B. Once this localization is determined for all frames, a rendered image is generated (Figure 2.16 C). Subsequently, a cross-correlation algorithm corrects for any drift that occurred during the acquisition time (Figure 2.16 D). Lastly, filtering is applied to enhance image quality (Figure 2.16 E). This filtering step is crucial as it combines experimental data (localization in the image) with prior knowledge of the sample, such as NP size. Samples are inherently imperfect, and unwanted signals may arise from imperfections during the

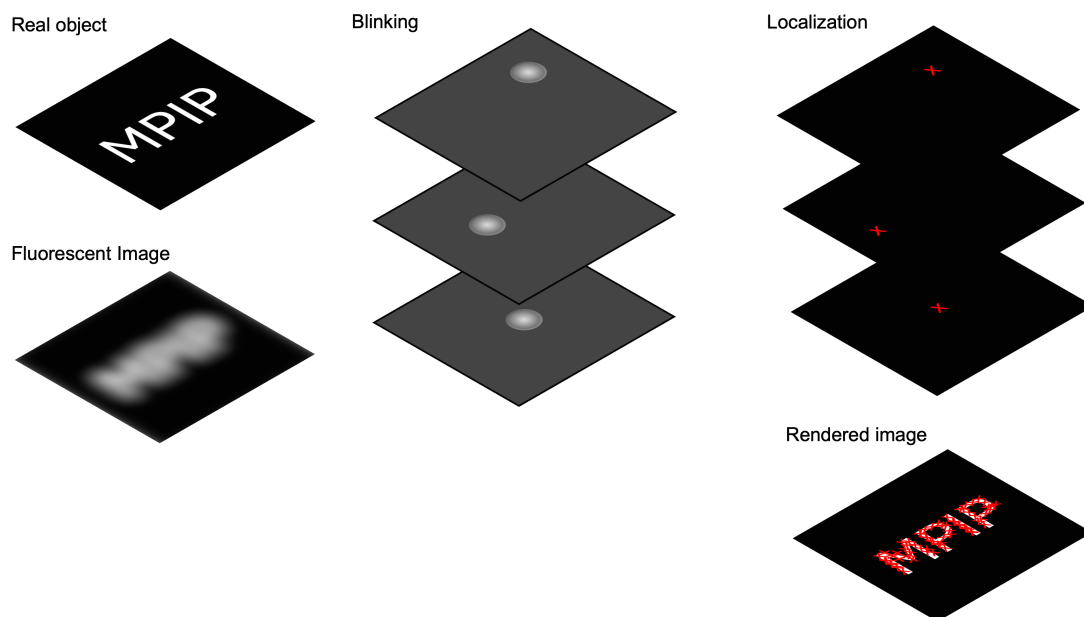


Figure 2.15: Single molecule localization microscopy. From left to right, schematic representation of the process in SMLM technique. A labeled object is observed with low resolution in FM. The object is allowed to stochastically blink. Each signal is localized. The localizations are summed to obtain the rendered image.

cleaning process, making it difficult to remove all unwanted dye signals entirely.

2.4.4 dSTORM: direct STochastic Optical Reconstruction Microscopy

The various SMLM techniques are distinguished by the mechanism of blinking. In dSTORM, this blinking is an inherent property of the dye, influenced by experimental factors such as laser power and buffer conditions.¹⁴¹

The process enabling this blinking phenomenon is illustrated in Figure 2.17. Initially, a dye absorbs a specific wavelength (depicted in dark green), causing an electron to transition to an excited state (S_1). From this state, the electron can return to the ground state (S_0), emitting fluorescence (depicted in light green, representing the "on" state). Alternatively, the excited state may undergo intersystem crossing (ISC), leading to its conversion into a triplet state ($_1$). In this triplet state, the dye can return to S_0 by emitting phosphorescence. Instead, in dSTORM, this state is reduced by introducing thiols, such as MEA (β -mercaptoethylamine),^{114,142–144} into the system. The thiol reduces the triplet state of the dye, converting it into a stable radical anion (as indicated by the orange arrow). This radical anion state represents the "off" state for the dye as it cannot emit fluorescence. The duration spent in the "on" and "off" states is governed by rate constants k_{on} and k_{off} , influenced by the dye's properties and the composition of the buffer. Typical buffers include thiols to induce dye reduction and oxygen scavenger systems to regulate oxygen levels. Oxygen plays a dual role in the system: it promotes the return of the dye to the "on" state, but it can also induce photobleaching, leading to the complete deactivation of the dye. A typical oxygen scavenger system is a mixture of Glucos Oxydase and Catalase (GODCAT).^{114,142–144} Buffer conditions are dependent

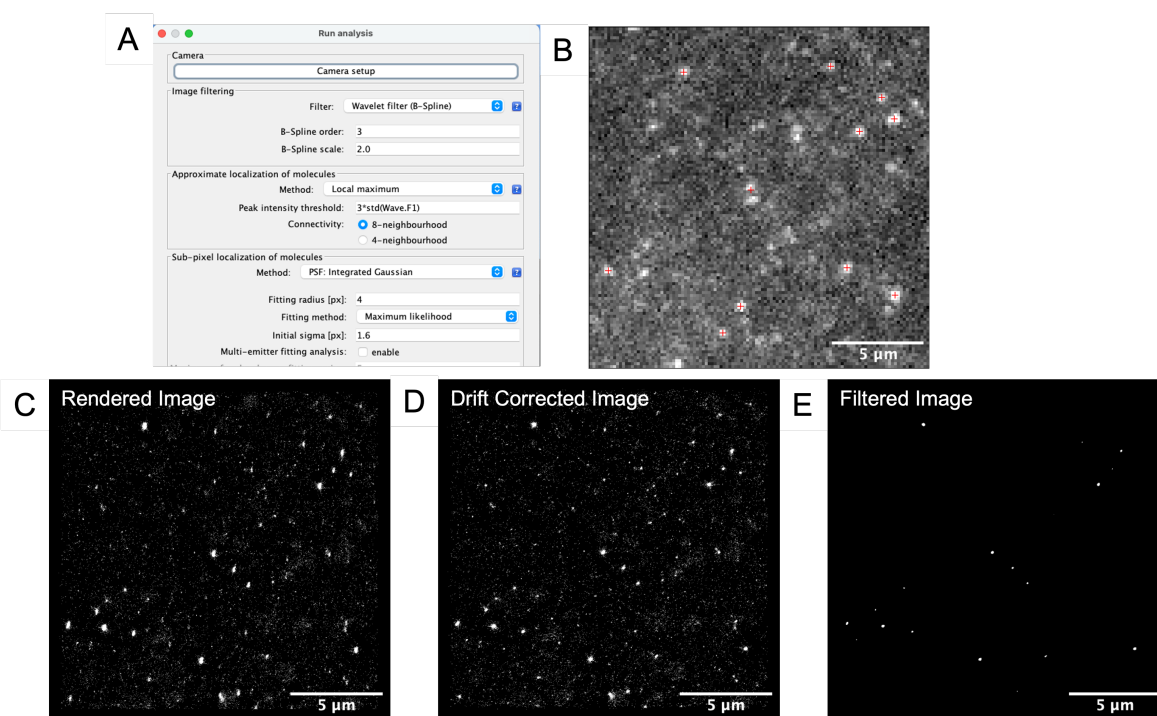


Figure 2.16: Example of an image analysis set up. (A) the ThunderSTORM plugin is called and the localization parameters are set. (B) the coordinates of each event are highlighted in each frame. (C) the localization are summed to produced a rendered image. (D) drift correction is applied. (E) filtering removes incorrect localization.

on the dye utilized and have been heavily characterized and optimize over the years.¹⁴⁵

2.4.5 DNA-PAINT: DNA Point Accumulation for Imaging in Nanoscale Topography

PAINT microscopy, unlike dSTORM, relies on the blinking events associated with the chemical features of the probe rather than their photophysical properties. Specifically, in DNA-PAINT, blinking is induced by transient interactions between two types of DNA strands: docking and imager. The imager strand is labeled with a highly fluorescent dye and it freely diffuses in solution. The docking strand, on the other hand, is conjugated to the object of interest, serving as the docking site for the imager strand. The kinetics of interaction between these strands, characterized by the association (k_{on}) and dissociation (k_{off}) rates, regulate the duration of blinking. Typically, such duration is controlled by the length of the DNA strand (8-10 bases).

Contrarily to dSTORM, DNA-PAINT is less susceptible to bleaching due to the rapid diffusion of the imager strand. As bleached strands diffuse out of the field of view, new ones can interact with the target. Hence there is no the need for additional solution components, like STORM buffer, to prevent bleaching and promote the dye's blinking. This diffusion-based blinking mechanism, along with the kinetic constants, allows for quantitative analysis of DNA binding events, leading to qPAINT. While this method offers advantages in quantitative analysis, it may present challenges in selectively labeling the target. Therefore, the choice of microscopy technique should be tailored to

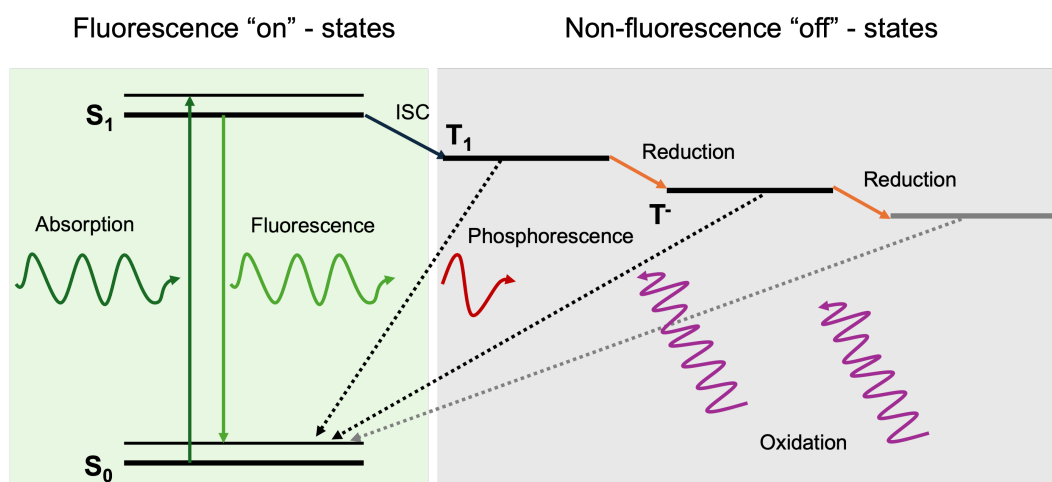


Figure 2.17: Jablonski diagram. The Jablonski diagram highlights the processes involved in dSTORM. This diagram is inspired by the one reported in the book chapter by Endesfelder and Heilemann.¹⁴¹

the specific scientific question at hand.

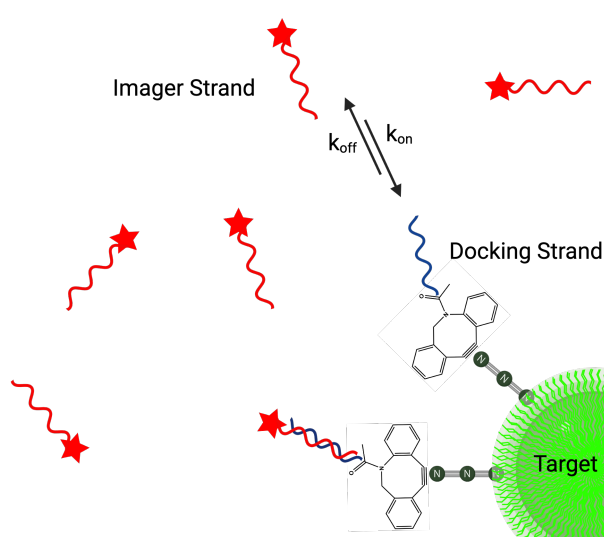


Figure 2.18: DNA-PAINT. Schematic representation of DNA-PAINT blinking mechanism between the imager strand (red) and the docking strand (blue) on the target (green).

Chapter 3

Unraveling Structural Characteristics of HONEY ASAP: Hexagonally Ordered Nanofibrils Easily Yielded by Amphiphilic Self Assembly Peptides

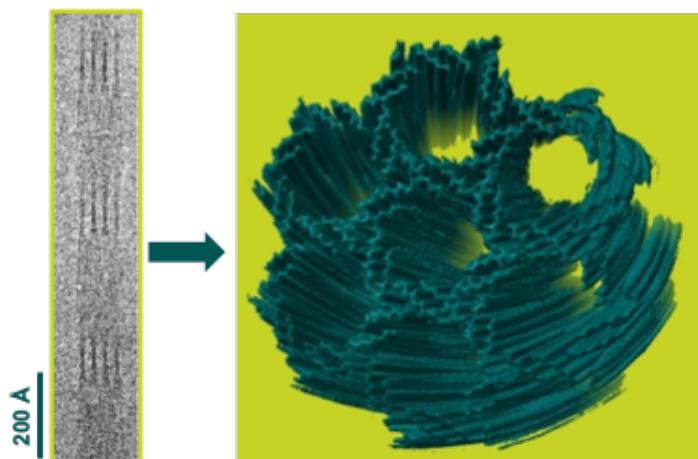
This chapter is primarily based on a collaboration project within the Max Plank Institute for Polymer Research. The work is reported in a manuscript in preparation:

“ Peptide Origami: 3D Hexagonal and Honeycomb Fibrils from Dual-Amphiphilic Peptides ”

Mazzotta Francesca*, Jasmina Gaćanin*, Baptista Luis Andre, Schmidt Matthias, Fändrich Marcus, Cortes-Huerto Robinson, Weil Tanja, Lierbrwirth Ingo, Landfester Katharina

Contributions:

I performed the cryogenic electron microscopy imaging together with the single particle volume reconstruction and atomic modeling. J.G. synthesized the peptide sequence, performed the characterization analysis. L.B.A performed the molecular dynamics simulations. I.L, M.S. and M.F. supervised F.M. in the single particle analysis studies. R.H. supervised L.B.A. in the simulations. F.M., J.G., L.B.A. and R.H contributed to the paper writing. M.F., T.W., K.L, and I.L. contributed to the final version of the manuscript.



Chapter Motivation

In this first chapter, my aim is to emphasize the critical importance of structure-function relationship studies in nanomaterial research. By diving into atomistic structural understanding, we pave the way for a targeted and tailored investigation into the applications of novel nanomaterials. Single particle analysis and cryogenic electron microscopy have been combined to provide an atomistic understanding of peptide-peptide interactions. This approach is essential to overcome the resolution limit imposed by beam-sensitive materials like peptide nanofibrils, which result in electron microscopy images with low signal-to-noise ratio and low resolution. Single particle cryogenic electron microscopy restores the Å-level resolution achievable with electron microscopy by averaging thousands of these images. The comprehensive analysis presented in this chapter sets the stage for elucidating the underlying interactions governing the formation of the material, which promises to unlock its full potential in diverse applications. More importantly, the results unlock a novel platform for tailored and selective interactions comparable to DNA-origami. Hence, our structural characterization brings us a step closer to the establishment of a novel function: peptide-origami.

ABSTRACT

Self-assembly represents nature’s elegant solution for effortlessly producing intricate supra-molecular structures from simple building blocks, such as peptides. Among these, amyloids stand out as rigid supramolecular assemblies characterized by a highly organized cross- β structure. These versatile systems serve important roles, functioning both in physiological contexts and pathological conditions, while also inspiring the development of synthetic analogs. While natural amyloids have been extensively studied at the atomic level to elucidate their formation mechanisms and correlate structure with function, synthetic amyloids remain relatively underexplored. Consequently, the relationship between synthetic amyloid fibrils and their structural properties remains only partially understood. To bridge the gap in understanding the structure of synthetic amyloids, our study focuses on the structural investigation of Hexagonally Ordered Nanofibrils Easily Yielded by Amphiphilic Self Assembly Peptides: HONEY ASAP. Employing cryogenic electron microscopy combined with single particle analysis, we obtained a high-resolution volume of 2.9 Å. Our investigations unveil that this easily synthesized peptide, characterized by a dual amphiphilic structure, gives rise to amyloid fibrils that not only conform to the typical cross- β motif but also exhibit a novel trimeric junction. This unique combination of motifs results in the formation of fibrils composed of hexagonal multi-compartment nanotubes, featuring 5 nm pores. Interestingly, the fibril structure is based on the interaction among 60 chains per plane. These findings introduce a novel interaction motif that holds promise for the development of innovative functional materials. Leveraging the dual amphiphilic nature of the peptide, we can now induce programmable interactions that can lead to the development of peptide-origami. Furthermore, the unexpected discovery of nanotubes underscores the importance of structural investigations in nanomaterials, highlighting the potential for tailoring specific applications based on their unique architectures.

3.1 Introduction

Self-assembly is a fundamental process in nature, where simple building blocks interact via non-covalent forces to form complex structures.²⁰ Among the notable examples of natural self-assembling systems are DNA and proteins. DNA interactions are remarkable for their simplicity and specificity, relying on just four nucleotide units that interact in a highly specific manner, with only two types of interactions. However, when arranged in the correct order, these interactions give rise to highly ordered structures, showcasing the remarkable self-assembly capabilities of DNA that originated the field of DNA origami.²¹ Similarly, these interactions have also been exploited to develop super-resolution microscopy techniques such as DNA-PAINT. As mentioned in Section 2.4.5, in this field, the interactions enable well-defined blinking events.

In contrast, proteins are composed of 20 natural amino acids, offering a significantly greater degree of variability. This diversity in amino acid composition enables proteins to exhibit a wide range of functions as well as a more complex array of interactions.¹⁴⁶ Despite this complexity, nature efficiently guides the self-assembly of proteins, highlighting its ability to harness the intricacies of molecular interactions.²⁰ Within these protein sequences, specific motifs play a crucial role in driving self-assembly.^{20,146} These motifs

serve as guiding principles to engineer peptide based self-assembling materials such as peptide nanofibrils (PNFs).^{96,147}

An important class of PNFs are amyloids, defined by their ability to form cross- β structures.²⁹ These structures have been observed in nature²⁴ both with a pathological^{104,148} and functional role.²⁸ By engineering peptide sequences to specifically assemble into similar amyloid structures, researchers have unlocked various applications, including the development of nanowires,¹⁴⁹ carbon dioxide capture,⁴³ catalysts,^{47,50} and platforms for viral transduction.^{42,105} However, unlike DNA origami, where folding and structure are well-defined and engineered, amyloid peptides pose challenges due to the variability in type of interactions. Structural studies have revealed that amyloid-forming proteins can assemble into structures that appear similar at the micro- and nano-meter scale due to the fundamental β -sheet structure, while exhibiting significant differences at the sub-nano scale owing to variations within side chain interactions.¹⁴⁸ This variability underscores the importance of tailoring interactions to produce specific assemblies with well-defined structures.

Although atomic-level understanding of amyloid assemblies has primarily focused on natural amyloids to elucidate their mechanisms of action, some studies have also explored synthetic amyloids used for catalysis⁵⁰ and retroviral transfection.¹⁰⁵ These studies underscore the significance of the structure-function relationship; understanding the atomistic disposition elucidates the optimal performance of the material. Hence, there is a need to expand such studies to encompass a broader range of synthetic amyloid nanomaterials. Enhanced understanding of these assemblies will not only advance our knowledge of self-assembly processes but also facilitate the rational design of novel amyloid materials. Moreover, a deeper understanding of structure can guide application research tailored to specific structural features.

In this study, we aim to address this gap by investigating the structural features of a novel Hexagonally Ordered Nanofibril Easily Yielded by Amphiphilic Self Assembly Peptides: HONEY ASAP. Through SPA, we uncover that the typical cross- β motif is accompanied by a trimeric interaction, so called junction. While trimer interactions are often observed in nature,^{26,36,150,151} here we demonstrated, by means of molecular dynamics (MD), the importance this interaction plays in the stabilization of the amyloid fibril. The combination of the junction and cross- β motifs, achievable via the dual-amphiphilic nature of the peptide, produces a complex and highly organized assembly structure. Contrarily to the typical amyloids, whose fundamental unit is based on two or three chains,¹⁵⁰ HONEY ASAP presents a minimum of 18 up to more than 60 chains per fibril plane. These chains assemble on the perimeter of one up to fourteen hexagons enabling the unexpected formation of multi-compartment nanotubes. With such insight into the material's architecture, we can rationally interpret the key design element in the peptides sequence. Such understanding stands as a step towards development of peptide origami.

3.2 Nanofibril Formation

This work is based a simple dual amphiphilic peptide (DAP) sequence with nine amino acids: KVKVSQINM (Figure 3.1 A). DAP was designed to easily self-assemble into PNFs (Figure 3.1 B). Assembly was promoted by hydrophobic interactions between

individual peptide chains to produce trimer interactions, so-called junctions (Figure 3.1 C). The molecular engineering was aimed at producing β -strand peptides with a dual amphiphilic feature. Thus, rather than presenting an evenly split surface, with hydrophilic and hydrophobic side chains on opposite sides, the sequence introduces an inversion in the central point. Such features leads to distinct assembly interactions where the hydrophobic patches, referred to as sticky-ends, are constantly exposed to water (Figure 3.1 C). This assembly produces a supramolecular structure termed HONEY ASAP: Hexagonally Ordered Nanofibrils Easily Yielded by Amphiphilic Self Assembly Peptides. Importantly, this system bridges multiple scales ranging from atomic to micron scales. Evidently, the simple peptidic molecular building block ($\text{\AA}$) assembles precisely in the plane (nm) and extends in depth (μm) to produce long (few μm) fibrils.

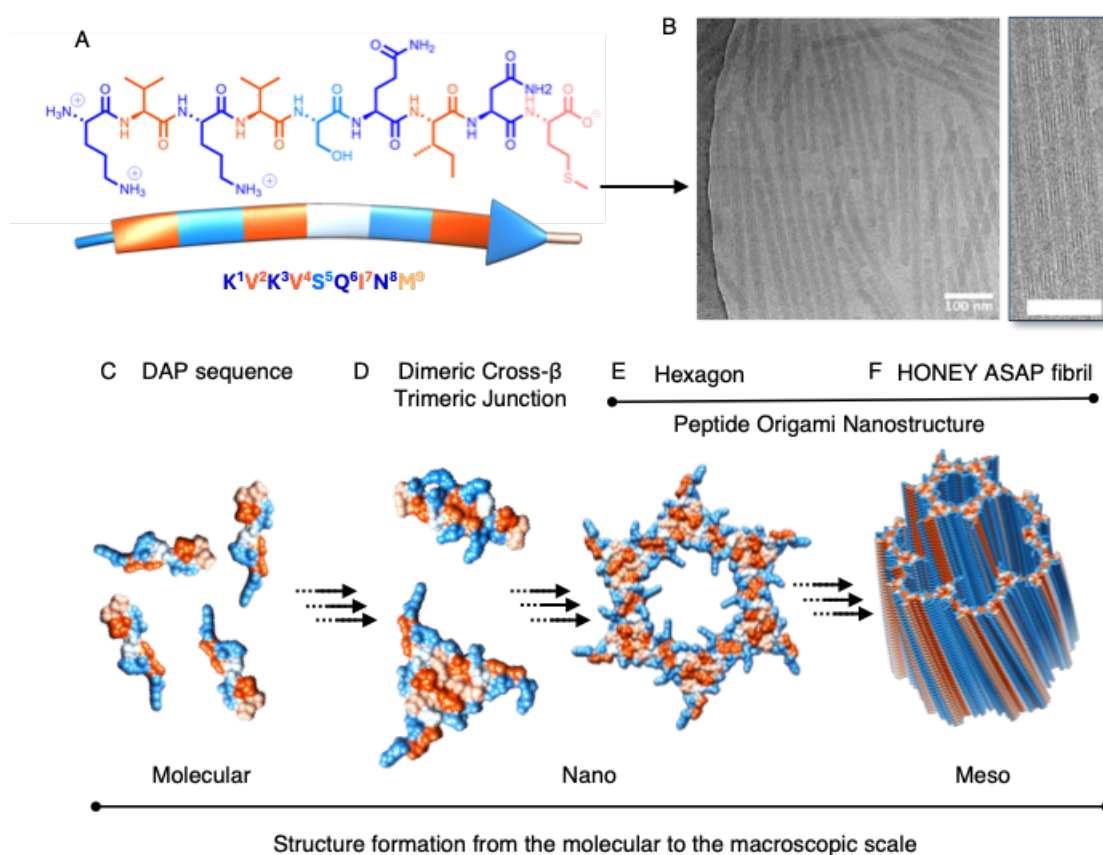


Figure 3.1: HONEY ASAP overview. Peptide sequence color coded based on the Kyte Doolittle parameters¹⁵² of each residue. Red to blue the hydrophobicity decreases (A). Exemplary CryoEM image highlighting the highly structured fibril projection with an close up on the formed fibrils. Scale bar = 100 nm. (B). Schematic representation of the fundamental components formed by the DAP sequence. DAP ranges from the atomic monomer (C), to a nanometric in plane assembly based on dimeric or triemric motifs (D) and a hexagon (E), to a micrometric-long fibril (F).

To determine optimal conditions for peptide assembly, the sequence was incubated in a variety of solutions (Table 3.1). The starting condition was to dissolve the fibrils in DMSO and subsequently dilute them in PBS for a final 10:90 solvent ratio. This condition enabled fibril formation (Figure 3.2 A). To establish the importance of PBS buffer the same procedure was replicated in water, yet no fibrils were detected. To verify

if DMSO compromises fibril formation, the DAP monomer was directly dissolved in PBS successfully forming fibrils (Figure 3.2 B). The experiment once again confirmed the importance of PBS, as in water no fibrils were formed. Additionally, no morphological difference was detected (Figure 3.2 B) thus we continued with the DMSO:PBS approach. A last test to confirm the specific importance of PBS, was to investigate fibril formation in acetate buffer. No fibrils were detected, probably due to the lower pH that affects the protonation state of the amino groups in the LYS.

DMSO %	Buffer Type	Buffer %	H2O %	[DAP] (mg/mL)	Fibril formation
10	PBS	90	-	1	
10	-	-	90	1	X
-	-	-	100	1	X
-	PBS	100	-	1	
1	PBS	9	90	0.1	
10	Acetate	90	-	1	X

Table 3.1: Fibril formation in different conditions. Overview of the experimental conditions that enable fibril formation after 24 h incubation at 22 °C.

Next we proceeded with determining experimental conditions in which preformed fibrils (after 24 h assembly) are stable. As mentioned, water hampered fibril formation, yet fibrils (after 24 h incubation) presented long term stability (up to 2 months) even after dilution in water (to 0.1 mg/mL) with no major morphological differences detected (Figure 3.2 C). Further stability on the preformed fibrils was tested under shear forces (sonication) and increased thermal energy (60, 85 or 95 °C). Fibers did not present any relevant difference in morphology after 15 min sonication in water bath (Figure 3.3) and when incubated at 60 °C (both for 15 and 60 min) (Figure 3.4 A,D). Similarly,

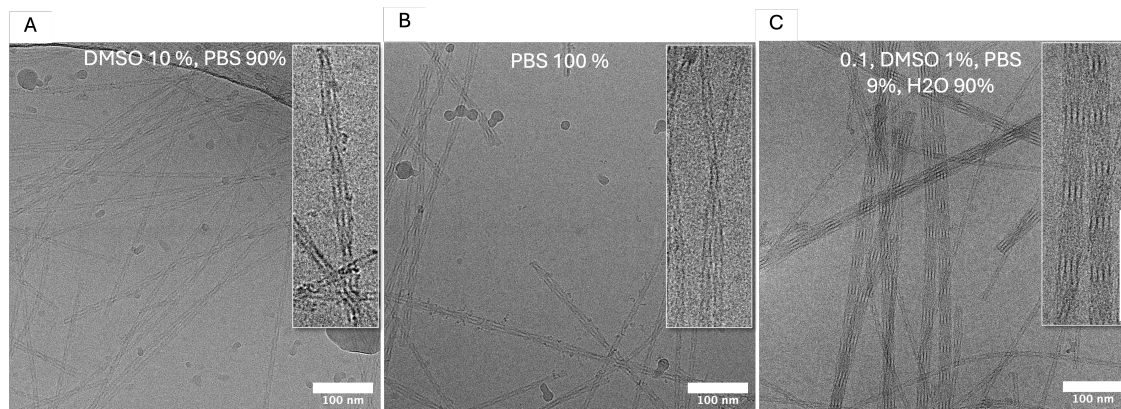


Figure 3.2: Fiber formation in different conditions. CryoEM micrographs of fibrils assembled in 10:90 DMSO:PBS, pure PBS and in a diluted state with 1:99 DMSO:PBS. Insert is a close up of the fibrils. Scale bar = 100 nm.

when incubated at 85 and 95 °C for 15 min, fibrils preserved their structure (Figure 3.4 B,C). Longer incubation times (60 min), while not destroying the fibrils, caused small morphological variations such as loss of clear and rigid patterns and increased curvature (Figure 3.4 E,F).

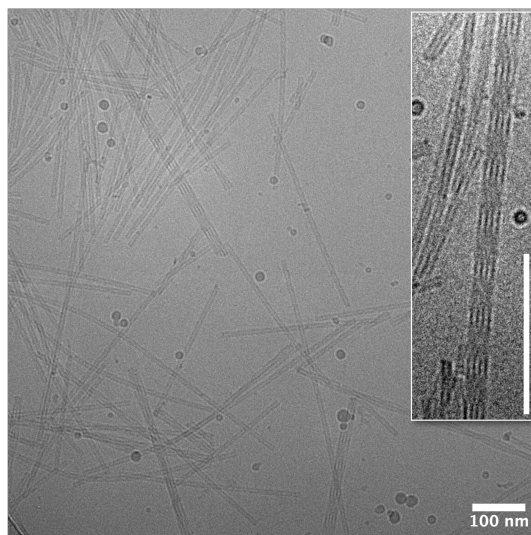


Figure 3.3: Shear stability. CryoEM micrograph of preformed fibrils after 15 min in a sonication bath demonstrating the fibril did not disassemble. Insert is a close up of the fibrils. Scale bar = 100 nm.

To assess relevant time points in fibril formation the peptide self-assembly process was followed over time (Figure 3.5). Preliminary fibril detection was observed after 30 min, yet clear morphology difference was detected over time. To ensure high quality reconstruction fibril morphology required sample homogeneity and reduced background. Hence fibrils were left to equilibrate for 2 months with intermittent imaging to verify fibril quality (Figure 3.6). Over time the background improved without external manipulation. We hypothesize that such difference in image background is associated to presence of soluble oligomers. Similarly to typical amyloid fibrils, the sequence might assemble into oligomeric structures¹⁵³ (e.g. short junctions) that could be present in solution and that over time equilibrate in mature fibrils.

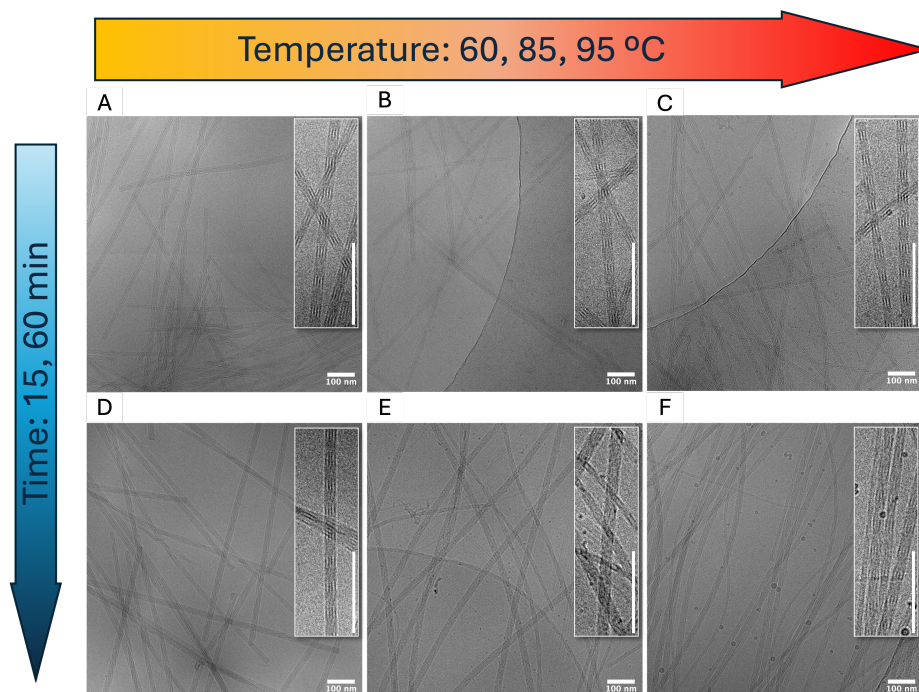


Figure 3.4: Thermal stability. CryoEM micrographs of preformed fibrils after 15 min at 60 (A), 85 (B), or 95 (C) °C and after 60 min at 60 (D), 85 (E), or 95 (F) °C. Fibers are detected in all conditions. Above 85 °C and 60 min the fibril's persistence length is decreased (E and F). Insert is a close up of the fibrils. Scale bar = 100 nm.

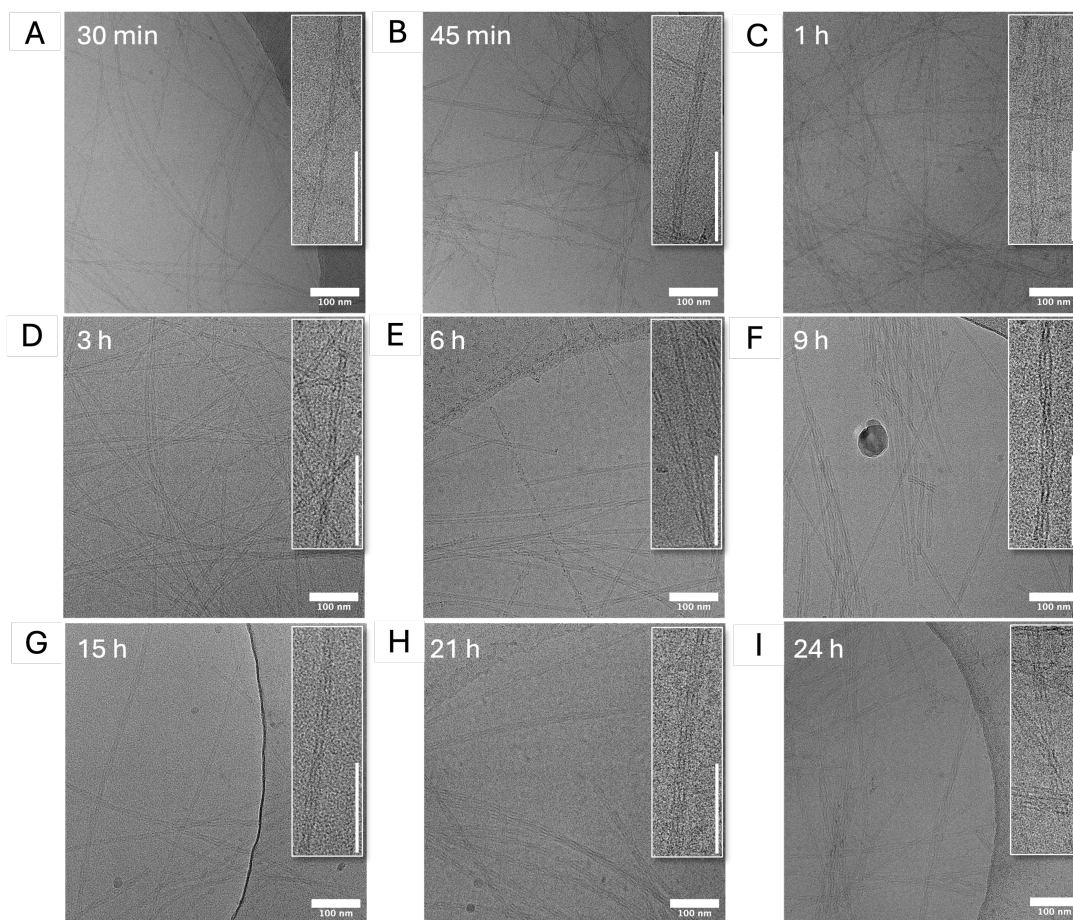


Figure 3.5: Early kinetics. CryoEM micrographs of fibrils in the initial assembly stages while shaking at 22 °C: 30 min, 45 min, 1 h, 3 h, 6 h, 9 h, 15 h, 21 h, and 24 h. Insert is a close up of the fibrils. Scale bar = 100 nm.

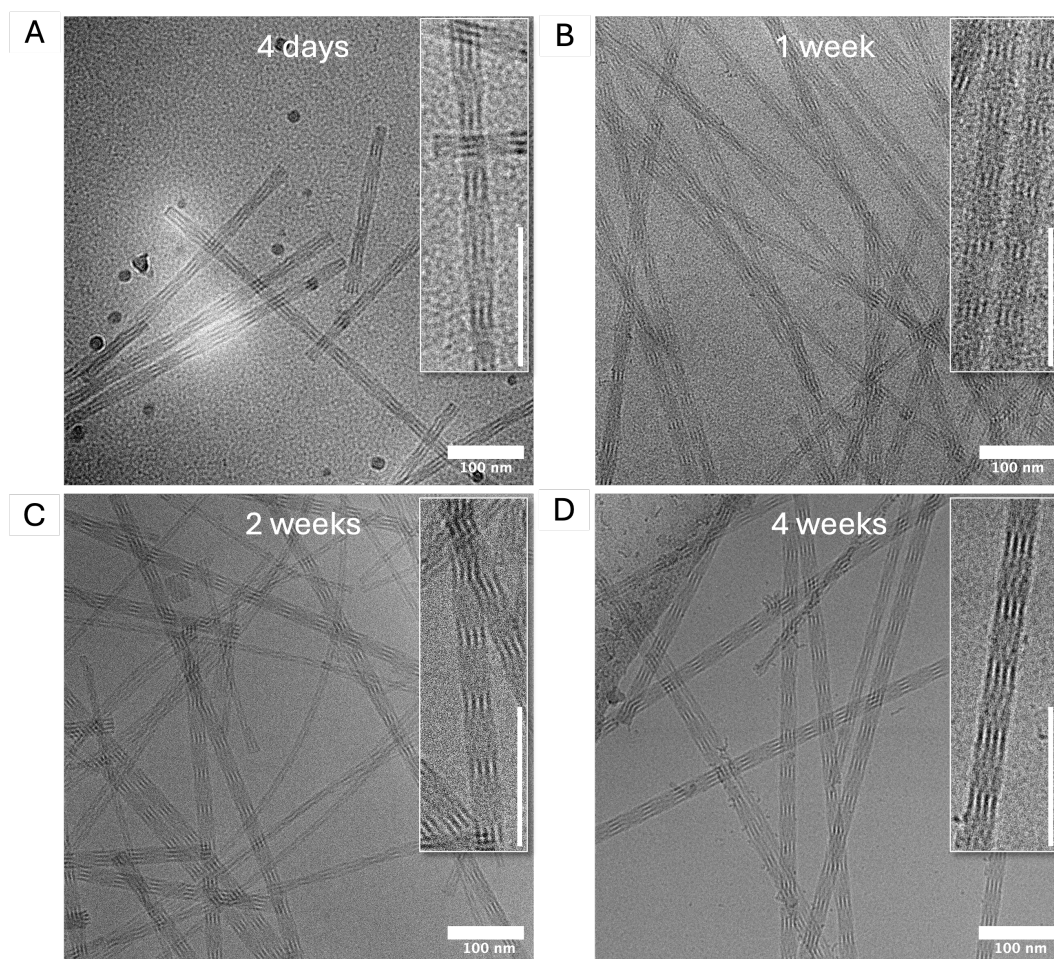


Figure 3.6: Long term stability. CryoEM micrographs of fibrils after 4 days, 1 week, 2 weeks and 4 weeks of incubation at 37 °C. Insert is a close up of the fibrils. Scale bar = 100 nm.

3.3 Morphology Analysis

To determine the underlying structural features associated with DAP fibrils, CryoEM images were investigated to identify predominant morphologies which were subsequently reconstructed via SPA. Specifically, nine morphologies (Figure 3.7) were detected. Each morphology presented the same fundamental repeating unit where a striped section was followed by a featureless one (stripes in Figure 3.7). Notably, the number of bright stripes varied in each morphology from one to a total of five. Each morphology was named after its characteristic stripe pattern and presents a three-fold nomenclature, which reflects the sequential number of stripes. For example, morphology 2-2-1 is composed by a total of three units of striped areas that are intermitted by featureless ones. Two of the striped areas feature two stripes (2-2) while the third one only one (1). The cross-over distances, measured as the length of these three fundamental units, varied from 80 and to 220 nm. The increase was complementary to the average width growth of the fibrils (Figure 3.8 A). Specifically, depending on the respective stripe pattern, the average width of the fibril varied between 60 and 250 Å.

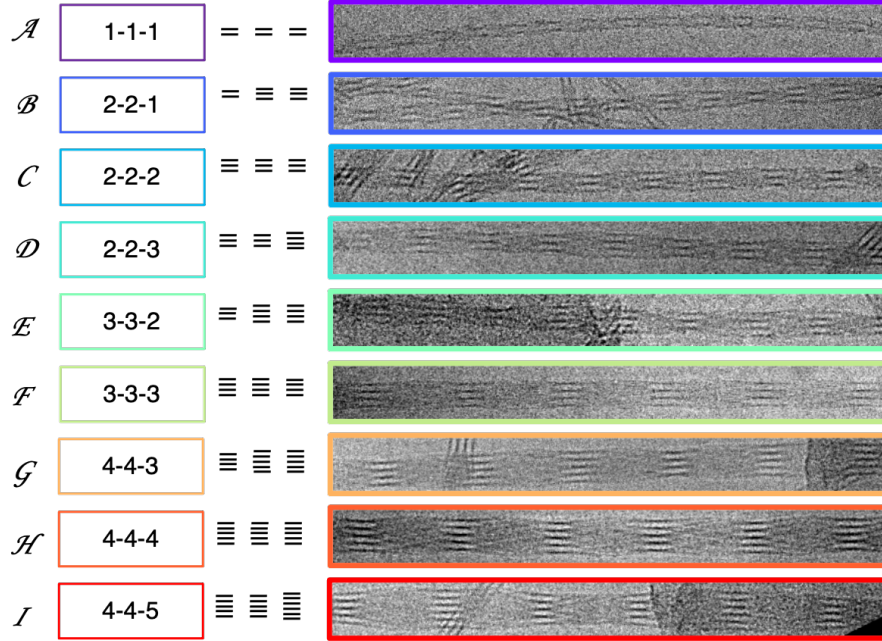


Figure 3.7: HONEY ASAP Morphologies. Closeup of the nine detected morphologies ($\mathcal{A}$ to $\mathcal{I}$) in the CryoEM micrographs. The nomenclature is based on the striped-unresolved pattern. The black lines guide the visualization of the morphology-specific pattern. . Scale bar = 50 nm.

Further analysis shed light on the morphology abundances (Figure 3.8 B). The quantification indicates that 3-3-3 (light green) and 1-1-1 (purple) present prevalent morphologies with a relative abundance respectively of 73% and 12%. Hence, the 3D volume of these morphologies was obtained via SPA and yielded 2.9 Å and 4 Å resolution volumes, respectively. In both cases the in-plane resolution is high enough to successfully identify the peptide backbone in the fibril plane. Hence, the in-plane density (cross-section) allowed successful identification of the peptide backbone (Figure 3.9) in both morphologies. Specifically, the bright areas, indicative of the electronic density, highlight an unprecedented, highly ordered, multi-hexagonal peptide disposition where the peptides

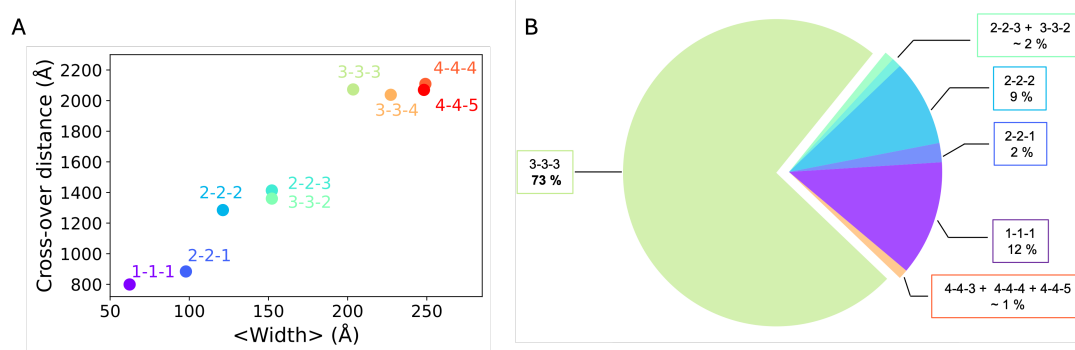


Figure 3.8: HONEY ASAP Morphologies. Cross-over VS Width of the morphologies (A). Relative quantification of the morphologies (B).

arrange on the hexagon's perimeter. Such perimetral disposition enables the formation of pores, which in the extended fibril structure introduces nanotube characteristics to the HONEY ASAP fibrils.

In addition to pore formation, the cross-section highlights interesting features in the peptide chain disposition. Specifically, the 1-1-1 morphology clearly presents 12 peptide chains (1 to 12 in Figure 3.9) which interact two-by-two in a cross- β manner, forming six β -sheets. Additionally, 6 less resolved densities are found to connect each cross- β pair in a triangular manner, forming the previously mentioned junction. These 6 densities assume a clear resolution in the 3-3-3 morphology (I to VI in Figure 3.9) chains. In this 3-3-3 morphology, it is possible to detect that these (6) junction-forming chains are the ones that also form cross- β pairing in the perimeter of a neighboring hexagon. Hence, starting from the single hexagon present in the 1-1-1 morphology, six new hexagons are connected via these bridging chains to obtain the 3-3-3 morphology. Such feature suggests an in plane lateral growth of the fibril where the terminal chain acts as a sticky surface.

Given this bridging between the two morphologies, we applied the hexagonal-growth concept to simulate the remaining morphologies (Figure 3.10). Hence, we selectively combined a different number of hexagons, based on the 1-1-1 morphology, in the fibril plane (Figure 3.11). By extending these multi-hexagonal dispositions along the fibril axis (center of mass) we successfully replicated the morphologies detected in CryoEM (Figure 3.7). In such a way the DAP building block scales from the atomic to the nanometric scale producing nanofibrils with one (1-1-1) up to 14 (4-4-5) multi-compartment nanotubes. While peptide-based nanotubes have been extensively investigated in the literature,⁵² only few studies successfully produced multi-compartment structures.^{154,155} In these instances, cyclic peptides were used as the building blocks, with specific side chains modified to facilitate lateral assembly. Consequently, the synthetic pathway inherently possesses greater complexity compared to the method outlined for DAP.

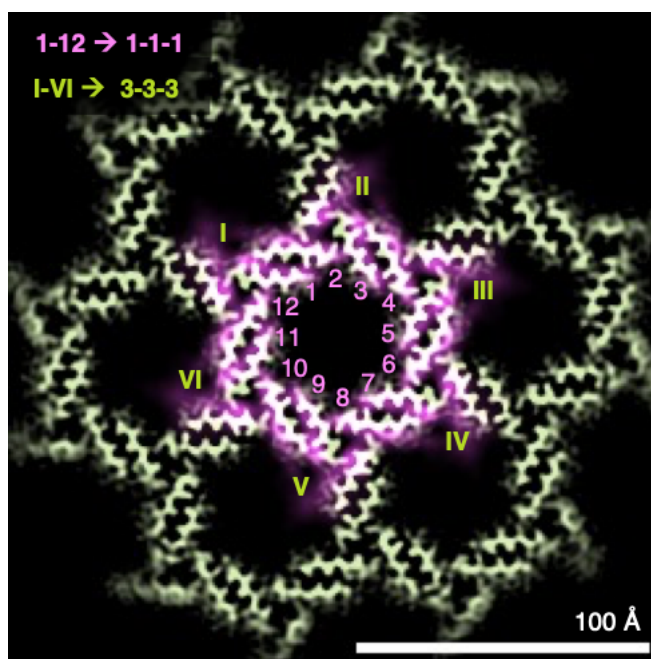


Figure 3.9: 1-1-1 and 3-3-3 overlap. Cross-section of the 1-1-1 morphology (green) overlaid with the 3-3-3 one (red). The numbers guide the reading of the central resolved densities (1 to 12) that interact in a cross- β manner and the bridging ones (I to VI) that connect the 1-1-1 to the 3-3-3 morphology by forming junction interactions. Scale bar = 100 Å.

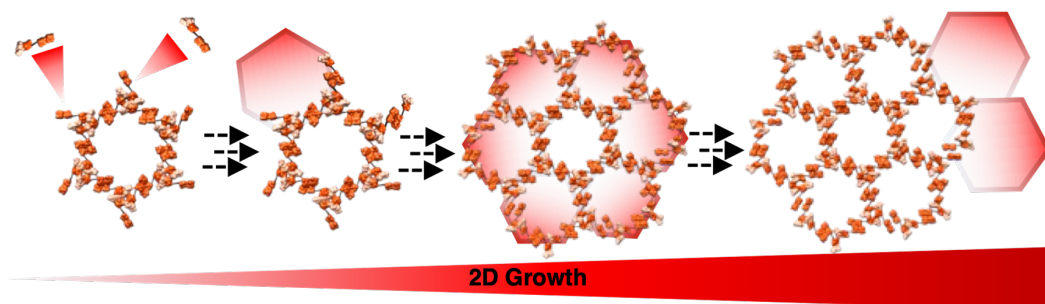


Figure 3.10: From 1-1-1 to 3-3-3. Conceptual scheme highlighting the bridging point that connect the single, central hexagon (1-1-1) with the seven hexagons (3-3-3) via sticky-end lateral extension.

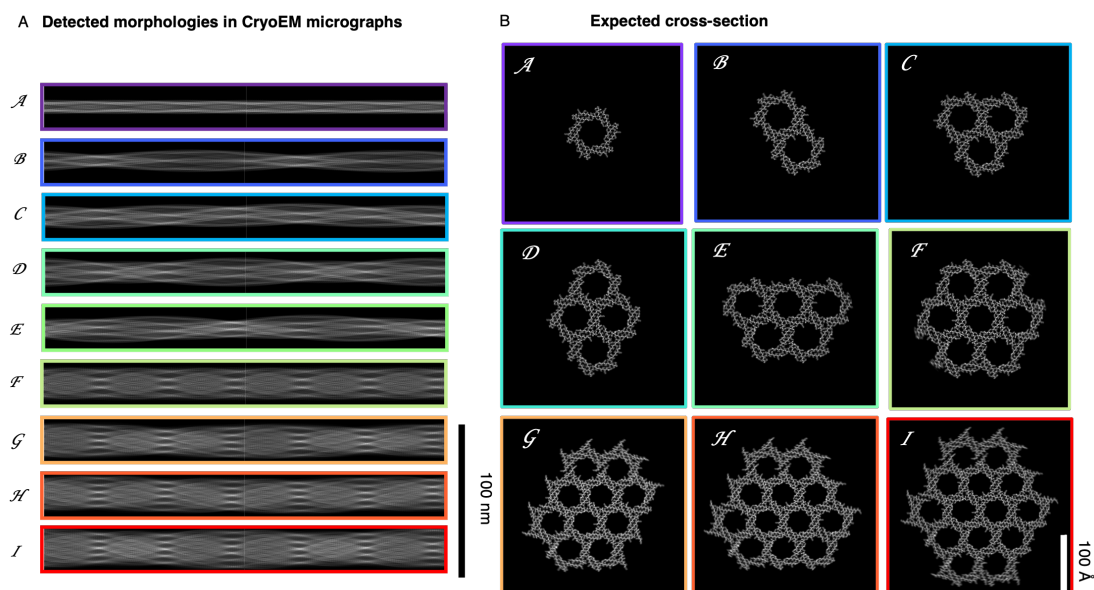


Figure 3.11: Simulated morphologies. Cross-section (A) and projection (B) of simulated morphologies 1-1-1 (purple), 2-2-1 (blue), 2-2-2 (light blue), 2-2-3 (cyan), 3-3-2 (light green), 3-3-3 (green), 4-4-3 (orange), 4-4-4 (red orange), and 4-4-5 (red). The morphologies were obtained by growing an hexagonal distribution of peptide chains around the central hexagon. To the center of mass a helical symmetry was applied with a 0.64° twist and a $4.77 \text{ \AA}$ rise. Scale bar = $100 \text{ \AA}$.

3.4 Atomic Modeling Reveals Key Interactions

To comprehend the lateral growth and interpret key features that stabilize the fibrils, atomic modeling of the 3-3-3 morphology was performed. Additionally, MD simulations were ran to aid the dynamic interpretation of the model and support the detected interaction. The reconstructed volume (Figure 3.12 A) produced a fibril with a total diameter of $210 \text{ \AA}$. The fundamental parameters that enable helical symmetry (discussed in Section 2.2.2) are the rise (Δz) and twist ($\Delta\theta$) which respectively present a value of $4.77 \text{ \AA}$ and a -0.64° (left handed helix). Fiber growth and stability in the z-axis was promoted by hydrogen bonds (H-bonds) between the backbone of each chain (Figure 3.13). In each chain, seven central amino acids (VKVSQIN) presented torsion angles consistent with β -sheet parameters, while the 2 external ones (LYS1 and MET9) were more flexible (line representation in Figure 3.13). Each layer of the fibril is composed by 60 chains. The C6 symmetry along the fibril axis reduces the modeling to 10 fundamental chains (A to J, Figure 3.14). As previously detected, the resultant structure is based on two types of interconnected interactions that can now be atomically described: the cross- β (rectangles in Figure 3.12) and the junction (triangles, Figure 3.12).

The cross- β interaction is composed by two chains in the same layer oriented in antiparallel manner to each other (C- to N- terminal vs N- to C- terminal). This interaction is mainly composed by hydrophobic contacts between the valine residues of each chain (orange, Figure 3.15). Additionally, the N-terminal amide group of both chains can interact with ASN6 of the opposite chain via H-bond. Additionally, these residues and GLN8 form H-bonds in an out of plane manner (Figure 3.15) providing additional stabilization along the fibril axis. In this area the CryoEM volume is not highly resolved,

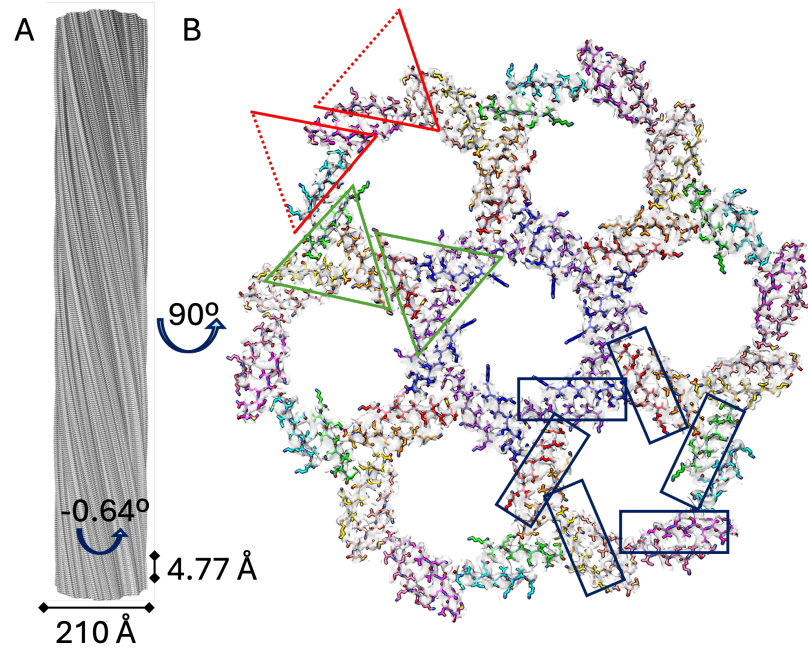


Figure 3.12: HONEY ASAP fibril. CryoEM volume (A) and atomic model (B) of the 3-3-3 morphology. The modeled chains are color coded based on their symmetry. The same color derives from the $C6$ symmetry where the chain has been rotated around the fibril axis. The color labeling is given in Figure 3.14. The blue rectangles highlight the fundamental cross- β interaction. The green triangles highlight the junction interactions. The red triangles show the incomplete junctions. The absence of a chain in these locations is comparable to the overlap highlighted in Figure 3.9.

thus indicating increased side chain mobility and the potential of forming H-bonds in a variety of conformations. The modeled atomic disposition in each of the six cross- β interactions presents small variations. Such minor differences do not highlight strong variations within the fibril layer. However, they emphasize side chain mobility which lead us to expect that all modeled variations co-exist in solution.

The junction interaction is composed by three chains from the same layer oriented in the sides of an equilateral triangle. In this arrangement, the C-terminal of each chain orients towards the LYS2 and SER5 of the neighboring chain. This interaction is based on the formation of the hydrophobic core composed by of a total of three ILE6 and three MET9 residues of each chain (orange, Figure 3.16). The carboxylic group in the C-terminal position establishes a H-bond with the LYS3 of the neighboring chain. The H-bonds are primary formed within the same fibril plane, yet some out of plane interaction are also modeled (top panel Figure 3.16). Each chain in the junction presents a slight tilt compared to the fibrils plane (Figure 3.17). Two chains are almost parallel to the plane, with an angle of $\sim -2^\circ$. While the third chain (* label in Figure 3.17), which closes the junction, shows a different behavior with a 10° tilt. Hence, the chains cooperate in order to properly interlock.

As for the cross- β , the junction interactions are preserved throughout the fibrils plane, yet slight modifications can be observed in each modeled junction. Larger difference can be observed in the outer hexagons, where the atomic model presents semi-

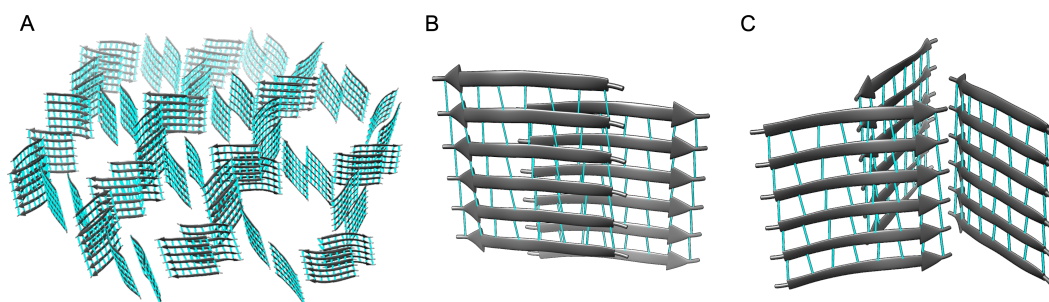


Figure 3.13: Out of plane interactions. H-bonds (light blue) along the fibril axis throughout the full fibril width (A), focused on the cross-β interaction (B), and in the junction interaction (C). the figure highlights that all residues present a β-sheet conformation and that each j chain forms with the $j+1$ (above chain) and $j-1$ (below chain) 8 H-bonds.

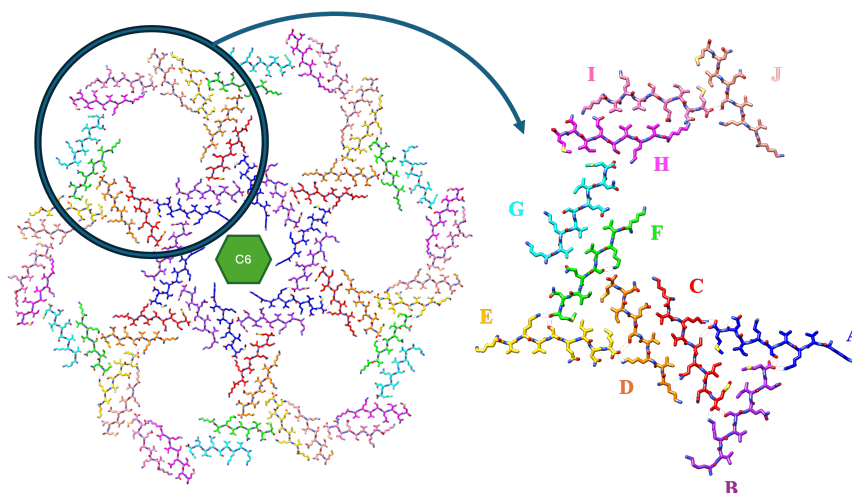


Figure 3.14: Atomic modeling. The full fibril is decomposed based on the C_6 symmetry to its 10 fundamental units A to J. A, B, and C compose the inner hexagon. C and D are the bridging chains that connect the inner hexagon to the outer ones. E, F, G, H, I and J produce the outer hexagons.

junctions (red triangles in Figure 3.12) that consist of only two chains positioned in the same triangular orientation. The lack of the third junction-forming chain is due to undefined CryoEM volume that hints to a possible presence of such chain yet lacks the resolution necessary to properly model the sequence, similarly to what was observed in the 1-1-1 morphology. The lower resolution information in this section of the fibrils can be explained in two ways. On one hand it can be intrinsically due to image alignment during the reconstruction process. During image analysis, the central 10% of the image is used for alignment, thus features in the outer section are neglected and cause improper alignments. On the other hand, it can be an intrinsic property of the fibril where single peptide chains can transiently interact with the fibril surface. Such interpretation correlates to the sticky surface and the hexagonal growth concept discussed in the previous section which correlates the different detected morphologies (Figure 3.10). The atomic analysis highlights that the junction forming residues, that bridge the connection between two hexagons, expose the hydrophobic Valines. Hence, these water exposed

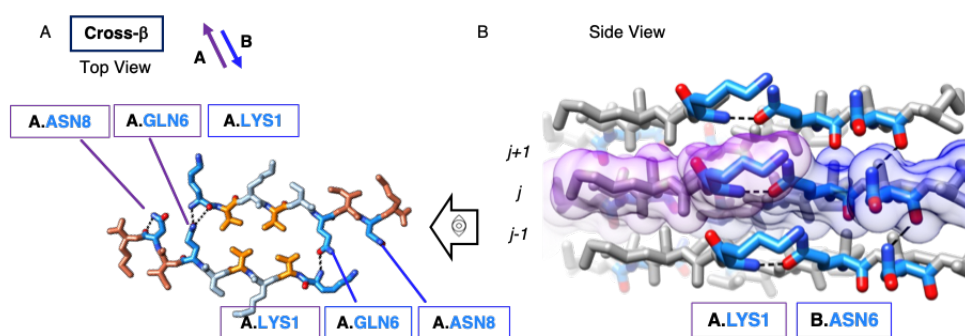


Figure 3.15: Cross- β interaction. Interaction between chain A and B, in the inner hexagon represented from a top view and a side view to highlight out of plane interactions. The side view shows the A-B interaction as highlighted by the electron density around the chain (purple for A and blue for B).

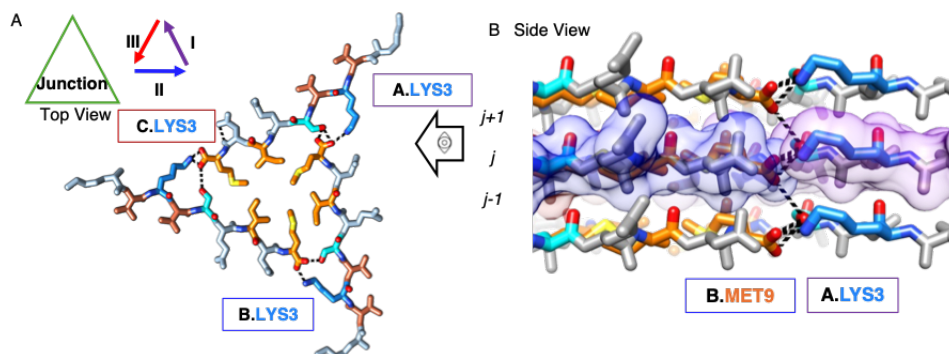


Figure 3.16: Junction interaction. Interaction between chain A, B, and C in the inner hexagon represented from a top view and a side view to highlight out of plane interactions. The side view shows the A-B interaction as highlighted by the electron density around the chain (purple for A and blue for B).

hydrophobic residues, which were expected given the dual amphiphilic nature of DAP, promot comparison to DNA sticky-ends¹⁵⁶ that can be engineered for subsequent programmable interactions.

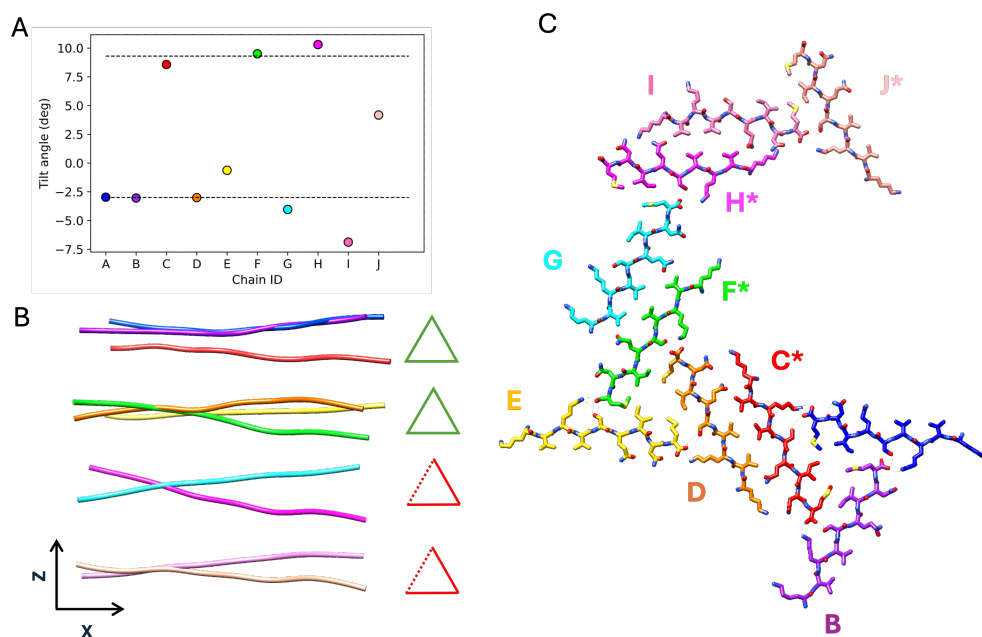


Figure 3.17: Chain Tilt Analysis. Tilt angle compared to the fibril plane of each of the 10 fundamental chains (A). The tilt values are visible when grouping each junction interaction (green for the full junction and red for the semi-junction) (B). The fundamental chains are reported once again to emphasize the starred chains (*) that present the 10 °tilt (C).

3.5 The Leading Role of Junction Stabilization

Given the novelty of the junction interaction, full atom MD simulation were carried out to comprehend key structural features. Specifically, the reconstructed atomic model was utilized as starting point for the simulation. Given the high number of atoms in the full fibril, the simulation focused on the central fibril that reflects the 1-1-1 morphology. Specifically, a total of 18 chains per layer was simulated, thus comprising both cross- β and junction interaction. Firstly, the minimum number of layers required for the fibril to be preserved was investigated (Figure A.2). A 100 ns simulation showed that the structure requires 5 layers to remain stable (Figure 3.18 A). Such results emphasizes the importance of β -stacking in the fibril stabilization.

Subsequently, a second simulation was conducted with a similar system where the junction interaction was removed. Hence, in each plane of the fibril only the 6 cross- β interaction were present, with a total of 12 chains per layer. Reduction of the hexagon to only 6 cross- β dimer motifs, lead the hexagon to rapidly disassemble (3.18 B) thus indicating the junction's leading role in fibril stabilization.

To further elucidate the junction's role, fifteen layers of the individual motifs were simulated resulting in quick disassembly of the cross- β (3.19 B) whereas the junction remained intact (3.19 A NAME). The strong junction stability indicates a possible intermediate specie formation. The trimeric junctions might be a precursor of the hexagon hinting at a mechanistic assembly approach where trimeric-junction-fibrils could independently form and laterally assemble via cross- β interactions leading to the final hexagon.

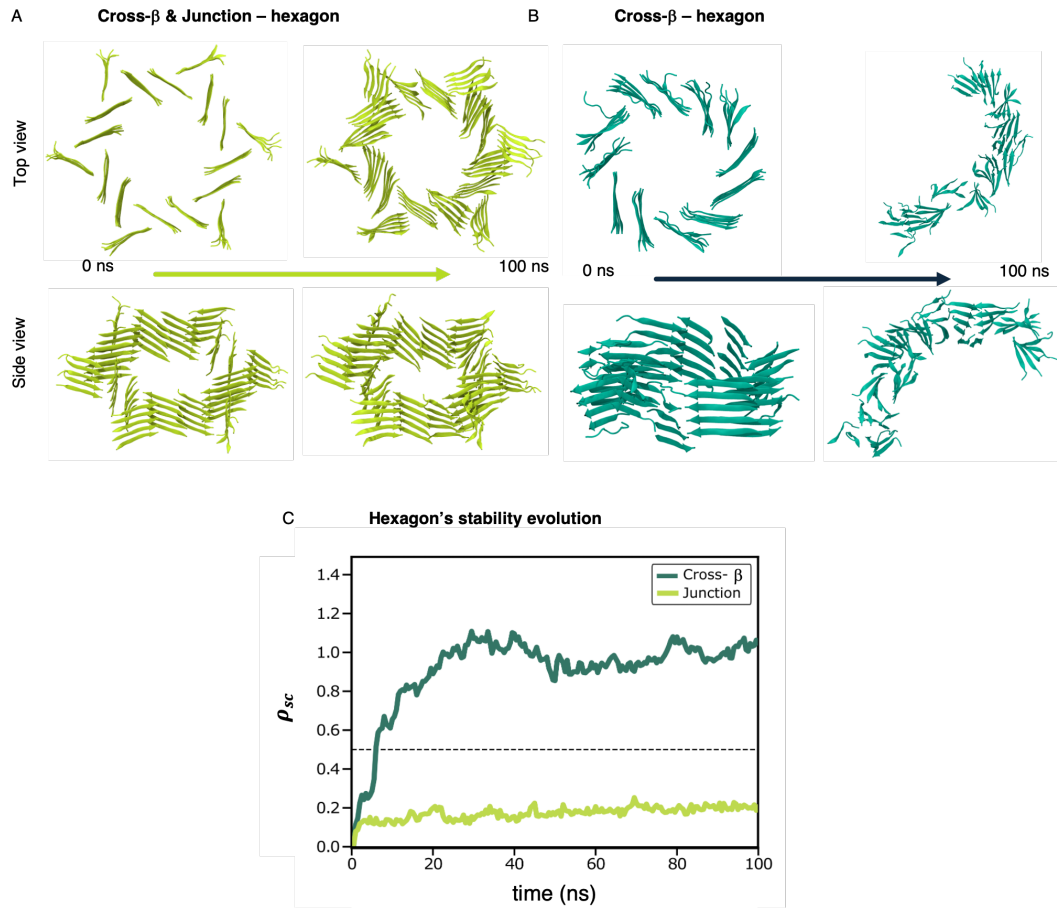


Figure 3.18: Hexagon stability. MD simulation at time 0 and after 100 ns of simulations of a five-layer hexagon composed by the combination of junction and cross- β motifs (A) or only via cross- β motif (B). After 100 ns of simulations the combination of cross- β and junction remains stable, while without the complete junction formation it disassembles. (C) Normalised root-mean-square deviation (ρ_{sc}) during the MD simulation indicating that the hexagon with junction and cross- β remains nearly unchanged (light green, $\rho_{sc} < 0.5$) while the one without cross- β quickly disassembles in the first 20 ns of simulation (dark green, $\rho_{sc} > 0.5$).

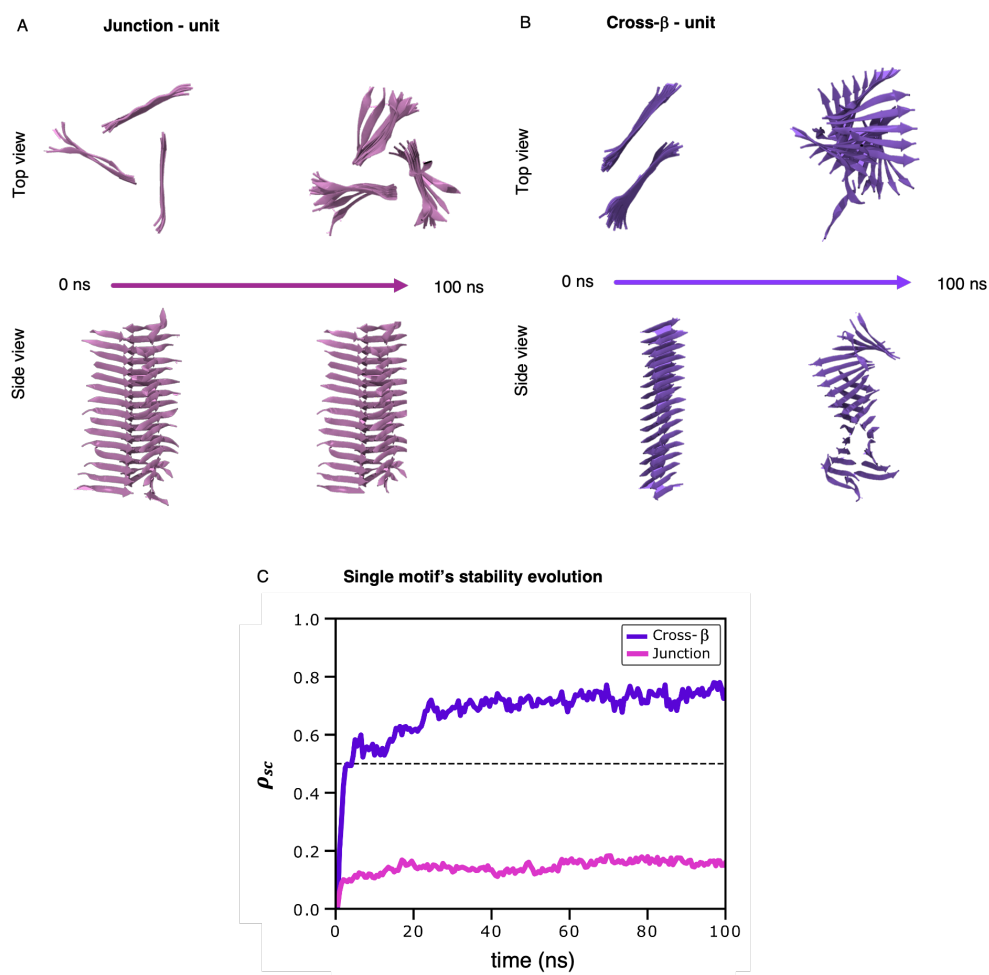


Figure 3.19: Single motif stability. MD simulation at time 0 and after 100 ns of simulations of a ten-layer junction (A) or cross-β (B) motifs. After 100 ns of simulations the junction remains stable, while the cross-β disassembles. (C) Normalised root-mean-square deviation (ρ_{sc}) during the MD simulation indicating that the junction remains nearly unchanged (pink, $\rho_{sc} < 0.5$) while the one without cross-β quickly disassembles in the first 20 ns of simulation (purple, $\rho_{sc} > 0.5$).

3.6 Interpretation of DAP's Key Interactions

Interpreting the atomic structure enables critical description of the DAP sequence. The central amino acid (SER5) introduces an inversion in the orientation of the hydrophilic and hydrophobic side chains leading to the dual amphiphilic pattern. Furthermore, the inversion causes the sequence to present two sticky ends: VAL2 - VAL4 and ILE7-MET9. The valines lead to the cross- β formation (motif 1, purple in Figure 3.20) while the ILE and MET promote the junction formation (motif 2, green in Figure 3.20). Additionally, the serine residue is involved in the junction interaction as it presents a transition residue in the 3D space. Its hydrophilicity value, reported by the Kyte Doolittle parameter,¹⁵² is close to zero (-0.8) and acts as a good transition between the MET (1.9) and LYS (-3.9). Thus, producing an environment with averaged hydrophilicity that accommodates both these residues and promotes the crucial interaction between the carboxylic group of the C-terminal and the LYS3 amino group.

In addition, GLN6 and ASN8 present further atomic relevance. Their role is known to produce polar zippers in amyloid fibrils,²⁴ and this feature is confirmed in HONEY ASAP. The presence of both a hydrogen donor and hydrogen acceptor enables multiple orientations that promote H-bond formation. Hence, we highlight the importance of carboxamide-containing residues in this portion of the sequence. Additionally, this interaction area involves LYS1. Indeed, a hydrophilic residue is necessary to stabilize the interaction of the protein with water. Yet, in the reconstructed volume, the side chain does not present strong electronic density features, contrarily the atomic model primarily highlights H-bond formation between the N-terminal amino group rather than the side chain. Thus, we believe that the nature of the side chain does not majorly influence the peptide-peptide interactions.

Therefore, the DAP sequence can be considered a platform based on five crucial features (Figure 3.20): i) trimeric junction (motif 1), ii) inversion point, iii) cross- β sheet (motif 2), iv) sticky ends, and v) hydrophilic pore. Each of these features can be summarized and commented.

i) The trimeric junction (hydrophobic core) is formed from isoleucine and methionine presents a steric zipper-like conformation with tightly interlocking residues. Such tight interlocking leads to a solvent restricted surface (maximum pore size ~ 5 Å, Figure 3.21 A). A similar trimeric formation has rarely been reported in literature. Notable comparison can be posed with uperin3.5³⁶ and Orb2²⁶ (Figure 3.22 B and C) amyloids where six-amino acid interact leading to the formation of trimeric cores. In the case of Orb2, the junction presents a 15 Å hydrophilic pore. On the other hand, uperin3.5 presents a positively charged and amphiphilic core.

ii) The inversion point is achieved by serine. SER5 defines the molecule's hydrophobicity pattern and the switch in secondary structure elements: from junction promoting amino acid patterns (motif 2) to cross β -sheet sticky ends (motif 1).

iii) The Cross- β sheet is commonly observed in amyloid fibrils.²⁹ In HONEY ASAP the cross- β is formed by interlocking valines. The presence of these residues (the KVKV section) was inspired by the MAX-1 peptide interaction motif (Figure 3.22 A).??

iv) Hydrophobic sticky ends are created at the aqueous solvent exposed side: either at the junction's interface, leading to the cross- β formation, or vice versa (from the

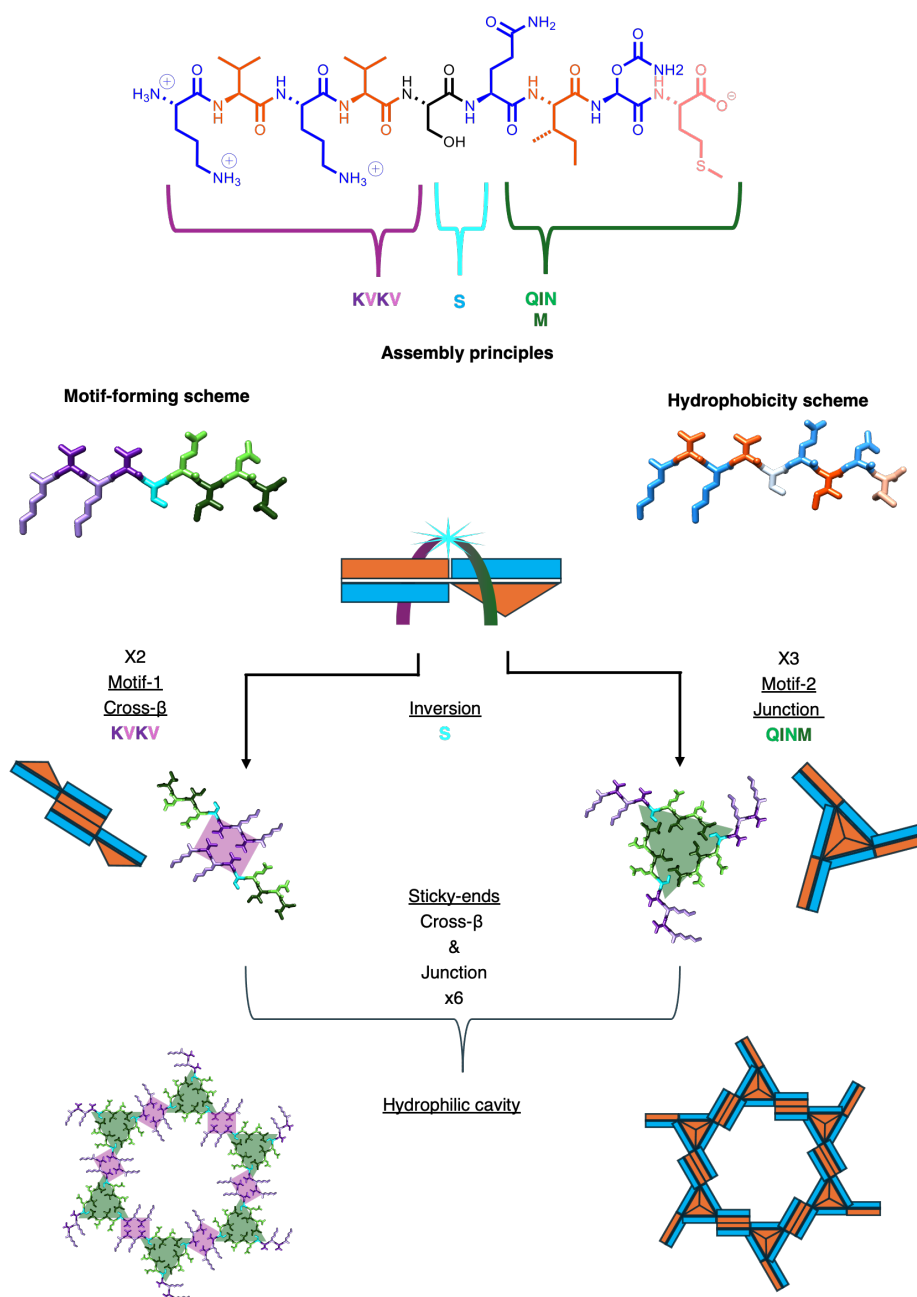


Figure 3.20: DAP sequence design and assembly motifs. Chemical structure of the DAP sequence (KVKKVSQIN), colour coded based on the Kyte-Doolittle hydrophobicity scale (orange for hydrophobic and blue for hydrophilic amino acids).¹⁵² Assembly principles schemes based on the sequence motif-forming design (KVKKV, S, and QIN) and the hydrophobicity design. The DAP sequence is designed with a cross-β forming motif (motif-1), an inversion point, and junction forming motif (motif-2). The sequence self-assembles via hydrophobic sticky-ends leading to the formation of a hydrophilic cavity.

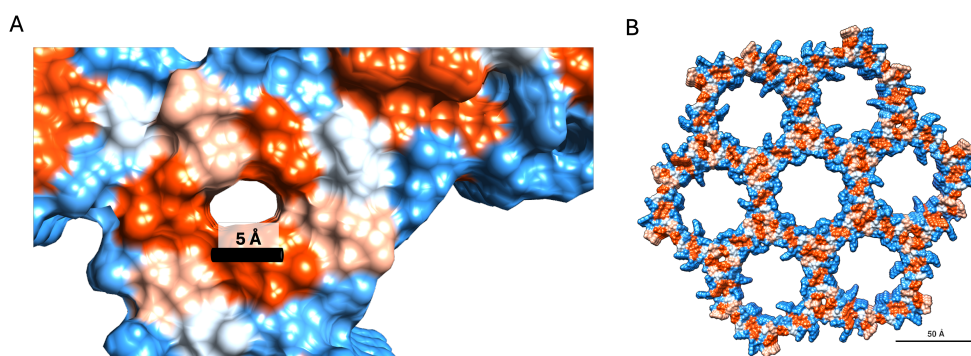


Figure 3.21: HONEY ASAP hydrophobicity surface. The surface of the atomic model is displayed in a color coding based on the Kyte Doolittle parameters¹⁵². Red is more hydrophobic and blue more hydrophilic. A close up to the external junction shows that the ILE and MET are oriented in a way that produces a small hydrophobic pore in the center of the junction (A). By zooming out and observing the full fibril the structure reveals seven hydrophilic pores with a 5 nm diameter.

cross- β leading to the junction). Notably, this pore size is inherently dictated by the assembly motif. Hence, each nanotube will present the same size and can lead to the formation of a well defined pours template.

v) Hydrophilic pore formation, sets as a distinctive surface feature of DAP. Here, carboxamide residues (glutamin, asparagin) and charged lysines face towards the same, solvent-exposed, side. In such a way, these residues create a hydrophilic, H-bond-promoting environment, leading to the formation of a solvated central channel. Importantly, the tailored DAP sequence interactions dictate the size of the hydrophilic pore (Figure 3.21 B). Such pore size, while similar in hydrophilicity to the glutamine-rich sequences in Orb2 (Figure ??), presents a larger size (50 Å instead of 15 Å).

The interaction between the five crucial residues leads to hexagonal lateral growth (Figure 3.10). A similar tendency, where the fibril can grow in plane, was reported for PNF-18 synthetic amyloids.¹⁰⁵ In this work, it was claimed that thicker morphologies are the thermodynamically preferred species, while kinetically, smaller intermediate species prevail in earlier time points. The explanation was associated to the increase of hydrophilic interactions in larger fibrils. While this claim is consistent and straightforward with their cross- β based system, it becomes more complex in DAP. PNF-18 is based on two peptides that interact in a cross- β manner; multiple units (two, three or four) can assemble linearly to produce larger fibrils. Contrarily, HONEY ASAP is based on two fundamental interactions that via mutual support form a complex assembly. Hence, the lateral growth is not based on adding a single unit but on multiple interactions. Regardless of the number of necessary interactions, classical nucleation theory would generally predict a similar outcome, where longer incubation times should result in increased lateral growth. Indeed, by assuming that lateral growth can be modeled as the aggregation of core-units structures, it is expected that fibrils with large cross-sections eventually appear in the experiment. Given the current limitations of our understanding of the assembly mechanism, whether and how fibrils grow to larger sizes within the fibril's plane remains an open question. However, within the time scales observed, it seems that this is not the case, and the fibrils' cross-section tends to reach a maximum size. Future work will require detailed investigation of the key physiochemical properties (e.g.

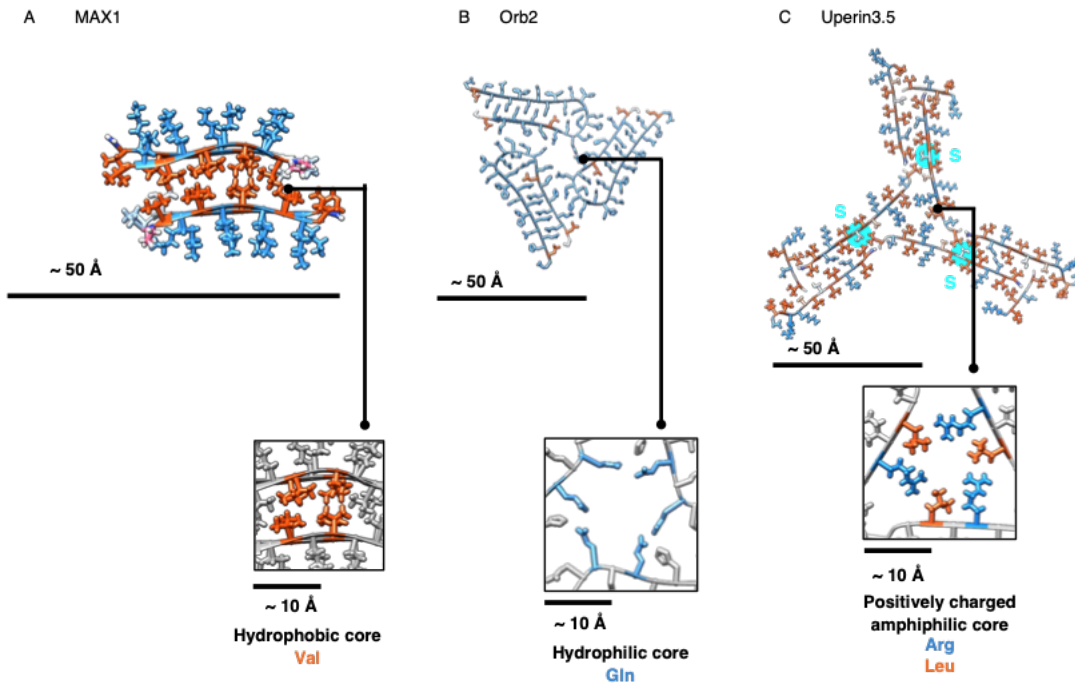


Figure 3.22: MAX1-peptide and trimeric amyloids. Atomic models that contribute to the key interpretation of the DAP design are (A) MAX1-peptide (PDB:2N1E)?? and trimeric-forming functional amyloids (B) Orb2 (PDB:6VPS)²⁶ and (C) Uperin 3.5 (PDB:7QV5)³⁶ colour coded based on the Kyte-Doolittle hydrophobicity scale,¹⁵² pick for the non-natural amino acid in the MAX1 structure. Insert highlights the respective cores hydrophobic for MAX1, hydrophilic for Uperin 3.5, and positively charged and amphiphilic for Orb2. Scale bars have been obtained via Chimera and, due to the three-dimensionality used in the software, are to be considered approximative.

incubation temperature) that guide the assembly and tune the pairing to result in high purity of geometrical arrangements (i.e. selectively producing the 2-2-1 morphology).

While the mechanism of lateral assembly is not yet elucidated, the surface properties still promote comparison with systems that present similar properties. Specifically, the water exposed hydrophobic residues present an anchor point similar to sticky ends in DNA-origami systems.^{156,157} In DNA the interaction is based on well-established binding between complementary bases and can be taken advantage to engineer specific sequences to promote material growth from these anchor points.¹⁵⁶ Similarly, it would be possible to design a building block that would interact in a complementary manner to the valine residues, taking advantage of hydrophobic interactions. Similar types of hydrophobic interactions have been reported using Nile Red.¹⁵⁸

3.7 Summary and Outlook

Only by shifting the SPA-CryoEM approach from biology to NMs, our study was able to determine that the DAP building block presents a novel platform for the development of future NMs. Specifically, we uncovered a peptide sequence that, by incorporating a residue that inverts the amphiphilic trend in the sequence's center point, presents

tailored and selective hybridization motifs: dimeric cross- β and trimeric junction. The interplay of these two motifs lead to a highly organized structure where an in plane hexagonal arrangement of the peptides was found. A single hexagonal arrangement, found in the 1-1-1 morphology, stands as the fundamental unit of the HONEY ASAP structures. The junction-forming peptides in the hexagon, in fact, act as a bridging component to produce additional external hexagonal arrangements. In such a way, the PNF can exist as a multi-compartment nanotube with 5 nm pores.

Such lateral (multi-compartment formation) and vertical (sheet formation) controlled assembly presents as a first step into lego-like interactions for the establishment of peptide-origami. Hence, while single-peptide based nanofibrils were investigated in this work, future studies will take inspiration from DNA-origami systems to exploit the surface bridging effect. In such a way we aim to tailor programmable interaction to modify the outer surface of the nanofibrils leading to the formation of multi-phase PNFs. Therefore, the DAP building-block poses as a blueprint guiding future research in the development of rationally designed multi-amphiphilic peptides for the formation of ordered 3D peptide origami nanostructures, analogous to the DNA origami technique.

However, further research is required to elucidate the impact of incubation temperature, counter ions and solvent mixtures on the assembly process and the formation of structural defects. In the early stages of DNA nanotechnology, it was challenging to assemble a distinct nanostructure with the desired level of purity thus temperature annealing protocols had to be established to address this issue. Exploring the necessary experimental protocols will additionally enable mechanistical understanding of hexagonal growth, potentially leading to a polymorphism control and a deeper insight in amyloid self-assembly pathways. Such understanding could potentially unravel a correlation with aggregation mechanism of amyloid-associated diseases and pave the way for the development of targeted therapeutic interventions.

We envision that the unexpected hydrophilic nanotube characteristics, with pre-defined and controlled size, can be implemented in ion channel flux, ideally to be incorporated in artificial cells' membranes. Hence, the system stands as an inspiration for the development of novel rationally designed nanomaterials.

3.8 Materials and Methods

Materials

Triisopropylsilane, α -cyano-4-hydroxycinnamic acid ($\geq 99.0\%$), and Dulbecco's phosphate buffered saline (PBS) were obtained from Sigma-Aldrich Chemie GmbH. Thioflavin T was obtained from Fluka Analytical, Sigma Aldrich. Tris-(2-carboxyethyl)-phosphate (TCEP) hydrochloride (99%) was purchased from Acros Organics. PyBOP, Fmoc-Asn-(tBu)-OH, Fmoc-Ile-OH, Fmoc-Gln(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Ser(tBu)-OH and Fmoc-Met-Wang resin were purchased from Novabiochem®, Merck. Piperidine ($\geq 99.5\%$ for peptide synthesis) and trifluoroacetic acid (TFA, $\geq 99.9\%$) were obtained from Carl Roth. Dimethylformamide (DMF, peptide synthesis), diethyl ether and acetonitrile (HiPerSolv Chromanorm for HPLC-gradient grade) were purchased from VWR Chemicals Prolabo. Dimethylsulfoxid (DMSO, $\geq 99.5\%$) was purchased from Sigma. Syringe filters Chromafil®Xtra RC-20/13 (0.20 μm) were obtained from Machery-Nagel.

Solid-Phase Peptide Synthesis

For peptide synthesis, the Fmoc-based synthesis strategy by R. B. Merrifield was applied with synthesis of the peptide from C to N-terminus via the N,N'-Diisopropylcarbodiimide (DIC) - ethyl cyano(hydroxyimino)acetate (Oxyma) strategy using an automated microwave peptide synthesizer (CEM, Liberty Blue™). The peptide was synthesized onto preswollen fluorenylmethyloxycarbonyl(Fmoc)-Met-Wang-resin (100 μ mol, 147 mg, substitution 0.68 mmole/g, PS, 100–200 mesh) by sequential coupling of activated N-Fmoc-amino acid in DMF (2.5 mL, 0.2 M) in the presence of DIC (1 mL, 0.5 M) and Oxyma (0.5 mL, 1.0 M) in DMF via microwave-assisted reaction at 75 °C (170 W) for 15 s and 90 °C (30 W) for 110 s followed by washing of the resins with DMF (2 mL). The Fmoc-amino acid side-chain protections were chosen as follows: Boc (Lys), tBu (Asn, Ser), Trt (Gln). Thereby, sequentially Fmoc-Asn(tBu)-OH, Fmoc-Ile-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ser(tBu)-OH, Fmoc-Val-OH, Fmoc-Lys(Boc)-OH, Fmoc-Val-OH, and Fmoc-Lys(Boc)-OH were coupled to the resin via cycling coupling, washing and N-Fmoc-deprotection steps. N-Fmoc deprotection was conducted by application of piperidine (3 mL, 20% v/v in DMF) under heating (75 °C, 155 W, 15 s, followed by 90 °C, 30 W, 50 s), followed by washing with DMF (2x 2 mL, 1x 3mL, 5min each). Subsequently, the peptides were cleaved from the resin using a cleavage cocktail (10 mL) comprising TFA (95% v/v), Milli-Q-water (2.5% v/v), and TIPS (2.5% v/v). After 2 h incubation on a shaker at room temperature, the peptides were precipitated with 4 °C cold diethyl ether (40 mL) and submitted to centrifugation at 4000 rpm for 10 min (x2, 0 °C). The resultant peptides were left for drying overnight at RT. The crude product was purified by preparative reverse-phase HPLC using a 0.1% TFA water–acetonitrile mixture as eluant and lyophilized for 48 h to yield the peptide as white amorphous powder. In one HPLC run, performing multiple manual injections, 5.8 mg (5.4 μ mol, 60.4%) product was isolated from 9.6 mg crude. The purified peptides were characterized by MALDI TOF MS and solids stored at -20 °C until use.

MALDI TOF Details

MALDI TOF MS: m/z calc. = 1045.60, m/z found: 1046 [M+H]⁺, 1062 [M+O+H]⁺, 1068 [M+Na]⁺, 1084 [M+K]⁺, 1100, 1106. Peptide was recorded on a Bruker Rapi-flex MALDI-TOF/TOF mass spectrometer (Bruker Corporation) using an -cyano-4-hydroxycinnamic acid matrix. The data were evaluated with mMass software.

HPLC Details

HPLC of the dissolved precipitate ($c = 1.3$ mg/mL, water with 0.1% TFA (v/v) and 5% ACN (v/v)) was performed via multiple manual injections (2-3x 8 mL) of the peptide crude solution (Shimadzu modules DGU-20A5R, LC-20AP, CBM-20A, SPD-M20A, SIL-10AP, and FRC-18A). Separation was performed by preparative reverse-phase HPLC column (Phenomenex Gemini 5 μ m NX-C18, 110 Å, 150 $\times$ 30 mm) using a 0.1% TFA water–ACN mixture as eluant (flow 25 mL/min), the applied gradient is summarized in Table 3.2. Chromatography was monitored with a UV absorption detector at 214 nm.

Time (min)	ACN, 0.1%TFA (%)	0.1%TFAaq (%)	Flow (ml/min)
0.01	5	95	25
5	5	95	25
17	70	30	25
19	100	0	25
24	100	0	25
28	5	95	25
30	5	95	25

Table 3.2: Gradient for peptide purification

Nanofibril Preparation

Nanofibril formation was induced by introducing the pre-dissolved peptide stock solution (10 mg/mL, DMSO) to DPBS (pH 7.4) yielding a 1 mg/mL solution. After short vortexing, the sample was left for incubation on a shaker (500 rpm, 22 °C) overnight. Further incubation was conducted without shaking at 37 °C for the indicated periods. The incubation times and temperatures were varied as indicated below with deviation from the general procedure:

I) Preformed fibrils were obtained by incubating at 22 °C for 24h with 500 rpm shaking. II) Nanofibril formation in water was examined by introducing the peptide stock solution (10 mg/mL, DMSO) into milliQ water yielding a 1 mg/mL solution with 10:90 DMSO:H₂O. III) Nanofibril formation without pre-dissolved DMSO peptide-stock was determined by dissolving the lyophilized peptide directly with DPBS (pH 7.4) or milliQ water yielding a 1 mg/mL solution. IV) Dilution stability test was performed diluting the preformed fibrils as described in (I) in water. 10 L of the preformed fibrils (1 mg/mL) were added to 90 L milliQ water (0.1 mg/mL) with 1:9:90 DMSO:DPBS:H₂O. This sample was used for the reconstruction for the 1-1-1 morphology. V) Early kinetics experiments were conducted by inducing nanofibril formation via the general method with incubation (22 °C 500 rpm) for either 30 min, 45 min, 1 h, 3 h, 6 h, 9 h, 15 h, 21 h, 24 h. VI) Late kinetics experiments were conducted using the preformed fibrils as described in (I) and transferring them into a 37 °C oven without shaking. The samples were then imaged after 4 days, 1 week, 2 weeks, and 56 days. The 56 day sample was used for reconstruction of the 3-3-3 morphology and for the polymorphism analysis. VII) Temperature stability test were performed by using the preformed fibrils as described in (I) and transferring them for 15 or 60 min in an oven at 60, 85 or 95 °C. VIII) Sonication stability test were performed by exposing preformed fibrils as described in (I) to sonication (15 min, sonication bath).

Cryo-EM Image Acquisition

A 4 μ L aliquot of the DAP fibrils (at different incubation times) or hydrogel were applied on a Quantifoil grid (Q R2/2, 400 mesh) that was glow-discharged in a 6:1

oxygen: hydrogen plasma (Diener Nano®, Diener electronic, Germany) for 30 s shortly before. The excesses solution was blotted for 4 s and force 0 at 4 °C and >80% humidity. The Vitrobot (FEI) was used for the cryo-plunging in liquid ethane. The sample was imaged using a Titan Krios transmission electron microscope (Thermo Fisher Scientific) at 300 kV, using a Gatan imaging filter with a 20 eV slit. Table 3.3 lists the data acquisition parameters. Automated data acquisition was performed using SerialEM.

Single Particle Analysis for Helical Reconstruction

Raw movies were processed with MotionCor2¹⁵⁹ to apply drift-correction. The corrected micrograph was processed with CTFFIND¹⁶⁰ to fit CTF parameters. Further image processing and 3D reconstructions were done with RELION-4.0.¹²⁷ Selection of 656 (out of 1761) micrographs was done manually by removing micrographs containing ice or lacking the 4.8 Å resolution information based on the CCTFIND results. From these micrographs, fibrils were manually picked by selecting homogenous looking filaments. Particles were extracted with a box size of 350 (or 250) and with an interbox distance of 32.9 Å. This was achieved by selecting a rise of 4.7 Å and 7 as the number

Data collection and helical processing	#1 3-3-3 morphology (EMD-51914) (PDB 9H7U)	#2 1-1-1 morphology
Fibrils preparation		
Incubation time (at 37 °C)	2 months	10 days
Solvent composition (DMSO:PBS:H2O)	10:90:0	1:9:90
Microscope	Titan Krios (Thermo Fisher Scientific)	Titan Krios (Thermo Fisher Scientific)
Camera	K2 (Gatan)	K2 (Gatan)
Magnification	81'000	81'000
Acceleration Voltage (kV)	300	300
Electron exposure (e-/Å ²)	39.28839	22.3206
Defocus range (m)	0.5-2.5	0.5-2.5
Number of movies per frame	25	25
Pixel size (Å)	0.8875	0.8875
Box size (pixel)	300	250
Inter box distance (Å)	32.9	23.5
Initial particle images (no.)	213'714	84'212
Final particle images (no.)	108'623	60'461
Map resolution (Å) 0.143 FSC threshold	2.95833	3.6972
Applied B-factor	-50	-
Helical rise (Å)	4.77	4.67
Helical twist (°)	-0.64	-0.885
Symmetry imposed	C6	C6

Table 3.3: Details on the CryoEM image acquisition and processing. Microscope set up for image acquisition and helical parameters used for helical reconstruction of morphology 3-3-3 and 1-1-1.

of asymmetrical units. 2D classification was performed to improve cross-over determination. Here a value of 440 Å was measured, thus the twist was estimated to be -1.8° . As an initial model for the 3D classification, a featureless cylinder was used with a 60 Å low pass filter.

During 3D auto-refinement, without imposing any additional symmetry (C1), a C2 symmetry was observed (Figure 3.23 C1). Hence, the reconstruction was performed by applying C2 symmetry (Figure 3.23 C2) which improved the cross-section alignment. Further 3D Auto-refinement with and C3 (Figure 3.23 C3) and C6 (Figure 3.23 C6) symmetries allowed the visualization of the single peptide chain in the cross-section. To check for artifacts deriving from the symmetry imposition a 3D Auto-Refine with a C4 symmetry was tested. In this case both the cross-section and projection worsened indicating the need for the C6 symmetry (Figure 3.23 C4). At this point a 4.3 Å resolution volume with a well aligned cross-section was obtained, yet the Z-projection was not clearly resolved. Therefore, the full dataset was re-processed via 3D classification and further 3D Auto-Refine to reach a 3 Å volume.

This map underwent Bayesian polishing and per-particle CTF estimation.¹²⁸ Thus producing an improved final map which was post processed in RELION by using a soft mask (10% of the central Z length) and a B-factor of -50. The masked post processed map yielded a final resolution of 2.96 Å.

Atomic Modeling

The atomic model of HONEY ASAP was built *de novo* into the post processed and masked 2.96 Å resolution map (Table 3.4). To do so, the initial peptide was built in Chimera.¹³³ The dihedral angles of the backbone were imposed to follow the β -strand parameters where $(\Phi, \Psi) = (-135^\circ, 135^\circ)$. The sequence was then iteratively processed between Chimera, COOT,¹³⁴ and Phenix¹³⁵. This iteration process aims to promote H-bond formation and address clashes between atoms positioned too closely to each other, which violates the expected spatial arrangement and indicates errors in the model. The

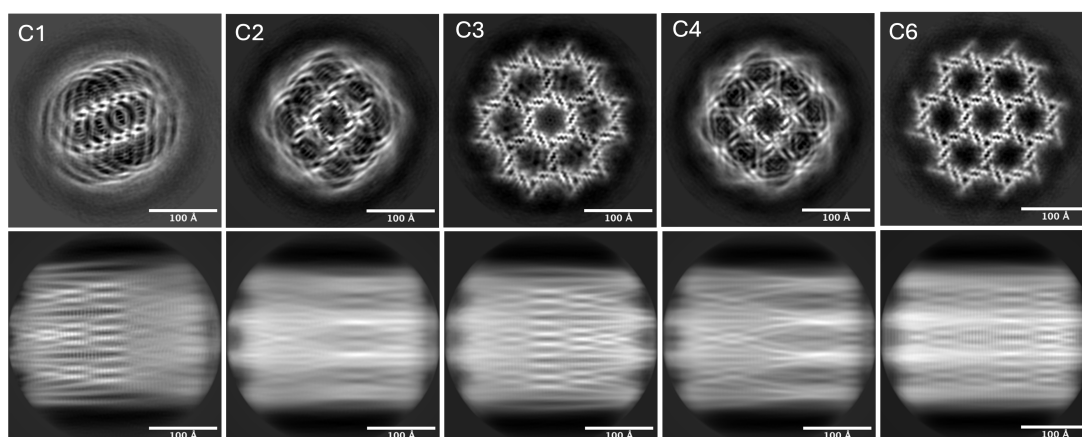


Figure 3.23: Symmetry study. Evolution of the cross-section (top) and projection (bottom) when using a featureless cylinder as reference and applying C1, C2, C3, C4 or C6 symmetries. C4 produced a artificial reconstruction while rationally increasing the symmetry operation from 1 to 6 properly improved image alignment.

extent of this error was quantified and monitored using Molprobity.¹³⁶ Do to the large number of sequences, two or three chains were modeled individually and each modeling was combined together requesting minor adjustments.

Polymorphism Analysis

The CTF corrected micrographs were used for the polymorphism analysis. A subset of 100 micrographs was randomly generated with the Relion “Subset Selection” function. Each of these micrographs were analyzed and the different morphologies were picked and extracted using a box size of 350 pixel, tube diameter of 220 Å, number of asymmetrical units 7 and rise of 4.7 Å. The number of extracted particles were used to quantify the different morphologies.

The polymorphism prediction was performed using Chimera,¹³³ RELION¹²⁸ and ImageJ.¹³⁹ Three layers of the atomic model of the central hexagon obtained from the 3-3-3 morphology was copied and shifted manually to create plausible hexagonal patterns. In Chimera, the atomic model was converted into a volume with a 3 Å resolution (Command: `molmap # 0 3`) and saved as an .mrc file. The file was then processed with RELION to create a prolonged volume. Specifically, the file was converted into a file with box size of 1750 pixels, a tube diameter of 250 Å, rise 4.77 Å and twist of -0.64°. The following commands were used:

```
relion_image_handler \
  --i Model_File.mrc \
  --new_box 1750 \
  --o Large_Box_File_BS1750.mrc

relion_helix_toolbox \
  --impose --i Large_Box_File_BS1750.mrc \
  --o Extended_Model_File_BS1750_4.77_t0.64.mrc \
  --cyl_outer_diameter 230 \
  --angpix 0.8875 \
  --rise 4.77 \
  --twist -0.64 \
  --z_percentage 0.1
```

The volume was then processed in ImageJ. The central 1511 slices of the volume were analyzed to visualize the projection. This number corresponds to the number of slices that generate a single cross over distance, hence the three-fold pattern. To visualize the full 360 °of rotation pattern the images was rotated by 180 °and the two projection images were combined.

Molecular Dynamics Simulations

The layer structures were built by rotating and translating the fundamental junction (for the cross- β -junction simulation), or the fundamental cross- β (for the cross- β simulation), unit along the z-direction. Five systems were simulated with 1, 3, 5, 10, and 15 layers of peptide chains, respectively. The peptides were centred in a 22.0 x 22.0

Refinement	#1 3-3-3 morphology (EMD-51914) (PDB 9H7U)
Initial model used	Featureless Cylinder
Model resolution (Å) FSC threshold	4.3
Map sharpening B factor (Å²)	- 50
Model composition Non-hydrogen atoms Protein residues	3'888 54
B factors (Å²) Protein	0
R.m.s. deviations Bond lengths (Å) Bond angles (°)	0.016 2.468
Validation MolProbity score Clashscore Poor rotamers (%)	1.62 5.41 0
Ramachandran plot Favored (%) Allowed (%) Disallowed (%)	95.24 4.76 0

Table 3.4: Atomic model parameters. Parameters deriving from the validation tool (MolProbity) for the full atomic model (eighteen chains and three layers) in the 3-3-3 morphology reconstructed volume.

x 22.0 nm³ box and solvated with a mixture of PBS:DMSO 9:1 %v/v. The composition of the Phosphate-buffered saline (PBS) was given by the systematic treatment of equilibrium¹⁶¹ at 298.15 K. The dissociation constant for the ionizable amino acids in the chains was assumed to be independent of each other. The final composition of each system is shown in Table 3.5.

Layers	Chains	Cl ⁻	H ₂ PO ₄ ⁻	HPO ₄ ²⁻	K ⁺	Na ⁺	DMSO	TIP ₃ P
1	18	928	63	11	27	1002	9392	318,938
3	54	992	63	11	29	992	9306	316,027
5	90	1056	62	11	28	983	71351	202,475
10	180	1216	61	11	29	960	9006	305,838
15	270	1375	59	11	26	938	8792	298,561

Table 3.5: Composition of the MD systems.

The simulations were performed using the GROMACS 2021.7 molecular dynamics package¹⁶² in the NPT ensemble. The temperature was imposed using a velocity rescale thermostat¹⁶³ at T = 298.15 K with a time constant = 1.0 ps⁻¹. The pressure was controlled at P = 1.0 bar using the Parrinello-Rahman barostat^{164,165} with a time constant = 2.0 ps⁻¹. The equations of motion were integrated using the leap-frog algorithm with a time step of 1.0 fs. Peptide chains and sodium, potassium, and chloride ions were modelled using CHARMM36m force field parameters,¹⁶⁶ and water was described by the TIP3P model.^{167,168} The H₂PO₄⁻ and HPO₄²⁻ ions parameters were taken from CHARMM FF on CHARMM-GUI.^{169,170} Electrostatic interactions were treated using the smooth particle mesh Ewald method.¹⁷¹ The solvent molecules and ions were equilibrated by the steepest descent energy minimization with a tolerance of 100.0 kJ mol⁻¹, with position restraints in all peptide atoms, followed by 25 ns of molecular dynamics simulation with position restraints only in the backbone atoms of the peptides. After the equilibration of the solvent, the systems were simulated by 100 ns without any restraint.

Chapter 4

Chemical Phase Distribution Within Polymeric Nanoparticles

This chapter is primarily based on a collaboration project which was performed both in an industrial (Covestro) and academic (Max Plank Institute for Polymer Research and Technical University of Eindhoven) setting. The work is reported in a manuscript ready for submission:

“Dual Color dSTORM: Unraveling Composition, Size, and Morphology in Multicomponent Nanoparticles”

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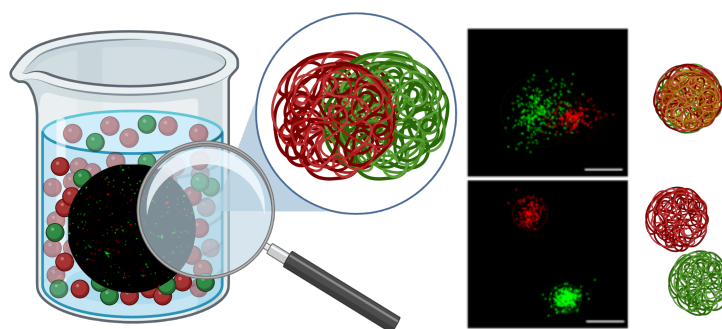
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Contributions:

I performed the CryoEM and dSTORM imaging together with the image analysis. B.M. and E.v.d.W. performed the NPs synthesis and ensemble characterization. Q.C. performed the AFM measurements. Q.C., P.S., D.v.d.Z., L.A. supervised the project. F.M. and B.M. wrote the manuscript. Q.C., P.S., D.v.d.Z., I.V., L.A., K.L., and I.L. contributed to the final version of the manuscript.



This chapter also incorporates data from reference⁷⁸.

[hb] “Aerobic Photobiocatalysis ~~Enabling the Confining~~ Core - Shell Nanophotoreactors

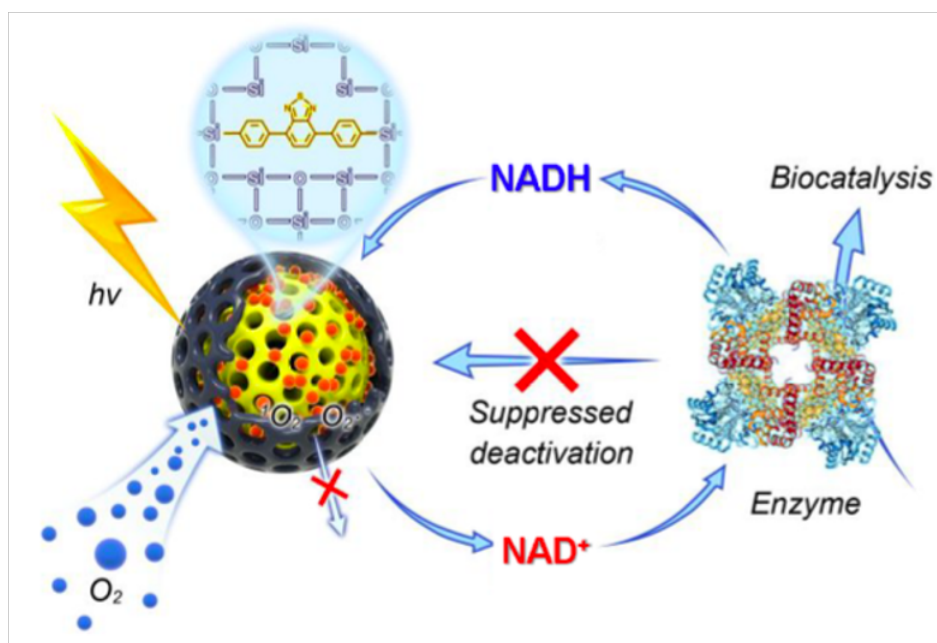
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Contributions:

I performed the EELS measurements and analysis. I.L. supervised the EELS results. W.W. designed and performed the main part of the experiments, analyzed the data and drafted the manuscript. W.W., C.F., K.Z. and K.L. contributed to the final version of the manuscript. C.F., K.Z., K.Z., and K.L. supervised the project.



Chapter Motivation

In this brief introduction, I aim to explain how the **“Chemical phase distribution within polymeric nanoparticles”** chapter connects to the research presented thus far. The previous chapter focused on unraveling the atomic structure of peptide nanofibers, providing detailed insights into how peptide chains are arranged within the nanomaterial. This understanding of the structural features not only sheds light on the material’s composition but also serves as inspiration for exploring its potential functions. By understanding how the structure relates to the material’s function, we can chemically design materials with tailor made functionality. For instance, the tubular structure observed within the fibers hints at a channel-like function, sparking interest in incorporating these fibers into membranes for applications in fields such as bottom-up assembly of an artificial cell. Thus, comprehending the structure-function relationship of materials stands as a foundation in scientific advancement.

Nanomaterials can be produced with various building blocks that affect the atomic, nano-scopic, and macroscopic properties of the final material. For instance, HONEY ASAP, derived from a homogeneous solution of a single-type of peptide, self-assembles into a highly ordered structure. Conversely, similar solutions composed of single-type peptide chains may lead to the formation of undefined clusters.¹⁷² This variance prompts the classification of nanomaterials into two categories: those with highly ordered structures and those existing in an amorphous state. While the previous chapter analyzes highly ordered structures, the current chapter shifts its focus to investigate materials exhibiting amorphous characteristics.

When characterizing materials with a high degree of order using microscopy techniques, averaging the objects of interest, as done in SPA, enhances SNR, improving visualization of such object. However, in amorphous materials, this approach blurs information, amplifying noise without enhancing signal clarity. Therefore, to understand the structure-function relationship in such amorphous materials, we need to refocus the structure search. This involves shifting from atomic-level analysis to characterizing nanomaterial morphology, thus moving from an atomic to a nanometer scale characterization. This morphology analysis includes evaluating particle size and shape. Furthermore, certain nanomaterials consist of multiple chemical components, such as asymmetrical NPs (e.g., Janus NPs), where two sides exhibit different chemical compositions or functional groups.^{84,86,111,112,173} In these types of materials, morphology analysis should also focus on detecting the specific distribution of these chemical components (moieties) within the material.

In this chapter, I employ dSTORM to comprehensively analyze the structural features of industrially relevant NPs for coating application. Additionally, as separate project, I performed EELS on photocatalytic NPs to prove the core-shell morphology. This characterization is crucial to demonstrate the efficient singlet oxygen deactivation. By zooming out from atomic-level structural analysis and focusing on morphology I aim to provide insights into the material’s structural features which enable their diverse functionalities.

ABSTRACT

Nanoparticles (NPs) composed of a mixture of chemical components are prone to energy derived phase separation. Such driving force leads to the formation of a different NP morphology, each offering opportunities to develop optimal materials needed in fields like biomedicine or coatings. In the latter, the combination of both polyurethane (PU) and polyacrylate (PA) properties within the same film is needed to introduce extra mechanical and chemical resistance. While the final film characterization has been extensively studied, investigating the initial dispersion presents challenges. Common imaging techniques often rely on dry-state analysis which can lead to artifacts due to NPs coalescence. In this work we introduce dual color dSTORM as a novel approach to overcome these limitations. We synthesized six different PU/PA systems and labeled them respectively with Cy3 and Cy5 dyes, thus enabling the chemical phase distinction. Our results demonstrate an overall phase separation in the samples, with less than 6% of the sample showing potential PU/PA composite NPs. Remarkably, this is the first application of dSTORM to image polymeric phases on 100 nm scale, thus demonstrating the suitability of this technique for such imaging purposes.

Depending on the NP of interest, chemical composition differentiation can also be performed via electron microscopy. More specifically, in this work I demonstrate the use of EELS to visualize a core-shell morphology. My results highlight a distinct carbon distribution within a silica NP that derives from the presence of a photocatalysis constrained in the NP core.

4.1 Introduction

NPs are used in a variety of applications, ranging from drug delivery systems,^{80,174} to nanoreactors,¹⁷⁵ biosensing,¹⁷⁶ coatings^{177–180} and so on.^{181,182} Multiple polymeric components are often mixed to manufacture composite NPs with complex structures and advanced functionalities. For instance, in the biological field, liposomes can be made of several lipids composing the membrane where each component plays a role in the particle flexibility, stability and interaction with the medium.¹⁸³ Polymersomes also illustrate this case, where multiple polymers can compose the same NP.^{174,184} Multicomponent polymersomes are an example of particles made for encapsulating diverse molecules of interest.¹⁸⁵ On the other hand, in industrial applications like coating, multi-polymeric suspensions are often employed to blend material properties. This allows to combine features such as the mechanical stability of polyurethane and the chemical stability of polyacrylate. The goal is to achieve more durable coatings at reduced costs.^{186,187}

The final composition of NPs formed by combining different chemicals is not always a simple blend of their components. In reality, their relation is strongly dependent on the synthetic strategy and processing conditions used to prepare the particles and many other factors, including the solubility and polymer's miscibility.¹⁸⁸ The latter is dependent on the (co)solvent composition, salinity, pH, and temperature. Two neutral, water-soluble polymers that are miscible in aqueous dispersions in low ionic strength at room temperature, for example, may become immiscible at elevated salt concentrations and temperatures.^{189,190} Polymer miscibility may thus vary as processing conditions are adjusted during particle synthesis.^{111,112} Our understanding of the particle forma-

tion pathways relies on a delicate interplay between thermodynamic and kinetic factors. Thus, predicting the final result can be challenging. This calls for a quantitative characterization strategy that is able to resolve the relative abundance and distribution of the various polymeric constituents within the particles.

Ensemble methods like DLS offer valuable insights into the averaged properties of particles within a system. However, achieving detailed information on individual NPs demands techniques capable of high-resolution single particle visualization. Conventional diffraction-limited microscopy struggles to discern the relative abundance and distribution of various polymeric components due to the submicron dimensions of these NPs. Consequently, there arises a need for high-resolution microscopies such as Atomic Force Microscopy (AFM) and EM.

However, while AFM and EM excel in providing high-resolution imaging, they often lack the chemical specificity required to distinguish between different types of polymers. Nevertheless, in selected cases, electron beam sensitivity in EM can be leveraged to discriminate between phases. Urban et al.¹¹¹ demonstrated this capability by synthesizing Janus NPs comprising poly-styrene and poly-lactic acid, where the beam-stable polymer (poly-styrene) remained intact under the electron beam, while the beam unstable polymer (poly-lactic acid) underwent degradation. Their analysis resulted in TEM micrograph with distinct black and white particles.

Moreover, EM can be accompanied with elemental analysis (energy dispersive X-ray spectroscopy EDX or electron energy loss spectroscopy EELS) to identify different moieties within an object, based on their chemical composition. However, these technique necessitates high-energy doses, leading to sample degradation. Consequently, it finds primary application in the characterization of highly stable objects such as metals¹⁹¹ and silica¹⁹² NPs.

Nevertheless, the aforementioned techniques rely on samples in a dry state. Such limitation can lead to evaporation induced assembly or alteration of the NP morphology due to a change in its environment. Therefore, complementary solvated methods are essential to discriminate single particles and to detect their morphology. One of these methods can be CryoEM where the solvated samples are immobilized in amorphous solvent environment thus preserving the original morphology. Yet, cryogenic conditions currently are not compatible with the necessary high electron doses used for chemical composition distinction. Hence, they lack ability to detect chemical subpopulations within the NPs. This limitation can be overcome with dSTORM.¹⁹³ dSTORM allows visualization of NPs in their native environment with spatial resolutions up to 20 nm. Additionally, thanks to selective labeling of two (or more) distinct phases with a fluorescent dye, it can distinguish chemical subpopulations.^{115,194}

In this chapter, I demonstrate the power of dSTORM as a method to quantitatively resolve the composition, composition-specific size, and morphology of NPs prepared from mixtures of (macro)molecular building blocks (Figure 4.1). To this end I selected a relevant industrial case of PU-PA colloidal mixture designed for coating applications. The polymer moieties were selectively labeled with green (CY3) and red (CY5) dyes, and the rendered images were efficiently analyzed to quantitatively characterize the systems (see Figure 1). Specifically, the chemical composition of single NPs was determined based on the clustering of the dye's localizations, enabling the identification of single-component or hybrid NPs. The size of each cluster was quantified, allowing for composition-specific

size determination. Lastly, for hybrid NPs, where both red and green components could be detected, a morphological estimation could be determined based on the cluster's overlap. Unexpectedly, we uncovered that the systems are predominantly phase separated and not core-shell as thought before,⁶⁷ emphasizing the importance of in-depth characterization of chemical composition within hybrid NPs systems. Moreover, switching to a silica based system, I use EELS to prove the core-shell structure of photocatalytically relevant NPs.

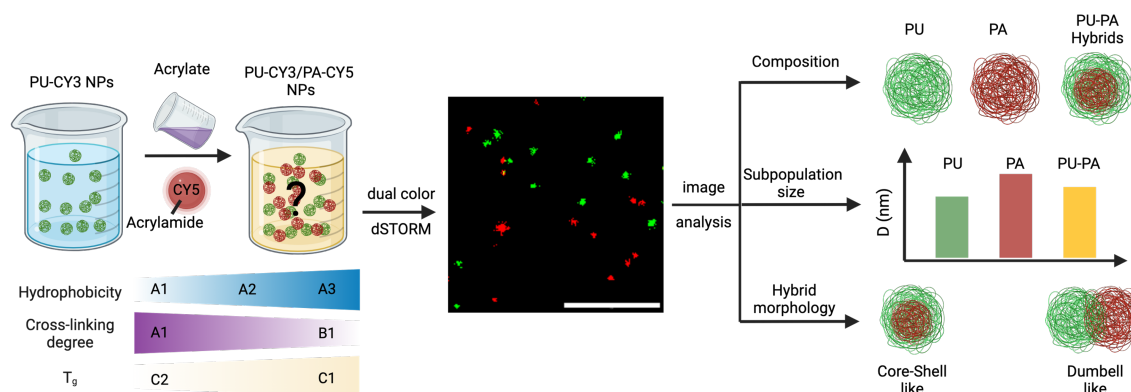


Figure 4.1: Schematic overview of the workflow. CY3 labeled PU seeds are used to polymerize acrylic monomers together with a CY5 dye. Six samples were produced by tuning the following parameters: hydrophobicity, cross-linking degree and T_g. The systems were imaged via dSTORM. Image analysis reveals the composition, composition-specific size distribution, and the hybrid's morphology of each system. Scale bar = 10 μ m.

4.2 Polyurethane-Polyacrylate Nanoparticle Synthesis and Bulk Characterization.

Polyurethane-Polyacrylate (PU/PA) NPs were synthesized by an organic solvent free synthesis (Figure 4.2). First a prepolymer of the urethane phase was obtained, followed by its dispersion in water. In this step, the N-Hydroxysuccinimide-Cy3 (NHS-Cy3) dye and the chain extender (either diethylene triamine (DETA) or Hydrazine) were added to the solution, thus selectively labeling the PU NPs. The latter was used for a seeded polymerization. Thus, the acrylate monomers together with the acrylamide functionalized Cy5 dye were added to the PU NPs and left to equilibrate. Subsequently, free radical polymerization was carried out. The final solution generated a system with NPs composed of Cy3 labeled PU phase and Cy5 labeled PA phase. To remove all unreacted material, especially free dye, samples were dialyzed over a week in a dark environment. The synthesis steps generated a total of six PU/PA samples where the hydrophobicity (A1, A2, A3), nature of the chain extender (A1, B1) and glass transition temperature (C1, C2) were tuned. Specifically, increasing hydrophobicity was addressed by utilizing different acrylate monomer n-butyl acrylate (BA), 2-ethylhexyl acrylate (EHA), and isodecyl acrylate (IA). The chain extender used in the PU phase was modified to present two (hydrazine) or three (DETA, diethylenetriamine) amine groups to test cross-linking effects. Lastly, glass transition temperature was increased from 20 to 105 $^{\circ}$ C by using

4.2. Polyurethane-Polyacrylate Nanoparticle Synthesis and Bulk Characterization.

respectively butyl methacrylate (BMA) and methyl methacrylate (MMA). It was additionally necessary to prepare three reference samples (controls). To this end, pure PU (with either hydrazine (D2) or DETA (D1)) or PA (D3) NPs were synthesized. The full description of the samples synthesized is reported in Table 4.1.

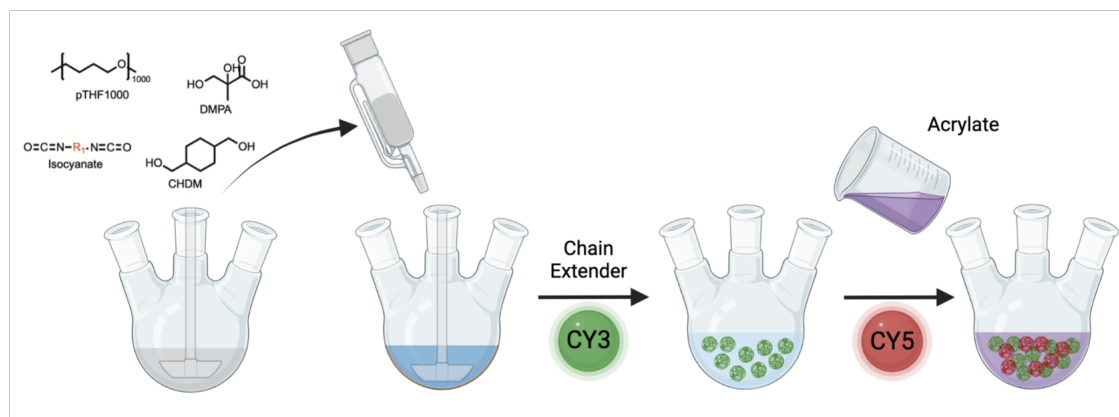


Figure 4.2: Synthesis scheme process for the PU-PA sample preparation. The cartoon shows the synthesis sequence including the prepolymer formation, subsequent chain extension and seeded polymerization of the acrylate moiety where each phase is tagged with a different dye, either Cy3 or Cy5.

The pH and viscosity of the samples were measured before and after dialysis to ensure no macroscopic changes occurred during the dialysis process (see table 4.1). The characterization detected pH values around 7 with slightly variations for all samples. In terms of viscosity all samples presented low values (< 5 mPa) except for C1. This sample, containing the MMA monomer, presented increased viscosity (60 mPa) due to the high T_g (105 °C) of the monomer. Additional ensemble characterization measured DLS and zeta potential values. Results are reported in Table 4.1. Overall, particle sizes for the DETA chain extension are approximately 100 nm while the hydrazine ones are slightly smaller, about 90 nm. For all NPs a negative zeta potentials was detected (around -45 mV) due to the carboxylic group in the PU phase that are responsible for the NPs electrostatic stabilization.

Sample Code	Acrylate Phase	Chain Extender	Tg (°C)	Dh* (nm)		PDI		pH		ζ (mV)		Viscosity (mPa)
				Before	After	Before	After	Before	After	Before	After	
A1	BA	DETA	-54	96.7	98.8	0.094	0.099	8.06	6.93	-52.8	-49	3.4
A2	EHA		-53	98.4	102.2	0.101	0.11	8.27	7.04	-53.6	-39.5	5.4
A3	IA		-41	99.8	99.9	0.089	0.102	7.82	6.93	-55.3	-49.5	4.8
B1	BA	Hydrazine	-54	84.1	82.8	0.083	0.083	7.57	7.52	-59.6	-53.7	4.5
C1	MMA	DETA	105	181.7	210.6	0.218	0.172	8.11	6.95	-46	-45.7	60.5
C2	BMA		20	103.5	100.8	0.132	0.096	7.9	7.05	-49.6	-50.5	4.9
D1	-	Hydrazine	-	78.4	77.6	0.081	0.087	7.04	7.02	-54.9	-54.9	-
D2	-	DETA	-	91.9	90.1	0.101	0.102	7.55	7.27	-50.5	-50.6	3.4
D3	MMA + AA	-	-54	81	82.4	0.024	0.024	2.85	3.98	-52.5	-38.7	3.6

Table 4.1: Sample characterization overview. Sample name with correspondent synthesis details and characterization via DLS, pH, ζ-potential, and viscosity measurements. *Hydrodynamic diameter measured via DLS.

4.3 Nanoparticle Characterization via Electron Microscopy.

After the bulk characterization is concluded, the morphology analysis can be initiated. Conventional dry state characterization techniques, such as Phosphotungstic acid (PTA) stained TEM (Figure 4.3), were carried out for fast and initial overview of the samples. The film forming nature of the material hampered a clear distinction of both the single NPs and of the PU and PA moieties while still confirming the presence of NPs in the aggregated state. Thus, the images are an indication that further characterization on the system can be carried out.

To visualize the solvated state of the sample, CryoEM was utilized. Figure 4.4 reports the collected micrographs which clearly distinguish single particles. The imaging detected a difference between the control systems: sample D2 (Figure 4.4 D2) where the PU NPs was crosslinked via DETA present a patchy morphology while sample D1 (Figure 4.4 D1), which was chain extended using hydrazine, shows a smooth and spherical surface. The surface morphology detected in the D1 samples is analogously found in the samples derived from its seeded polymerization (B1 and B2 in Figure 4.4 B1 and B2). While for the NPs deriving from the crosslinked PU samples (A1, A2, and A3 in Figure

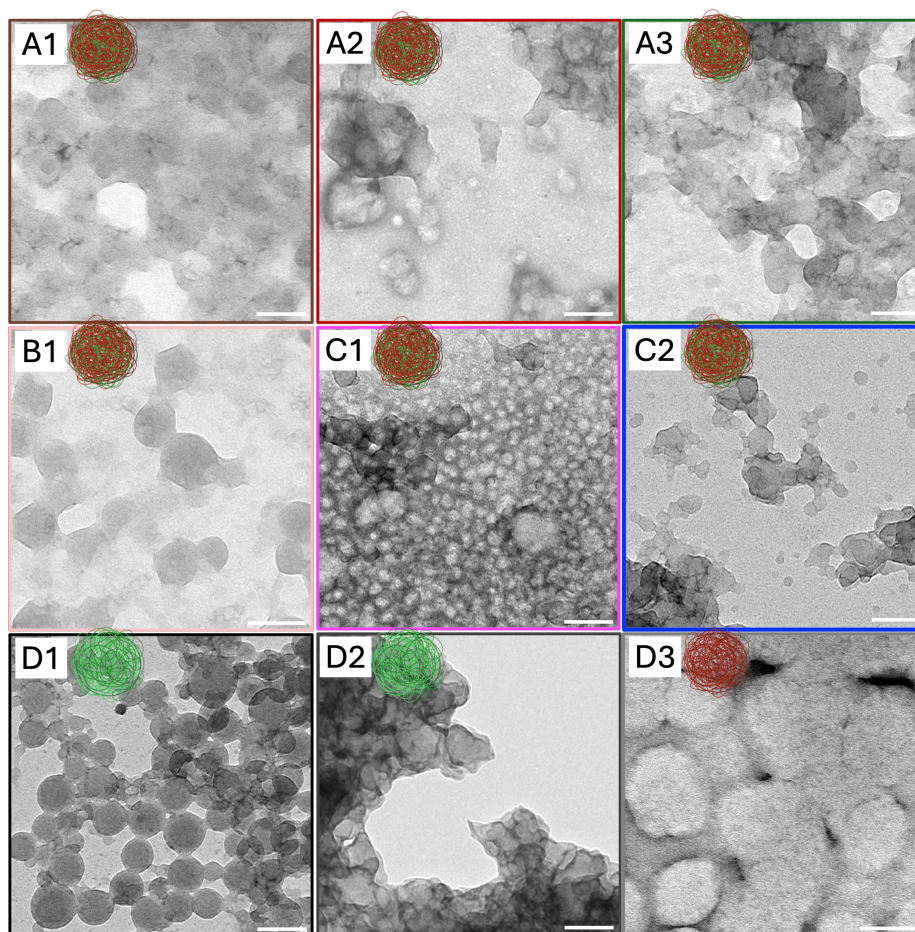


Figure 4.3: PTA stained TEM of the NPs. Micrographs of A1, A2, A3, B1, C1, C2 and the control experiments of pure PU or PA (D1, D2, D3). Cartoon representations are overlaid to highlight the different type of samples. Scale bar = 100 nm.

4.4 A1-A3) the particle morphology is different; additionally to patchy NPs, smooth, spherical, and smaller (about 30 nm) NPs were also detected. The smooth surface is comparable to the PA control samples (D3, Figure 4.4 D3) that presents similar morphology. Thus, the images of the PU-PA crosslinked systems could be an indication of phase separation, yet in these experimental conditions no chemical specificity can be detected.

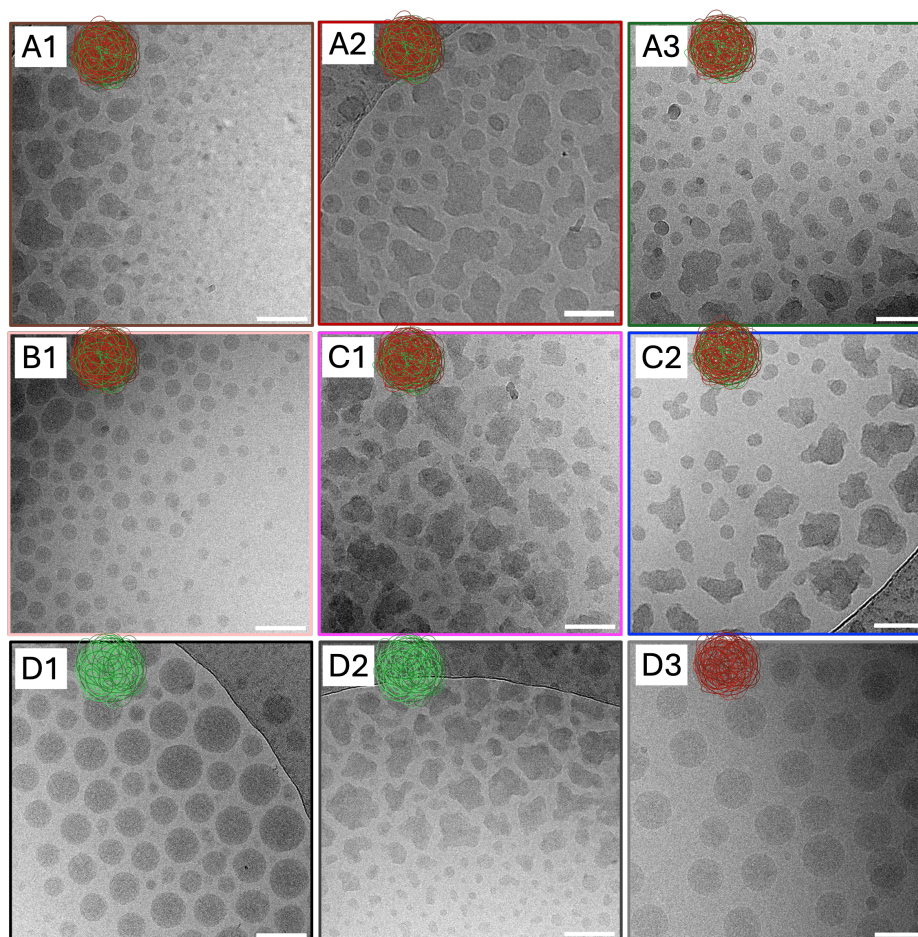


Figure 4.4: *CryoEM of the NPs. Micrographs of A1, A2, A3, B1, C1, C2 and the control experiments of pure PU or PA (D1, D2, D3). Cartoon representations are overlaid to highlight the different type of samples. Scale bar = 100 nm.*

4.4 Nanoparticle Polymer Composition Detection via dSTORM

Once the particle morphology is determined, the subsequent step is to detect whether single NPs contain either one or both polymer types. dSTORM was employed to achieve such characterization in the NPs native environment, at high resolution and with a color distinction between the PU (green) and PA (red) moieties. To achieve the rendered dSTORM images (Figure 4.5), the data required a chromatic aberration correction.

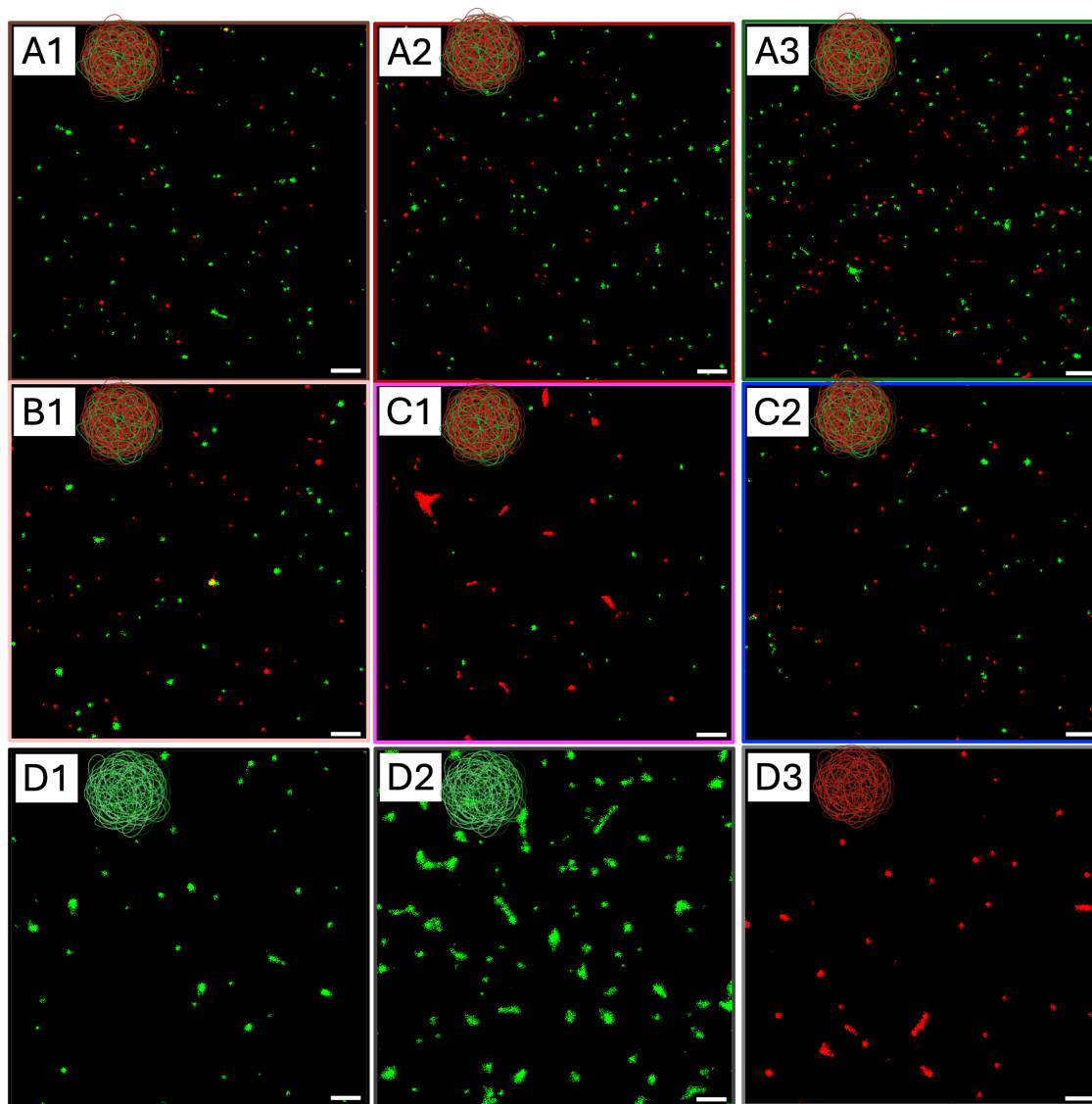


Figure 4.5: Rendered dSTORM images. Representative reconstructed dSTORM images of the hydrophobicity study (A1, A2, A3), chain extender effect (B1) and T_g dependency (C1, C2) compared to the control experiments (D1, D2, D3). Scale bar = 100 nm.

As discussed in Section 2.4.1, correcting chromatic aberration is essential for accurate dual-color dSTORM imaging. During subpixel analysis, even small differences (e.g., 1 pixel) detected by the camera can be greatly magnified. Consequently, this can lead to incorrect shifts in object localization, necessitating correction to properly overlay the

localization. This correction is achieved using fiducial markers that are NPs containing both green ($\lambda_{em} = 561$ nm) and red ($\lambda_{em} = 647$ nm) dyes. The markers are deposited across the field of view and allow for the detection of shifts in the x (Δx) and y (Δy) directions from green to red localizations. The resulting shifts were plotted (see Figure 4.6, blue) and fitted with a linear curve (magenta). The parameters from this fit were then applied to correct the 561 nm localizations, yielding new shifts (orange). This fitting process effectively reduced shifts in both the x and y directions, resulting in an average shift of 0 nm. Examples of chromatic aberration correction applied to a fiducial marker are shown in Figures 4.6 C and D. Before correction, the particle exhibits Janus-like morphology, while after correction, despite minor imperfections, it demonstrates a high degree of overlap, accurately representing a composite particle.

Given the corrected dSTORM images, it is possible to then quantitatively analyze them to detect single NPs polymer composition. The analysis was performed as follows. Large densities of Cy3 or Cy5 localization within a 100 nm radius were clustered and represented by a circle. The distance (d) between each NP was measured using Equation 4.1:

$$d = C2C - (GU + RA) \quad (4.1)$$

where $C2C$ is the center-to-center distance between the PU and PA particle, GU is the measured PU size while RA is the PA's one. The d of the particles was evaluated in order to classify each cluster as: single PA NP, single PU NP or, as hybrid-like NP for d values below 10 nm (Figure 4.7). Once each cluster has been classified, the overall NPs system composition can be quantitatively described based on the PU, PA or hybrid

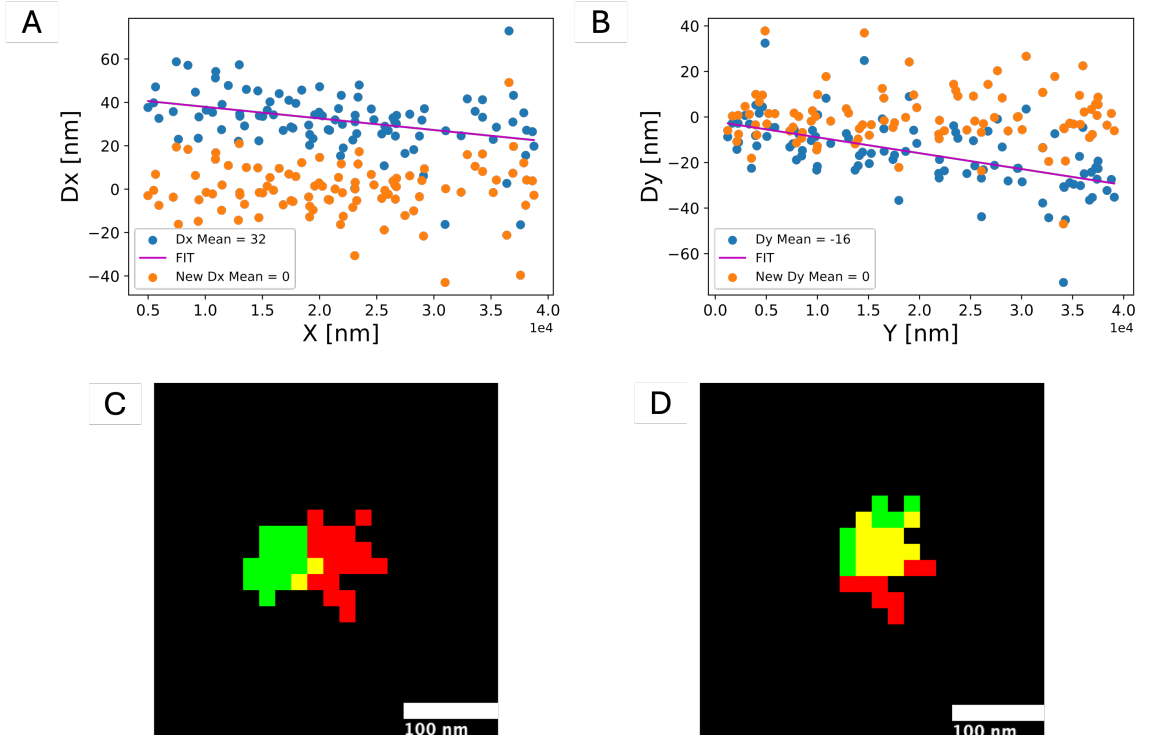


Figure 4.6: Chromatic aberration correction. Scatter plot of the shift before and after chromatic aberration correction in x (A) and y (B). Example on the fiducial marker before (C) and after (D) chromatic aberration correction.

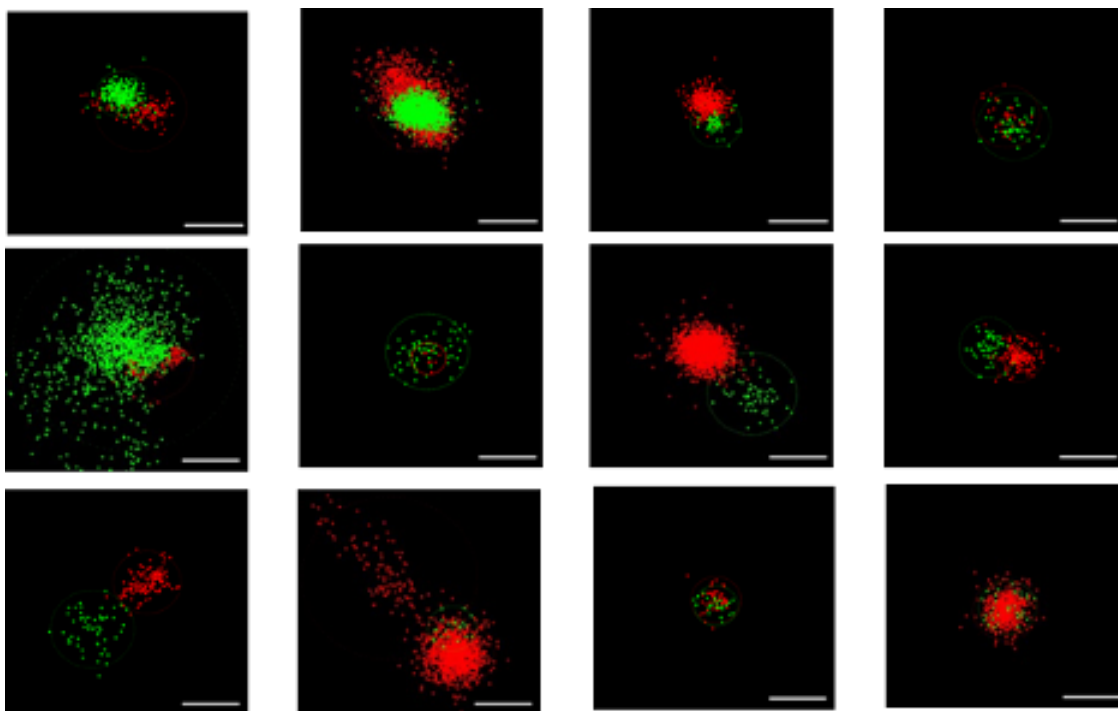


Figure 4.7: Hybrid NPs. Examples of detected hybrid NPs where $d < 10$ nm. Scale bar = 100 nm.

percentage ($H\%$). $H\%$ is defined with Equation 4.2:

$$H\% = \frac{\#Red\ Hybrids}{\#Green\ NPs + \#Red\ NPs - \#Green\ Hybrids} * 100 \quad (4.2)$$

The bar plot in Figure 4.8 summarizes the composition on the systems investigated in this work. The results indicates that less than 6% of the NPs are detected as a hybrid-like particles. Interestingly, none of the parameters evaluated (hydrophobicity, chain extender, and T_g) significantly affected the final hybrid content. A summary of all the dSTORM analysis data is reported in Table A.2.

The detected demixing result is associated to the chosen NPs synthesis approach. Contrarily to other works,¹⁸⁰ the PU NPs used for seeded polymerization did not contain a covalently conjugated acrylate monomer. Thus, did not present a linking group to grow an acrylate moiety. In this work, the selected synthesis approach relies on the stabilization of the acrylate monomers by the PU NPs. The presence of such NPs plays a key role due to the formation of hydrophobic areas (i.e. the PU core) and due to the carboxylic groups on the NPs surface which affect the pH and ionic strength of the solution. Therefore, the pure PA NPs (sample D3) required a different experimental approach and could not be synthesized by simply removing the PU seeds form the setup. Overall, the PU stabilization of the acrylate monomers does not directly imply the formation of blended PU-PA NPs. In this work, the presented result indicate that the analyzed PU and PA moieties are incompatible with polymer blending.

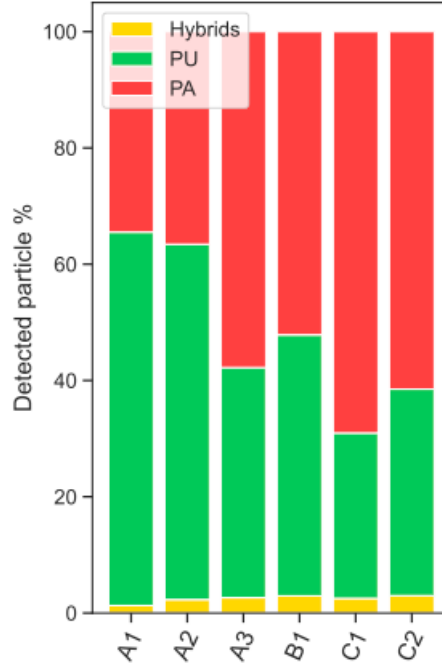


Figure 4.8: Percentage of NP-type in each PU/PA system. Bar plot indicating the detected percentage of hybrid NPs (yellow), PU NPs (green), and PA NPs (red).

4.5 Subpopulation Size Determination

Having classified the NPs as PU, PA, or hybrid, it is then possible to study the size of these particle's subpopulations within each of the investigated samples as a complementary information compared to the one detected by DLS and CryoEM. To do so we analyzed the radius of the detected circles, for either PA (red), PU (green), or hybrid (yellow), and compared their distributions and average size (Figure 4.9). Hybrid radius (HR) was determined based on the comparison between the two radii (GU and RA) and the $C2C$, specifically the enclosedness parameter (discussed later in Equation 4.5). Positive enclosedness values defined HR as the largest particle:

$$HR = |GU, RA|_{max} \quad (4.3)$$

while negative values defined HR as a combination of the two particles:

$$HR = \frac{C2C + RA + GU}{2} \quad (4.4)$$

The detected PU NPs produce monodisperse distributions (green, 100-110 nm) with only minor difference between the samples. Contrarily, PA distributions present variations both in the average particle size (98-159 nm) and in the dispersion of the distribution. Lastly, the hybrids produced larger average sizes together with non-Gaussian distributions.

The differences detected between the PU and PA distributions are representative of the particle synthesis. On the one hand, the PU phase- excluding sample B1- has an identical synthesis protocol for all systems, thus reducing variability between series.

Contrarily, the PA phase in each system was obtained by polymerization of monomers with distinct properties, thus introducing large differences across the series. For instance, low T_g samples (A1, A2, A3, B1) present a narrow Gaussian-like distributions centered around 100 nm. Contrarily, samples with higher T_g values (C1 and C2), show a broad distribution with values ranging from 100 nm to more than 150 nm. Such feature could be associated with the increased viscosity of these materials at room temperature. In glass-deposition techniques such as dSTORM, higher T_g values could lead to the polymer spreading on the coverslip.

On the other hand, hybrids (yellow cross in Figure 4.9 and Figure A.3) consist of a combination of green and red circles, their size was determined either as a combination of the two particles or as the largest particle (for enclosed systems, details in SI). The presence of larger particle sizes suggests existence of dumbbell-like structures that require larger circles to effectively describe their configuration. Therefore, while size analysis is enough to characterize the single PU and PA NPs, it only provides partial insight into the complexity of multicomponent NPs such as hybrids. That is why, in addition to size, further studies are required to properly investigate the morphology of hybrids. Specifically, morphology will be dictated by the way the green and red circles are arranged.

Additionally, it should be noted that the composition-specific size information, extracted from the circle representation of each cluster, is complementary to the one detected via DLS and CryoEM. While DLS offers the advantage of rapidly estimating sample size, it tends to bias toward larger particles, resulting in the oversight of 30 nm nanoparticles in this system. In contrast, CryoEM clearly detected the NPs size distribution and its morphology. However, this technique lacks the chemical specificity to detect hybrid NPs. Therefore, while dSTORM's resolution limit overestimated the NPs size, it remained an asset in improving the characterization of the system.

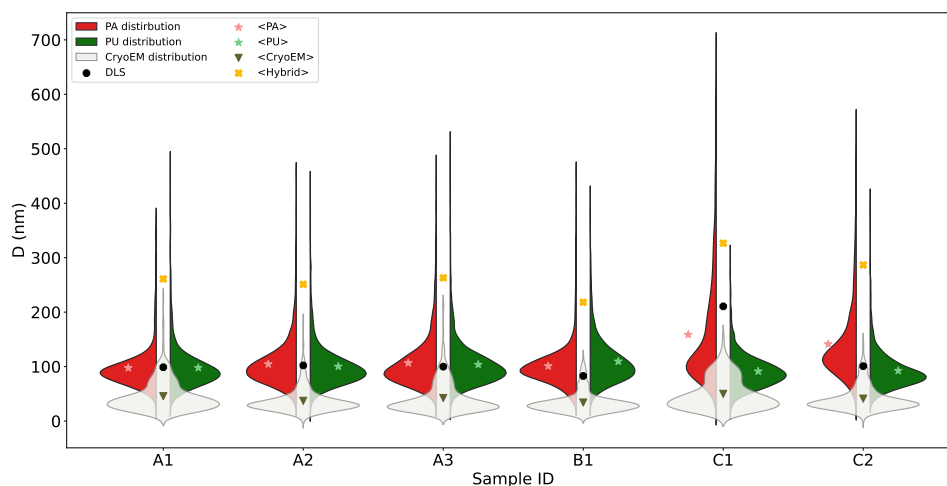


Figure 4.9: Sub-population size determination via dSTORM. Violin plots of PA (red), PU (green) and CryoEM (gray) NP size distribution. The plots are overlaid with the average values for each distribution (PA - red star, PU - green star, Cryo - gray arrow) together with the hybrids values (yellow cross, the hybrid's violin plot is reported in Figure A.3). The data is compared to the DLS-measured (black octagon) hydrodynamic radius.

4.6 Hybrid Morphology Analysis Based on Localization Overlap.

Upon identifying the hybrid particles containing both PU and PA (Figure 4.7, the dSTORM localizations were employed to elucidate NP morphology. Specifically, the analysis allowed investigation of the relative distribution of the PU and PA moieties within individual NPs. To achieve this, we analyzed the relative position of Cy3 and Cy5 clusters, which respectively represent the PU and PA domains, within the detected hybrid NPs. To this end, the distribution was investigated in terms of Enclosedness and C2C distance between the two clusters. The Enclosedness concept is schematized in Figure 4.10 was defined as reported in Equation 4.5.

$$Enclosedness = |GU - RA| - C2C \quad (4.5)$$

The enclosedness parameter is representative of the level of overlap between the two circles. Values above 0 are representative of interconnecting circles, while values below 0 are produced for enclosed ones.

The determined Enclosedness and C2C of each hybrid NPs, were reported in a scatter plot (Figure 4.11). Each sample presents a continuous distribution along the plot, examples of hybrids in the upper half (Enclosedness > 0) and lower half (Enclosedness < 0) are reported in Figure 4.12. Specifically, in the first case the distribution of Cy3 and Cy5 circles produces core-shell morphologies, hence a smaller circle is enclosed within a larger one. While in the second half the distribution corresponds to dumbbell morphologies, where the circles do not completely overlap but produce an intersection.

Moving along the x-axis, the differences in C2C serve to accentuate the distance between the two clusters. In the case of core-shell morphologies, the presence of small C2C values (e.g. below 100 nm) indicates that the two clusters are in a state of centered overlap (Figure 4.12 A) while larger values describe an off-centered system (Figure 4.12 B). In the case of dumbbell morphologies, the differences in C2C are indicative of the degree of overlap. Smaller values (e.g. below 100) are indicative of a high degree of

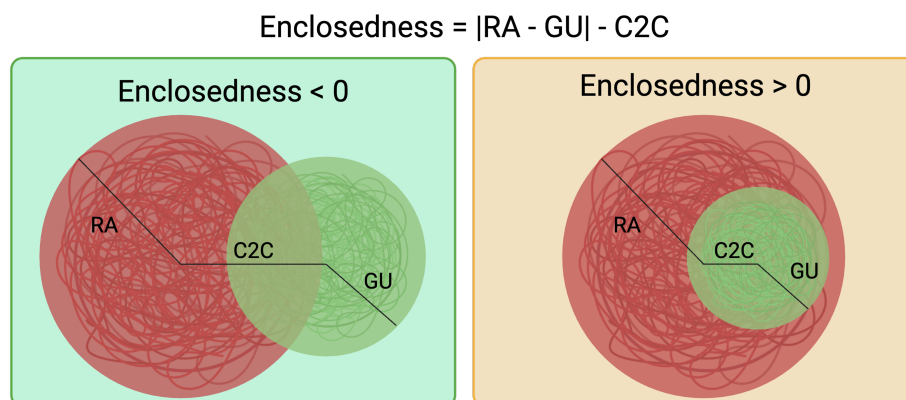


Figure 4.10: Enclosedness concept. Schematic representation of the enclosedness concept. When the value is below 0 the two circles are not enclosed but interconnected. Contrarily, values above 0 are representative of enclosed circles.

overlap (Figure 4.12 C). Contrarily, larger numbers are indicative of a high degree of phase separation (Figure 4.12 D).

The scatter plots demonstrate that the hybrids predominantly exhibit dumbbell morphologies. However, the continuous distribution indicates lack of preferred morphology. Given that the hybrids are only a minor component in each of the investigated sample, the observed trend can be considered representative of the de-mixing tendency of the selected PU and PA moieties.

Regardless, this type of scatter plot offers a significant advantage in characterizing hybrid morphologies, as they provide a quick overview of the sample's features. By proposing a semi-quantitative characterization of the detected morphologies, it overcomes the need of image-by-image and user-biased analysis. Furthermore, while the method was applied on an industrial showcase example, it can be translated not only on similar hybrid nano-systems, such as multiphase nanotubes,¹⁹⁵ but the concept can be extended on larger micro-meter systems, such as phase changing coaervates.¹²¹

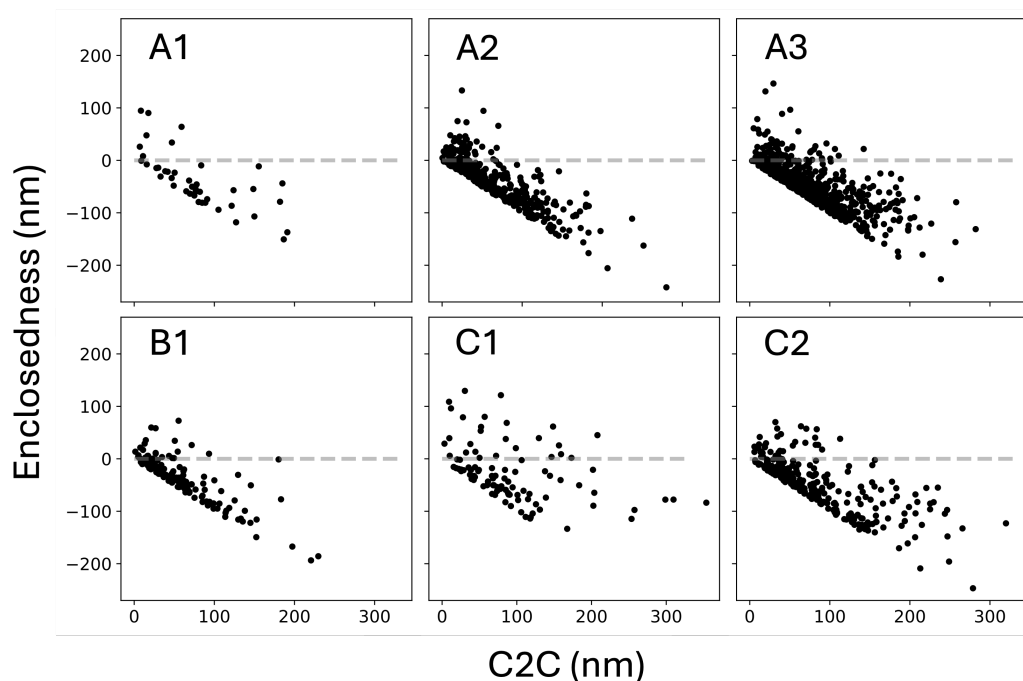


Figure 4.11: Morphology analysis within hybrid NPs. Scatter plot of the C2C distance VS Enclosedness value. The gray dotted line emphasizes the threshold between enclosed and interconnected circles. Form left to right and top to bottom the scatter plot report on sample: A1, A2, A3, B1, C1, and C2.

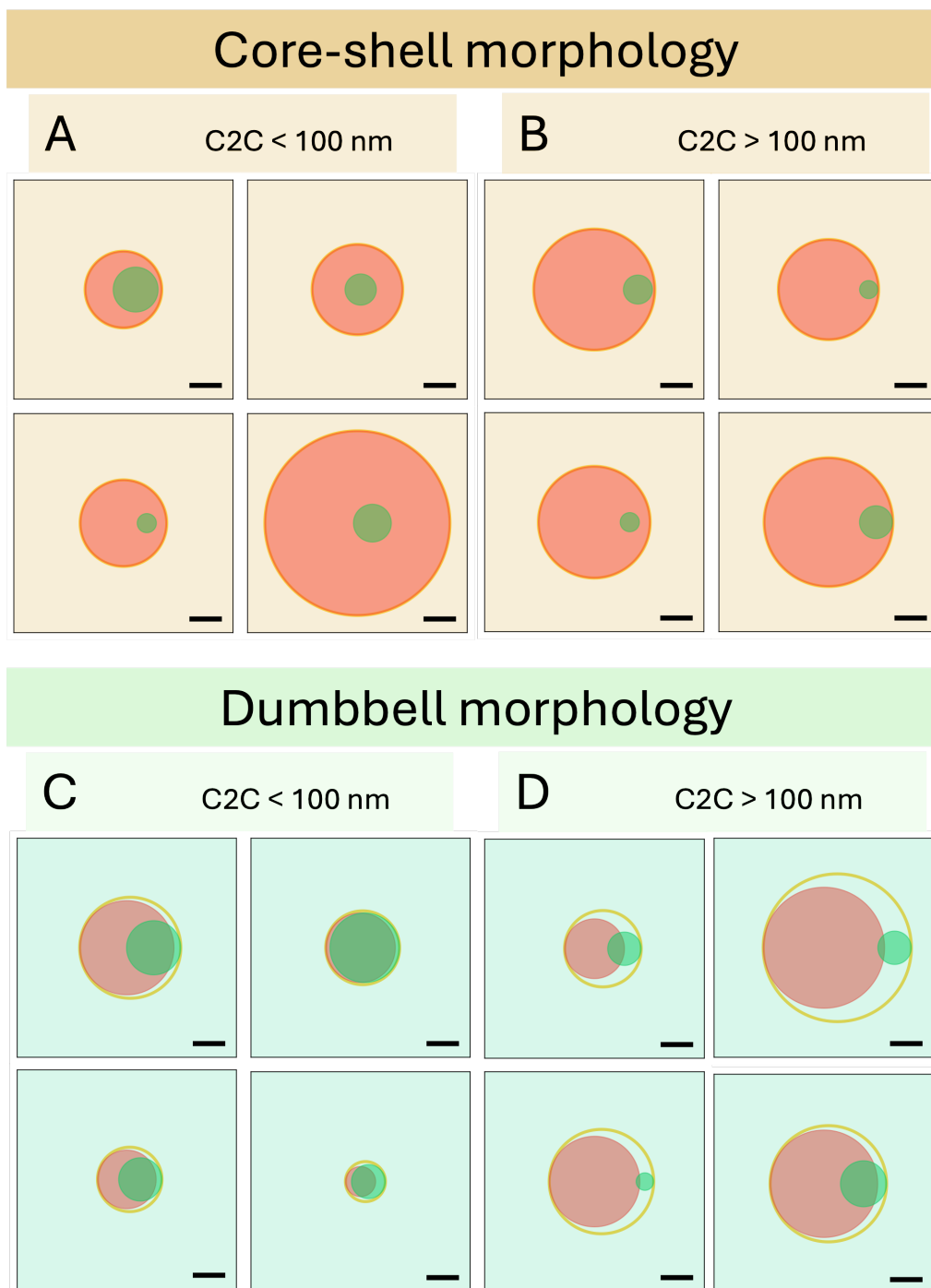


Figure 4.12: Morphology examples. Examples of core-shell and dumbbell morphologies taken from sample C1. The yellow circle shows the circular hybrid representation used to measure the hybrid size (HR). Scale bar = 100 nm.

4.7 Morphology determination via Elemental Analysis

Up to now, in this chapter, the morphology of a PU-PA system has been investigated. In the work, the PU and PA were not covalently conjugated. What would happen if instead they were? I now introduce a case where covalent conjugation was employed in the NP synthesis. Wei *et al* in reference⁷⁸ produced a NPs system where a photocatalytic unit was covalently conjugated to a silica component. His work predicted the formation of a core-shell structure. I performed EELS measurements to support this claim.

4.7.1 Overview of the Nanoparticle Fabrication

Wei's objective, as outlined in reference⁷⁸, was to synthesize photocatalytically active silica NPs. While the detailed synthesis procedure is documented in the published work, a summarized overview is presented in Figure 4.13 and elucidated below.

In the initial step (Figure 4.13 A), a photocatalyst (BTDATS) modified with two organosilane groups was introduced into an aqueous solution containing tetraethoxysilane (TEOS) and a surfactant (CTAB). Under these experimental conditions, a condensation reaction occurred, resulting in the formation of NPs comprising both silica from TEOS and the photocatalytic unit (BTDATS). At this stage, the NP is denoted as photocatalytic core NP (NP-C).

Subsequently, the NP-C served as a seed for the second synthesis step (Figure 4.13 B). The NP-C in an aqueous solution with a surfactant (CTAC) was combined with TEOS in cyclohexane. These conditions facilitated polymerization at the interface between the NP-C and the oil phase. The polymerization process induced the growth of a silica shell around the NP-C, thereby giving rise to the photocatalytic core-shell NP (NP-CS).

The proposed synthesis route anticipates a core-shell structure, which is pivotal for achieving efficient photocatalytic reactivity. However, conventional ensemble characterization methods struggle to discern such structural formations. Techniques like DLS have revealed differences in size between NP-C (200 nm) and NP-CS (250 nm), indicating particle growth. Nonetheless, this data alone does not confirm the presence of a core-shell structure. Thus, necessitating a microscopy-based approach for further investigation.

4.7.2 EELS Characterization

To comprehensively characterize the NP-CS, electron microscopy coupled with elemental analysis was employed. An overview of the samples was obtained in bright field TEM (Figure 4.14), where the NP-C and NP-CS were compared. At this stage, minor difference were observed, with both samples exhibiting the characteristic porous structure that are typical in silica NPs.

To confirm the successful formation of the core-shell structure in the NP-CS, elemental mapping of the NP-CS sample was performed via EELS. Initially, an annular dark-field image was acquired to localize the NPs in STEM mode (Figure 4.15 A). Once a particle of interest was selected (white box in Figure 4.15 A), elemental mapping was conducted for Silicon (Si), Oxygen (O), Carbon (C), and Chlorine (Cl).

The elemental maps revealed a distribution of Si and O throughout the entire particle

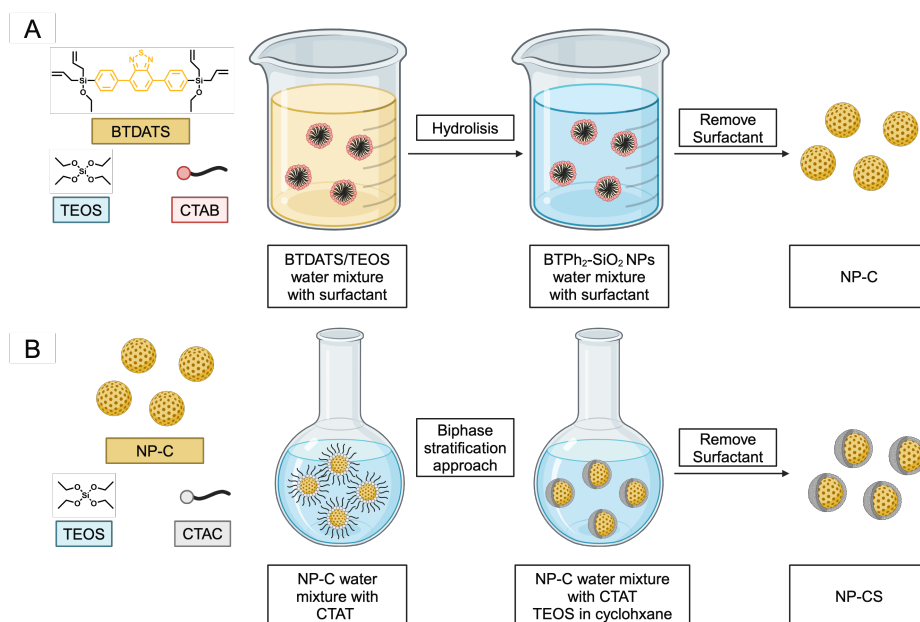


Figure 4.13: NP-C and NP-CS synthesis. Schematic of the synthetic route for photocatalytic core NP (NP-C) (A) and photocatalytic core-shell NP (NP-CS) (B). Figure adapted from reference⁷⁸.

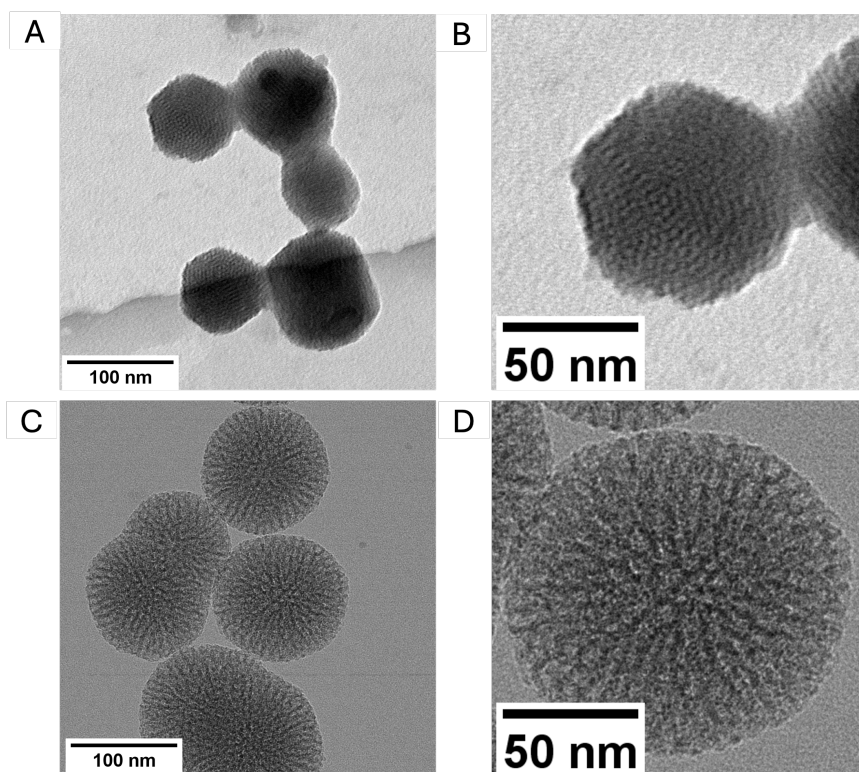


Figure 4.14: TEM of NP-C and NP-CS. TEM micrographs of NP-C (A,B) and NP-CS (C,D). Figure adapted from reference⁷⁸.

(Figures 4.15 B, C), consistent with the presence of the SiO_2 fundamental unit in both the NP-C and NP-CS. In contrast, C was not homogeneously distributed (Figure 4.15 D), exhibiting signal in the core of the NP, indicative of the desired core-shell structure.

Additionally, a C signal was detected on NP's surface. The signal was attributed to the presence of the surfactant (CTAC), employed in the synthesis process. CTAC is composed of a hydrophobic chain containing C and a hydrophilic tail with chloride as a counter ion. The surfactant's role is to stabilize the NP by adhering to its surface thus causing C detection on the NP's outer layer. To confirm the presence of the surfactant, Cl mapping was performed, revealing an overlap of C and Cl signals (Figure 4.15 E). Specifically, Cl was distributed on a thin layer on the outer side of the C signal, consistent with the distribution of the surfactant's chloride counter ion. Such feature is highlighted on the overlaid map in Figure 4.16 A.

Furthermore, both C and Cl exhibited high intensity on the left side of the NP, a result associated to the characteristics of the lacy grid used for analysis. Unlike typical TEM grids, lacy grids exhibit non-homogeneous C deposition that create pores. In this study, the grid facilitated particle clustering near the C layer while minimizing signal interference deriving from the C substrate. Yet, this clustering process, occurred also for the surfactant which adhered to the C layer and deposited on top of it. This phenomenon explains the observed signals of both C and Cl, highlighting the role of the lacy grid in facilitating NP clustering and surfactant accumulation.

The EELS map provides qualitative insights into the elemental distribution within the NP. However, for a comprehensive comparison of each signal, it is beneficial to analyze a line profile across the NP (Figure 4.16). Although the line profiles are not intended for quantitative analysis, which requires thin specimens and proper fitting of the EELS spectra, they serve as indicators of the relative abundance throughout the specimen. Thus, the line plots serve to highlight the distribution of signal intensity within each map.

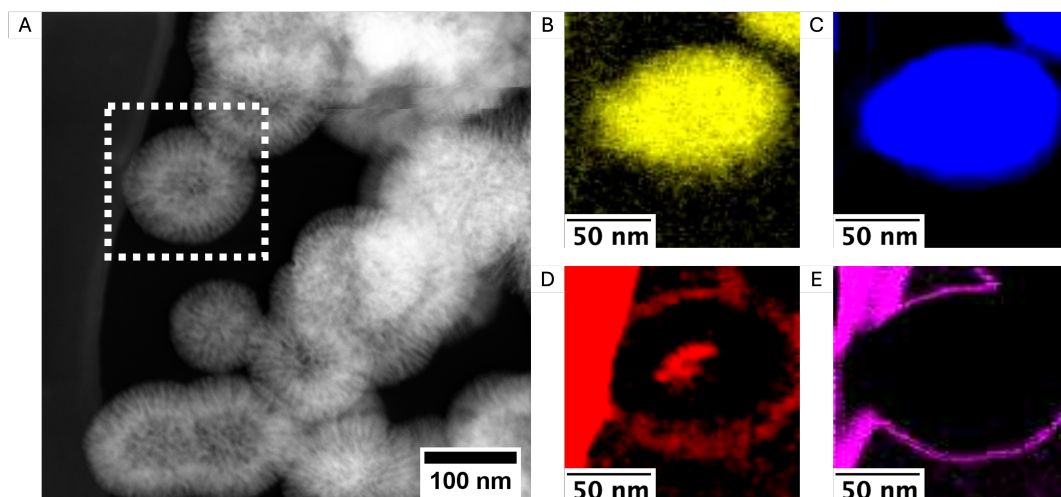


Figure 4.15: EELS of NP-CS (A) Annular dark-field (ADF) image showing the selected NP for elemental mapping of (B) Si, (C) O, (D) C and (E) Cl. The results highlight enriched contents of C in the core while Si and O are more evenly distributed in the whole NP. Figure adapted from reference⁷⁸.

In the case of Si and O (Figure 4.16 B and C), the signal is relatively uniform throughout the 100 nm NP, with higher intensity observed towards the center of the NPs. This increase in intensity is representative of the particle's shape. In spherical NPs, the central section is thicker compared to the sides, resulting in a larger amount of atoms and thus an increase in the detected EELS signal.

The more intriguing line plots are those of C and Cl (Figure 4.16 D and E). The Cl results delineate the NP's surface, where the O and Si signal drop and the Cl signal begins. The C line plot accentuates the core dimension, which appears to have a width of approximately 20 nm. However, the map reveals that the core is not uniformly distributed but exhibits an ellipsoid shape. Consequently, measuring the core in a different direction doubles its size (e.g., Figure A.4).

The ellipsoidal distribution of C may be inherently linked to the NP synthesis process. However, the annular dark-field image of the NP before and after elemental mapping highlights beam-induced deformation (Figure 4.17) that transforms the NP from a circular to an ellipsoidal shape. As discussed in the introduction (Section 2.3.1), EELS necessitates longer acquisition times compared to conventional TEM imaging and is typically performed on highly electron-beam stable materials. Nonetheless, even highly stable materials like silica NPs can undergo beam damage, resulting in the detected deformation. Consequently, while the mapping can still provide valid information regarding the presence of the core, it should not be utilized for size determination due to both beam-induced damage and drying artifacts, which may cause NP collapse. For size-related information, techniques such as DLS are more suitable.

Furthermore, the before and after images (Figure 4.17) are of critical importance in confirming that the NP were not lost during acquisition and in characterizing particle deformation. Such detection was not straightforward, as with similar NPs, the measurement led to complete particle burning and loss of signal.

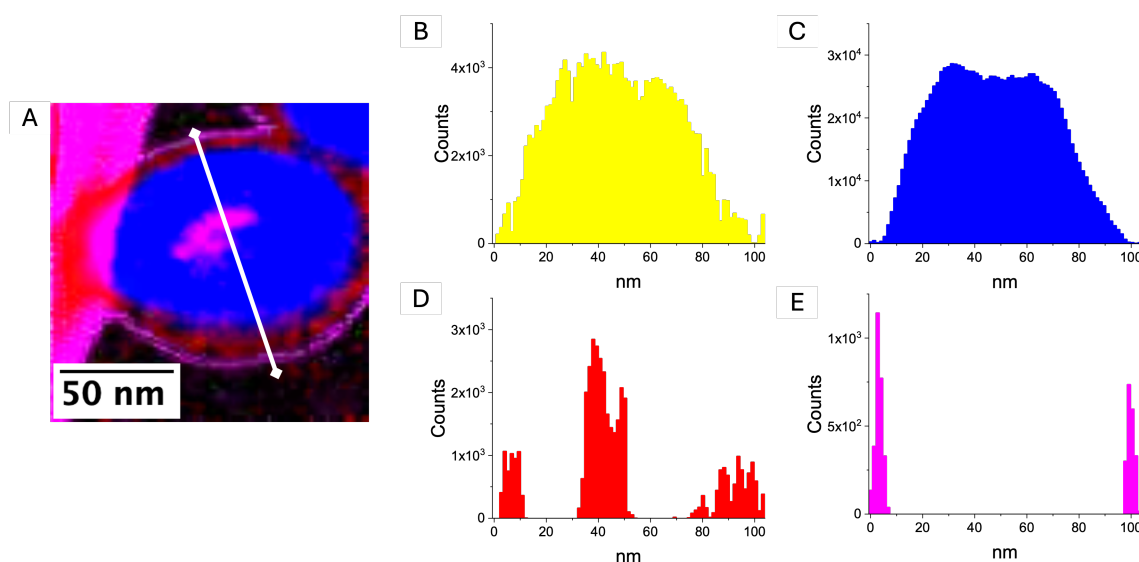


Figure 4.16: Intensity profile of NP-CS (A) combined elemental mapping with the line (white) highlighting the location where the cross section elemental distribution of NP-CS is analyzed for (B) Si, (C) O, (D) C and (E) Cl. Figure adapted from reference⁷⁸.

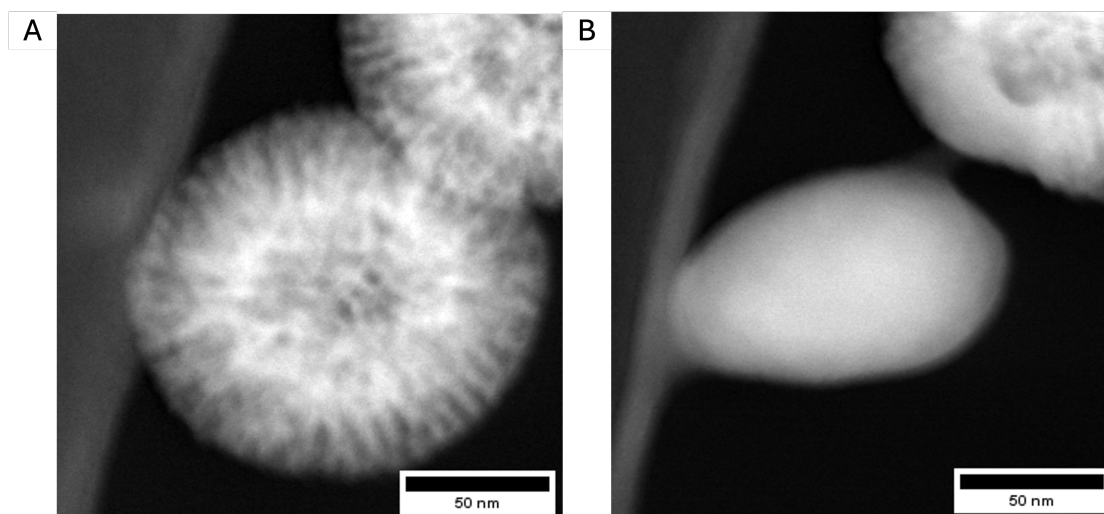


Figure 4.17: *Beam damage effect on NP-CS. Annular dark-field (ADF) micrograph of the particle of interest (A) before and (B) after a 15 min acquisition highlighting beam damage. Figure adapted from reference ⁷⁸.*

4.8 Summary and Outlook

In this chapter two types of multicomponent NPs were characterized to determine their morphology. A major part of the chapter employs dSTORM as a technique to quantify single NPs composition, alongside composition-specific size distribution and, for hybrid NPs, morphology determination. The analysis was performed on 100 nm NPs deriving from a relevant industrial system. In contrast to previous hypotheses regarding core-shell morphology formation, the findings indicate that the samples exhibit an overall phase separation of PU and PA. The unexpected limited formation of hybrids, presents an opportunity for future investigation into factors influencing the formation of more robust films (e.g. combination of polydisperse systems). Furthermore, composition-specific size distribution highlighted how different acrylic monomers influence the PA NPs size distribution. Lastly, for the hybrids, size characterization was coupled with morphology investigation. Morphological analysis was based on relative localization of the green and red clusters. While largely distributed, the analysis could semi-quantitatively distinguish core-shell and dumbbell morphologies. The suggested approach eliminates user bias in single particle classification while facilitating the analysis of large number of particles through single scatter plot representation. The results presented, help shine light into chemical characterization and morphology of a relevant PU-PA systems. Further research of analogues systems, such as liposomes, composed by multiple chemical components, hold promise in elucidating moieties behaviors at the single particle level.

Moreover, the final section employs EELS elemental mapping to investigate the formation of a core-shell NP in a photocatalytically relevant system. The finding prove the formation of the predicted core-shell morphology.

In this chapter the goal was to answer the following question: "*can we differentiate chemical components within small NPs?*". The answer is: "*yes, but...*". Indeed the goal can be achieved but the technique needs to be tuned to the material in question. EELS

was not a suitable method to characterize the PU/PA system for two reasons: 1) the chemicals involved in the synthesis are not stable under strong energy source (i.e., the electron beam) and 2) the PU and PA moieties present similar elemental composition hampering a chemical distinction. Contrarily, with proper synthesis and analysis tuning, dSTORM might have been a suitable technique for the characterization of the NP-CS. Therefore, I believe further research can and should be conducted in analogous systems where NPs are composed by multiple moieties. Additional work can highlight and improve such characterization tool and provide further insights into NPs morphologies at the single particle level.

4.9 Material and Methods

4.9.1 dSTORM Relevant Nanoparticles

Materials

All reagents (Dimethylol propionic acid (DMPA), Cyclohexane-dimethanol (CHDM), poly THF 1000 (pTHF), DABCO T-9, Nonyl phenol, diethylene triamine (DETA), Hydrazine 16%, 1-butylpyrrolidin-2-one (NBP), tert-butylhydroperoxide (tBHPO), N-butyl acrylate (BA), 2-ethylhexyl acrylate (EHA), isodecyl acrylate (IA), meta methacrylate (MMA) and butyl methacrylate (BMA), Sodium lauryl sulphate (SLS) 30%, Acrylic acid, Sodium dithionite (SD), Triethylamine (TEA), Iron III, Isoascorbic acid (iAA), Amonium persulfate (AP), glucose, glucose oxidase, catalase, ethanolamine (MEA)) were obtained from common laboratory chemical suppliers such as VWR Inc., Sigma-Aldrich or Thermo Fisher Scientific Inc., except for isophorone diisocyanate (IPDI), which was obtained from Covestro A.G., TEGO® Foamex 805, which was obtained from Evonik Industries AG, Libratex AS-10, which was obtained from Libra Speciality Chemicals, Cyanine N-Hydroxysuccinimide (Cy3-NHS), which was obtained from Lumiprobe GmbH and Cyanine 5-Acrylamide (Cy5), which was obtained from SyMo-Chem B.V.

Prepolymer Mixture Synthesis

Into a flask reactor the following items were added: 42 g DMPA, 28 g CHDM, 279.77 g pTHF and 350.23 g of IPDI, and mixed under air conditions at 50 °C. Three drops of DABCO T-9 were added to the solution and a slowly heated to 87 °C, followed by 1h mixing. Two more drops of DABCO T-9 were added and the solution slowly cooled to 75°C while mixing. Subsequently, 14.6 g TEA solubilized in 30 g of demineralized water was added, the solution was stirred for 1h and stored until use.

Polyurethane Dispersion Synthesis

In a reactor flask 3.3 g of Nonyl Phenol, 776.3 g demineralized water and 0.86 g Foamex were added. The solution was mixed for 15 min at room temperature. 1786 g of the prepolymer mixture were slowly fed to the reactor flask. In a container, 14.6 g of DETA or 22.2 g of Hydrazine were solubilized in 30.0 g of demineralized water containing 2 mg of Cy3-NHS dye. This mixture was added to the reactor and mixed for 30 min.

Synthesis of Polyurethane/Polyacrylate Dispersion

In a reactor, 4.1 g of Libratex and 350 g of polyurethane dispersion were mixed for 15min. Separately, 1 mg of Cy5-acrylamide was diluted into 1 mg of NBP. The reactor flasks were put under nitrogen flow and the dye mixture was added followed by the acrylate monomers (38.5 mg). 0.5 g of Iron III diluted into 1 mL of water was added and mixed for 1 h at RT. A dilution of 1:10 tBHPO in water was made and added to the reactor. Separately, 1:100 dilution of iAA was prepared and fed in steps of 1 mL to the reactor within 4 5min while the exotherm was observed. After the final addition of iAA, the mixture stirred for another 15 min and cooled down to 25 °C. Finally, samples were filtered with a cloth filter of 400 mesh and stored in the dark until use.

Synthesis of Polyacrylate Control

Samples containing only PA dispersion was also prepared (D3). In a reactor 95.76 g of BA was added to 1.65 g of SLS, 40.48g of demiwater and 3.4 mg of Cy5 acrylamide. The mixture was left to homogenize for 15 min. Then 2 mg of Fe (III) diluted in 1mL demiwater was added to the solution followed by 0.49 g of AP diluted in 9.38 g of demiwater. Finally 0.3 g of SD diluted in 5.63 g of water was added to the solution. The mixture was allowed to stir for 15 min plus the time required to reach room temperature. Lastly, the solution was filtered and stored until use.

Nanoparticle Characterization

All samples in their original concentration were dialyzed for 7 days (1kDa dialysis tubes from Spectrum labs) before imaging. Solid content, pH and viscosity were measured on the sample before and after dialysis. DLS and ζ -potential measurements were performed using Malvern Zetasizer on a diluted solution (0.5 wt%, in 10 mM KCl).

Samples were diluted into a solution of 10mM KCl (300 time) to measure both hydrodynamic size, polydispersity and zeta potential using a Malvern Zetasizer.

dSTORM Imaging Setup

dSTORM imaging was performed with a Nikon N-STORM system configured for TIRF imaging and equipped with a perfect focus system. The Nikon 100X, 1.4 NA oil immersion objective was used. The imaging was performed using the 561 nm and 647 nm lasers to image respectively the CY3 and CY5 dyes. No UV activation was used. After adjusting the TIRF angle, the fluorescence signal passed through a quad-band-pass dichroic filter (97335, Nikon) and was recorded using an Andor EMCCD camera (Nixon3) with pixel size of 160 nm and a region of interest of 256 x 256 pixels. Coverslips (22 mm x 22 mm, #1.5) were immersed in methanol and sonicated in a water bath for 20 min, then dried with nitrogen flow. While the microscopy slides (76 mm x 26 mm, 1 mm) were cleaned with acetone-soaked microscopy tissue. The imaging chamber (20 μ L) was prepared using double sided tape to attach the coverslip to the microscopy slide. The samples were added to the chamber and left to incubate in high humidity box for 5 h by flipping the chamber and sealing it with scotch to limit solvent evaporation. The

undeposited particles were then flushed with 180 μ L of dSTORM buffer (10 wt% glucose, 0.5 mg/mL glucose oxidase, 40 μ g/mL catalase, 1 mM MEA). Lastly, the chamber was sealed with nail polish. Imaging was performed using 40% laser power for both the 647 nm and 561 nm channels with an exposure time of 40 ms. A total of 20 000 frames were acquired by sequentially alternating the lasers (10 frames with 647 nm, 10 frames with 561 nm, looped 100 times) to optimally compare the PU and PA overlap and to reduce possible errors associated to drift or NPs detachment.

dSTORM Data Analysis

The .nd2 movies collected by the microscope were then analyzed with ThunderSTORM, the ImageJ plugin. The localizations were detected using a β -spline wavelet filter with scale 2 and order 3, a local maxima finder with a threshold set to 3 times (or 2 if fiducial markers were present) the standard deviation of wave F1 of the filter. Localizations with uncertainty less than 1 were filtered out and then a drift correction was obtained using the fiducial marker method. The drift correction was calculated using the 647 nm signal and then saved to be applied to the 561 nm data. Lastly, a density filter was applied with a radius of 70 nm and a minimum number of 20 events. The result table was exported and further analyzed by first applying a chromatic aberration correction on the 561 nm data. Subsequently, the data was analyzed with a MATLAB code. Briefly, the code runs a mean shift clustering algorithm on both the 561 nm and 647 nm data to detect clusters with a bandwidth of 100 nm and a minimum of 8 localization events. The code then fits a circle around 95 % of the localizations within the cluster to estimate each particles size (radius) and its center.

4.9.2 EELS Relevant Nanoparticles

Materials

Triethylamine (TEA), tetraethylorthosilicate (TEOS), hexadecyltrimethylammonium chloride (CTAT), hexadecyltrimethylammonium bromide (CTAB), bis(acetonitrile) (1,5-cyclooctadiene) rhodium(I) tetrafluoroborate, tetrakis(triphenylphosphine)palladium(0) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). 4,7-bis(4-allylethoxysilylphenyl)-2,1,3-benzothiadiazole (BTBADS) was synthesized in house.

Preparation of NP-C and NP-CS

NP-C: A mixture of CTAB (25 mg), distilled water (12 mL), and sodium hydroxide (87.5 μ L, 2 M) was stirred at 80 °C for 50 min at 700 rpm in a 25 mL round bottom flask. Then, TEOS (0.1 mL, 0.45 mmol) was added along with 4,7-bis(4-allylethoxysilylphenyl)-2,1,3-benzothiadiazole (BTBADS, 26.7 mg in 1 mL of dry THF). The mixture was treated ultrasonically for 20 min to disperse the hydrophobic BTBADS in the aqueous solution, and the condensation process was carried out for 12 h. Afterwards, the solution was cooled to room temperature with stirring. Fractions were gathered in propylene tubes and collected by centrifugation.¹⁹⁶

NP-CS: 60 mg NP-C, 2.4 mL of (25 wt %) CTAC solution and 18mg of TEA were added to 3.6 mL of water and stirred gently at 60 °C for 1 h in a 10-mL round

bottom flask, then 2 mL of (20 v/v %) TEOS in cyclohexane was carefully added to the water-CTAC-TEA solution and kept at 60 °C in an oil bath under a magnetic stirring. The stirring rate was set to be 150 rpm. The reaction was then kept at a constant temperature with continuous stirring for 16 h to obtain the products. The products were collected by centrifugation and washed for several times with ethanol to remove the residual reactants. Then, the collected products were extracted with a 0.6 wt % ammonium nitrate (NH_4NO_3) ethanol solution at 60.0 °C for 6 h twice to remove the template and dried in vacuum.¹⁹⁷

EELS Imaging Setup

A lacy grid was used to deposit 10 μL of sample. The sample was left to dry for 10 minutes. To guarantee full drying of the sample, the grid was additionally back blotted with Whatmann paper. The grid was imaged in a Titan Krios G4, (Thermo-Fisher Scientific) in cryogenic temperature to avoid carbon contamination during image acquisition. The microscope was operated at 300 kV. Data was acquired using a GIF Continuum K3 system. The elemental maps were extracted from the spectral images using Digital Micrograph.

Chapter 5

Functional Group Distribution within Nanoparticles

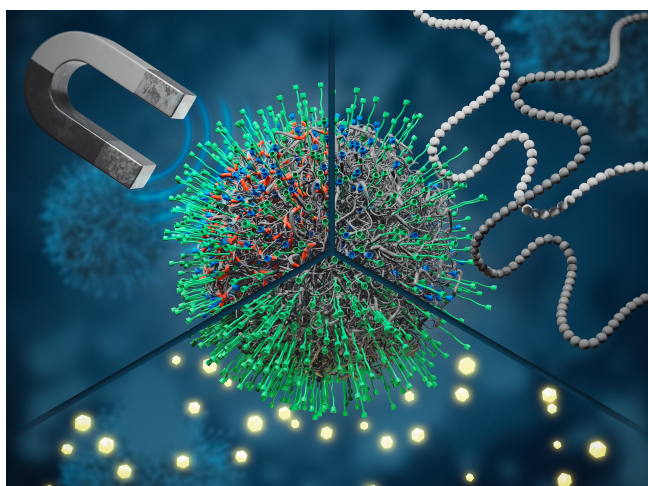
This chapter is based on a work that was carried out primarily in the Max Plank Institute for Polymer Research. DNA-PAINT was performed in Technical University of Eindhoven. The text reported in the sans serif font explicitly references the work published in RSC Polymer Chemistry:¹⁹⁸

“Tailoring reactive handles on the surface of nanoparticles for covalent conjugation of biomolecules”

Francesca Mazzotta, Sharafudheen Pottanam Chali, Ingo Lieberwirth,* Calum T. J. Ferguson^{†,*} Katharina Landfester*

Contributions:

I performed all the experiments, data analysis and wrote the manuscript. S.P.C., I.L., C.F. and K.L. supervised my work and contributed to the final version of the manuscript.



Chapter Motivation

In the previous chapter, I investigated the distribution of chemical phases within NPs. Comprehending this distribution is crucial for understanding their structural details and the material properties. However, in this final chapter, the focus shifts towards the distribution of functional groups (FGs) within individual polymer-type NPs. Thus, the emphasis moves away from polymer-polymer distribution and into the variations within the polymer itself. For this purpose, polymers were synthesized to incorporate two types of monomers: a primary one serving as the NP's building block and a co-monomer containing FGs.

Understanding the distribution of FGs within NPs plays a crucial role in determining their stability. As highlighted in the previous chapter, I discussed the need for an adaptation during the synthesis process of the poly-acrylate control sample (D3). This adaptation involved the co-polymerization of methyl methacrylate monomer (MMA) with acrylic acid (AA), allowing for the introduction of carboxylic groups that stabilize the NPs and enable control experiments. The importance of understanding the distribution of FGs becomes evident as these groups play pivotal roles in tuning material applications. For example, FGs can be utilized to conjugate antibodies on NPs surface, thus facilitating targeted delivery to specific areas in the body.^{81–83,120} Products of such conjugation have been investigated in detail with super resolution imaging techniques (DNA-PAINT or dSTORM). In contrast, the examination of unmodified NPs and their FGs distribution is less extensive, relying on ensemble-averaged measurements that overlook both the particle-to-particle differences and the in-particle distribution.

Therefore, in this chapter, I investigate the distribution of various FGs – including amino, carboxylic, and azide – within polymeric NPs. My primary focus is on discerning the presence of FGs inside the NP versus those on its surface. Additionally, I aim to provide insights into the density of reactive FGs at the level of single particles, allowing for an examination of particle-to-particle differences by means of DNA-PAINT. This investigation offers a close examination of atomically relevant species and how they can be utilized to enhance particle function.

ABSTRACT

Surface modification of nanoparticles (NPs) utilizes numerous types of active molecules such as DNA, antibodies, enzymes, or carbohydrates. These modifications usually require reactive handles like amines, carboxylic acids, or azides on the NPs. In this work, utilizing poly-benzyl methacrylate based NPs as model NP system, the amount of FGs was tuned with functional co-monomers: amino ethyl methacrylate (AEMA) for the amino groups (NH_2), methacrylic acid (MAA) for the carboxylic groups (COOH) or azido ethyl methacrylate (AzMA) for the azide groups (N_3). Herein, a systematic study is presented where the FGs in the NPs are differentiated between total, visible and accessible FGs. The concentration of each type of FGs is compared using various methods. Polymers synthesized using free radical polymerization were analyzed using ^1H -NMR to obtain the total number of FGs. Via miniemulsion-solvent evaporation technique, these polymers were used to synthesize the NPs. Zeta potential, pH value and particle charge detection (PCD) were used to determine the number of visible FGs. The number of accessible FGs were quantified by conjugating small fluorescent dyes. The fluorescence measurements were directly performed on the system under investigation, hence eliminating errors associated to indirect measurements and detecting very low concentrations (e.g. 80 nM). Additionally, human serum albumin (HSA) was conjugated to investigate the effect of a bulky molecule on the accessibility of these reactive handles. Lastly, DNA-PAINT imaging was applied to shift from an ensemble averaged analysis to a particle specific visualization.

5.1 Introduction

Surface modification of NPs is of great interest in order to create novel and functional nanomaterials. Their application extends from the biomedical field,^{80,199} to material science.²⁰⁰ For example, NPs in nanomedicine have utilized their ability to encapsulate therapeutic molecules. Thus, allowing to protect such molecules from binding to plasma protein, suppressing the clearance rate, increasing biodistribution, tissue uptake and more.⁶² To achieve targeted cargo delivery, NP surfaces are often functionalized with molecules such as antibodies,^{81–83,120} enzymes,^{84–86,201} carbohydrates,^{87,88} peptides^{89,90} or other small molecules.⁹¹ For this purpose, a precise control of the reactive molecules on the NPs surface is required. These ligands are primarily introduced by using covalent conjugation exploiting reactive handles such as amines,¹⁹⁹ carboxylic acids,²⁰² azides,²⁰³ maleimides,²⁰⁴ or alkynes⁸¹ that can be directly incorporated on the NPs surface during the synthesis process. Various conjugation strategies can be applied to couple molecules of interest to such reactive handles. A cheap and commonly implemented reaction is the amide bond formation by using EDC/NHS (N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide / N-hydroxysuccinimide) chemistry to either conjugate amines to carboxylic functionalized surfaces or vice versa, making this a versatile tool for the coupling of many molecules, proteins as an example.^{199,205} Alternatively, the Nobel Prize-winning click chemistry reaction has emerged as a convenient method for surface functionalization.^{200,203,206–208} Therefore, the assessment of FGs on the NP surface is essential for facilitating their effective application, providing critical insights into the selection and optimization of surface functionalization strategies.

Various strategies are employed to characterize both the surface of modified and unmodified NPs. The number of FGs on the unmodified NPs can be distinguished between the

total (e.g. in the whole particle), the visible (e.g. those that affect the NPs physiochemical properties) and the accessible (e.g. the reactive handles that can be used for subsequent conjugation) amounts. The total number of FGs can be determined in a straight forward way via NMR spectroscopy.^{116,209} A reliable strategy to determine the visible groups is with electrochemical tools, such as potentiometric titrations, which can use the smallest probes available (protons or hydroxide ions) to quantify all the surface charges associated to the FGs. However, these methods require tedious work and long equilibration times.¹¹⁶ Optical, photometric and fluorimetric techniques, such as the toluidine blue assay can be used as well.^{116,118,209} While these methods focus on visible groups, quantifying reactive handles, and thus accessible FGs, relies on indirect measurement. This entails analyzing the unbound reagent instead of the bound one. Consequently, these methods involve multiple cleaning steps, which can introduce errors into the final results. Alternatively, Moser et. al.¹¹⁹ reported cleavable probes for amines and carboxylic acid quantification.

Moving on to surface-modified NPs, quantification of the surface modifications varies depending on the specific type of modification. For protein modified NPs, the quantification of surface conjugation can be done by using standard protein assays such as the BCA,²¹⁰ Bradford, or Pierce⁸³ ones. However, their result can often be subject to errors associated with interfering substances.²¹¹ Alternatively, depending on the type of protein, its characteristics can be taken advantage of for quantification purposes: for enzymes, their enzymatic activity,²¹² while for antibodies their interaction with antigens.²¹³ Instead, for carbohydrates conjugated NPs, the primary quantification strategy is based on thermogravimetric analysis.^{87,214} Moreover, recent advancements in super-resolution microscopy have introduced innovative quantification techniques such as quantitative PAINT (Points Accumulation for Imaging in Nanoscale Topography). Such techniques can be tuned for different types of surface modification. Their use enables the analysis of single NPs, providing valuable insights into particle distribution and composition.^{86,120,212,215}

While the particles can be characterized utilizing the above mentioned methods, to the best of our knowledge there are no reports regarding two very important aspects that affect the final product: 1) the bulkiness of the active molecules and 2) the number of reactive handles on the NPs surface. Herein, we report a systematic study on the characterization of the NPs from the raw material to the final product by tuning and investigating the number of total, visible and accessible FGs (amines, carboxylic, and azide groups) on polymeric NPs. For this purpose, statistical polymers were synthesized with precise functional monomer composition and NMR was used to quantify the total number of FGs. The polymers were then used to synthesize NPs via the miniemulsion-solvent evaporation technique. Next, the visible FGs were investigated using a quick potentiometric tool: particle charge detection (PCD). The accessible FGs were determined by applying the commonly used EDC/NHS reaction or via copper catalyzed click chemistry to couple a small fluorescent molecule. The conjugated dye is directly quantified on the system under investigation by drying the polymeric particles and subsequently dissolving them in an organic solvent. In this way, common errors associated to indirect measurements (e.g. supernatant studies) were avoided. Additionally, we conjugated a model biomacromolecule, human serum albumin, to analyze the effect of a bulky protein as an active molecule. Lastly, we conjugated a DNA strand for DNA-PAINT imaging to detect particle-to-particle differences in FGs densities.

5.2 Polymer and Nanoparticle Synthesis

The objective in this chapter is to produce NPs with a reactive surface containing specific functional groups (FGs) such as amino, carboxylic, or azide handles. This was achieved by utilizing statistical functional co-polymers. These polymers were synthesized through free radical polymerization of benzyl methacrylate (BzMA) as the primary monomer (99 or 90 wt%) with 2-aminoethyl methacrylate (AEMA), methacrylic acid (MAA), or 2-azidoethyl methacrylate (AzMA) as functional co-monomers (1 or 10 wt%). Maintaining the 1:99 or 10:90 ratio between functional co-monomer and monomer was crucial for this study. Hence, high monomer conversion (>92%, see Table A.4) was required. BzMA was chosen as the primary monomer over the more commonly used styrene monomer due to its lower activation energy,²¹⁶ ensuring a higher conversion rate and affecting the final polymer percentage.

The polymer synthesis process required slight modifications depending on the monomers used. P(BzMA-co-AEMA) (Figure 5.1) was synthesized by solubilizing AEMA in MeOH and subsequently adding an acetone solution containing BzMA and the initiator. This mixture of MeOH and acetone as solvents was necessary to efficiently combine the hydrophilic solid form of AEMA with the hydrophobic BzMA. Conversely, P(BzMA-co-MAA) and P(BzMA-co-AzMA) (Figure 5.1) were more easily obtained by dissolving all monomers (BzMA with MAA or AzMA) and the initiator in a single solvent: DMSO.

To produce functional NPs an miniemulsion-solvent evaporation process was performed (Figure 5.2). Specifically, the polymers were dissolved in ethyl acetate and emulsified with an aqueous solution containing the non-ionic surfactant Lutensol AT50, necessary to stabilize the NPs in media with increased ionic strength. Specifically, P(BzMA-co-AEMA) with either 1% and 10% functional monomers was used to synthesize respectively NH₂-NPs-1% and NH₂-NPs-10%. Similarly, COOH-NPs-1% and COOH-NPs-10% were obtained using 1% or 10% P(BzMA-co-MAA) polymer, while N₃-NPs-10% was produced using P(BzMA-co-AzMA)10% polymer.

After miniemulsion and solvent evaporation, the NPs were purified and characterized via DLS and TEM. All NPs exhibited a narrow size distribution and a spherical morphology (see Figure 5.3), with a diameter (D_h) of approximately 200 nm. However, to achieve these dimensions, NH₂-NPs-10% required additional polymer (450 mg VS 150 mg) and reduced surfactant (0.1 VS 0.4 wt%) (see Table 5.1), as the original parameters would produce smaller NPs. This necessity may be attributed to the charged amino group in P(BzMA-co-AEMA), resulting in electrostatic stabilization in addition to steric stabilization, which could reduce the particle size.

Initial detection of the visible FGs was performed through ζ -potential and pH measurements of a 0.1 wt% particle sample (Table 5.1). The data confirms the presence of amino and carboxylic FGs and their varying concentrations. For NH₂-NPs, there was an increase in ζ -potential from 32.5 mV to 38.8 mV and a decrease in pH from 4.7 to 4.3 when comparing NPs-1% and NPs-10%. Conversely, with COOH-NPs, there was a decrease in both ζ -potential and pH, with values varying from -15.4 mV and pH 6.1 to -26.1 mV and pH 5.7, respectively.

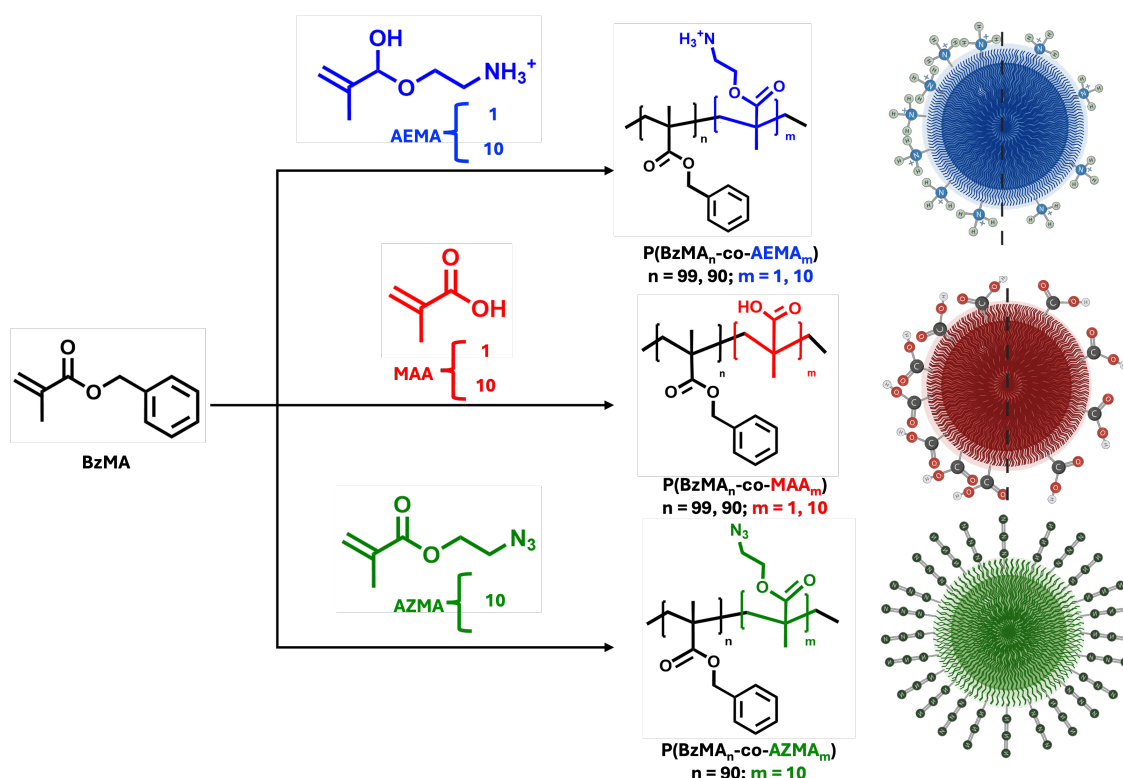


Figure 5.1: Sample overview. Schematic overview of the samples. From left to right, the monomer (BzMA) was reacted with the specific functional monomer (AEMA, MAA or AzMA) to produce the functional polymers ($P(\text{BzMA-co-AEMA})$, $P(\text{BzMA-co-MAA})$, and $P(\text{BzMA-co-AzMA})$). The functional polymer is then utilized to produce the functional NPs. The polymer's final composition was calculated based on mol% while the nomenclature is associated to the initial wt% of the monomers added. Adapted from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

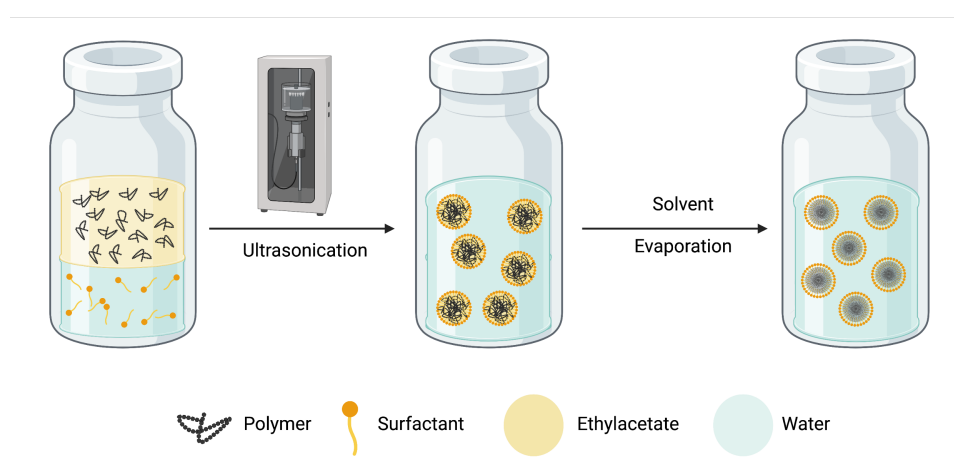


Figure 5.2: Miniemulsion - solvent evaporation. Schematic representation of the miniemulsion process. The polymers are dissolved in ethylacetate (oil phase), ultrasonicated and oil phase is lastly left to evaporate. Reproduced from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

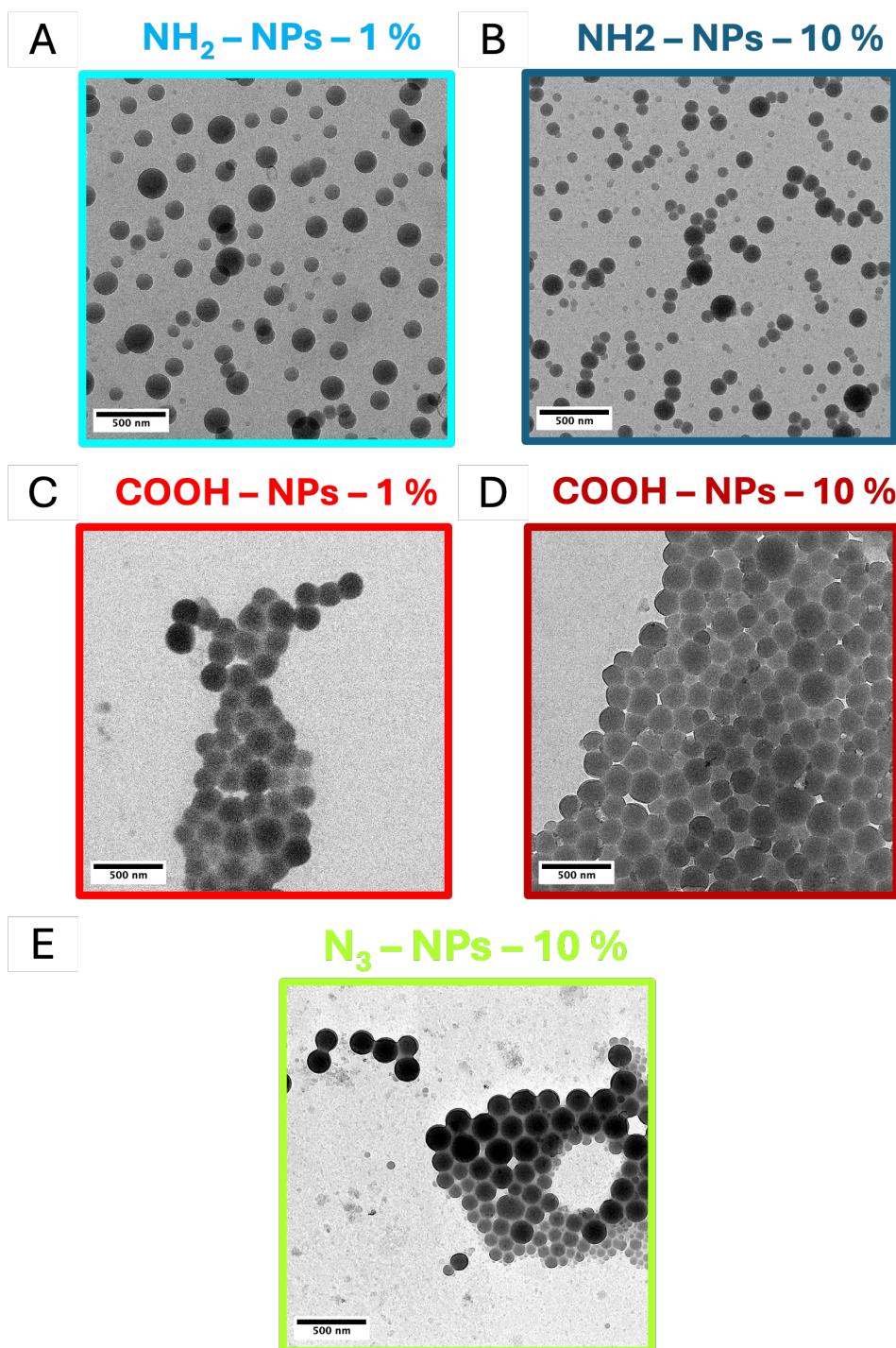


Figure 5.3: TEM characterization. Representative TEM images of NH₂-NPs-1% (A), NH₂-NPs-10% (B), COOH-NPs-1% (C), COOH-NPs-10% (D), N₃-NPs-10% (E). Adapted from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

NPs	FGs %	Polymer (mg)	Lutensol AT50 (wt%)	Dh* (nm)	PDI	ζ (mV)	pH
NH ₂	10	450	0.1	276.9	0.099	38.8	4.3
	1	150	0.4	199	0.111	32.5	4.7
COOH	10	150	0.4	206.7	0.034	-26.1	5.7
	1	150	0.4	206	0.031	-15.4	6.1
N ₃	10	150	0.4	219.6	0.034	-6.78	-

Table 5.1: NPs synthesis details and characterization via DLS, ζ -potential and pH measurements. *Dh: Hydrodynamic diameter measured via DLS. Adapted from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

5.3 Total Functional Group Detection

After NP synthesis a thorough characterization of the FGs was performed. The total amount of FGs directly correlates to the polymer composition as the amount of functional monomers (FMs) in the statistical co-polymer is equivalent to the number of FGs in the NPs. Hence, it is necessary to quantify the amount of functional monomers in the final polymer. To quantify this number, each final and purified polymer was analyzed via ¹H-NMR. In each spectrum the molar ratio between the FM and BzMA was determined and converted into a molar concentration.

For the P(BzMA-co-AEMA), the CH₂ peak of the BzMA ($\delta \approx 4.4$ –5, c in Figure A.5A, A.6, A.7) was compared to the CH₂ peak of the AEMA ($\delta \approx 2.6$ –3.2, g in Figure A.5A, A.6, A.7). Specifically, the AEMA's mol % was calculated as reported in Equation 5.1:

$$\text{AEMA mol \%} = \frac{(\text{CH}_2\text{AEMA}/2)}{(\text{CH}_2\text{AEMA}/2) + (\text{CH}_2\text{BzMA}/2)} \times 100 \quad (5.1)$$

For the P(BzMA-co-MAA), in each spectrum, the aromatic peak of the BzMA ($\delta \approx 6.5$ –7.5, d in Figure A.5 A, A.8, A.9) was compared to the COOH peak of the MAA ($\delta \approx 12.1$ –12.7, g in Figure A.5 A, A.8, A.9). Specifically, the MAA's mol% was calculated as reported in Equation 5.2:

$$\text{MAA mol \%} = \frac{(\text{COOH}_{\text{MAA}}/1)}{(\text{COOH}_{\text{MAA}}/1) + (\text{Aromatic}_{\text{BzMA}}/5)} \times 100 \quad (5.2)$$

For the P(BzMA-co-AzMA), the CH₂ peak of the BzMA ($\delta \approx 4.6$ –4.9, c in Figure A.5 C, A.10) was compared to the CH₂ peak of the AzMA ($\delta \approx 3$ –3.27, g in Figure A.5 A)). Specifically, the AzMA's mol % was calculated as reported in Equation 5.3:

$$\text{AzMA mol \%} = \frac{(\text{CH}_2\text{AzMA}/2)}{(\text{CH}_2\text{AzMA}/2) + (\text{CH}_2\text{BzMA}/2)} \times 100 \quad (5.3)$$

To determine the molar concentration relative to 1 g of system, we have introduced an “average-monomer” (AM) molecular weight (MW). In this sense, the polymer will be composed by a new “average-monomer”. The molecular weight of this “average-monomer” is dictated by the mol% of each component:

$$\text{MW of the "average-monomer"} = \frac{(\text{MW}_{\text{BzMA}} \times \text{BzMA mol \%} + \text{MW}_{\text{FM}} \times \text{FM mol \%})}{100} \quad (5.4)$$

Given the calculated mol% the MW of the AM are: 174 g/mol for P(BzMA-co-MAA) 1 wt%, 163 g/mol for P(BzMA-co-MAA) 10 wt%, 176 g/mol for P(BzMA-co-AEMA) 1 wt%, 175 g/mol for P(BzMA-co-AEMA) 10 wt%, 174 g/mol for P(BzMA-co-AzMA) 10 wt%.

Given the MW of the AM, the moles of FM per gram of AM can be calculated using Equation 5.5:

$$\text{Moles of FM per g of AM} = [\text{FM}]_{\text{mol}} = \frac{\text{FM mol \%}}{\text{MW}_{\text{AM}}} \quad (5.5)$$

Notably, as mentioned above, the FM mol% is equivalent to the FG mol%. This is true given that each FM presents a single FG, either an amino, carboxylic, or azido group. Finally, given a selected concentration of NPs (e.g. 0.1 wt% which corresponds to a solid content (SC) of 1 g/L) the concentration of FGs in solution can be calculated:

$$\text{FGs concentration} = [\text{FGs}] = [\text{FM}]_{\text{mol}} \times \text{SC} \quad (\text{S5}) \quad (5.6)$$

The FGs concentration for each system is reported in the bar plot in 5.4. It can be observed, that all NP systems (NH₂, COOH) present a large difference when comparing the NPs-10% (1641 μM or 452 μM) with the NPs-1% (194 μM, 163 μM). Notably, the calculation steps described above enable the determination of the total number of FGs, i.e. the maximum amount of reactive handles that can be present on the surface of the NPs. However, the method does not determine the location of the FGs in the NPs, thus no distinction can be made between FGs trapped in the core of the NPs and those exposed to the aqueous solvent.

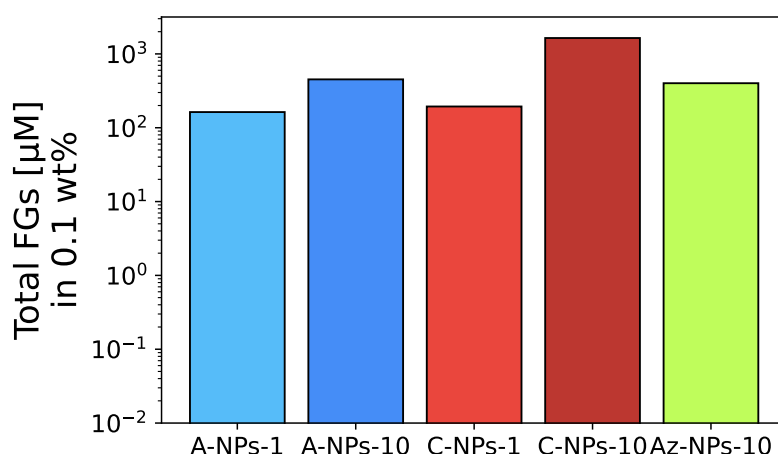


Figure 5.4: Total FGs. Quantification of total FGs via ^1H -NMR analysis in a 0.1 wt% solution of NPs. Adapted from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

5.4 Visible Functional Group Detection

Next, the visible FGs quantification can be performed. Visible FGs refers to the FGs located on the surface of the NPs. These affect the NPs physicochemical properties such as the ζ -potential. Hence ζ -potential measurements were performed using particle charge detection (PCD). This technique relies on the charged state of FGs, which allows for electrostatic interactions with a counter charged electrolyte. Consequently, the NPs must be in a charged state for accurate analysis. To ensure this, the solution's pH was adjusted to pH 2.5 for NH_2 -NPs and pH 8 for COOH -NPs. Unfortunately, N_3 -NPs could not be analyzed via PCD due to the impossibility of protonating this FG.

The NPs were titrated with oppositely charged polyelectrolytes: sodium polyethylene sulfonate (PES-Na) for NH_2 -NPs and poly(diallyldimethylammonium chloride) (Poly DAD-MAC) for COOH -NPs. The results (Figure 5.5) revealed a disparity between the quantifications obtained via PCD and NMR. In all systems, the visible FGs were consistently fewer than the total FGs. This expected discrepancy arises from the inherent dissimilarities between the two analysis methods. While PCD focuses solely on the NPs surface, NMR studies the entire system, thereby unable to distinguish between FGs trapped within the NP core and those present on the surface.

Moreover, the data highlights that the visible NH_2 concentration (182 and 24 μM) is closer to the total concentration compared to COOH -NPs. This observation can be attributed to the protonated state of the NH_2 groups in the P(BzMA-co-AEMA) starting material. The pronounced hydrophilicity of the charged NH_2 groups in the polymer induces a driving force for FGs to migrate to the NP surface during synthesis. Thus, allowing the PCD to detect higher NH_2 concentrations. Conversely, the carboxylic groups in the P(BzMA-co-MAA) polymer are not ionized, leading to reduced hydrophilicity and consequent entrapment of FGs within the particle core. Consequently, lower concentrations were detected for COOH -NPs: 53 and 24 μM .

Furthermore, an additional observation can be made. Unlike NH_2 -NPs, COOH -NPs-10% and COOH -NPs-1% exhibit no ten-fold decrease. To interpret this result, it is essential to consider that the PCD technique assumes accessibility to all FGs²¹⁷ and the possibility for

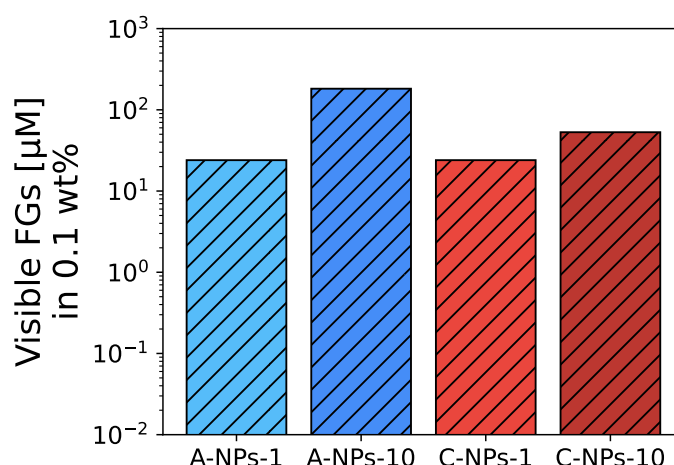


Figure 5.5: Visible FGs. Quantification of visible FGs via particle charge detection. Adapted from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

simplex formation between the polyelectrolyte and all FGs on the NP surface²¹⁸. However, some FGs may adopt sterically less available conformations, resulting in a trapped conformation that hinders accurate PCD interpretation. Such trapping is representative of NPs deriving from statistical copolymers that form a “shell-like” surface layer.²¹⁹ This shell, by lacking a single FG-layer conformation, traps some of the FGs, hampering proper PCD’s interaction. Consequently, the results may be attributed to structural differences in the functional monomers: AEMA possesses a short linker, opposed to MAA. This chemical disparity may cause the COOH groups to be closer to the NP surface, leading to constraints and limited accessibility, thereby hampering detection. Thus, different COOH concentration on the NP surface might be present but cannot be readily detected due to the groups conformation.

5.5 Accessible Functional Group Detection

The subsequent focus of our study is now shifted towards quantification of the accessible FGs via chemical reactions with small ligands. To accomplish this, we utilized model molecules (~ 400 g/mol) for covalent conjugation to the reactive handles present on the NPs. For the model molecules, fluorescent probes were selected: either free acid CY3, amino-modified bodipy (BDP FL amine), or alkyne-modified Rhodamine B. The conjugation of the first two dyes employed EDC/NHS chemistry to NH_2 -NPs or COOH -NPs, respectively. On the other hand, Rhodamine was conjugated to N_3 -NPs via copper-catalyzed click reaction.

Typical strategies for quantifying labeled particles involve either flow cytometry or analysis of the supernatant solution obtained during cleaning steps. However, the size of our NPs falls below the detection limit of flow cytometry. Alternatively, the supernatant assay could be utilized, yet it introduces errors associated with indirect measurement approaches, such as imprecision in the collected volume due to repetitive pipetting.

Therefore, in our study, we quantified the conjugated dye directly on the system under investigation. To do so, purified fluorescently labeled particles were dried and dissolved in an organic solvent to record the fluorescence spectrum. Such spectrum was then compared to a calibration curve of the dye in the same solvent. The amount of dye conjugated to the

polymer (polymer-dye system) was extrapolated from this calibration curve.

Solubilizing the particle-dye complex i) facilitates increased mobility of the polymer-dye compared to the particle-dye, ii) reduces environmental and solvent effects, iii) and eliminates NP scattering. All these parameters can interfere with the final fluorescent signal. However, it is crucial to ensure that the fluorescence spectra obtained are comparable to those of the free dye to confirm the absence of spectral shape differences (Figure 5.6). Such difference could indicate changes in dye properties thus hampering the correlation with the calibration curve. In this context, we opted for BDP FL Amine dye over the cheaper and more commonly used fluorescein due to significant spectral differences between fluorescein and the polymer-fluorescein system (Figure 5.7)^{220–222}.

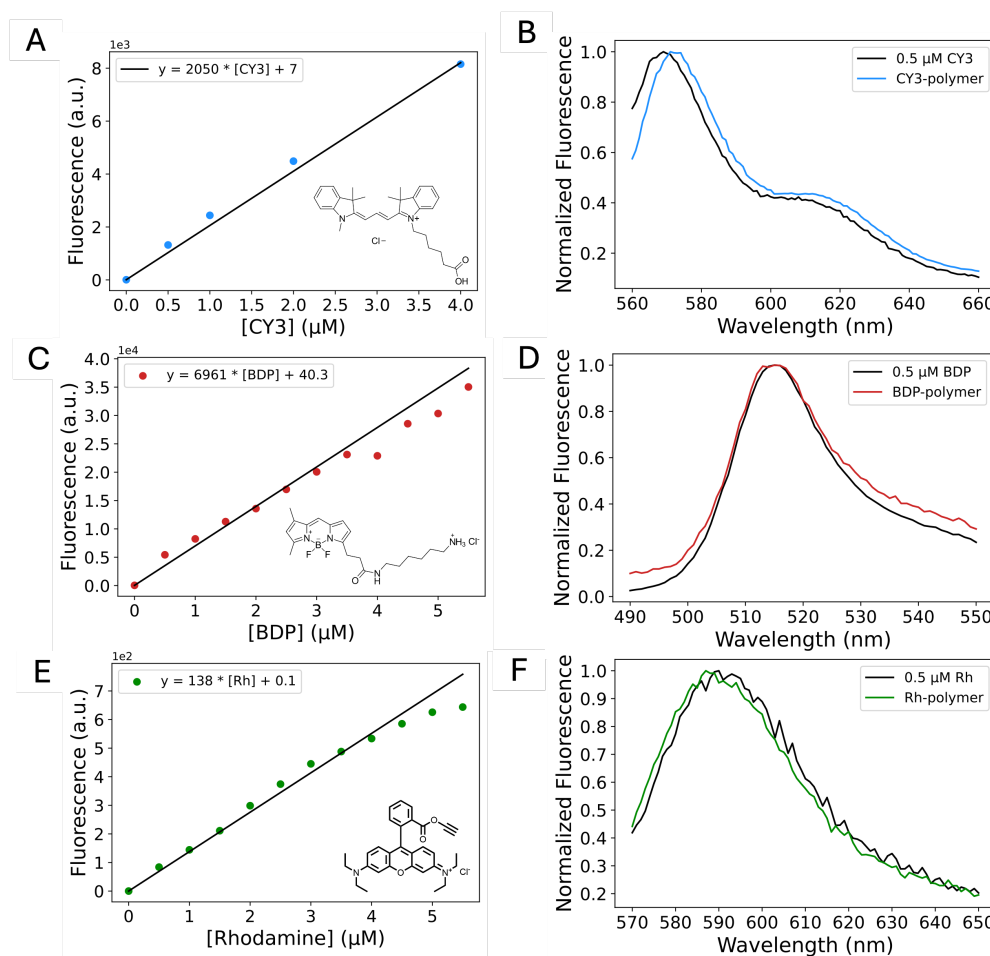


Figure 5.6: Calibration curves. Calibration curve of CY3 in DMSO:THF (1:1) (A), BDP (C) and Rh (E) in DMSO. The inset shows the chemical structure of CY3, BDP and Rh. Comparison between the normalized fluorescence spectra of CY3 and CY3-Polymer (B), BDP and BDP-Polymer (D), and Rh and Rh-Polymer (F). Adapted from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

The results, reported in Figure 5.8, were obtained by conjugating to a 0.1 wt% solution of NPs, varying concentrations of dye to generate titration curves. In all NP systems, a plateau was reached within the margin of error. The NH_2 -NPs and N_3 -NPs exhibited clearer plateaus due to a pronounced increase in bound dye with increasing added dye concentration.

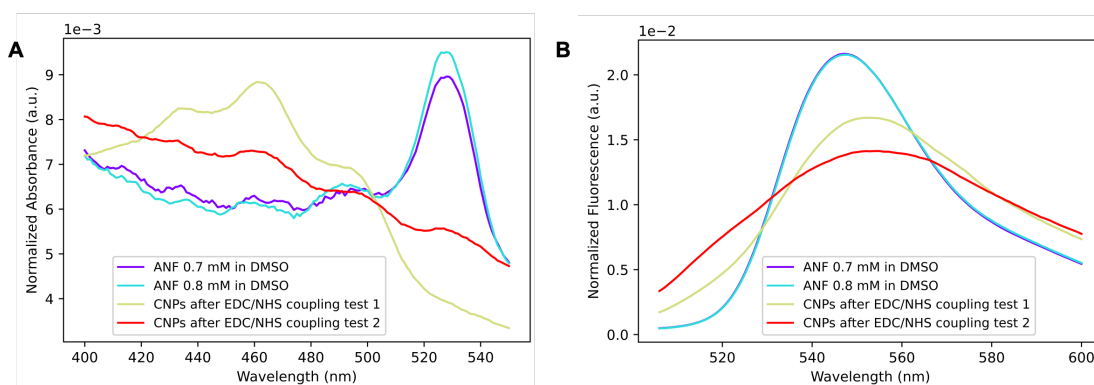


Figure 5.7: ANF spectral differences. Absorbance (A) and fluorescence (B) spectra of ANF and ANF-polymer system showing the large difference, which hamper the dye quantification. Reproduced from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

Conversely, the curve for COOH-NPs was noisier and required higher dye concentrations (a minimum of $150\ \mu\text{M}$ for COOH-NPs versus a maximum of $110\ \mu\text{M}$ for NH_2 -NPs or even $30\ \mu\text{M}$ for N_3 -NPs) to saturate. This noise may be attributed to the small bound concentrations (less than $0.1\ \mu\text{M}$) detected in these systems, which would be challenging to measure with a supernatant assay due to the minimal difference in concentration between the added dye (e.g., $200\ \mu\text{M}$) and the unbound dye (e.g., $199.8\ \mu\text{M}$), potentially masked by human errors (e.g., small pipetting mistakes).

The final plateaus were utilized to determine the total number of accessible FGs (Figure 5.9). As anticipated, the accessible FGs showed an increase when comparing NPs with 1% and 10% functional monomers. COOH-NPs exhibited concentrations of $0.07\ \mu\text{M}$ and $0.22\ \mu\text{M}$ COOH, while NH_2 -NPs showed concentrations of $0.67\ \mu\text{M}$ and $16.82\ \mu\text{M}$ NH_2 . Lastly, N_3 -NPs displayed a value of and $0.69\ \mu\text{M}$.

The higher concentration for NH_2 -NPs may be associated with the presence of more visible FGs, as measured with PCD. Alternatively it can be indicative of a simpler conjugation reaction. Indeed, the carboxylic group on CY3 dye, being a small and mobile molecule, can readily interact with coupling reagents (EDC/NHS) and be activated for amide bond formation. Conversely, carboxylic groups on COOH-NPs, constrained to the NP surface, may experience mobility hindrance and steric hindrance, limiting their activation and resulting in reduced conjugation product. Notably, the N_3 -NPs require the least amount of dye to reach the plateau. This is attributed to the fact that click chemistry has a higher reaction efficiency. Contrarily, EDC/NHS chemistry in water is known to present low yield due to the easily hydrolysable NHS group.²²³

Fluorescamine assay to quantify NH_2 groups

In the case of NH_2 -NPs, often the fluorescamine assay is employed to measure amino groups on various types of NPs, including silica NP,^{80,199} liposomes²⁰⁰, and protein nanocapsules.⁶² Hence, this rapid assay was tested with the NPs synthesized in this work. However, adjustments were needed due to a large increase in fluorescence for the fluorescamine-NP system, compared to the calibration curve (Figure A.12 A). Such increase could be due to the polymer in the NP becoming part of the chromophore¹¹⁹ and altering the fluorescent prop-

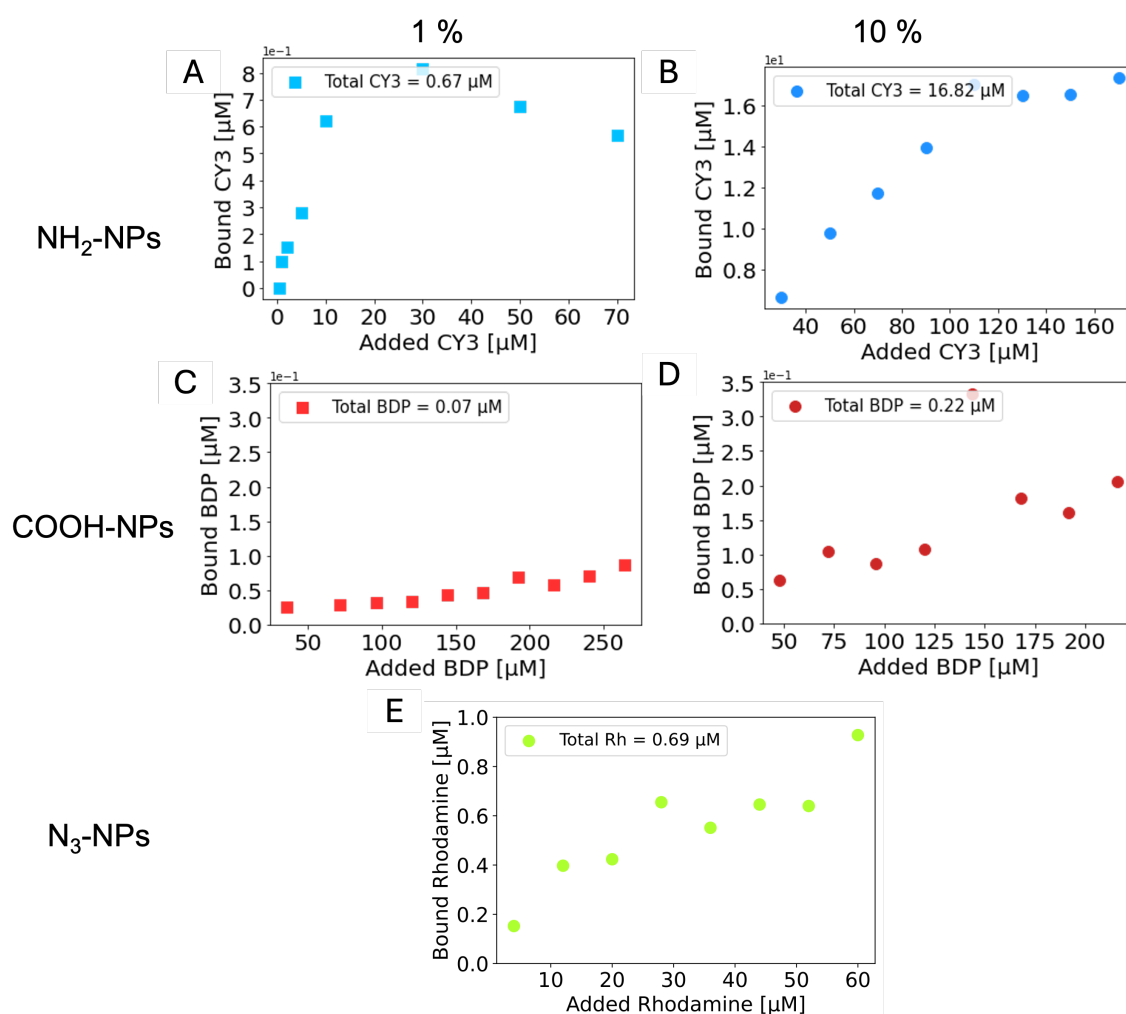


Figure 5.8: Titration curves. Fluorescence titration curves obtained via conjugation of CY3 on NH₂-NPs-1% (A), NH₂-NPs-10%, of BDP on COOH-NPs-1% (D) and COOH-NPs 10% (E), and of Rhodamine on N₃-NPs-10% (E). Adapted from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

erties of the dye. Therefore, the NPs were titrated by sequential addition of fluorescamine until the fluorescent signal reached a plateau (Figure A.12 B,C).

The results detected an amino concentration of 660 μM for the NPs-10% and 50 μM for the NPs-1% (Figure 5.10) which are higher numbers compared to the PCD and CY3 quantification results. Such high numbers can be interpreted in two ways. First of all, the use of DMSO as solvent for the fluorescamine can affect the measurement. While not swelling the NPs (Figure A.13), it can modify the outer conformation of the NPs. Thus it can favor the detection of previously hidden amino groups. Additionally, fluorescamine conjugation has a high conversion yet it is never 100 % (80-95%²²⁴ based on theoretical calculations). Hence, in each titration step there is a 5-20% error on the amount of conjugated dye. Thus, summing each error can lead to overestimation of conjugated dye.

Therefore, for the PBzMA based NPs presented in this work, the fluorescamine assay is not suitable for accessible amino quantification. Yet, it can be used for a quick (30 min)

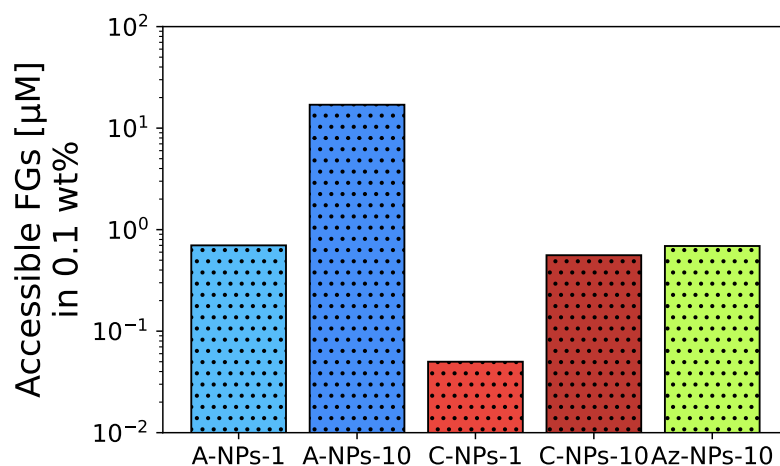


Figure 5.9: Accessible FGs. Quantification of the accessible FGs via conjugation of NPs with small dyes. Adapted from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

overview of the NH_2 -NPs system.

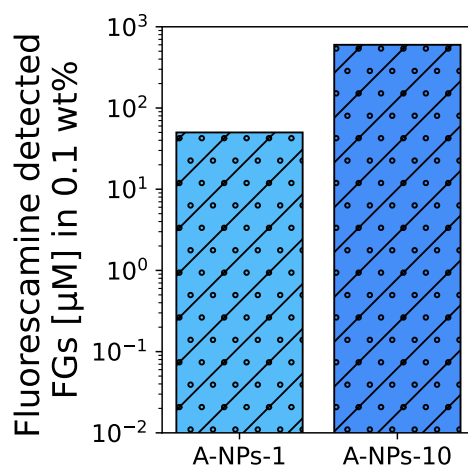


Figure 5.10: Fluorescamine quantification. Quantification of amino FGs via fluorescamine titration. Adapted from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

5.6 Albumin Conjugation to COOH-Nanoparticles

To investigate the effect of bulky molecules on the NPs coverage, human serum albumin (HSA, 66 kg/mol) was conjugated to the COOH-NPs and the bound amount was investigated. Protein quantification is often performed using the Pirece, Brad-ford or BCA assays. However, the quantification can be strongly influenced and hampered by interfering substances in solution such as PBS and MES buffers, thus incompatible with detection on NPs (Figure A.14). Therefore, in this work the previously described dye quantification protocol was adapted for the protein detection. The HSA was fluorescently labeled with Rhodamine B to be treated as a bulky dye molecule and conjugated to the COOH-NPs. Unfortunately, the polymer-HSA-Rhodamine spectra in DMSO showed a 11 nm red-shift (Figure A.15) com-

pared to the reference (HSA-Rhodamine in DMSO), thus the protein quantification based on the calibration curve was not possible. Nevertheless, the fluorescence intensity can be utilized to qualitatively compare the NPs-1% and NPs-10%. For the NPs-1% the fluorescence was approximately 2 time lower than the one in the NPs-10% (Figure 5.11). The value is consistent with the difference in conjugated dye and in the PCD results.

The interesting aspect is that an increase in probe size (from 425 g/mol to 66000 g/mol) strongly modifies the steric hindrances contribution to the conjugation, thus, the large probe (e.g. protein) should have less space for interaction compared to the small probe (e.g. dye). Given two NPs with the same morphology and size, as for the systems herein presented, the surface area should be fully covered thus conjugating approximately 4000 proteins and obtaining similar fluorescence intensity. However, based on the dye quantification results, on the NPs-10% and NPs-1% respectively 720 and 220 FGs per particle were measured. These smaller numbers prevent the NPs from being completely covered by HSA and can explain the detected difference in fluorescence intensity. The results emphasize the importance of quantifying the available reactive handles, whereas the bulkiness of the probe is of secondary importance. Therefore, thoroughly investigating the morphology and characteristics of the unmodified NPs is crucial before conjugating the larger active molecules to them.

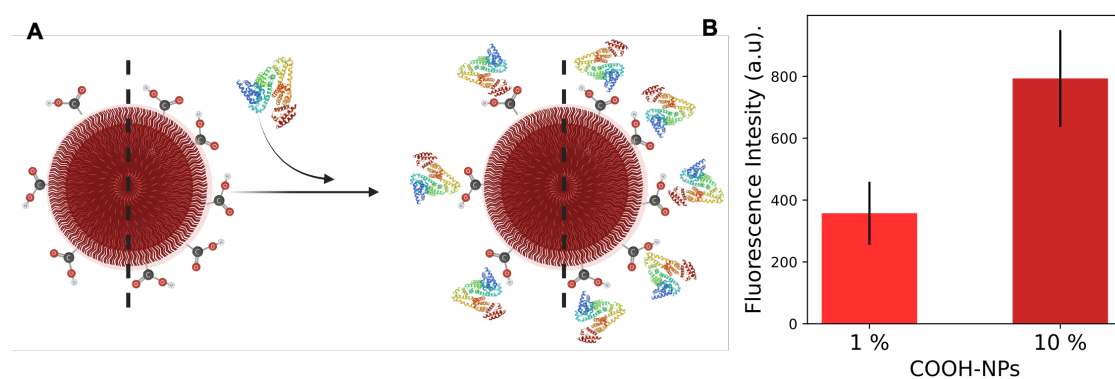


Figure 5.11: Large molecule conjugation. Schematic representation of the HSA (PDB ID 1AO6)²²⁵ conjugation on the COOH-NPs (A). Fluorescence intensity at the plateau for both the NPs-1% and NPs-10% NPs (B). Reproduced from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

5.7 DNA-PAINT Imaging

So far, the NPs have been characterized using ensemble quantification methods. Our attention is now turned to detecting differences between individual particles. This is necessary because the synthesis strategy used in this work may lead to considerable heterogeneity in both single polymer chains and the final NP surface composition. To identify and quantify this heterogeneity, we employed DNA-PAINT imaging.

To characterize the particles via super resolution microscopy, adaptations were made during the particle synthesis. During the miniemulsion-solvent evaporation process, the oil phase (ethyl acetate) was mixed with Bodipy 523/535. The hydrophobic nature of the dye led to its entrapment in the NP core, thereby fluorescently labeling the NPs for easy tracking during microscopy imaging.

Subsequently, the NPs surfaces needed to present an azide reactive handle for DNA strand conjugation. While the N_3 -NPs required no modifications, the NH_2 -NPs and $COOH$ -NPs underwent chemical modification. The amino and carboxylic FGs were utilized to conjugate a short linker; (NHS-PEG₄-N₃), and 3-Azido-1-Propylamine (NH_2 -CH₂CH₂-N₃), respectively for the NH_2 -NPs and $COOH$ -NPs. For NH_2 -NPs, the conjugation was a single-step process. Contrarily, for $COOH$ -NPs, it involved an initial activation step of $COOH$ groups followed by conjugation with the amine linker, using the same EDC/NHS protocol used to conjugate the BDP dye to the $COOH$ -NPs.

The docking DNA, which needs to be conjugated to the NPs, presents a DBCO group at the 5' extremity, enabling copper-free click chemistry for DNA-NP conjugation. The DNA strand had the following sequence: *DBCON-TT TCT CCT AAT TCA CTC ATC TCA A*. It comprised 24 bases, with the last 8 bases required for DNA-PAINT imaging as they are complementary to the imager strand (*ATTO-655-TT GAG ATG*), inducing blinking. The initial 16 bases spaced out the DNA and facilitated interaction with the imager.

While successful for N_3 -NPs and $COOH$ -NPs, this functionalization step failed for NH_2 -NPs. The strong positive charge of NH_2 -NPs destabilized this system leading to NPs aggregation. This aggregation was induced by the high salt concentrations required for the clicking step. The charges that increase the ionic strength are attributed to both DNA and salt-induced increase in ionic strength.

Subsequently, DNA-PAINT imaging was exclusively performed on N_3 -NPs and $COOH$ -NPs with either 1% or 10% FGs. Rendered images are reported in Figure 5.12. The green signal rises from the NP-core locations, while the red signal results from detected blinking events (imager-docking interaction) on NP surfaces. The DNA-PAINT image was initially processed by clustering the NP signals (green) into a distinct cluster. Subsequently, the blinking events (red) around each cluster were quantified. Hence, the image analysis reports on 1) the number of NPs per field of view (i.e. number of clusters) and 2) the number of blinking events per cluster. The data can be plotted as a histogram (Figure 5.13) to highlight particle-to-particle differences.

The DNA-PAINT results highlight differences in number of events: larger numbers indicate greater DNA densities due to higher probability of interaction with the imager strand. Thus, the average number of events mirrors accessible FG quantifications (Figure 5.9) where the number decrease from $COOH$ -NPs-10% and lastly $COOH$ -NPs-1%. However, the disparity in accessible groups detected via DNA-PAINT was more pronounced than in the quantification results, likely attributed to the complexities in the DNA-functionalization steps. While N_3 -NPs required a single reaction, $COOH$ -NPs necessitated two steps for azide group introduction, plus the click reaction. Every reaction step can lead to a decrease in the final amount of DNA.

Moreover, significant differences were observed in number of detected NPs between N_3 -NPs (~ 300 clusters) and $COOH$ -NPs (~ 60 clusters) despite all samples being deposited with the same particle concentration (0.25 mg/mL), this discrepancy raised questions. We hypothesize that coverslips preparation might contribute to such difference. To deposit NPs, the coverlip were passivated with a Poly(L-Lysine)-Poly(Ethylenglycol) (PLL-PEG). Such strategy utilizes PEG's antifouling properties to reduce unspecific binding of the DNA imager, thus improving signal to noise ratio (SNR) and analysis.²²⁶ The positive charges of PLL in the coverslips preparation could promote deposition of

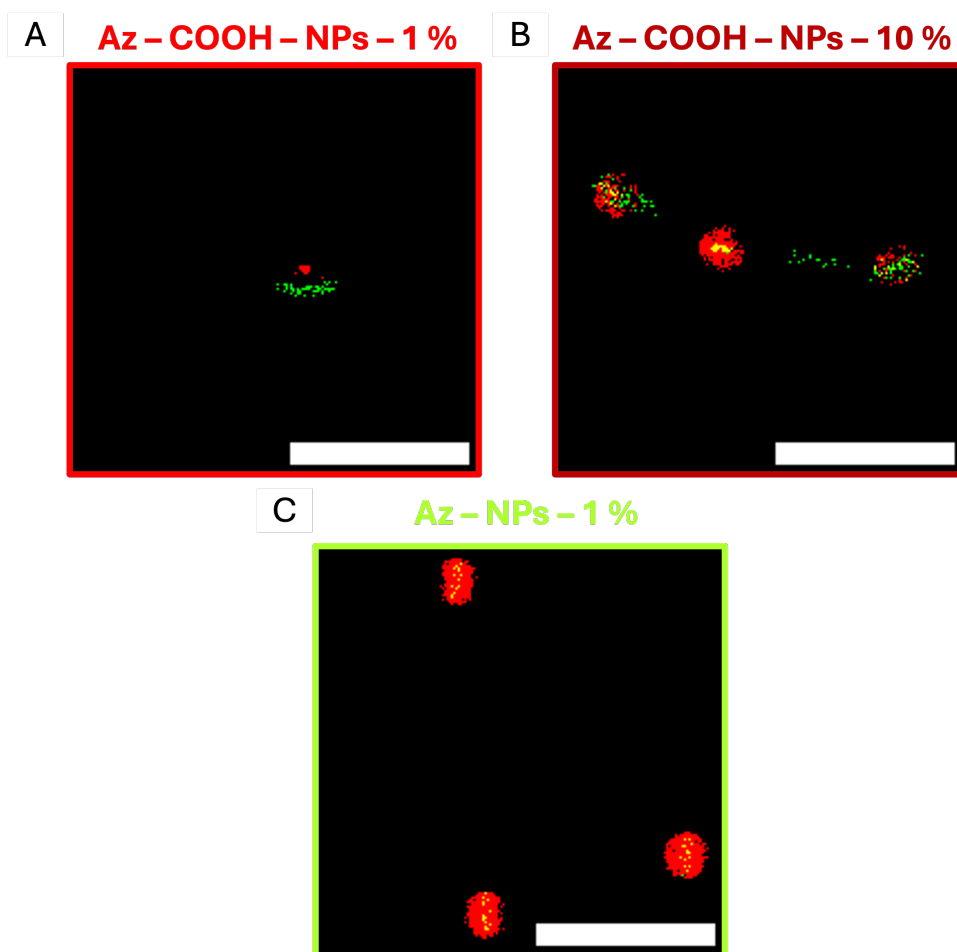


Figure 5.12: DNA-PAINT imaging. Representative DNA-PAINT rendered images with a close up of detected NPs (green) with the correspondent imager interactions (red). Samples imaged are: COOH-NPs-1% (A), COOH-NPs-10% (B), N_3 -NPs-10% (C).

negatively charged NPs. Consequently, increased DNA density in N_3 -NPs promotes deposition, whereas COOH-NPs, with lower DNA amounts, primarily experience the antifouling effect of PEG.

Due to the low NP count in COOH-NPs, statistical analysis for particle-to-particle differences was unfeasible. Conversely, such analysis was viable for N_3 -NPs. N_3 -NPs-10% exhibited a Gaussian distribution. Contrary to expectations, the event distribution in both systems did not exhibit significant broadness. Therefore, the distribution of DNA content on the NP-surface appears to mirror the intrinsic polydispersity observed through DLS. Consequently, the number of DNA molecules per particle seems to correlate not only with the FMs in the initial co-polymer but also with NP size, resulting in larger particles exhibiting higher DNA densities.

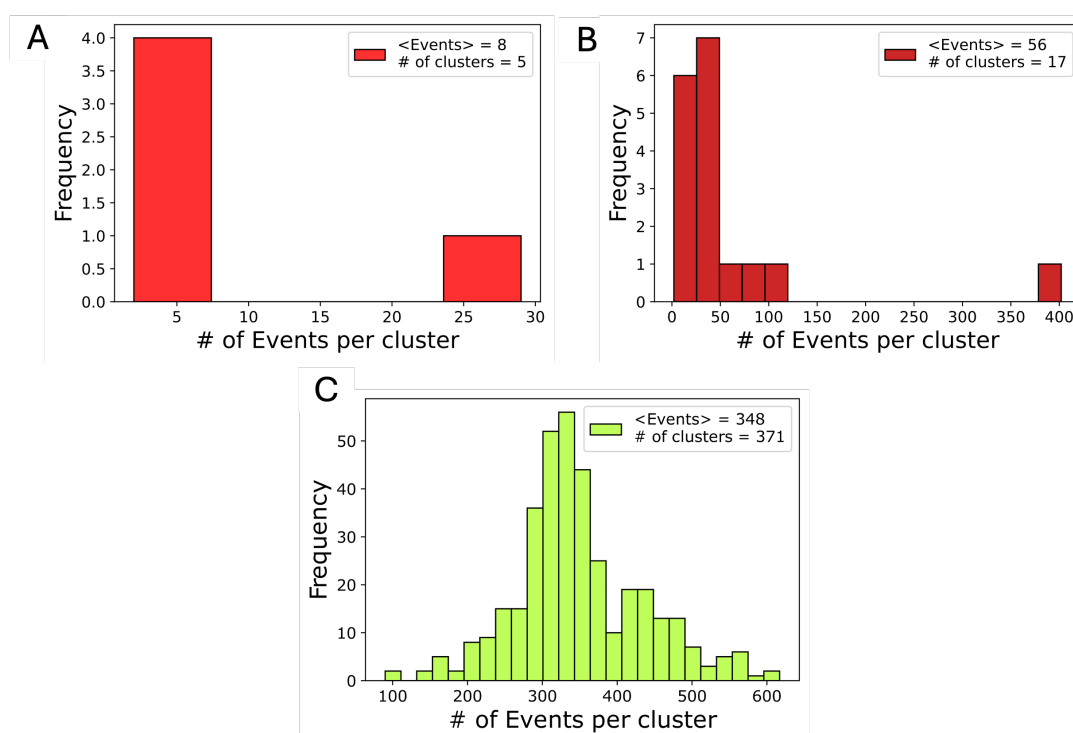


Figure 5.13: DNA-PAINT events. Histograms of the number of detected localizations around the NPs (Clusters) for the COOH-NPs-1% (A), COOH-NPs-10% (B), N₃-NPs-10% (C).

5.8 Summary and Outlook

This study provides a comprehensive analysis of total, visible, and accessible FGs on NP surfaces. Statistical copolymers with 1% or 10% AEMA, MAA, or AzMA functional monomers were utilized in a miniemulsion-solvent evaporation method to fabricate NPs with NH₂, COOH, or N₃ FGs. Total FGs were quantified via NMR, visible FGs via PCD, and accessible FGs via fluorescence. The accessible quantification employed a direct measurement approach to mitigate errors associated with indirect quantification studies. Therefore, fluorescence was not measured in the supernatant, but measured after drying and subsequently dissolving the NPs in an organic solvent. Conjugation methodologies based on EDC/NHS or click chemistry were employed, offering versatile application to various systems, including protein-NP conjugation. This method is adaptable to any dissoluble NPs (e.g., polymer NPs, liposomes) with diverse FGs, facilitating comprehensive characterization of both unmodified and modified NPs. Results indicated a sequential decrease in FGs from total to visible and accessible, emphasizing the significance of tuning reactive handle numbers and locations.

Moreover, HSA conjugation onto NPs explored the impact of molecule size on reactive handle accessibility. The investigation revealed that, in the herein presented systems, the amount of available reactive handle prevailed over the bulkiness effect. Hence, the use of a larger molecule in the conjugation to NPs did not limit the amount of conjugated HSA.

Lastly, DNA-PAINT experiments were conducted to discern particle-to-particle differences. Despite successful functionalization of N₃-NPs and COOH-NPs, NH₂-NPs exhibited destabilization during the clicking step due to their strong positive charge. The DNA-PAINT results revealed differences in DNA density on the NP surface, yet

the particle-to-particle analysis could only be performed on N₃-NPs. The analysis shows heterogeneity comparable to NPs size distribution. Hence, larger NPs presented higher DNA densities.

5.9 Material and Methods

Materials

Benzyl methacrylate (BzMA, Sigma-Aldrich, 96%), methyl methacrylate (MAA, Merck, 99%), 2-amino-ethyl methacrylate hydrochloride (AEMA, Sigma-Aldrich, 90%), azobisisobutyronitrile (AIBN), Lutensol AT50 (poly(ethylene glycol)-hexadecyl ether), dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), ethanol (EtOH), methanol (MeOH), acetone, ethyl acetate, dimethylformamide (DMF), N-ethyl-N'- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC-HCl), N-hydroxy sulfosuccinimide (sulfo-NHS), poly (diallyl-dimethylammonium chloride) (Poly DADMAC), sodium polyethylene sulfonate (PES-Na), human serum albumin (HAS, Sigma-Aldrich). Fluorescamine, 6-amino-fluoresceine (ANF, Sigma-Aldrich), amino derivative of boron dipyrromethene FL dye (BDP, Lumiprobe), free Cyanine3 carboxylic acid (CY3, Lumiprobe), Rhodamine B isocyanate (Sigma-Aldrich). BzMA and MAA were purified using an aluminum oxide column prior to use. Alkyne-functionalized rhodamine B and 3-azidopropyl methacrylate (AzMA) were kindly synthesized by Angelika Manhart following respectively protocols reported in reference¹¹² and²²⁷. DNA strands were purchased by Integrated DNA Technology (IDT). The docking strand was modified on the 5' with dibenzocyclooctyne (DBCO) unit (sequence: /5DBCON/TT TCT CCT AAT TCA CTC ATC TCA A). The imager strand was modified on the 5' with ATTO 655 (sequence: /5ATTO655N/ TTGAGATG).

Polymer Synthesis

Polymers were synthesized via free radical polymerization. The amino functionalized polymers were obtained by mixing AEMA (1 or 10 wt%) in MeOH (1 mL) with BzMA (99 or 90 wt%) in acetone (4 mL) in a 15 mL glass vial with AIBN (1 wt% of the monomers). The carboxylic and azide functionalized polymers were prepared by adding MAA or AzMA (1 or 10 wt%) and BzMA (99 or 90 wt%) in a 15 mL glass vial with DMSO (4 mL) and AIBN (1 wt% of the monomers). All samples were degassed with nitrogen flow for 10 min under vigorous stirring and the reactions were activated by heating to 60 °C in oil bath for 24 h. Subsequently the product was analyzed via ¹H-NMR spectroscopy and purified via precipitation in MeOH (P(BzMa-co-MAA) and P(BzMA-co-AzMA)) or EtOH (P(BzMa-co-AEMA)), dried at 40 °C in a vacuum oven, the steps were repeated three times. The final polymer was analyzed via ¹H-NMR spectroscopy (Figure A.5) and via GPC (Figure A.11) using DMF as eluent and polystyrene as reference.

Nanoparticle Synthesis and Characterization

NH₂-NPs, COOH-NPs, and N₃-NPs were synthesized based on previously reported protocol.¹¹¹ Briefly, the polymer was dissolved in 3 mL of EA and mixed with 6 mL of 0.1 wt% Lutensol AT50. The solution was stirred with a T25 Ultra-Turrax for 1 min at 5 krpm and

ultrasonicated with a Branson Ultrasonics Sonifier for 3 min (pulsed 20 s on, 10 s off) with a $\frac{1}{2}$ ' tip at 70% amplitude. The miniemulsion was left to evaporate for 16 h at 40 °C at 1 krpm on a heat plate (Figure 5.2), the obtained NPs were centrifuged for 20 min at 2 krpm and the supernatant was collected. The solid content of the NPs was determined gravimetrically by drying 100 μ L of the particle solution. NPs size (Dh) was measured by means of dynamic light scattering (DLS) at a fixed angle of 175° using a Malvern Zeta Nanosizer, (Malvern Instruments GmbH, Herrenberg, Germany). The same instrument was used to measure the ζ -potential by diluting 5 μ L of NPs with 2 mL of a 0.1 M KCl solution. TEM (transmission electron microscopy) samples were prepared by drop casting 5 μ L of diluted NPs solution onto a carbon coated copper grid, back-blotting with Whatman paper excess solution and lastly depositing a 2 nm layer of carbon. Grids were imaged using a FEI Tecnai (120 kV) transmission electron microscope with a 4k x 4k Gatan CCD camera at low electron dose.

Particle Charge Detection

Particle Charge Detection was performed as follows. 10 mL of a 0.1 wt% solution of the NPs samples was prepared in MilliQ water. The solution was then introduced in the PCD 02 (Mütek GmbH, Herrsching, Germany) and titrated using 702 SM Titrino automated titrator (Metrohm AG, Herisau, Switzerland) with 1 mM solution of PDADMAC, each sample was analyzed in triplicates. As a result, the volume of titrant necessary to reach the point of zero charge of the sample was detected ($V_{Titrant}$) and used to calculate a molar concentration of FGs (C_{FGs}) in solution as follows (Equation 5.7):

$$C_{FGs} = \frac{V_{titrant} * C_{titrant} * Wt\%}{SC} \quad (5.7)$$

where $C_{titrant}$ is the titrant's molarity, SC is the solid content of the particles in solution and $wt\%$ is the samples density (1 mg/mL, 0.1 wt%).²²⁸

EDC-NHS Chemistry on COOH-Nanoparticles

COOH-NPs were conjugated with BDP, HSA or 3-axido-1-propylamine via a two-step reaction. 0.1 wt% solution of COOH-NPs was activated using EDC-HCl (14.4 mM) and sulfo-NHS (14.4 mM) in 200 μ L 10 mM PBS pH 5.4 for 10 min. Subsequently the NPs were centrifuged 10 min at 4 °C with a 15.5 krcf, the supernatant was discarded and the pellet was quickly redissolved in 10 mM PBS at pH 8 with the desired concentration of amino groups. Finally, the samples were left to react for 2.5 h at room temperature after which the product was isolated by repeated centrifugation (10 min at 4°C with a 15.5 krcf for 4 times) and resuspension in 200 μ L of a 0.1 wt% Lutensol AT50 solution. On the last purification step, the pellet was left to dry at 40 °C and dissolved in 200 μ L DMSO. The fluorescent spectrum was collected with the Infinite M1000 plate reader (Tecan, Männedorf, Switzerland) ($\lambda_{excitation} = 430$ nm, $\lambda_{emission} = 490 - 550$ nm). Schematic illustration and details can be found in the Figure 5.14.

The EDC/NHS conjugation required an initial screening for optimal buffer conditions and reaction times (Figure 5.15 A) resulting in the need for a two-step reaction (carboxylic activation at pH 5 and amino addition at pH 8) and a 2.5 h reaction time. Additionally, the proper EDC and NHS concentrations were investigated (Figure 5.15 B), finding that a 14.4 mM concentration was optimal to saturate the curve at lower dye concentrations. More in

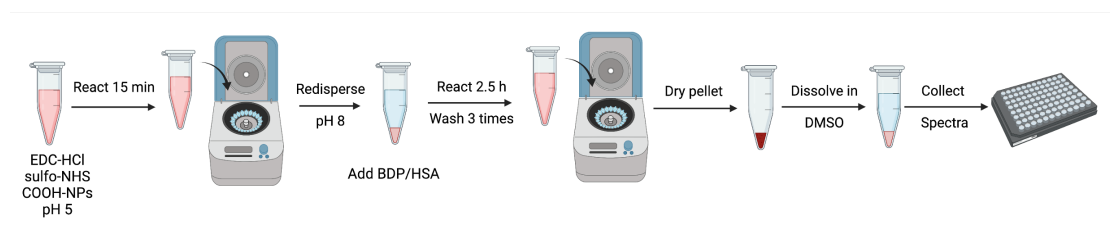


Figure 5.14: *EDC/NHS on the COOH-NPs* Schematic representation of the EDC/NHS protocol on the COOH-NPs.

detail, in this work samples were prepared as follows: first the COOH-NPs were diluted to a 0.11 wt% solution in a 0.1 wt% solution of Lutensol AT50 in 10 mM PBS at (pH 5) for a final volume of 180 μL . Then EDC-HCl and sulfo-NHS were collected from their storage location (-20°C) and left to equilibrate at room temperature for 15 min. The chemicals were quickly weighed to obtain a large excess (14.4 mM) and dissolved with the COOH-NPs solution. To reduce hydrolysis, which would hamper the amide bond formation, the particles were left to react for only 10 min and then centrifuged for 10 min at 15.5 krcf at 4°C . The pellet was redispersed in 200 μL of 10 mM pH 8 PBS buffer with the proper amine (dye or protein) concentration. At this pH NHS hydrolyzes within 2.5 h thus the procedure has no need for longer reaction times.

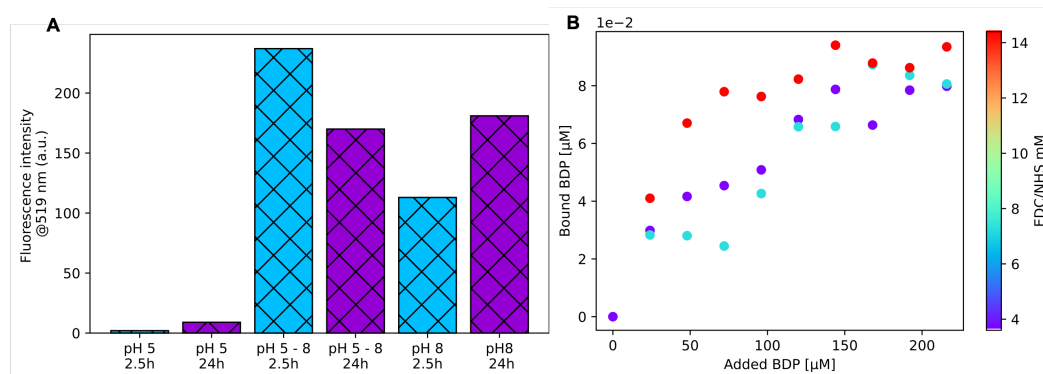


Figure 5.15: *EDC/NHS chemistry optimization.* BDP fluorescence signal after EDC/NHS conjugation, where time (2.5 h in blues and 24 h in purple) and buffer conditions were investigated (A). The COOH-NPs-10% were reacted with BDP in different experimental conditions, the final dried polymer was dissolved in DMSO and the fluorescence intensity is reported in this plot. BDP conjugation to COOH-NPs-10% with different EDC and NHS concentrations to optimize dye conjugation conditions (B).

EDC-NHS Chemistry on NH_2 -Nanoparticles

NH_2 -NPs were covalently conjugated via EDC/NHS chemistry similarly to the above described protocol. EDC-HCl (1.4 mM) and sulfo-NHS (1.4 mM) were dissolved in a final volume of 20 μL of a 10 mM PBS pH 5.4 solution and mixed with various concentration of CY3 or HSA. The sample was mixed on a moving plate for 10 min. Subsequently 180 μL of a 0.11 wt% solution of the NH_2 -NPs in 10 mM PBS at pH 8 were added and the reaction was carried out at room temperature for 2.5 h. The product was isolated by repeated

centrifugation (10 min at 4°C with a 15.5 krcf for 4 times) and resuspension in 200 μL of a 0.1 wt% Lutensol AT50 solution. On the last purification step, the pallet was left to dry and dissolved in a 1:1 DMSO:THF solution to collect the fluo-rescent spectrum ($\lambda_{\text{excitation}} = 550 \text{ nm}$, $\lambda_{\text{emission}} = 570\text{-}660 \text{ nm}$). Schematic illustration and details can be found in Figure 5.16.

The EDC/NHS conjugation on NH_2 -NPs was slightly modified compared to the COOH -NPs. First the carboxylic dye (CY3) in DMSO or the protein (HSA) was added to each of the eppendorf and diluted, to a final volume of 10 μL , to the proper concentration using 10 mM PBS (pH 5). Then EDC and NHS (1.4 mM) were weighed and dissolved in the same buffer. The conjugating reagent solution (10 μL) is then added to the dye and the samples were left to react for 10 min. In the mean time a 0.11 wt% solution of the NH_2 -NPs was prepared in a 10 mM PBS pH 8 buffer solution. Lastly, 180 μL of NH_2 -NPs were added to each eppendorf and were left to react for 2.5 h.

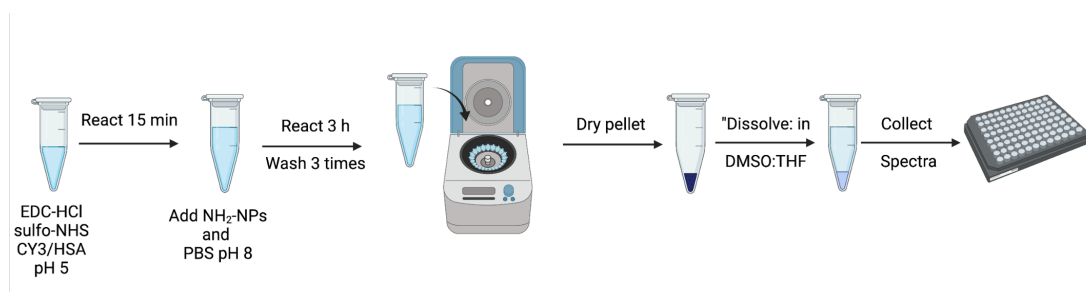


Figure 5.16: *EDC/NHS on NH_2 -NPs.* Schematic representation of the EDC/NHS protocol on NH_2 -NPs.

Click Chemistry on N_3 -Nanoparticles

N_3 -NPs were covalently conjugated via click chemistry to Alkyne modified Rhodamine following an adaptation of the protocol reported in reference¹¹². Sodium ascorbate (3 mM), PMDETA (0.25 mM), CuSO_4 (0.07 mM), and dye (various concentrations) were mixed with a 0.1 wt% solution of the N_3 -NPs. The reaction was carried out for 24 h at 50°C on a thermo shaker (500 rpm). The product was isolated by repeated centrifugation (10 min at 4°C with a 15.5 krcf for 4 times) and resuspension in 200 μL of a 0.1 wt% Lutensol AT50 solution. On the last purification step, the pallet was left to dry, weighted and dissolved in 100 μL THF solution to collect the fluorescent spectrum ($\lambda_{\text{excitation}} = 550 \text{ nm}$, $\lambda_{\text{emission}} = 570\text{-}660 \text{ nm}$). Schematic illustration and details can be found in Figure 5.17.

Fluorescamine Titration of NH_2 -Nanoparticles

NH_2 -NPs were characterized via adaptation of the fluorescamine assay. To 100 μL of a 0.01 wt% solution of the NH_2 -NPs in 10 mM PBS buffer at pH 8, 0.5 μL of a fluorescamine stock solution (5 or 0.5 mM in DMSO) were added sequentially, stirred for 1 min and fluorescence spectrum ($\lambda_{\text{excitation}} = 400 \text{ nm}$, $\lambda_{\text{emission}} = 425\text{-}525 \text{ nm}$) was collected at each step until there was no evident fluoerescence intensity change. The titration data is reported in Figure A.12. Schematic illustration and steps can be found in Figure 5.18.

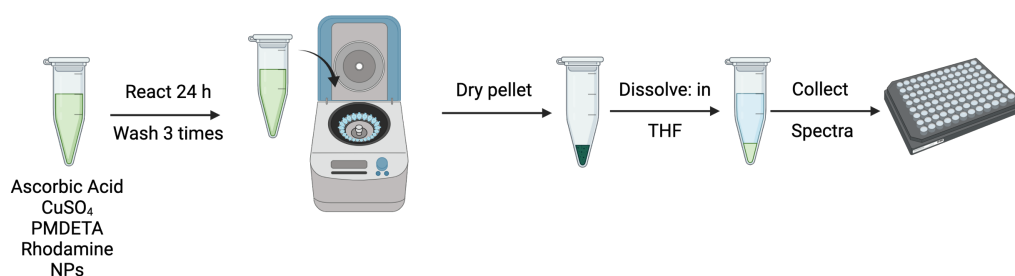


Figure 5.17: Click on N_3 -NPs. Schematic representation of the click chemistry protocol on N_3 -NPs.

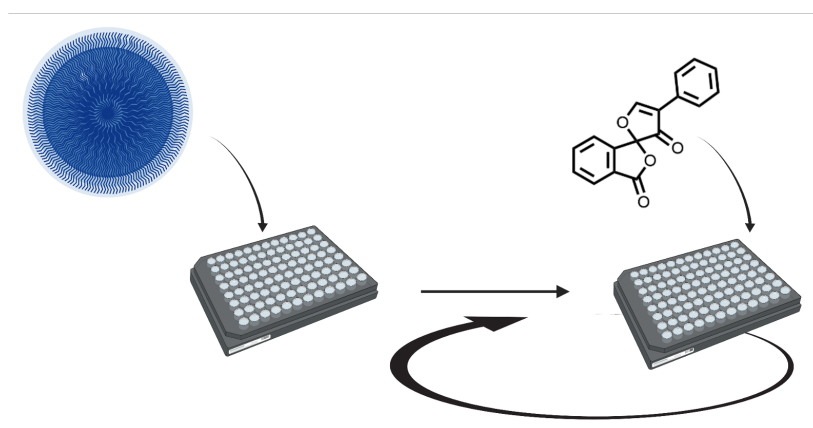


Figure 5.18: Fluorescamine assay. Schematic representation of the adapted Fluorescamine assay on NH_2 -NPs.

Calculation for Protein Conjugation on the Surface of Nanoparticles

Maximum NPs coverage with HSA. Based on the work of Yu *et al.*²²⁹, we have assumed that albumin has an equilateral triangular prism volume with the following dimensions: $8 \times 8 \times 3.5$ nm. Overall we will have two faces with a triangle area ($\frac{8nm \times 6.7nm}{2} = 27nm^2$) and three rectangular areas ($8nm \times 3.5nm = 28nm^2$). Assuming a random orientation of the protein on the NPs surface we can average the protein surface area and assume that the protein interacts with a 27.6 nm^2 surface area. Given that our NPs have a diameter of 200 nm, their surface area is $130'000 \text{ nm}^2$. Thus we can calculate how many proteins would fit on the surface area with Equation :

$$N_{protein} = \frac{Area_{NPs}}{Area_{Protein}} = \frac{130'000}{27.6} = 4'800 \quad (5.8)$$

obtaining approximately 4'800 proteins on each NPs.

From μM concentration to groups per particle. The FGs concentration obtained from the different experiments can be converted to the average number of groups per particle as follows (Equation 5.9):

$$FG_{particle} = C * \frac{1}{SC} * \frac{g_{polymer}}{particle} * N_A \quad (5.9)$$

where C is the molar concentration, SC is the solid content which in this work was $1g/L$.

The grams of polymer per NPs ($\frac{g_{polymer}}{particle}$) were be calculated with Equation 5.10:

$$\frac{g_{polymer}}{particle} = V * \rho \quad (5.10)$$

where V is the volume of the NPs treated as a sphere and ρ is the polymer density (1.17 g/L).

DNA conjugation

NPs with azide FGs were conjugated to the DBCO modified docking DNA strand via copper free click chemistry. The DNA was stored at -20°C in a TE buffer (110 mM of Tris-Cl and EDTA). The NPs were diluted to 0.1 wt% in TE buffer with 0.1 wt% Titron X and 375 mM NaCl and mixed with 6.4 mM DNA in a low binding eppendorf tube. Unbound DNA was removed by repeated (3x) centrifugation (10 min at 4°C with a 15.5 krcf for 4 times) and resuspension in TE buffer.

Optical Setup

DNA-PAINT imaging was performed with a Oxford Nanoimager microscope (ONI, Oxford, UK) system configured for TIRF imaging and equipped with a perfect focus system. The signal was recorded using a 100X, 1.4 NA oil immersion objective, passed through a beam splitter to obtain a green and a red channel. The imaging was performed using the 561 nm (5 %) and 647 nm lasers (30 %) to image respectively the BODIPY and ATTO-655 dyes. Images were acquired onto a 427×520 pixel region (pixel size 0.117 μ m) of a sCMOS camera.

DNA-PAINT Imaging

Coverslips (22 mm x 22 mm, #1.5) were immersed in methanol and sonicated in water bath for 20 min. The coverslips were then dried with nitrogen flow and treated with a plasma cleaner for 1 min. While the microscopy slides (76 mm x 26 mm, 1 mm) were cleaned with acetone-soaked microscopy tissue. The imaging chamber (20 L) was prepared using double sided tape to attach the coverslip to the microscopy slide. The coverslip was passivated with PLL-PEG by incubating a 0.5 mg/mL solution for 10 min. Subsequently, the chamber was flushed with TE buffer.

The samples were added to the chamber and left to incubate in high humidity box for about 1 h by flipping the chamber and sealing it with tape to limit solvent evaporation. The undeposited particles were then flushed with 180 L of TE buffer and subsequently with 180 μ L of a 10 pM TE solution containing the imager DNA strand. Lastly, the chamber was sealed with nail polish. The imaging was performed with an exposure time of 80 ms. The lasers were sequentially activated: the green laser (647 nm) for one frame and the red laser for 100 (561 nm), looped 65 times. The movies were saved as .tiff files.

DNA-PAINT Analysis

The .tiff movies collected by the microscope were analyzed with ThunderSTORM, the ImageJ plugin. The localizations were detected using a β -spline wavelet filter with scale 2 and order 3, a local maxima finder with a threshold set to 1.3 times the standard deviation of wave F1 of the filter. Localizations with uncertainty less than 1 were filtered. The result table was exported and further analyzed by first clustering, with a MATLAB code, the 561 nm localizations. The clusters were used to determine the drift and apply drift correction to the full data set. The center of each cluster was then used to determine the 647 nm localizations around each NP.

Chapter 6

Summary

This thesis stands as an interdisciplinary project, spanning from polymer synthesis to NPs synthesis, from ensemble characterization to atomic and structure investigation and characterization of particle-to-particle differences. Therefore, diverse projects have been investigated with a communal theme: detailed and high-resolution characterization of soft NMs. As such each project required the adaptation of the characterization approach based on the system under investigation.

I first introduced a semi-crystalline material, HONEY ASAP. It's structural repetitiveness and homogeneity allowed me to apply an averaging approach to establish atomic resolution. Taking advantage of SPA-CryoEM, I uncovered the structural characteristic of the system, which enable a rational description of the peptide and peptide-peptide interaction. The novelty of in the discovered structure stands as proof of how important it is to investigate at an atomic level NM structures.

I then moved onto amorphous materials: multi-phase NPs. Lack of high order in the system requires shifting the characterization approach. Hence, I zoomed out of from the atomic scale and shifted in the nanometer scale. At this resolution I was able to characterize the ability to produce phase separated or co-localized NPs. Implementing a quantitative characterization of dSTORM images I was able to characterize the behavior of different polymers within single NPs establishing particle-to-particle differences.

Lastly, I continued with amorphous material- polymeric NPs- and investigated the distribution of functional groups.¹⁹⁸ In this section the scientific question zoomed back into the atomic level, yet, contrarily to the first project, the lack of high order impeded the application of averaging tools. Hence, the scientific question was approached in an ensemble averaged way, leading to a loss in high resolution information. To re-establish the lost information, dynamic investigation of the sample was tested. Here, DNA-PAINT, via transient interaction between the probe and docking strand, highlighted particle-to-particle differences. The investigation lead to a comprehension of FG distribution within the NPs at an ensemble level and a particle-to-particle surface distribution.

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Abbreviations

Abbreviation	Full Form
AEMA	2-amino-ethyl methacrylate hydrochloride
AFM	Atomic Force Microscopy
ANF	6-amino-fluorescein
AzMA	3-azidopropyl methacrylate
BDP	Boron dipyrromethene
BTDATS	4,7-bis(4-allylethoxysilylphenyl)-2,1,3-benzothiadiazole
CryoEM	Cryogenic Electron Microscopy
CTAB	hexadecyltrimethylammonium bromide

Abbreviations

CTAT	hexadecyltrimethylammonium chloride
CTF	Contrast Transfer Function
Cy3	Cyanine3
Cy5	Cyanine5
DAP	Dual Amphiphilic Peptide
DBCO	Dibenzocyclooctyne
DMSO	Dimethyl sulfoxide
dSTORM	direct STochastic Optical Reconstruction Microscopy
EDC	N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide
EDX	Energy dispersive X-Ray spectroscopy
EELS	Electron Energy Loss Spectroscopy
FCS	Fourier Shell Correlation
FFT	Fast Fourier Transfer

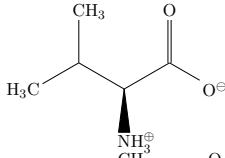
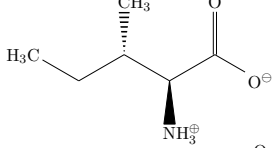
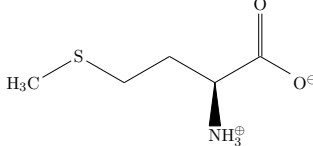
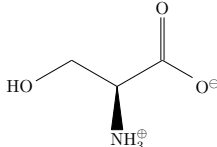
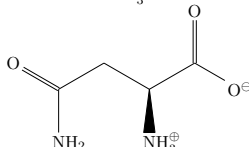
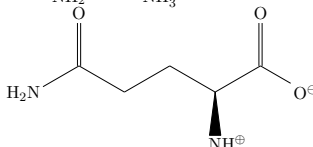
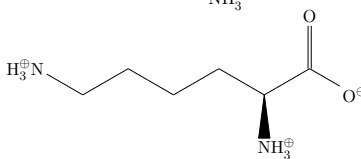
HSA	Human Serum Albumin
MD	Molecular Dynamics
MMA	methyl methacrylate
NHS	N-Hydroxysuccinimide
NM	Nanomaterials
NP	Nanoparticle
PBzMA	Polybenxylmethacrylate
PA	Polyacrylate
PAINT	Point accumulation for imaging in nanoscale topography
PNF	Peptide Nanofibril
PTA	Phosphotungstic acid
PU	Polyurethane
Rh	Rhodamine B

Abbreviations

SML	Single Molecule Localization
SNR	Signal to noise ratio
SPA	Single Particle Analysis
SRM	Super Resolution Microscopy
STORM	STochastic Optical Reconstruction Microscopy
sulfo-NHS	N- hydroxy sulfosuccinimide
TEM	Transmission electron microscopy
T_g	Glass transition temperature

Amino Acids

Table 6.1: Amino Acid Table

Name	3-Letter Code	1-Letter Code	Structure
Valine	Val	V	
Isoleucine	Ile	I	
Methionine	Met	M	
Serine	Ser	S	
Asparagine	Asn	N	
Glutamine	Gln	Q	
Lysine	Lys	K	

Materials

Instruments

Instrument	Manufacturer
Titan Krios 300kV transmission electron microscope	Thermo Fischer Scientific
Vitrobot	Thermo Fischer Scientific
Oxford Nanoimager microscope (ONI)	Oxford, UK
Nikon N-STORM	Nikon Instruments Inc.
Plate Reader	Tecan, Männedorf, Switzerland
Particle Charge Detection	Mütek GmbH, Herrsching, Germany
NMR	400 MHz Bruker AMX system at 298.3 K

Software

The following software were used in collecting the data and writing this thesis. SerialEM was used to automate data collection of CryoEM images. Relion 3.1 was used to analyze the large data sets and obtain 3D volumes. Chimera and ChimeraX were utilized to visualize the 3D volume, for atomic modeling and for image creation. Phenix was utilized to run Molprolity and to interlock with the Coot software for proper atomic modeling. ImageJ was used to process most microscopy images in all of the chapters in this thesis. DigitalMicrograph was used to process and visualize the EELS data. SRM images were analyzed using Thunder-STORM. MatLAB and Python were used to analyze and process the SRM data. Python was used to plot data. The thesis was written in LateX using Textstudio. Non-sensitive text, such as the one in the introduction, was polished using artificial intelligence language tools, these tools were never used to generate new text. Some images were created using Biorender.com. Multipanel figures or schemes were organized in PowerPoint. Belender and Photoshop were used by Stefan Schuhmacher to create the TOC figure on page 97.

Appendix

Use of artificial intelligence tools

In this thesis Artificial Intelligence (AI) tools were never used to generate text from blank (e.g. Write an introduction paragraph (200 words) on amyloid fibrils). AI tools were used as assistance tools as follows:

- Semantic Scholar was used for additional literature research in the introduction sections.
- ResearchRabbit was used to track important literature and to identify related papers that were relevant and needed to be cited. The tool was used throughout the whole thesis.
- DeepL Writing and ChatGPT were used to reformulate my text to improve readability. This was done in most of the sections of the thesis, prior to colleagues-related feedback on the text. Examples of improved readability, extracted from Chapter 1, are reported in Table A.1.
- ChatGPT was used to test readability of the text (e.g. What do you understand from this paragraph?). This was done in the Chapter 1 and 2.
- ChatGPT was used to format the thesis in Latex. This was done in the preamble section and in most of the tables.
- DeepL Translate was used to translate the abstract from English to German.

Version	Content	AI Usage
1	The above described NMs are of course not visible to the naked eye. Therefore, it is important to understand in detail the distinctive nature of these materials. To do so, characterization techniques are utilized. Over the years a myriad of techniques have been developed and not all of them can and should be listed in this short introduction. Contrary, characterization should be properly used by phrasing the question: what do we want to know or see? That is why I will now describe, in reference to amyloid nanofibers and polymeric NPs, the typical questions that people ask about materials and what techniques can be used to address them.	Fully written by me
2	The nanomaterials discussed thus far are imperceptible to the naked eye, underscoring the importance of detailed characterization to discern their distinctive properties. A plethora of characterization techniques have emerged over the years, each tailored to address specific inquiries about these materials. In this brief introduction, rather than exhaustively listing all available techniques, the focus lies on articulating the fundamental questions researchers seek to answer and the corresponding techniques employed to address them. Specifically, I will delineate the key inquiries pertinent to amyloid nanofibers and polymeric nanoparticles (NPs), elucidating the techniques utilized to decipher their structural and functional attributes. By framing characterization within the context of these essential questions, we can glean insights into the intricate nature of nanomaterials and their potential applications.	Major AI usage
3	The NMs discussed thus far are imperceptible to the naked eye, underscoring the importance of detailed characterization to discern their distinctive properties. A plethora of characterization techniques have emerged over the years, each tailored to address specific scientific questions about these materials. In this brief introduction, rather than listing all available techniques, the focus lies on describing the fundamental questions researchers seek to answer and the corresponding techniques employed to address them. Specifically, I will elucidate how to understand the structural and morphological characteristics of PNFs and polymeric NPs (Figure 1.4).	AI version merged with my version

Table A.1: Example of AI tools used for text revision. Comparison of different content versions for describing nanomaterials and their characterization.

Unraveling Structural Characteristics of HONEY ASAP: Hexagonally Ordered Nanofibrils Easily Yielded by Amphiphilic Self Assembly Peptides

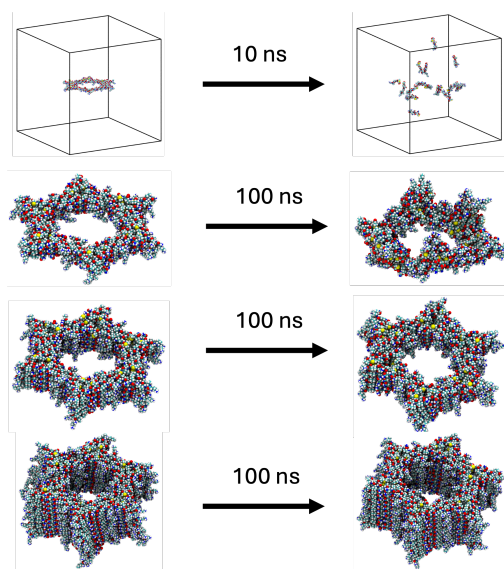


Figure A.2: Layer analysis. Molecular dynamics overview showing 1, 3, 5, and 10 layers of the central morphology. On the left, there is a time 0 structure, from the atomic model, and on the right, the simulation after 100 ns. The figure clearly depicts deformation for the fiber with less than 5 layers.

Chemical Phase distribution within Polymeric Nanoparticles

Code	Number of Images	Number of Green NPs	Number of Red NPs	Number of Green Hybrids	Number of Red Hybrids	Average number of Hybrids	Average Green Radius (nm)	Average Red Radius (nm)	Hybrid %
A1	4	2201	1201	43	43	43	47.12	47.29	1.3
A2	13	9283	5683	341	335	338	48.31	53.07	2.3
A3	9	8090	11572	506	505	506	49.58	52.18	2.6
B1	5	2392	2752	148	146	147	51.28	51.04	2.9
C1	9	1227	2830	101	98	100	45.93	77.96	2.5
C2	10	3796	6361	290	297	294	48.64	55.05	3.0

Table A.2: *dSTORM* table with summary results.

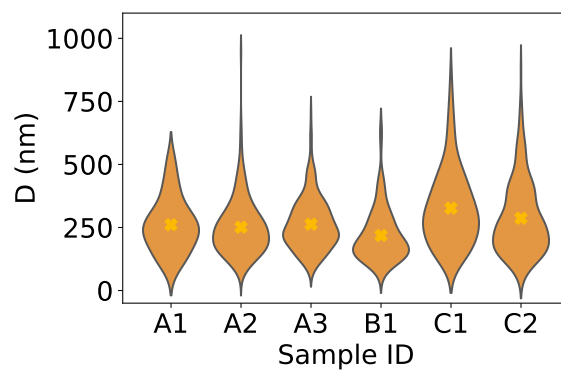


Figure A.3: Violin plot of the size distribution of the detected hybrids for sample A1, A2, A3, B1, C1 and C2.

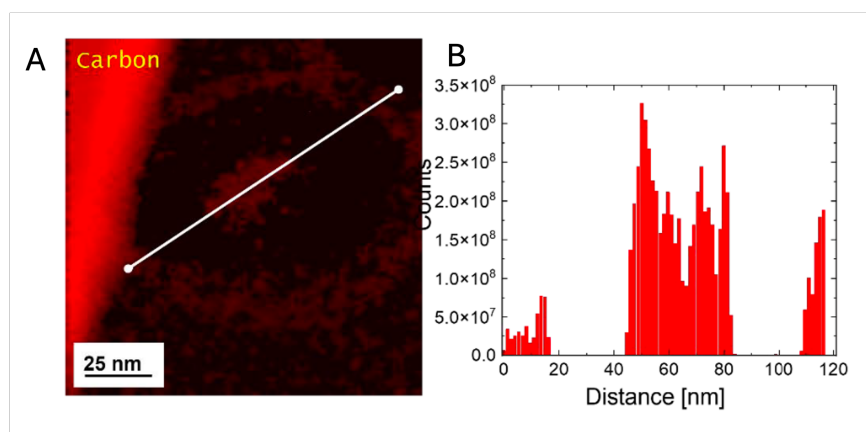


Figure A.4: Elemental map of C (A) highlighting the line of NP-CS from major axis of ellipse which produces the (B) cross section elemental distribution. Adapted from Ref.⁷⁸.

Functional Group Distribution within Nanoparticles

Error evaluation on μM quantification

For all described methods, experimental and mathematical errors can arise. Specifically, the experimental errors are those associated to the intrinsic nature of the method applied. Such errors affect the experimentally determined value. On the other hand, mathematical errors are those that arise from applying a conversion from the experimental value to a concentration value. For instance, the experimental error will be associated to the fluorescence detection limit in the plate reader and will affect the fluorescence intensity. The mathematical error will be converting the fluorescence intensity into dye concentration based on a calibration curve. Therefore, quantification should be performed in triplicate measurement to investigate error effects. In addition to experimental and mathematical errors, quantification differences could also arise from reaction conditions. Such effect plays a predominate role in accessible FG quantification where variation in temperature, pH buffer, or reaction times can strongly affect the reaction's yield (Figure S9). In this section we discuss in more detail the primary errors associated to each of the quantification methods presented in this work. Notably, many errors can arise in each method, details can be found in works such as Bharti *et al.*²³⁰ and Böckenhoff *et al.*¹¹⁷

Total FGs

The quantification of total of FGs is determined through the application of $^1\text{H-NMR}$ spectroscopy. Specifically, the relative molar composition between the BzMA and the FM was converted into molar concentration of FGs in a NP solution with a 1 mg/mL concentration of polymer (Equation 5.2). Therefore, the primary sources of error in the quantification are associated to NMR peak integration and sample reproducibility. Firstly, the NMR peak integration is an experimental error, which is inherent to the technique itself. In this case, error can arise due to inadequate baseline correction or the broadness of the peak in selected regions. For instance, when comparing the CH_2 and aromatic signals of the BzMA, the integrals should report a 2:5 ratio. However, small variations in the selected region could lead to divergencies such as 2:4.8. Such an error would be easily detected in the CH_2 :BzMA

ratio; yet, when comparing unknown ratios (e.g. BzMA:FM), this error can result in a significant variation.

The second major error is the sample reproducibility. To obtain triplicate measurements, the produced polymer was introduced into three separate NMR tubes. Notably, the polymer presented here was achieved via free radical polymerization, which has poor control over the statistical polymer produced. Therefore, it is possible that each polymer chain may present large deviation not only in polymer length but also in monomer ratio. The presence of large heterogeneity in the polymer composition may result in different molar %.

Visible FGs

The quantification of visible FGs is based on the PCD, which is conducted via titration method. Specifically, the volume of titrant utilized ($V_{titrant}$) is converted into a molar concentration in a NP solution with a 1 mg/mL concentration of polymer using Equation 5.7, as reported in the main text. The degree of reproducibility is dictated by the ability of determining the $V_{titrant}$. It should be noted that the streaming currents¹¹⁷ within the sample may affect the results of the investigation. Therefore, the discrepancies in the measurements can be considered an intrinsic experimental error.

Accessible FGs

The quantification of accessible FGs is based on a dye conjugation process. Specifically, a dye is coupled to the NPs. Unreacted dye is removed via repeated centrifugation and re-suspension steps. The final product is dissolved in an organic solvent and a fluorescence spectrum is acquired. The fluorescence signal can be converted into a dye concentration by using a calibration curve. The reaction is repeated in different conditions to obtain a titration curve where the detected dye increases until reaching a plateau. In this plateau, increasing the free dye for conjugation does not affect the amount of bound dye. Hence, averaging the values in this range enables detection of the maximum number of dyes that can be conjugated to the NP solution. Furthermore, given that the solid content of the polymer is known in this solution, the dye concentration can be correlated to the concentration of dye in NP solution with a polymer concentration of 1 mg/mL. The errors in this measurement derive from multiple factors, a greater number than those identified in the aforementioned methods. The errors can be attributed to various factors, including the reaction itself (e.g., yield reproducibility), cleaning steps (e.g., pipetting), calibration curve errors, and polymer interference with chromophore. The primary factors affecting the quantification are the reaction yield and the cleaning steps. In the first case, the variability of the reaction yield will result in large discrepancies in quantification, due to inherent variability in the amount of conjugated dye. Specifically, the EDC/NHS coupling is challenging²³¹ and requires detailed optimization of reaction conditions to improve the yield.²³² Notably, such reaction-bound error will be found in any chemical-conjugation based approach. Therefore, it is of great importance to select reactions with high yields (e.g. substitute EDC/NHS coupling with click chemistry reactions) and with limited reaction steps. However, this is not always possible and will strongly depend on the type of FGs that needs to be quantified. Cleaning errors, on the other hand, are highly user dependent. Therefore, the direct method presented in this work stands to limit such manual limitations.

Fluorescamine Assay

Fluorescamine assay quantification was determined using titration curves. The concentration of added dye which allow the fluorescence to plateau is associated to the accessible group in the specific experimental conditions. Such determination is based on user-biased selection of the plateau point. This point is clearly detectable in the averaged values (black dots in Figure A.12) but less pronounced in the individual titration curves. Therefore, in this case the error is not associated to the triplicate measurements but rather the amount of dye added between each titration point. In the presented experiment each titration point consists of a 0.5 or 5 μM fluorescamine addition, respectively for the 1% and 10% systems. These concentrations can be considered the error in the measurement. Given that the experiment was performed on a 0.01 wt% solution, the determined concentration was scaled by a factor of 10. It is then necessary to apply the same scaling factor on the error.

Micro BCA

Micro BCA was performed following an adaptation of the publish method²³³ using the Pierce Micro BCA Protein Assay Kit (Thermo Scientific). After the last step of purification, the purified NPs were re-suspended in 150 μL of MilliQ water at a final concentration of 0.13 wt%. The solution was mixed with 150 μL of the working reagent. The reaction was carried out for 2 hours at 37 $^{\circ}\text{C}$ on a thermoshaker at 500 rpm. The particles were then centrifuged for 10 min at 4 $^{\circ}\text{C}$ with 15 krcf. 200 μL of the supernatant was collected and the absorbance was measured at 562 nm in a plate reader.

Supplementary Table and Figures

Polymer Type	BzMA wt%	FMs wt%	FM initial mol %	Polymer yield (%)	Mn (g/mol)	Mw (g/mol)	$\bar{D}$
BzMA-AEMA	90	10	1	33	38416	109734	2.86
BzMA-AEMA	99	1	11	43	40629	113572	2.8
BzMA-MAA	90	10	2	84	38000	120382	3.17
BzMA-MAA	99	1	19	85	51323	180743	3.52
BzMA-AzMA	90	10	11	85	58325	420626	10

Table A.4: Monomer conversion and final polymer composition measured via NMR and GPC. Adapted from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

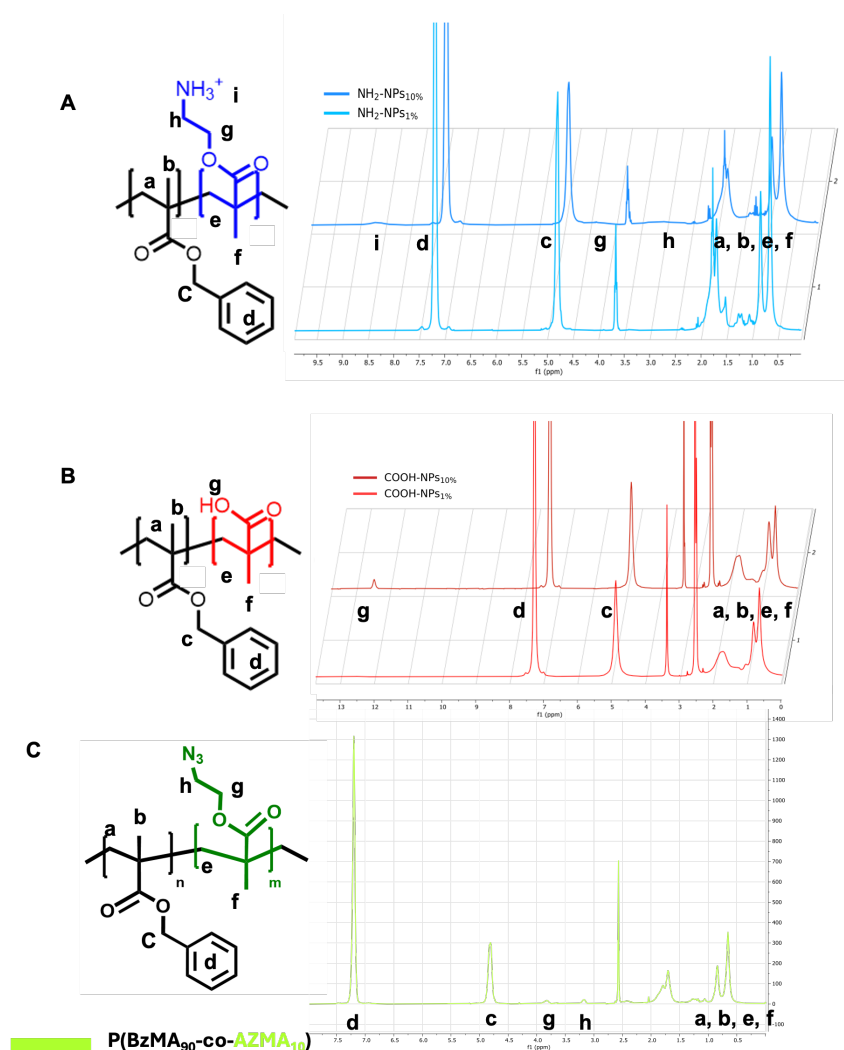


Figure A.5: ^1H -NMR of the cleaned $P(\text{BzMA-co-AEMA})$ (A), $P(\text{BzMA-co-MAA})$ (B), and $P(\text{BzMA-co-AzMA})$ (C) polymers in d_6 -DMSO (400 MHz, at 298 K). Adapted from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

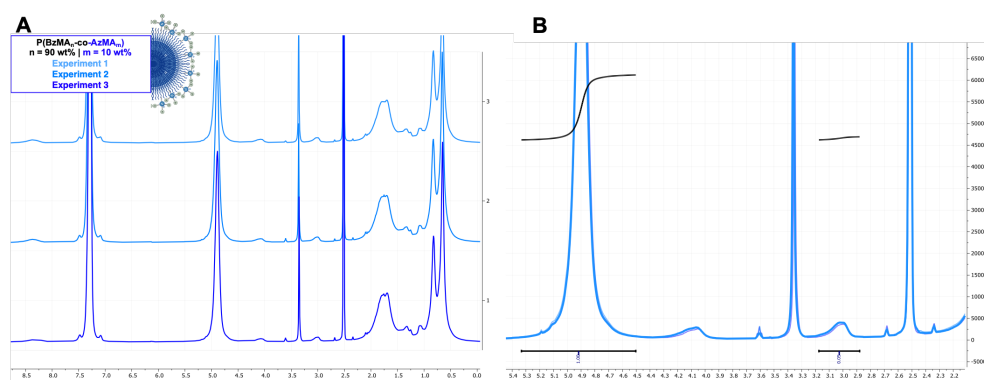


Figure A.6: (A) ^1H -NMR spectra of the purified $P(\text{BzMA}_{90\text{wt}\%}\text{-co-AEMA}_{10\text{wt}\%})$ in d_6 -DMSO (400 MHz, at 298 K). (B) Close up on the regions of interest where the normalized integrals of the BzMA ($\delta \sim 4.9$) and AEMA ($\delta \sim 3$) CH_2 peaks are reported. Reproduced from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

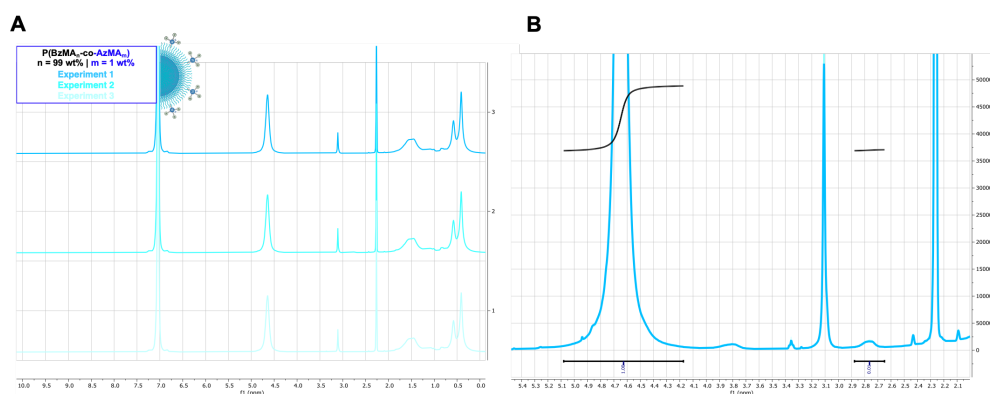


Figure A.7: (A) ^1H -NMR spectra of the purified $P(\text{BzMA}_{99\text{wt}\%}\text{-co-AEMA}_{1\text{wt}\%})$ in d_6 -DMSO (400 MHz, at 298 K). (B) Close up on the regions of interest where the normalized integrals of the BzMA ($\delta \sim 4.9$) and AEMA ($\delta \sim 3$) CH_2 peaks are reported. Reproduced from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

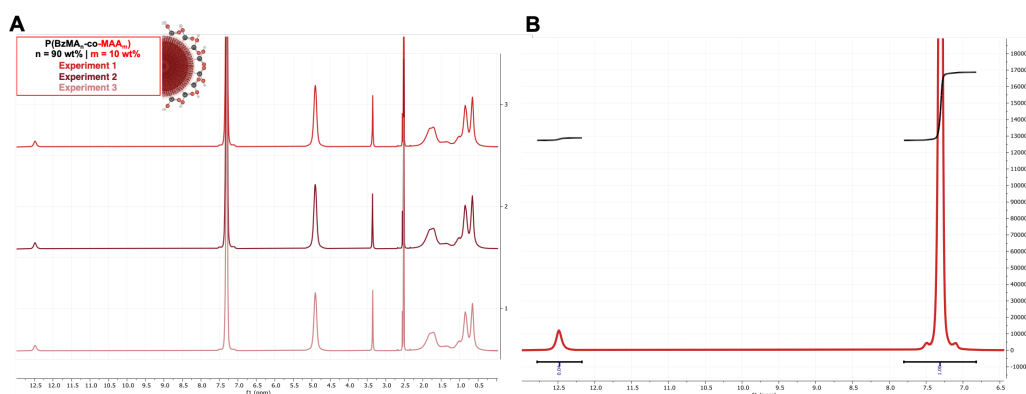


Figure A.8: (A) ^1H -NMR spectra of the purified $P(\text{BzMA}_{90\text{wt}\%}\text{-co-MAA}_{10\text{wt}\%})$ in d_6 -DMSO (400 MHz, at 298 K). (B) Close up on the regions of interest where the normalized integrals of the COOH peak ($\delta \sim 12.5$) and aromatic peak ($\delta \sim 7.3$) are reported. Reproduced from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

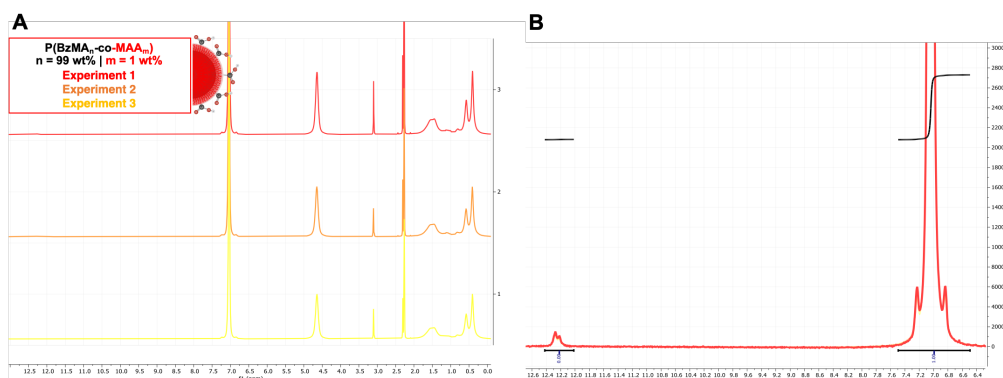


Figure A.9: (A) ^1H -NMR spectra of the purified $P(\text{BzMA}_{99\text{wt}\%}\text{-co-MAA}_{1\text{wt}\%})$ in d_6 -DMSO (400 MHz, at 298 K). (B) Close up on the regions of interest where the normalized integrals of the COOH peak ($\delta \sim 12.5$) and aromatic peak ($\delta \sim 7.3$) are reported. Reproduced from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

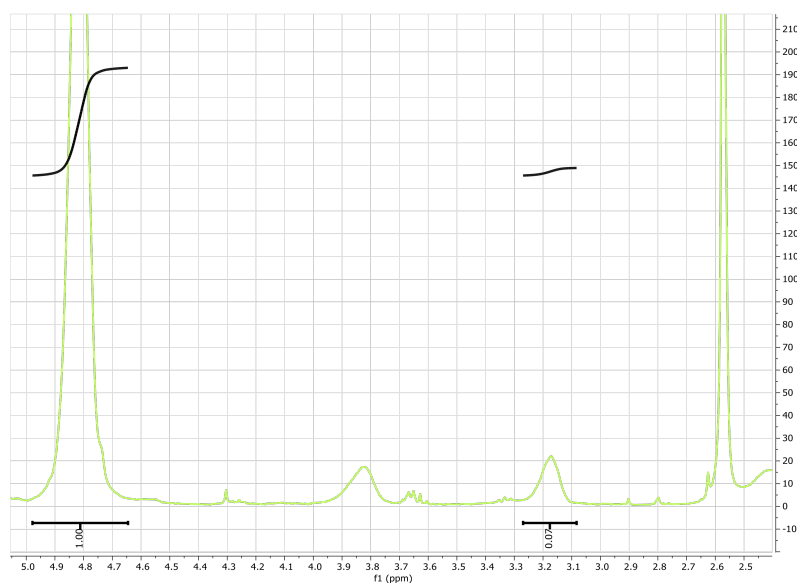


Figure A.10: (A) ^1H -NMR spectra of the purified $P(\text{BzMA}_{90\text{wt}\%}\text{-co-AzMA}_{10\text{wt}\%})$ in d_6 -DMSO (400 MHz, at 298 K). The spectra focuses on the regions of interest where the normalized integrals of the BzMA ($\delta \sim 4.8$) and AzMA ($\delta \sim 3.2$) CH_2 peaks are reported.

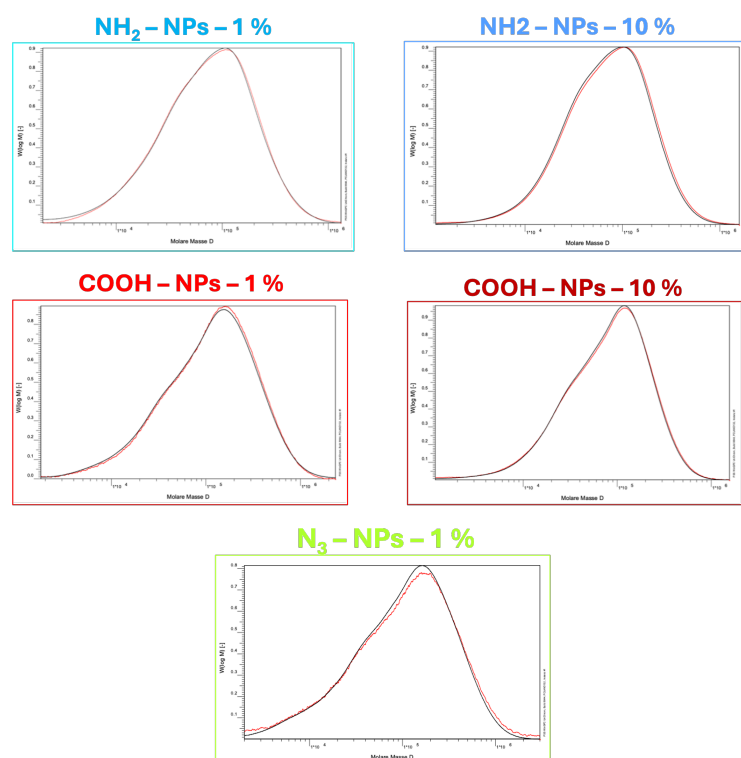


Figure A.11: UV absorbance at 280 nm (red) and RI (red) spectrum obtained via GPC for the (A) $\text{NH}_2\text{-NPs-10\%}$, (B) $\text{COOH-NPs-10\%}$, (C) $\text{NH}_2\text{-NPs-1\%}$ and (D) $\text{COOH-NPs-1\%}$. Adapted from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

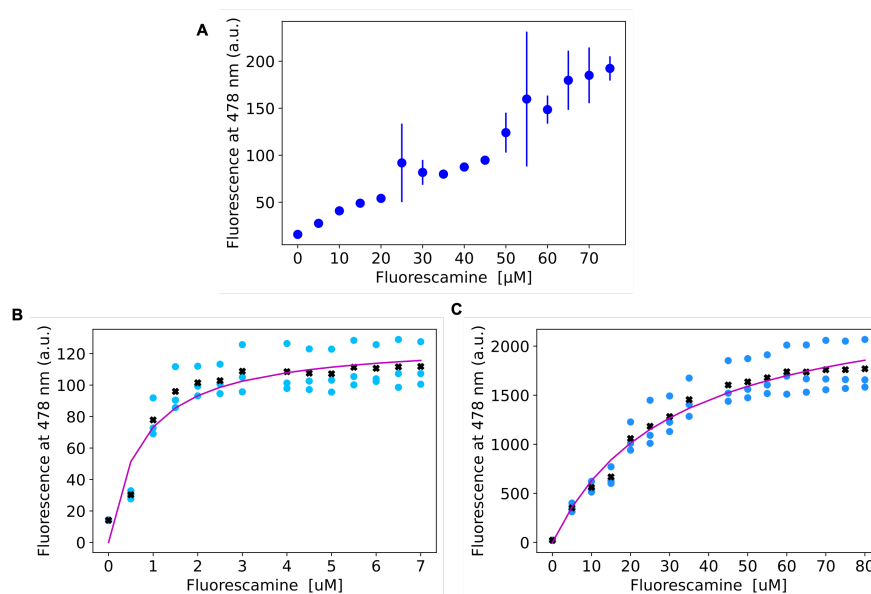


Figure A.12: (A) Comparison between the fluorescence intensity in the calibration curve system (10 mM of propylamine and increasing concentration of fluorescamine) and the fluorescamine titration on (B) NH₂-NPs-1% or (C) NH₂-NPs-10% in a 0.01 wt% NPs solution. The plots report the triplicate measurement and, in black, the average result used for the quantification. Reproduced from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

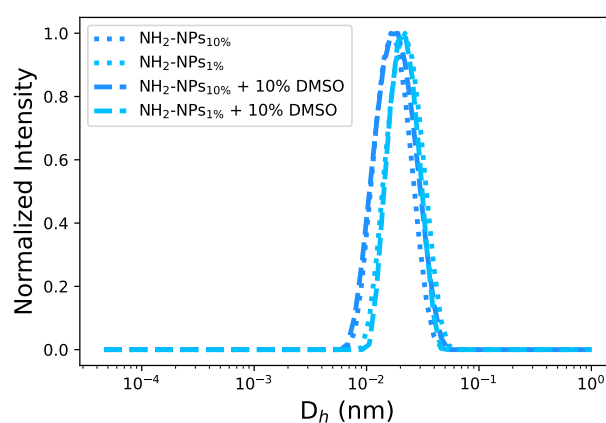


Figure A.13: DLS of the NH₂-NPs before and after the addition of 10% DMSO to illustrate lack of detectable swelling. Reproduced from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

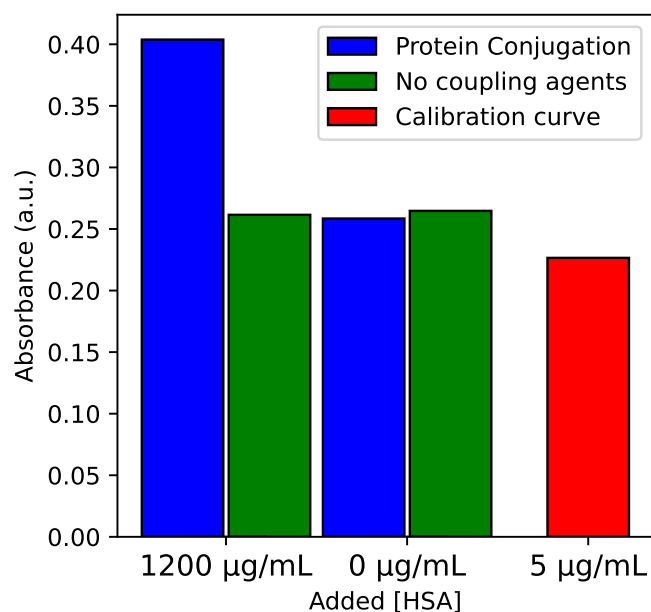


Figure A.14: The bar plot indicates the absorbance values obtained with Micro BCA Assay. In blue, the absorbance data of COOH-NPs activated with EDC/NHS then reacted with 1200 or 0 g/mL of HSA and lastly cleaned three times with a 0.1 wt% solution of Lutensol AT50 and one time with 2 wt% SDS. In green, the absorbance control data where the COOH-NPs were not activated but otherwise treated identically to the blue samples. In red, the absorbance of 5 g/mL of HSA obtained from the calibration curve data. Reproduced from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

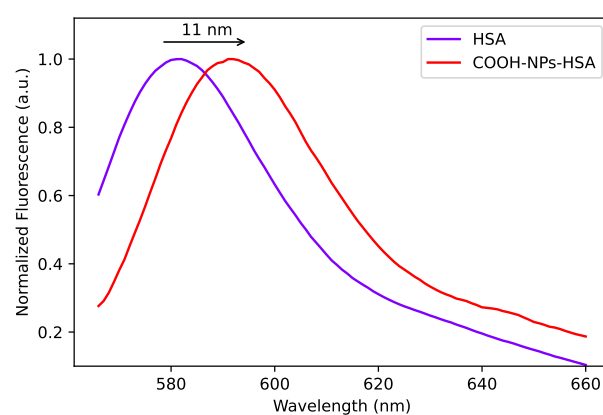


Figure A.15: Overlay of HSA-Rhodamine B in DMSO and polymer-HSA-Rhodamine B spectra. The 11 nm red-shift hampers the quantification protocol. Reproduced from Ref.¹⁹⁸ with permission from the Royal Society of Chemistry.

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Curriculum Vitae

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Education

- October 2020 - Ongoing **Research Assistant Chemistry**, *Max Plank Institute for Polymer Research, Mainz, Germany*, SUPERCOL project, supported by the Innovative Training Network, funded by the H2020 Marie Curie Actions of the European Commission, Title: Soft matter characterization beyond resolution limit.
Supervisors: Prof. Katharina Landfester and Dr. Ingo Lieberwirth
- January - April 2023 **Guest PhD Student**, *Covestro, Waalwijk, Netherlands*, Second industrial secondment for the SUPERCOL project, Objective: Using dSTORM for dual color imaging of multiphase nanoparticles. Manuscript in preparation.
Supervisors: Dr. Chen Qi, Dr. Stals Patrick and Dr. van der Zwaag Daan
- March - June 2022 **Guest PhD Student**, *Technical University of Eindhoven, Eindhoven, Netherlands*, First university secondment for the SUPERCOL project, Objective: Quantitative-DNA-PAINT for functional group quantification on nanoparticle surface.
Supervisor: Prof. Ilja Voets
- 2018–2020 **Master in Chemistry**, *University of Rome Tor Vergata*, 110/110 cum Laude, Thesis project carried out in **Lund University**, Sweden, Thesis Title: Detection of A β 42 aggregation intermediates.
Supervisors: Dr. Mattias Törnquist, Prof. Sara S. Linse, Prof. Lorenzo Stella
- 2015-2018 **Bachelor of Chemistry**, *University of Rome Tor Vergata*, 110/110 cum Laude, Thesis Title: Synthesis and Characterization of PVA-based hydrogel for paper restoration applications.
Supervisors: Dr. Claudia Mazzuca, Prof. Antonio Palleschi and Prof. Gaio Paradossi

Symposium and Conference contributions

- October 2024 **CryoNET**, *CryoNET symposium, Aarhus*,
Selected short talk from poster submission: "Unraveling structural characteristics of hexagonally ordered amphiphilic peptide nanofibrils: HONEY ASAP!".
- September 2024 **MSE**, *Material Science and Engineering, Darmstadt*,
Selected talk: "Unraveling structural characteristics of hexagonally ordered amphiphilic peptide nanofibrils: HONEY ASAP!".
- September 2023 **IMC-20**, *International Microscopy Conference, Busan*,
Poster presentation: "Unraveling Structural Characteristics of Self-Assembling Peptide Nanofibrils",
Poster presentation: "Dual color imaging for the detection of polymeric NP composition".
- September 2023 **ECIS**, *European Colloid and Interface Science, Naples*,
Poster presentation: "Dual color imaging for the detection of polymeric NP composition".
- June 2023 **EMR-S**, *European Materials Research Society, Strasbourg*,
Oral presentation: "Tailoring reactive handles on the surface of nanoparticles for covalent conjugation of biomolecules".

List of Publications

First author

- **Mazzotta Francesca***, Gaćanin Jasmina*, Baptista Andre Luis, Schmidt Matthias, Cortes-Huerto Robinon, Lieberwirth Ingo, Weil Tanja, Landfester Katharina. Peptide Origami: 3D Hexagonal and Honeycomb Lattices from Dual-Amphiphilic Peptides. Manuscript in preparation
- **Mazzotta Francesca***, Malheiros Barbara*, van der Woude Erika, Lieberwirth Ingo, Landfester Katharina, Chen Qi, Stals Patrick J. M., van der Zwaag Daan, Albertazzi Lorenzo, Voets, Ilja K. Dual Color dSTORM: Unraveling Composition, Size, and Morphology in Multicomponent Nanoparticles. Manuscript in preparation.
- **Mazzotta, Francesca**, Sharafudheen Pottanam Chali, Ingo Lieberwirth, Calum TJ Ferguson, and Katharina Landfester. Tailoring reactive handles on the surface of nanoparticles for covalent conjugation of biomolecules. *Polymer Chemistry*, 2024. DOI <https://doi.org/10.1039/D4PY01119H>

Co-author

- Iain Harley, **Mazzotta, Francesca**, Xhorxhina Shauli, Frank Scheffold, Katharina Landfester, and Ingo Lieberwirth. Practical considerations for plunge freezing samples over 40 °C for cryo-em. *Micron*, page 103745, 2024
- Wenxin Wei, **Mazzotta, Francesca**, Ingo Lieberwirth, Katharina Landfester, Calum TJ Ferguson, and Kai AI Zhang. Aerobic photobiocatalysis enabled by combining core-shell nanophotoreactors and native enzymes. *Journal of the American Chemical Society*, 144(16):7320–7326, 2022