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Controlling reaction pathways to modulate assembly processes

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Abstract

Regulation of the assembly and disassembly of peptide nanostructures is essential to create complex and dynamic systems that exhibit pathway selectivity. This thesis demonstrates the design of synthetically modified peptides that are responsive to stimuli such as light, pH, or redox, thereby controlling their assembly and disassembly behavior. In addition, the concept of stimulus-controlled assembly was investigated in complex environments.

In order to achieve control over the disassembly of nanostructures two short anthracene-containing peptides were designed that also feature a pH sensitive lysine. They assembled into nanosheets or nanoribbons with a distinct secondary structure. These morphologies could further be modulated by pH, leading to different amount of charges on the peptide. Upon irradiation the light-responsive anthracene units performed a bimolecular [4+4] cycloaddition, which disrupted the π - π stacking by distortion of the previously flat aromatic groups and therefore lead to disassembly of the supramolecular architecture. In contrast to monomolecular reactions such as photocleavage, the preorganization of the anthracene units within the supramolecular architecture exerted a notable influence on the kinetics of the bimolecular photodimerization. This highlights the interplay between the supramolecular structure and the molecular chemistry and showcased the controlled disassembly of diverse nanostructures.

The assembly process can also be precisely controlled using light-responsive systems. In conjunction with redox-responsive cysteine, a photolytic reaction cascade was engineered, yielding a disulfide that exhibited pathway selectivity during the assembly process. This was achieved by starting from a caged isopeptide, which released the linear peptide upon photocleavage and subsequently was oxidized by the side products of the cage to a disulfide. The modulation of this process was possible through the introduction of the reducing agent DTT, which affords control over the production and lifetime of the supramolecular monomers. The disulfide displayed short, twisted fibers when directly assembled, yet revealed short and thin fibers when assembled as part of the cascade. By cycling between the redox states, a third morphology of long and thin fibers emerged, whose secondary structure could

be tuned by the amount of DTT. This project demonstrated how a combination of light and redox responsiveness enables control over the assembly process with several distinct pathways giving rise to different nanostructures.

A glutathione-responsive isopeptide was then used to control the assembly of nanostructures in a complex environment such as the cell. Elevated levels of glutathione within the cytosol compared to the extracellular matrix could selectively cleave the cage, initiate the reaction cascade and release the linear, assembling peptide. The formation of nanostructures and their effect on MDA-MB-231 breast cancer cells was observed through live-cell imaging and fluorescence spectroscopy, as well as the oxygen consumption of the cell. The supramolecular assembly led to a disruption of mitochondrial integrity and inhibited energy homeostasis. Notably, the observed effects were not confined to two-dimensional cell culture, they could also result in a reduction in tumor size in a three-dimensional tumor spheroid model.

In summary, this thesis presented the modulation of assembly processes by controlling their reaction pathways. This was achieved by incorporating stimuli-responsive groups and tuning of supramolecular interactions. Furthermore, the interplay between molecular chemistry and the supramolecular architecture and vice versa is highlighted. These results should facilitate the discovery of next-generation biomaterials with control over the formation of complex nanostructures.

Zusammenfassung

Die Regulierung der Assemblierung und Disassemblierung von Peptid-Nanostrukturen ist für die Schaffung komplexer und dynamischer Systeme, die eine Wegelektivität aufweisen, von entscheidender Bedeutung. Diese Arbeit zeigt das Design synthetisch modifizierter Peptide, die auf Stimuli wie Licht, pH-Wert oder Redox reagieren und so ihr Auf- und Abbauverhalten steuern. Darüber hinaus wurde das Konzept der durch Stimuli kontrollierten Assemblierung von Peptiden in komplexen Umgebungen untersucht.

Zur Erlangung der Kontrolle über die Disassemblierung von Nanostrukturen wurden zwei kurze, anthracenhaltige Peptide entwickelt, die zudem ein pH-sensitives Lysin enthalten. Die daraus resultierenden Strukturen ordneten sich zu Nanoblättern oder Nanobändern mit einer unterschiedlichen Sekundärstruktur an. Die Morphologien konnten durch den pH-Wert moduliert werden, was zu unterschiedlichen Ladungen am Peptid führte. Bei Bestrahlung führten die lichtresponsiven Anthracen-Einheiten eine bimolekulare [4+4]-Cycloaddition durch, die die π - π -Wechselwirkungen durch Verzerrung der zuvor flachen aromatischen Gruppen störte und somit zur Auflösung der supramolekularen Architektur führte. Im Gegensatz zu monomolekularen Reaktionen, wie der Photospaltung, übte die Präorganisation der Anthracen-Einheiten innerhalb der supramolekularen Architektur einen bemerkenswerten Einfluss auf die Kinetik der bimolekularen Photodimerisierung aus. Dies unterstreicht die Wechselwirkung zwischen der supramolekularen Struktur und der molekularen Chemie und demonstriert die kontrollierte Disassemblierung verschiedener Nanostrukturen.

Der Assemblierungsprozess kann ebenfalls mithilfe lichtresponsiver Systeme präzise gesteuert werden. In Verbindung mit Redox-responsivem Cystein, wurde eine photolytische Reaktionskaskade entwickelt, die ein Disulfid freisetzte, dessen Assemblierungsprozess eine Reaktionswegelektivität aufwies. Dieser Effekt wurde durch den Einsatz eines blockierten Isopeptids erzielt, welches das lineare Peptid nach der Photospaltung freisetzte und anschließend durch die Nebenprodukte der Schutzgruppe zu einem Disulfid oxidiert wurde. Die Modulation dieses Prozesses wurde durch die Einführung des Reduktionsmittels DTT erreicht, wodurch eine

Kontrolle über die Produktion und Lebensdauer der supramolekularen Monomere möglich wurde. Das Disulfid wies kurze, verdrehte Fasern auf, wenn es direkt assembliert wurde, zeigte jedoch kurze und dünne Fasern, wenn es als Teil der Kaskade assembliert wurde. Durch Schalten zwischen den Redoxzuständen entstand eine dritte Morphologie aus langen und dünnen Fasern, deren Sekundärstruktur durch die Menge an DTT angepasst werden konnte. Dieses Projekt demonstrierte, dass eine Kombination aus Licht und Redoxreaktion die Kontrolle über den Assemblierungsprozess mit mehreren unterschiedlichen Pfaden ermöglicht, die zu unterschiedlichen Nanostrukturen führen.

In der Folge wurde ein Glutathion-reaktives Isopeptid genutzt, um den Aufbau von Nanostrukturen in einer komplexen Umgebung wie der Zelle zu steuern. Erhöhte Glutathionwerte im Zytosol im Vergleich zur extrazellulären Matrix konnten den Käfig selektiv spalten, die Reaktionskaskade einleiten und das lineare, assemblierende Peptid freisetzen. Die Bildung von Nanostrukturen und ihre Wirkung auf MDA-MB-231-Brustkrebszellen wurden durch die Methoden der Lebendzell-Bildgebung und Fluoreszenzspektroskopie sowie durch die Messung des Sauerstoffverbrauchs der Zelle beobachtet. Die supramolekulare Anordnung führte zu einer Störung der mitochondrialen Integrität und hemmte die Energiehomöostase. Bemerkenswerterweise waren die beobachteten Effekte nicht auf zweidimensionale Zellkulturen beschränkt, sondern konnten auch in einem dreidimensionalen Tumorsphäroidmodell zu einer Verringerung der Tumorgroße führen.

In der vorliegenden Arbeit wurde die Modulation von Assemblierungsprozessen durch die Steuerung der Reaktionswege vorgestellt. Dies wurde durch die Einbindung von auf Stimulus-responsiven Gruppen und die Anpassung der supramolekularen Wechselwirkungen erreicht. Darüber hinaus wird das Zusammenspiel zwischen der molekularen Chemie und der supramolekularen Architektur und umgekehrt hervorgehoben. Die Ergebnisse der Forschung sollten die Grundlage für die Entwicklung neuer Biomaterialien der nächsten Generation bilden, mit denen die Bildung komplexer Nanostrukturen kontrolliert werden kann.

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1 Introduction

1.1 Supramolecular peptide assemblies in nature

Supramolecular assembly is crucial to life because it is a core mechanism in which biomolecules regulate many of our body's functions. This starts with the dimerization of the DNA double helix, which stores the blueprint of every cell and goes to complex proteins that are only active when assembled and folded in the correct way. Proteins are particularly interesting because of the great diversity in structure and function that has evolved over the course of evolution.^[1] Some researchers estimate that there may be up to several billion protein species that keep the human body functioning.^[2] This complexity stems from the 20 canonical amino acids that function as the basic building blocks. These amino acids differ mainly by their side chain, which can be either basic, acidic, polar or unpolar. The combination of these properties as a primary structure combined with the hydrogen-bonds between the peptide backbone then gives rise to a three-dimensional tertiary structure.^[1] These subunits then assemble into hierarchical quaternary structures, either homomeric, by repeating the same subunit or heteromeric, resulting in a structure composed of several different subunits.^[3]

The diversity of proteins is also reflected in their morphology. These range from simple structures such as fibrils (Figure 1a),^[4] rings^[5] or tubes^[6] to very complex structures like mechanically-interlocked catenanes (Figure 1b),^[7] knots^[8] or even protein cages, that allow further compartmentalisation within the cell (Figure 1c).^[9]

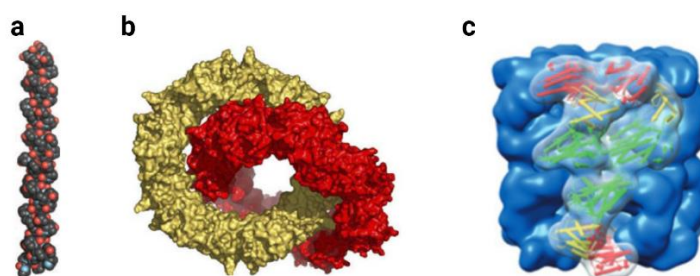


Figure 1: **a)** High-resolution crystal structure of the (ProHypGly)₄-(ProHypAla)-(ProHypGly)₅ collagen triple helix. Reprinted with permission from ref.^[4] Copyright 2010 Annual Reviews, Inc. **b)** Surface diagram of SP-22 C168S exhibiting a catenane structure. Reprinted with permission from ref.^[7] Copyright 2005 Elsevier. **c)** Structure of the unliganded (apo) GroEL with the different subunits colored in red, green and yellow. Reprinted with permission from ref.^[9] Copyright 2013 Springer Nature.

The formation of these distinct structures is contingent on the interplay between non-covalent interactions, which act over various length scales, each extending up to several angstroms, and exhibit varying strengths. The energy of these interactions is influenced not only by the nature of the interaction itself but also by the surrounding chemical environment, as these interactions can exhibit a high degree of cooperativity.^[10] Hydrogen bonds are particularly notable in peptides due to amount of possible interactions between the amides in the peptide's backbone, in addition to the polar side chains. Furthermore, hydrogen bonds exhibit a high degree of directionality, which facilitates the formation of secondary structures, such as β -sheets and α -helices.^[11,12] The nature of electrostatic interactions is dictated by coulomb forces, which govern the attraction or repulsion between charges. The strength of these interactions is approximately $\sim 500 \text{ kJ mol}^{-1}$, which is comparable to covalent bonds.^[12] The driving force of hydrophobic interactions is to minimize the contact with water. They are therefore governed by a favorable entropy instead of a favorable enthalpy. These interactions are contingent upon the polarity of the surrounding solvent, exhibiting no directionality and often lead to a formation of a micellar structure. A distinctive form of hydrophobic interactions is represented by π - π interactions that display a strength of up to 50 kJ mol^{-1} .^[13] These interactions are found in aromatic and conjugated peptides and lead to directional growth by stacking of the π -systems. Depending on the solvent and the chemical environment, the π - π stacks can adopt different conformations, such as face-to-face or edge-to-face, which in turn can also lead to distinct photophysical properties of the aromatic groups compared to the single molecule.^[14,15] Furthermore, aromatic groups can interact with charge side-chains of basic amino acids and form cation- π interactions.^[16]

These interactions can be dynamic, as can the structures they form. This is crucial because misfolded or misaggregated proteins can cause diseases such as Alzheimer's, Parkinson's and Huntington's.^[17,18] Nature has therefore developed methods to either disassemble and degrade proteins or to refold them into their correct conformation. The ubiquitin-proteasome system (UPS) regulates proteolysis in the cell by tagging misfolded proteins with the protein ubiquitin in an enzymatic cascade, usually through an isopeptide bond with a lysine side chain. The tagged protein is then delivered to the proteasomes, which first unfold the peptide and then degrade it into individual amino acids or oligopeptides.^[19,20] Another proteostasis mechanism involves chaperones. These macromolecular machines have a wide

range of substrates and assist in protein folding or disassemble proteins and prevent their aggregation. For example, the bacterial chaperonin GroEL (Figure 1c) can bind to proteins with exposed hydrophobic surfaces in its chamber, unfold them by exerting mechanical forces, and then allow them to refold in a hydrophilic chamber.^[9]

To drive the system out of equilibrium and make it dynamic, these processes require the addition of energy in form of ATP or GTP. Instead of performing a specific function when energy is added, it can also be used to generate transient assembled structures that fall apart when the energy is depleted. The cytoskeleton is made up of actin monomers and tubulin dimers, which form filaments and microtubules when they are driven out of equilibrium by energy dissipation.^[21] Monomeric actin (G-actin) binds ATP and can then polymerize into filaments (F-actin). It is then hydrolyzed to ADP-actin and depolymerized by the loss of phosphate. The filament has two biochemically distinct sites as growth occurs at the barbed end and disassembly occurs at the pointed end, creating a treadmill-like movement.^[22] These ends display different conformations, as the barbed end adopts a flat F-actin conformation and the pointed end a twisted G-actin conformation. This leads to a structural asymmetry and polarity at the barbed end, which favors the addition of additional monomers.^[23] Microtubules are assembled from α -/ β -tubulin heterodimers by hydrolysis of the attached GTP to GDP. The structures have a plus and a minus end, with only the plus end propagating, although both sides are chemically identical. Microtubules therefore exhibit structural plasticity.^[24,25] The observed kinetic disparities are attributed to variations in polarity and conformation at the respective interface, as tubulin heterodimers assemble in a head-to-tail configuration.^[26] The assembly and disassembly at the same end of the microtubule can be explained by a stress-storage-crack propagation model and is a result of different preferred conformations of GTP- and GDP-bound subunits. GTP-subunits add to open sheets at the plus-end, which are then straightened and closed to form the tubular structure. Upon hydrolysis to the GDP-subunit, an outwardly curved structure is preferred, which causes the accumulation of strain within the tube and results in cracks between the filaments. This sequence of events culminates in the disassembly of the filaments and the subsequent release of stored energy.^[24]

1.2 Synthetic supramolecular assemblies.

These biological assemblies were replicated, in parts, within synthetic cell models, often with short amphiphilic or cyclic peptides. One of the earliest examples of this was the synthesis of uniform hollow tubular structures by the assembly of cyclic peptides in 1993. In this study, the researchers used alternating L- and D-amino acids to create a flat ring-shaped conformation, which was then elongated by stacking in an antiparallel fashion through hydrogen bonds.^[27] Synthetic peptide assemblies offer numerous advantages, including biocompatibility and biodegradability, as well as diverse structural motifs and dynamics resulting from non-covalent interactions.^[14,28] The dynamic behavior of these systems can be tuned by the lifetimes of the supramolecular interactions, with too short lifetimes ($\tau < 1 \mu\text{s}$) preventing assembly, while too long lifetimes ($\tau > 1 \text{ min}$) resulting in a static behavior.^[29] In addition, the physical dimensions of the nanostructures can be modulated by controlling the assembly process.^[30]

Covalent polymerization is a process that is usually kinetically controlled due to the irreversibility of the covalent bond formation. In contrast, supramolecular polymerization is often characterized by its thermodynamic control. This means that reaction parameters, including conversion and the molecular weight of the resulting polymer, are directly dependent on concentration, temperature, and pressure. There are three main mechanisms that describe the growth of supramolecular polymers or assemblies. The isodesmic polymerization (Figure 2a) is distinguished by the reversible formation of a single non-covalent bond, where the association constant remains constant during the polymerization process. This independence from neighboring groups, the absence of cyclic intermediates, and the correlation between the degree of polymerization and the association constant are notable features. This mechanism is analogous to the step polymerization observed in conventional covalent polymers. The second mechanism is the ring-chain supramolecular polymerization (Figure 2b). This process entails the use of a ditopic monomer, which is characterized by a flexible tether linking the functional groups. The monomer exhibits a dynamic equilibrium between an open and linear form and a cyclic form, with the rate of intramolecular cyclization being contingent on the conformation of the linker.^[31]

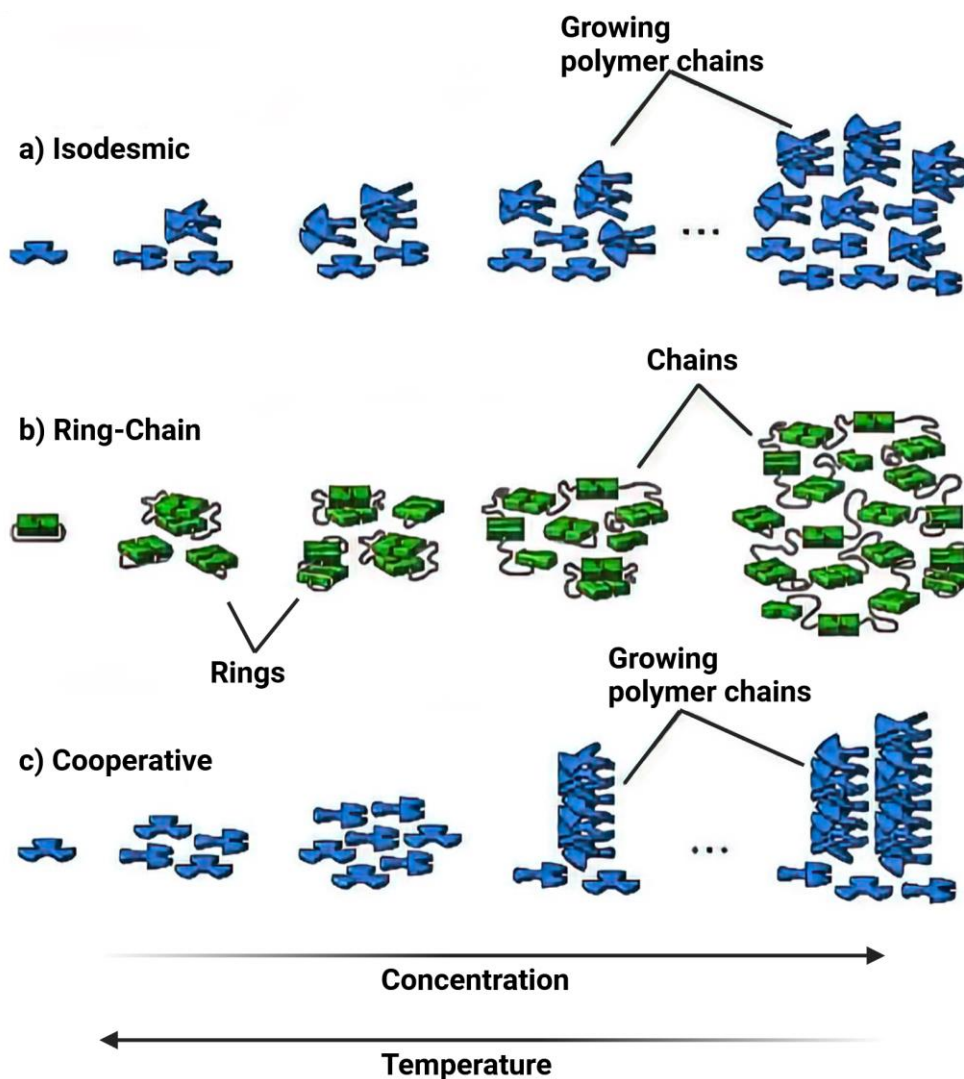


Figure 2: Schematic illustration of the three distinct mechanisms of supramolecular polymerization: **a)** isodesmic polymerization. **b)** ring-chain mediated polymerization. **c)** cooperative polymerization. Adapted with permission from ref.^[31] Copyright 2009 American Chemical Society.

The third category is the cooperative supramolecular polymerization (Figure 2c), which is a prevalent method for the assembly of peptides and proteins, including the peptide hormone islet amyloid polypeptide.^[32] This mechanism comprises two stages, which result in either cooperative or anticooperative growth. In the initial stage, a polymer is formed by isodesmic polymerization with a specific association constant, K_n , until a nucleus with a certain degree of polymerization is formed. In the subsequent phase, monomers are introduced to this nucleus, with an association constant of K_e being higher compared to the initial value of K_n , resulting from cooperative effects, which will be explained further in this chapter. In the case of anticooperative polymerizations, the association constants are reversed, resulting in K_n being larger than K_e . This leads to low polydispersity.

In general, cooperative supramolecular polymerizations are distinct from isodesmic polymerizations in several aspects. Firstly, a time-dependent lag phase is observed during polymerization, as the formation of the nucleus is a prerequisite. This lag phase is attributed to the fact that the nucleus, being the least prevalent species in solution, functions as a bottleneck. This challenge, and consequently the lag phase, can be circumvented by incorporating a preformed nucleus as a seed. Additionally, a critical concentration must be attained to shift the equilibrium between monomers and polymer towards the latter. Within the cooperative mechanism, there are two subsets: nucleated and downhill supramolecular polymerization. The distinction between these two processes arises from the variation in Gibbs free energy during the initial step. In instances where the Gibbs free energy increases and attains its maximum at the formation of the nucleus, the process is classified as nucleated polymerization. Conversely, in the case of downhill polymerization, the monomer possesses the maximum free energy, and the nucleation step is merely slower than the elongation step.^[33] However, this differentiation is concentration dependent, as the nucleated polymerization can become downhill upon increasing the monomer concentration.^[34]

The degree of cooperativity, denoted by σ , can be quantified using the ratio K_e/K_n . In the context of polymerization, σ values less than unity are indicative of a cooperative process, values greater than unity correspond to an anti-cooperative process, and values equal to unity correspond to an isodesmic process. The mole fractions of assembled molecules, denoted by Φ , plotted against the dimensionless concentration KC_t (Figure 3a) demonstrate a constant increase in polymer for the isodesmic process ($\sigma = 1$), while in the cooperative process, there is negligible polymerization for concentrations below the critical concentration. Above this concentration, high molecular weight polymers are produced within a narrow concentration range. It can thus be concluded that an increase in the degree of polymerization can be achieved by increasing the concentration or decreasing the σ (Figure 3b). Isodesmic polymerization exhibits simultaneous growth of equilibrium monomer and polymer concentration upon increasing the overall concentration. Conversely, in a cooperative polymerization, the monomer concentration rises until it reaches a critical assembly concentration, at which point polymer formation commences, while the monomer concentration reaches a plateau (Figure 3c). A key feature that differentiates these mechanisms is the mass distribution pattern. Isodesmic polymerization is characterized by a broad monomodal distribution, whereas cooperative

polymerization is typified by a bimodal distribution comprising monomers or polymers with high polymerization degrees (Figure 3d).^[31]

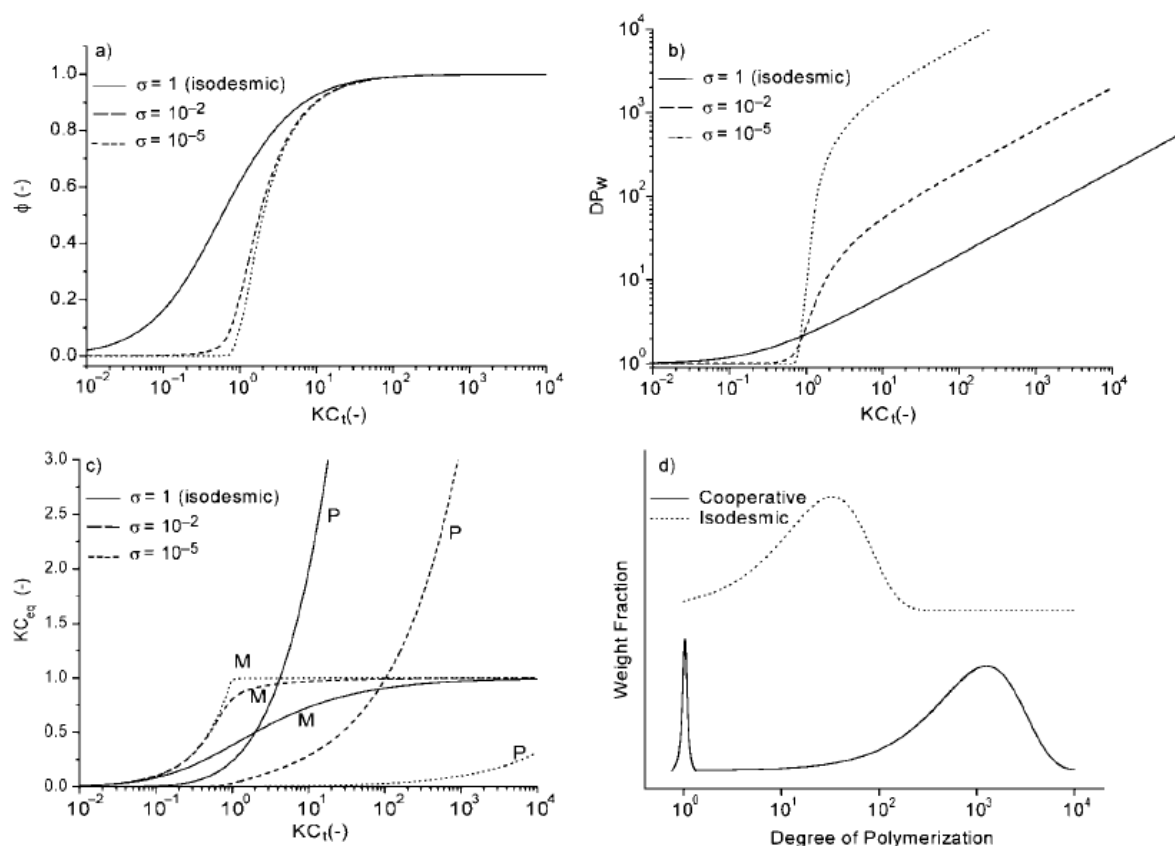


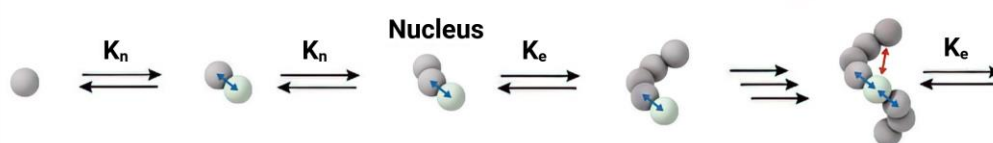
Figure 3: Concentration-dependent properties of isodesmic and cooperative supramolecular polymerizations with different values of σ . **a)** Fraction of polymerized material, Φ , as a function of the dimensionless concentration KC_t . **b)** Weight-averaged degree of polymerization (DP_w) as a function of KC_t . **c)** Dimensionless equilibrium concentration of monomer and polymer as a function of KC_t . **d)** Molecular weight distribution as a function of the degree of polymerization for isodesmic and supramolecular polymerizations.^[31] Reprinted with permission from ref.^[31] Copyright 2009 American Chemical Society.

In addition to the critical concentration, cooperative assemblies demonstrate a temperature dependence. This phenomenon is evident in the floor temperature for entropy-driven systems, where polymerization is absent below it. Conversely, enthalpy-driven polymerizations are constrained by the ceiling temperature. Once this threshold is surpassed, the supramolecular polymer disassembles.^[35] While synthetic systems are usually dominated by the enthalpic component, most biological systems such as collagen fibrils or the bovine brain tubulin instead exhibit a floor temperature and are therefore entropy driven.^[36,37] Despite its seemingly counterintuitive appearance, the entropy of the system is increased upon assembly when the water shell around the single molecule is taken into account. The water molecules form a

cage-like structure around the hydrophobic patches, thereby minimizing the disruption of the hydrogen bond network. Within this cage, the mobility of the water molecules is restricted and therefore their entropy is low. Conversely, aggregation of the hydrophobic surfaces results in the release of these water molecules, thereby achieving an entropy gain.^[38,39] However, the aggregation requires the attainment of a specific volume-to-surface area ratio.^[40]

The cooperativity of these polymerizations is attributed to a combination of electronic, structural, and hydrophobic effects. Electronic effects play a predominant role in linear polymers that assemble via hydrogen bonds, where the addition of a monomer to an existing polymer is entropically favored in comparison to the formation of a dimer by two monomers. For instance, the formation of formamide chains is governed by pairwise long-range dipole-dipole electrostatic interactions, short-range non-pairwise polarization and resonance-assisted hydrogen bonding. However, these effects are only present when the hydrogen bonding end groups are not divided by a flexible spacer.^[31,41]

a) Single stranded helical supramolecular polymers



b) Multi stranded helical or tubular supramolecular polymers

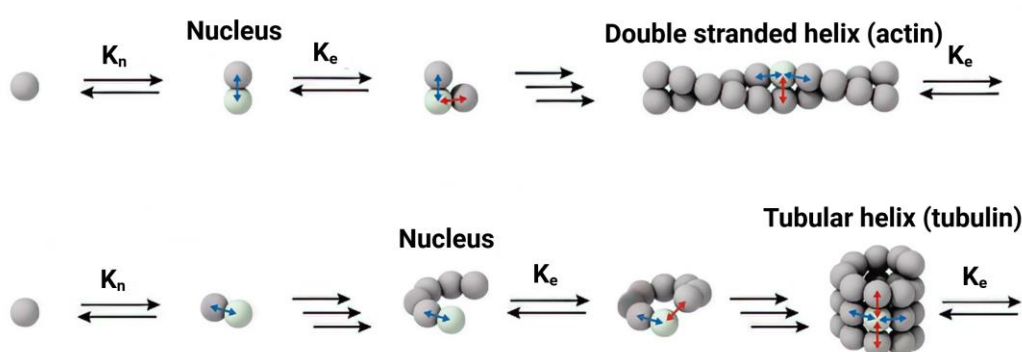


Figure 4: Schematic illustration of the cooperativity of helical or tubular in a) single-stranded or b) multi-stranded supramolecular polymers. Red arrows denote the secondary interactions that facilitate cooperative growth. Adapted with permission from ref.^[31] Copyright 2009 American Chemical Society.

Structural cooperative effects are dependent on the morphology of the assembly as they are predominantly present in helical or tubular structures. Upon completion of

the initial turn of a helix or tube, a new monomer engages not only with the preceding molecule but also with the molecule situated on the opposing side of the helix (Figure 4a). This effect is particularly pronounced in multi-stranded assemblies or tubes, which exhibit enhanced interstrand interactions (Figure 4b).^[31] This phenomenon is particularly pronounced in biological systems, such as actin or tubulin, where such structures are prevalent.^[42,43] Furthermore, additional cooperativity emerges due to allosteric effects, which refer to the conformational changes of monomers and polymers into a state favorable for polymerization.^[44]

1.2.1 Assembly of short aromatic peptide amphiphiles

In order to gain a deeper understanding of the supramolecular interactions and design principles presented in the previous section, minimalistic peptide sequences were developed, that offer a similar array of structures and functions compared to their larger counterparts. These short peptides, however, are easier to synthesize and modify and therefore enable the systematic programming of interactions.^[45–47] As these peptides contain a limited number of amino acids to enable sufficient number of hydrogen bonds to promote assembly, synthetic hydrophobic groups are often incorporated, either as aliphatic chains or aromatic groups, to supplement hydrophobic interactions.^[48,49] While the aliphatic version, with its structure similar to synthetic covalent block-copolymers, typically forms spherical and cylindrical micelles,^[50,51] aromatic systems exhibit directionality caused by the π - π stacking of the planar aromatic moieties, leading to geometric restrictions, that in turn lead to an increased order within the nanostructure (Figure 5a).^[45,52]

The Fmoc-group is a particularly notable aromatic moiety due to its prevalent usage as a protecting group in peptide synthesis.^[53,54] The first example of this was Fmoc-LD, which formed a thermoresponsive gel and was reported in 1995.^[55] Comparison between a series of dipeptides with Fmoc and Boc groups at the N-terminus, respectively, showed that the Fmoc peptides formed gels in more cases, highlighting the importance of the π - π stacking.^[56] Besides the Fmoc group, there are several other aromatic groups used such as naphthalene,^[57] naphthalene diimides,^[58] azobenzene^[59] or pyrene.^[60] However, there is no conclusive correlation between the relative hydrophobicity together with a certain peptide sequence and the assembly behavior.^[45] Apart from changing the N-terminal substituent, it is also possible to

modify it on the aromatic ring. For instance, using a peptide capped with pentafluorophenyl leads to hydrogels, while the non-fluorinated analogues did not.^[61] The linker, which connects the aromatic group with the peptide, plays a pivotal role in assembly, determining the relative orientation between the two groups and thereby dictating the extent of intermolecular interactions in each segment. A study employing multiple naphthalene amphiphiles, each with a distinct linker, revealed that a single variant exhibited gelation, characterized by a particularly linear geometry (Figure 5b).^[57] It is noteworthy that the presence of hydrogen bond donors or acceptors within the linker system can concomitantly promote assembly. In general, a rigid linker system with a linear conformation provides the optimal scaffold for the formation of nanostructures; nevertheless, it offers options for modification of the whole system in a complex way, for instance by using different numbers of methylene units, which causes alterations to the handedness of the supramolecular chirality.^[62]

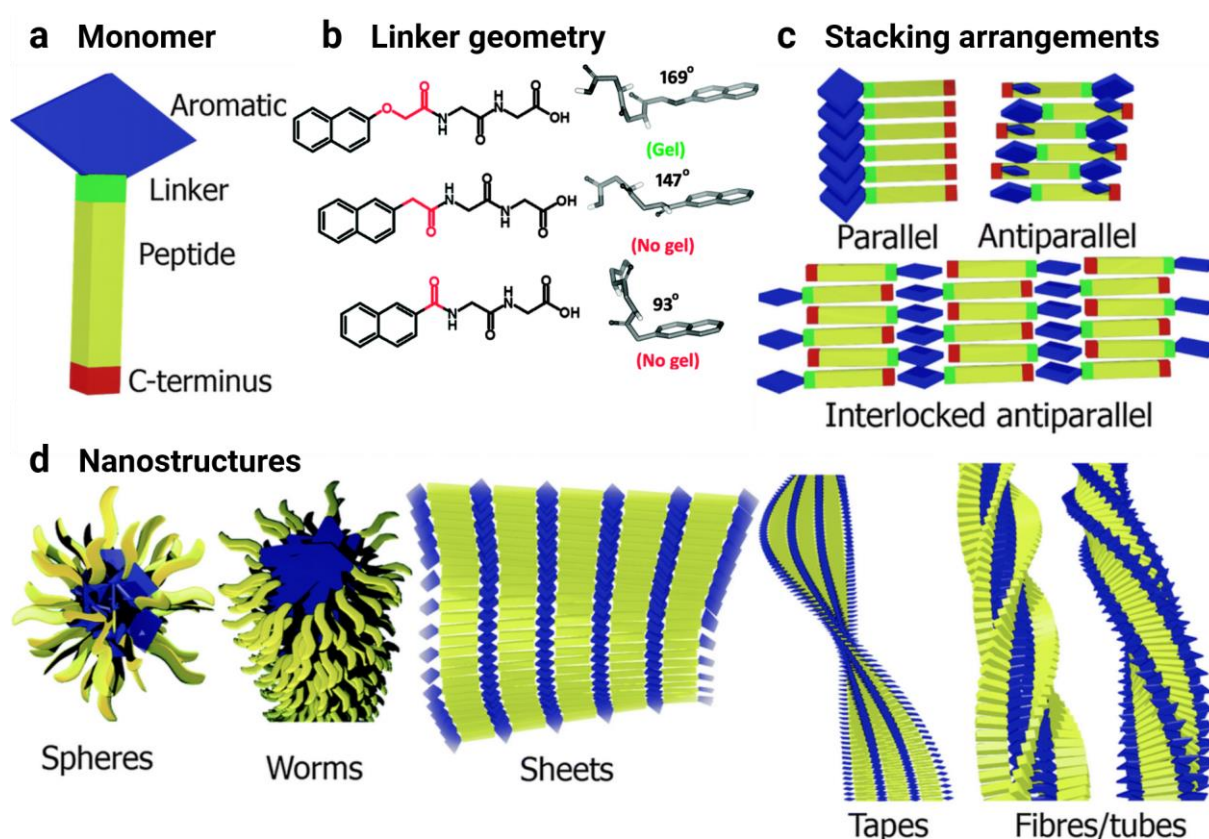


Figure 5: **a)** Schematic illustration of the different components of a short amphiphilic peptide. **b)** Potential stacking arrangements. **c)** The influence of the linker and its geometry on the gelation properties of a naphthalene-containing peptide. **d)** Supramolecular nanostructures assembled from a short aromatic peptide amphiphile. Adapted with permission from ref.^[45] Copyright 2014, Royal Society of Chemistry.

Other than the terminal modifications and linker design, the peptide sequence can also be customized to tune the non-covalent interactions and the morphology of the assembly. Glycine, an achiral amino acid, contributes to the system's flexibility, whereas proline, with its atypical secondary amide bond, imparts rigidity.^[63] The peptide sequence is instrumental in balancing the hydrophobic aromatic part with hydrophilic segments, thereby preventing precipitation.^[45] A common approach to facilitate the formation of β -sheets involves the incorporation of alternating polar and non-polar amino acids in the sequence. This approach is believed to result in a balanced interior/exterior geometry of the side-chain residues, thereby shielding the hydrophobic parts from the water.^[64] The C-terminus has the capacity to provide additional hydrophilicity, though this property is contingent upon factors such as pH and modification. The potential charge associated with the carboxylic acid can serve as a pH trigger within the system, leading to either assembly upon lowering or disassembly upon increasing the pH.^[65] Methylation of the C-terminus has been observed to induce solubility challenges, underscoring the necessity for incorporating additional hydrophilic amino acids to address these concerns.^[66]

As previously outlined, the assembly of these short aromatic peptides is governed by the amphiphilicity of the system.^[67] However, these subunits can stack in different conformations, thereby resulting in different structures. Parallel stacking of the aromatic groups is commonly seen in side-chain halogenated Fmoc-F and Fmoc-Y derivatives. In this parallel stacking conformation, the fibrils associate, thus ensuring the burial of the hydrophobic Fmoc-group in the peptide's core, while the hydrophilic C-terminal group is oriented towards the solvent.^[68] This phenomenon bears resemblance to the assemblies observed in aliphatic amphiphilic peptide systems.^[51] Conversely, antiparallel stacking is observed in peptides with aromatic side chains. This enables the complementary stacking of the aromatic end group and aromatic side chains (Figure 5c).^[69] In the absence of aromatic side chains, the peptide can also adopt an antiparallel conformation by interlocking with adjacent assemblies, thereby forming larger 2D-structures, such as sheets.^[45,70] In instances where the hydrophilic or hydrophobic part exerts a dominant influence, this imbalance frequently gives rise to the formation of worm-like or micellar structures, which exhibit a reduced degree of order in comparison to their stacked counterparts (Figure 5d).^[62]

Covalent synthesis

Single step

Multi step

Diagram illustrating covalent synthesis pathways. The single step pathway involves the reaction of nitrobenzene (c1ccccc1[N+](=O)[O-]) to form a complex molecule. The multi step pathway involves the reaction of styrene (C=Cc1ccccc1), HCl, and acetic acid (CC(=O)O) to form the same complex molecule, with reagents HNO3, NaOH, and H2O indicated.

Noncovalent synthesis

Diagram illustrating noncovalent synthesis pathways. The single step pathway involves the reaction of a complex molecule to form a large, multi-ring structure. The multi step pathway involves the reaction of a complex molecule to form the same large, multi-ring structure, with reagents H2O and HCl indicated.

In order to accentuate the intricacies inherent in this subject matter, Vantomme and Meijer reintroduced the term 'noncovalent synthesis' in 2019 with regard to the assembly of complex structures.^[72] Although this concept had been utilized by Whitesides and Reinhoudt in the 1990s, it had not been the focus of significant attention.^[73,74] This is primarily due to the fact that balancing entropic energy with non-

covalent interactions to achieve novel structures is a challenging task. Orthogonal assembly methods, compartmentalization, and selective catalysts can be used to direct the assembly towards different pathways.^[72] Furthermore, it is also possible to combine covalent and non-covalent polymerization techniques to achieve structures that are unachievable by either of the two components alone.^[75]

In general, three distinct thermodynamic regimes have been identified for supramolecular systems: equilibrium assemblies, kinetically trapped assemblies, and far-from-equilibrium assemblies. Equilibrium assemblies are at the thermodynamic minimum according to their respective Boltzmann distribution and are therefore stable over time. Kinetically trapped assemblies, on the other hand, are trapped in a local minimum and would convert to their thermodynamic minimum by the addition of an activation energy. Consequently, their stability is only transient. Finally, far-from-equilibrium systems require the constant addition of energy; otherwise, they would revert to their thermodynamic minimum or a kinetic trap. These systems are the most complex, but also offer the possibility of developing completely new functions (Figure 7).^[76]

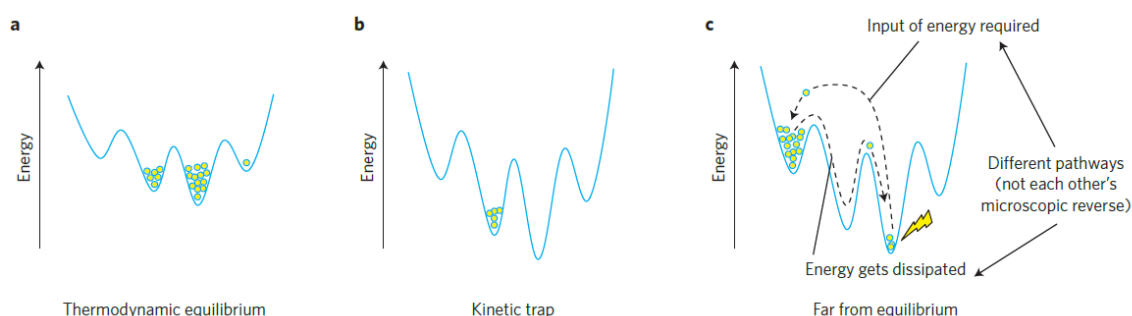


Figure 7: Energy diagrams of different assemblies. **a)** Thermodynamic equilibrium assemblies: Final state is determined by the Boltzmann distribution. **b)** Kinetically trapped assemblies: Final state is determined by the synthetic pathway. **c)** Far-from-equilibrium assemblies: Final state is determined by the balance between continuous synthesis and degradation pathway as well as the dissipation of energy. Reprinted with permission from ref.^[76] Copyright 2015 Springer Nature.

Structures in their thermodynamic equilibrium adopt the properties of their constituent parts. Through the judicious tuning of the chemical design of the monomers and their interactions, a variety of architectures can be synthesized. The employment of templates during the assembly process facilitates the creation of mechanically interlocked structures under thermodynamic control.^[77] The discovery of new systems is enabled through dynamic combinatorial chemistry, wherein building blocks

employing the same dynamic non-covalent interaction are mixed and then the relative abundance of products is analyzed. This process exemplifies the Boltzmann distribution within the library.^[78] This approach has been employed in the context of discovering the most stable host-guest interactions.^[79] It should be noted, however, that once these systems attain their thermodynamic minimum, any alteration to them is only possible by creating a new thermodynamic minimum. This can be achieved, for instance, by modulating the pH, thereby facilitating the protonation/deprotonation of groups within the assembly.^[76,80]

While these systems possess only one thermodynamic minimum and consequently manifest a single structure, numerous kinetically trapped assemblies can be observed within a single system. Each assembly of this kind possesses a distinct set of properties and functions, deviating from the equilibrium state. It is therefore crucial to select the pathway with the appropriate conditions in order to achieve the desired kinetic state.^[76] Once trapped, transitions become possible only downhill to a state with lesser thermodynamic energy, provided there is no external input of energy.^[81,82] This property can be leveraged to regulate the emergence and longevity of metastable states during the assembly process.^[83–86] This phenomenon was exemplified by a supramolecular polymer that assembles into helical fibers. Depending on the experimental conditions, two distinct helicities were observed, with the right-handed representing the kinetically trapped pathway and the left-handed representing the thermodynamic pathway (Figure 8a).^[71]

Catalysts have been shown to facilitate access to kinetically trapped systems through autocatalysis or the addition of external catalysts. Subtilisin, for instance, catalyzes the cleavage of ester bonds and has been employed to modulate the strength of supramolecular hydrogels through different extent of kinetically trapped structures. In this instance, Fmoc-dipeptides were modified with a methylester on the C-terminus, a modification that prevented assembly. Once the ester is cleaved by subtilisin, fibers composed of the dipeptide began to grow from the enzyme. By altering the amount of enzyme, it was possible to obtain structurally different gels of variable strengths while maintaining complete conversion of the precursor and therefore a constant concentration of gelator (Figure 8b).^[87] Autocatalytic systems have proven to be instrumental in the study of the origins of life, particularly in the context of the emergence of homochirality from a small initial enantiomeric excess.^[88] Furthermore,

kinetically driven dynamic combinatorial libraries have been employed to analyze the autocatalysis of supramolecular structures through an elongation and breakage mechanism. In this regard, thiol-functionalized peptides have been shown to form diverse ring structures through disulfide formation via air oxidation. Initially, the formation of trimers and tetramers predominates; however, over time, hexameric cycles become the predominant structural motif. This transition is attributed to the formation of hexameric fibers through a nucleation-growth mechanism. The application of mechanical force to the system results in the fragmentation of fibers, with the resulting fragments autocatalytically forming additional fibers (Figure 8c).^[89,90]

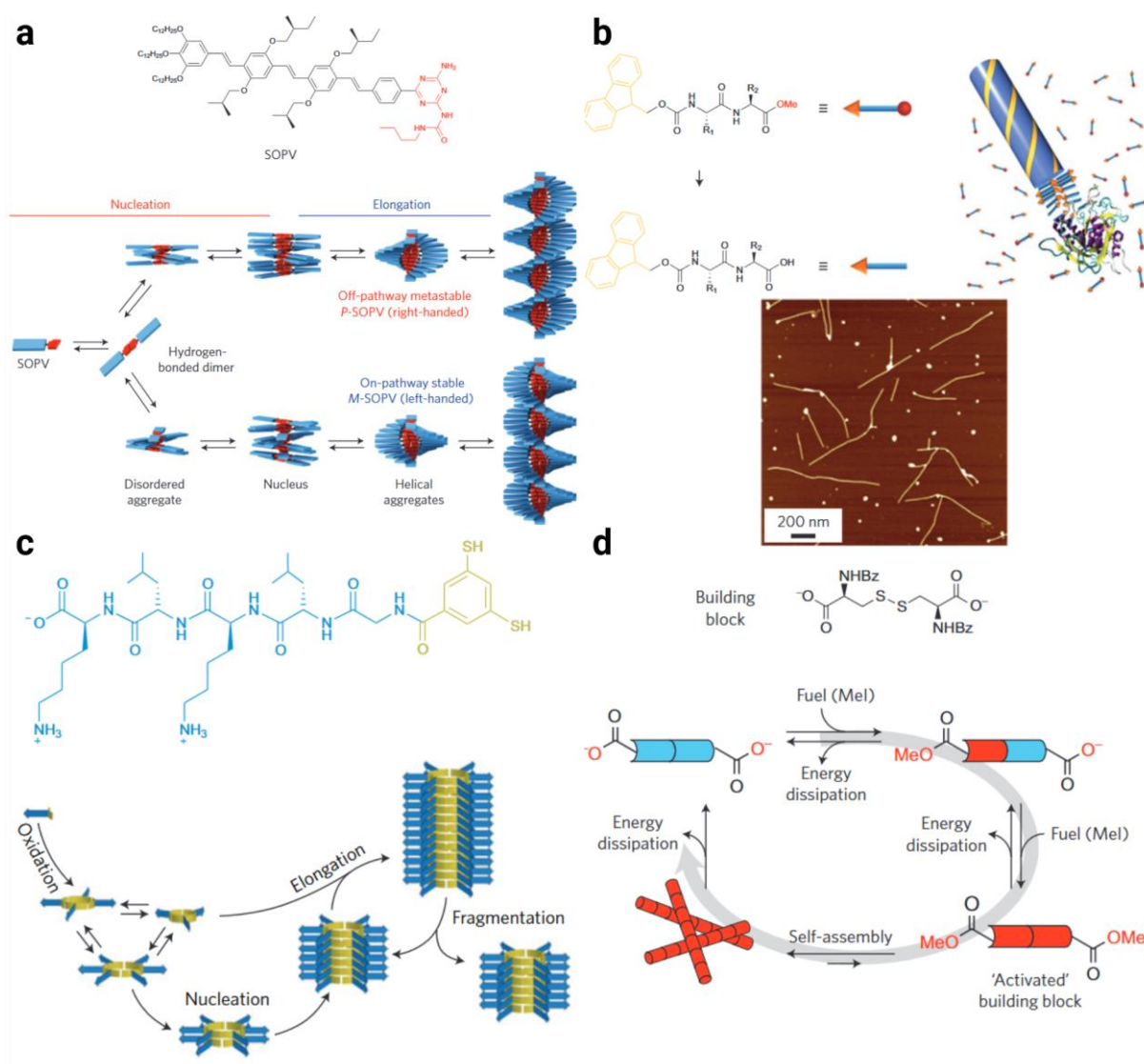


Figure 8: **a)** Supramolecular polymer consisting of the *S*-chiral oligo(*p*-phenylenevinylene) (SOPV) with two distinct helicities depending on the pathway. The right-handed helix is only metastable and is transformed to the left-handed over time.^[71] **b)** Kinetically trapped assembly by enzymatic catalysis, where a methyl ester is converted to the gelating carboxylic acid. By adjusting the enzyme concentration, the gel strength is modified.^[87] **c)** Dynamic combinatorial

library of a thiol-functionalized peptide building block show the emergence of autocatalytic behavior through a fibril elongation and breakage mechanism.^[76] d) Reaction scheme of the dissipative assembly of a dicarboxylate precursor. The carboxylates are methylated with methyl iodide resulting in the formation of a gel. Once the fuel is consumed the gel disassembles again.^[91] Reprinted with permission from ref.^[76] Copyright 2015 Springer Nature.

Another example of pathway selectivity that results in distinct structures is the assembly of peptide amphiphiles in 20% HFIP in water. Despite maintaining equivalent solvent composition and peptide concentration, molecular organization and aggregate size are contingent on the sequence of additions of the peptide stock and HFIP. Adding the peptide stock first leads to an assembly of larger β -sheets, compared to smaller random coils for the addition of HFIP first. This outcome is attributed to variations in nucleation speed within the solvent compositions.^[92]

Far-from-equilibrium systems require the continuous dissipation of energy to preserve their structural integrity. This dissipation enables transitions between various thermodynamic states, a phenomenon that is absent in systems constrained within kinetic traps or in thermodynamic equilibrium. However, analyzing these structures necessitates a steady state and, consequently, a constant flow of matter if a chemically fueled system is employed.^[76] To circumvent this necessity, the utilization of light as a source of energy has emerged as a prominent approach, exemplified by the employment of azobenzene-switches. These azobenzenes, when attached to nanoparticles, undergo a Z- to E-isomerization. This process generates a dipole moment in the azobenzenes, leading to their aggregation. Conversely, in the absence of continuous irradiation, the assembly reverts to its original molecular state.^[93] Despite the increased complexity inherent in the setup, the utilization of chemical fuels for far-from-equilibrium assemblies is indeed feasible. This phenomenon was exemplified through the utilization of methyl iodide as the fuel source, which facilitated the alkylation of the carboxylic acid end groups of a diacid. Consequently, the electrostatic repulsion that had previously hindered assembly was neutralized, resulting in the formation of a gel. However, upon the depletion of the fuel, the system undergoes disassembly due to the continuous hydrolysis of the ester. Notably, the reintroduction of fuel into the system results in the formation of a new gel, once again exhibiting far-from-equilibrium behavior (Figure 8d).^[91]

1.3.1 Controlling the assembly of nanostructures

The formation of complex structures often necessitates the ability to regulate both the assembly and disassembly processes. This can be achieved by incorporating responsive groups that modify the morphology of the system in response to a stimulus.^[94] Nature has demonstrated numerous instances of this phenomenon, including the allosteric shift between two conformations of hemoglobin with different affinities for oxygen, depending on the presence of small molecules such as protons, carbon dioxide, or 2,3-bisphosphoglycerate.^[95] The assembly of supramolecular structures can be influenced by various stimuli, including light, pH and redox reactions. These stimuli can either modulate the noncovalent interactions between the subunits or the chemical structure of the monomers.^[94]

The utilization of light as a stimulus offers numerous advantages, including low biological toxicity, spatiotemporal resolution, non-invasiveness, and the absence of waste products. The employment of different wavelengths enables the execution of orthogonal reactions. However, this process necessitates the synthetic modification of targeted peptides with light-responsive groups, which can be categorized into three main types: Photocleavable, photoswitchable, and photodimerizable units, each of which exhibits a distinct mode of action and, consequently, a unique effect on the assembly (Figure 9).^[94,96]

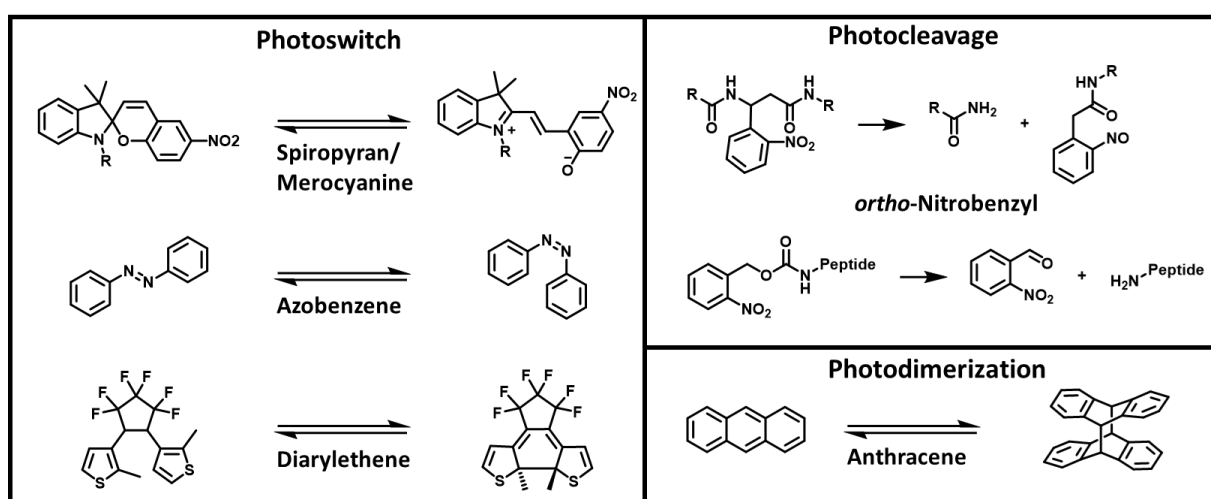


Figure 9: Three classes of photoresponsive used for the controlled assembly of peptides.

Photocleavable units are designed to undergo an irreversible reaction upon exposure to light, with their integration occurring within the peptide backbone, at the terminal

end of the peptide, or via attachment to a side-chain. Notable examples include *ortho*-nitrobenzyl derivatives, which were first utilized in 1978 to cage ATP.^[97] These units are cleaved when irradiated between 300-365 nm, a range that can be tuned by the addition of methoxy groups to the aromatic ring. The cleavage process is characterized by a Norrish-type II mechanism, which involves an intramolecular rearrangement.^[98–100] This process initially involves the transfer of a benzylic proton to the oxygen of the nitro group. Subsequent to reprotonation, the formation of a cyclic intermediate is postulated, which subsequently dissociates into a nitroso-compound and the now uncaged species (Figure 10).^[101–103] The decay of the aci-nitro anion has been identified as the rate-determining step in this mechanism, exhibiting a pH dependency.^[104]

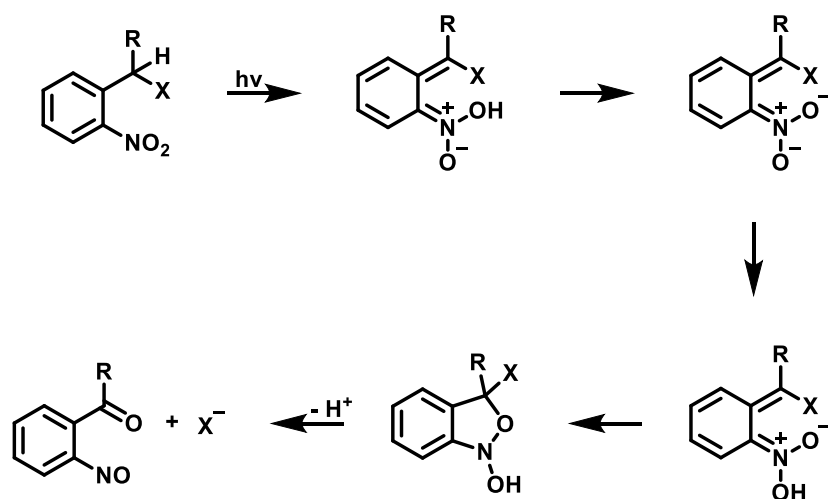


Figure 10: Mechanism of the cleavage of the light-responsive ortho-Nitrobenzylgroup.^[99]

The *ortho*-nitrobenzyl can be attached to an unnatural amino acid, thereby functioning as a linker between two fragments. By employing a polycationic fragment on one side of the linker and an assembling peptide sequence on the other, control over assembly is achieved. The polycationic side initially impedes the assembly process through electrostatic repulsion; however, following irradiation, this hydrophilic portion is cleaved, leading to the formation of supramolecular peptide fibers after an incubation period of several hours.^[105] Alternatively, the nitrobenzyl group can be attached as a side-chain, resulting in its self-elimination and alteration of the system's hydrophobic/hydrophilic balance. This approach has been utilized to modulate the morphology of a peptide amphiphile, transitioning from quadruple helical fibers with high aspect ratios to non-helical single fibers after a 5-minute irradiation at 350 nm (Figure 11a).^[106] It is important to note that the photocleavage has the potential to

initiate a cascade reaction. The photoresponsive group was installed on the N-terminus of an isopeptide, which is connected by an ester bond rather than an amide. Irradiation of the system leads to the release of a free amine, which can undergo an O-N acyl shift and thereby create the linear peptide. In contrast to the kinked isopeptide, the linear peptide is able to assemble.^[107]

Photoswitches are dependent on the reversible isomerization between *cis* and *trans* or open and closed forms, respectively, which exhibit different polarities. Azobenzenes are the most common *cis/trans* photoswitch due to their accessible synthesis and modification.^[108,109] They can function not only as a switch but also as a guest molecule for certain macrocyclic hosts, such as cyclodextrins.^[110,111] The reverse transformation/isomerization of azobenzenes is possible upon irradiation with visible light as well as thermally.^[96,108] The azobenzene-switch has been utilized as a linker in the peptide backbone of amyloid- β 42, where it has been observed that the *trans* state results in fibrous assemblies, while the *cis* state leads to amorphous aggregates. This morphological switch has been shown to translate into differential toxicity, with the *trans* version exhibiting similar toxicity to the wild-type amyloid, while the *cis* state demonstrates no toxicity.^[112]

Spiropyranes transition from an uncharged state to the zwitterionic merocyanine form upon irradiation with 365 nm light, thereby exerting a substantial structural influence on the system. The observed structures are contingent upon the configuration of the system and the prevailing experimental conditions, with structures either forming in the spiropyran or the merocyanine form.^[113,114] For instance, the chaperone GroEL has been observed to assemble into nanotubes when modified with merocyanine, a process facilitated by the coordination of Mg^{2+} . However, these nanotubes undergo dissociation upon exposure to visible light, as the photoswitch undergoes an isomerization back to the uncharged spiropyran.^[115] Conversely, the short amphiphilic peptide Fmoc-KK^{SP}KF-NH₂, featuring spiropyran (SP) sidechain modification, undergoes β -sheet fiber formation under the influence of visible light. However, removal of the light source results in disassembly due to the thermally induced isomerization of the switch to its more stable zwitterionic merocyanine form, which exhibits augmented charge repulsion.^[116]

A third class of photoswitches comprises diarylethene derivatives, which undergo a transition between an open form characterized by a flexible, twisted conformation and

a closed form distinguished by a rigid, planar structure. In contrast to the other switches, the polarity of the system is minimally altered, rather, the capacity to stack the planar, hydrophobic rings is enhanced. Moreover, the reverse reaction is thermally inaccessible.^[94,117,118] The incorporation of diarylethene within an amphiphilic molecule has been demonstrated to facilitate a reversible transition between nanofibers and spheres.^[94]

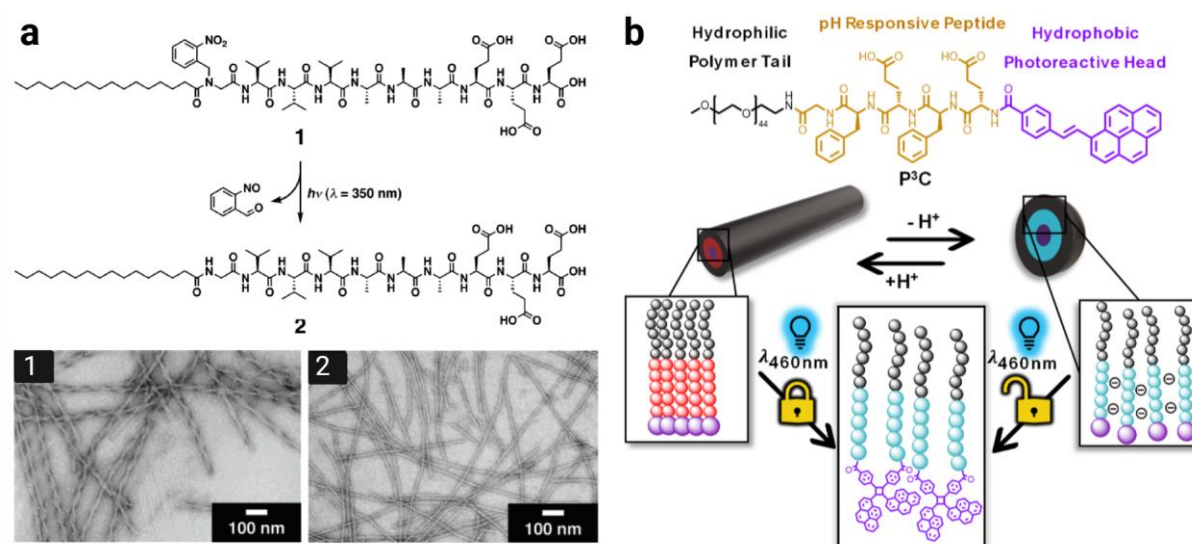


Figure 11: a) Cleaving of the ortho-Nitrobenzyl group off a peptide amphiphile leads to a morphology change from quadruple helical fibers to single fibers as shown in the TEM images. Adapted with permission from ref.^[106] Copyright 2008, American Chemical Society. **b)** Light- and pH-responsive amphiphilic peptide requires higher pH values to achieve a preorganization of the peptides that facilitate the [2+2] photocycloaddition. In an acidic environment as well as in solution there is no photoreaction. Reprinted with permission from ref.^[119] Copyright 2023, American Chemical Society.

A particular instance of photoresponsiveness is photodimerization, which requires an intermolecular reaction, in contrast to the previously demonstrated examples. There are multiple aromatic or unsaturated groups that are compatible with this category of reactions, each requiring distinct wavelengths.^[96] These reactions are not thermally allowed according to the Woodward-Hoffmann rules and therefore require light for the excitation of an electron and subsequently to achieve an orbital overlap between the two reaction partners. Therefore, these intermolecular reactions frequently necessitate a specific spatial orientation to achieve the suprafacial conformation and elevated concentrations to efficiently dimerize.^[120–122] Consequently, they can exhibit selectivity through preorganization within an assembled structure. This phenomenon was exemplified in the [2+2] photocycloaddition of a styrylpyrene unit that was

modified with a pH-responsive peptide. In acidic conditions, this monomer assembles into 1D fibers, but only exhibits minor dimerization. However, upon increasing the pH to 10, the pH-responsive glutamic acids induce electrostatic repulsion, thereby altering the morphology of the structure to micellar clusters, where a high degree of dimerization efficiency is achieved. It is noteworthy that dimerization did not occur in an unassembled state in DMF, even at higher concentrations (Figure 11b).^[119]

Anthracene can undergo a reversible [4+4] cycloaddition upon irradiation with 365-nm light, and it possesses a large aromatic π -system, capable of exhibiting π - π stacking. This property renders anthracene a promising candidate for substituting the Fmoc-group in short amphiphilic peptides, while concurrently facilitating efficient photodimerization. Upon exposure to light, the dianthracene adopts a bent conformation, which can result in a change in morphology. This phenomenon was observed in the context of nanotubes, where the dimerization of anthracene resulted in a bending of the structure.^[123] A further example is the reversible transition between nanofibers and nanoparticles in a peptide with a sidechain anthracene modification.^[124]

pH-responsive systems depend on ionizable groups in their structure, such as carboxylic acids or amines. Alternatively, the incorporation of pH-cleavable groups, such as hydrazones or imines, can be employed.^[94] Given the prevalence of basic or acidic groups in the primary structure of peptides, the incorporation of a pH trigger often obviates the necessity for additional functional synthetic groups. Peptide amphiphiles, for instance, comprising multiple glutamic acid residues, exhibit a pH-dependent transition between fibers and micelles, as well as single molecules, as a function of peptide concentration. The relationship between these variables can be mapped out in a phase diagram through a combination of circular dichroism and the determination of the critical assembly concentration (Figure 12a).^[125] Furthermore, it was found that by isomerizing the peptide sequence, the pH required for initiation of the supramolecular polymerization could be tuned.^[126] In this particular instance, the formation of fibers is initiated by the acidification of the system, which in turn leads to the protonation of the carboxylic acid side chains. Conversely, the utilization of basic side chains has been observed to induce assembly when the pH is elevated above 7.^[127] Due to the importance of pH gradients in cells, the employment of pH-cleavable groups has been a recurring theme in the domain of drug delivery applications. An

acid labile aconityl linkage is incorporated between a polyethyleneglycol chain and a peptide, which form drug-loaded micelles. Upon acidification, the micelle undergoes degradation, resulting in the release of the encapsulated drug.^[128]

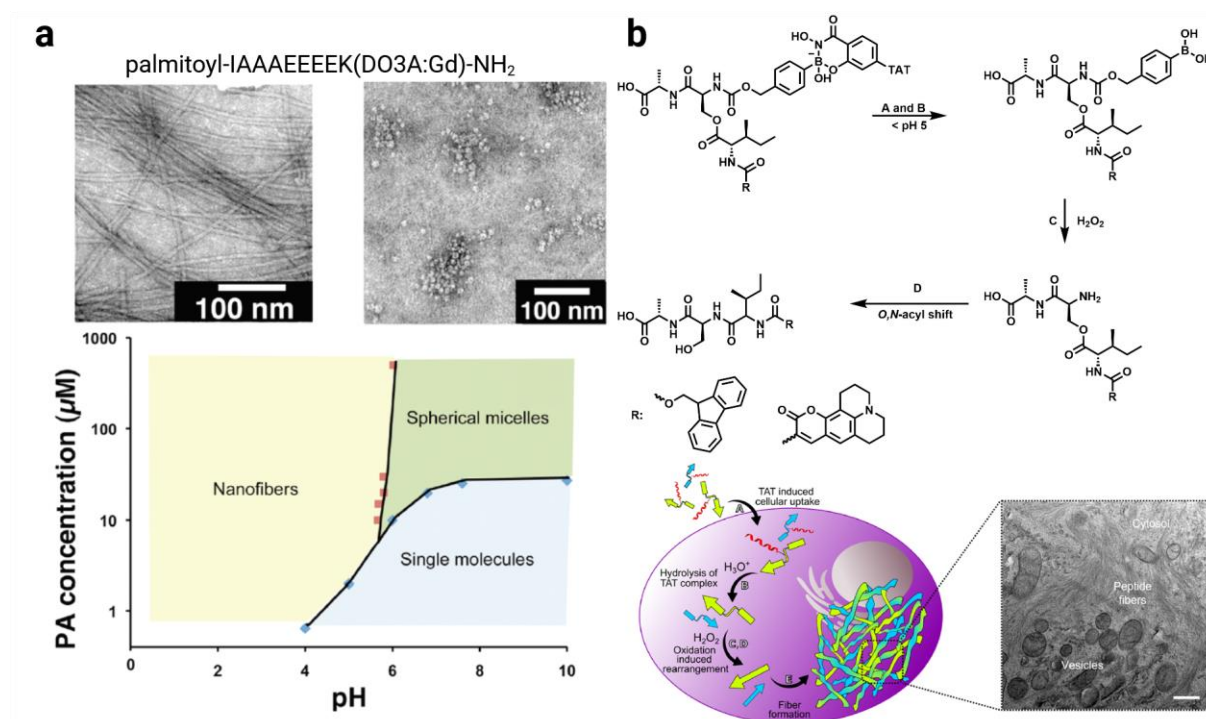


Figure 12: a) TEM images of a pH-responsive peptide amphiphile showing either nanofibers or spherical micelles. By a combination of CAC determination and CD spectroscopy a phase diagram of the peptide assembly is measured. Reprinted with permission from ref.^[125] Copyright 2012. American Chemical Society. **b)** Reaction cascade of a redox- and pH-responsive system that generates peptide fibers inside cancer cells due to elevated concentrations of reactive oxygen species. Adapted from ref.^[129] Copyright 2020 American Chemical Society, licensed under CC-BY.

Redox reactions are also employed to regulate the assembly process, with the capacity to target either redox-active proteinogenic amino acids, such as cysteine, selenocysteine, methionine, or synthetic functional groups such as boronic acids or phenothiazines.^[130–132] An oxidative environment is frequently induced by H₂O₂ or other reactive oxygen species (ROS), while mono- and dithiols, such as GSH or DTT, are utilized for reductive conditions.^[133–136] The integration of pH and redox-triggered systems has been demonstrated to result in an enhanced selectivity with respect to the location of fiber assembly. The dynamic covalent bond between salicylhydroxamate and phenylboronic acid is disrupted upon acidification, thereby releasing the phenylboronic acid. Subsequent oxidation of the phenylboronic acid by H₂O₂ occurs in a self-immolative process to release the amine of an isopeptide where

it rapidly undergoes an O-N acyl shift to form the linear peptide. The linear amphiphilic peptide then proceed to self-assemble into nanofibers (Figure 12b).^[129] Peptide fibers can also be obtained in a reductive environment through the employment of a disulfide linker, which functions as a connector, joining a hydrophilic polyethyleneglycol (PEG) chain to a hydrophobic peptide sequence. With the flexible PEG attached, the polymer-peptide conjugate form micelles in solution. Cleavage of the disulfide bond of the linker by GSH leads to the release of the peptide and subsequent assembly into fibers.^[137] Similar design strategies and operating mechanisms using enzymes,^[138–141] ionic strength,^[142] temperature,^[143,144] or dilution^[145,146], have since been used to gain control over the assembly of nanostructures.

1.3.2 Controlling the disassembly of nanostructures

The capacity to regulate the disassembly of nanostructures is imperative in both naturally occurring processes, such as the recycling of misfolded proteins, and in synthetic peptide materials research. These structures can be utilized to study the disassembly of complex proteins in a more simplified and controlled environment, thereby facilitating the acquisition of insights into the treatment of Alzheimer's disease, a condition that arises from the aggregation of misfolded proteins.^[147] Furthermore, the utilization of peptide assemblies as a drug delivery mechanism necessitates the precise modulation of disassembly and release at designated locations.^[148] This approach would also minimize adverse effects when if a therapeutically active peptide structure could be disassembled into less active monomers at off-target sites and therefore enhance the biocompatibility.^[149]

The disassembly process is predominantly initiated by modifications of the non-covalent intermolecular interactions. This can result in alterations to the balance between hydrophobic and hydrophilic components or the increase in geometric frustration within the system. This can be conceptualized as a defect arising from the incompatibility of local interactions with global geometric constraints. In the event that this exceeds the attractive interactions of the system, disassembly occurs.^[150–152]

The merits of light as a stimulus have previously been documented, thereby establishing its suitability for triggering disassembly. The photocleavable ortho-Nitrobenzyl derivative Nvoc functions as a cage for the sidechain amine of lysine,

thereby converting it into a hydrophobic state. Consequently, it can substitute for phenylalanine in the amyloid- β derived sequence KLVFFAE without altering its propensity to assemble into fibers. Upon irradiation, the charged lysine is released, perturbing the amphiphilic balance of the assembly as well as the β -sheet formation, and consequently causing the structure to irreversibly disassemble. Notably, this process remains feasible even when utilizing 40% of the unmodified peptide.^[147]

In addition to its utilization as a sidechain modification, the photocage has also been employed in the backbone of an assembling peptide. The sequence CKFK-nvoc-FQF has been observed to assemble into amyloid-like fibrils; however, the fragments on each side of the photocleavable linker have been found to lack sufficient supramolecular interactions to assemble independently, resulting in their disassembly upon irradiation. The employment of a photomask on a surface coated with preformed fibrils has been demonstrated to facilitate the generation of patterns of nanostructures. These nanostructures have been utilized to define areas of cell adhesion, as they adhere exclusively to the non-irradiated regions containing fibrils (Figure 13a).^[153]

The disassembly due to geometric frustration was demonstrated for a supramolecular nanotube that is assembled by aromatic interactions and contains two azobenzene photoswitches per monomer. Irradiation of the nanotube leads to the transformation of the azobenzene photoswitches into the cis-configuration, thereby inducing strain within the system. Once a threshold is attained, the strain is relieved through the disassembly of the tube. While the switches may thermally isomerize back to their trans-state, the supramolecular structure remains irreversible, as a persistent increase in the trans-version fails to generate the same cooperative effects as if all monomers are uniformly present in their trans-isomer (Figure 13b).^[154]

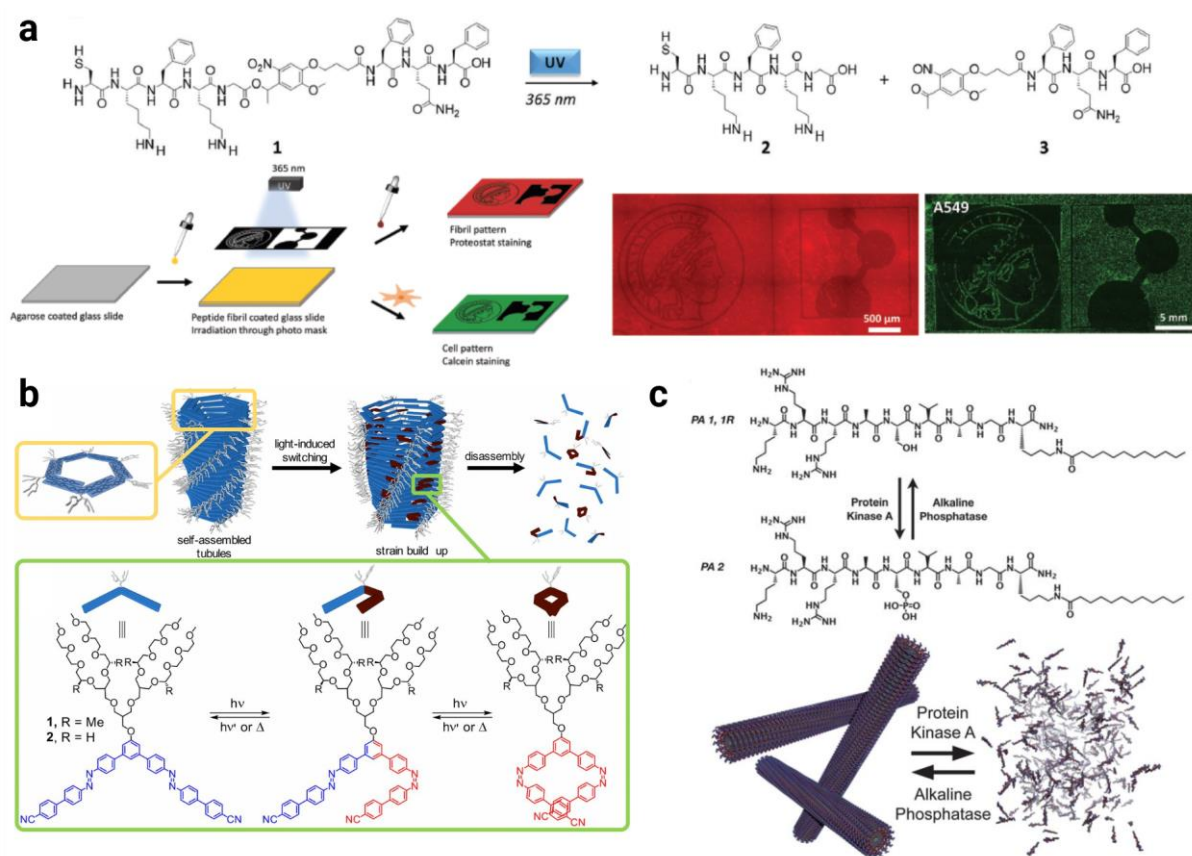


Figure 13: a) Light-responsive cleavage of the ortho-Nitrobenzyl group incorporated in a peptide backbone causes disassembly and thereby enables the patterning of fibrils and cells through a photomask. Adapted from ref.^[153] Licensed under CC-BY 4.0 **b)** Disassembly of a synthetic supramolecular tube is achieved by the build up of strain due to light-triggered switching of azobenzene units within the tube. Reprinted with permission from ref.^[154] Copyright 2017, National Academy of Sciences. **c)** Reversible disassembly of peptide nanofibers by enzymatic phosphorylation. Reprinted with permission from ref.^[155] Copyright 2012 Royal Society of Chemistry.

The combination of two stimuli frequently results in an accelerated disassembly. For instance, the pH-responsive peptide F20H can assemble into long nanofibrils at pH 9.5 due to the incorporation of a histidine residue. Conversely, decreasing the pH to 5 initiates a disassembly process by protonation of the basic amino acid. However, this process requires a duration of 5 days. The ultrasonication of the sample following acidification has been shown to accelerate this process, resulting in disassembly within a span of six hours.^[156]

The incorporation of methionine can serve as a disassembly trigger within a nanostructure due to its redox sensitivity. The cross- β -sheet forming sequence KIKISQINM displays methionine on the side of the fibril; however, it can be oxidized to the corresponding sulfoxide by hydrogen peroxide. This reaction renders the amino

acid hydrophilic, thereby disrupting the hydrophobic interactions and inducing the disassembly of the fiber.^[157]

The employment of enzyme-responsive groups for disassembly confers certain advantages, including the ability to operate under mild conditions and achieve selectivity depending on enzyme expression levels. The Stupp group has generated nanofibers from a peptide amphiphile containing the sequence RRXSO, where X can be any residue and O is a hydrophobic residue. The use of the enzyme protein kinase A facilitated the disassembly of this structure due to the phosphorylation of the serine residue, rendering the subunits too hydrophilic to remain assembled. Notably, the serine, which was situated within the central part of the structure, remained accessible to the enzymes. However, when the serine was shifted by one amino acid (RRSAV), no phosphorylation occurred, thereby demonstrating a high selectivity of this process (Figure 13c).^[155]

1.4 Application of supramolecular peptide biomaterials

Peptide nanostructures have demonstrated considerable potential as responsive materials within the domain of biomedicine, exhibiting applications in various fields including drug delivery, imaging, tissue engineering, and therapeutics. In comparison to small molecules, these biomaterials possess several advantageous properties, including reduced side effects, enhanced biocompatibility and biodegradability, prolonged circulation time as a carrier, and reduced propensity for drug resistance. Furthermore, these materials possess the capacity to respond to specific intracellular stimuli, such as an increase in GSH concentration in the cytosol or a decrease in pH in endosomes. This property enables them to exhibit a targeting effect.^[133,158–161]

In general, the assemblies can be divided into ex situ and in situ constructed structures, depending on whether they are formed before or after reaching their target site. The latter category is contingent upon a previously described stimuli responsiveness to trigger the assembly.^[161] Ex situ materials offer high stability, and their tumor uptake can be facilitated by the enhanced penetration and retention effect (EPR).^[161] These assemblies find application in medical imaging due to their capacity to incorporate multiple imaging and targeting units within a single nanostructure. This functionality has been demonstrated with a peptide containing a Gd-complex that

functions as an MRI contrast agent. These nanostructures assemble into nanofibers, resulting in an increase in the T_1 relaxation time compared to conventional contrast agents.^[162] Peptide nanostructures have also demonstrated suitability as therapeutic agents themselves, exhibiting a wide range of functions and defined morphologies while being amenable to synthesis. Hydrogels composed of a 20-amino acid sequence, predominantly valine and lysine, exhibited a broad spectrum of antibacterial properties due to the disruption of bacterial membranes at the surface of these nanostructures. Notably, this effect was selective against bacteria, as mammalian cells could still proliferate on the surface.^[163]

Peptides have garnered significant attention as therapeutic agents as well as adjuvants for retroviral gene delivery. One such example involves the assembly of an amphiphilic block-like peptide into positively charged core-shell fibrils, which are located on the cell membrane and facilitate virus adhesion to the cell. These fibrils also exhibit good biodegradability through enzymatic cleavage.^[164]

Regenerative materials represent an emerging field within the biomedical context, and peptide nanomaterials have the potential to serve as a scaffold for tissue growth due to their broad range of biocompatible structures. This property was exemplified by three fiber-forming peptides identified through a screening approach, which have the capacity to provide a bioactive scaffold for the regeneration of the peripheral nervous system. These active peptides are distinguished by their propensity to form fibers with a substantial cross-sectional diameter and a high intermolecular β -sheet content, a property attributable to the alternating pattern of hydrophobic and hydrophilic positively charged amino acids.^[165]

In situ assembled supramolecular nanostructures have been employed in cancer therapy due to their capacity to induce cell death by disrupting cellular structures through mechanical stress. The intrinsic propensity of monomers to assemble is enhanced by the crowding of the cellular environment by soluble proteins or phase-separated components. The judicious selection of appropriate stimuli in conjunction with targeting units can ensure subcellular selectivity.^[133,166,167] One of the earliest reported instances of stimuli-responsive peptide nanostructures was in 2007, where the assembly was initiated by esterase enzymes that cleaved the hydrophilic part, leading to intracellular assembly and subsequent cell death.^[139]

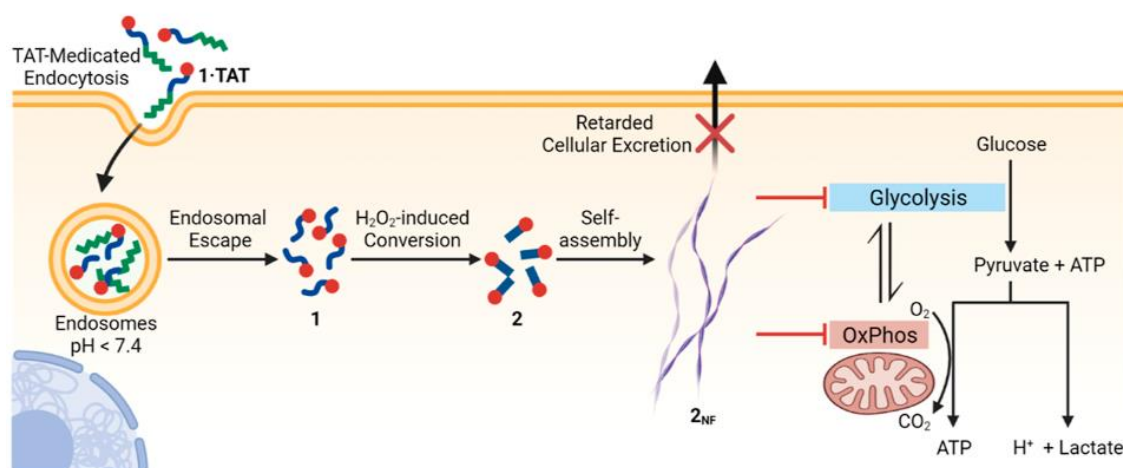


Figure 14: Schematic representation of the intracellular H₂O₂-induced formation of linear platinum(II)-containing peptides that assemble into nanofibers and disrupt the cellular metabolism. Reprinted from ref.^[168] Licensed under CC-BY 4.0.

This pioneering concept of utilizing a pro-assembling precursor was further advanced and integrated with an imaging component to visualize the process. Using the isopeptide strategy, a platinum (II) containing tripeptide was caged with a boronic acid linker. The linker is then further functionalized with the trans-activator of transcription (TAT), which is responsible for cellular uptake and endosomal release. It is subsequently cleaved by an acid-sensitive trigger that utilizes the lowered pH in endosomes. Following this, the boronic acid is removed by endogenous H₂O₂, and the NIR-emitting tripeptide is linearized and assembles into nanofibers. The resulting nanofibers exert an inhibitory effect on cellular metabolism, culminating in the termination of ATP production and, consequently, cell death. This strategy has been shown to circumvent the resistance of cancer cells to small-molecule inhibitors (Figure 14).^[168]

2 Motivation

Evolution has led to the development of a sophisticated system for the precise regulation of assembly and disassembly processes, both within and outside of cells, even though the cellular environment is packed with a multitude of potential reaction partners. This regulatory system possesses multiple layers with different complexity starting from the tuning of interactions with responsive groups, such as redox-responsive cysteines or pH-responsive glutamic acids up to the employment of chaperones and enzymes to regulate the assembly and disassembly of these structures.

The assembly of structures such as the cytoskeleton forming proteins actin and microtubule is not thermodynamically controlled and therefore the morphology of these systems is not inherited by the chemical structure of their components. Instead, the assembly is reliant on the assembly pathway that can generate different conformations and morphologies without altering the chemical structure. This is achieved by kinetically controlled or far-from-equilibrium assemblies, which require the constant dissipation of energy to maintain the transient structures. Chemists are striving to replicate the control over assembly with stimuli-responsive systems and in contrast to natural systems, synthetic modulation of the chemical structure of amino acids and peptides allows for a wide range of potential triggers and morphologies. However, it remains challenging to achieve complex synthetic systems with control over the selected pathway as well as completely understanding their design principles.

This thesis aims to address this challenge by expanding our toolbox for modulating precise nanostructures with control over pathway selectivity. This control can be achieved by carefully designing stimulus-responsive peptides that facilitates the interplay between molecular and supramolecular chemistry.

While there are many options for stimuli-responsive groups in a chemical or biological context, light offers many advantages such as spatiotemporal resolution, minimal waste products or the possibility to perform orthogonal reactions with different wavelengths. The irreversible photocleavage of 2-nitrobenzyl units by UV-irradiation, for instance, can initiate cascade reactions, while concurrently releasing nitroso compounds that act as an oxidation agent.^[99,169] This can create different assembly

pathways when employing redox-responsive amino acids, such as cysteine. To gain a deeper understanding of the controlled assembly, these concepts can also be applied to a more complex environment such as cells, while also increasing the toolbox of suitable stimuli-responsive groups. Disulfide, for instance, offer a responsivity towards characteristics of the cellular environment, such as increased levels of GSH, making the assembly selective towards the cytosol.

Although nature usually shows precise control over the assembly process, there are still structures that misfold and can subsequently induce harm. Therefore, there are additional mechanisms that can either assist in refolding the structures or disassembling and degrading them. For instance, the ubiquitin-proteasome system can tag misfolded proteins and degrade them enzymatically by proteasomes. However, this is not successful for all misfolded structures such as the cross- β -sheet amyloid networks, which can lead to the formation of neurodegenerative amyloid plaques and subsequently cause Alzheimer's disease. Hence, it is necessary to improve our knowledge of the disassembly process to develop solutions in the long-term for the treatment of aggregation related diseases and further establish an end-of-lifetime trigger for the disassembly of synthetic nanostructures used as therapeutics. This requires precise control over the disassembly process.

This thesis aims to address that challenge by using photodimerization as a new trigger for disassembly. In contrast to monomolecular isomerizations or cleavage reactions, the bimolecular photodimerization renders its kinetics dependent on the chemical environment, thus allowing for modulation through preorganization within a nanostructure.

In Chapter 3, the [4+4] photodimerization of anthracene is utilized to regulate the disassembly of two supramolecular structures that form either a 2D-sheet or twisted ribbons. The two peptides demonstrate analogous structural motifs, comprising a pH-responsive lysine group, but showing different effects upon increasing the pH. The preorganization within the supramolecular structure is capable of leading to either accelerated or decelerated reaction kinetics, as well as influencing the conversion of the anthracene moiety. Therefore, it is possible to modulate the molecular chemistry required for disassembly by the supramolecular architecture.

In Chapter 4, a pro-assembling iso-peptide is demonstrated, that exhibits pathway selectivity. This is achieved by combining the light-cleavable caging group Nvoc with a redox-responsive cysteine in the peptide sequence. UV-irradiation initiates a reaction cascade that linearizes the peptide and releases a nitroso moiety from the caging group, which, in turn, is capable of oxidizing the thiol group of cysteine to a disulfide. This reaction cascade leads to different morphology of the disulfide assembly compared to the assembly by dissolution of the disulfide. Furthermore, the oxidation reaction can be modulated by the introduction of dithiothreitol (DTT) as reducing agent, thereby cycling between the redox states and creating a third morphology of one supramolecular monomer. Moreover, modifying the DTT concentration allows for control over the transience and interconversion of the peptide in the respective oxidation state within the cascade.

In Chapter 5, a glutathione-responsive iso-peptide is utilized as a precursor for the intracellular formation of nanostructures. This peptide is equipped with a cell-penetrating peptide and assembles selectively in cells due to the elevated levels of GSH in comparison to the extracellular environment. This process is observed in real-time by live-cell and fluorescent lifetime imaging and therefore providing insight in the assembly of nanostructures in the complex environment of the cytosol. Furthermore, the resulting nanostructures induce metabolic disruption and mitochondrial degradation in drug-resistant MDA-MB-231 triple negative breast cancer cells, culminating in cell death both in two-dimensional (2D) cell culture and three-dimensional (3D) tumor spheroid models.

3 Light triggered disassembly of peptide nanostructures

Authors

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Contributions

Raphael Meyer:	Conceived the project, performed the synthesis of the peptides, performed UV-VIS spectroscopy to generate the calibration curve, performed the irradiation kinetic experiments in solution and in assembly and analyzed the remaining monomer concentration with the calibration curve, performed the NMR irradiation experiments at different pH values, analyzed the secondary structure of both peptides at different pH before and after irradiation using Circular Dichroism, performed the Proteostat assay to analyzed the β -sheet content of the assemblies, prepared the samples for TEM measurements, data analysis and interpretation of all corresponding results, and wrote and edited the manuscript.
Julian Link:	Performed TEM measurements and interpretation of results.
Lucas Gunkel:	Performed FTIR measurements and interpretation of results.
Albin Lahu:	Performed TEM measurements and interpretation of results.

Hakan Demirezen:	Performed TEM measurements and interpretation of results.
Tanja Weil:	Acquiring funding for the project, design and discussion of the concept and results. Editing of the manuscript.
David Y. W. Ng:	Design and discussion on the concept and results. Editing of the manuscript.

Light-Triggered Disassembly of Peptide Nanostructures

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Abstract:

While the assembly of supramolecular peptide nanostructures with diverse functions and morphologies has been extensively studied, the controlled disassembly of these architectures remains less understood. To address this, we designed two short amphiphilic peptides incorporating anthracene as a light-responsive unit and lysine as a pH-sensitive residue. These peptides self-assembled into nanosheets or nanoribbons with distinct secondary structures, which were further tunable by pH through modulation of peptide charge. Upon irradiation at 365 nm, the anthracene moieties underwent a bimolecular [4+4] cycloaddition, disrupting the π - π stacking interactions by distorting the planarity of the aromatic units. This photoreaction led to disassembly of the supramolecular architectures within ten minutes. Unlike monomolecular reactions such as photocleavage, the kinetics of this bimolecular photodimerization were significantly influenced by the degree of preorganization within the assemblies. These findings underscore the critical interplay between supramolecular architecture and molecular photochemistry, enabling light-triggered, structure-dependent disassembly of diverse peptide nanostructures.

Introduction

Supramolecular peptide nanostructures have emerged as versatile biomaterials^[1,2], addressing a broad range of applications in biomedicine^[3] and tissue engineering^[4] as they offer scaffolds that can be customized with diverse functionalities. Moreover, their synthesis routes are well-

advent of modern biorthogonal chemistries, thus bringing forth the next generation of materials that exhibit various depths of adaptivity, mechanochemical and viscoelastic properties.^[5–7] Despite these seminal advancements in the supramolecular assembly of peptide materials,^[8–13] their disassembly upon completion of their designated tasks, while equally critical, is much less explored.

One of the main reasons is that a large proportion of supramolecular peptide materials operates dominantly on β -sheet type interactions found in neurodegenerative amyloid plaques and pathogenic protein aggregates.^[14–16] The combined synergy between hydrogen bonding, π - π stacking and hydrophobic forces result in the aggregate or material exhibiting high thermodynamic stability. Hence, disassembly strategies would need to overcome a tremendous energy barrier to break apart the strong attractive forces holding them together. Using the concept of inducing molecular, geometric frustration to the aggregate by multiple strong local interactions, the development of small molecular β -sheet breakers^[17] or molecular tweezers^[18,19] have found preliminary successes. However, while these disassembling molecules are attractive as therapeutic solutions, they are cumbersome for material-based applications where a more integrative design is required for processing.

In this regard, supramolecular peptide scaffolds containing stimulus-responsive groups (pH,^[20] temperature,^[21] enzymes,^[22] redox reactions,^[23] or light^[24,25]) that chemically react, cleave or isomerize have been exploited to disrupt intermolecular forces on demand. Among these, light offers a unique advantage in self-assembly processes as photons do not directly interfere with intermolecular forces and the resultant photochemical reactions can be precisely controlled spatiotemporally.^[26] In this regard, photo-switches (i.e. azobenzenes, spiropyranes) and photo-cleavable groups have been developed to control the aggregation threshold of a targeted self-assembling peptide.^[26] The resultant light-induced chemical change occurs within the self-assembling molecule (monomolecular) where structural isomerization or cleavage destabilizes the formed aggregate, favoring its dissolution. Using these motifs allow reactions to occur homogeneously, with every molecule exposed to light behaving in a similar fashion.^[27]

In contrast, bimolecular photo-cycloaddition reactions, such as the [4+4] photodimerization of anthracene, are dependent on how molecules are pre-organized within the solid-state aggregate. Anthracene molecules that are stacked and sufficiently oriented to facilitate the cycloaddition will afford successful dimerization, leading to a distortion of the planar anthracene groups and disrupting the constructive π - π interactions. The geometric frustration generated as a consequence have recently shown to induce morphological switching in the nanoscale, between fibers and vesicles or the bending of nanotubes.^[28,29] Nonetheless, the extent of how 1D and 2D nanoscale structures succumb to the molecular deformation to allow structure-selective disassembly have not been explored.

In this study, we designed two anthracene-containing, short amphiphilic peptides Anthracene-GISVK (Ant-GISVK) and Ant-GISVKAE to assemble different pH-dependent β -sheet nanostructures, imparted

by the lysine (K) and glutamic acid (E) residues (Figure 1a). At pH 7.8 and pH 11, the overall charge on the peptides can be modulated, which in turn dictate molecular preorganization within the assembly. Subsequently, the kinetics of the photodimerization of anthracene are monitored via UV-Vis spectroscopy to correlate the reaction efficiency and the extent of structural disruption. The loss of secondary structure integrity and eventual disassembly are investigated via circular dichroism (CD) spectroscopy and transmission electron microscopy (TEM).

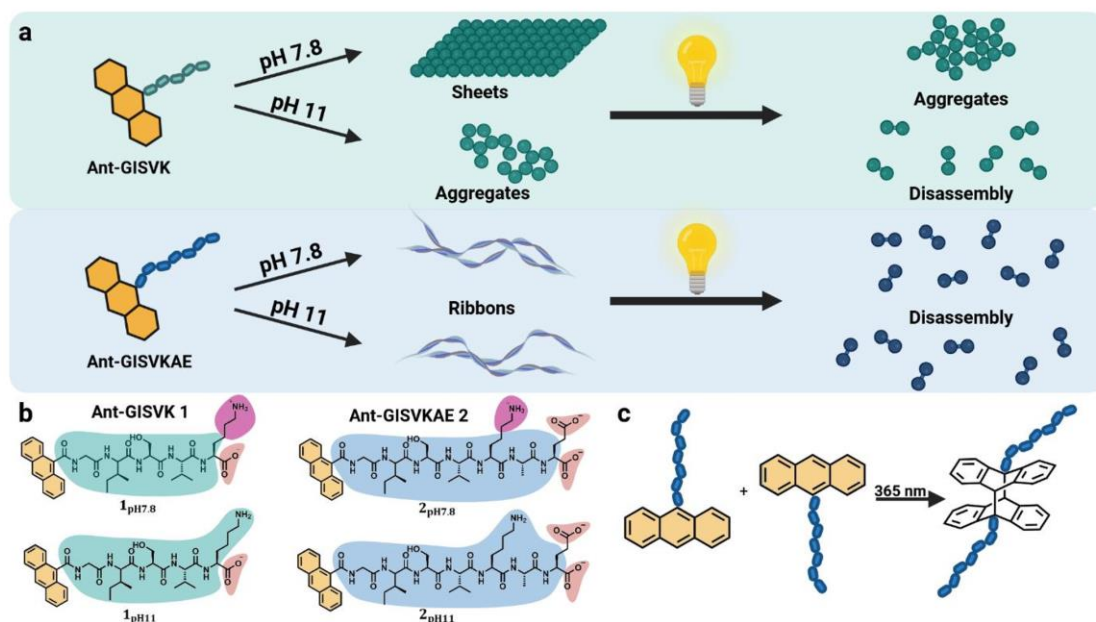


Figure 1: a) Schematic illustration of the assembly pathways of **1** and **2** at different pH and the disassembly pathway upon irradiation. b) Chemical structure of **1** and **2**, respectively at pH 7.8 or pH 11, showing the different charged states. c) [4+4] photodimerization of anthracene showing the bent configuration of the dimer.

Results and Discussion

Ant-GISVK **1** and Ant-GISVKA2 **2** (Figure 1b) were synthesized using Fmoc-solid phase peptide synthesis on Wang resin using *N,N'*-diisopropylcarbodiimide (DIC) and ethyl (2*Z*)-2-cyano-2-(hydroxyimino)acetate (OxymaPure) for the iterative peptide coupling steps and benzotriazol-1-yloxytripyrrolidino-phosphonium hexafluoro-phosphate (PyBOP) and diisopropylethylamine (DIPEA) for the coupling of 9-anthracenecarboxylic acid to the peptide, respectively. Purification was achieved by HPLC and the peptides subsequently analyzed by NMR and LCMS (Supplementary Figures 1-6).

The peptides were then assembled in a solvent mixture of 5% DMSO in 95% NH₄HCO₃ buffer (10 mM) at pH 7.8 and pH 11 (adjusted with 1 M NaOH), respectively. Raising the pH to 11 results in the deprotonation of the side chain lysine (pK_a=10.4),^[30] thereby causing the overall charge of both peptides

to become more negative compared to pH 7.8, where the lysine is positively charged (Figure 1b). Subsequently, we performed TEM of the peptides at both pH values and analysed the influence of charge on the assembled nanostructure.

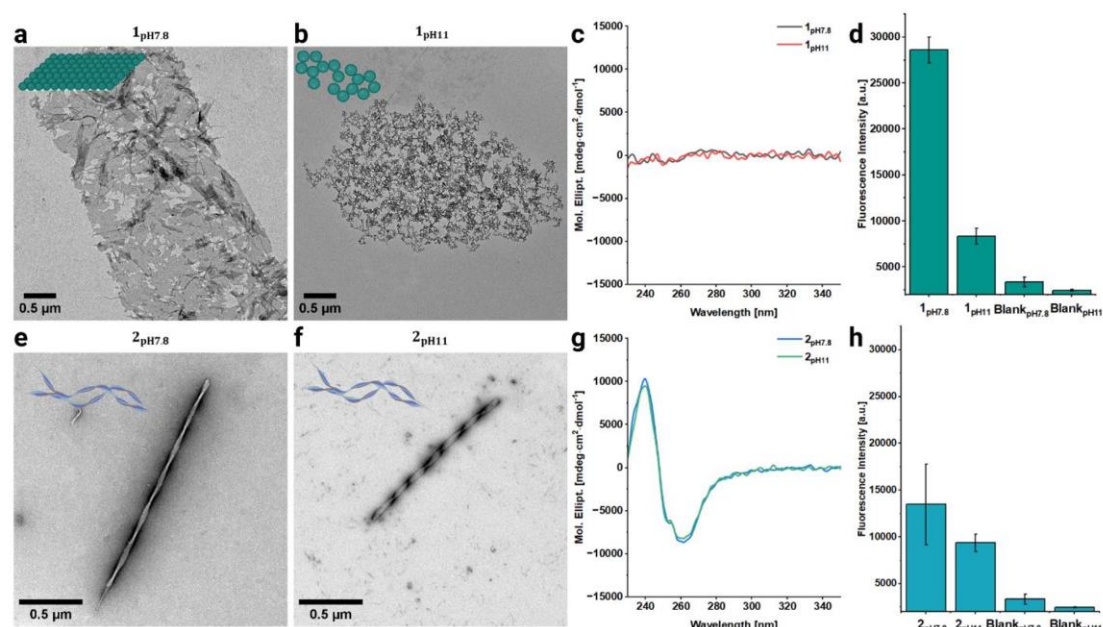


Figure 2: a) TEM image of $1_{pH7.8}$ revealing 2D-sheets. b) TEM image of 1_{pH11} revealing unordered aggregates. c) CD spectra of $1_{pH7.8}$ and 1_{pH11} indicating that there is no coupling of the anthracene chromophores. d) Proteostat assay showing increased levels of β -sheets for the sheets of $1_{pH7.8}$ compared to the aggregates of 1_{pH11} . e) TEM image of $2_{pH7.8}$ revealing single twisted ribbons. f) TEM image of 2_{pH11} revealing single twisted ribbons. g) CD spectra of $2_{pH7.8}$ and 2_{pH11} indicating interactions of the anthracene according to the bisignate curve. h) Proteostat assay showing similar levels of β -sheets in the nanostructures of $2_{pH7.8}$ and 2_{pH11} . All TEM scale bars are 0.5 μ m.

$1_{pH7.8}$ was observed to form freestanding, non-uniform 2D-sheets with irregular edges (Figure 2a) whereas 1_{pH11} did not assemble into ordered structures and formed only aggregates (Figure 2b). The increase in pH leads to a shift from an overall charge of 0 (pH 7.8), due to the zwitterion, to -1 (pH 11). This results in an enhanced electrostatic repulsion between the carboxylic acids of the C-terminus, leading to the destabilization and the formation of aggregates instead.^[31] In comparison to **1**, **2** is extended by an alanine (A) and glutamic acid (E), resulting in an additional negative charge at pH 7.8 while preserving the alternating hydrophobic/hydrophilic pattern. The extended peptide sequence $2_{pH7.8}$ assembles into single twisted ribbons. While the increase in pH to 11 increases the overall charge by -1, the same twisted ribbon morphology is retained for 2_{pH11} (Figure 2e,f).

Although both peptides demonstrate an analogous increase in negative charge at elevated pH levels, the position of the lysine within the sequence varies. Specifically, peptide **1** features lysine positioned at the C-terminus, exhibiting a pronounced alteration in morphology upon an increase in pH. Conversely, in peptide **2**, the lysine is positioned at the sequence's center and, therefore, there is no change in the local chemical environment at the peptide terminus and no change in morphology at elevated pH. This observation indicates that the local chemical environment at the C-terminus, rather than the overall charge of the peptide, plays a pivotal role in determining its structural morphology.

The secondary structure was analyzed CD spectroscopy from 230 nm onwards as the presence of DMSO in the solvent composition shields the far UV region. **2_{pH7.8}** and **2_{pH11}** exhibit a bisignate curve with a negative cotton effect at 261 nm and a positive ellipticity at 240 nm, corresponding to the π - π^* transition of the anthracene. (Figure 2g). This observation suggests that the anthracenes are in close spatial proximity and possess a chiral orientation within the structure.^[32–34] Conversely, **1_{pH7.8}** and **1_{pH11}** exhibit no coupling of the anthracene chromophores, indicating that the interactions between the aromatic groups are not the dominant driving force for assembly (Figure 2c). To further analyze the structural differences between the sheets of **1_{pH7.8}** and the twisted ribbons of **2_{pH7.8}**, an FTIR analysis was also conducted. The spectrum of **1_{pH7.8}** exhibits a peak at 1625 cm^{-1} , while **2_{pH7.8}** displays peaks at 1610 and 1625 cm^{-1} (Supplementary Figure 13). These peaks can be attributed to intermolecular β -sheets, showing additional differences in secondary structure between **1_{pH7.8}** and **2_{pH7.8}**.^[35,36]

The β -sheet content and molecular order are subsequently quantified with the fluorogenic Proteostat[®] assay.^[27,37] **1_{pH7.8}** exhibited the highest fluorescence intensity, suggesting a high degree of order within the 2D, sheet-like structures. In contrast, **1_{pH11}** which predominantly formed lower structural order aggregates exhibit reduced fluorescence intensity (Figure 2d). **2_{pH7.8}** and **2_{pH11}** showed comparable fluorescence intensities, which is in line with the CD and TEM results showing no difference in secondary structure or morphology upon increasing the pH (Figure 2h).

To establish the photochemistry of the anthracene units, we monitored the photodimerization reaction of peptides **1** and **2** using UV-VIS spectroscopy, where the characteristic anthracene signal at 365 nm diminishes upon dimerization (Figure 1c). A quantitative framework for monitoring the dimerization is established by a calibration curve using different concentrations of anthracene to correlate the change in absorption and the remaining anthracene concentration. Using the calibration plot as a reference, the reactivity of both peptides (**1** and **2**) in their molecular forms (100 μM) was analyzed in a solvent mixture of 1:1 DMSO/ NH_4HCO_3 buffer (10 mM) at pH 7.8 and pH 11 (adjusted with 1 M NaOH). Under these conditions, the monomers remain dissolved and no assembly takes place.

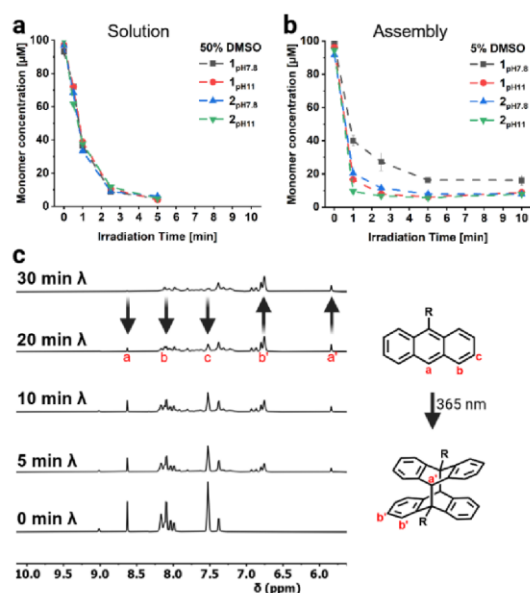


Figure 3: a) Consumption of the anthracene moiety of **1** and **2** in the non-assembled state with increasing irradiation time in 50/50 DMSO:Buffer (pH 7.8 or pH 11). b) Consumption of the anthracene moiety of **1** and **2** in the assembled state with increasing irradiation time in 5/95 DMSO:Buffer (pH 7.8 or pH 11). c) ¹H-NMR kinetic analysis of **1** at pH 7.8 at different irradiation times showing the decline in the anthracene signals while new signals of the dimer emerge.

In the molecular state, the monomer concentration was found to be less than 10% of its initial value after 5 min of 365 nm irradiation for peptides **1** and **2** across both pH values (Figure 3a). Subsequently, the solvent composition was changed to 5:95 DMSO/NH₄HCO₃ buffer (10 mM) at pH 7.8 and pH 11 to initiate assembly, and the irradiation kinetics was repeated under the new conditions (Figure 3b). **1**_{pH7.8} exhibited slower kinetics compared to when it is dissolved in 50% DMSO, with substantial amount of monomer (16%) remaining even after 10 min irradiation. Conversely, **1**_{pH11} and **2**_{pH7.8} showed a lower remaining monomer concentration after 1 min and therefore a faster initial rate but still required 5 min to reach below 10%, similar to the irradiation in the non-assembled state. Peptide **2**_{pH11} demonstrated the most accelerated kinetics in the assembled state compared to its solution state, with less than 10% monomer remaining already after 1 min of 365-nm irradiation (Figure 3b).

The collective secondary structure information revealed the kinetic discrepancies observed during irradiation. **1**_{pH7.8} demonstrates the highest degree of molecular order with no proximity coupling of the aromatic moieties, thereby preventing a substantial proportion of anthracene molecules from adopting the orientation needed for the dimerization reaction. This considerably slows the photochemical reaction and prevents the complete conversion of the anthracene peptides. In contrast, the preorganization in **2**_{pH11}

leads to an accelerated reaction, as the anthracenes are already in close spatial proximity within the assembly.

The [4+4] photodimerization was further confirmed by ^1H -NMR, which shows the emergence of a distinctive bridge proton signal at 5.8 ppm with the concomitant decrease of the respective anthracene signal at 8.6 ppm across all samples upon irradiation (Figure 3c). However, it was observed that only up to 60% conversion to the dimer was achieved, while the remaining monomer was found to be below 10% of the initial concentration (Supporting Figure 12). While this result suggests the occurrence of side reactions upon irradiation, it is crucial to note that the overall concentration was 20-fold higher than the assembly conditions in order to achieve a sufficient signal-to-noise ratio. Therefore, these experiments were conducted in a solution of 80% DMSO- d_6 and 20% NH_4HCO_3 buffer (10 mM) at pH 7.8 and pH 11, a composition that was found to ensure the solubility of all compounds at higher concentration while maintaining control over pH. The difference in concentration and solvent composition can, in principle, alter the reactivity due to different mobility and electrostatic repulsion.

We hypothesize that the different dimerization kinetics and efficiency as a consequence of molecular preorganization within the supramolecular structure can lead to different nanoscale outcomes. Using TEM, each nanostructure ($\mathbf{1}_{\text{pH}7.8}$, $\mathbf{1}_{\text{pH}11}$, $\mathbf{2}_{\text{pH}7.8}$, $\mathbf{2}_{\text{pH}11}$) was subjected to 10 min irradiation, a duration that has been shown to ensure maximum consumption of anthracene units (Figure 3b). In general, irradiated samples are denoted with λ . TEM analysis of $\mathbf{1}_{\text{pH}7.8}^\lambda$ revealed unordered aggregates, contrasting with the 2D-sheets observed for the non-irradiated $\mathbf{1}_{\text{pH}7.8}$ (Figure 4a). The dimer, with its bent conformation of the phenyl moieties, impedes the π - π stacking of the planar aromatic groups, thereby causing a structural change towards aggregates. However, the reduction in attractive forces, as well as the geometric frustration induced by the dimerization, proves insufficient to cause full disassembly. Irradiation exhibits similar structural destabilization compared to pH increase, as evidenced by the unstructured aggregates observed in both the irradiated $\mathbf{1}_{\text{pH}7.8}^\lambda$ and the non-irradiated $\mathbf{1}_{\text{pH}11}$ at higher pH. The combination of pH and irradiation as destabilizing effects for $\mathbf{1}_{\text{pH}11}^\lambda$, results in a complete disassembly of the nanostructure, due to the decrease of π - π stacking coupled with the increase in electrostatic repulsion (Figure 4b). The twisted ribbons of $\mathbf{2}_{\text{pH}7.8}$ exhibit a distinct response to irradiation as evidenced by the absence of both assemblies and aggregates in $\mathbf{2}_{\text{pH}7.8}^\lambda$ (Figure 4c). This emphasizes the necessity of balancing hydrophobic and hydrophilic moieties to facilitate the dissolution of assembling units, as opposed to the formation of aggregates due to the collapse of hydrophobic regions, as $\mathbf{2}$ features a greater number of hydrophilic charged groups such as glutamic acids in comparison to $\mathbf{1}$. Furthermore, the observation of disassembly in $\mathbf{2}_{\text{pH}11}^\lambda$ upon irradiation suggests that the morphology of the monomer and dimer assemblies of $\mathbf{2}$ is pH-independent (Figure 4d).

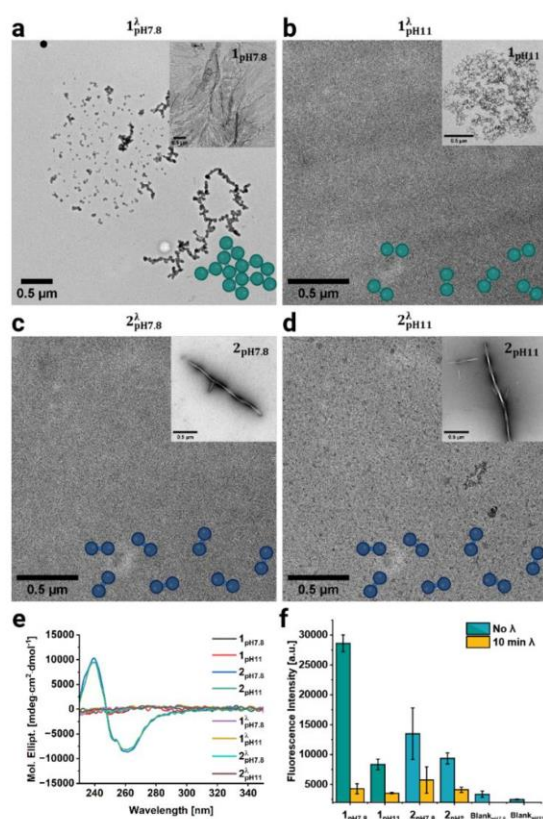


Figure 4: a) TEM image of $1_{pH7.8}^{\lambda}$ revealing unordered aggregates. Inset: TEM image of $1_{pH7.8}$ showing 2D-sheets. b) TEM image of 1_{pH11}^{λ} revealing the absence of nanostructures. Inset: TEM image of 1_{pH11} showing unordered aggregates. c) TEM image of $2_{pH7.8}^{\lambda}$ revealing the absence of nanostructures. Inset: TEM image of $2_{pH7.8}$ showing twisted ribbons. d) TEM image of 2_{pH11}^{λ} revealing the absence of nanostructures. Inset: TEM image of 2_{pH11} showing twisted ribbons. e) CD spectra of **1** and **2** at pH 7.8 and 11 before and after irradiation. The CD spectra of the irradiated peptides 1_{pH11}^{λ} , $1_{pH7.8}^{\lambda}$, $2_{pH7.8}^{\lambda}$ and 2_{pH11}^{λ} show no signal overall. f) Proteostat assay of **1** and **2** in different pH with and without irradiation at 365 nm showing the disassembly upon irradiation.

The effect of irradiation on the supramolecular structure was further confirmed by circular dichroism and Proteostat[®] assay. The CD signals for $2_{pH7.8}^{\lambda}$ and 2_{pH11}^{λ} are removed, thereby confirming the disassembly of the structure. $1_{pH7.8}^{\lambda}$ and 1_{pH11}^{λ} continue to demonstrate an absence of CD signal (Figure 4e). Proteostat confirmed these observations as the fluorescence intensity decreased upon irradiation in all cases (Figure 4f).

Conclusion

In summary, we have demonstrated the assembly of short anthracene-containing peptides into different 1D and 2D morphologies by pH-dependent charge modulation. Each morphology expresses different levels of molecular order and preorganization, leading to distinct kinetic responses towards the [4+4]

photodimerization of anthracene units on the self-assembling peptide. As a result, the extent of geometric frustration upon irradiation becomes selective towards nanostructures that promote proximity π - π interactions of the anthracene moieties. Our results highlight that bimolecular photochemistry is a highly attractive avenue to integrate supramolecular selectivity into complex responsive systems, while offering the unique advantages of light. We believe that the technology will provide a new dimension towards light-driven supramolecular materials and expand the toolbox for controlling disassembly processes.

Experimental Section

General synthesis procedure

The preloaded Wang resin was swollen in 5 mL DMF at room temperature for 1 h before transferring it into the peptide synthesizer. DMF was removed via a draining process and the resin swollen with 20 mL DMF for 20 s. Prior to each coupling step, as well as during the final deprotection, the N-terminal Fmoc group was removed using two deprotection steps: 20% piperidine in DMF was applied for 15 seconds at 75 °C, followed by 50 seconds at 90 °C. Fmoc-protected amino acids were coupled in a 5-fold molar excess for 15 seconds and 110 seconds at 75 °C and 90 °C, respectively, using a solution of DIC (0.25 M) and Oxyma (0.5 M) in DMF. The solution was drained, and the resin washed with DMF three times. Anthracen was then coupled to the peptide by adding 9-anthracene carboxylic acid (2 equiv.), PyBOP (2 equiv.) and DIPEA (4 equiv.) in DMF to the resin and shaking overnight. The peptide was cleaved from the resin by adding 10 mL of cleavage cocktail (95% TFA, 2.5% TIPS, 2.5% MilliQ water) to the resin and shaking at room temperature for 2 h. The cleavage cocktail was drained, and the resin washed with TFA. The TFA containing solutions were combined and the liquids removed under reduced pressure. The peptide was purified via HPLC with a YMC-Actus Triart C18 column (flowrate 20 mL/min). After lyophilisation, the peptide was received as a colourless solid.

General transmission electron microscopy procedure

Peptide **1** or **2** (100 μ M) was dissolved in 5% DMSO and 95% NH_4HCO_3 buffer (10 mM) and incubated overnight at 25°C while shaking at 300 rpm. The solutions for irradiation were then transferred to a glass vial equipped with a stirring bar and covered with a blanket of argon. They were then irradiated at 365 nm for 10 min while stirring at 300 rpm and again incubated overnight at 25°C. Then TEM grids were prepared by pipetting 3 μ L of irradiated or non-irradiated peptide solution onto a Formvar-coated copper grid and incubating for 5 min. After the incubation, the solutions were removed with filter paper, and the

grids were stained with 7 μ L 4% uranyl acetate solution for 2.5 min. The grids were washed three times with MilliQ water and dried before being measured.

Keywords: peptide amphiphile • supramolecular assembly • disassembly • Light • Anthracene

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Supporting Information

Light-Triggered Disassembly of Peptide Nanostructures

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1 Materials and Instruments

1.1 Materials

All chemicals were purchased from commercial sources and were used without further purification. Solvents and reagents used for peptide synthesis were obtained in peptide grade. Purification with HPLC was performed with water obtained from a Millipore purification system and HPLC grade acetonitrile.

1.2 Instruments

1.2.1 Microwave Peptide Synthesizer

Peptides and peptide fractions were synthesized with a Liberty Blue Automated Microwave Peptide Synthesizer by CEM Corporation.

1.2.2 High-Performance Liquid Chromatography (HPLC)

The synthesized peptides were purified by preparative HPLC using a Shimadzu setup. A YMC-Actus Triart C18 column (150 x 20 mm, 5 μ m) with a flowrate of 20 mL/min or a Phenomenex Kinetex EVO C18 100 (150 x 30 mm, 5 μ m) with a flowrate of 25 mL/min was used for purification.

All purification steps were performed by using gradients of MilliQ water and acetonitrile, each acidified with 0.1% formic acid. Analytical measurements were performed by using gradients of MilliQ water and acetonitrile, each acidified with 0.1% trifluoroacetic acid. The HPLC data were processed with the software LabSolutions by Shimadzu and OriginPro 2024b by OriginLab.

1.2.2 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR spectra were recorded on a Bruker Avance III 700 MHz NMR spectrometer. For ^1H spectra, the chemical shifts are reported in parts per million (ppm) from high to low frequency using the residual solvent peak as the internal reference ($\text{DMSO-}d_6 = 2.50$ ($\text{CHD}_2\text{SOCD}_3$) ppm). All ^1H resonances are reported to the nearest 0.01 ppm. The multiplicity of ^1H signals are indicated as: s = singlet; d = doublet; t = triplet; q = quartet; p = pentet; m = multiplet; br = broad; or combinations of thereof. Coupling constants (J) are quoted in Hz and reported to the nearest 0.1 Hz. Where appropriate, averages of the signals from peaks displaying multiplicity were used to calculate the value of the coupling constant. The data was processed in MestReNova.

1.2.3 1.2.4 Liquid Chromatography – Mass Spectrometry (LC-MS)

Peptides were analysed by HPLC-ESI-MS on a LC-MS 2020 setup by Shimadzu using a Kinetex EVO C18 100 Å LC column (50 x 2.1 mm, 2.6 μ m). Acetonitrile (ACN) and MilliQ water acidified with 0.1% formic acid were used as solvents for all measurements. The solvent gradient

started with 5% ACN and 95% water, while the ACN content was linearly increased to 95% in 12 min. Data were processed in LabSolutions and OriginPro 2024b by OriginLab.

1.2.4 Circular Dichroism Spectroscopy (CD)

CD spectra of the peptides were recorded on a JASCO J-1500 spectrometer in a 0.1 cm High Precision Cell by HellmaAnalytics. The recorded data were processed in Spectra Analysis by JASCO and OriginPro 2024b by OriginLab.

1.2.5 Fluorescence Spectroscopy

To record the fluorescence intensity, a SPARK 20M microplate reader by Tecan Group Ltd. was used. The samples were measured in a Greiner 384 flat black well plate and data was processed in OriginPro 2024b by OriginLab. The absorbance was recorded on the same instrument with a Greiner UV-STAR® plate, 384 well, F-Bottom, clear.

1.2.6 Transmission Electron Microscopy (TEM)

TEM images of the peptide samples were recorded on a JEOL 1400 transmission electron microscope at a voltage of 120 kV. Formvar/carbon-film coated copper grids (300 mesh) by Plano GmbH were used to prepare the samples. The images were processed in Fiji ImageJ.

1.2.7 Irradiation setup

Irradiations were performed with a Thorlabs M365L3 lamp with a nominal wavelength of 365 nm, a bandwidth (FWHM) of 9 nm and a current of 1000 mA.

1.2.8 Fourier-transform Infrared spectroscopy

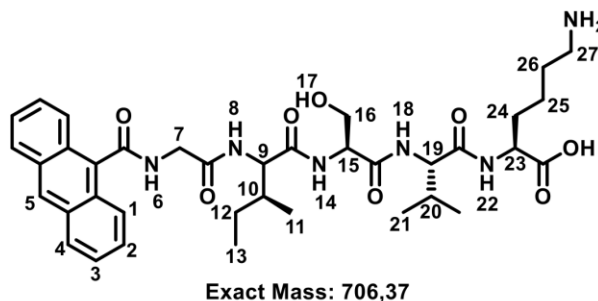
Infrared spectra were recorded in transmission geometry using a Bruker Vertex 70 IR spectrometer. The spectrometer has a resolution of 4 cm⁻¹ in a range from 400 cm⁻¹ to 4000 cm⁻¹.

2 Synthesis

2.1 Ant-GISVK

The Wang resin preloaded with Fmoc-Lys(Boc) (0.1 mmol) was swollen in 5 mL DMF at room temperature for 1 h before transferring it into the peptide synthesizer. DMF was removed via a draining process and the resin swollen with 20 mL DMF for 20 s. Prior to each coupling step, as well as during the final deprotection, the N-terminal Fmoc group was removed using two deprotection steps: 20% piperidine in DMF (3 mL) was applied for 15 seconds at 75 °C, followed by 50 seconds at 90 °C. Fmoc-protected amino acids (Fmoc-Val, Fmoc-Ser(tBu), Fmoc-Ile and Fmoc-Gly) were coupled in a 5-fold molar excess (5 eq. in 1.25 mL) for 15 seconds and 110 seconds at 75 °C and 90 °C, respectively, using a solution of DIC (0.25 M) and Oxyma (0.5 M) in 2.75 mL DMF. The solution was drained, and the resin washed with DMF three times. Anthracen was then coupled to the peptide by adding 9-anthracene carboxylic acid (44.45 mg, 2 equiv.), PyBOP (104 mg, 2 equiv.) and DIPEA (68 μ L, 4 equiv.) in 5 mL DMF to the resin and shaking overnight. The peptide was cleaved from the resin by adding 10 mL of cleavage cocktail (95% TFA, 2.5% TIPS, 2.5% MilliQ water) to the resin and shaking at room temperature for 2 h. The cleavage cocktail was drained, and the resin washed with TFA. The TFA containing solutions were combined and the liquids removed under reduced pressure.

The peptide was purified via HPLC with a YMC-Actus Triart C18 column (flowrate 20 mL/min). After lyophilisation, the product Ant-GISVK was received as a colourless solid (14.13 mg, 0.02 mmol, 20%).



$^1\text{H-NMR}$ (700 MHz, DMSO-d_6) δ [ppm] = 9.05 (t, J = 6.1 Hz, 2H, **6**), 8.65 (s, 1H, **5**), 8.25 (d, J = 36.1 Hz, 2H, **1**), 8.13–8.10 (m, 3H, **4,8**), 8.08 (d, J = 7.6 Hz, 1H, **14**), 8.03 (d, J = 8.2 Hz, 1H, **18**), 7.58–7.51 (m, 4H, **2,3**), 7.48 (d, J = 7.1 Hz, 1H, **22**), 4.51 (q, J = 6.6 Hz, 1H, **15**), 4.46 (dd, J = 9.0, 7.6 Hz, 1H, **9**), 4.13 (d, J = 49.6, 16.1, 6.0 Hz, 2H, **7**), 4.05 (dd, J = 8.2, 5.4 Hz, 1H, **19**), 3.83 (q, J = 6.2 Hz, 1H, **23**), 3.63 (qd, J = 10.7, 6.1 Hz, 2H, **16**), 2.71 (t, J = 9.6 Hz, 2H, **27**), 2.12 (m, dd, J = 13.0, 6.5 Hz, 1H, **20**), 1.79–1.73 (d, 1H, **10**), 1.59 (d, J = 8.9 Hz, 1H, **26**), 1.51 (dd, J = 14.4, 7.4 Hz, 4H, **12, 24, 26**), 1.36–1.19 (m, 2H, **25**), 0.910.82 (m, 12H, **11, 13, 21**).

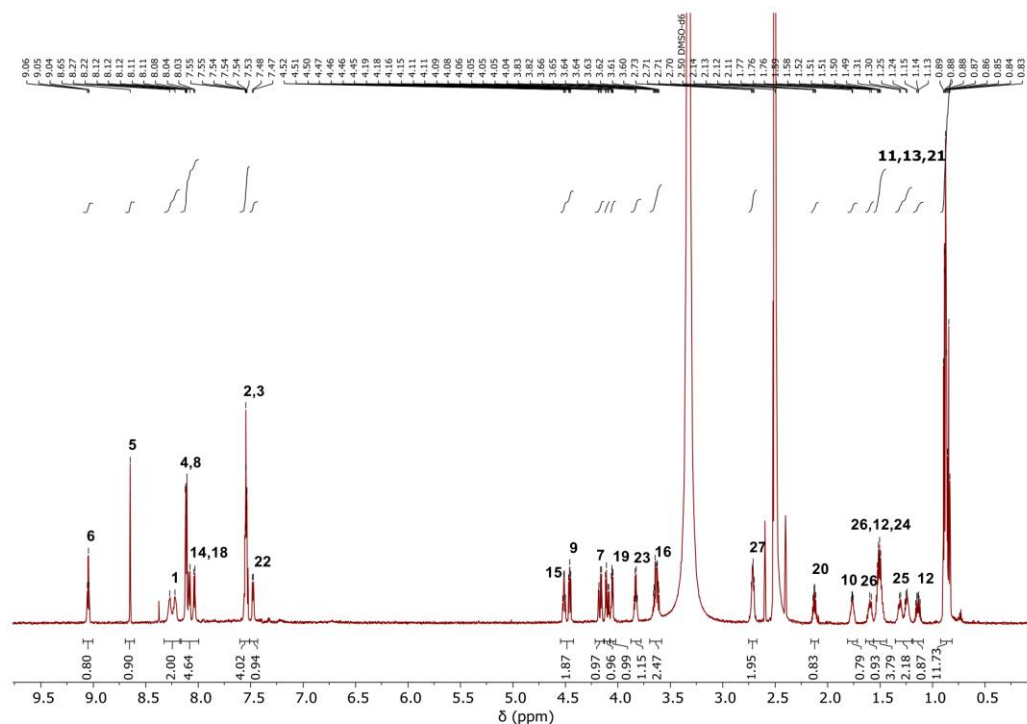


Figure 1: ^1H NMR spectrum (700 MHz, DMSO-d_6 , 298 K) of compound **1**

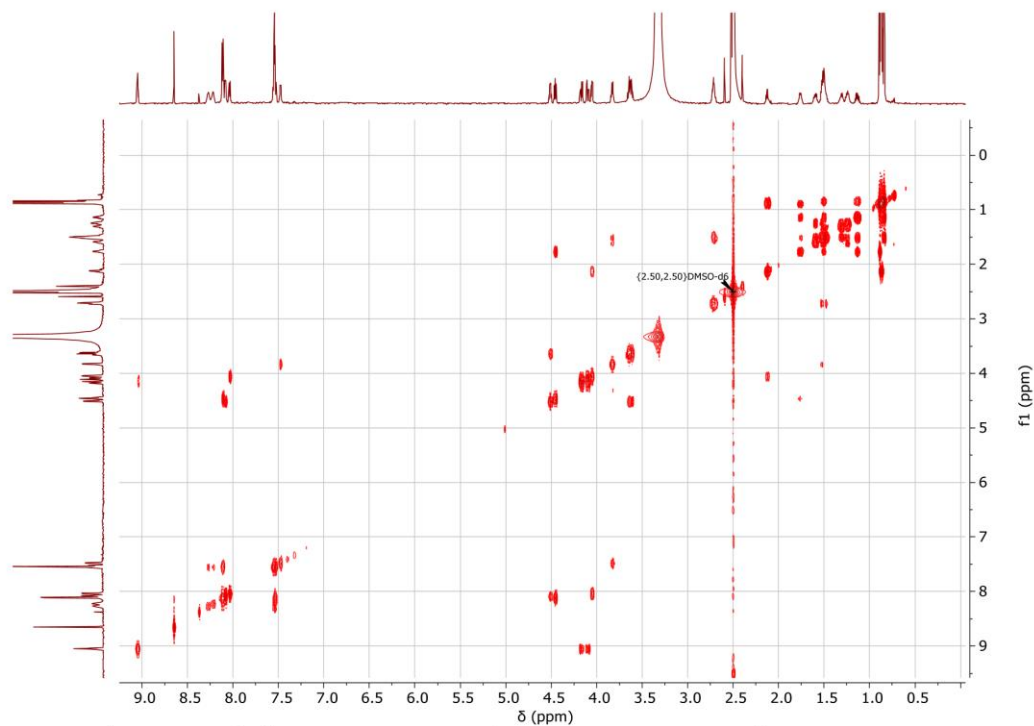


Figure 2: ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra (700 MHz, DMSO-d_6 , 298 K) of compound **1**

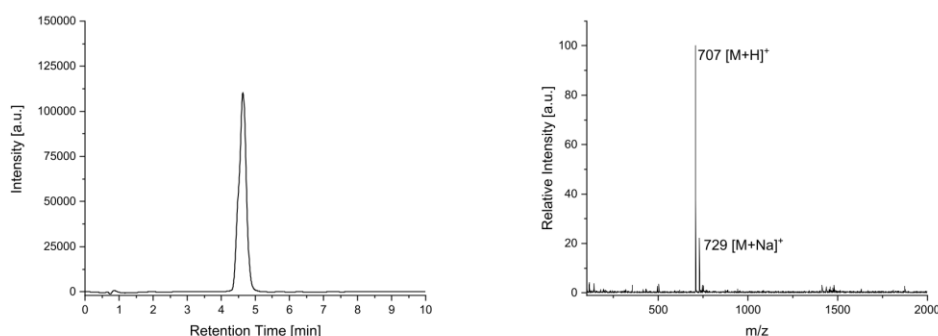
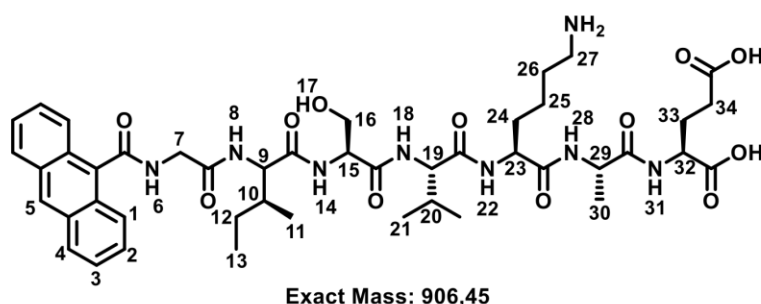


Figure 3: LC spectrum (left) and ESI MS (positive mode, right) of **1**. m/z calculated: $[M+H]^+ = 707$, found: $[M+H]^+ = 707$, $[M+Na]^+ = 729$.

2.2 Ant-GISVKAE

The Wang resin preloaded with Fmoc-Glu(OtBu) (0.25 mmol) was swollen in 5 mL DMF at room temperature for 1 h before transferring it into the peptide synthesizer. DMF was removed via a draining process and the resin swollen with 20 mL DMF for 20 s. Prior to each coupling step, as well as during the final deprotection, the N-terminal Fmoc group was removed using two deprotection steps: 20% piperidine in DMF (3 mL) was applied for 15 seconds at 75 °C, followed by 50 seconds at 90 °C. Fmoc-protected amino acids (Fmoc-Ala, Fmoc-Lys(Boc), Fmoc-Val, Fmoc-Ser(tBu), Fmoc-Ile and Fmoc-Gly) were coupled in a 5-fold molar excess (5 eq. in 1.25 mL) for 15 seconds and 110 seconds at 75 °C and 90 °C, respectively, using a solution of DIC (0.25 M) and Oxyma (0.5 M) in 2.75 mL DMF. The solution was drained, and the resin washed with DMF three times. Anthracen was then coupled to the peptide by adding 9-anthracene carboxylic acid (111 mg, 2 equiv.), PyBOP (260 mg, 2 equiv.) and DIPEA (170 μ L, 4 equiv.) in 5 mL DMF to the resin and shaking overnight. The peptide was cleaved from the resin by adding 10 mL of cleavage cocktail (95% TFA, 2.5% TIPS, 2.5% MilliQ water) to the resin and shaking at room temperature for 2 h. The cleavage cocktail was drained, and the resin washed with TFA. The TFA containing solutions were combined and the liquids removed under reduced pressure. The peptide was purified via HPLC with a YMC-Actus Triart C18 column (flowrate 20 mL/min). After lyophilisation, the product Ant-GISVKAE was received as a colourless solid (50.6 mg, 0.06 mmol, 24%).



^1H NMR (700 MHz, DMSO) δ 9.02 (t, J = 6.2 Hz, 1H, **6**), 8.65 (s, 1H, **5**), 8.53 (d, J = 7.6 Hz, 1H, **28**), 8.27 (d, J = 7.7 Hz, 2H, **1**), 8.23 (s, 1H, **14**), 8.14 – 8.10 (m, 2H, **4**), 8.08 (d, J = 9.0 Hz, 1H, **8**), 8.04 (d, J = 7.7 Hz, 1H, **31**), 7.69 (d, J = 8.8 Hz, 1H, **18**), 7.54 (dd, J = 7.1, 3.0 Hz, 4H, **2**, **3**), 7.33 (d, J = 6.5 Hz, 1H, **22**), 4.47 (t, J = 8.0 Hz, 1H, **9**), 4.41 (q, J = 6.2 Hz, 1H, **15**), 4.29 (q, J = 7.6 Hz, 1H, **32**), 4.24 – 4.05 (m, 4H, **7**, **29**, **19**), 3.81 (q, J = 6.4 Hz, 1H, **23**), 3.59 (d, J = 6.1 Hz, 3H, **16**), 2.83 – 2.66 (m, 2H, **34**), 2.26 – 2.06 (m, 2H, **27**), 1.96 (q, J = 6.8 Hz, 1H, **20**), 1.85 – 1.65 (m, 4H, **26'**, **24**, **10**), 1.60 – 1.27 (m, 6H, **26'**, **33**, **12'**, **25**), 1.22 (d, J = 7.3 Hz, 3H, **30**), 1.14 (dt, J = 14.0, 7.7 Hz, 1H, **12'**), 0.98 – 0.67 (m, 12H, **11**, **13**, **21**).

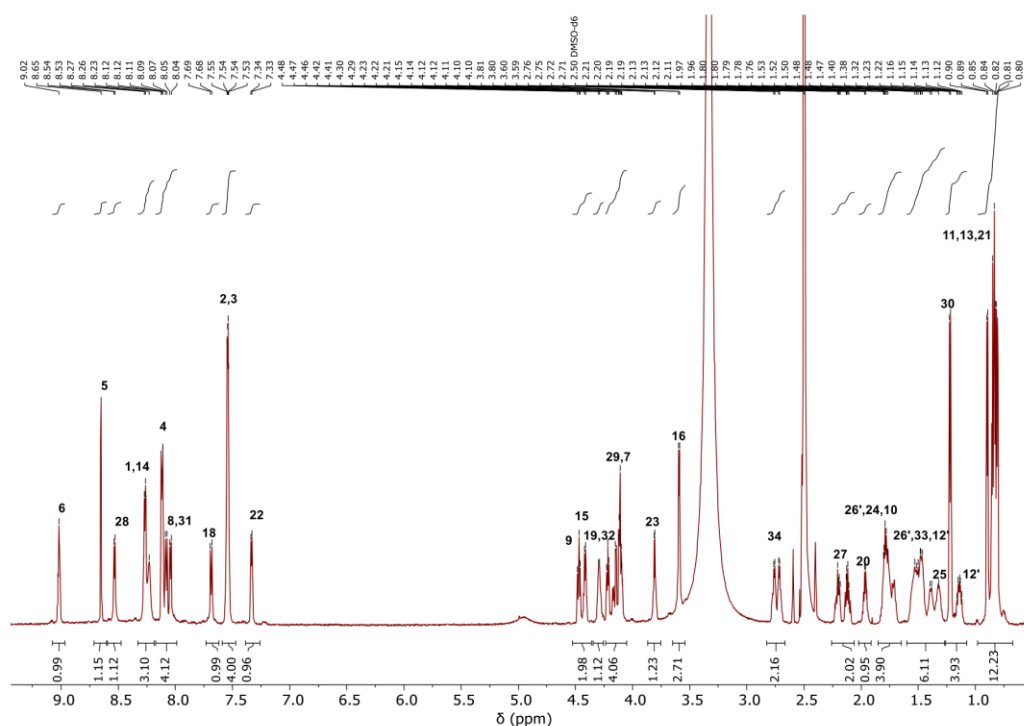


Figure 4: ^1H NMR spectrum (700 MHz, DMSO- d_6 , 298 K) of compound **2**

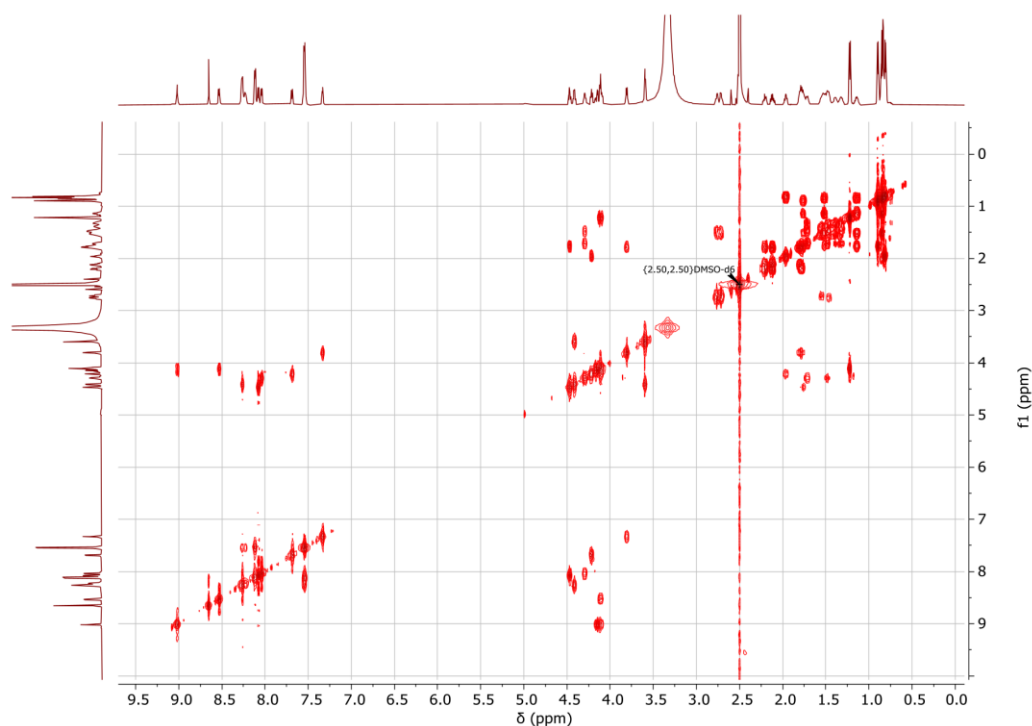


Figure 5: ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra (700 MHz, DMSO-d_6 , 298 K) of compound **2**

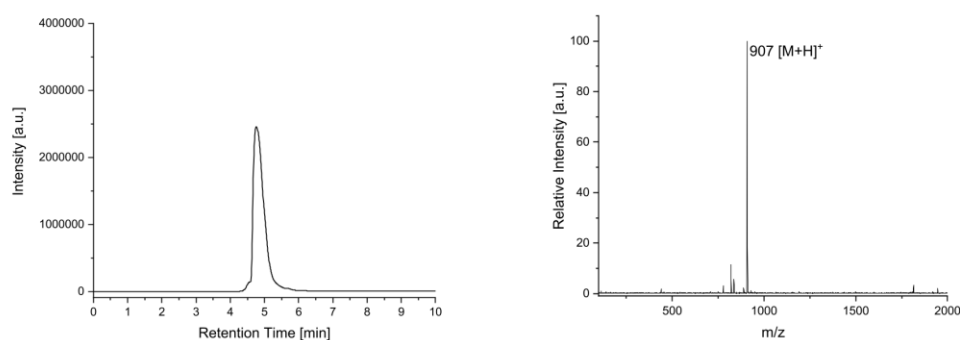


Figure 6: LC spectrum (left) and ESI MS (positive mode, right) of **2**. m/z calculated: $[\text{M}+\text{H}]^+ = 907$, found: $[\text{M}+\text{H}]^+ = 907$.

3 Characterization

3.1 Absorbance and Fluorescence spectroscopy

3.1.1 Calibration curves

1 was dissolved in 50% DMSO and 50% NH_4HCO_3 buffer (10 mM, pH 7.8) in the following concentrations: 100, 50, 25, 12.5, 6.25 and 0 μM . Then for each concentration three times 60 μL were transferred to a Greiner 384 UV-STAR® clear wellplate with flat bottom and the absorbance at 366 nm was measured. Absorbance was then plotted against the concentration and the points fitted linearly. The same procedure was conducted for **2**.

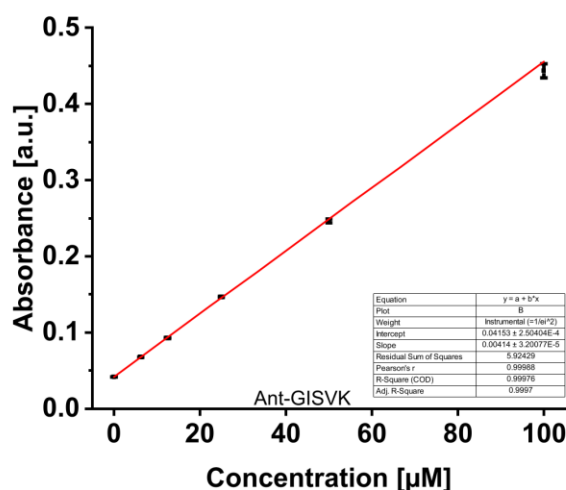


Figure 7: Linear fit of the absorbances of **1** at 366 nm with different concentrations in 50% DMSO and 50% NH_4HCO_3 buffer (10 mM, pH 7.8).

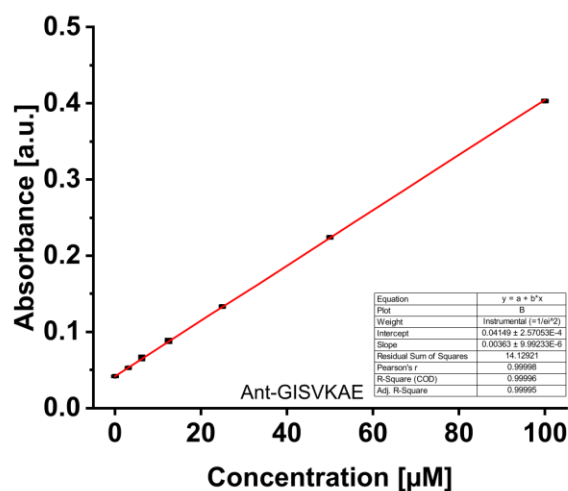


Figure 8: Linear fit of the absorbances of **2** at 366 nm with different concentrations in 50% DMSO and 50% NH_4HCO_3 buffer (10 mM, pH 7.8).

3.1.2 Irradiation conversion in 50% DMSO

1 (100 μM) was dissolved in 500 μL of 50% DMSO and 50% NH_4HCO_3 buffer (10 mM, pH 7.8) in a glass vial equipped with a stirring bar and covered with a blanket of argon. The solution was then irradiated at 365 nm for 0.5, 1, 2.5 and 5 min while stirring at 300 rpm. After each irradiation timepoint the absorbance at 366 nm was measured with three times 60 μL using a Tecan plate reader and Greiner 384 UV-STAR® clear wellplates with flat bottom. After each measurement the solution was filled back to the glass vial to keep the volume consistent. This procedure was repeated for **1** in 50% DMSO and 50% NH_4HCO_3 buffer (10 mM, pH 11 (adjusted with 1 M NaOH solution)) and for **2** at both pH values. The concentration of remaining **1** or **2** was then calculated with the absorbance values using the calibration curves of **3.1.1**.

3.1.3 Irradiation conversion in 5% DMSO

1 (100 μM) was dissolved in 150 μL of 5% DMSO and 95% NH_4HCO_3 buffer (10 mM, pH 7.8) and incubated overnight at 25°C while shaking at 300 rpm. The solution was then transferred to a glass vial equipped with a stirring bar and covered with a blanket of argon. The solution was then irradiated at 365 nm for 1 min while stirring at 300 rpm and then diluted with 135 μL DMSO to achieve a DMSO content of 50%. The absorbance at 366 nm was measured with three times 60 μL using a Tecan plate reader and Greiner 384 UV-STAR® clear wellplates with flat bottom. This process was repeated for irradiation times of 0, 2.5, 5 and 10 min of irradiation at 365 nm.

The procedure was conducted for **1** in 5% DMSO and 95% NH_4HCO_3 buffer (10 mM, pH 11) and for **2** at both pH values.

The concentration of remaining **1** or **2** was then calculated with the absorbance values using the calibration curves of **3.1.1** taking into account the dilution with DMSO.

3.1.4 Proteostat assay

1 (100 μ M) was dissolved in 5% DMSO and 95% NH_4HCO_3 buffer (10 mM, pH 7.8) and incubated overnight at 25°C while shaking at 300 rpm.

The Proteostat Protein Aggregation Assay Kit was purchased from *Enzo Life Sciences, Inc.* 0.52 μ L of the Proteostat stock solution was diluted with 98.48 μ L MilliQ water and 1 μ L assay buffer. 1 μ L of this solution was added to 9 μ L peptide solution in a Greiner 384 flat black wellplate three times for each sample. The solutions were incubated in the dark for 15 min while shaking at 500 rpm. The fluorescence intensity was measured at an excitation wavelength of 550 nm and emission wavelength of 600 nm and with a bandwidth of 20 nm. The procedure was conducted for **1** in 5% DMSO and 95% NH_4HCO_3 buffer (10 mM, pH 11) and for **2** at both pH values.

Samples for irradiation were transferred to a glass vial equipped with a stirring bar and covered with a blanket of argon. They were each irradiated at 365 nm for 10 min while stirring at 300 rpm and again incubated overnight at 25°C.

3.2 Nuclear magnetic resonance

1.4 mg of **1** was dissolved in 600 μ L DMSO- d_6 and 150 μ L NH_4HCO_3 buffer in H_2O (10 mM, pH 7.8). ^1H -NMR was then measured with water suppression. The sample was then covered with a blanket of argon and irradiated for 5 min at 365 nm while stirring at 300 rpm. ^1H -NMR with water suppression was again measured and this process repeated for a total of 10, 20 and 30 min of irradiation at 365 nm. The integral of the 1H-singulett of the anthracene monomer at 8.63 ppm and of the anthracene-dimer at 5.84 ppm were compared to each other while using the peptide signal at 2.69 ppm as a standard.

The procedure was conducted for **1** in 80% DMSO- d_6 and 20% NH_4HCO_3 buffer in H_2O (10 mM, pH 11) and for **2** at both pH values.

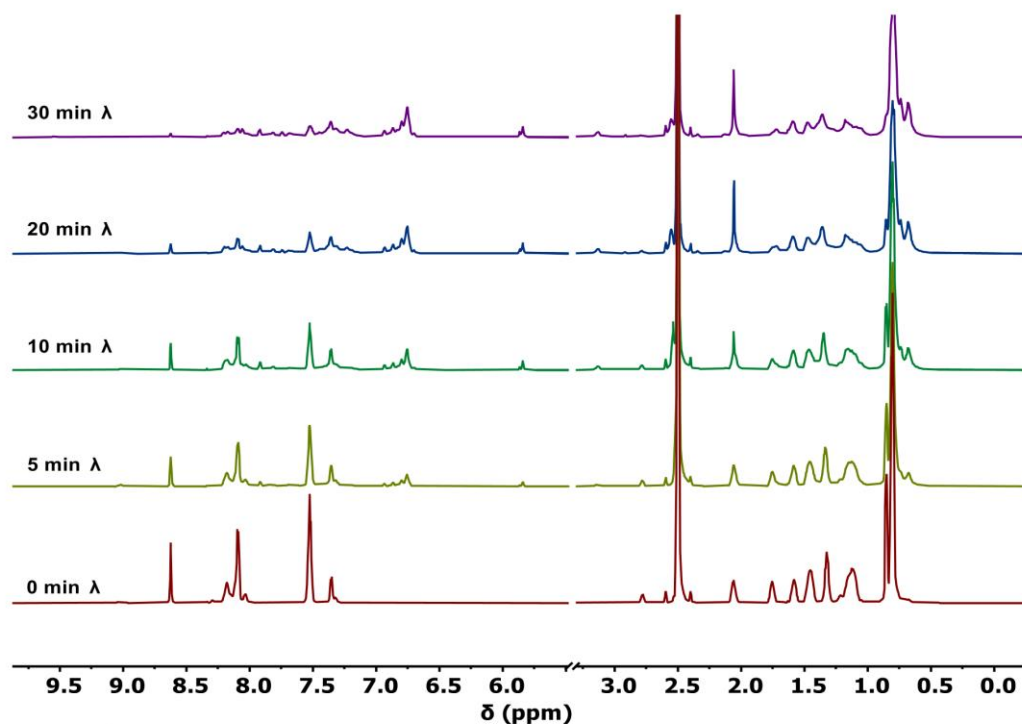


Figure 9: ^1H NMR kinetic (700 MHz, 298 K) of **1** at different irradiation times in 80% DMSO-d_6 and 20% NH_4HCO_3 buffer in H_2O (10 mM, pH 11).

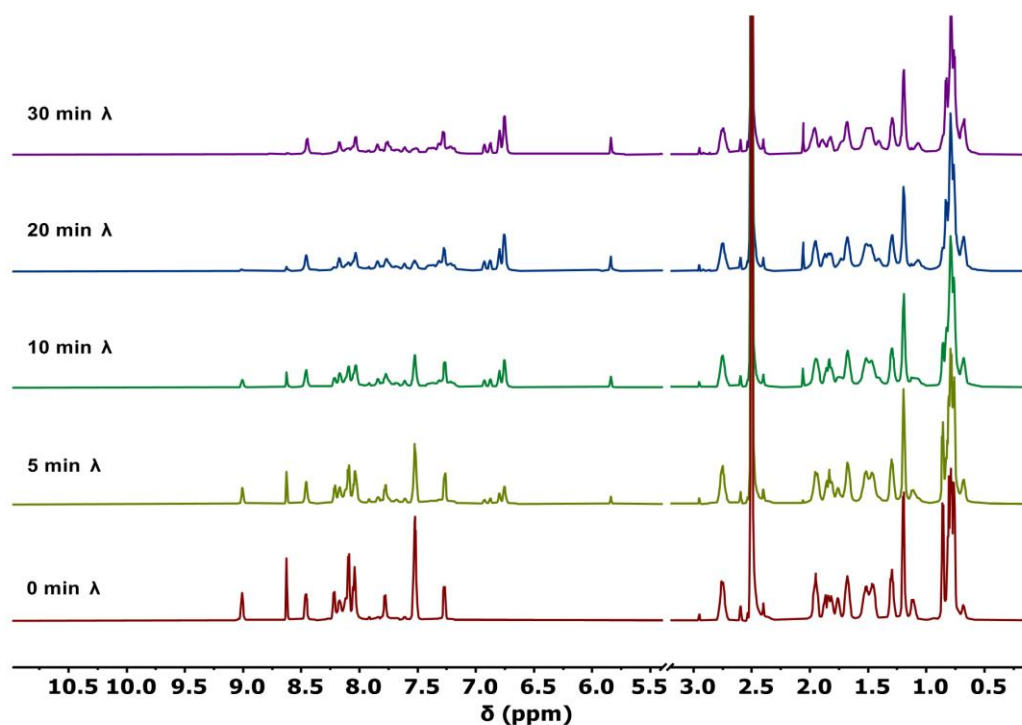


Figure 10: ^1H NMR kinetic (700 MHz, 298 K) of **2** at different irradiation times in 80% DMSO-d_6 and 20% NH_4HCO_3 buffer in H_2O (10 mM, pH 7.8).

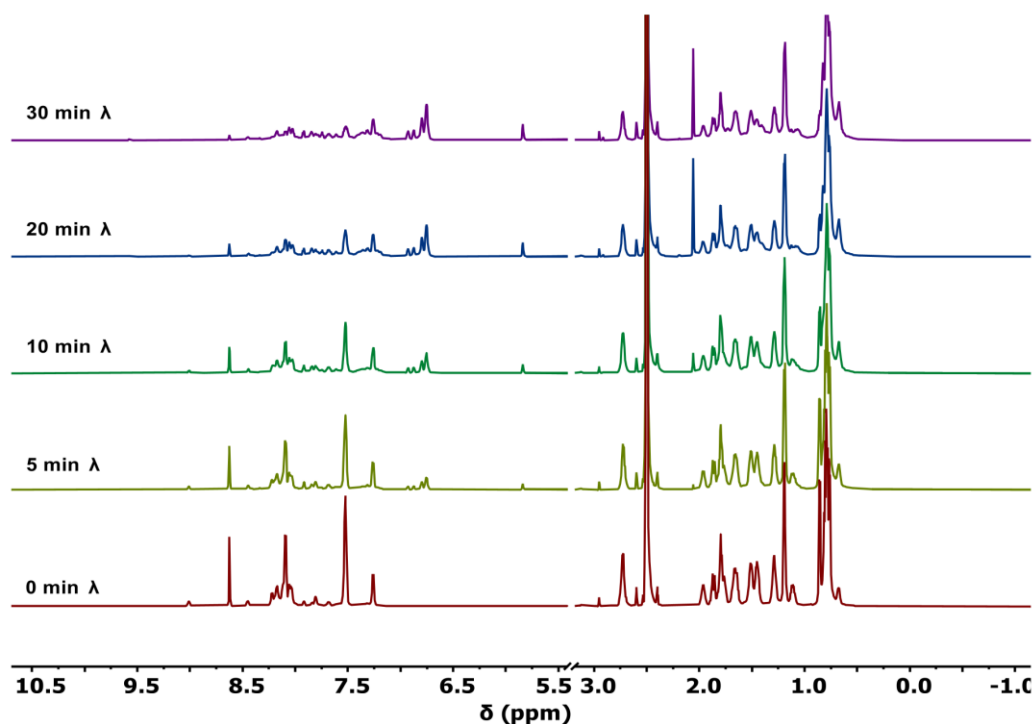


Figure 11: ^1H NMR kinetic (700 MHz, 298 K) of **2** at different irradiation times in 80% DMSO-d_6 and 20% NH_4HCO_3 buffer in H_2O (10 mM, pH 11).

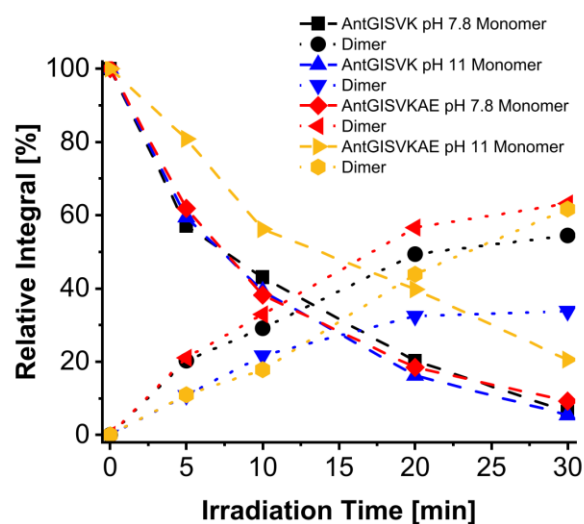


Figure 12: Relative integral of the anthracene monomer and dimer signals of **1** and **2** in 80% DMSO-d_6 and 20% NH_4HCO_3 buffer in H_2O (10 mM, pH 7.8 or pH 11) by ^1H NMR (700 MHz, 298 K).

3.3 Transmission electron microscopy

3.3.1 Non-irradiated samples

1 (100 μ M) was dissolved in 5% DMSO and 95% NH_4HCO_3 buffer (10 mM, pH 7.8) and incubated overnight at 25°C while shaking at 300 rpm. Then TEM grids were prepared by pipetting 3 μ L solution onto a Formvar-coated copper grid and incubated for 5 min. After the incubation, the solutions were removed with filter paper, and the grids were stained with 7 μ L 4% uranyl acetate solution for 2.5 min. The grids were washed three times with MilliQ water and dried before being measured. The procedure was conducted for **1** in 5% DMSO and 95% NH_4HCO_3 buffer (10 mM, pH 11) and for **2** at both pH values.

3.3.2 Irradiated samples

1 (100 μ M) was dissolved in 5% DMSO and 95% NH_4HCO_3 buffer (10 mM, pH 7.8) and incubated overnight at 25°C while shaking at 300 rpm. The solution was then transferred to a glass vial equipped with a stirring bar and covered with a blanket of argon. It was then irradiated at 365 nm for 10 min while stirring at 300 rpm and again incubated overnight at 25°C. Then TEM grids were prepared by pipetting 3 μ L solution onto a Formvar-coated copper grid and incubated for 5 min. After the incubation, the solutions were removed with filter paper, and the grids were stained with 7 μ L 4% uranyl acetate solution for 2.5 min. The grids were washed three times with MilliQ water and dried before being measured. The procedure was conducted for **1** in 5% DMSO and 95% NH_4HCO_3 buffer (10 mM, pH 11) and for **2** at both pH values.

3.4 Circular Dichroism

1 (100 μ M) was dissolved in 5% DMSO and 95% NH_4HCO_3 buffer (10 mM, pH 7.8) and incubated overnight at 25°C while shaking at 300 rpm. CD spectra were recorded at wavelength from 300 to 185 nm with a bandwidth of 1 nm, data pitch of 0.2 nm and a scanning speed at 20 nm/min from 20°C. Spectra were measured three times. The procedure was conducted for **1** in 5% DMSO and 95% NH_4HCO_3 buffer (10 mM, pH 11) and for **2** at both pH values.

Samples for irradiation were transferred to a glass vial equipped with a stirring bar and covered with a blanket of argon. They were each irradiated at 365 nm for 10 min while stirring at 300 rpm and again incubated overnight at 25°C. The samples were measured as described above.

3.5 Fourier-transform Infrared spectroscopy

1 (500 μM) or **2** (500 μM) was dissolved in 5% DMSO and 95% NH_4HCO_3 buffer (10 mM, pH 7.8) and incubated overnight at 25°C while shaking at 300 rpm. Each sample was then measured in a cell between two CaF_2 windows separated by a 100 μm Teflon spacer.

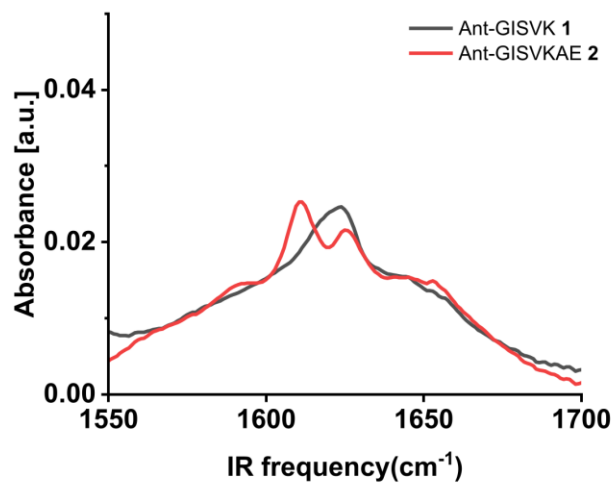


Figure 13: FTIR of **1**_{pH7.8} and **2**_{pH7.8} in 5% DMSO and 95% NH_4HCO_3 buffer (10 mM)

4 Supramolecular assembly guided by photolytic redox cycling

Authors

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Contributions

Patrick Roth: Conceived the project, performed the synthesis of the peptides, conducted structure analysis with CD and TEM, performed the Proteostat assay, and wrote the manuscript.

Raphael Meyer: Performed the synthesis of the linear peptides, isopeptides and nvoc-derivative, conducted and analyzed kinetic HPLC experiments of the isopeptide with equivalents of DTT or benzyl mercaptan as reducing agent ranging from 0 to 100 equiv., elucidation of reaction mechanism by decoupling the redox processes and analyzing them by HPLC, characterization of the nitroso byproduct by HPLC and MS, performed the NMR analysis

for characterization of the peptides and NMR temperature gradient analysis of the assembly of monomer and dimer, performed the Proteostat assay to analyze the critical assembly concentration of the isopeptide, monomer and dimer, data analysis and interpretation of all corresponding results, and wrote the manuscript.

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In living systems, the formation of structures relies on balancing kinetic and thermodynamic influences powered by reversible covalent bond chemistry. Although synthetic efforts have replicated these processes to some extent, elucidating their combination is necessary to identify mechanisms that confer nature's structural precision and flexibility within a complex environment. Here we design a photolytic reaction cascade where competing redox pathways control the transience, interconversion and production rates of thiol/disulfide supramolecular monomers *in situ*. In contrast to direct assembly by dissolution, cascade generation of the same monomers formed hierarchical assemblies with different structural order. Redox-induced cycling between thiol–disulfide formation led to the emergence of new secondary structures and chirality within the final assemblies. These multiple structural states found within the same molecular system demonstrate the concept of assembly plasticity engaged frequently in biology. We demonstrate the importance of reaction complexity in controlling supramolecular propagation and in expanding the library of nanoarchitectures that can be created.

Hierarchical structures in biology are driven by complex reaction pathways with corrective mechanisms to ensure their precision and function within the often-crowded living system. In cells, the number of reactive intermediates and partners engaged in the propagation of a single superstructure far exceeds that of synthetic supramolecular systems, often raising the question of whether such complexity is necessary from a structural perspective. The microtubule of the cytoskeleton, for example, is made up of simple tandem repeats of α - and β -tubulin proteins but involves chemical reactions by seven groups of post-translational modification¹ and a proteome of >500 microtubule-associated proteins that collectively regulate its structure^{2,3}. At first glance, the effort to build the microtubule seems to be excessive but recent findings in synthetic systems have found that supramolecular assembly processes are highly delicate^{4–9}. Seemingly minimal (<0.01%) changes in liquid–liquid interfaces and composition and in fluid dynamics are as important in directing structure formation as well-established aspects such as concentration, temperature, molecular design and solvent polarity^{10–12}. Given that the former set of

factors are dynamic and heterogeneous within the cells, the magnitude and variety of control mechanisms necessary to guide structure formation in cells appear more reasonable.

Within the crowded cellular environment, the production of assembly precursors and their quantity are tightly regulated by interconnected reaction pathways. In this way, the reaction kinetics are coupled to the assembly landscape where the local chemical state and concentration influence the probability of metastable or kinetically trapped structures¹³. The importance of chemical reaction kinetics in controlling supramolecular assemblies has since been demonstrated in synthetic dissipative systems where advances in mimicking cellular homeostasis have showed recent success^{14–23}. However, unlike synthetic analogues, biological precursors also modulate polymerization dynamics through structural plasticity, which reflects changes in the structural state without altering its chemical identity²⁴. In microtubules, α -/ β -tubulin at the polymerization terminal or the receding end are chemically identical yet they create opposite effects upon the global structure. Separate pathways then control the immediate

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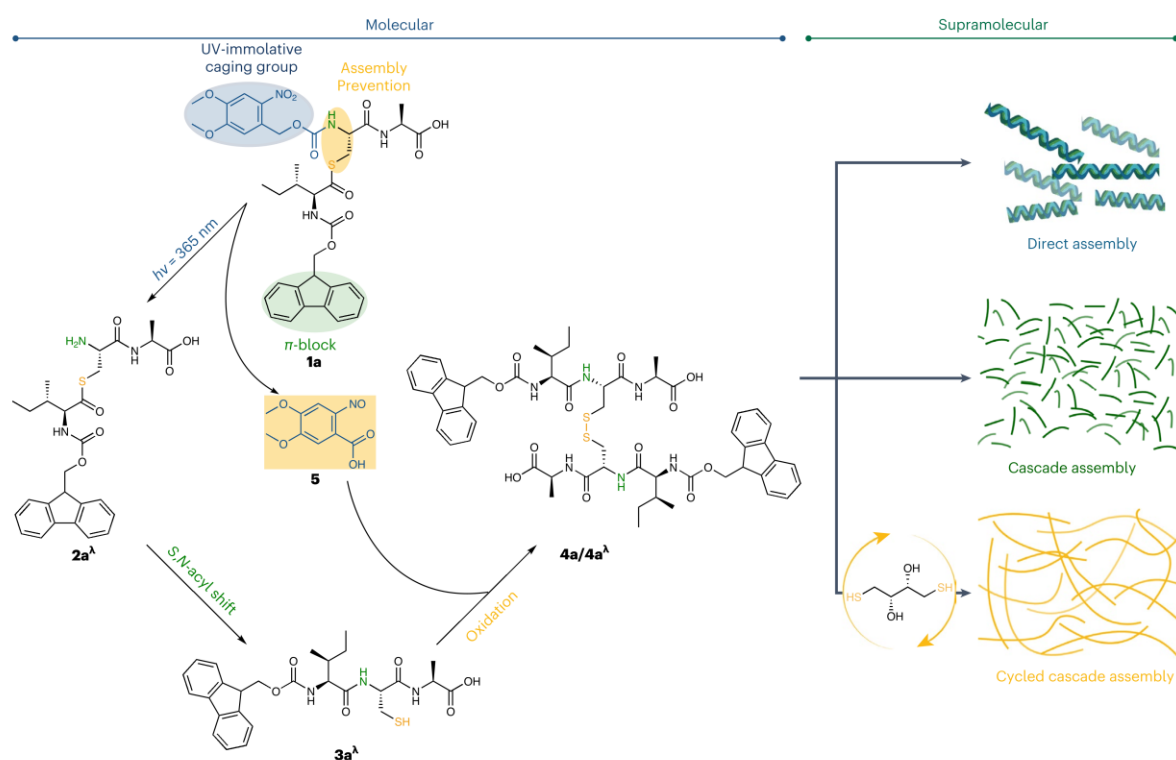


Fig. 1 | Photolytic cascade generation of molecular components and its effect on the formation of different supramolecular nanostructures. The molecular section describes a series of covalent reactions, the combined kinetics of which determine how the assembly precursor is produced in solution. By exposing the precursors to different combinations of reactions, the supramolecular pathway is altered, producing several morphologies despite having the same chemical identity. The reaction pathway of **1a** after ultraviolet irradiation leads to the

decayed isopeptide **2a^A**, and rearrangement/linearization and oxidation of the thiol **3a^A** to the disulfide **4a^A**. The nvoc side product **5** enters the cycle as the oxidant to fuel the final step. The addition of DTT reverses the oxidation, causing **3a^A** and **4a^A** to rapidly interconvert, thereby delaying the production of **4a^A**. Depending on the assembly pathway (direct, cascade, cycled cascade) of **4a/4a^A**, different supramolecular nanostructures and chirality are produced.

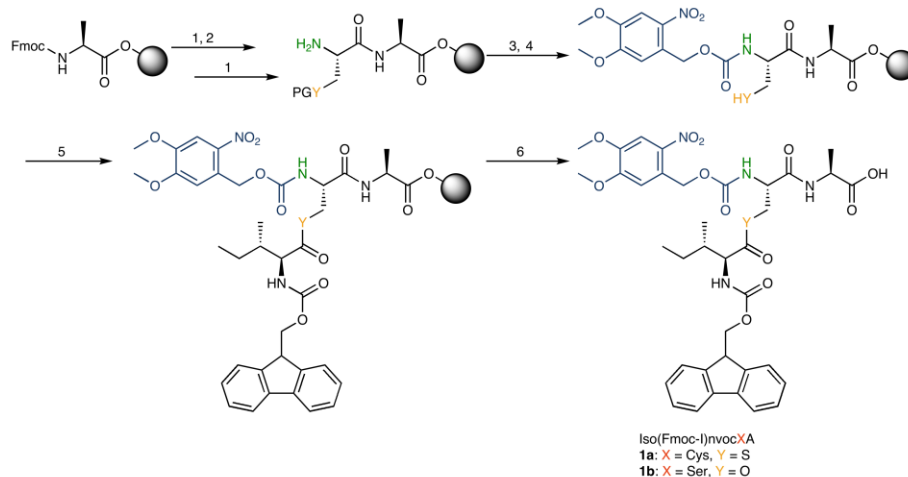


Fig. 2 | Solid-phase synthesis of the isopeptide derivatives. Synthesis of iso(Fmoc-I)nvocXA by solid-phase peptide synthesis: (1) piperidine; (2) Fmoc-X (protecting group)-OH, DIC, ethyl cyanohydroxyiminoacetate (oxyma); (3) 6-nitroveratryl chloroformate (nvoc-Cl), triethylamine; (4) dichloromethane, TIPS, TFA; (5) Fmoc-Ile-OH, DIC, 4-dimethylaminopyridine; (6) TFA, TIPS, H₂O.

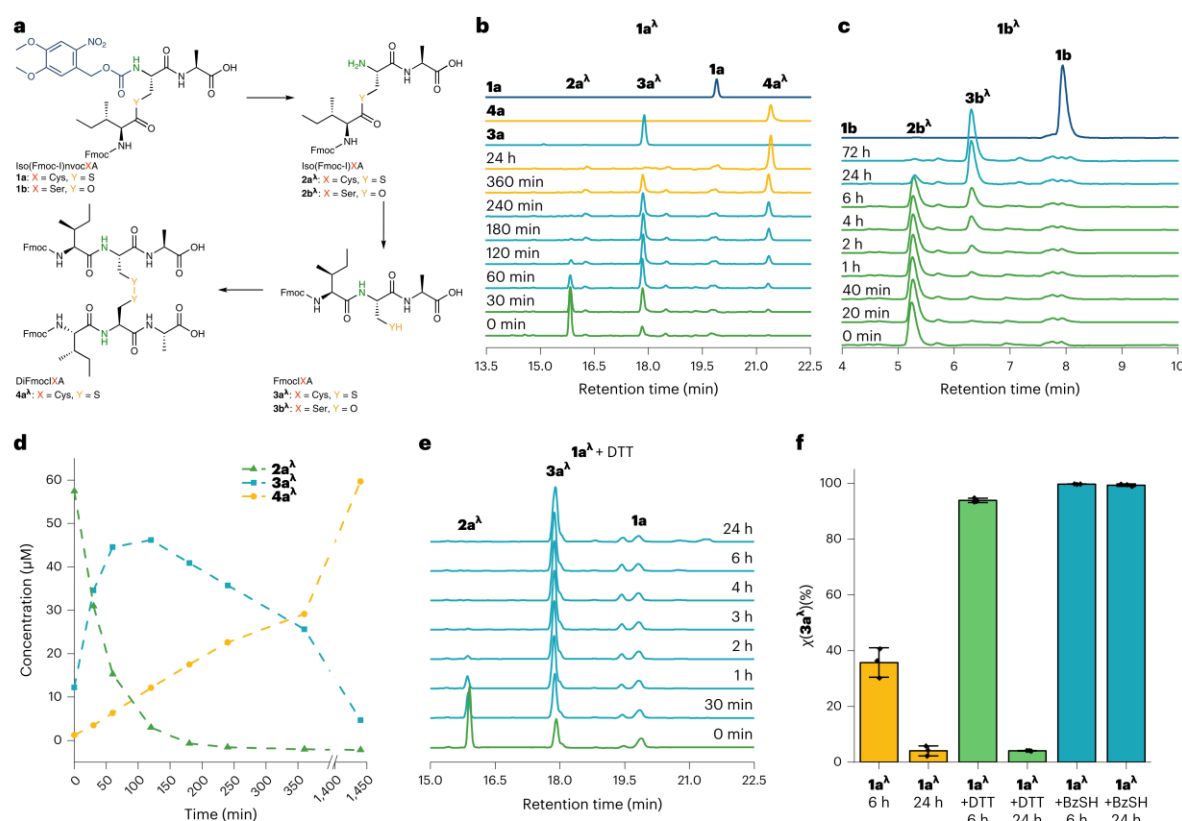


Fig. 3 | Kinetic profile of iso(Fmoc-I)nvocXA derivatives showing accelerated conversion for 1a and recycling pathway. a, Reaction pathway of the two isopeptides after irradiation. **b**, HPLC kinetics of iso(Fmoc-I)nvocCA 1a in methanol:NH₄HCO₃ buffer (1:1) over the 24 h after irradiation. After deprotection, iso(Fmoc-I)CA 2a rearranges into the linear peptide Fmoc-ICA 3a and oxidizes into the dimer DiFmoc-ICA 4a. **c**, HPLC kinetics of iso(Fmoc-I)nvocSA 1b in methanol:NH₄HCO₃ buffer (1:1) over the 72 h after irradiation. After

deprotection, iso(Fmoc-I)SA 2b rearranges into the linear peptide Fmoc-ISA 3b. **d**, Concentrations of the intermediates and the product over time (1a) calculated from the calibration curves of 3a and 4a. The intermediate 2a was calibrated with 3a. **e**, HPLC kinetics of iso(Fmoc-I)nvocCA 1a 100 μM irradiated with DTT 10 mM in methanol:NH₄HCO₃ buffer (1:1). **f**, Molar proportion (χ) of 3a versus 4a after 6 h and 24 h for 1a, 1a + 10 equiv. of DTT and 1a + 10 equiv. of benzyl mercaptan. Data are presented as $\pm$ s.d., $n = 3$.

environments surrounding the terminals where the cell can regulate the level of order/disorder within the superstructure. By creating additional nodes of complexity in reaction networks, biology gains access to another dimension of supramolecular features.

Inspired by this concept, we first establish a light-promoted molecular transformation cascade that organizes reversible and non-reversible covalent reactions to generate assembly precursors. The reaction kinetics will therefore dictate the availability of precursors over time while the reversible segment will modulate the lifetime of the precursors switching between active states. By controlling these parameters in the reaction cascade, we aim to demonstrate that assembly precursors can access different superstructures that are unavailable via conventional, direct supramolecular assembly.

As the first activator of the reaction cascade, we take the photoprotecting group (PPG) 2-nitroveratryloxycarbonyl (nvoc), for which the mechanism of photolysis and broad substrate capacity are well established²⁵. The nvoc group absorbs ultraviolet light (365 nm) to form a zwitterionic excited state followed by an N=O_{nitroso} bond scission and the expulsion of CO₂ (ref. 25). The resultant aromatic N=O_{nitroso} group can oxidize thiols into disulfides via sequential addition and elimination steps²⁶. By combining both reactions, we propose that

thiol-containing molecules protected with the nvoc group would undergo a self-promoted transformation into disulfides in situ. As the thiol component, we design iso(Fmoc-I)nvocCA 1a, consisting of a masked cysteine connected via a thioester to form an isomerized backbone that temporarily blocks self-assembly. The nvoc group is installed at the N-terminus of Cys, thus regulating both the isomerization and self-promoted oxidation.

While the nvoc group undergoes a Norrish type II reaction to form the N=O_{nitroso} group 5, the peptide performs an S_N-acyl rearrangement aligning the backbone and thus liberating the Cys-SH group, yielding Fmoc-ICA (3a, Fig. 1)²⁵. The N=O_{nitroso} by-product 5 promotes the main reaction sequence, oxidizing the free thiol of Fmoc-ICA 3a into its disulfide form DiFmoc-ICA 4a. We regulate the redox reversibility between 3a and 4a by introducing dithiothreitol (DTT) into the cascade, where the interconversion between both species is determined by the stoichiometry between N=O_{nitroso} and DTT. The presence of both oxidation and reduction pathways therefore regulates the lifetime of 3a and 4a in solution, with the prevailing reaction defining the final species. To clarify the nomenclature, products formed within the cascade are denoted as 3a for Fmoc-ICA and 4a for DiFmoc-ICA, whereas the independently synthesized control

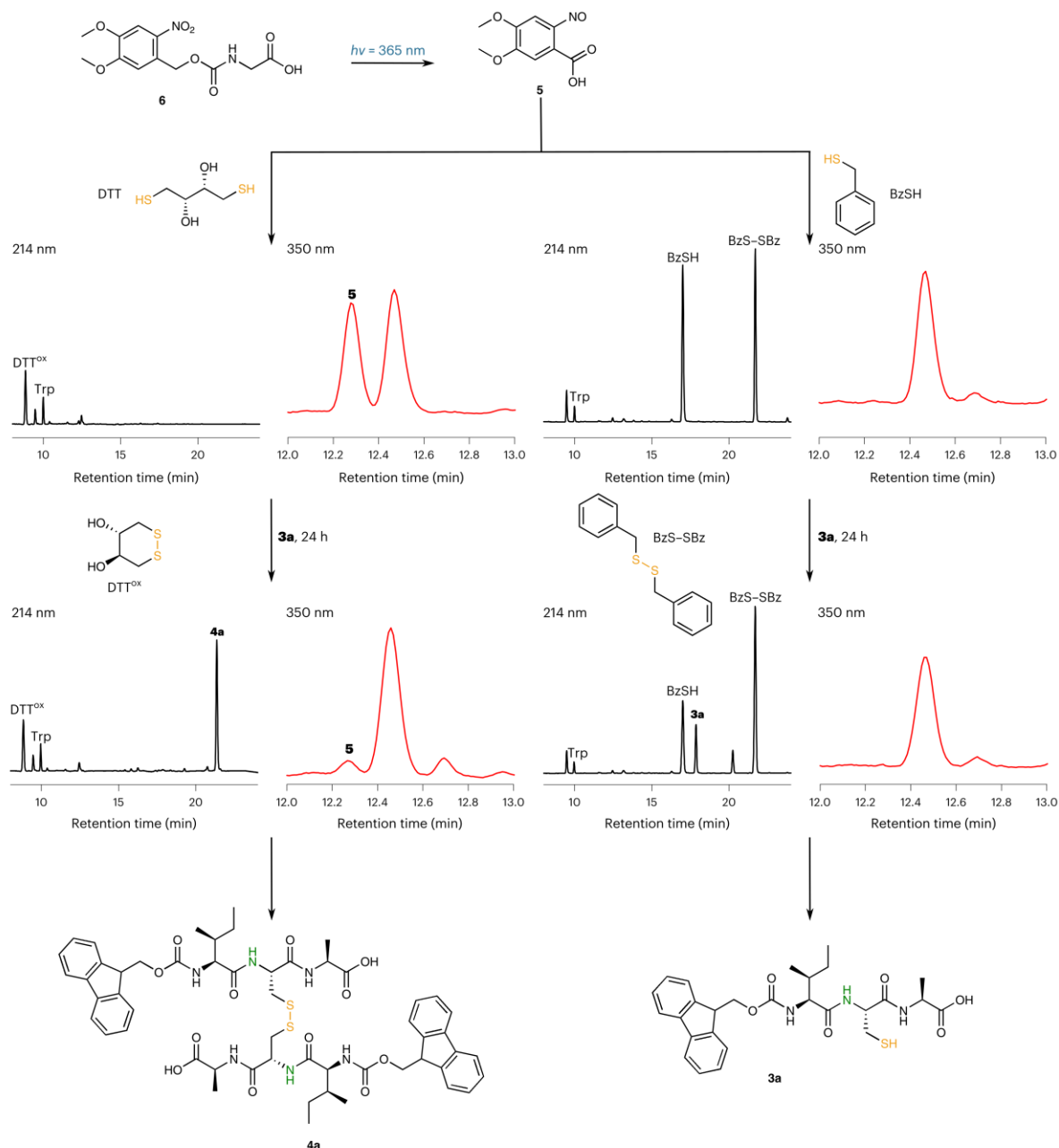


Fig. 4 | Decoupling the redox process by pretreating N=O_{nitroso} with different thiols. HPLC chromatograms of 6-nitroveratryl glycine (nvoc-Gly) **6** irradiated with 10 equiv. DTT (left) or BzSH (right) after 24 h. The intermediate N=O_{nitroso} **5** is consumed by BzSH to produce BzS-SBz, but is not consumed by DTT/DTT^{ox}.

Fmoc-ICA **3a** (1 equiv.) was then added and after a further 24 h samples were analysed by HPLC again. Oxidation into **4a** was observed for the DTT-pretreated reaction due to the presence of N=O_{nitroso} **5**. LC traces at 214 nm (black) and 350 nm (red) are shown for each step.

peptides are termed **3a** and **4a**, respectively. Structure formation is compared by (1) direct assembly where precursors are diluted in a poor solvent, (2) cascade assembly where **1a** is activated by light to eventually form the assemblies of **4a^h** and (3) cyclic cascade assembly where DTT is added to interconvert **3a^h** and **4a^h** (Fig. 1). Hence, the control peptides and their cascade counterparts possess identical

chemical structures but differ in the way they are made available for assembly in solution.

Results and discussion

Peptide synthesis was carried out using Fmoc solid-phase peptide synthesis on Wang resin using *N,N'*-diisopropylcarbodiimide (DIC) and

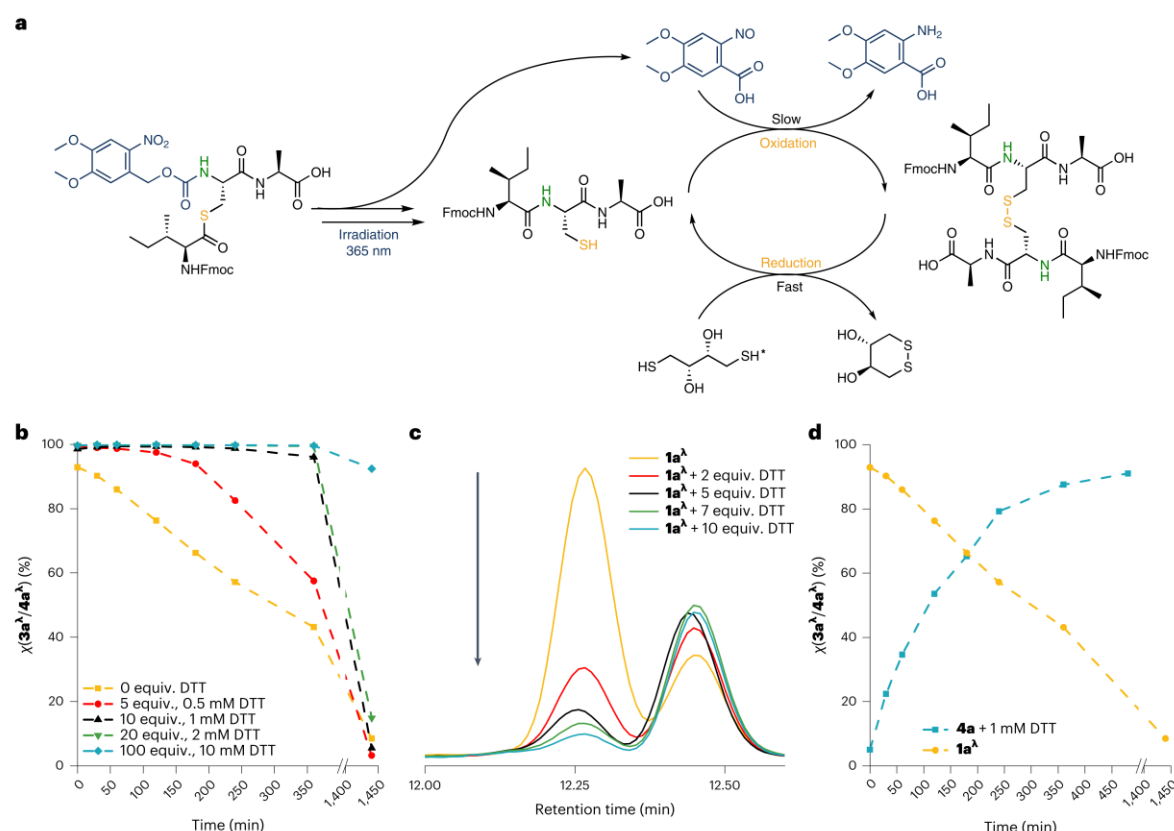


Fig. 5 | Modulation of the cascade by DTT. **a**, Reaction pathway of $1a^A$ with DTT. $N=O_{\text{nitroso}}$ **5** and DTT form a redox cycle between $3a^A$ and $4a^A$ where they undergo multiple redox reactions. Denoted by DTT*, DTT also undergoes a separate, radical oxidation pathway with **5**. **b**, Molar proportion of $3a^A$ versus $4a^A$ with different concentrations of DTT over 24 h. **c**, HPLC chromatograms of $1a^A$ with

different concentrations of DTT. The peak of **5** is shown in the 350 nm trace.

d, Molar proportion of $3a^A$ versus $4a^A$ for the reduction of 50 μM $4a^A$ with 1 mM DTT and the cascade of 100 μM $1a^A$ with 1 mM DTT over time. Note that 50 μM disulfide **4a** is equivalent to 100 μM of thiol $1a^A$.

ethyl (2Z)-2-cyano-2-(hydroxyimino)acetate (OxymaPure) for the iterative coupling steps (Fig. 2). To construct an isomerized Cys backbone, Fmoc-cysteine-monomethoxytrityl (Fmoc-Cys(Mmt)-OH) was used to enable orthogonal installation of the nvoc group via 6-nitroveratryl chloroformate on the N-terminus and Steglich esterification at the S-terminus. The so-called isopeptide was cleaved off from the resin with trifluoroacetic acid (TFA):triisopropyl silane (TIPS): H_2O (95:2.5:2.5) and purified by high-performance liquid chromatography (HPLC) to afford **1a**. As reference compounds, Fmoc-ICA **3a** and DiFmoc-ICA **4a** were synthesized using modified protocols (Supplementary Information). The serine analogue, where the cysteine of **1a** was substituted by serine to form **1b**, was also synthesized to elucidate the role of the thiol group in the oxidation reaction.

The molecular transformation of each step in the cascade was characterized by HPLC in methanol: NH_4HCO_3 buffer (1:1) at pH 7.4. This condition provides pH control and ensures that reaction intermediates and products remain in their molecular state and can therefore be quantified, using tryptophan as an internal standard. The optimum ultraviolet irradiation (365 nm) time of 180 s gave a deprotection conversion of 94% (Supplementary Fig. 20). Upon irradiation, the formation of each intermediate—the deprotected isopeptide **2a** and the *S,N*-rearranged peptide **3a**—and oxidation to the disulfide **4a** was monitored over 24 h (Fig. 3b). Separately synthesized **3a** and **4a** were used as references for

the retention times. Upon successful photodeprotection, the *S,N*-acyl shift to **3a** began immediately and proceeded with a $t_{1/2}$ (time required to attain 50% conversion) of 30 min. In comparison with the *O,N*-acyl shift²⁷ from **2b** to **3b** with $t_{1/2} > 6$ h (Fig. 3c), the thiolate was demonstrated to be a better leaving group with an accelerated rearrangement kinetics. Although no further chemical changes were detected with the serine analogue **3b**, the cysteine derivative **3a** was further oxidized by the $N=O_{\text{nitroso}}$ into **4a** with a $t_{1/2} = 5$ h (Fig. 3d). The control reference **3a** remains stable in its reduced form for 24 h under ambient conditions, confirming that its oxidation into **4a** was the result of the cascading step of the $N=O_{\text{nitroso}}$ by-product (Supplementary Fig. 25). We also studied the cascade reaction in an aprotic solvent, acetonitrile, in which the oxidation step of **3a** was delayed by more than a factor of 4 ($t_{1/2} > 24$ h) (Supplementary Fig. 26).

The mechanism was investigated in parts by decoupling the cascade, separating the $N=O_{\text{nitroso}}$ production step and the thiol oxidation. Therefore, nvoc-glycine **6** was synthesized and, via irradiation, found to produce the $N=O_{\text{nitroso}}$ intermediate **5** (retention time, $R_t = 12.27$ min) with quantitative conversion (Supplementary Fig. 33). In contrast to most findings that observed the nitrosobenzaldehyde to be the cleavage product, we found intermediate **5** to be the carboxylic acid derivative that was reported to form in oxygenated aqueous conditions (Supplementary Fig. 34)²⁸. The addition of separately synthesized **3a**

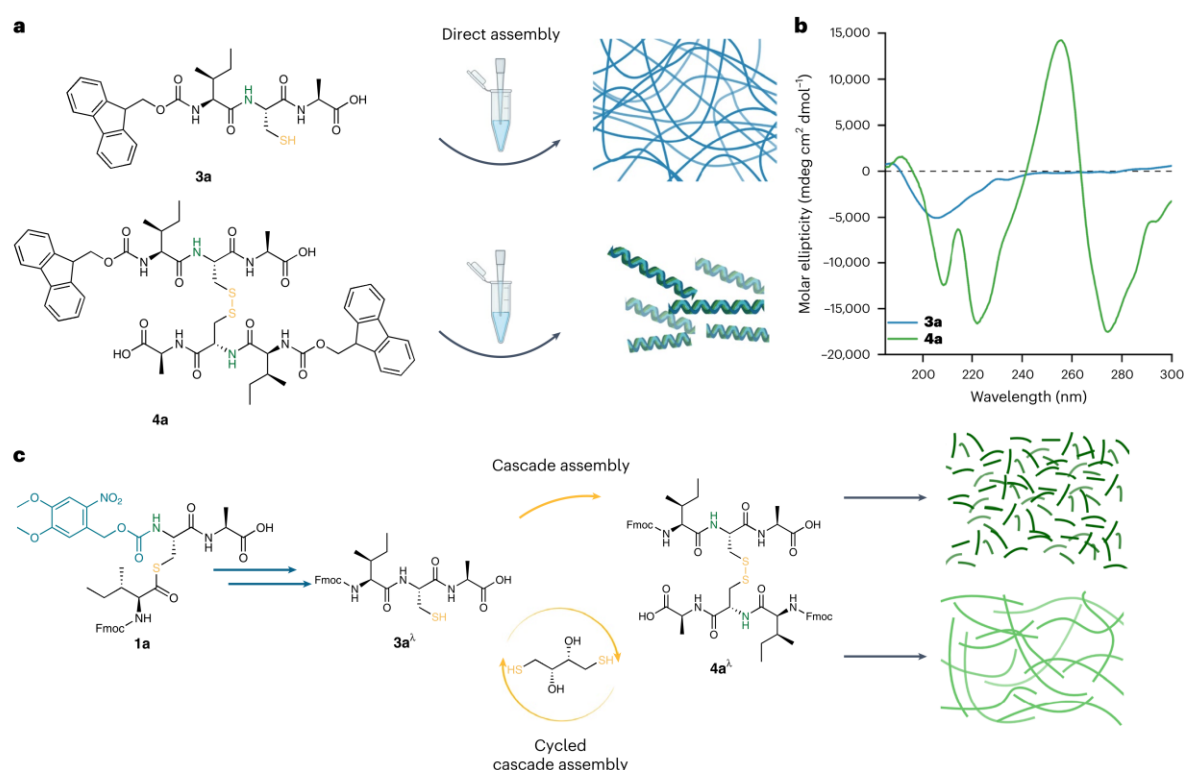


Fig. 6 | Influence of assembly pathways (direct, cascade, cyclic cascade) on structure formation, chirality and long-range order. a, Direct assembly of **3a** and **4a**. **b**, CD spectra of **3a** and **4a** indicating distorted α -helical and atypical twisted β -sheet structures, respectively. **c**, Schematic of the cascade and cyclic cascade assembly.

into the reaction successfully consumed the intermediate **5** to form **4a** (Supplementary Fig. 35). This reaction pathway was further confirmed by using benzyl mercaptan (BzSH, $R_f = 17.01$ min) as a substitute for **3a**, which resulted in the formation of the oxidized BzS–SBz ($R_f = 21.70$ min) (Supplementary Fig. 36). Each molecule of $N=O_{\text{nitroso}}$ **5**, apart from side reactions, is capable of oxidizing up to 4 equiv. of thiols²⁶.

From these findings, we postulate that the cascade from **1a** to **4a** can be modulated by interfering with the redox-sensitive species. As expected, complete inhibition of step **3a** to **4a** was observed by introduction of excess BzSH (10 equiv.), which reacted competitively with $N=O_{\text{nitroso}}$ **5** (Fig. 3f). Next, we further increase the complexity of the system by adding a selective reductant (DTT) into the cascade which reduces disulfide **4a** back to thiol **3a**. The coexistence of DTT and $N=O_{\text{nitroso}}$ **5** therefore forms a redox cycle, causing molecules of **3a** and **4a** to rapidly interconvert, with the prevailing stoichiometry and their kinetics determining the final redox state of the peptide at equilibrium. Hence, as long as DTT is present in solution, full oxidation by $N=O_{\text{nitroso}}$ **5** could not be achieved and would be observed as a net delay in the formation of **4a** (Fig. 3e). Although DTT also possesses thiol groups, its intramolecular cyclization favoured an oxygen-promoted radical elimination from the $N=O_{\text{nitroso}}$ **5** core in the presence of water and oxygen^{29,30}. The radical mechanism proposed would cycle the aryl nitroso compound, which preserves the oxidation capacity of **5**. This was confirmed by treating $N=O_{\text{nitroso}}$ **5** with excess DTT (10 equiv.) for 24 h before adding 1 equiv. of thiol **3a** for a further 24 h incubation. The first 24 h of pretreatment, complete cyclization of DTT occurred, and in the following 24 h, complete oxidation of **3a** to **4a** showed the

activity of $N=O_{\text{nitroso}}$ **5** (Fig. 4). In contrast, pretreatment with excess BzSH (10 equiv.) completely consumed $N=O_{\text{nitroso}}$ **5** in the first phase, and hence no subsequent oxidation of **3a** was observed when added in the second phase (Fig. 4).

To understand the interplay of DTT and $N=O_{\text{nitroso}}$ in the redox cycle, we ran the full cascade over 24 h with varying amounts of DTT (5–100 equiv., 0.5–10 mM) and elucidated the reaction kinetics (Fig. 5a). By plotting the molar proportion of **3a**/**4a**, we observed a time-dependent suppression of the formation of disulfide **4a** because the reduction by DTT initially dominated the reaction pathway and rapidly reduced **4a** as soon as this was formed (Fig. 5b). Eventually, at lower DTT ratios, oxidation to **4a** dominated as DTT supply decreased over time. Consequently, by increasing the amounts of DTT and thus the reduction rate, the delay in the formation of **4a** was prolonged as long as sufficient $N=O_{\text{nitroso}}$ **5** was present (Fig. 5b,c). For example, at 1 mM of DTT, synthesized **4a** was reduced to **3a** with a $t_{1/2} = 2$ h, whereas the DTT-free cascade oxidation into **4a** by $N=O_{\text{nitroso}}$ **5** occurred with $t_{1/2} = 5$ h (Fig. 5d). Comparing these reaction rates at 1 mM DTT, the reduction by DTT controls the initial phase of the redox cycle, where **4a** was only transiently present due to its rapid reconversion to **3a**. Therefore, steady production of **4a** could only occur when the rate of oxidation by $N=O_{\text{nitroso}}$ **5** began to dominate after 6 h (Fig. 5b).

Next, we studied the impact of the cascade pathway and redox cycling on structure formation through self-assembly at a standardized condition of methanol: NH_4HCO_3 buffer (1:9) at pH 7.4. Reducing the organic solvent content to 1:9 (from 1:1 used in the reaction cycle in Fig. 3) was necessary to promote self-assembly. To confirm that the cascade proceeded efficiently under reduced amounts of methanol,

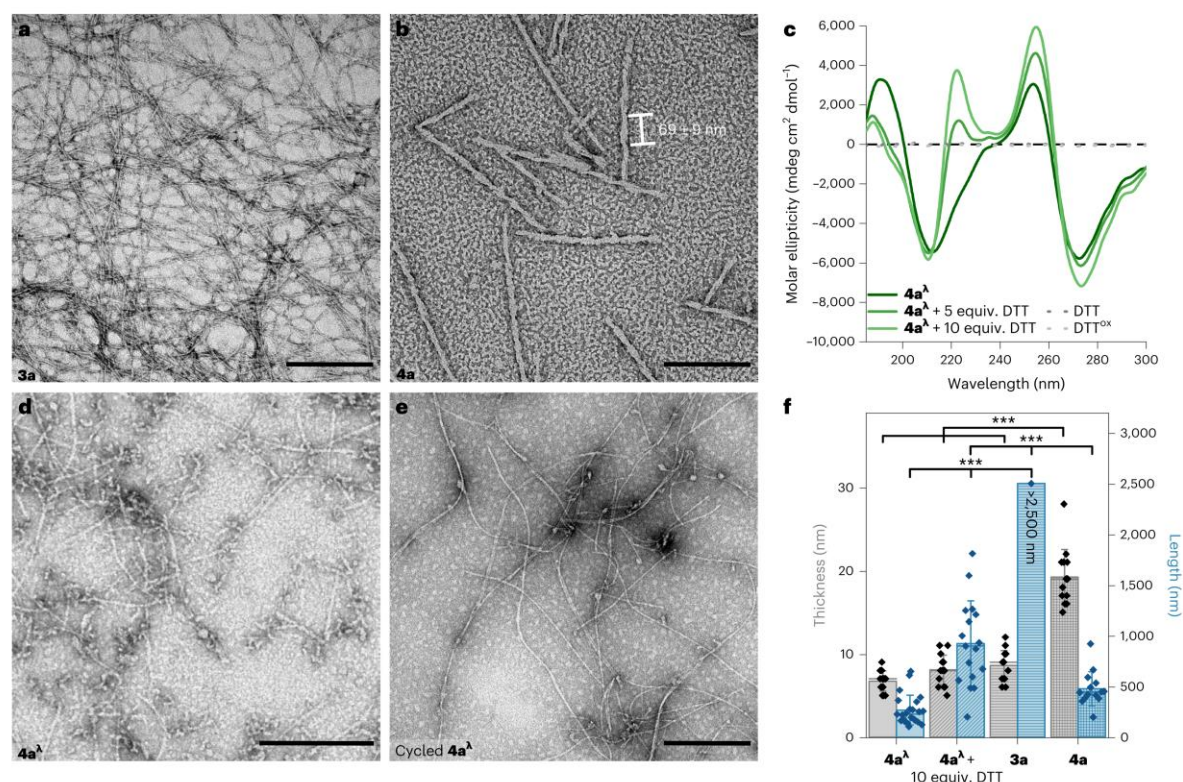


Fig. 7 | Influence of assembly pathways (direct, cascade, cycled cascade) on structure formation, chirality and long-range order. a, TEM image of **3a** revealing a network consisting of thin (9 ± 2 nm) fibres. **b,** TEM image of **4a** revealing twisted fibres with defined lengths (480 ± 170 nm) and periodicity (69 ± 9 nm). **c,** CD spectra of **4a^a** with different equivalents of DTT indicating the emergence of secondary chirality at 222 nm and overall order as a function of redox cycling. DTT in its reduced and oxidized form (2 mM) as controls

showed no contribution to the CD signature. **d,** TEM image of **4a^a** showing fragmented fibres with average lengths of 260 ± 150 nm. **e,** TEM image of **4a^a** with 10 equiv. DTT revealing the emergence of longer fibres (920 ± 420 nm). Scale bars in **a, b, d, e,** 250 nm. **f,** Thickness and length of the individual peptide fibres of monomers **3a**, **3a^a**, **4a** and **4a^a** obtained from TEM. Data are presented as mean $\pm$ s.d., $n = 14$. Statistical significance was calculated by analysis of variance with a Tukey post-hoc test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

we performed a reverse analysis (by redissolving the assemblies in 50% methanol) and we showed that the conversion of **1a^a** to **4a^a** was complete at 24 h (Supplementary Fig. 24). The critical assembly concentration (CAC) was determined by Proteostat assay, a general indicative fluorescence probe for peptide nanostructures (Supplementary Figs. 44–46)³¹. The control peptides **3a** and **4a** showed a CAC of 140 μ M and 50 μ M, respectively, whereas **4a^a** showed a CAC of 30 μ M. The higher fluorescence intensity produced by **4a** suggested that its superstructures contain higher molecular order than those of **3a**. By considering the findings of the CAC, we henceforth standardized the monomer concentrations to 200 μ M, 24 h incubation time at room temperature for all subsequent structural studies to ensure that the formed superstructures are at equilibrium. Temperature-dependent ¹H NMR spectroscopy studies revealed that the assemblies of **3a** and **4a** (Supplementary Figs. 47 and 48) remained stable and did not depolymerize within 20–60 °C, taking into consideration the boiling point of methanol.

Circular dichroism (CD) spectroscopy analysis of **3a** and **4a** revealed major differences in the secondary structures of both assembled nanostructures. The control peptide **3a** showed a positive ellipticity at 187 nm and a negative Cotton effect at 205 nm attributed to the $n \rightarrow \pi^*$ transitions of the peptide backbone typical of a distorted α -helical structure (Fig. 6b)³². Assemblies of **4a** exhibit different secondary structural features that are indicative of higher order.

Positive ellipticity at 191 nm and negative ellipticities at 208 nm and 222 nm indicated an atypical twisted β -sheet structure, corroborating the increased fluorescence detected by the Proteostat assay (Fig. 6b)³². Additionally, the characteristic $\pi \rightarrow \pi^*$ transition of the Fmoc group at 255, 274 and 294 nm could only be found in the corresponding spectra of **4a**, indicating that the Fmoc group played a critical role in the emergence of supramolecular chirality during the assembly process. Having elucidated the supramolecular assembly characteristics by the direct assembly of **3a** and **4a**, we then studied how the reaction cascade would guide the assembly landscape. The reaction cascade to form monomer **4a^a** affords self-assembled superstructures with combined secondary signatures of **3a** in the region < 240 nm, and Fmoc interactions of **4a** in the region > 240 nm. The possibility that the cascade did not drive to completion was excluded because post-assembly analyses by HPLC confirmed the total conversion of **4a^a** and the absence of **3a^a** (Supplementary Fig. 24). It is intriguing that **4a** and **4a^a**, although molecularly identical, adopted different secondary structures within the same solvent. When the cascade was performed with redox cycling through DTT, the final superstructures revealed the emergence of a new positive ellipticity at 222 nm and an increase in the intensity of the Fmoc contribution at > 240 nm (Fig. 7c). These observations increased in magnitude with increasing amounts of DTT.

Transmission electron microscopy (TEM) studies were conducted under the same conditions, that is, methanol: NH_4HCO_3 buffer (1:9) at pH 7.4 (Fig. 6), to investigate the morphologies of the formed supramolecular structures. TEM samples from the cascade were prepared, in situ, from the reaction mixture after 24 h, similar to the CD experiments. Each variation (for example, by DTT) was cross-checked via post-assembly HPLC analyses to ensure complete conversion to **4a**^h. For the control peptides, direct assembly of **3a** resulted in the formation of a network consisting of thin (9 ± 2 nm) peptide fibres (Fig. 7a), whereas **4a** formed short and unusually defined fibres (Fig. 7b). Statistical analyses revealed that **4a** appears to have a critical length (480 ± 170 nm) with a twist periodicity of 69 ± 9 nm (Fig. 7f). Compared to **4a**^h produced by the cascade, the resulting morphologies were largely fragmented fibres with a strong tendency to form localized clusters among few mature fibres (Fig. 7d). Without the irradiation step to initiate the reaction cycle, no assemblies were observed (Supplementary Fig 49). With redox cycling via DTT (10 equiv.), the delayed production of **4a**^h showed a longer growth (>3-fold) in the length of the fibres (920 ± 420 nm) (Fig. 7e). In addition to the bulk analyses from CD, these TEM studies demonstrated that reaction pathways for the in situ generation of supramolecular assemblies can be used to modulate long-range structural order.

Conclusion

We have developed a reaction cascade that highlights the trademark structural plasticity in nature that enables different hierarchical states to be formed by one type of chemical identity. By merging reaction dynamics with a modulated global redox environment, a disulfide assembly precursor (**4a**^h) has been found to exhibit different nanostructural orders, lengths and chirality. The disulfide bond is important in coordinating the aromatic contribution of the monomer towards structure propagation and the overall secondary structure of fibrils. Based on this principle, redox cycling between the thiol (**3a**^h) and disulfide (**4a**^h) results in the emergence of unique chiral signatures, ultimately forming different assembly states of **4a**^h. The presented approach encompasses a number of strategies adopted in nature to regulate hierarchical structures through controlled and reversible production of assembly intermediates. We believe that this integrative chemical concept provides a fresh perspective on the bottom-up synthesis of nanostructures and expands the repertoire of dynamic supramolecular architectures that can be formed under mild conditions.

Methods

Light-induced *S,N*-acyl shift/*O,N*-acyl shift (HPLC)

The isopeptides were dissolved in 300 μL methanol (200 μM) and combined with 300 μL of an aqueous NH_4HCO_3 solution (5 mM, pH 7.4) to yield a 100 μM peptide solution. The peptide solutions were irradiated with ultraviolet light (365 nm) for 3 min while stirring at 300 r.p.m. and tryptophan (10 μM) was added as an internal standard. Aliquots of 10 μL were analysed at certain time points in an HPLC set-up. The kinetics were monitored for 24 h (**1a**) and 72 h (**1b**).

CD spectroscopy

For the measurement of the irradiated iso(Fmoc-I)nvocCA, the peptide was dissolved in 60 μL methanol (2 mM) and combined with 540 μL of an aqueous NH_4HCO_3 solution (5 mM, pH 7.4) to yield a 200 μM peptide solution. The solution was then irradiated with ultraviolet light (365 nm) for 10 min and incubated for 24 h. CD spectra were recorded at wavelengths from 300 to 185 nm with a bandwidth of 1 nm, data pitch of 0.2 nm and a scanning speed at 20 nm min⁻¹ at 20 °C.

TEM

The isopeptide was dissolved in 60 μL methanol at a concentration of 2 mM and diluted with 540 μL NH_4HCO_3 buffer (5 mM, pH 7.4) to yield 100 μL solutions (200 μM). Then, the peptide was irradiated for 10 min

(365 nm) and 100 μL of this solution incubated for 24 h. TEM grids were prepared by pipetting 3 μL solution onto a Formvar-coated copper grid and incubating these for 5 min. After the incubation, the solutions were removed with filter paper, and the grids were stained with 7 μL 4% uranyl acetate solution for 5 min. The grids were washed three times with MilliQ water and dried before being measured.

Data availability

The data that support the findings of this study are available in the Supplementary Information, and all data sets can be found in the Zenodo open access repository (<https://doi.org/10.5281/zenodo.7836264>).

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Author contributions

P.R., D.Y.W.N. and T.W. conceived the project. P.R. and R.M. performed the synthesis experiments and kinetics. P.R. conducted CD analysis and TEM with the advice of I.H., I.L. and K.L. R.M. and M.W. performed the NMR analysis. R.M. and P.R. performed the Proteostat assay. P.R., R.M., D.Y.W.N. and T.W. wrote the manuscript. D.Y.W.N. and T.W. supervised the project. All authors have read and approved the final manuscript.

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Supramolecular assembly guided by photolytic redox cycling

In the format provided by the
authors and unedited

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1. Materials and Instruments

1.1 Materials

All chemicals were purchased from commercial sources and were used without further purification. Solvents and reagents used for peptide synthesis were obtained in peptide grade. Purification with HPLC was performed with water obtained from a Millipore purification system and HPLC grade acetonitrile.

Fmoc-ISA was purchased from GenScript Biotech (Netherlands) B.V.

1.2 Instruments

1.2.1 Microwave Peptide Synthesizer

Peptides and peptide fractions were synthesized with a Liberty Blue Automated Microwave Peptide Synthesizer by CEM Corporation.

1.2.2 High-Performance Liquid Chromatography (HPLC)

The synthesized peptides were purified by preparative HPLC using a Shimadzu setup. A YMC-Actus Triart C18 column (150 x 20 mm, 5 μ m) with a flowrate of 20 mL/min or a Phenomenex Kinetex EVO C18 100 (150 x 30 mm, 5 μ m) with a flowrate of 25 mL/min was used for purification. For analytical measurements an Atlantis T3 column (4.6 x 100 mm, 5 μ m) was used at a flowrate of 1 mL/min.

All purification steps were performed by using gradients of MilliQ water and acetonitrile, each acidified with 0.1% formic acid. Analytical measurements were performed by using gradients of MilliQ water and acetonitrile, each acidified with 0.1% trifluoroacetic acid. Absorbance was recorded at 214, 254, 350 and 400 nm wavelength. The HPLC data were processed with the software LabSolutions by Shimadzu and OriginPro 2019b by OriginLab.

1.2.3 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR spectra for compound **4a**, **1b** and other experiments were recorded on a Bruker Avance III 700 MHz NMR spectrometer. The spectra of compound **1a** and **3a** were recorded on a Bruker Avance III 850 MHz NMR spectrometer. For ^1H spectra, the chemical shifts are reported in parts per million (ppm) from high to low frequency using the residual solvent peak as the internal reference (D_2O δ = 4.79 (HDO) ppm, $\text{DMSO}-d_6$ = 2.50 ($\text{CHD}_2\text{SOCD}_3$) ppm, CD_3CN = 1.94 (CHD_2CN) ppm. All ^1H resonances are reported to the nearest 0.01 ppm. The multiplicity of ^1H signals are indicated as: s = singlet; d = doublet; t = triplet; q = quartet; p = pentet; m = multiplet; br = broad; or combinations of thereof. Coupling constants (J) are quoted in Hz and

reported to the nearest 0.1 Hz. Where appropriate, averages of the signals from peaks displaying multiplicity were used to calculate the value of the coupling constant. The data was processed in MestReNova and TopSpin.

All protons were conducted in different solvent and the temperature was kept at 20°C (or variable temperature for self-aggregation kinetic) and defined with a standard ^1H methanol NMR sample for all the magnet systems. The control of the temperature was realized with a VTU (variable temperature unit) and an accuracy of $\pm 0,1\text{K}$, which was checked with the standard Bruker Topspin 3.6.2 software.

1.2.4 Liquid Chromatography – Mass Spectrometry (LC-MS)

Peptides were analysed by HPLC-ESI-MS on a LC-MS 2020 setup by Shimadzu using a Kinetex EVO C18 100 Å LC column ($50 \times 2.1\text{ mm}$, $2.6\text{ }\mu\text{m}$). Acetonitrile (ACN) and MilliQ water acidified with 0.1% formic acid were used as solvents for all measurements. The solvent gradient started with 5% ACN and 95% water, while the ACN content was linearly increased to 95% in 12 min. Data were processed in LabSolutions and OriginPro 2019b by OriginLab.

1.2.5 Circular Dichroism Spectroscopy (CD)

CD spectra of the peptides were recorded on a JASCO J-1500 spectrometer in a 0.1 cm High Precision Cell by HellmaAnalytics. The recorded data were processed in Spectra Analysis by JASCO and OriginPro 2019b by OriginLab.

1.2.6 Fluorescence Spectroscopy

To record the fluorescence intensity, a SPARK 20M microplate reader by Tecan Group Ltd. was used. The samples were measured in a Greiner 384 flat black well plate and data was processed in OriginPro 2019b by OriginLab.

1.2.7 Transmission Electron Microscopy (TEM)

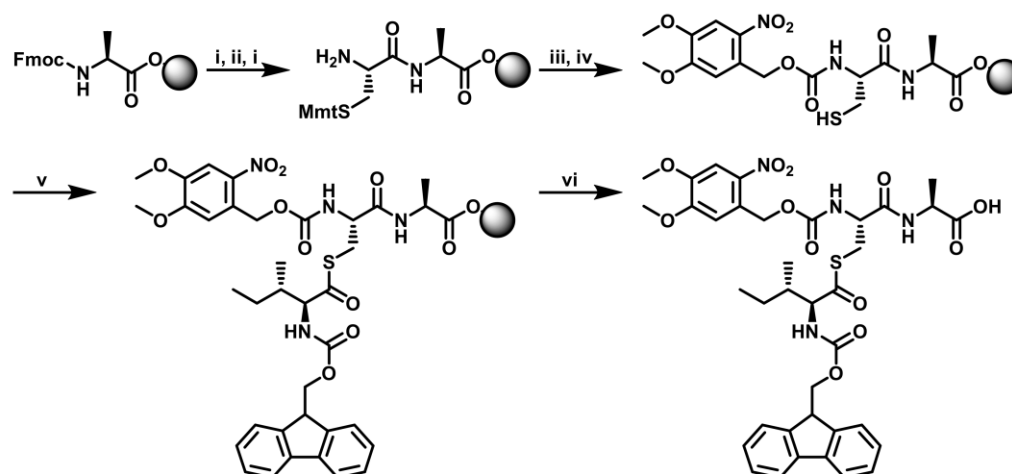
TEM images of the peptide samples were recorded on a JEOL 1400 transmission electron microscope at a voltage of 120 kV. Formvar/carbon-film coated copper grids (300 mesh) by Plano GmbH were used to prepare the samples. The images were processed in Fiji ImageJ.

1.2.8 Irradiation setup

Irradiations were performed with a Thorlabs M365L3 lamp with a nominal wavelength of 365 nm, a bandwidth (FWHM) of 9 nm and a current of 1000 mA.

2. Synthesis

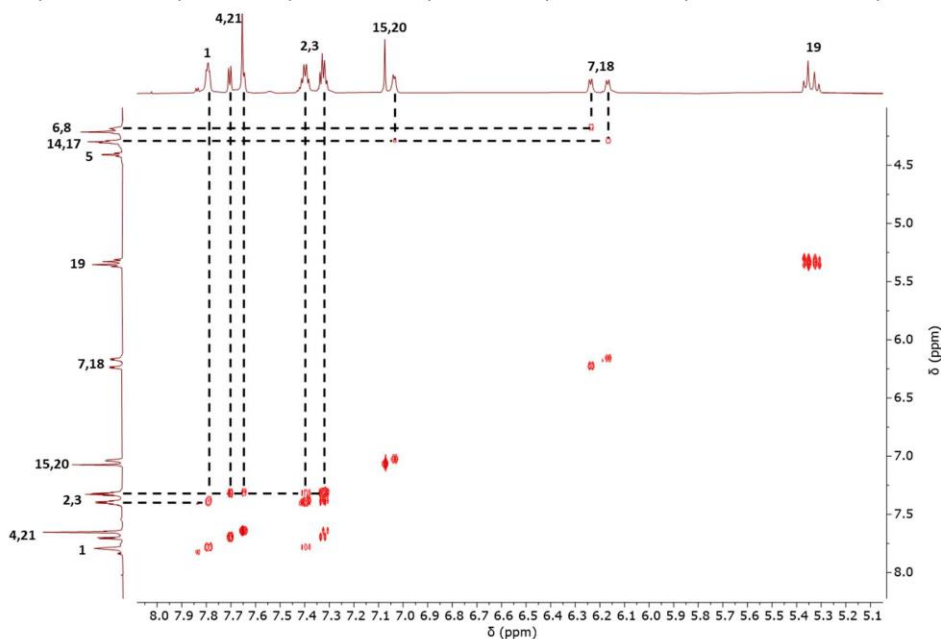
2.1 *Iso*(Fmoc-I)nvocCA



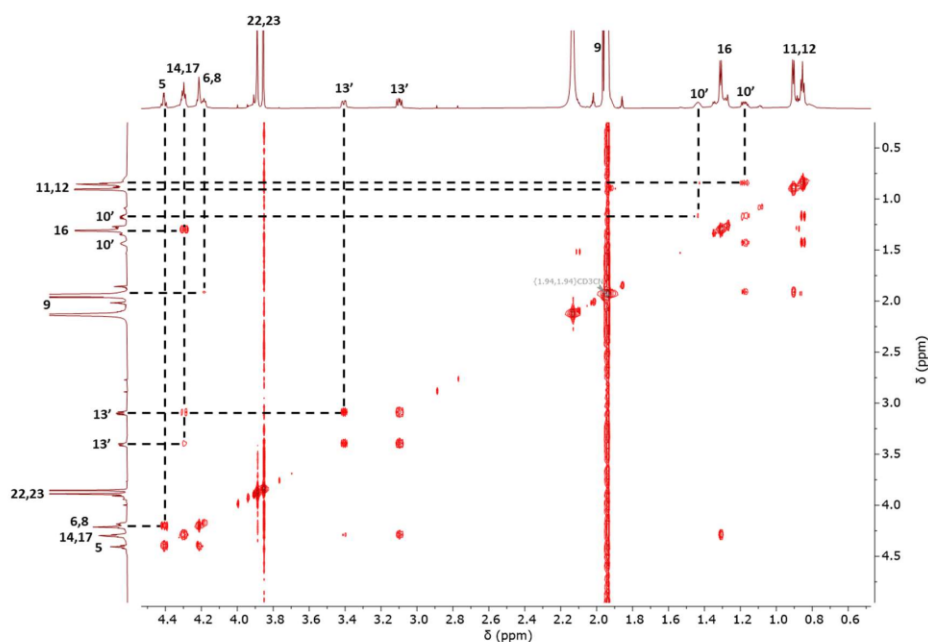
Scheme 1: Synthesis of *iso*(Fmoc-I)nvocCA by solid-phase peptide synthesis. (i) 20% v/v Piperidine in DMF, (ii) Fmoc-Cys(Mmt)-OH, DIC, Oxyma in DMF, (iii) Nvoc-Cl, TEA in DMF, o.n., rt, (iv) DCM, TIPS, TFA, (v) Fmoc-Ile-OH, DIC, DMAP in DMF, o.n., rt, (vi) TFA, TIPS, H₂O, 2 h, rt.

Peptides were synthesized in a microwave assisted peptide synthesizer using the Fmoc solid-phase peptide synthesis strategy, synthesizing the peptide from the C- to N-terminus.^[1] The Wang resin preloaded with Fmoc-Ala (0.5 mmol) was swollen in 5 mL DMF at room temperature for 1 h before transferring it into the peptide synthesizer. DMF was removed via a draining process and the resin swollen with 20 mL DMF for 20 s. After the draining of the DMF, the Fmoc group was removed with 10 mL 20% v/v piperidine in DMF and a heating cycle at 70°C for 25 s followed by another cycle at 90°C for 65 s. The solution was drained, and the resin washed with DMF three times (i). After the last washing step, Fmoc-Cys(Mmt)-OH (0.2 M in DMF, 10 mL), DIC solution (0.5 M in DMF, 4 mL) and OxymaPure solution (1.0 M in DMF, 2 mL) was added to the resin and the solution heated to 70°C for 30 s followed by a heating cycle to 90°C for 120 s (ii). The solution was drained, the Fmoc group removed as described before and washed with DMF three times (i). The free amine was protected with nvoc by dissolving nvoc-Cl (1.1 equiv.) and TEA (2 equiv.) in 5 mL dry DMF under argon atmosphere and shaking at room temperature overnight (iii). After the coupling, the solution was removed, and the resin washed with DCM and dry DMF five times. The thiol function was deprotected with a DCM:TIPS 95:5 solution, containing 1% TFA. For this, 10 mL solution was added to the resin and shaken at room temperature for 10 min. The solution was removed, and the process repeated 8 times (iv). After the deprotection, the resin was washed with DCM and dry DMF five times. To form the thioester, Fmoc-Ile-OH (10 equiv.) and DMAP (1 equiv.) were dissolved in 5 mL dry DMF under argon atmosphere and added to the resin. DIC (10 equiv.) was added to the resin and the mixture shaken at room temperature under argon atmosphere overnight (v). The solution was removed, and the resin washed with DCM five times. The peptide was cleaved from the resin by adding 10 mL of cleavage cocktail (95% TFA, 2.5% TIPS, 2.5% MilliQ water) to the resin and shaking at room temperature for 2 h. The cleavage cocktail was

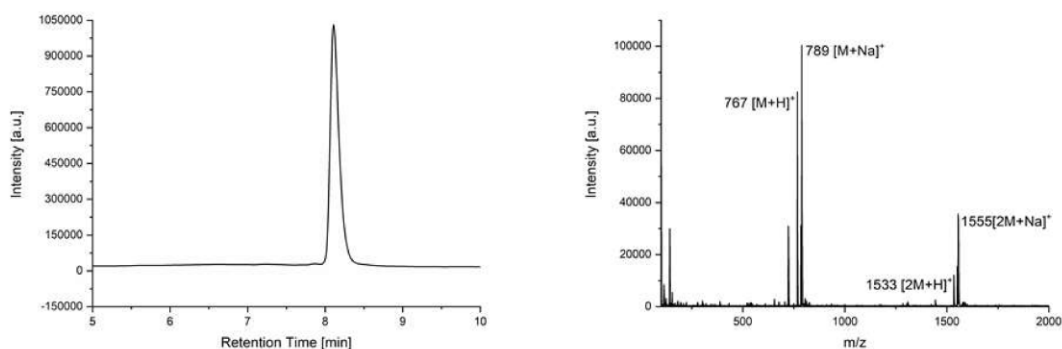
^1H -NMR (850 MHz, CD_3CN) δ [ppm] = 7.79 (t, J = 6.2 Hz, 2H, **1**), 7.71–7.64 (m, 3H, **4** and **21**), 7.40 (q, J = 8.2 Hz, 2H, **2**), 7.33 (q, J = 8.0 Hz, 2H, **3**), 7.07 (s, 1H, **20**), 7.04 (d, J = 7.1 Hz, 1H, **15**), 6.24 (d, J = 8.7 Hz, 1H, **7**), 6.17 (d, J = 8.8 Hz, 1H, **18**), 5.37–5.31 (m, 2H, **19**), 4.42–4.40 (m, 1H, **5**), 4.31–4.29 (m, 2H, **14** and **17**), 4.22–4.18 (m, 3H, **6** and **8**), 3.89 (s, 3H, **22**), 3.86 (s, 3H, **23**), 3.41 (dd, J = 14.0, 4.3 Hz, 1H, **13'**), 3.10 (dd, J = 14.1, 8.9 Hz, 1H, **13'**), 1.96 (s, 1H, **9**), 1.44 (m, 1H, **10'**), 1.31–1.27 (m, 3H, **16**), 1.19–1.16 (m, 1H, **10'**), 0.91–0.85 (m, 6H, **11** and **12**).



Supplementary Fig. 2: Partial ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra (850 MHz, CD_3CN , 298 K) of compound **1a** (aromatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectrum.



Supplementary Fig. 3: Partial ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra (850 MHz, CD_3CN , 298 K) of compound **1a** (aliphatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectrum.



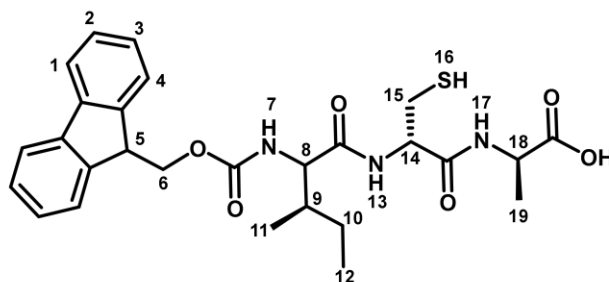
Supplementary Fig. 4: LC spectrum (left) and ESI MS (positive mode, right) of *iso*(Fmoc-I)nvocCA. m/z calculated: $[\text{M}+\text{H}]^+ = 767$, found: $[\text{M}+\text{H}]^+ = 767$, $[\text{M}+\text{Na}]^+ = 789$, $[2\text{M}+\text{H}]^+ = 1533$, $[2\text{M}+\text{Na}]^+ = 1555$.

2.2 Fmoc-ICA

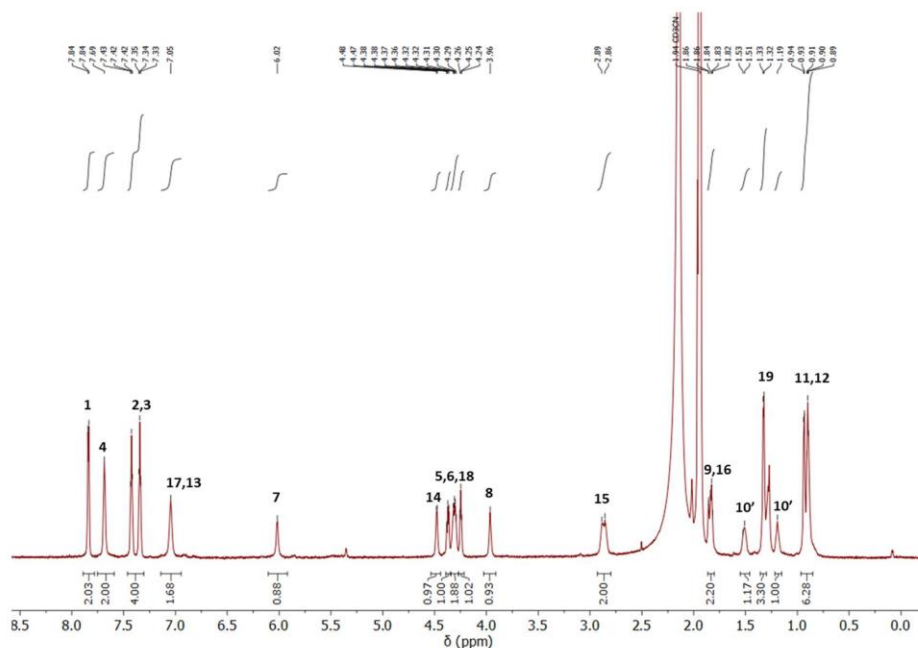
The Wang resin preloaded with Fmoc-Ala (0.5 mmol) was swollen in 5 mL DMF at room temperature for 1 h before transferring it into the peptide synthesizer. DMF was removed via a draining process and the resin swollen with 20 mL DMF for 20 s. After draining of the DMF, the Fmoc group was removed with 10 mL 20 % v/v piperidine in DMF and a heating cycle at 70°C for 25 s followed by another cycle at 90°C for 65 s. The solution was drained, and the

resin washed with DMF three times. After the last washing step, Fmoc-Cys(Trt)-OH (0.2 M in DMF, 10 mL), DIC solution (0.5 M in DMF, 4 mL) and OxymaPure solution (1.0 M in DMF, 2 mL) was added to the resin and the solution heated to 70°C for 30 s followed by a heating cycle to 90°C for 120 s. The solution was drained, the Fmoc group removed as described before and washed with DMF three times. After the last washing step, Fmoc-Ile-OH (0.2 M in DMF, 10 mL), DIC solution (0.5 M in DMF, 4 mL) and OxymaPure solution (1.0 M in DMF, 2 mL) was added to the resin and the solution heated to 70°C for 30 s followed by a heating cycle to 90°C for 120 s. The solution was drained, and the resin washed with DMF three times. The peptide was cleaved from the resin by adding 10 mL of cleavage cocktail (95% TFA, 2.5% TIPS, 2.5% MilliQ water) to the resin and shaking at room temperature for 2 h. The cleavage cocktail was drained, and the resin washed with TFA. The TFA containing solutions were combined and the liquids removed under reduced pressure.

The peptide was purified via HPLC with a YMC-Actus Triart C18 column (flowrate 20 mL/min). The gradient started at 20% ACN content and was linearly increased to 75% over 20 min. The retention time of the product was 18.1 min. After lyophilisation, the product Fmoc-ICA was received as a colourless solid (33.80 mg, 0.06 mmol, 12.8%).

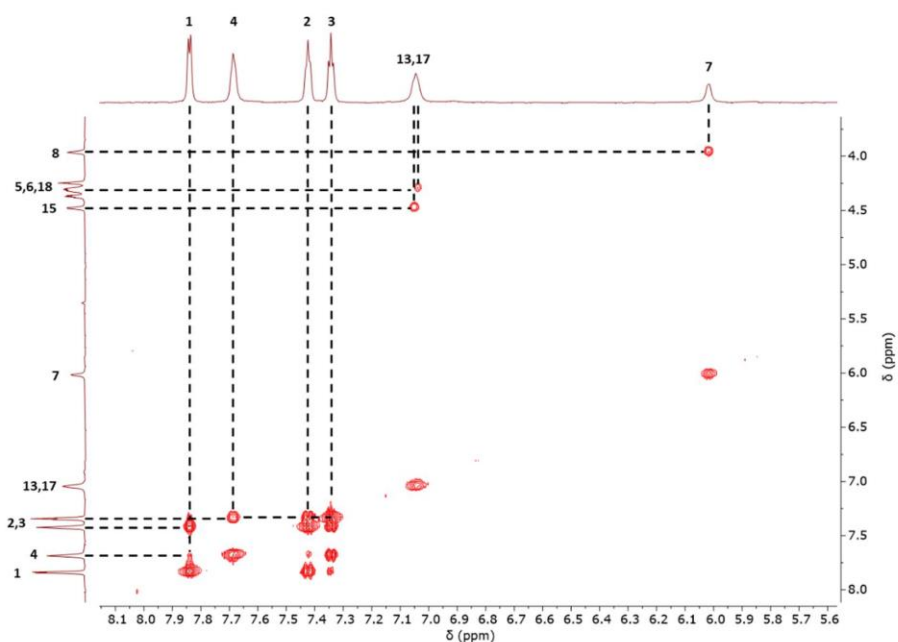


Exact Mass: 527.21

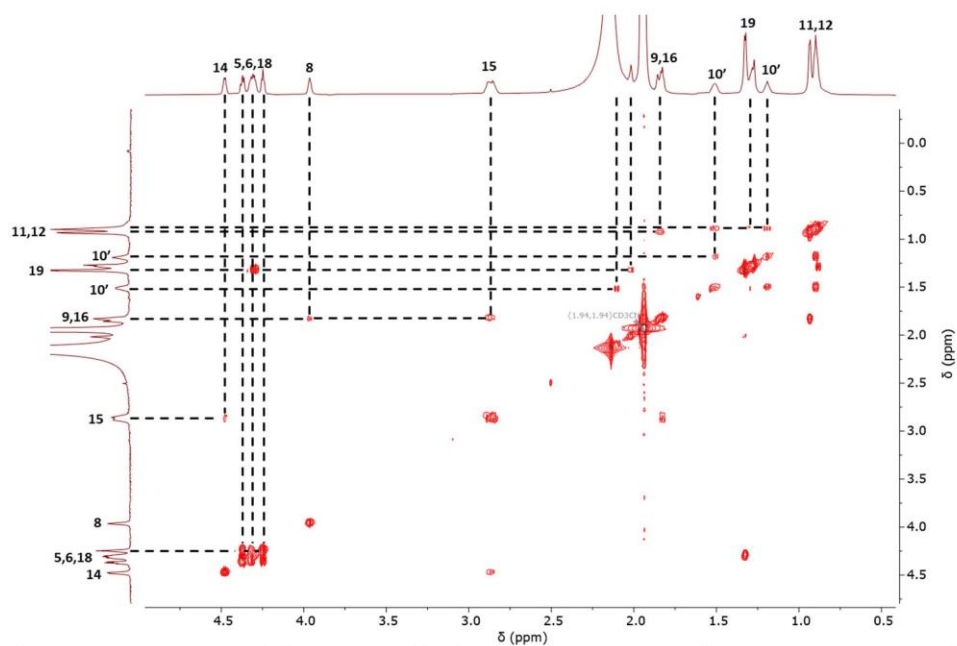


Supplementary Fig. 5: ^1H NMR spectrum (850 MHz, CD_3CN , 298 K) of compound **3a**

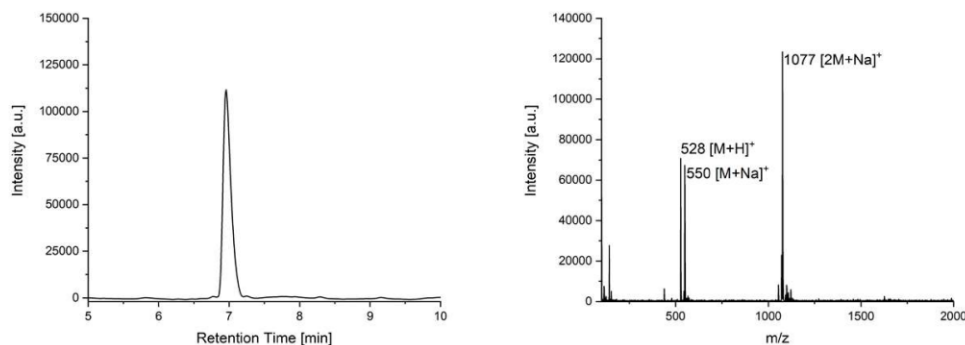
^1H -NMR (850 MHz, CD_3CN) δ [ppm] = 7.79 (d, J = 7.5 Hz, 2H, **1**), 7.69 (s, 2H, **4**), 7.42 (t, J = 7.3 Hz, 2H, **2**), 7.34 (t, J = 7.4 Hz, 2H, **3**), 7.05 (s, 2H, **13** and **17**), 6.02 (s, 1H, **7**), 4.48 (d, J = 7.1 Hz, 1H, **14**), 4.38 (t, J = 8.8 Hz, 1H, **5**), 4.32–4.24 (m, 3H, **6** and **18**), 3.96 (s, 1H, **8**), 2.88 (m, 2H, **15**), 1.86–1.82 (m, 2H, **9** and **16**), 1.53–1.51 (m, 1H, **10'**), 1.33 (d, J = 7.3 Hz, 3H, **19**), 1.19 (m, 1H, **10'**), 0.94–0.89 (m, 6H, **11** and **12**).



Supplementary Fig. 6: Partial ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra (850 MHz, CD_3CN , 298 K) of compound **3a** (aromatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectrum.



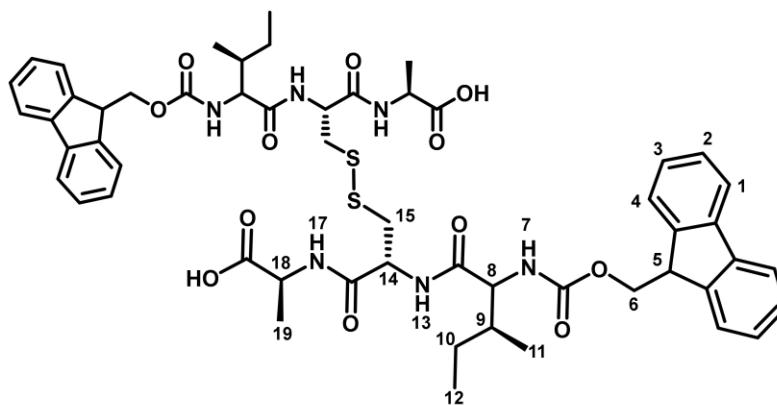
Supplementary Fig. 7: Partial ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra (850 MHz, CD_3CN , 298 K) of compound **3a** (aliphatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectrum.



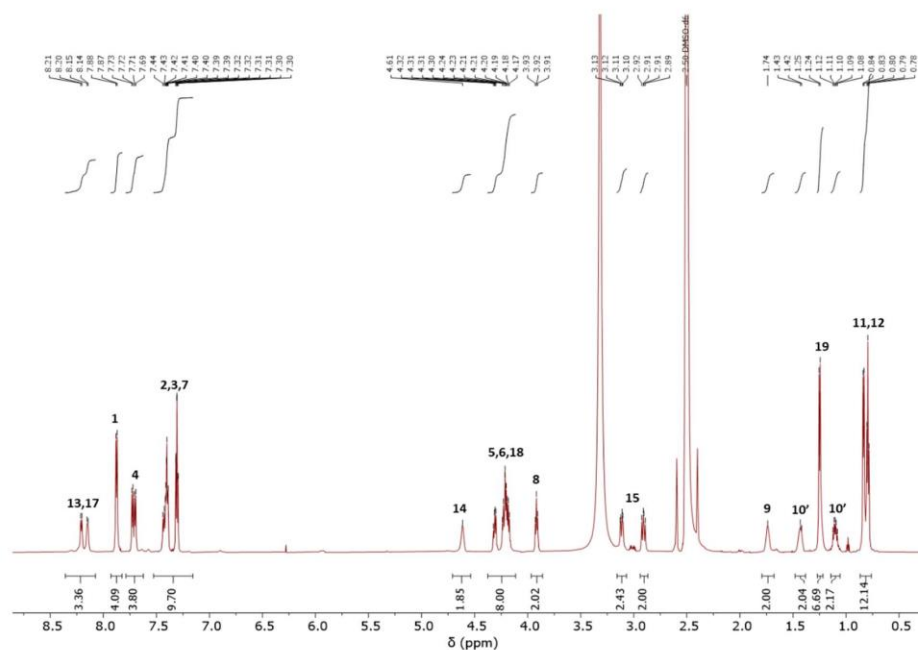
Supplementary Fig. 8: LC spectrum (left) and ESI MS (positive mode, right) of FmocICA. m/z calculated: $[M+H]^+ = 528$, found: $[M+H]^+ = 528$, $[M+Na]^+ = 550$, $[2M+Na]^+ = 1077$.

2.3 DiFmoc-ICA

Fmoc-ICA was dissolved in DMSO and diluted with MeOH to yield a 20% DMSO in methanol (v/v) solution. The solution was stirred at room temperature for 72 h. MeOH was removed by reduced pressure and the peptide solution diluted with MilliQ water. After the dilution, the solution was lyophilized and the peptide dissolved in DMF:MilliQ water 1:1. The peptide was purified via HPLC with a YMC-Actus Triart C18 column (flowrate 20 mL/min). The gradient started at 50% ACN content and was linearly increased to 65% over 10 min. After this, the gradient was increased to 100% ACN within 1 min and kept for 5 min. The retention time of the product was 14.1 min. After lyophilisation the product DiFmoc-ICA was received as a colourless solid (6.5 mg, 6.2 μ mol, 65%).

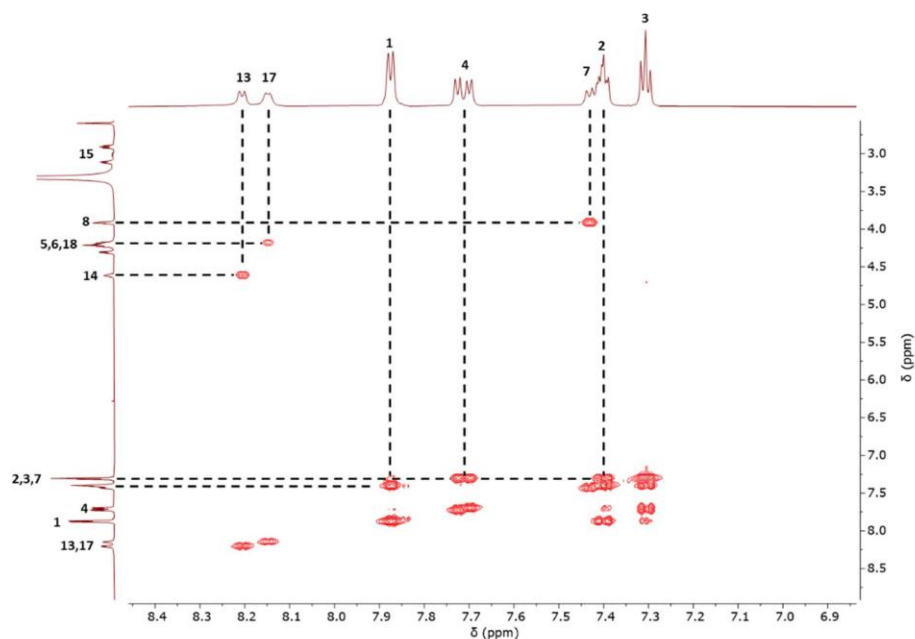


Exact Mass: 1052.40

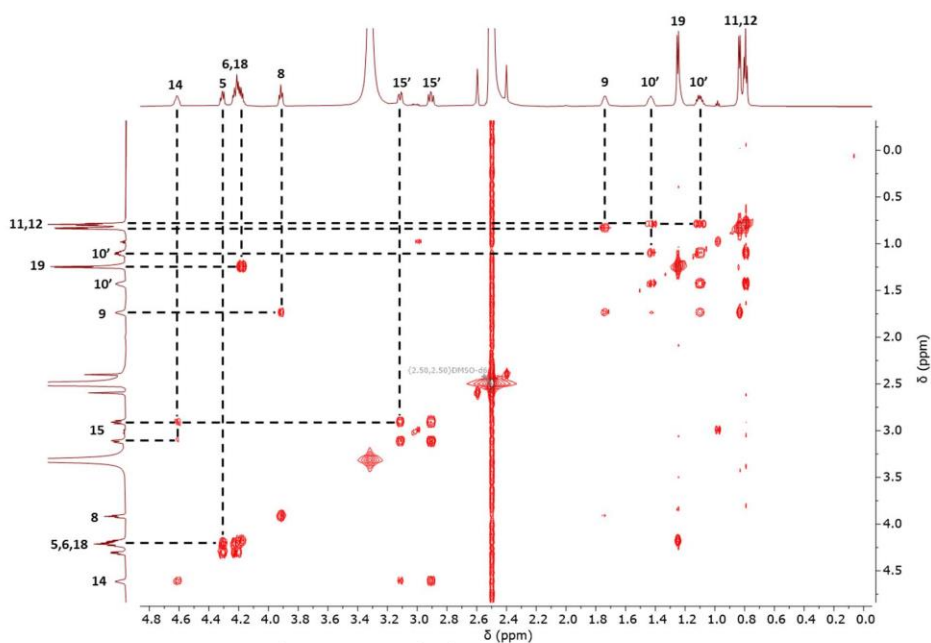


Supplementary Fig. 9: ^1H NMR spectrum (700 MHz, DMSO-d_6 , 298 K) of compound **4a**

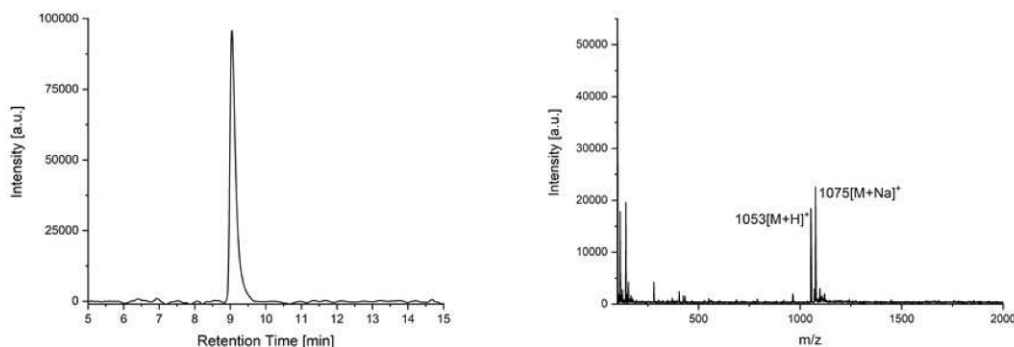
^1H -NMR (700 MHz, DMSO-d_6) δ [ppm] = 8.21 (d, J = 8.2 Hz, 2H, **13**), 8.15 (d, J = 7.1 Hz, 2H, **17**), 7.88 (d, J = 7.5 Hz, 4H, **1**), 7.72 (dd, J = 18.0, 7.5 Hz, 4H, **4**), 7.44–7.39 (m, 6H, **2** and **7**), 7.31 (m, 3H, **3**), 1.85 (m, 2H, **14**), 4.32–4.30 (dd, J = 9.8, 6.8 Hz, 2H, **5**), 4.24–4.17 (m, 6H, **6** and **18**), 3.92 (t, J = 7.9 Hz, 2H, **8**), 3.12 (dd, J = 13.4, 4.5 Hz, 2H, **15'**), 2.91 (dd, J = 13.5, 9.6 Hz, 2H, **15'**), 1.74 (m, 2H, **9**), 1.43 (m, 2H, **10'**), 1.25 (d, J = 7.3 Hz, 6H, **19**), 1.12–1.08 (m, 2H, **10'**), 0.84 (d, J = 6.8 Hz, 6H, **11**), 0.79 (t, J = 7.4 Hz, 6H, **12**).



Supplementary Fig. 10: Partial ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra (850 MHz, CD_3CN , 298 K) of compound **4a** (aromatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectrum.

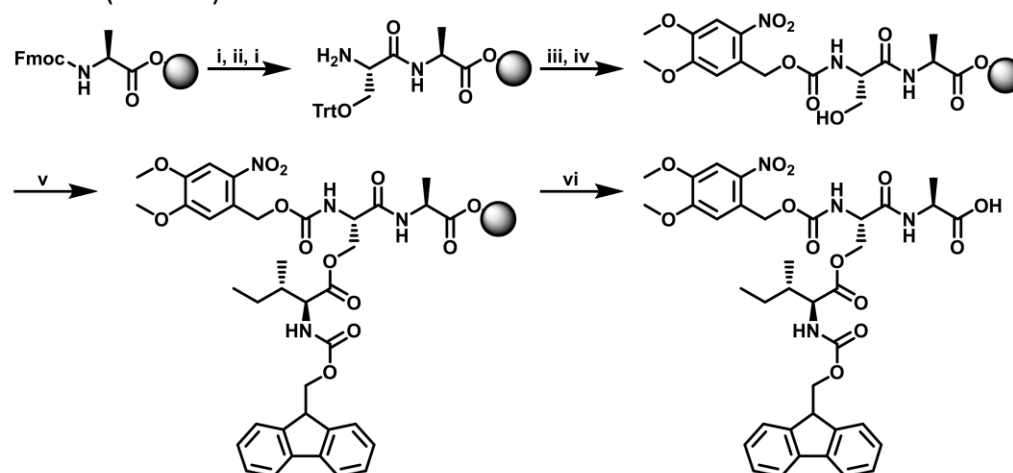


Supplementary Fig. 11: Partial ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra (850 MHz, CD_3CN , 298 K) of compound **4a** (aliphatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectrum.



Supplementary Fig. 12: LC spectrum (left) and ESI MS (positive mode, right) of DiFmocI. m/z calculated: $[M+H]^+ = 1053$, found: $[M+H]^+ = 1053$, $[M+Na]^+ = 1075$.

2.4 Iso(Fmoc-I)nvocSA

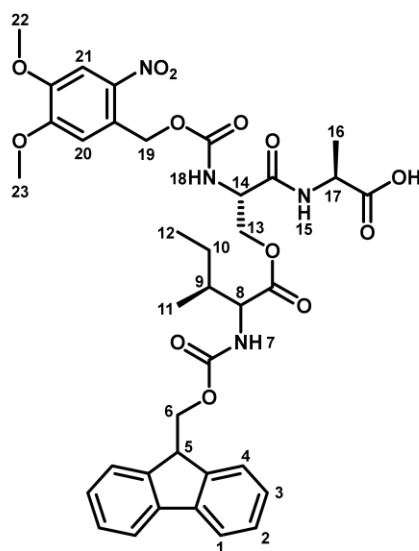


Scheme 2: Synthesis of iso(Fmoc-I)nvocSA by solid-phase peptide synthesis. (i) 20% v/v piperidine in DMF, (ii) Fmoc-Ser(Trt)-OH, DIC, Oxyma in DMF, (iii) Nvoc-Cl, TEA in DMF, o.n., rt, (iv) DCM, TIPS, TFA, (v) Fmoc-Ile-OH, DIC, DMAP in DMF, o.n., rt, (vi) TFA, TIPS, H₂O, 2 h, rt.

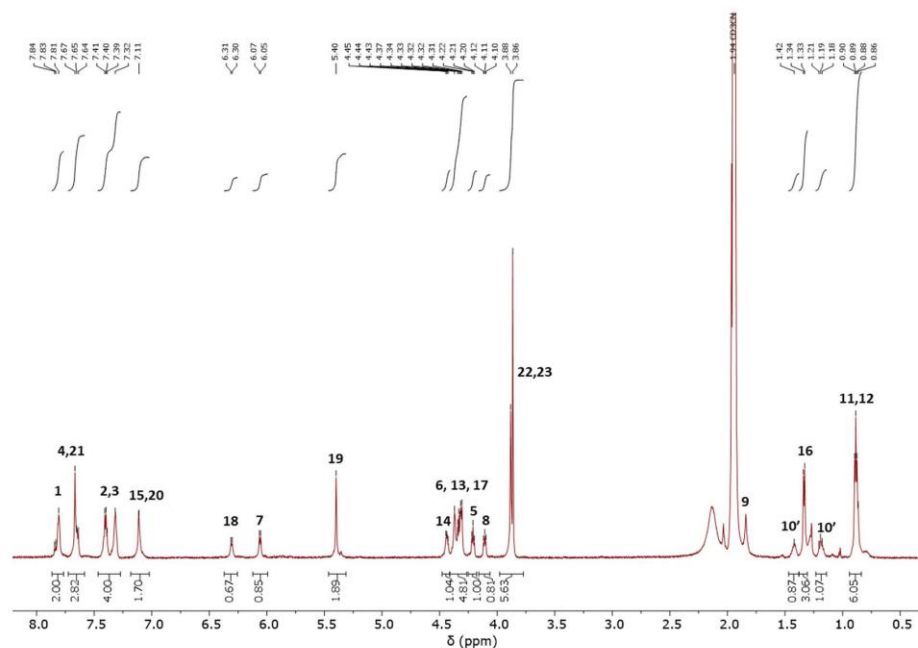
Peptides were synthesized in a microwave assisted peptide synthesizer using the Fmoc solid phase peptide synthesis strategy by Merrifield, synthesizing the peptide from the C- to N-terminus. The Wang resin preloaded with Fmoc-Ala (0.25 mmol) was swollen in 5 mL DMF at room temperature for 1 h before transferring it into the peptide synthesizer. DMF was removed via a draining process and the resin swollen with 20 mL DMF for 20 s. After draining of the DMF, the Fmoc group was removed with 10 mL 20 % v/v piperidine in DMF and a heating cycle at 70°C for 25 s followed by another cycle at 90°C for 65 s. The solution was drained, and the resin washed with DMF three times (i). After the last washing step, Fmoc-Ser(Trt)-OH (0.2 M in DMF, 10 mL), DIC solution (0.5 M in DMF, 4 mL) and OxymaPure solution (1.0 M in DMF, 2 mL) was added to the resin and the solution heated to 70°C for 30 s followed by a heating cycle to 90°C for 120 s (ii). The solution was drained, the Fmoc group removed as described before and washed with DMF three times (i). The free amine was protected with

nvoc by dissolving nvoc-Cl (1.1 equiv.) and TEA (2 equiv.) in 5 mL dry DMF under argon atmosphere and shaking at room temperature overnight (iii). After the coupling, the solution was removed, and the resin washed with DCM and dry DMF five times. The hydroxy function was deprotected with a DCM:TIPS 95:5 solution, containing 1% TFA. For this, 10 mL solution was added to the resin and shaken at room temperature for 10 min. The solution was removed, and the process repeated eight times (iv). After the deprotection, the resin was washed with DCM and dry DMF five times. To form the ester, Fmoc-Ile-OH (10 equiv.) and DMAP (1 equiv.) were dissolved in 5 mL dry DMF under argon atmosphere and added to the resin. DIC (10 equiv.) was added to the resin and the mixture shaken at room temperature under argon atmosphere overnight (v). The solution was removed, and the resin washed with DCM five times. The peptide was cleaved from the resin by adding 10 mL of cleavage cocktail (95% TFA, 2.5% TIPS, 2.5% MilliQ water) to the resin and shaking at room temperature for 2 h. The cleavage cocktail was drained, and the resin washed with TFA. The TFA containing solutions were combined and the liquids removed under reduced pressure (vi).

The *iso*(Fmoc-I)nvocSA was purified via HPLC with Phenomenex Kinetex EVO C18 100 column (flowrate 25 mL/min). The gradient started at 20% ACN content and was linearly increased to 75% over 25 min. The retention time of the product was 27.1 min. After lyophilisation the product *iso*(Fmoc-I)nvocSA was received as a colourless solid (7 mg, 0.01 mmol, 4%).

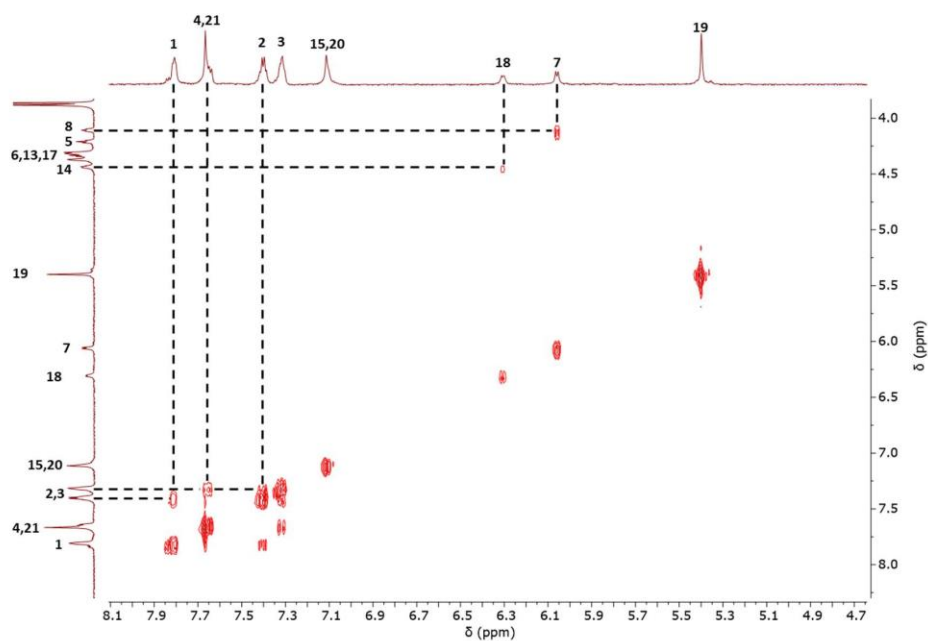


Exact Mass: 750,27

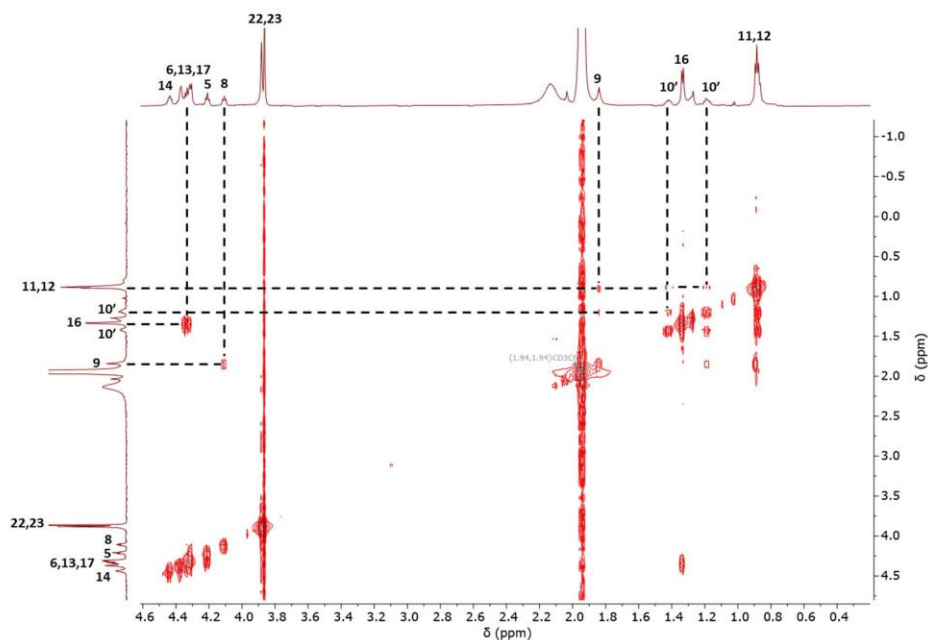


Supplementary Fig. 13: ^1H NMR spectrum (700 MHz, CD_3CN , 298 K) of compound **1b**

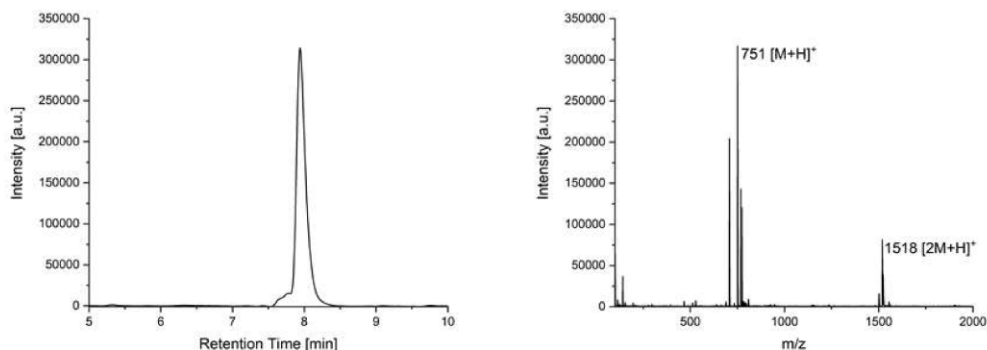
^1H -NMR (700 MHz, CD_3CN) δ [ppm] = 7.84–7.81 (m, 2H, **1**), 7.67–7.64 (m, 3H, **4** and **21**), 7.41–7.39 (m, 2H, **2**), 7.32 (m, 2H, **3**), 7.01 (s, 1H, **20**), 6.31 (d, $J = 8.3$ Hz, 1H, **18**), 6.06 (d, $J = 8.2$ Hz, 1H, **7**), 5.40 (s, 2H, **19**), 4.45–4.43 (m, 1H, **14**), 4.37–4.31 (m, 5H, **6** and **13** and **17**), 4.21 (t, $J = 7.3$ Hz, 1H, **5**), 4.11 (t, $J = 7.2$ Hz, 1H, **8**), 3.89 (s, 3H, **22**), 3.86 (s, 3H, **23**), 1.42 (m, 1H, **10'**), 1.33 (m, 3H, **16**), 1.21–1.18 (m, 1H, **10'**), 0.90–0.86 (m, 6H, **11** and **12**).



Supplementary Fig. 14: Partial ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra (850 MHz, CD_3CN , 298 K) of compound **1b** (aromatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectrum.

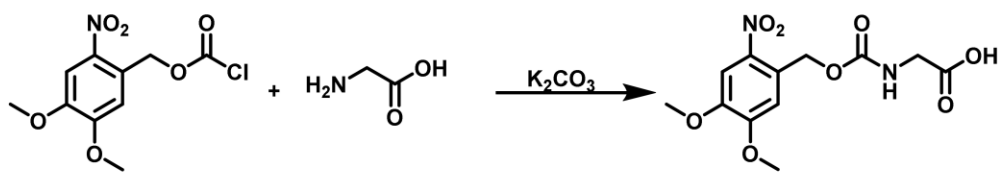


Supplementary Fig. 15: Partial ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra (850 MHz, CD_3CN , 298 K) of compound **1b** (aliphatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectrum.

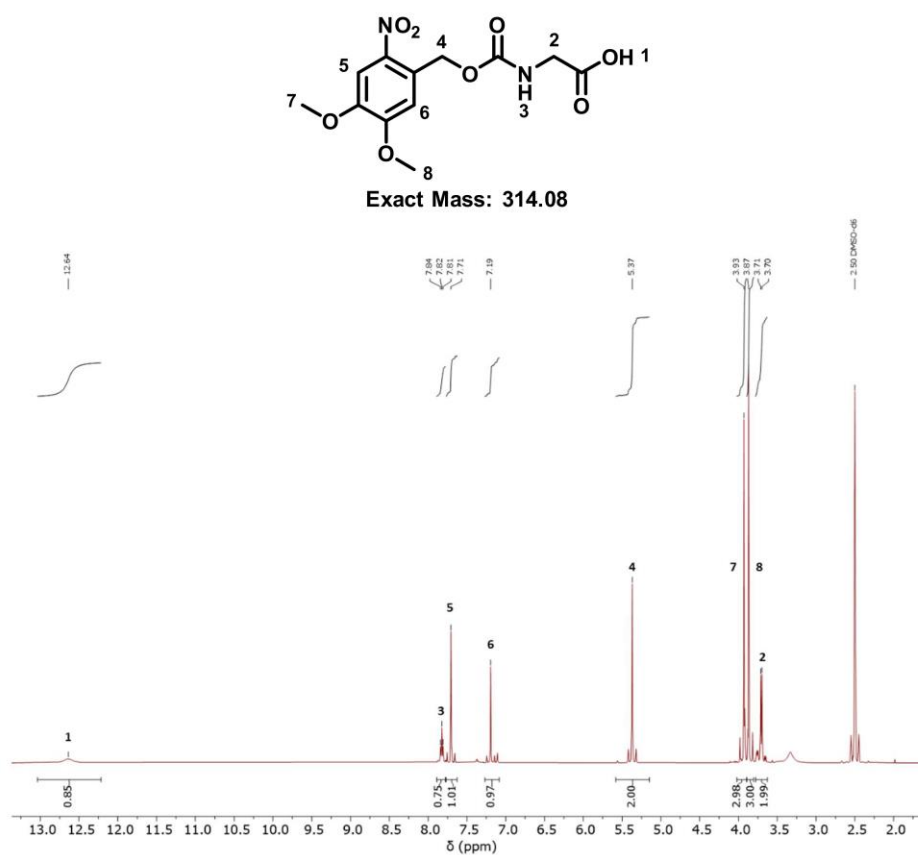


Supplementary Fig. 16: LC spectrum (left) and ESI MS (positive mode, right) of *iso*(Fmoc-I)nvocSA. m/z calculated: $[M+H]^+ = 751$, found: $[M+H]^+ = 751$, $[2M+H]^+ = 1518$.

2.5 Nvoc-Gly

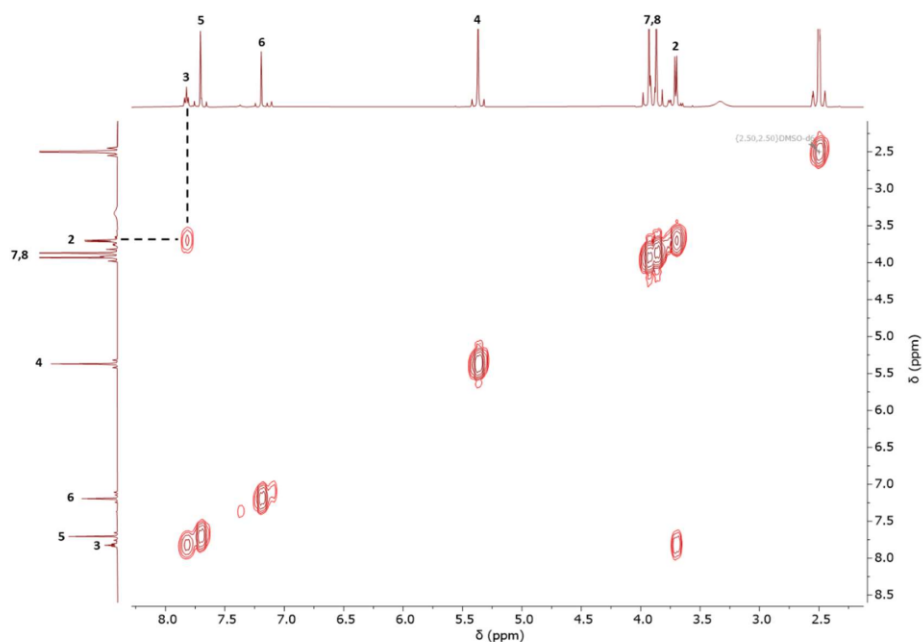


Glycine (24.7 mg, 0.33 mmol, 1 equiv.) was dissolved in a solution of K_2CO_3 (91.16 mg, 0.66 mmol, 2 equiv.) in 2.5 mL H_2O and at 0°C nvoc-Cl (100 mg, 0.36 mmol, 1.1 equiv.) in 3 mL 1,4-dioxan was added dropwise. The mixture was stirred overnight, and the organic solvent was removed under reduced pressure. Then, the remaining aqueous phase was first washed with ethyl acetate and subsequently acidified with 1 M HCl. The acidic aqueous phase was then extracted with ethyl acetate and the combined organic phases were washed with brine and dried over sodium sulfate. The solvent was then removed under reduced pressure to yield the product as an orange solid (84.6 mg, 0.27 mmol, 82 %)

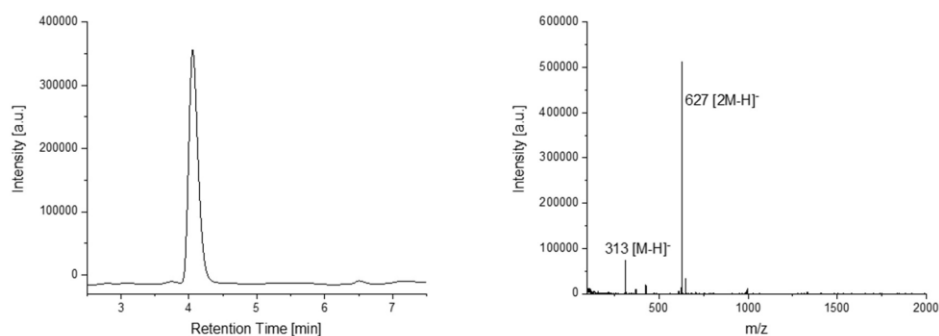


Supplementary Fig. 17: ^1H NMR spectrum (400 MHz, DMSO- d_6 , 298 K) of nvoc-Gly.

^1H -NMR (400 MHz, DMSO- d_6) δ [ppm] = 12.64 (s, 1H, **1**), 7.82 (t, J = 6.2 Hz, 1H, **3**), 7.71 (s, 1H, **5**), 7.19 (s, 1H, **6**), 5.37 (s, 1H, **4**), 3.93 (s, 3H, **7**), 3.87 (s, 3H, **8**), 3.71 (d, J = 6.2 Hz, 2H, **2**).



Supplementary Fig. 18: Partial ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra (400 MHz, DMSO-d_6 , 298 K) of nvoc-Gly. The correlations between ^1H signals are highlighted with dashed lines in the COSY spectrum.



Supplementary Fig. 19: LC spectrum (left) and ESI MS (negative mode, right) of nvoc-Gly. m/z calculated: $[\text{M-H}]^- = 313$, found: $[\text{M-H}]^- = 313$, $[2\text{M-H}]^- = 627$.

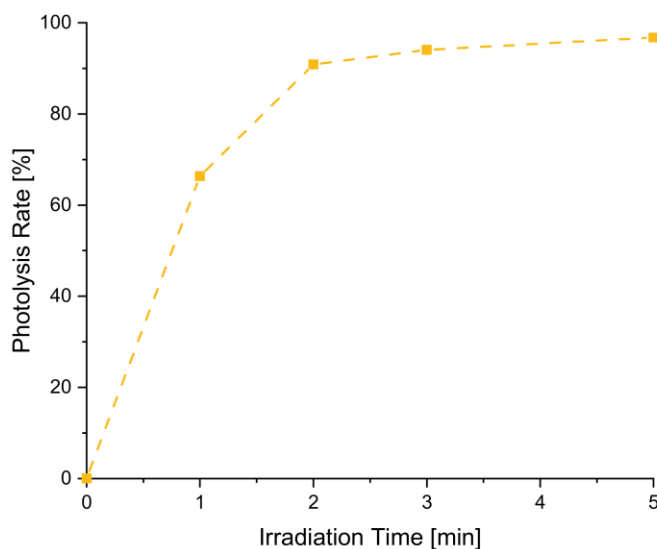
3. Characterization

3.1 High Performance Liquid Chromatography

3.1.1 Photolysis rate

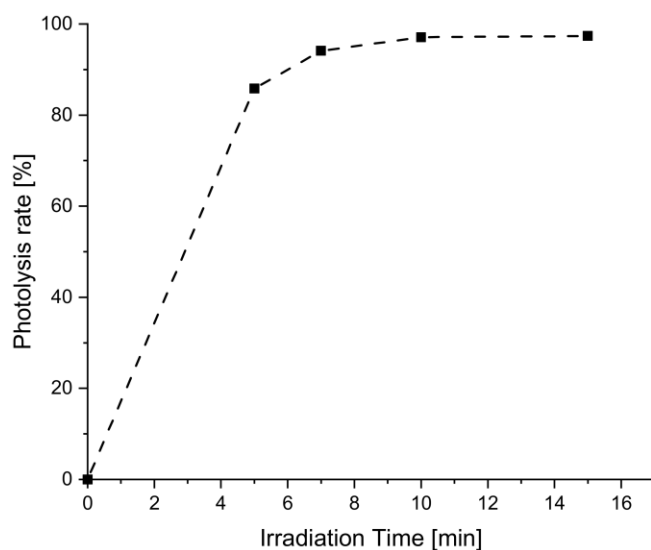
Iso(Fmoc-I)nvocCA (100 μM) was dissolved in 1:1 MeOH/ NH_4HCO_3 buffer (5 mM, pH 7.4), irradiated with light (365 nm) for 60 s, 120 s, 180 s, 300 s while stirring at 300 rpm. One sample was not irradiated for reference. The integral of the peak between 13.33–13.58 min (*iso*-

peptide) versus the integral of the non-irradiated sample was used to calculate the conversion rate of the deprotection.



Supplementary Fig. 20: Conversion rates of *iso*(Fmoc-I)nvocCA **1a** in 1:1 MeOH/ NH_4HCO_3 buffer with increasing irradiation time.

Photolysis rate for assembly studies was determined by dissolving *iso*(Fmoc-I)nvocCA (200 μM) in 1:9 MeOH/ NH_4HCO_3 buffer (5 mM, pH 7.4) and irradiating with light (365 nm) for 5 min, 7 min, 10 min, 15 min while stirring at 300 rpm. One sample was not irradiated for reference. The samples were incubated for 24 h and then diluted to 50% methanol to prevent clogging of the column. Aliquots of 20 μL were then analysed in an HPLC setup. The integral of the peak between 19.73–19.97 min (*iso*-peptide) was used to calculate the conversion rate of the deprotection.

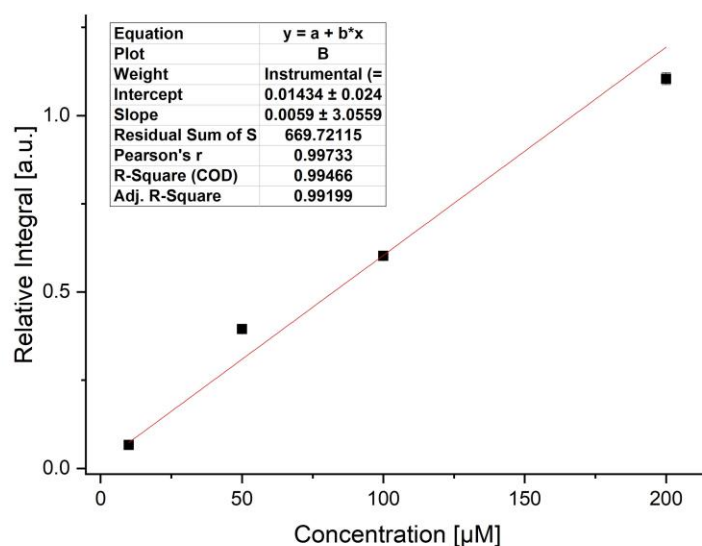
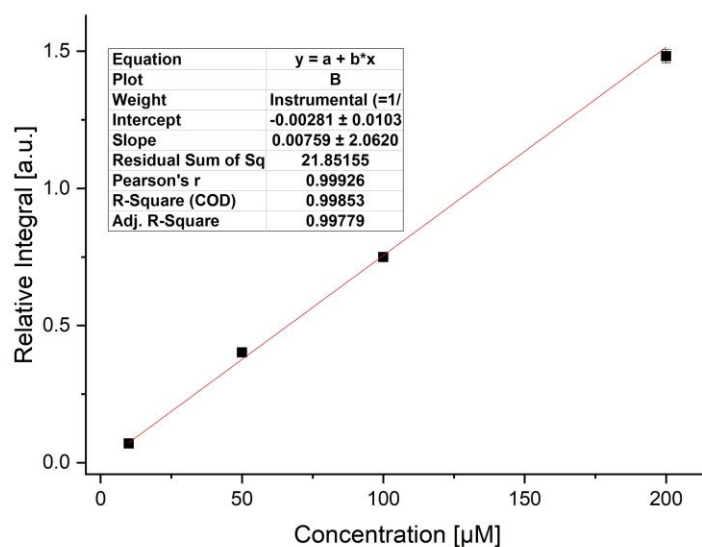


Supplementary Fig. 21: Conversion rates of *iso*(Fmoc-I)nvocCA **1a** in 1:9 MeOH/ NH_4HCO_3 buffer with increasing irradiation time.

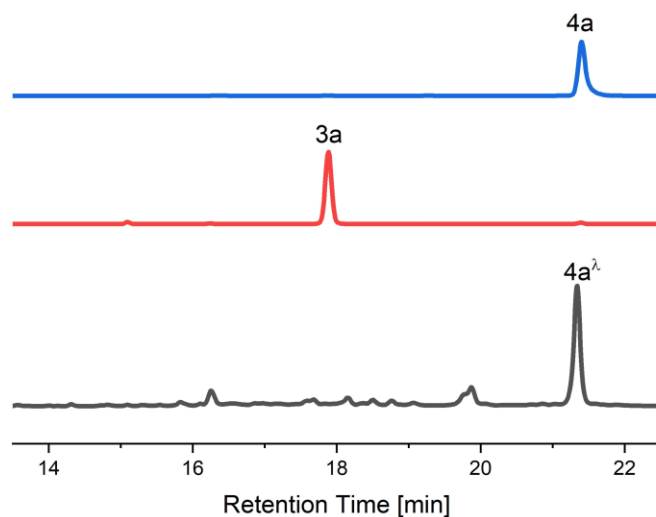
3.1.2 Light-induced *S,N*-acyl shift / *O,N*-acyl shift

The *iso*-peptides were dissolved in 300 μL MeOH (200 μM) and combined with 300 μL of an aqueous NH_4HCO_3 solution (5 mM, pH 7.4) to yield a 100 μM peptide solution. The peptide solutions were irradiated with light (365 nm) for 3 min while stirring at 300 rpm and Tryptophan (10 μM) was added as an internal standard. Aliquots of 10 μL were analysed at certain time points in an HPLC setup. The kinetic was monitored for 24 h (**1a**) and 72 h (**1b**).

For the calculation of concentrations calibration curves of **3a** and **4a** were recorded. **3a** and **4a** were dissolved in MeOH/ aqueous NH_4HCO_3 solution (5 mM, pH 7.4) with Tryptophan (10 μM) as internal standard in concentrations of 200, 100, 50 and 10 μM , respectively. Samples were measured in an HPLC setup, the Trp peak normalized to 1 and the integral of each peak was measured.


 Supplementary Fig. 22: Calibration curve of **3a**.

 Supplementary Fig. 23: Calibration curve of **4a**.

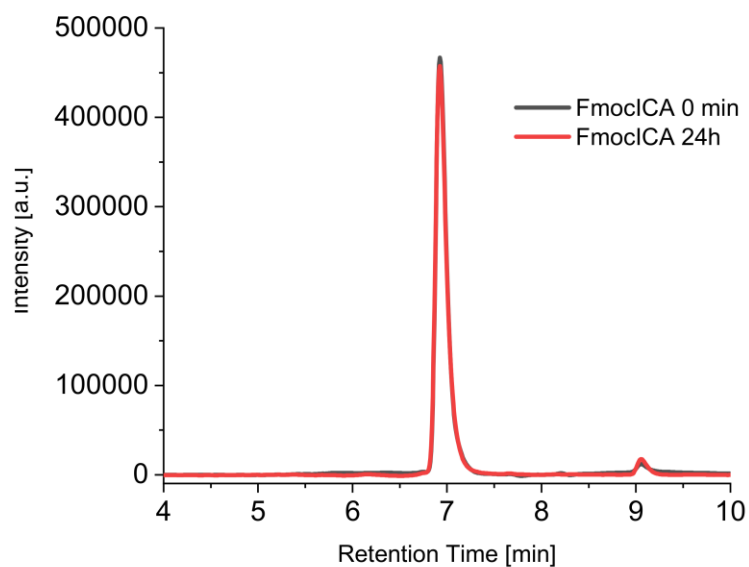
Iso(Fmoc-I)nvocCA (200 μ M) was dissolved in 1:9 MeOH/ NH_4HCO_3 buffer (5 mM, pH 7.4) and irradiated with light (365 nm) for 10 min while stirring at 300 rpm. The sample was incubated for 24 h and then diluted to 50% methanol to prevent clogging of the column. An aliquot of 20 μ L were then analysed in an HPLC setup. The spectra of **3a** and **4a** are shown for reference.



Supplementary Fig. 24: HPLC trace (black) of **1a^λ** (200 μ M) in 1:9 MeOH/ NH_4HCO_3 buffer after 24 h. The solution was diluted to 50% Methanol before HPLC injection.

3.1.3 Control of Fmoc-ICA

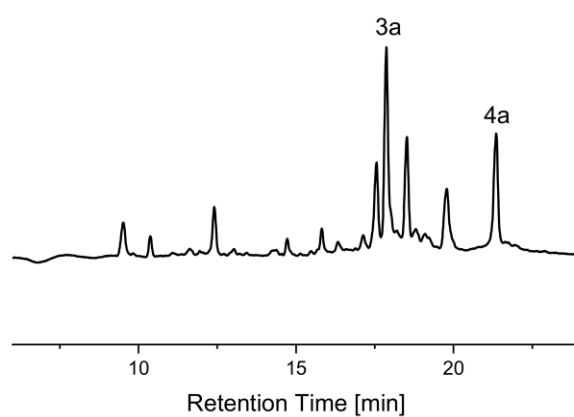
Fmoc-ICA (50 $\mu\text{g/mL}$) was dissolved in 1:1 MeOH/ NH_4HCO_3 buffer (5 mM) and incubated for 24 h. LC-MS was measured at the start and end of that time period.



Supplementary Fig. 25: LCMS trace of Fmoc-ICA before and after standing alone in 1:1 MeOH/NH₄HCO₃ buffer (5 mM) over 24 h.

3.1.4 Irradiation of *iso*(Fmoc-I)nvocCA in acetonitrile

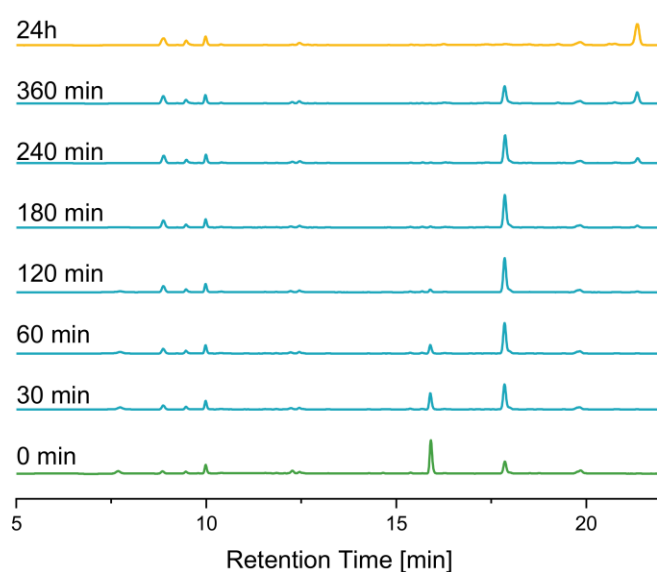
iso(Fmoc-I)nvocCA (100 μ M) was dissolved in dry acetonitrile and irradiated with light (365 nm) for 5 min while stirring at 300 rpm. The solution was stirred for 24 h and then an aliquot of 10 μ L was analysed in an HPLC setup.



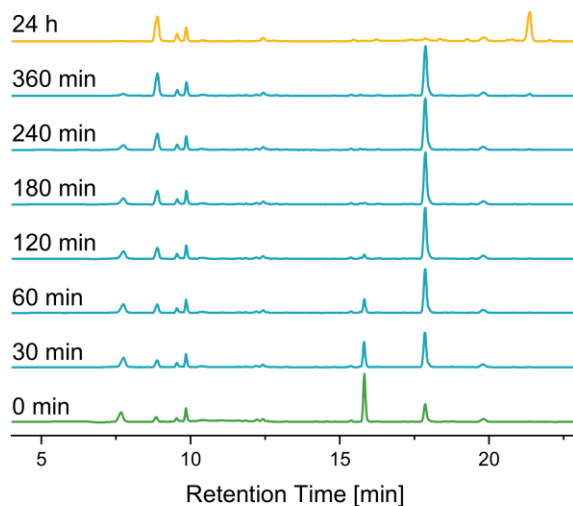
Supplementary Fig. 26: HPLC trace of irradiation of **1a^λ** after 24 h in acetonitrile.

3.1.5 Reductive irradiation of *iso*(Fmoc-I)nvocCA with DTT

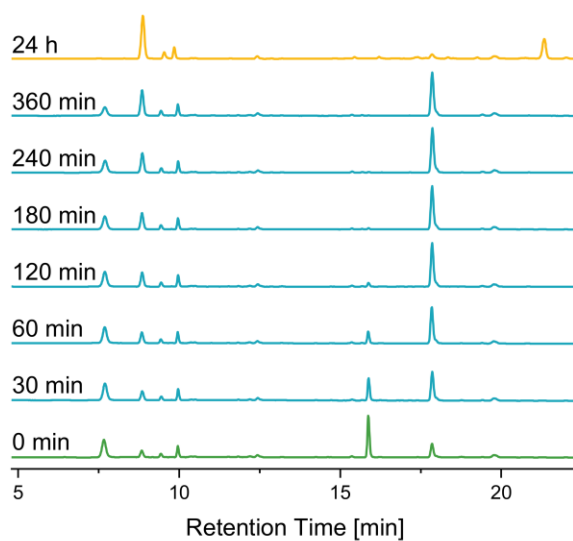
Iso(Fmoc-I)nvocCA was dissolved in 300 μL methanol and combined with 300 μL of an aqueous solution of DTT in NH_4HCO_3 buffer (5 mM, pH 7.4) to yield an 100 μM peptide solution with 5/10/20/100 equiv. DTT. The peptide solution was irradiated with light (365 nm) for 180 s while stirring at 300 rpm and then Tryptophan (final concentration 10 μM) was added. Aliquots of 10 μL were analysed at certain time points in an HPLC setup and the kinetic was monitored for 24 h. For each DTT concentration the molar percentage of the integral of **3a** versus **4a** was plotted at every time point.



Supplementary Fig. 27: HPLC kinetic of *iso*(Fmoc-I)nvocCA **1a** 100 μM irradiated with DTT 500 μM .



Supplementary Fig. 28: HPLC kinetic of *iso*(Fmoc-I)nvocCA **1a^A** 100 μM irradiated with DTT 1 mM.

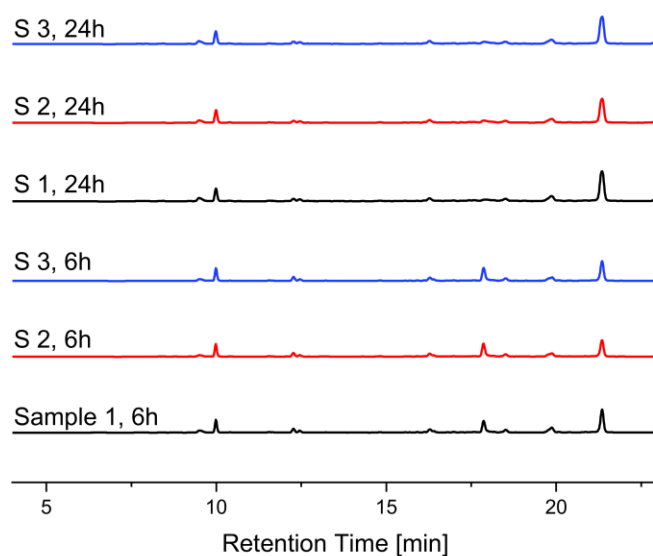


Supplementary Fig. 29: HPLC kinetic of *iso*(Fmoc-I)nvocCA **1a^A** 100 μM irradiated with DTT 2 mM.

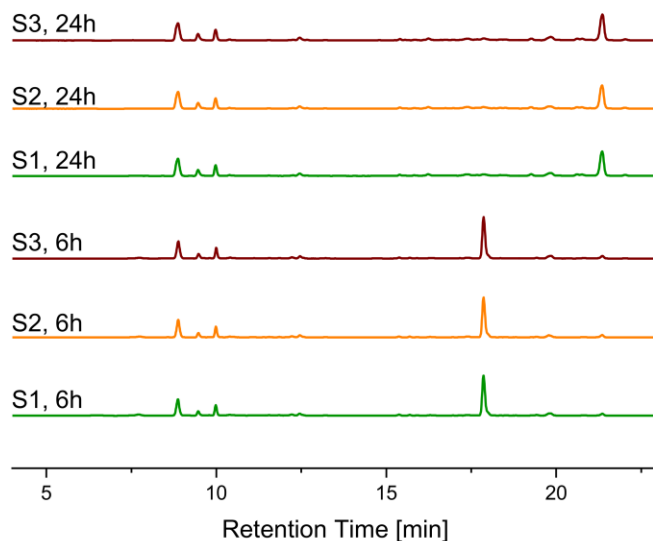
3.1.6 Comparison irradiation of *iso*(Fmoc-I)nvocCA with DTT/BzSH

iso(Fmoc-I)nvocCA (100 μM) was dissolved in 1:1 MeOH/ NH₄HCO₃ buffer (5 mM, pH 7.4), irradiated with light (365 nm) for 180 s while stirring at 300 rpm and then Tryptophan (10 μM)

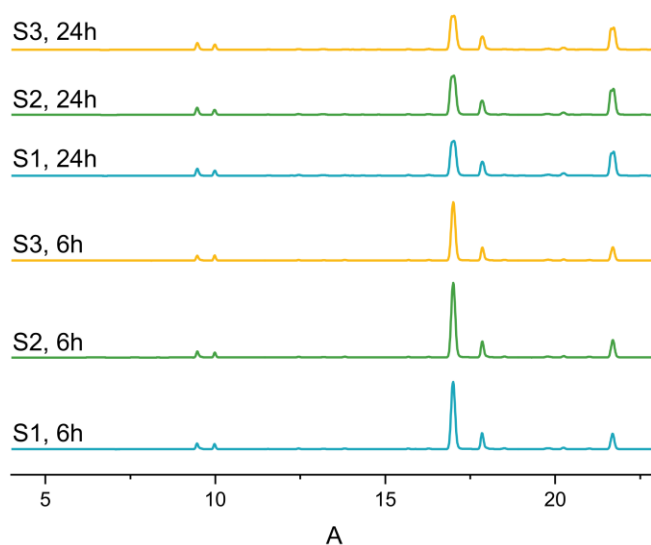
was added. Three of those samples were analysed in an HPLC setup after 6 h and 24 h. The molar percentage of the integral of **3a** versus **4a** was plotted at every time point. This procedure was conducted as well for *iso*(Fmoc-I)nvocCA (100 μ M) with DTT (1 mM) and *iso*(Fmoc-I)nvocCA (100 μ M) with benzyl mercaptan (BzSH) (1 mM).



Supplementary Fig. 30: HPLC of 3 samples *iso*(Fmoc-I)nvocCA **1a**^λ 100 μ M irradiated after 6 h and 24 h



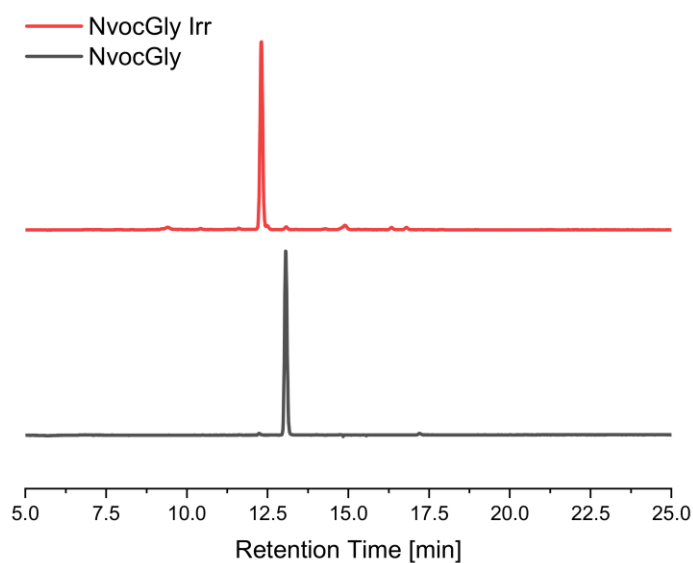
Supplementary Fig. 31: HPLC of 3 samples *iso*(Fmoc-I)nvocCA **1a**^λ (100 μ M) and DTT (1 mM) irradiated after 6 h and 24 h



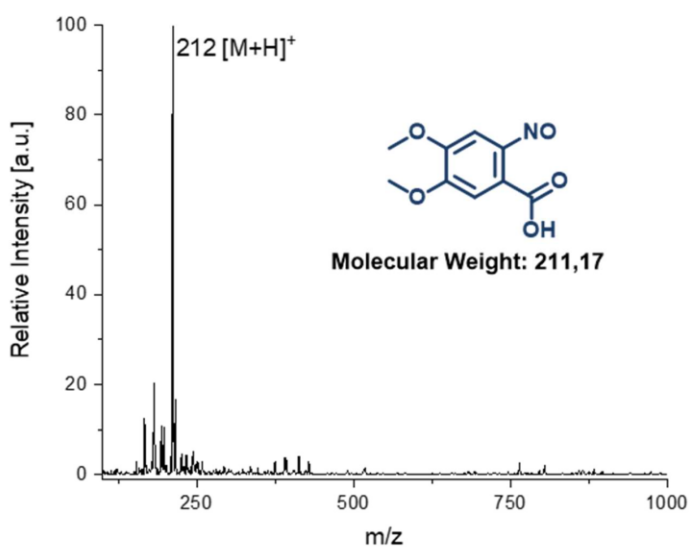
Supplementary Fig. 32: HPLC of 3 samples *iso*(Fmoc-I)nvocCA **1a**^λ (100 μM) and BzSH (1 mM) irradiated after 6 h and 24 h

3.1.7 Irradiation of nvoc-Gly

Nvoc-Gly (100 μM) was dissolved in 1:1 MeOH/ NH₄HCO₃ buffer (5 mM, pH 7.4) and irradiated with light (365 nm) for 180 s while stirring at 300 RPM. Aliquots of 10 μL of the solution were analysed in an HPLC setup before and after irradiation. The sample was then further analysed by direct injection in the LC-MS.



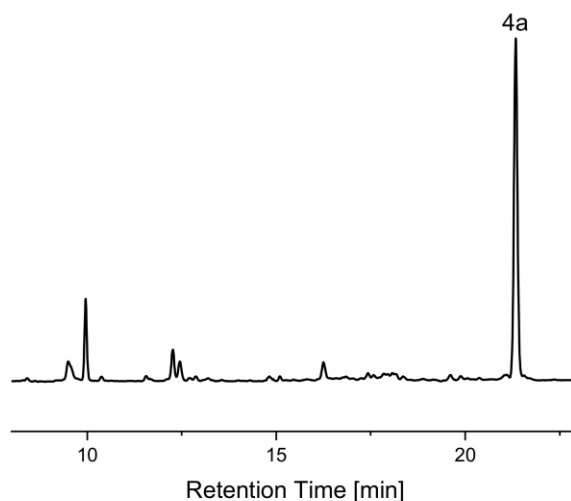
Supplementary Fig. 33: HPLC trace (400 nm) of nvoc-Gly **6** before and after irradiation.



Supplementary Fig. 34: ESI MS (positive mode,) of irradiated nvoc-Gly. m/z calculated: $[M+H]^+ = 211.17$, found: $[M+H]^+ = 212$.

3.1.8 Oxidation with nvoc-Gly

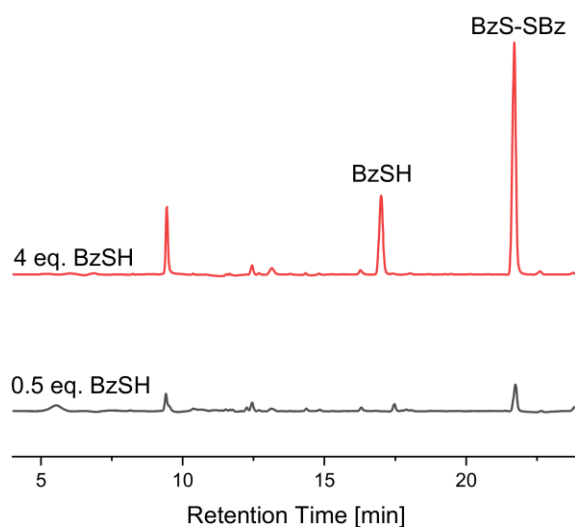
Nvoc-Gly (100 μM) and Fmoc-ICA (100 μM) were dissolved in 1:1 MeOH/ NH_4HCO_3 buffer (5 mM, pH 7.4), irradiated with light (365 nm) for 180 s while stirring at 300 rpm and then Tryptophan (10 μM) as internal standard was added. After incubating for 24 h an aliquot of 10 μL was analysed in an HPLC setup.



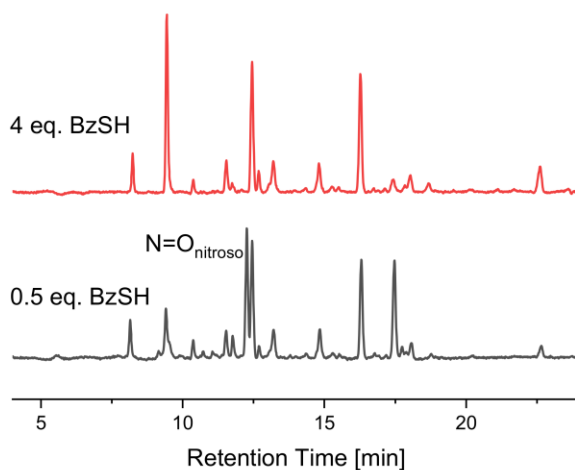
Supplementary Fig. 35: HPLC trace (214 nm) of nvoc-Gly **6** and Fmoc-ICA **3a** after irradiation and incubating for 24 h.

3.1.9 Oxidation with nvoc-Gly and BzSH

Nvoc-Gly (100 μM) was dissolved in 1:1 MeOH/ NH_4HCO_3 buffer (5 mM, pH 7.4) and irradiated with light (365 nm) for 180 s while stirring at 300 rpm. Benzyl mercaptan (400 μM , 4 equivalents) were added and the solution stirred for 24 h. An aliquot of 10 μL was then analysed in an HPLC setup. The experiment was also performed with 0.5 equivalents of benzyl mercaptan (50 μM)



Supplementary Fig. 36: HPLC trace (214 nm) of nvoc-Gly **6** (100 μ M) with 0.5/4 equivalents of benzyl mercaptan after 24 h.

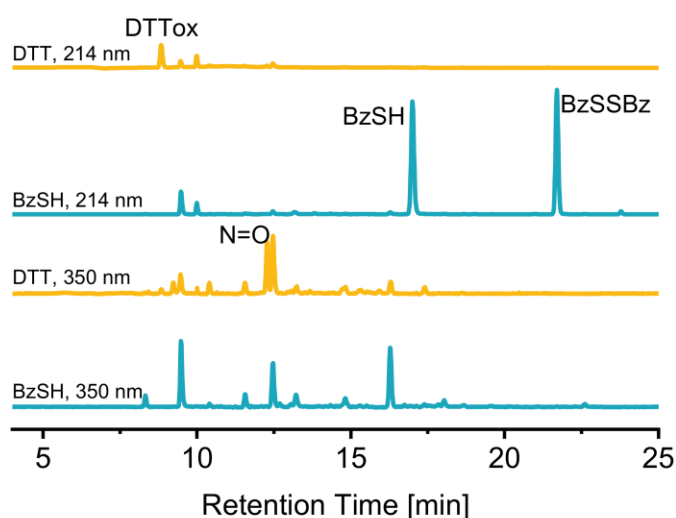


Supplementary Fig. 37: HPLC trace (350 nm) of nvoc-Gly **6** (100 μ M) with 0.5/4 equivalents of benzyl mercaptan after 24 h.

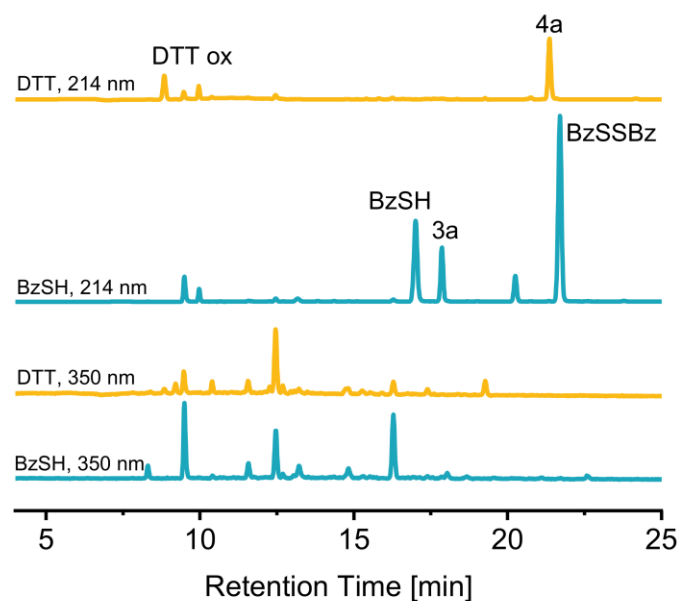
3.1.10 Delayed addition of Fmoc-ICA to nvoc-Gly + DTT/BzSH

Nvoc-Gly (111 μ M) and DTT (1.11 mM, 10 equivalents) were dissolved in 200 μ L MeOH and 250 μ L NH_4HCO_3 buffer (5 mM, pH 7.4) and irradiated with light (365 nm) for 180 s while

stirring at 300 rpm. Tryptophan (10 μ M) was added as a standard and after incubating for 24 h an aliquot of 10 μ L was analysed in an HPLC setup. Then, Fmoc-ICA in 50 μ L MeOH was added to yield concentrations of 100 μ M for Fmoc-ICA and nvoc-Gly and 1 mM for DTT. The solution was incubated for another 24 h and an aliquot of 10 μ L was analysed in an HPLC setup. This experiment was also conducted with 10 equivalents of benzyl mercaptan instead of DTT.



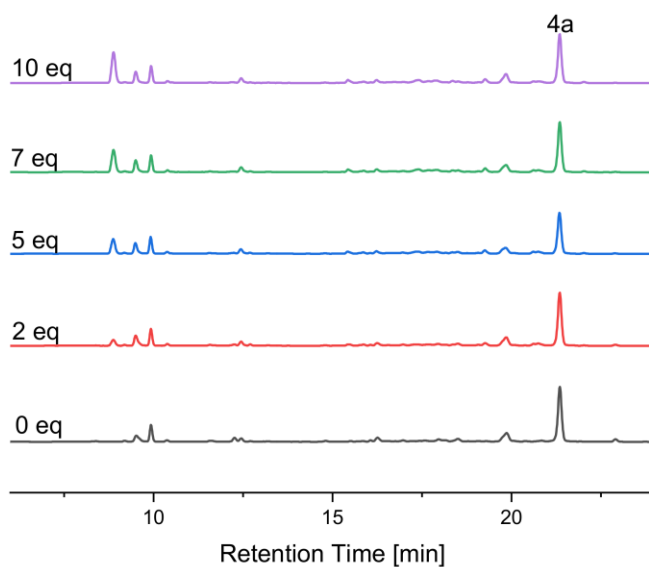
Supplementary Fig. 38: HPLC trace (214 nm, 350 nm) of nvoc-Gly **6** with BzSH (10 equiv., blue curve) / DTT (10 equiv., yellow curve) after 24 h.



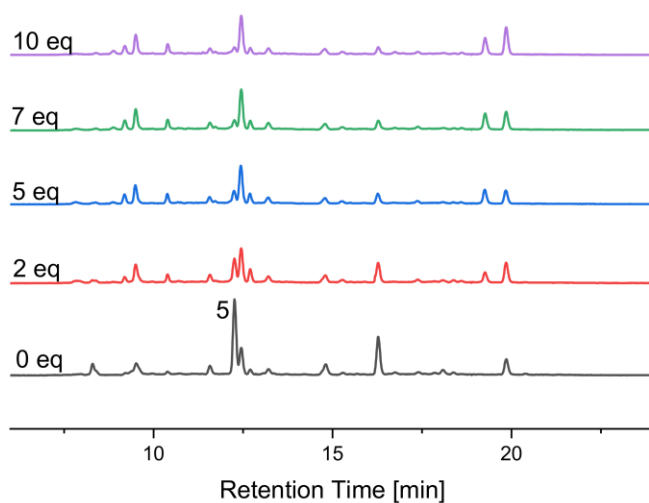
Supplementary Fig. 39: HPLC trace (214 nm, 350 nm) of nvoc-Gly **6** with BzSH (10 equiv., blue curve) / DTT (10 equiv., yellow curve) 24 h after the addition of Fmoc-ICA (1 equiv.) to each sample.

3.1.11 Reductive irradiation of *iso*(Fmoc-I)nvocCA with DTT after 24 h

iso(Fmoc-I)nvocCA **1a^λ** was dissolved in 300 μ L methanol and combined with 300 μ L of an aqueous solution of DTT in NH_4HCO_3 buffer (5 mM, pH 7.4) to yield an 100 μ M peptide solution with 0/2/5/7/10 equiv. DTT. The peptide solution was irradiated with light (365 nm) for 180 s while stirring at 300 rpm and then Tryptophan (final concentration 10 μ M) was added. After incubating for 24 h aliquots of 10 μ L were analysed at certain time points in an HPLC setup.



Supplementary Fig. 40: HPLC trace (214 nm) of of *iso*(Fmoc-I)nvocCA **1a** 100 μ M with DTT.



Supplementary Fig. 41: HPLC trace (350 nm) of of *iso*(Fmoc-I)nvocCA **1a** 100 μ M with DTT.

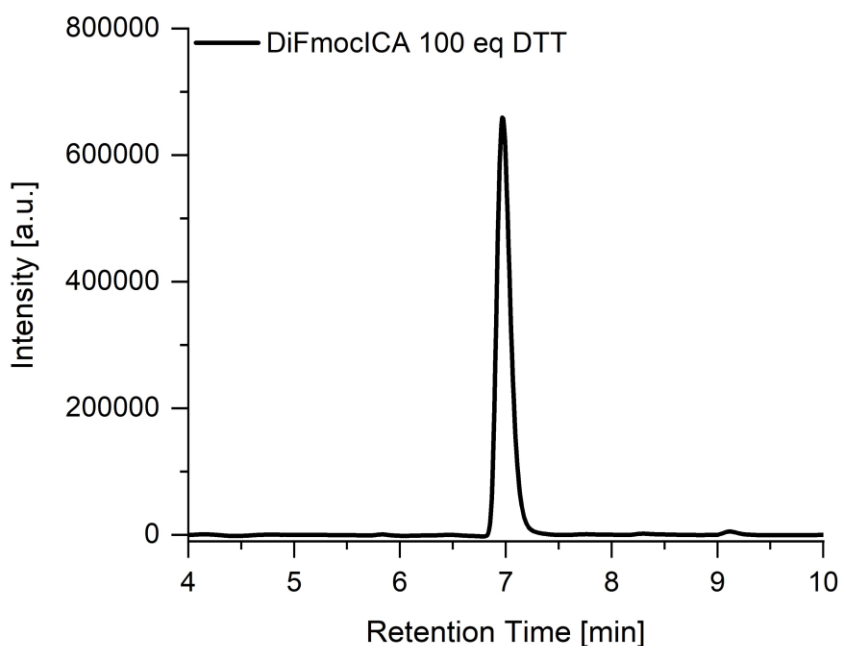
3.1.12 Reduction kinetic of DiFmoc-ICA with DTT

DiFmoc-ICA was dissolved in an aqueous 250 μ L NH_4HCO_3 buffer (5 mM, pH 7.4) and combined with 250 μ L of an aqueous solution of DTT in NH_4HCO_3 buffer (5 mM, pH 7.4) to yield an 50 μ M peptide solution with 1 mM DTT. Aliquots of 10 μ L were analysed at certain time points in a

HPLC setup. The kinetic was monitored for 8 h. For each DTT concentration the molar percentage of the integral of **3a** versus **4a** was plotted at every time point.

3.1.13 Reduction of DiFmoc-ICA with DTT

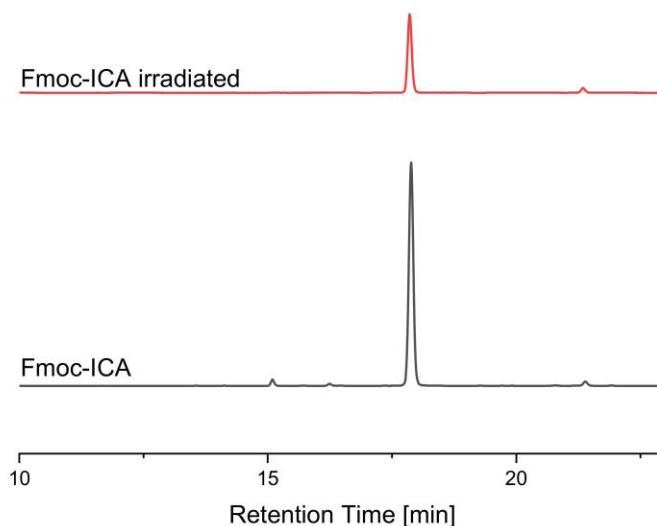
DiFmoc-ICA was dissolved in an aqueous 300 μL NH_4HCO_3 buffer (5 mM, pH 7.4) and combined with 300 μL of an aqueous solution of DTT in NH_4HCO_3 buffer (5 mM, pH 7.4) to yield an 100 μM peptide solution with 10 mM DTT. After 24 h the solution was analysed in a LC-MS setup.



Supplementary Fig. 42: HPLC trace of 100 μM DiFmocICA with 100 equiv. DTT after 24 h.

3.1.14 Irradiation of Fmoc-ICA

Fmoc-ICA (100 μM) was dissolved in 1:1 MeOH/ NH_4HCO_3 buffer (5 mM, pH 7.4) and irradiated with light (365 nm) for 180 s while stirring at 300 rpm. An aliquot of 10 μL was then analysed in an HPLC setup.

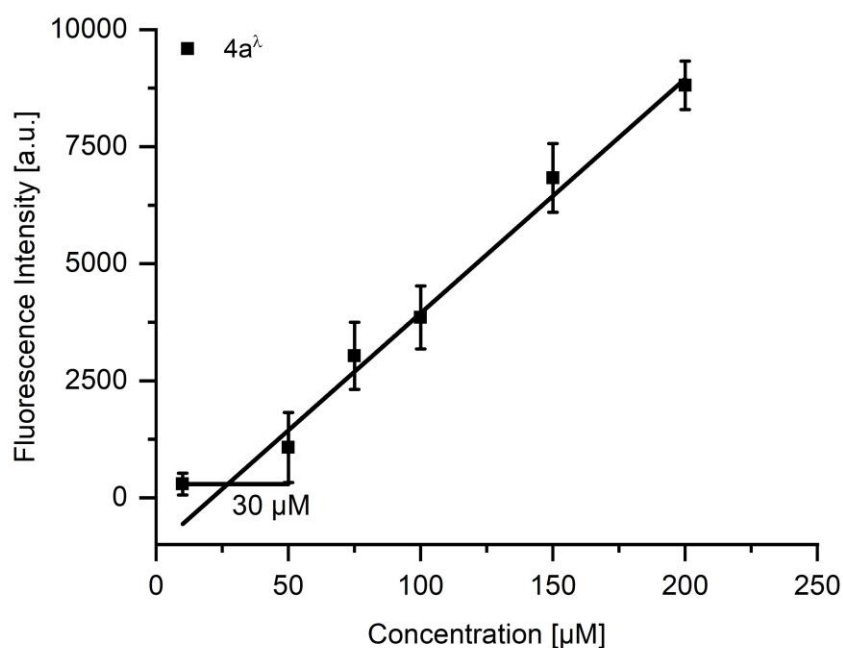


Supplementary Fig. 43: HPLC trace of FmocICA irradiated and non-irradiated.

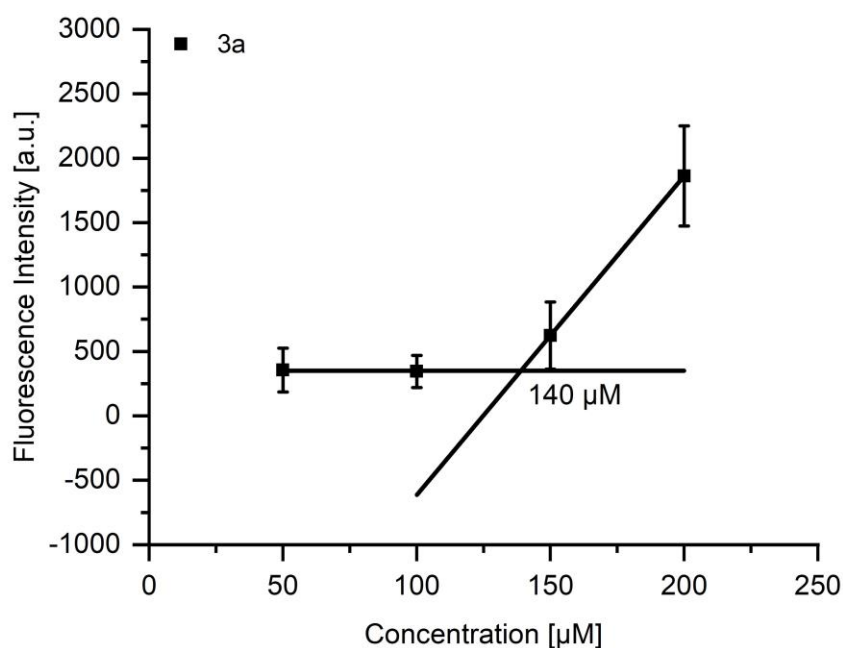
3.2 Critical Assembly Concentration (Proteostat Assay)

3a was dissolved in MeOH (4 mM) and diluted 1:9 with an aqueous NH_4HCO_3 buffer (5 mM, pH 7.4) to 200 μM . This stock was further diluted to 150, 100 and 50 μM with MeOH : buffer 1:9. **4a** was dissolved in MeOH (2 mM) and diluted 1:9 with an aqueous NH_4HCO_3 buffer (5 mM, pH 7.4) to 200 μM . This stock was further diluted to 150, 100, 75 and 10 μM with MeOH : buffer 1:9. **1a** was dissolved in 60 μL MeOH (2 mM) and diluted 1:9 with 540 μL of an aqueous NH_4HCO_3 buffer (5 mM, pH 7.4) to 200 μM . This stock was irradiated for 10 min (365 nm). Then, this stock was further diluted to 150, 100, 75, 50 and 10 μM with MeOH : buffer 1:9. All prepared samples were incubated with 100 μL for 24 h and used in the assay as peptide solutions.

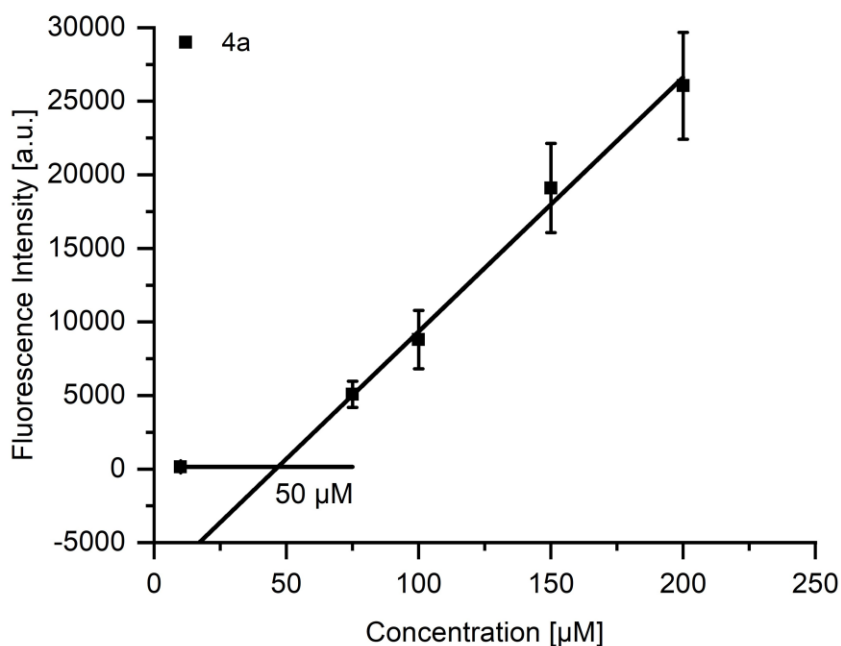
The Proteostat Protein Aggregation Assay Kit was purchased from *Enzo Life Sciences, Inc.* 0.52 μL of the Proteostat stock solution was diluted with 98.48 μL MilliQ water and 1 μL assay buffer. 1 μL of this solution was added to 9 μL peptide solution in a Greiner 384 flat black wellplate. The solutions were incubated in the dark for 15 min while shaking at 500 rpm. The fluorescence intensity was measured at an excitation wavelength of 550 nm and emission wavelength of 600 nm and with a bandwidth of 20 nm. The experiment was done four times.^[2]



Supplementary Fig. 44: Proteostat assay of *iso*-FmocInvocCA (**4a λ**) after 24 h of incubation. Fluorescence intensity plotted against different concentrations of the peptide. Fit of high concentrations and baseline (lines). Intersection of both determines the critical assembly concentration (30 μ M). Data are presented as $\pm$ s.d., $n = 5$.



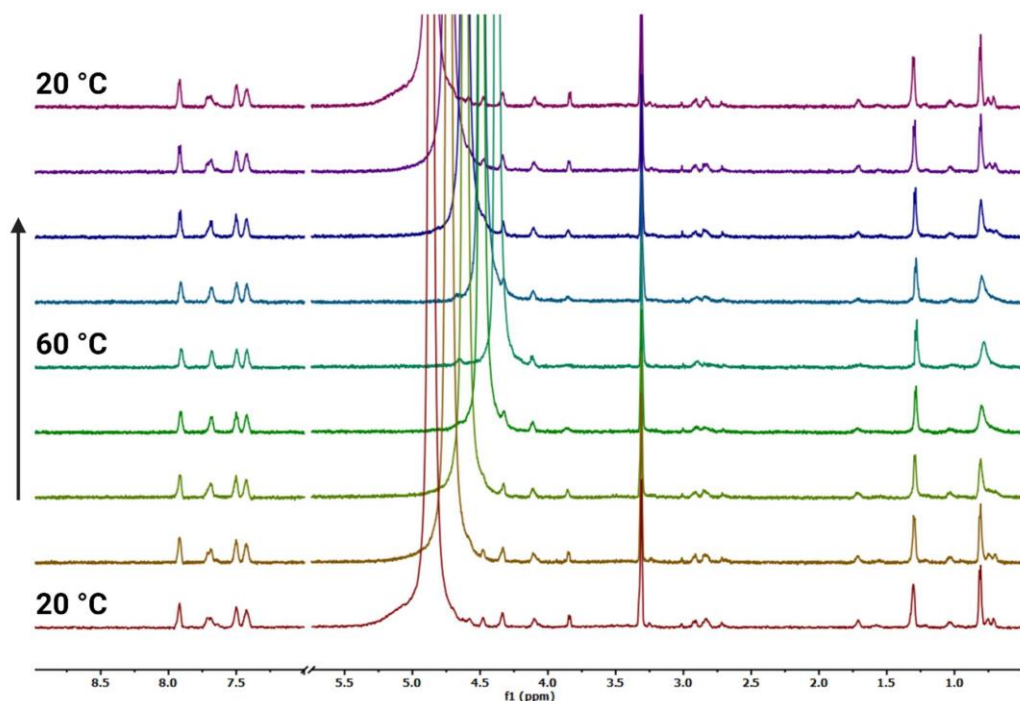
Supplementary Fig. 45: Proteostat assay of FmocICA (**3a**) after 24 h of incubation. Fluorescence intensity plotted against different concentrations of the peptide. Fit of high concentrations and baseline (lines). Intersection of both determines the critical assembly concentration (140 μ M). Data are presented as $\pm$ s.d., $n = 5$.



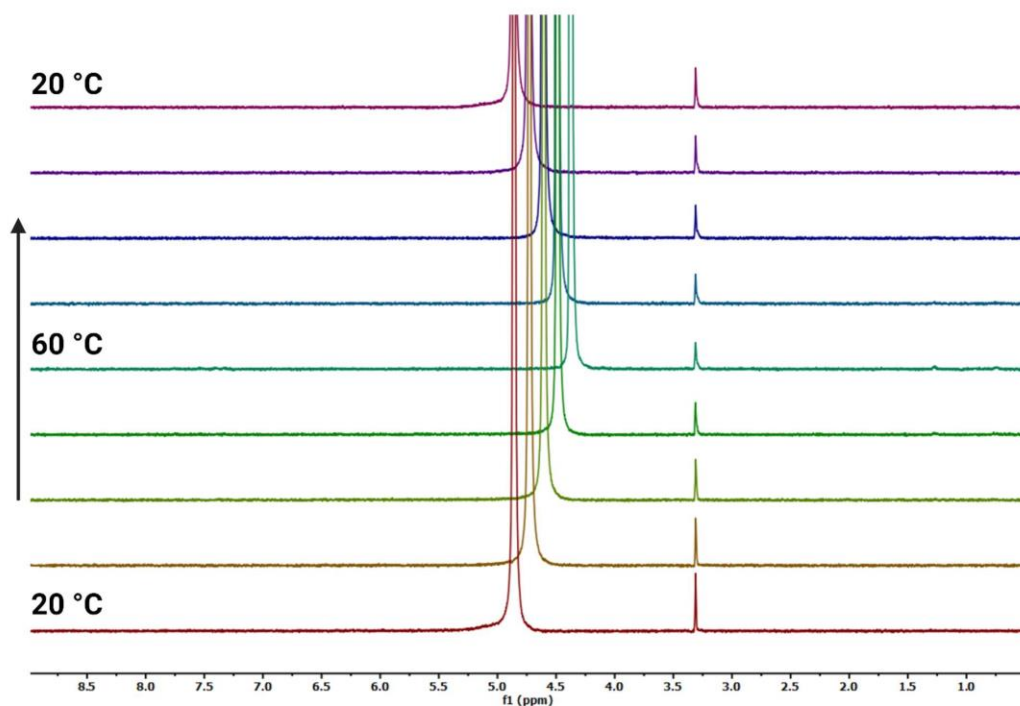
Supplementary Fig. 46: Proteostat assay of DiFmocICA (**4a**) after 24 h of incubation. Fluorescence intensity plotted against different concentrations of the peptide. Fit of high concentrations and baseline (lines). Intersection of both determines the critical assembly concentration (50 μM). Data are presented as $\pm$ s.d., $n = 5$.

3.3 NMR Temperature gradients

Fmoc-ICA or DiFmoc-ICA were dissolved in 60 μL of methanol- d_4 to yield a 2 mM solution and was diluted with 540 μL of NH_4HCO_3 -buffer (5 mM) in D_2O to 200 μM . The sample was then immediately analyzed via ^1H -NMR spectroscopy at different temperatures. Measurements were started at 20°C, going up to 60°C and back to 20°C in steps of 10°C. At each temperature the sample was equilibrated for 10 min before the measurement.



Supplementary Fig. 47: Temperature dependent ^1H -NMR spectra of Fmoc-ICA **3a** with an arrow indicating measurement sequence.



Supplementary Fig. 48: Temperature dependent ^1H -NMR spectra of DiFmoc-ICA **4a** with an arrow indicating measurement sequence.

3.4 Circular Dichroism

Peptides were dissolved in 60 μL MeOH at 2 mM and diluted with 540 μL NH_4HCO_3 buffer (5 mM, pH 7.4) to a concentration of 200 μM and incubated for 24 h. CD spectra were recorded at wavelength from 300 to 185 nm with a bandwidth of 1 nm, data pitch of 0.2 nm and a scanning speed at 20 nm/min from 20°C. Spectra were measured three times. For the measurement of the irradiated *iso*(Fmoc-I)nvocCA, the peptide was dissolved in 60 μL MeOH (2 mM) and combined with 540 μL of an aqueous NH_4HCO_3 solution (5 mM, pH 7.4) to yield a 200 μM peptide solution. After this, the solution was irradiated with light (365 nm) for 10 min incubated for 24 h and measured at 20°C.

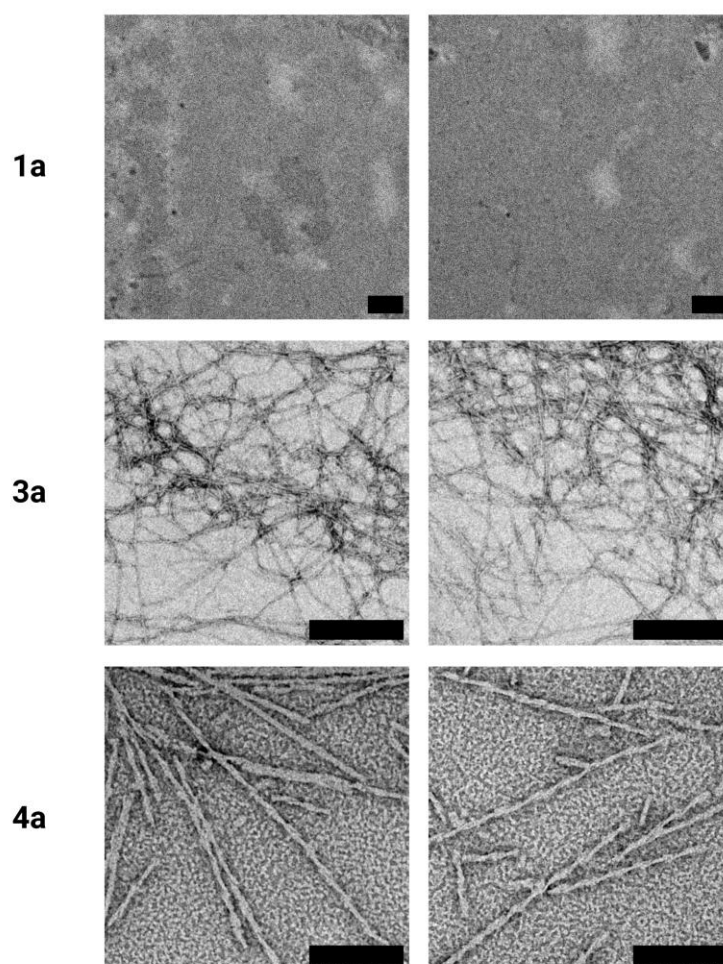
For CD measurements with DTT, *iso*(Fmoc-I)nvocCA was dissolved in 60 μL MeOH and added to an aqueous NH_4HCO_3 solution (5 mM, pH 7.4) with different equivalents of DTT (0, 5 and 10 equiv.) to yield a 200 μM peptide solution. The final solution contains a ratio 1 : 9 (MeOH : NH_4HCO_3). The mixture was irradiated with light (365 nm) for 10 min and incubated for 24 h. The measurements were conducted after the incubation period.

3.5 Transmission electron microscopy

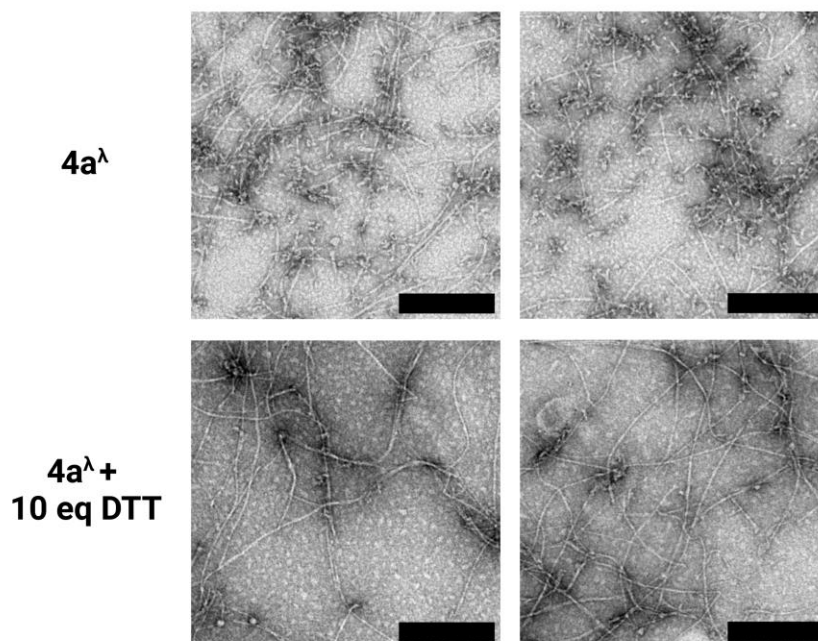
The control peptides (**3a** and **4a**) were dissolved in 10 μL MeOH at a concentration of 2 mM and diluted with 90 μL NH_4HCO_3 buffer (5 mM, pH 7.4) to yield 100 μL solutions (200 μM). The solutions were incubated for 24 h and TEM grids were prepared by pipetting 3 μL solution onto a Formvar-coated copper grid and incubated for 5 min. After the incubation, the solutions were removed with filter paper, and the grids were stained with 7 μL 4% uranyl acetate solution for 5 min. The grids were washed three times with MilliQ water and dried before being measured.

The *iso*-peptide was dissolved in 60 μL MeOH at a concentration of 2 mM and diluted with 540 μL NH_4HCO_3 buffer (5 mM, pH 7.4) to yield 100 μL solutions (200 μM). Then, the peptide was irradiated for 10 min (365 nm) and 100 μL of this solution incubated for 24 h. The TEM grids were prepared as described above without purification.

Iso(Fmoc-I)nvocCA was dissolved in 60 μL MeOH and added to 540 μL of an aqueous NH_4HCO_3 solution (5 mM, pH 7.4) with 10 equivalents of DTT to yield a 200 μM peptide solution. The mixture was irradiated with light (365 nm) for 10 min and incubated for 24 h. The TEM samples were prepared as described above without purification.



Supplementary Fig. 49: TEM images of the control peptides *iso*-FmocInvocCA (**1a**), FmocICA (**3a**) and DiFmocICA (**4a**). Scale bar = 250 nm.



Supplementary Fig. 50: TEM images of irradiated *iso*-FmocInvocCA (**4a^λ**) without DTT (top) and with 10 eq. DTT (bottom). Scale bar = 250 nm.

- [1] E. Atherton, H. Fox, D. Harkiss, C. J. Logan, R. C. Sheppard, B. J. Williams, *J. Chem. Soc. Chem. Commun.* **1978**, 537–539.
- [2] A. Apicella, P. Heunemann, L. Dejace, M. Marascio, C. J. G. Plummer, P. Fischer, *Colloids Surfaces B Biointerfaces* **2017**, 156, 221–226.

5 Intracellular Formation of Synthetic Peptide Nanostructures Causes Mitochondrial Disruption and Cell Death in Tumor Spheroids

Authors

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Contributions

Sarah Chagri:	Conceptualization, molecular design of peptides and planning of synthesis and experiments. Synthesis and characterization of peptides and associated molecules. CD measurements and NMR analysis. Cell uptake experiments and cell viability assays. Data analysis and interpretation of all corresponding results. Writing of the manuscript.
Konrad Maxeiner:	Cell uptake experiments and cell viability assays. Metabolic analysis and tumor spheroid experiments. Data analysis and interpretation of all corresponding results. Writing of the manuscript.
Maria J. S. A. Silva	Tumor spheroid experiments. Data analysis and interpretation.

Lisa Förch:	Tumor spheroid experiments.
Julian Link:	Synthesis of peptides.
Patrick Roth:	TEM measurements for the analysis of peptide structure formation and interpretation of results. Responsible for the TEM structure analysis part of the manuscript.
Raphael Meyer:	Investigation of the peptide nanostructure by secondary structure analysis of the assemblies of Fmoc- and C343-functionalized linear peptides with circular dichroism, showing aromatic-aromatic π - π^* at 260 nm and carbonyl-backbone n- π^* hydrogen-bonding transitions at 224 nm for Fmoc-ISA, while C343-ISA only shows the carbonyl-backbone n- π^* transitions and the absence of aromatic Fmoc-Fmoc interactions. Measurements of the co-assembly of the Fmoc- and C343-functionalized linear peptides in a ratio of 5:1 revealed the presence of Fmoc-Fmoc π - π^* transitions at 260 nm, however, with a slightly lower intensity compared to the assembly of Fmoc-ISA.
Jana Fetzer:	Synthesis of peptides.
Anke Kaltbeitzel:	CLEM measurements and interpretation of results.
Ingo Lieberwirth:	CLEM measurements and interpretation of results.
Katharina Landfester:	CLEM measurements and interpretation of results.
Manfred Wagner:	Measuring and interpretation NMR spectra.
David Y. W. Ng:	Design and discussion on the concept and results. Editing of the manuscript.
Tanja Weil:	Acquiring funding for the project, design and discussion of the concept and results. Editing of the manuscript.

RESEARCH ARTICLE

Intracellular Formation of Synthetic Peptide Nanostructures Causes Mitochondrial Disruption and Cell Death in Tumor Spheroids

Sarah Chagri, Konrad Maxeiner, Maria J. S. A. Silva, Lisa Förch, Julian Link, Patrick Roth, Raphael Meyer, Jana Fetzer, Anke Kaltbeitzel, Ingo Lieberwirth, Katharina Landfester, Manfred Wagner, David Y.W. Ng,* and Tanja Weil*

Supramolecular assemblies found in nature demonstrate the concept of creating functionality through structure formation. In recent years, these complex natural architectures have inspired the development of materials for the formation of synthetic nanostructures within living cells. These intracellular assemblies have the potential to modulate cellular processes, yet their specific effects on cellular metabolism and 3D cell networks, such as tumor spheroids, still remain underexplored. Herein, the study correlates the glutathione-induced formation of synthetic nanostructures inside MDA-MB-231 triple-negative breast cancer cells to the metabolic disruption and mitochondrial degradation observed in 2D cell culture, as well as to cell death and size decrease in a 3D tumor spheroid model. In 2D cell culture, material-cell interactions are examined through live-cell imaging and by quantifying changes in mitochondrial respiration. By studying the interplay between glutathione-responsive cytosolic peptide assembly and the implications on the integrity of the mitochondrial network, as well as on 3D cell networks, the work advances the understanding of how synthetic intracellular nanofibers impact vital functions of living cells.

for intracellular structure formation. The creation of synthetic architectures within mammalian cells represents a milestone in synthetic biology and nanomedicine, as it offers a bottom-up understanding of how natural intracellular assemblies can be replicated based on molecular design principles. This knowledge offers new avenues for understanding the impact of such assemblies on cellular integrity, function, and viability.^[2]

Given the prevalence of the resistance to various chemotherapeutics in healthcare, the development of alternative strategies for effective treatment represents a vital objective in biomedical sciences.^[3] Supramolecular approaches that involve forming synthetic nanostructures inside cells represent a unique strategy for mechanically inducing cell death by disrupting vital cellular processes.^[4] The accumulation of larger aggregates inside cancer cells enhances the retention of synthetic materials, thereby improving pharmacokinetic properties and circumventing common drug resistance mechanisms, such as clearance driven by the overexpression of efflux pumps. However, preventing the premature assembly of the nanomaterial outside cells is crucial in the material design process. Tailoring synthetic nanomaterials to display bioresponsive properties ensures that the pro-assembling compounds transform into active monomers only in the presence of specific endogenous cues, enhancing spatiotemporal control over assembly formation.^[2] Physiological stimuli, such as pH gradients^[5] intracellular enzymes^[6] and the high concentration of the reductive glutathione in the cytosol^[7] can be exploited to selectively induce structure formation. In cancerous cells, the intracellular glutathione concentration is often increased,^[8,9] which contributes to multidrug resistance due to the glutathionylation and subsequent elimination of small molecule drugs.^[10,11] This biological phenomenon can be used to design pro-assembling precursors by introducing glutathione-responsive trigger groups with cleavable disulfide bridges,^[6,12,13] enabling the controlled formation of synthetic nanostructures inside living cells upon reduction.

1. Introduction

Intracellular nanostructures, such as the cytoskeleton, are naturally occurring protein assemblies that regulate fundamental processes involved in cell metabolism, division, and motility.^[1] These intricate and dynamic protein assemblies span the cytosol, serving as a blueprint for the design of synthetic nanomaterials

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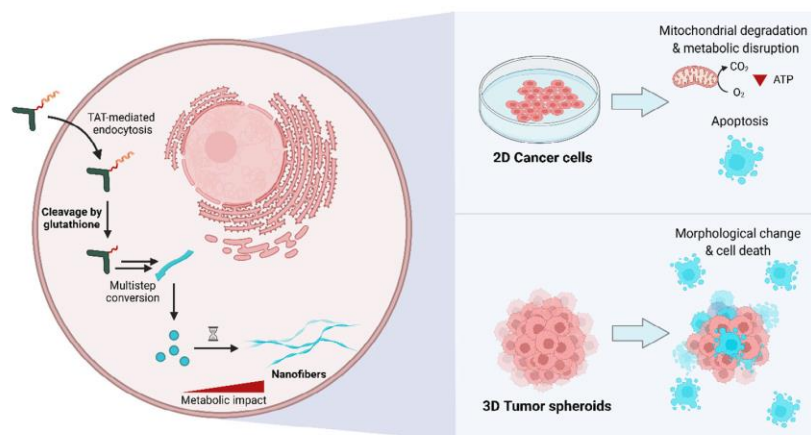


Figure 1. Schematic representation of glutathione-induced intracellular peptide assembly inside 2D-cultured cancer cells and 3D tumor spheroids. Glutathione-responsive isopeptides enter the cells via transactivator of transcription (TAT)-mediated endocytosis and are subjected to glutathione-induced cleavage in the cytosol, followed by a multistep conversion into linear self-assembling peptides. This intracellular transformation leads to the formation of nanostructures that disrupt the mitochondrial network causing cancer cell death and size decrease of tumor spheroids.

In recent years, our group has developed a series of peptide-based pro-assembling precursors that undergo a multistep conversion process in response to two specific environmental cues: the acidifying environment inside endosomes upon cellular uptake and the presence of reactive oxygen species (ROS) in the cytosol upon endosomal escape.^[4,14,15] Building upon this foundation, we have expanded the portfolio toward a glutathione-responsive system that activates immune cells through the formation of intracellular peptide nanostructures.^[16] The glutathione-induced peptide assembly within activated cytotoxic T cells stimulates T cell effector function, thereby increasing their cytotoxic activity toward cancer cells.^[16] Despite this progress, detailed studies on the concentration- and time-dependent biological impacts of synthetic intracellular assemblies—particularly regarding their effects on cancer cell metabolism—have only recently emerged. It is therefore essential to investigate the uptake, distribution, and intracellular conversion of assembly precursors over time to elucidate how the timeline of in situ structure formation correlates with the impact on cellular function.

To date, the analysis of in situ intracellular structure formation has been mostly focused on cells cultured in conventional 2D monolayers.^[15,16] However, findings derived from 2D cell cultures often fail to accurately replicate in vivo conditions, primarily due to their oversimplified cell-cell and cell-extracellular matrix interactions. In contrast, 3D cell networks more closely emulate the complex interactions observed in vivo. Notably, multicellular tumor spheroids exhibit key characteristics of solid tumors, including gradients in oxygen and nutrients from the outer cell layers to the hypoxic core, as well as high cell density.^[17]

Herein, we present the in situ formation of metabolism-disrupting nanostructures inside 2D-cultured cancer cells and 3D tumor spheroids using glutathione-responsive pro-assembling isopeptides. These peptides undergo a multistep reaction cascade into self-assembling linear peptides upon

cellular uptake (Figure 1). This study introduces live-cell and fluorescent lifetime imaging to observe real-time changes in the mitochondrial network and integrity during the formation of fluorescent peptide nanostructures for the first time. With similar time resolution, the impact on mitochondrial respiration is quantified by measuring the cellular oxygen consumption rate (OCR) via an extracellular flux analyzer. These measurements allow us to discern the effect of intracellular structure formation on the oxidative phosphorylation and energy homeostasis of multidrug-resistant MDA-MB-231 triple-negative breast cancer cells. Additionally, we investigate the toxicity of the glutathione-responsive isopeptides in 3D MDA-MB-231 tumor spheroids, monitoring their size reduction to assess the biological impact of the in situ formed nanostructures.

2. Results and Discussion

2.1. Design and transformation of Glutathione-Responsive isopeptides

The glutathione-responsive pro-assembling isopeptides **1a** and **1b** consist of three main structural components: 1) the cell-penetrating peptide sequence TAT (transactivator of transcription) derived from human immunodeficiency virus (HIV); 2) a glutathione-responsive trigger group capable of reductive degradation; and 3) the pro-assembling isopeptide with an aromatic N-terminal group that is necessary for self-assembly (Fmoc) and fluorescence (Coumarin 343), respectively (Figure 2a).^[16] To assess the effects of glutathione-induced transformation without self-assembly, we synthesized the control isopeptide **1c** (Figure 2a). This control includes a nitrobenzodiazole (NBD) fluorophore at the N-terminus and is designed to enter cells and undergo glutathione-induced transformation but does not produce self-assembling peptide monomers (Figure S21, Supporting Information). Additionally, non-cell-penetrating control isopeptides **4a**

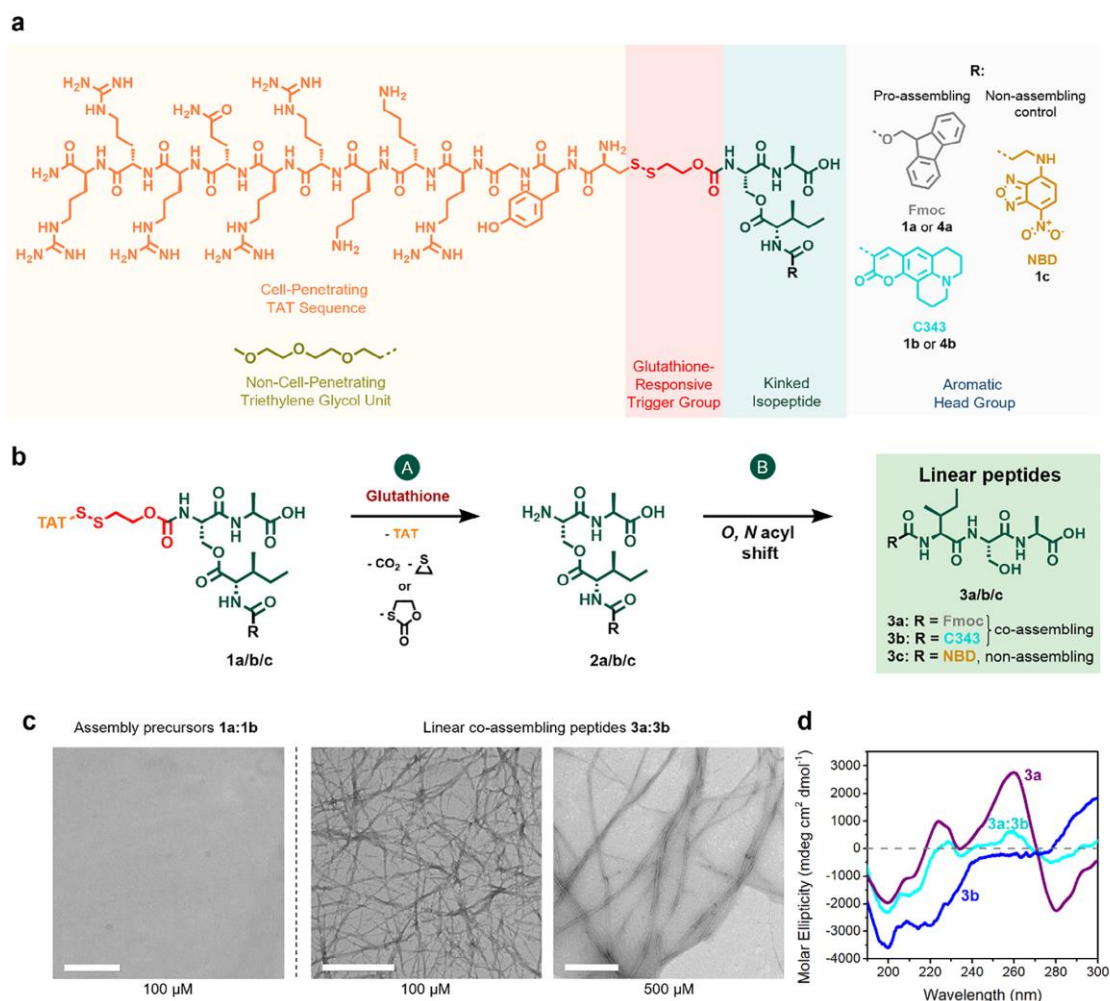


Figure 2. Chemical structures and multistep conversion of glutathione-responsive isopeptides into fibrillating peptide nanostructures. a) Chemical structure of glutathione-responsive pro-assembling isopeptides **1a** (with a Fmoc (9-fluorenylmethoxycarbonyl) group) and **1b** (with a C343 (Coumarin 343) moiety), as well as chemical structures of non-assembling control isopeptides **1c** (with an NBD (nitrobenzodiazole) unit), and triethylene glycol-modified non-cell-penetrating isopeptides **4a** and **4b**. b) Reaction scheme of the multistep conversion of glutathione-responsive isopeptides **1a/b/c** via the deprotected isopeptides **2a/b/c** into the co-assembling linear peptide monomers **3a** and **3b** or the non-assembling linear peptide **3c**. c) Transmission electron micrographs (TEM) of nanofibers formed by co-assembling peptides **3a** and **3b** (5:1 ratio at 500 and 100 μm) and TEM images of non-assembling assembly precursors **1a** and **1b** (5:1 ratio at 100 μm) in DPBS (pH 7.4) and DMSO (99:1). Scale bar 500 nm. d) Circular dichroism (CD) spectra of linear peptide **3a** (purple curve), linear peptide **3b** (blue curve), and linear co-assembling peptides **3a** and **3b** (5:1 ratio) (cyan curve) at 500 μm in phosphate buffer (10 mM, pH 7.4).

(with Fmoc) and **4b** (with Coumarin 343) were synthesized with a triethylene glycol unit replacing the cell-penetrating TAT sequence (Figure 2a).

To investigate the structure formation of the linear co-assembling peptides **3a** and **3b**, which are the final products of the glutathione-induced transformation of **1a** and **1b**, we used dry-state transmission electron microscopy (TEM) (Figure 2c; Figure S21, Supporting Information). The TEM images revealed that the co-assembly of Fmoc-ISA **3a** and Coumarin 343-ISA **3b**

formed elongated fibrillar peptide nanostructures at concentrations of 100 and 500 μm in a 5:1 ratio of **3a:3b** in DPBS (pH 7.4) with 1% DMSO. In contrast, the kinked pro-assembling isopeptides **1a** and **1b** did not form such supramolecular structures under similar conditions, underscoring their role as assembly precursors that require a stimulus to transform into the self-assembling monomers.

The circular dichroism (CD) spectra of the self-assembling monomer Fmoc-ISA **3a** exhibited nanoscale chirality^[18] featuring

a peak at 260 nm indicative of $\pi \rightarrow \pi^*$ transitions in the aromatic head groups (see Figure 2d). Additionally, a secondary peak at 224 nm was observed, corresponding to the $n \rightarrow \pi^*$ transition of the carbonyl groups in the peptide backbone, which can be attributed to hydrogen bonding.^[19,20] In contrast, the spectrum of the Coumarin 343-functionalized linear peptide **3b** lacks the 260 nm signal entirely, confirming the absence of Fmoc-Fmoc interactions. The signal indicative of backbone hydrogen bonding at 224 nm is present in the CD spectrum of **3b** but less pronounced compared to **3a**, suggesting weaker peptide backbone interactions. Notably, the CD spectrum of the co-assembled **3a:3b** mixture showed a slightly lower intensity at 260 nm compared to the spectrum of the self-assembling peptide **3a**. This reduction can be explained by the fact that the aromatic interactions between the Fmoc- and Coumarin 343-functionalized peptides **3a** and **3b** are weaker than the interactions solely between the Fmoc groups in peptide **3a**.

To complement these structural analyses and to assess the critical aggregation concentration (CAC) of the co-assembling peptides **3a** and **3b** (5:1 ratio), we conducted a Proteostat aggregation assay. This assay detects assemblies through an increase in fluorescence upon binding the Proteostat dye. The analysis indicated a CAC of 9.2 μ M for the linear peptides **3a** and **3b** in a 5:1 ratio in DPBS (pH 7.4) with 1% DMSO (Figure S23, Supporting Information).

2.2. In Situ Formation of Peptide Nanostructures Inside Cancer Cells

Next, we investigated the cellular uptake of the glutathione-responsive isopeptides, their subcellular distribution, and the in situ formation of intracellular nanofibers. For this purpose, we incubated MDA-MB-231 breast cancer cells with a 5:1 mixture of Fmoc-substituted isopeptide **1a** and Coumarin 343-substituted isopeptide **1b** and examined the emergence of fluorescent intracellular nanostructures via confocal laser scanning microscopy (CLSM) (Figure 3).

Time-lapsed live-cell imaging revealed that the TAT-modified isopeptides **1a** and **1b** (5:1 ratio, 50 μ M total peptide concentration) rapidly entered the cells and formed fluorescent peptide aggregates already within the first 15 min of incubation (Figure 3a; Video S1, Supporting Information). First, upon cell entry, we observed Coumarin 343-associated fluorescence in localized round areas distributed throughout the cytosol (Figure 3a). After 10 min of incubation with **1a** and **1b**, we noticed fibrillar and directed peptide nanostructures growing in an aster-like pattern from the round areas of increased fluorescence extending into the surrounding cellular space (Figure 3a, ROI). The cells exhibiting these intracellular structures showed a decrease in overall size and change in shape after 15 min (Figure 3a).

Further analysis of MDA-MB-231 breast cancer cells treated with the assembly precursors **1a:1b** at 100 and 50 μ M for 4 h revealed the presence of highly fluorescent structures throughout the cytosol (Figure 3b, second and third row). Co-staining of the nucleus showed a strong Coumarin 343-fluorescence signal in the perinuclear and nuclear regions (Figure 3b, second and third row). This observation of co-localization could be explained by the inherent nucleus-targeting properties of TAT^[24]

which may result in residual non-cleaved assembly precursors accumulating in the proximity of the nucleus. Additionally, we observed that in the cells treated with **1a:1b** at 100 μ M, the nucleus stain was most intense in small, condensed areas of the nuclear region (Figure 3b, second row). These observations are indicative of nuclear fragmentation, which is associated with decreased cell viability (Figure S35, Supporting Information). For the lower isopeptide concentration of 10 μ M, the peptides remained contained within small areas for 4 h (Figure 3b, bottom row), and no significant disruption of cellular integrity was evident.

The formation of intracellular peptide architectures was further investigated using correlative light and electron microscopy (CLEM) (Figure S28, Supporting Information). CLEM analysis of MDA-MB-231 cells treated with 100 μ M of **1a:1b** for 4 h revealed fluorescent peptide assemblies within the cytosol, especially in the nuclear and perinuclear regions (Figure S28, Supporting Information). In close proximity to fluorescent peptide nanostructures, mitochondria with disrupted membranes were observed, indicating that cytosolic assembly has negative impacts on the integrity of these organelles (Figure S28, Supporting Information). The subcellular distribution observed via CLEM was consistent with CLSM analysis (Figure 3; Video S1, Supporting Information), confirming the ability of isopeptides **1a:1b** to form nanostructures within living cells.

As a control, cells treated directly with the linear co-assembling peptides **3a:3b** (5:1 ratio), the final products of the glutathione-induced reaction cascade of **1a:1b**, did not show any uptake or impact on cellular morphology, underlining the importance of the TAT-mediated cell entry and the in situ intracellular transformation (Figure 3b, top row). For the non-assembly-inducing control isopeptide **1c**, internalization was observed but without impact on cellular integrity (Figure S25, Supporting Information). This further supports that the in situ formation of supramolecular peptide nanostructures is necessary to elicit a biological response.

2.3. Peptide Nanostructures Disrupt the Cytoskeleton of Cancer Cells

The cytoskeleton, particularly the actin filament network, plays a vital role in maintaining cell structure, movement, and organization.^[1] As we observed rapid morphological changes in cells in response to the formation of the peptide nanostructures, we seek to understand the relationship between the formation of synthetic peptide nanostructures and the loss of cytoskeletal integrity. Therefore, MDA-MB-231 cells were treated with varying concentrations of the isopeptides **1a:1b** for 4 h, and their actin filaments were subsequently stained with Alexa Fluor 555 Phalloidin (Figure 4). At high concentrations of 100 or 50 μ M of **1a:1b**, significant fragmentation of the actin filaments was observed (Figure 4, second to fourth row; Figure S25, Supporting Information). Notably, in areas of very high Coumarin 343 fluorescence, there was a marked absence of phalloidin-stained actin filaments, suggesting that peptide nanostructure formation disrupts the cytoskeleton (Figure 4, second and third row, ROI). In cells incubated with 10 μ M of the isopeptide mixture **1a:1b** (Figure 4, bottom row), no apparent changes to the integrity and density of the actin filaments were observed compared to the control cells treated with the linear peptides **3a:3b** (Figure 4, top row). To as-

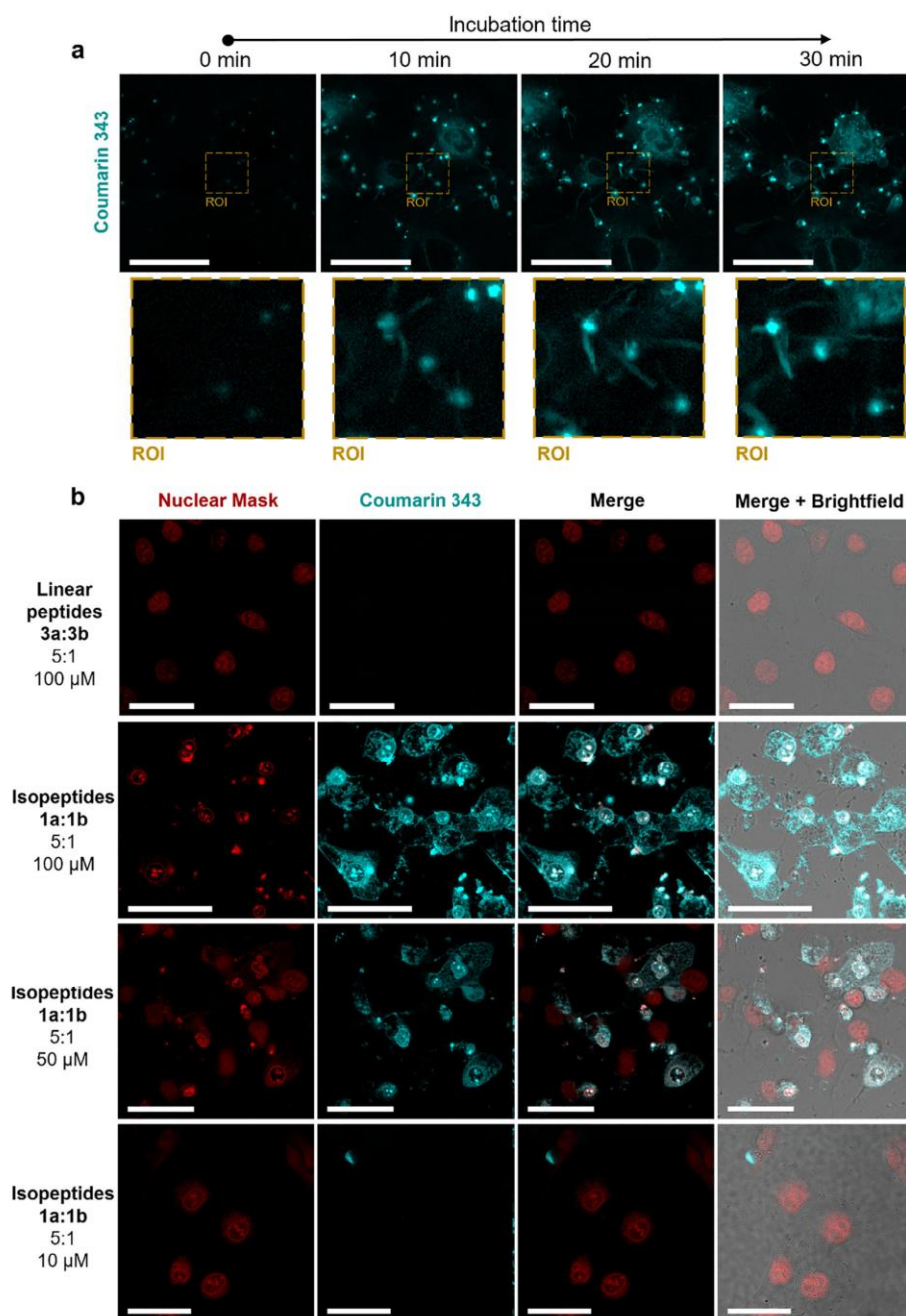


Figure 3. Cellular uptake and intracellular peptide assembly in cancer cells. a) Time-dependent live-cell imaging of MDA-MB-231 cells treated with 50 μM of the glutathione-responsive isopeptides **1a** and **1b** (5:1) (cyan). The rectangle indicates a region of interest (ROI), in which structure transformation from globular peptide structures to fibrous nanostructures can be observed. Scale bars 50 μm . b) Confocal laser scanning microscopy (CLSM) images of MDA-MB-231 breast cancer cells after 4 h of incubation with glutathione-responsive isopeptides **1a** and **1b** (5:1) at 100, 50, and 10 μM (cyan) (second to fourth row). As a control, cells were incubated with the linear peptides **3a** and **3b** (top row) at 100 μM . The nucleus was stained with HCS Nuclear Mask Deep Red (red). Scale bars 50 μm .

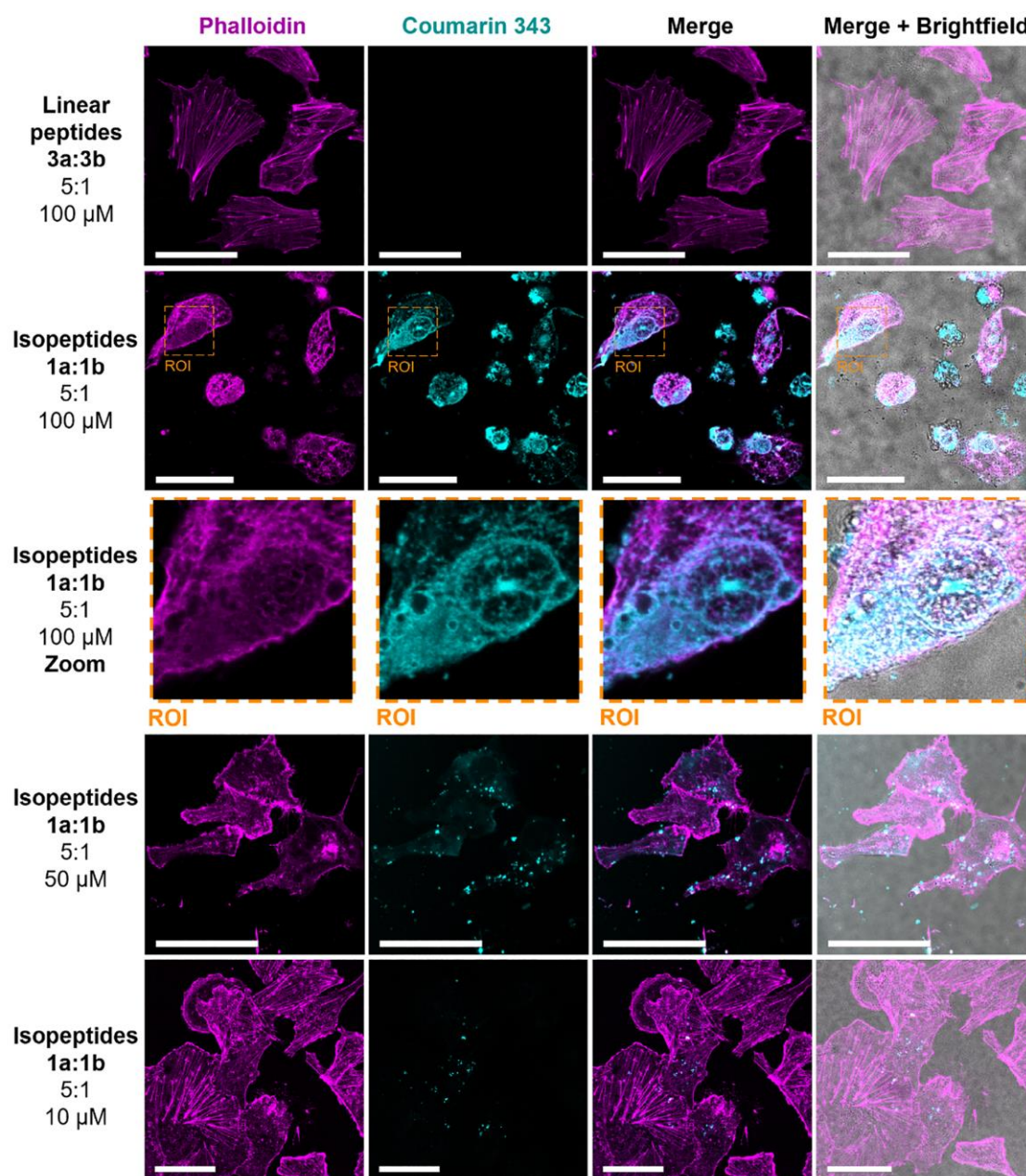


Figure 4. Disruption of actin filaments by in situ formed nanostructures. Confocal laser scanning microscopy (CLSM) images of MDA-MB-231 cells after 4 h of incubation with glutathione-responsive isopeptides **1a** and **1b** (5:1) (cyan) at different concentrations or the linear peptides **3a:3b** and additional staining with Alexa Fluor 555 Phalloidin (pink). Scale bars 50 μ m.

sess the time-dependent effects on cytoskeletal integrity, we performed phalloidin staining on cells treated with the assembly-inducing isopeptides **1a:1b** (at 100 and 10 μM) and the linear peptides **3a:3b** (at 100 μM) as a control. Imaging at 1, 2, 4, and 24 h treated with isopeptides **1a:1b** at 100 μM revealed that structural disruption of the cytoskeleton occurs rapidly, with pronounced effects already evident within 1 h of incubation (Figures S31–S33, Supporting Information).

To quantify the degree of actin cytoskeletal disruption, we conducted a western blot analysis of MDA-MB-231 cell lysates treated with either isopeptides **1a:1b** or linear control peptides **3a:3b** at 50 μM , comparing the G-actin/F-actin ratio after a 2 h incubation. G-actin (globular actin) represents the unpolymerized, soluble form of actin, while F-actin (filamentous actin) is the polymerized, structural form that contributes to the stability of the cytoskeleton.^[21] Cells treated with **1a:1b** (50 μM) exhibited a G-actin/F-actin ratio of 80.9% to 19.1%, whereas cells treated with **3a:3b** (50 μM) or the untreated control showed ratios of 62.9% to 37.1% and 62.2% to 37.8%, respectively (Figure S34, Supporting Information). This indicates that treatment with isopeptides causing intracellular structure formation leads to a measurable increase in the proportion of G-actin relative to F-actin, further suggesting cytoskeletal disruption.

2.4. Intracellular Structure Formation Impacts Metabolism, Disrupts Mitochondrial Integrity, and Causes Apoptosis

Analyzing the synthetic structure formation inside cells via CLSM and CLEM revealed morphological changes upon intracellular peptide assembly, which include blebbing, cell rounding and fragmentation of both nuclei and actin filaments (Figures 3 and 4; Video S1, Supporting Information). These findings indicate an effect on cell viability, which was determined in the next step using CellTiter-Glo Assay after a 4 h incubation with the pro-assembling precursors **1a:1b** at different concentrations. A concentration dependent effect on cell viability was observed and an IC_{50} of $36.4 \pm 3.1 \mu\text{M}$ was calculated (Figure S35, Supporting Information). In combination with the CLEM images, which revealed a localization of peptide nanofibers in close proximity to mitochondria with disrupted membranes, these results indicate that the peptide nanofibers impact energy metabolism of the cells. To gain further insights into the mechanism of how synthetic peptide nanostructures effect cell integrity, mitochondria function was investigated in more detail, as these organelles are essential for cell metabolism and dynamics, and maintaining energy homeostasis. To assess the impact of peptide assembly on mitochondrial structure and function, we performed live-cell imaging with cells that had been transfected with CellLight Mitochondria-RFP (Figure 5a; Figure S26 and Video S2, Supporting Information). We observed that within the first 30 min of incubation with 50 μM of the isopeptides **1a:1b** the mitochondria of the cancer cells underwent significant changes: starting from an interconnected network of tubular-shaped organelles, their morphology transformed into shrunken, fragmented, and more punctiform mitochondria (Figure 5a; Video S2, Supporting Information). These observations point toward mitochondrial fragmentation, which could be a result of changes in the rates of mi-

tochondrial fusion and fission events^[25] indicative of early stages of cell death.²⁶

To further investigate the mitochondrial disruption, we quantitatively assessed various aspect of mitochondrial respiration as a function of the formation of the peptide nanostructures. Using a Seahorse XFe96 extracellular flux analyzer, we measured the oxygen consumption rate (OCR) of cells treated with various electron transport chain (ETC) modulators, providing further insights into cellular respiration (Figure 5c,e).^[22] When adding the pro-assembling isopeptides **1a:1b** to the cells, we measured a concentration-dependent decrease in OCR already within the first 30 min of incubation (Figure 5b). For the lowest concentration of 10 μM , no effect on the OCR was found indicating cellular tolerance to this amount of assembly precursors. This correlates with the cell viability data, where no negative effect was observed at this concentration (Figure S35, Supporting Information).

However, starting at 25 μM total isopeptide concentration, a rapid reduction in OCR was observed, which was even more pronounced at 50 and 100 μM (Figure 5b). This showed that the addition of the assembly precursors **1a:1b** at concentrations capable of causing intracellular structure formation led to a significant decrease in basal respiration within <30 min. These quantitative findings aligned with the visual data from live-cell imaging of mitochondria-stained cells (Figure 5a), confirming mitochondrial dysfunction as a consequence of intracellular peptide assembly. Besides examining the cellular response to **1a:1b** in terms of basal respiration, we also studied the impact on ATP production and the spare respiratory capacity, as both properties are indicative of mitochondrial health and metabolic function (Figure 5d). Here we also noticed a concentration-dependent effect of the isopeptides **1a:1b** with cells being treated with $\geq 25 \mu\text{M}$ overall peptide concentration exhibiting a significantly reduced ATP production and spare respiratory capacity (Figure 5d). In contrast, no effect on mitochondrial function was observed for the linear peptides **3a:3b**, underscoring the importance of intracellular accumulation of assembly precursors and the propagation of resulting peptide assemblies to elicit a biological response.

To expand our analysis, we also investigated the effects of treatment with isopeptides **1a:1b** on A549 cells, a non-small cell lung cancer (NSCLC) line, on cell viability and mitochondrial respiration. Treatment with **1a:1b** for 4 h resulted in an IC_{50} value of $81.7 \pm 7.1 \mu\text{M}$ for A549 cells (Figure S36, Supporting Information), showing comparable but slightly lower toxicity than in MDA-MB-231 cells (IC_{50} : $36.4 \pm 3.1 \mu\text{M}$, Figure S35, Supporting Information). Seahorse metabolic analysis of A549 cells revealed that incubation with $\geq 25 \mu\text{M}$ of **1a:1b** caused a rapid decrease in basal respiration and disruption of mitochondrial functions (Figure S43, Supporting Information), similar to effects observed in MDA-MB-231 cells (Figure 5b). These findings suggest that the toxicity from intracellular structure formation is evident in both cancer cell lines.

In addition to examining mitochondrial fragmentation via genetically encoded fluorescence labeling of the organelle, MitoTracker DeepRed was used as a co-staining reagent (Figure 6; Video S3, Supporting Information). Unexpectedly, we observed that the dye underwent a change in fluorescence lifetime (FL) within cells exposed to the pro-assembling isopeptides **1a:1b** during live-cell imaging. Initially, the FL signal of the stain was uniform with signal component decay at 0.65 ns and could be ob-

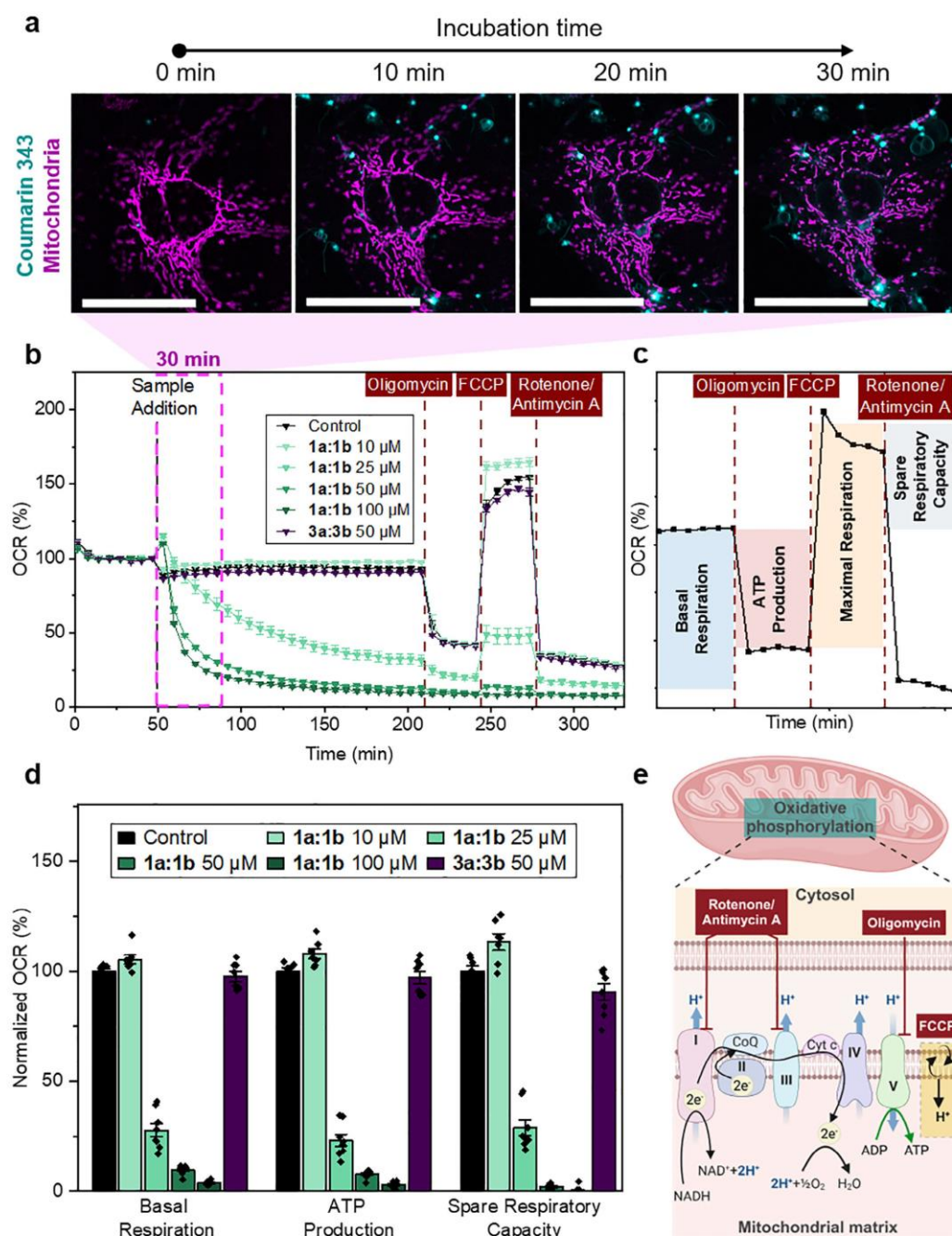


Figure 5. Effect of glutathione-responsive isopeptides on mitochondrial integrity and respiration. a) CLSM images of live-cell imaging of MDA-MB-231 cells treated with 50 μM of **1a:1b** (cyan). Cells were previously transfected with CellLight Mitochondria-RFP (pink). Scale bars 50 μm. b) Effect of isopeptide mixture **1a:1b** and of control compounds on the oxygen consumption rate (OCR) of MDA-MB-231 cells during the Mito Stress Test Assay. The last measurement before treatment injection of the compound is set as 100%. $n = 7$ c) Representative OCR trace from the Mito Stress Test. Specific electron transport chain (ETC) modulators are added at designated time points to assess mitochondrial function: 1) oligomycin inhibits ATP synthase,

2) FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) disrupts the mitochondrial membrane potential, 3) rotenone inhibits complex I and antimycin A inhibits complex III of the ETC.^[27] d) Effect of isopeptide mixture **1a:1b** and of control compounds on basal respiration, ATP production, and spare respiratory capacity. Values are normalized toward the untreated control group. Data are presented as mean $\pm$ s.e.m., $n \geq 7$. Statistical significance was calculated by ANOVA with a Tukey post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. e) Schematic representation of oxidative phosphorylation and the electron transport chain (ETC) in mitochondria, highlighting the effects of specific ETC modulators (oligomycin, FCCP, rotenone/antimycin A).

served homogeneously in all examined cells. However, the addition of the isopeptides **1a:1b** at 50 μM caused a shift in the FL of MitoTracker DeepRed to 1.20 ns, particularly in those cells exhibiting high Coumarin 343 fluorescence of the peptide nanostructures (Figure 6; Video S4, Supporting Information). The observed shift in the photochemical properties of the dye is likely caused by changes in its chemical environment, which appear to correlate with the degradation of the mitochondrial network as a result of the intracellular formation of peptide nanostructures.

To investigate the activation of apoptosis-related enzymes in relation to intracellular structure formation, we conducted a quan-

titative analysis of caspase-3/7 activation in cells treated with isopeptides **1a:1b** as well as live cell imaging using a caspase-3/7-sensitive fluorogenic dye. Caspase-3 and -7 are key executioner caspases in apoptosis, responsible for cleaving structural and regulatory proteins to facilitate controlled cellular disassembly.^[23] Therefore, their activation serves as a marker of apoptosis, linking upstream signaling events to the morphological and biochemical changes characteristic of programmed cell death.^[24]

Live-cell imaging of MDA-MB-231 cells treated with 50 μM isopeptides **1a:1b** revealed caspase-3/7 activation coinciding with the formation of fluorescent intracellular peptide structures. This

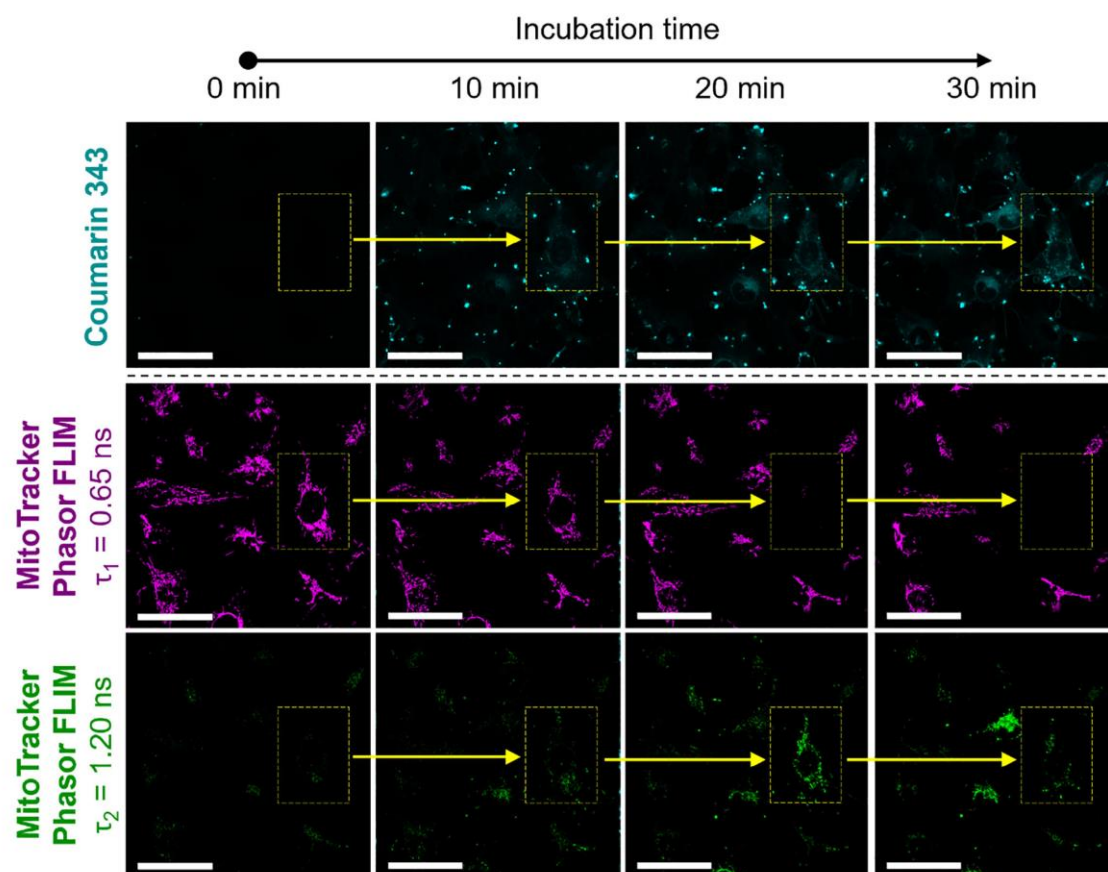


Figure 6. Changes in fluorescence lifetime of mitochondrial stain following intracellular structure formation. CLSM images show live-cell imaging of MDA-MB-231 cells treated with 50 μM of **1a:1b** (peptide fluorescence shown in cyan). Cells were previously stained with MitoTracker DeepRed. Fluorescence lifetime measurements of the stain were conducted concurrently with fluorescence imaging, revealing two distinct fluorescence lifetime signals for MitoTracker DeepRed over time. The corresponding phasor plots are provided in the Supporting Information (Figure S27, Supporting Information). Scale bars 50 μm .

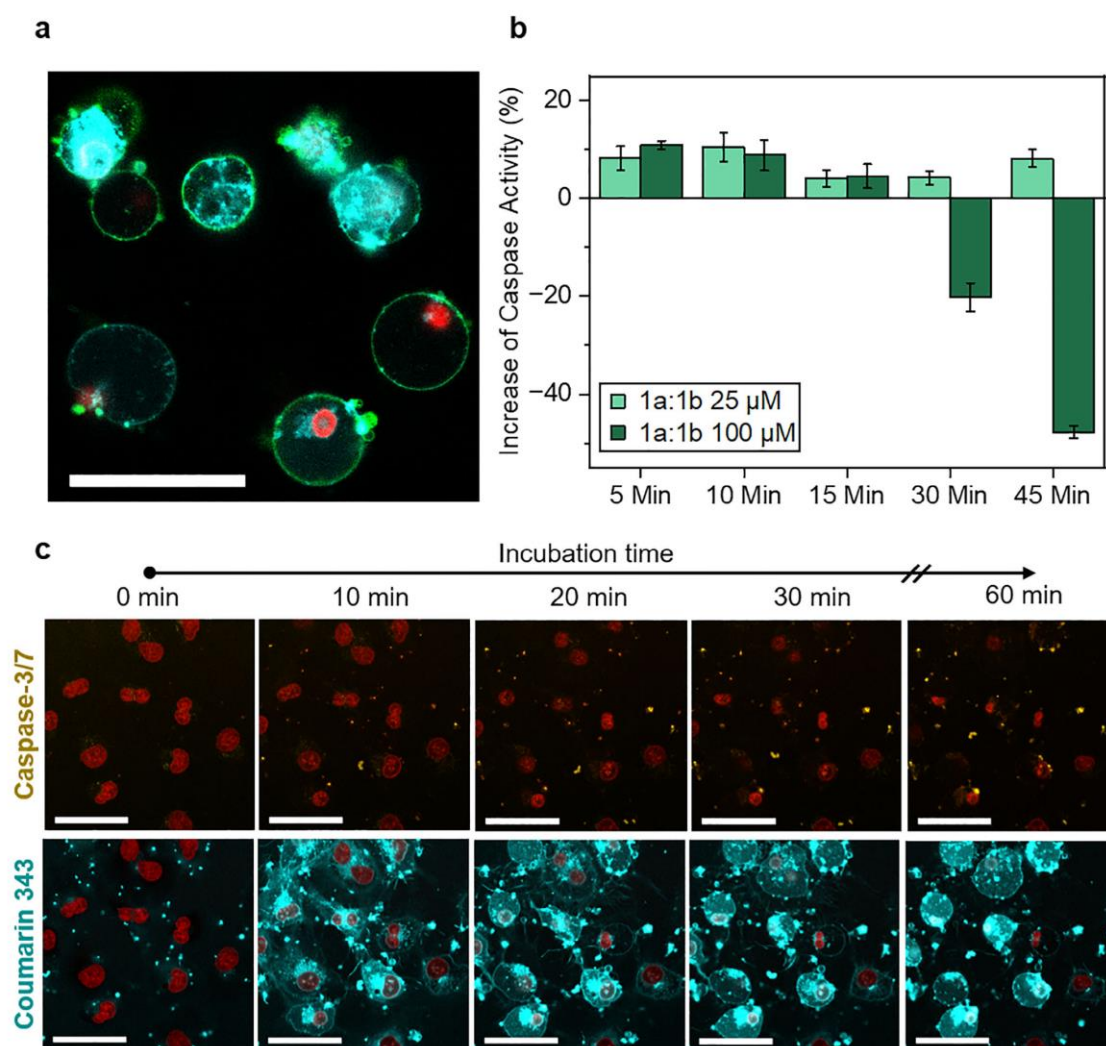


Figure 7. Apoptosis induced by intracellular peptide structure formation. a) Confocal laser scanning microscopy (CLSM) image of MDA-MB-231 cell after 1 h of incubation with isopeptides **1a** and **1b** (5:1) (cyan) at 50 μ M and additional staining with Annexin V (green) and propidium iodide (red). Scale bars 50 μ m. b) Caspase-Glo Assay results showing caspase activation in MDA-MB-231 cells after incubation with isopeptides **1a:1b** at 25 or 100 μ M at different timepoints. Increased caspase-3/7 activity indicates increased apoptosis compared to untreated control cells. Data represent mean $\pm$ s.e.m. ($n \geq 3$). c) Live cell imaging of MDA-MB-231 cells treated with isopeptides **1a:1b** (5:1) (cyan) at 50 μ M. Cells were pre-stained with Caspase-3/7 sensitive fluorogenic dye (yellow) and Nuclear Deep Red Mask (red). Scale bars 50 μ m.

activation was detectable within 10 min, as evidenced by the appearance of fluorescent areas in the cytosol resulting from the cleavage of the fluorogenic caspase-3/7-sensitive dye (Figure 7c, upper row). The caspase-3/7-induced fluorescence signal increased over time, up to 60 min of incubation, indicating accumulation of the fluorescent caspase cleavage product. The quantitative caspase-3/7 assay further confirmed this rapid activation, with enzyme activity rising within the first 5 to 10 min of isopeptide treatment (Figure 7b). Notably, at a high concentra-

tion of 100 μ M of **1a:1b**, caspase-3/7 activity declined again after 30 min, likely due to excessive toxicity (Figure S35, Supporting Information) and subsequent fast cellular degradation. In contrast, treatment with linear peptides **3a:3b** showed no measurable effect on caspase-3/7 activity (Figure S38, Supporting Information).

To further characterize apoptotic progression, we also performed Annexin V staining, which detects phosphatidylserine externalization, an early apoptosis marker resulting from impaired

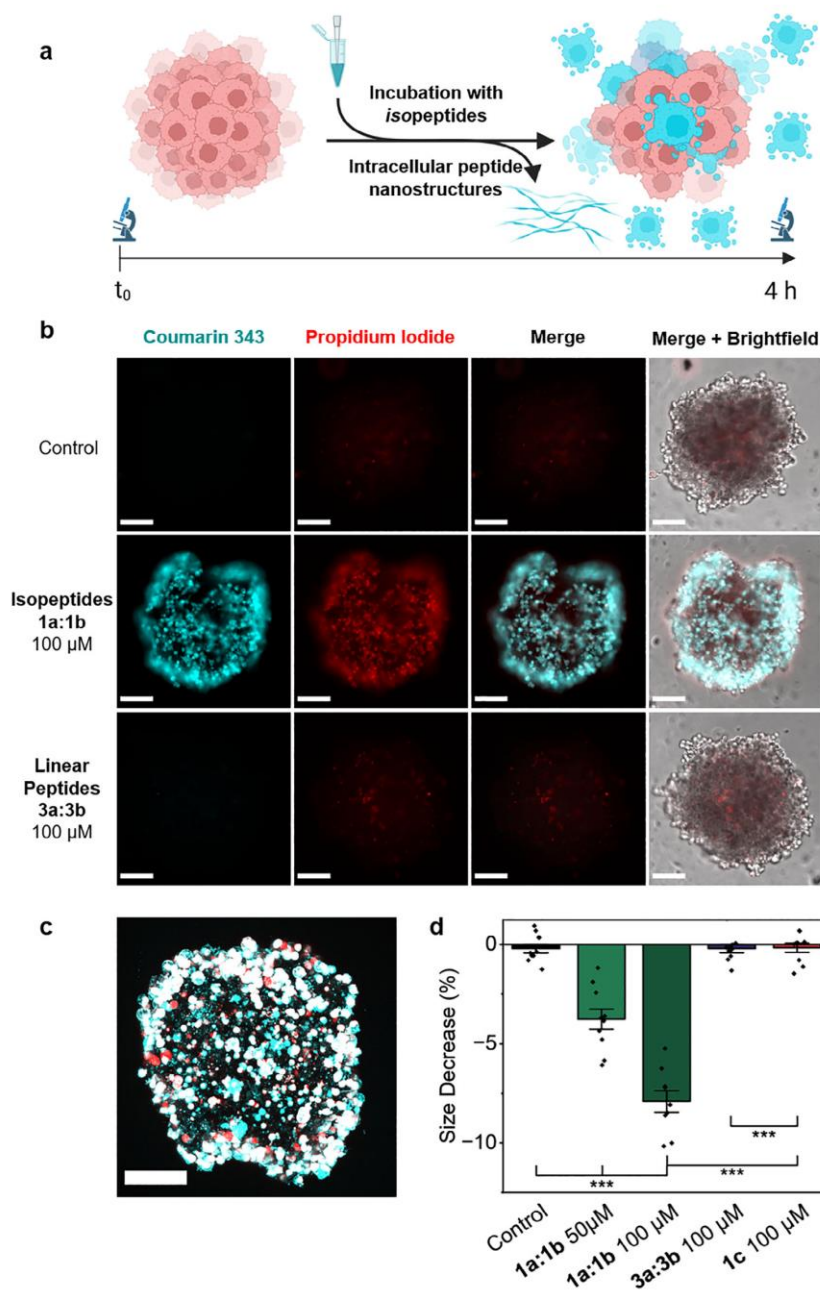


Figure 8. Intracellular peptide assembly disrupts tumor spheroid integrity. a) Schematic representation of tumor spheroid treatment with glutathione-responsive isopeptides and subsequent toxic intracellular assembly formation. b) Fluorescence microscopy images of uptake and dead staining of tumor spheroids. Coumarin 343 fluorescence is shown in cyan, and propidium iodide-stained dead cells are red. Scale bars 100 μ m. c) Maximum projection of a z-stack of a spheroid incubated with **1a:1b** at 100 μ M for 4 h. d) Spheroid size was measured before and after incubation with glutathione-responsive isopeptides using brightfield images in ImageJ. Diameter of each spheroid was measured at four different places (Figure S46, Supporting Information). Data are presented as mean $\pm$ s.e.m., $n = 10$. Statistical significance was calculated by ANOVA with a Tukey post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

flippase activity.^[25] Cells treated with 50 μM of **1a:1b** for 1 h exhibited Annexin V staining at the plasma membrane, confirming apoptotic induction (Figure 7a).

Together, these complementary analyses of caspase-3/7 activity and Annexin V staining provide strong evidence that apoptosis is a key mechanism underlying the cytotoxic effects of **1a:1b**.

2.5. Intracellular Peptide Nanostructures have a Toxic Effect on Tumor Spheroids and Cause Size Reduction

After observing the impact of intracellular assemblies on cellular organelles such as the cytoskeleton and mitochondria in 2D cell culture and their ability to induce apoptosis, we next investigated their effects on 3D cell networks. To assess the impact of intracellular peptide assembly on cell networks, we treated tumor spheroids derived from MDA-MB-231 breast cancer cells with glutathione-sensitive assembly precursors **1a:1b** and monitored their uptake after 4 h with fluorescence microscopy. After the incubation with the peptide precursors, spheroids were stained with propidium iodide (PI), which is unable to pass the membrane of healthy cells, to further investigate the impact on cell viability (Figure 8a,b).

Consistent with the 2D cell experiments, incubation with the **1a:1b** precursors at 100 μM resulted in a strong Coumarin 343 fluorescence signal within the spheroids (Figure 8b, second row). Cellular uptake was observed uniformly on the surface of the spheroids (Figure 8b,c). Additionally, slicing and subsequent imaging of tumor spheroids also revealed distribution of fluorescent peptides into the core of the spheroid, highlighting efficient penetration and intracellular localization throughout the 3D tumor spheroid (Figure S53, Supporting Information). In contrast, spheroids treated with the linear peptides **3a:3b** did not show any internalization, showing the necessity for the TAT-facilitated cellular uptake and intracellular activation cascade to obtain intracellular structures that directly affect cell integrity (Figure S45, Supporting Information). Spheroids treated with the non-assembling isopeptide **1c** showed a weak fluorescence signal, indicating uptake without subsequent assembly formation (Figure S45, Supporting Information).

In comparison to the control spheroids, those incubated with **1a:1b** at 50 or 100 μM exhibited a significant decrease in size after 4 h of incubation (Figure 8d; Figures S44–S46, Supporting Information). While spheroids incubated with **1a:1b** at 100 and 50 μM decreased in size by $7.9 \pm 0.5\%$ and $3.8 \pm 0.5\%$, respectively, spheroids incubated with the linear peptides **3a:3b** or non-assembling NBD-labeled peptides **1c** did not show any size changes during the incubation period (Figure 8d). This suggests that the uptake of the assembly precursors into cells leads to cell death, resulting in a decrease in tumor spheroid size, whereas no effects on the spheroid stability were observed for the treatment with the linear or non-assembling control peptides **3a:3b** and **1c** (Figure 8d). To further investigate the effects of prolonged isopeptide treatment on 3D tumor spheroids, we extended incubation to 24 and 48 h, assessing morphological changes and growth inhibition. Spheroid size further decreased by 13.9% at 24 h and 16.9% at 48 h after treatment with 100 μM of **1a:1b** (Figure S47, Supporting Information). Fluorescence imaging re-

vealed progressive disintegration of outer cell layers (Figure S48, Supporting Information).

Dead staining with propidium iodide (PI) revealed a stronger fluorescence signal for spheroids incubated with the glutathione-responsive isopeptides **1a:1b** at 100 μM for 4 h compared to the spheroids incubated with **1a:1b** at 50 μM or the spheroids incubated with **3a:3b** at 100 μM (Figure 8b). The residual fluorescence signal for propidium iodide for untreated and control spheroids is due to the natural necrotic core of the spheroids.^[17] The co-localization of Coumarin 343 fluorescence and the PI stain for spheroids incubated with the isopeptides **1a:1b** at 100 μM confirms that uptake and intracellular transformation of the isopeptides causes cell death, thereby disrupting the overall spheroid integrity (Figure 8b,d).

Aside from tumor spheroids grown from MDA-MB-231 cells, we additionally investigated 3D A549 tumor spheroids. Treatment with **1a:1b** at 100 μM for 4, 24, and 48 h also demonstrated uptake of fluorescently labeled isopeptides and resulted in cytotoxicity, as shown by propidium iodide staining (Figure S52, Supporting Information). Spheroid slicing further confirmed successful isopeptide uptake and distribution of fluorescent peptides into the core of A549 tumor spheroids (Figure S54, Supporting Information).

3. Conclusion

In conclusion, using glutathione-responsive isopeptides as assembly precursors has enabled us to assemble intracellular peptide nanostructures that disrupt the energy homeostasis of cancer cells and cause rapid cell death. The efficient cellular uptake and the fast glutathione-induced transformation of the kinked isopeptides into the linear self-assembling monomers allows the controlled formation of synthetic architectures within the complex cellular environment, both in 2D-cultured cells and 3D tumor spheroids. These nanostructures effectively disrupt essential subcellular structures and processes, such as mitochondrial function and the actin cytoskeleton, which results in apoptotic cell death and a significant reduction in spheroid size. By combining qualitative time-dependent microscopy with quantitative metabolic analysis of oxygen consumption rates and mitochondrial stress, we established a clear link between structural alterations in the mitochondrial network and the functional decline in mitochondrial respiration. Furthermore, using tumor spheroids as a model for 3D cellular networks enabled us to study the disruptive effects of intracellular synthetic nanostructures in a more complex and biologically relevant context. By continuing to explore the interactions between artificial and natural structures, we can gain important insights into the fundamental principles that govern cellular responses to synthetic assemblies.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

S.C. and K.M. contributed equally to this work. S.C., D.Y.W.N., and T.W. conceived the research concept. S.C. conducted the synthesis and characterization of peptides and associated molecules. J.L. and J.F. aided in the synthesis of peptides. S.C. and K.M. performed cell uptake experiments and cell viability assays. K.M. conducted the metabolic analysis and the tumor spheroid experiments. M. J. S. A. S. and L.F. aided in the tumor spheroid experiments. P.R. performed the TEM measurements. S.C. and R.M. conducted the CD measurements. A.K., I.L., and K.L. contributed CLEM measurements and analysis. S.C. and M.W. performed the NMR analysis. D.Y.W.N. and T.W. supervised the project and acquired funding. All authors have read and approved the final manuscript. All the authors have read and commented on the manuscript.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

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Supporting Information

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1. General Information

1.1. Materials

Reagents and solvents were purchased from commercial sources and were used without further purification. Peptide synthesis grade reagents were used for synthesizing the peptides. HPLC was performed using acetonitrile (CH₃CN) in HPLC grade (containing 0.1% trifluoroacetic acid (TFA)) and water for HPLC (containing 0.1% TFA) and reactions was obtained from a Millipore purification system. Flash column chromatography was carried out using Macherey-Nagel silica gel 0.04–0.063 mm.

1.2. Instruments

1.2.1. Microwave Peptide Synthesizer

Linear peptides were synthesized in a Liberty Blue Automated Microwave Peptide Synthesizer by CEM Corporation.

1.2.2. Liquid Chromatography-Mass Spectrometry (LC-MS)

Compounds were analyzed by HPLC-ESI-MS on a LC-MS 2020 by Shimadzu using a Kinetex 2.6 µm EVO C18 100 Å LC 50 × 2.1 mm column. MilliQ water acidified with 0.1% formic acid and CH₃CN were used as solvents for all measurements. The solvent gradient started with 5% CH₃CN and 95% water. This solvent ratio was kept constant for 2 min, then the CH₃CN content was linearly increased to 95% in 14 min. Data were processed in LabSolutions and OriginPro 9.

1.2.3. Atmospheric-Pressure Chemical Ionization Mass Spectrometry (APCI-MS)

All APCI-MS spectra were recorded on a Advion Expression-L Compact mass spectrometer. Data processing was performed in Advion Data Express.

1.2.4. Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR spectra of small molecules and peptides were recorded on a Bruker Avance II 300 MHz spectrometer and a Bruker Avance 400 MHz spectrometer. The solvent signal was used as a reference (deuterated chloroform CDCl₃ δ = 7.26 ppm for ¹H, 77.16 ppm for ¹³C and for DMSO-d₆ 2.50 ppm and 39.52 ppm respectively). The data was processed in MestReNova.

1.2.5. Transmission Electron Microscopy (TEM)

TEM images of the peptide samples were recorded on a JEOL 1400 transmission electron microscope at a voltage of 120 kV. Formvar/carbon-film coated copper grids (300 mesh) by Plano GmbH were used to prepare the samples. The images were processed in Fiji ImageJ or Icy.

1.2.6. 2D Cell Culture

MDA-MB-231 cells were cultured at 37 °C and 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM, high glucose), supplemented with 10% fetal bovine serum (FBS). The cells were cultured in T75 culture flask and subcultivated two to three times per week.

1.2.7. Tumor Spheroid Culture

MDA-MB-231 cells were seeded in Ultra-Low-Attachment (ULA) 96-well plates (Corning, Ref. 4520) at a cell density of 10,000 cells/well. The plate was centrifuged (1,200 rpm, RT, 5 min) to allow formation of pre-clusters facilitating sphere development over time. Spheroids were cultured for 7 days in complete medium (DMEM high glucose with glutamine supplemented with 10 % FBS) at controlled humidified atmosphere (37 °C, 5% CO₂).

1.2.8. Fluorescence Microscopy

Fluorescence images of the tumor spheroids were taken using a Keyence BZ-X810 fluorescence microscope using a 20x LD PH lens. Propidium iodide staining for dead imaging of tumor spheroids was analyzed using Cy5 filter with the following wavelength settings: Ex. 470/40 nm, Em. 525/50 nm. For Coumarin 343 imaging of the peptide co-assemblies a GFP filter was used: Ex.: 470/40 nm, Em.: 525/50 nm.

1.2.9. Confocal Laser Scanning Microscopy (CLSM)

Cells were imaged live using an incubator-equipped Leica Stellaris® 8 microscope (40x/1.25 glycerol immersion objective) with fast lifetime contrast (FALCON) module (Leica Microsystems GmbH). The incubator (okolab) is set and held constant at 37 °C, 5% CO₂ and a relative humidity of 90% throughout all measurements. For the detection of Coumarin 343 fluorescence, samples were excited using a 40 MHz pulsed white light laser tuned to 440 nm. Emitted photons were detected using HyD® S2 (GaAsP hybrid photocathode) detector with a filter window at 475 nm – 550 nm. The IRF standard used is fluorescein sodium salt.

Fluorescence lifetime imaging (FLIM) was performed on living cells at 2.5-minute intervals, with a scanning resolution of 1024 x 1024 pixels at 200 Hz, accumulating 15 frames per acquisition. Adaptive focusing was applied to each frame and well position. A total of 100 frames were captured. Photon counting was conducted using a FALCON-modified time-correlated single photon counting (TCSPC) method.

Each pixel was converted into a phasor plot using the following equation:

$$g_{i,j}(\omega) = \frac{\int_0^T I(t) \cdot \cos(n\omega t) dt}{\int_0^T I(t) dt}$$

$$s_{i,j}(\omega) = \frac{\int_0^T I(t) \cdot \sin(n\omega t) dt}{\int_0^T I(t) dt}$$

In this equation, $g_{i,j}(\omega)$ and $s_{i,j}(\omega)$ represent the x and y coordinates of the phasor plot, n and ω are the harmonic and angular frequencies of excitation, respectively, and T is the acquisition's repeat frequency. The frequency domain data acquired from each pixel can be transformed into phasor points using the following transformations:

$$g_{i,j}(\omega) = m_{i,j} \cdot \cos(\phi_{i,j})$$

$$s_{i,j}(\omega) = m_{i,j} \cdot \sin(\phi_{i,j})$$

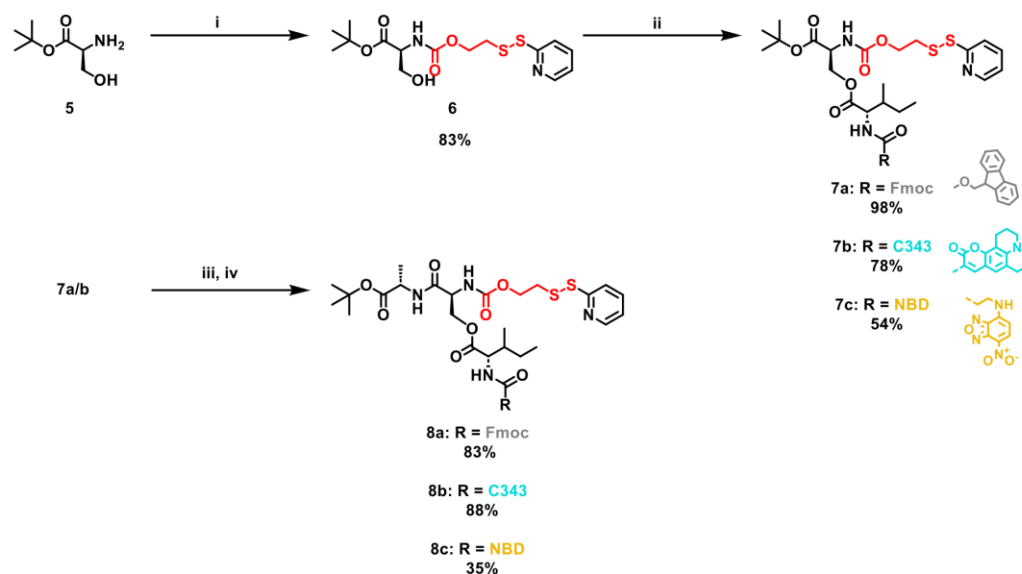
In these transformations, $m_{i,j}(\omega)$ and $\phi_{i,j}(\omega)$ represent the modulation and phase shift of the frequency domain measurement at pixel i,j . The decay at each pixel is thus mapped to a point in the phasor plot. Phasor components were identified and separated using LAS X software, and photons outside the defined phasor region were excluded from the images.

2. Synthesis

2.1. Synthesis of glutathione-responsive isopeptides

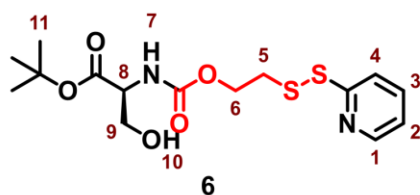
The synthesis of the glutathione-responsive isopeptides described in the following subsections have been reported previously by our group.¹

2.1.1. Synthesis of isopeptides with activated disulfide bond



Scheme S1 Synthesis of isopeptides with activated disulfide-bonds. i) 4-nitrophenyl 2-(pyridin-2-yl)disulfanylethyl carbonate, DIPEA, DCM, overnight, RT; ii) Fmoc/C343/NBD-Ile, DIC, DMAP, DCM, overnight, RT; iii) TFA, DCM, 4 h, RT; iv) Ala-tBuO, PyBOP, DIPEA, DCM, 3 h, RT.

2.1.1.1. Synthesis of compound 6



In a round-bottom flask, *tert*-butyl protected serine hydrochloride (200 mg, 1.01 mmol, 1 eq.) was dissolved in dry DCM (10 ml), and dry DIPEA (654 mg, 881 μ L, 5.06 mmol, 5 eq.) was added to the solution. Separately, 4-nitrophenyl 2-(pyridin-2-yl)disulfanylethyl carbonate was dissolved in dry DCM (5 ml) and slowly added to the reaction mixture. The solution was stirred at room temperature overnight, then washed twice with water and brine. The aqueous layer was extracted with ethyl acetate (EA), and the combined organic layers were dried over sodium sulfate. After solvent removal under reduced pressure, flash column chromatography (cHex:EA, 1:1) afforded compound **6** as a colorless amorphous solid (316 mg, 0.84 mmol, 83% yield).

¹H NMR (400 MHz, CDCl₃) δ 8.43 (d, *J* = 4.9 Hz, 1H, H-1), 7.74 – 7.64 (m, 3H, H-3, H-4), 7.10 (ddd, *J* = 6.8, 4.9, 1.4 Hz, 1H, H-2), 5.73 (d, *J* = 7.6 Hz, 1H, H-7), 4.40 – 4.21 (m, 3H, H-6, H-6', H-8), 3.90 (dd, *J* = 3.6, 2.3 Hz, 1H, H-9), 3.32 (s, 1H, H-10), 3.10 – 2.94 (m, 2H, H-5), 1.46 (s, 9H, H-11).

¹³C NMR (101 MHz, CDCl₃) δ 169.51, 163.21, 159.90, 155.89, 149.41, 137.59, 126.22, 121.08, 120.21, 115.82, 82.80, 63.41, 63.38, 56.74, 37.70, 28.09.

ESI-MS: *m/z* 375.1 (calculated for [M+H]⁺: 375.10)

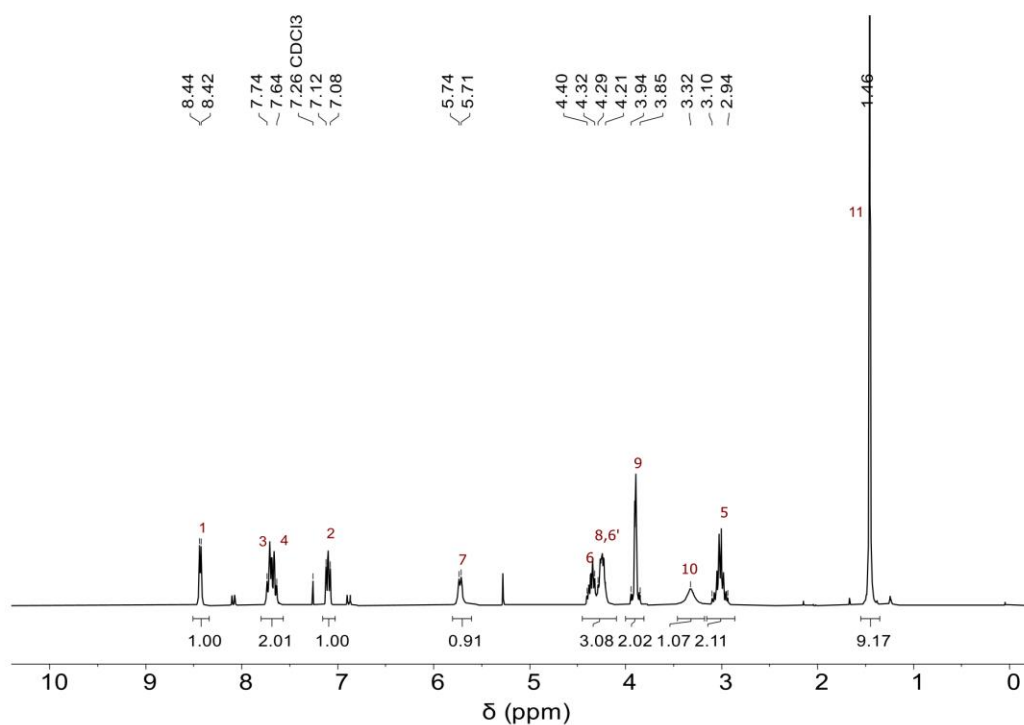


Figure S1 ¹H NMR spectrum (300 MHz, CDCl₃, 298 K) of compound **6**.

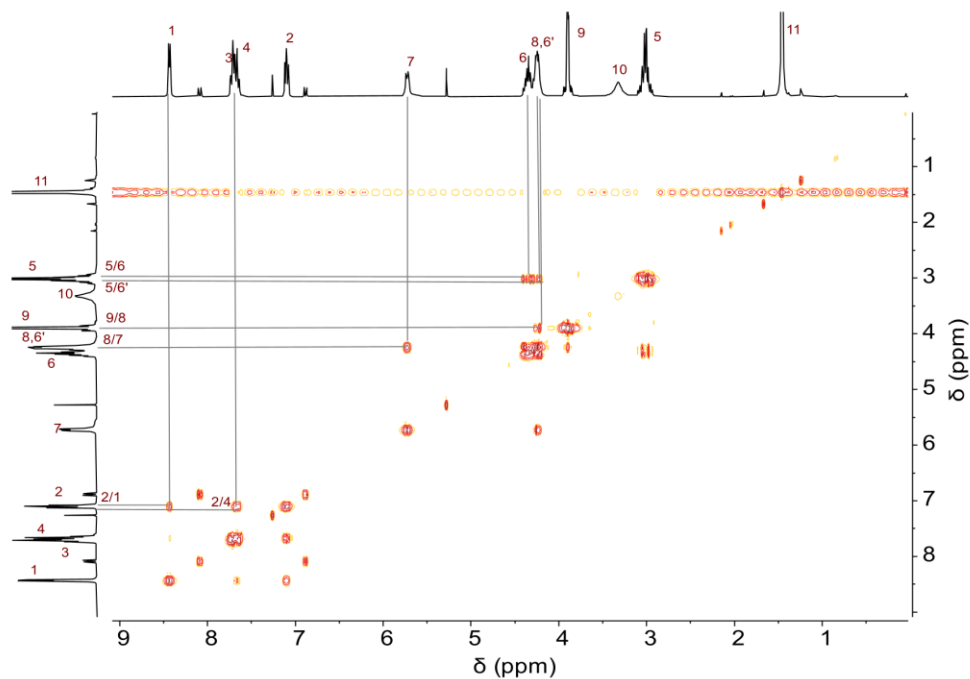


Figure S2 ^1H , ^1H COSY NMR spectrum (300 MHz, CDCl_3 , 298 K) of compound **6**.

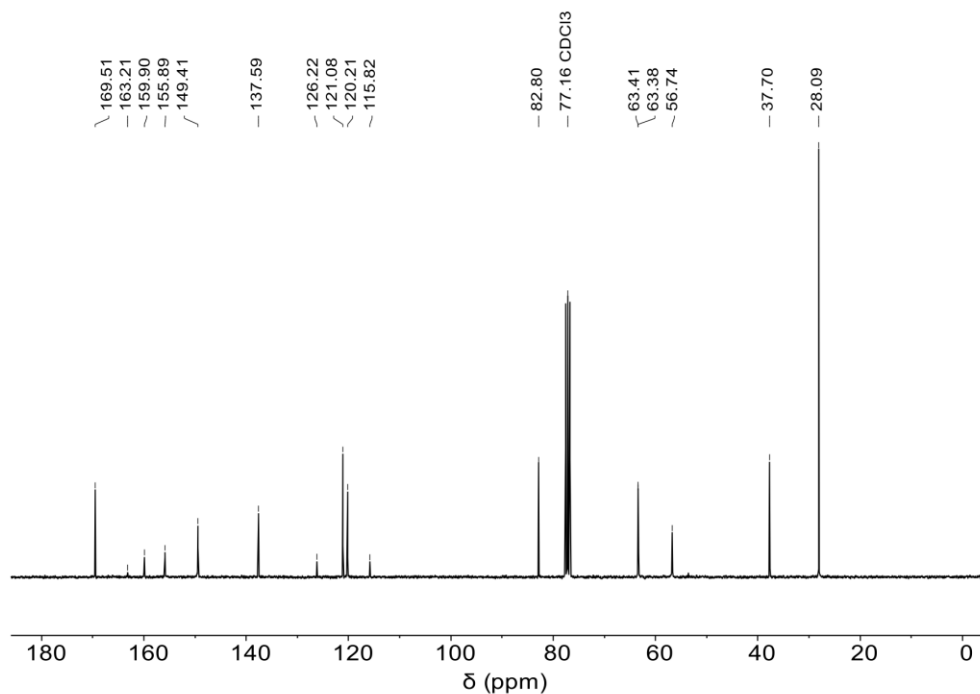
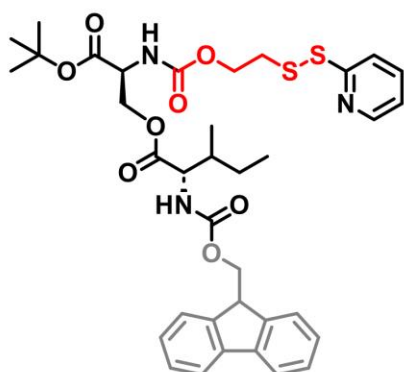


Figure S3 ^{13}C NMR spectrum (101 MHz, CDCl_3 , 298 K) of compound **6**.

2.1.1.2. Synthesis of compounds **7a**



7a

In a round-bottom flask, compound **6** (157 mg, 419 μmol , 1 eq.) was dissolved in dry DCM (5 ml) under a nitrogen atmosphere. Fmoc-Ile (274 mg, 774 μmol , 1.8 eq.), DMAP (23.6 mg, 193 μmol , 0.5 eq.), and DIC (98 mg, 774 μmol , 1.8 eq.) were dissolved in dry DCM (10 ml) and added dropwise to the solution of compound **6**. The reaction mixture was stirred at room temperature overnight. After solvent removal under reduced pressure, flash column chromatography (cHex:EA, 3:1) afforded compound **7a** as a colorless oil (291 mg, 410 μmol , 98% yield).

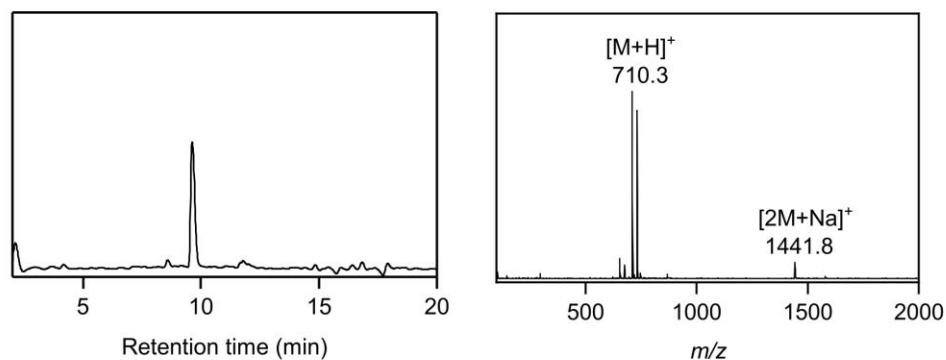
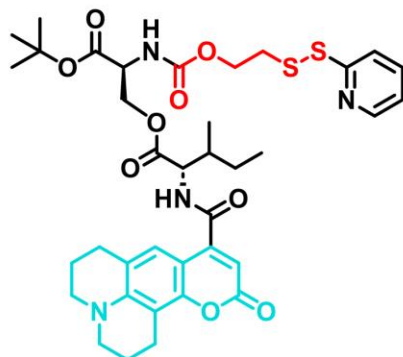


Figure S4 LC-MS data of compound **7a**. Left: LC trace (214 nm) with t_R (**7a**) = 9.63 min. Right: Convoluted ESI-MS spectrum showing peaks of $[M+H]^+$ (calculated: 710.26) and $[2M+Na]^+$ (calculated: 1441.49).

2.1.1.3. Synthesis of compounds **7b**



7b

In a round-bottom flask, compound **6** (80 mg, 214 μ mol, 1 eq.) was dissolved in dry DCM (4 ml) under a nitrogen atmosphere. Coumarin 343-Ile (126 mg, 316 μ mol, 1.5 eq.), DMAP (26.1 mg, 214 μ mol, 1 eq.), and DIC (40 mg, 320 μ mol, 1.5 eq.) were dissolved in dry DCM (5 ml) and added dropwise to the solution of compound **6**. The reaction mixture was stirred at room temperature overnight. After solvent removal under reduced pressure, flash column chromatography (cHex:EA, 3:2) afforded compound **7b** as a bright green oil (126 mg, 167 μ mol, 78% yield).

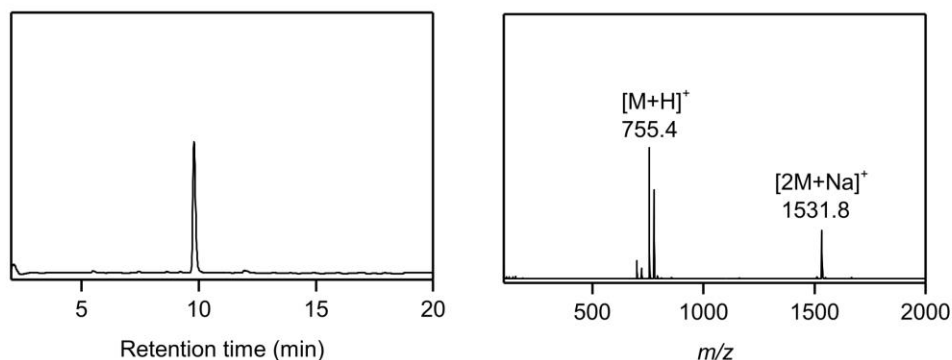
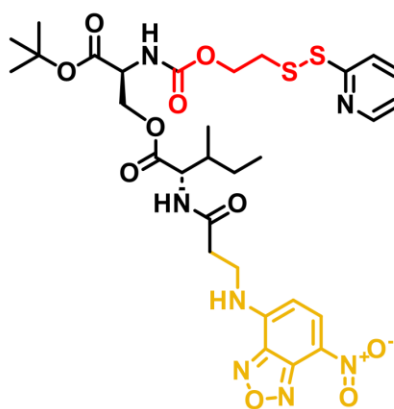


Figure S5 LC-MS data of compound **7b**. Left: LC trace (214 nm) with t_R (**7b**) = 9.80 min. Right: Convolution ESI-MS spectrum showing peaks of $[M+H]^+$ (calculated: 755.28) and $[2M+Na]^+$ (calculated: 1531.53).

2.1.1.4. Synthesis of compounds 7c



7c

In a round-bottom flask, compound **6** (162 mg, 433 μmol , 1.2 eq.) was dissolved in dry DCM (4 ml) under a nitrogen atmosphere. Nitrobenzoxadiazole (NBD)- β -Ala-Ile (132 mg, 361 μmol , 1 eq.), DMAP (88 mg, 722 μmol , 2 eq.), and DIC (91 mg, 722 μmol , 2 eq.) were dissolved in dry DCM (5 ml) and added dropwise to the solution of compound **6**. The reaction mixture was stirred at room temperature overnight. After solvent removal under reduced pressure, flash column chromatography (EA:cHex, 2:1) afforded compound **7c** as a bright yellow oil (141 mg, 195 μmol , 54% yield).

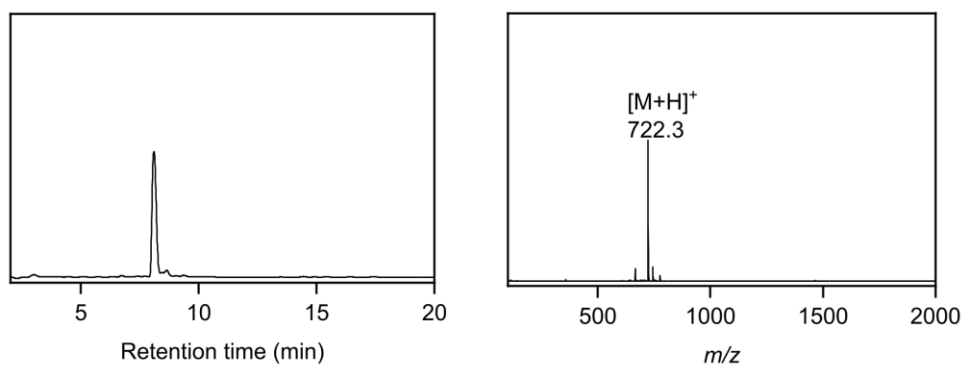
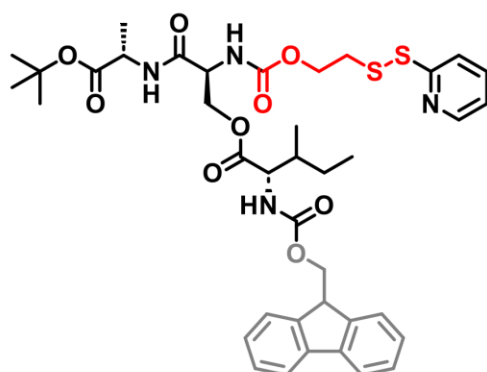


Figure S6 LC-MS data of compound **7c**. Left: LC trace (214 nm) with t_R (**7c**) = 8.63 min. Right: Convolution ESI-MS spectrum showing the peak of $[M+H]^+$ (calculated: 722.23).

2.1.1.5. Synthesis of compound **8a**



8a

In a round-bottom flask, compound **7a** (280 mg, 394 μ mol, 1 eq.) was dissolved in a 1:1 mixture of TFA and DCM (4 ml:4 ml) and stirred for 6 hours. The solvents were removed under reduced pressure. The resulting colorless oil was dissolved in dry DCM (15 ml), and DIPEA (329 mg, 443 μ L, 2.54 mmol, 6 eq.) was added under a nitrogen atmosphere. PyBOP (441 mg, 847 μ mol, 2.2 eq.) and *tert*-butyl-protected alanine hydrochloride (154 mg, 847 μ mol, 2.2 eq.) were then added, and the reaction mixture was stirred for 3 hours. The solution was washed with water, and the organic phase was extracted with ethyl acetate (EA). The combined organic layers were dried over sodium sulfate, and the solvents were evaporated. Flash column chromatography (EA:CHex, 4:3) yielded compound **8a** as a colorless oil (255 mg, 327 μ mol, 83% yield).

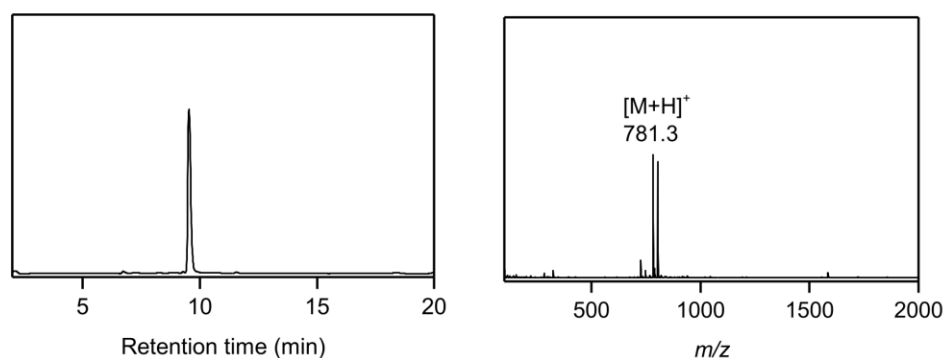
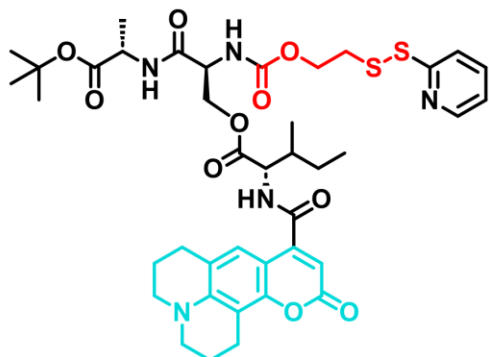


Figure S7 LC-MS data of compound **8a**. Left: LC trace (214 nm) with t_R (**8a**) = 9.53 min. Right: Convolved ESI-MS spectrum showing peaks of $[M+H]^+$ (calculated: 781.29).

2.1.1.6. Synthesis of compound **8b**



8b

In a round-bottom flask, compound **8b** (126 mg, 167 μ mol, 1 eq.) was dissolved in a 1:1 mixture of TFA and DCM (2 ml:2 ml) and stirred for 4 hours. The solvents were removed under reduced pressure. The resulting bright green oil was dissolved in dry DCM (10 ml), and DIPEA (297 mg, 400 μ L, 2.30 mmol, 13.7 eq.) was added under a nitrogen atmosphere. PyBOP (718 mg, 1.38 mmol, 8.2 eq.) and tert-butyl-protected alanine hydrochloride (267 mg, 1.47 mmol, 8.8 eq.) were then added, and the reaction mixture was stirred for 3 hours. The solution was washed with water and brine, and the organic phase was extracted with ethyl acetate (EA). The combined organic layers were dried over sodium sulfate, and the solvents were evaporated. Flash column chromatography (EA:cHex, 4:1) afforded compound **8b** as a bright green oil (122 mg, 148 μ mol, 88% yield).

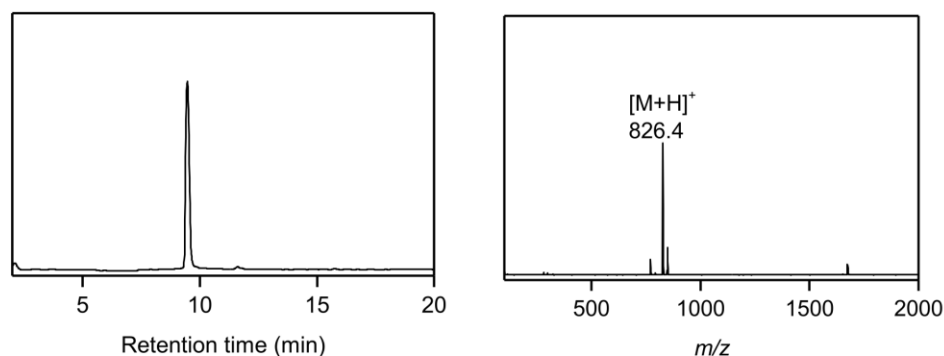
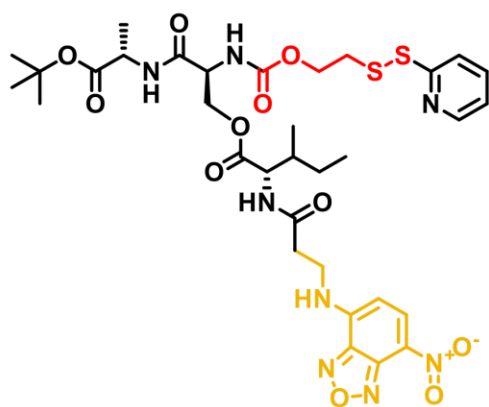


Figure S8 LC-MS data of compound **8b**. Left: LC trace (214 nm) with t_R (**8b**) = 9.47 min. Right: Convoluted ESI-MS spectrum showing peaks of $[M+H]^+$ (calculated: 826.32).

2.1.1.7. Synthesis of compound **8c**



8c

In a round-bottom flask, compound **7c** (141 mg, 195 μ mol, 1 eq.) was dissolved in a 1:1 mixture of TFA and DCM (2 ml:2 ml) and stirred for 4 hours. The solvents were removed under reduced pressure. The resulting bright yellow oil was dissolved in dry DCM (10 ml), and DIPEA (153 mg, 206 μ L, 1.18 mmol, 6 eq.) was added under a nitrogen atmosphere. PyBOP (205 mg, 393 μ mol, 2 eq.) and *tert*-butyl-protected alanine hydrochloride (71 mg, 393 μ mol, 2 eq.) were then added, and the reaction mixture was stirred for 3 hours. After solvent evaporation, the product was purified via HPLC using a Phenomenex Gemini column. The gradient began with 10% CH₃CN in H₂O (+0.1% TFA), held for 3 minutes, followed by a linear increase of CH₃CN to 100% over 12 minutes. This purification afforded compound **8c** as a bright yellow solid (55 mg, 69 μ mol, 35% yield).

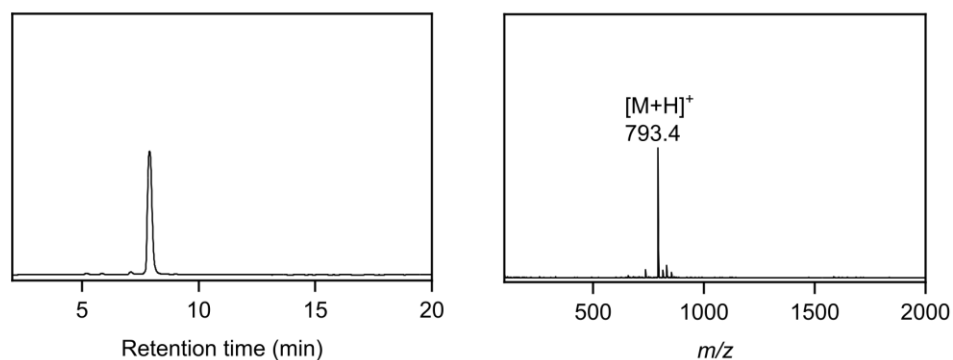
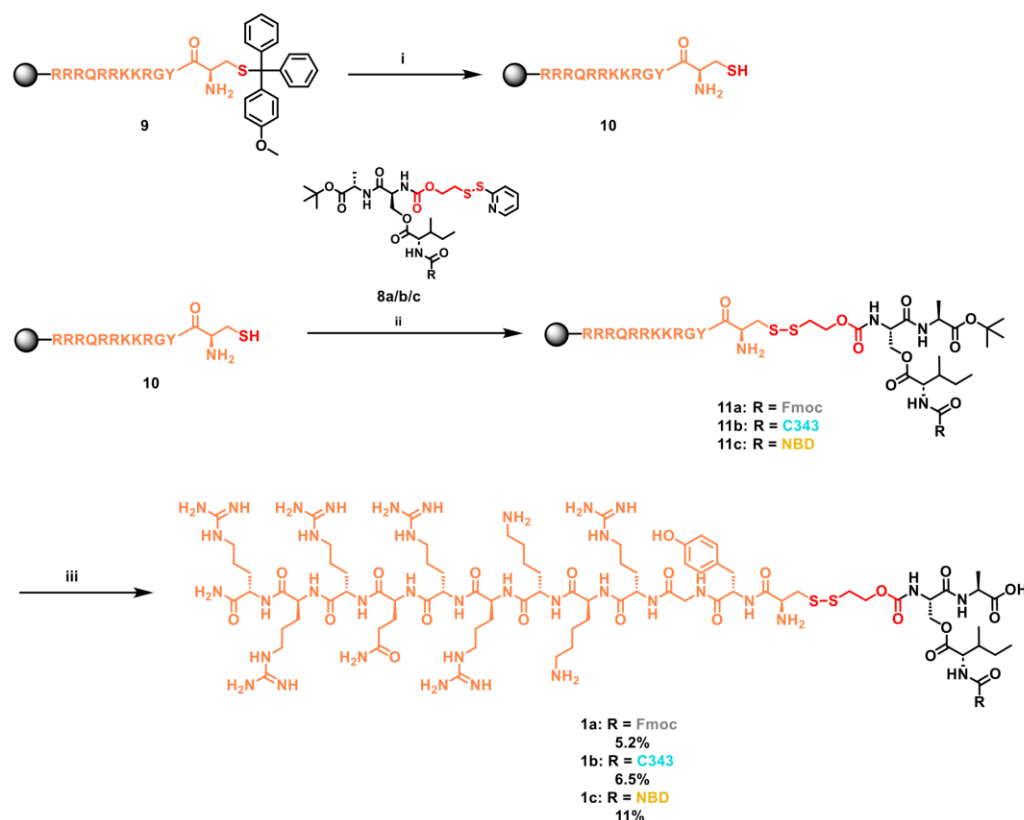


Figure S9 LC-MS data of compound **8c**. Left: LC trace (214 nm) with t_R (**8c**) = 7.89 min. Right: Convolved ESI-MS spectrum the showing peak of $[M+H]^+$ (calculated: 793.26).

2.1.2. Solid phase supported synthesis of TAT-modified glutathione-responsive isopeptides



Scheme S2 Synthesis of TAT-modified glutathione-responsive isopeptides. i) TFA, TIPS, DCM (2% : 5% : 93%, 5x8 min, RT); ii) **8a/b/c**, DMF, 2 h, RT; iii) TFA, TIPS, H₂O (95% : 2.5% : 2.5%), 2.5 h, RT.

The peptide TAT, with an additional N-terminal cysteine **9**, was synthesized via solid-phase peptide synthesis (SPPS) on a Rink amide resin at a 0.05 mmol scale. The resin was first swollen in DMF for 1 hour before starting the synthesis. Prior to each coupling step, as well as during the final deprotection, the N-terminal Fmoc group was removed using two deprotection steps: 20% piperidine in DMF (3 ml) was applied for 15 seconds at 75 °C, followed by 50 seconds at 90 °C. Fmoc-protected amino acids (Fmoc-Gln(Trt), Fmoc-Lys(Boc), Fmoc-Gly, Fmoc-Tyr(tBu), and Fmoc-Cys(Mmt)) were coupled in a 5-fold molar excess (5 eq. in 1.25 ml) for 15 seconds and 110 seconds at 75 °C and 90 °C, respectively, using a solution of DIC (0.25 M) and Oxyma (0.5 M) in 2.75 ml DMF. Fmoc-Arg(Pbf) was coupled using double coupling steps for better efficiency.

The acid-labile Mmt-protecting group on the cysteine side chain was removed by treating the resin with a mixture of 2% TFA and 5% TIPS in 93% DCM (5 ml each time). This mixture was added to the resin and shaken for 8 minutes under nitrogen before draining the solution. This process was repeated five times (step i). The resin was then thoroughly washed with DCM, and dry DMF (5 ml) was added

under nitrogen. Compound **8a** (82 mg, 0.11 mmol, 2.1 eq.), **8b** (56 mg, 0.068 mmol, 1.4 eq.), or **8c** (56 mg, 0.068 mmol, 1.4 eq.) was dissolved in dry DMF (5 ml) and added to the resin, with the mixture shaken for 2 hours at room temperature (step ii). The solution was drained, and the resin was washed with DMF and DCM. To cleave the peptide from the resin, a cleavage solution (5 ml of 95% TFA, 2.5% TIPS, 2.5% H₂O) was added, and the mixture was shaken for 2.5 hours at room temperature (step iii). The peptide solution was collected by filtering out the resin, and the resin was washed with 1 ml of TFA. The combined solutions were added dropwise to ice-cold diethyl ether to precipitate the product. After centrifugation at 4,000 rpm for 20 minutes at 0 °C, the supernatant was removed, and the pellet was dried.

The resulting products (**1a**, **1b** and **1c**) were each purified via HPLC using a Phenomenex Gemini column at a flow rate of 25 ml/min. The gradient began with 5% CH₃CN in H₂O (+0.1% TFA), held for 4 minutes, followed by an increase in CH₃CN to 50% over 31 minutes. Compound **1a** was eluted after 23.69 minutes and was obtained as a white powder after lyophilization (5.9 mg, 5.2% overall yield). Compound **1b** eluted after 23.87 minutes and was obtained as a yellow powder (7.6 mg, 6.5% overall yield). Compound **1c** eluted after 20.78 minutes and was obtained as a yellow powder (9.0 mg, 11% overall yield).

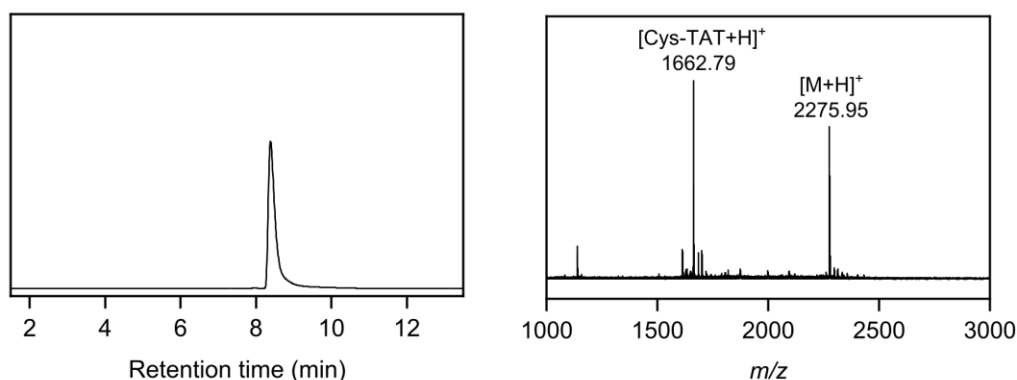


Figure S10 Analytical data of compound **1a**. Left: LC trace (214 nm) with t_R (**1a**) = 8.38 min. Right: MALDI TOF spectrum showing peaks of $[M+H]^+$ (calculated: 2276.70) and the product of disulfide cleavage $[Cys-TAT+H]^+$ (calculated: 1661.98).

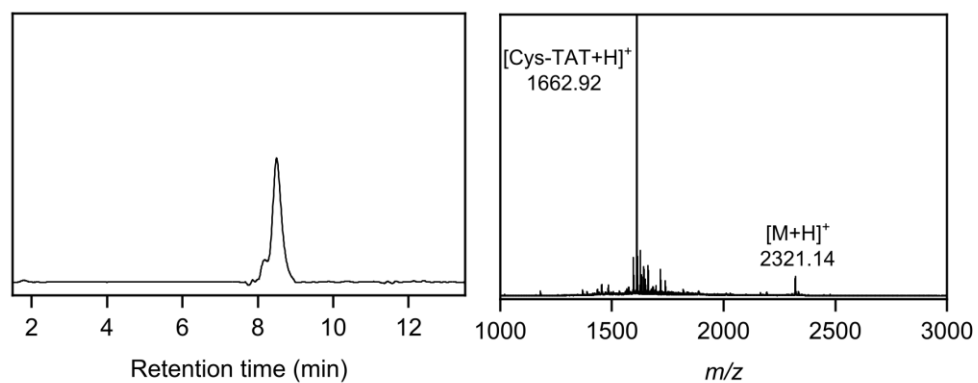


Figure S11 Analytical data of compound **1b**. Left: LC trace (214 nm) with t_R (**1b**) = 8.50 min. Right: MALDI TOF spectrum showing peaks of $[M+H]^+$ (calculated: 2321.74) and the product of disulfide cleavage $[Cys-TAT+H]^+$ (calculated: 1661.98).

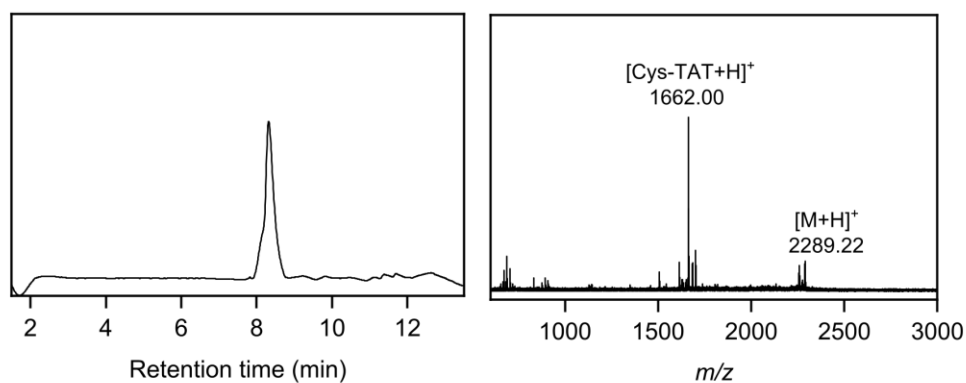
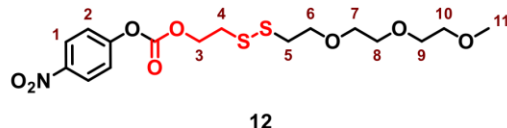


Figure S12 Analytical data of compound **1c**. Left: LC trace (214 nm) with t_R (**1c**) = 8.31 min. Right: MALDI TOF spectrum showing peaks of $[M+H]^+$ (calculated: 2288.63) and the product of disulfide cleavage $[Cys-TAT+H]^+$ (calculated: 1661.98).

2.2. Synthesis of control compounds

2.2.1. Synthesis of triethylene glycol-modified control isopeptides

2.2.1.1. Synthesis of compound **12**



In a round-bottom flask 4-nitrophenyl (2-(pyridin-2-yl)disulfaneyl)ethyl carbonate (287 mg, 815 μmol , 1.4 eq.) was dissolved in a mixture of EtOH:MeOH:AcOH (85:15:5 ml) under N_2 . 2-(2-(2-methoxyethoxy)ethoxy)ethane-1-thiol (106 mg, 588 μmol , 1.0 eq.) was dissolved in EtOH:AcOH (40:2 ml) under N_2 and the mixture was added dropwise to the solution of 4-nitrophenyl (2-(pyridin-2-yl)disulfaneyl)ethyl carbonate. The resulting reaction mixture was stirred for 24 h at room temperature. The solvents were removed in vacuo and subsequent flash column chromatography (cHex:EA 2:1) yielded compound **12** as a colorless oil (221 mg, 524 μmol , 89%).

^1H NMR (300 MHz, CDCl_3) δ 8.29 (d, $J = 9.2$ Hz, 2H), 7.39 (d, $J = 9.2$ Hz, 1H), 4.55 (t, $J = 6.5$ Hz, 2H), 3.75 (t, $J = 6.5$ Hz, 2H), 3.66 – 3.53 (m, 9H), 3.38 (s, 3H), 3.03 (t, $J = 6.6$ Hz, 2H), 2.94 (t, $J = 6.5$ Hz, 2H).

^{13}C NMR (176 MHz, CDCl_3) δ 155.56, 152.46, 145.58, 125.49, 121.94, 121.91, 70.72, 70.53, 69.77, 69.58, 67.05, 66.87, 59.20, 38.74, 38.50, 36.87, 36.70.

APCI-MS: m/z 439.1 (calculated for $[\text{M}+\text{NH}_4]^+$: 439.12).

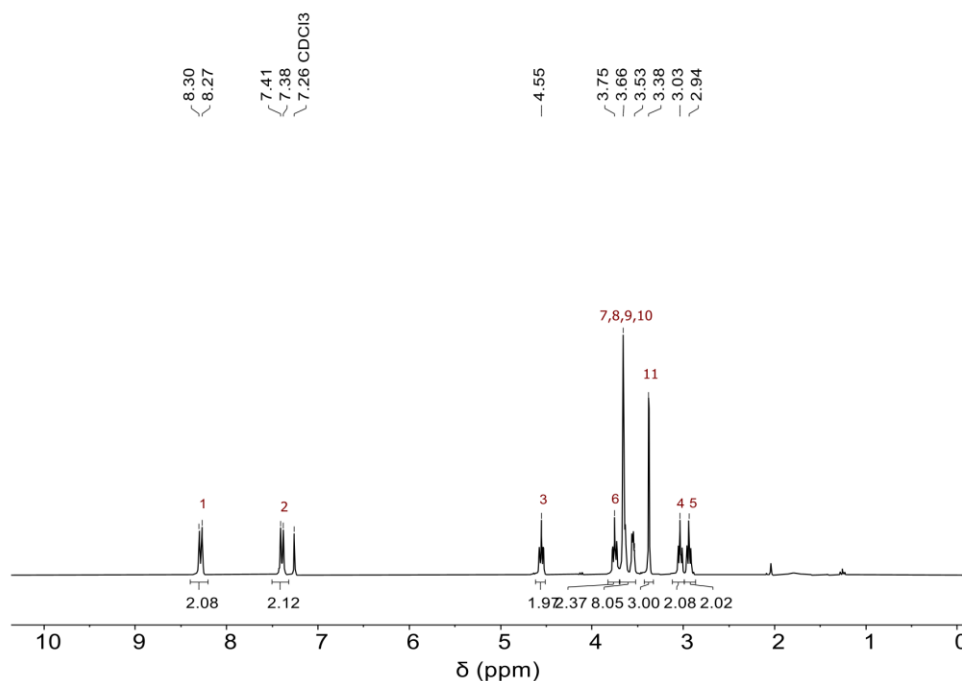


Figure S13 ^1H NMR spectrum (300 MHz, CDCl_3 , 298 K) of compound **12**.

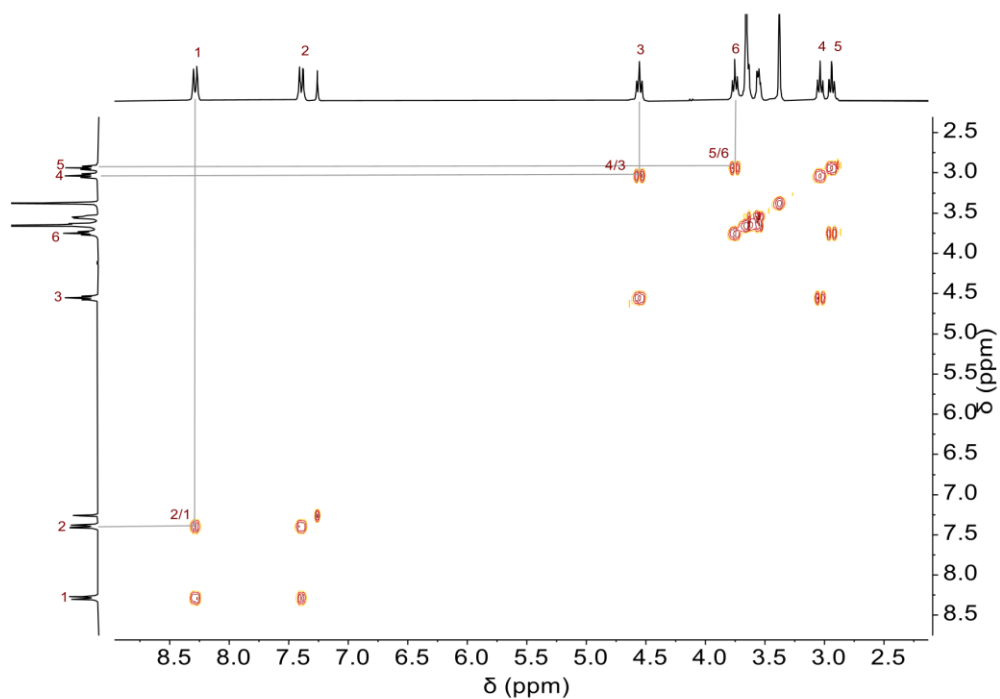


Figure S14 $^1\text{H},^1\text{H}$ COSY NMR spectrum (300 MHz, CDCl_3 , 298 K) of compound **12**.

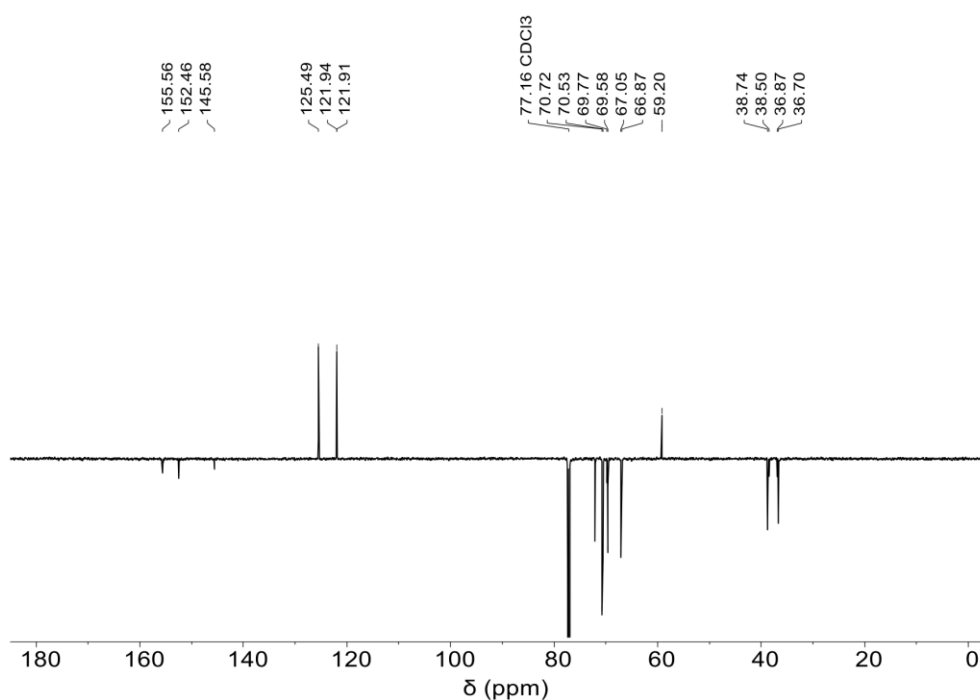
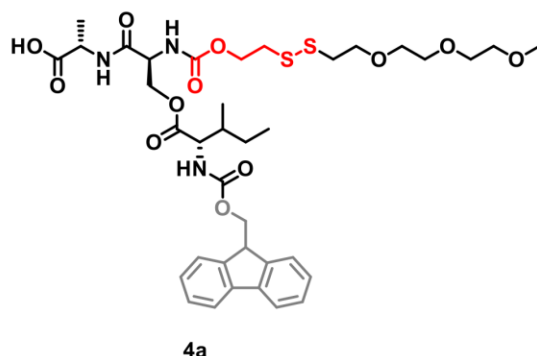


Figure S15 ^{13}C NMR spectrum (176 MHz, CDCl_3 , 298 K) of compound **12**.

2.2.1.2. Synthesis of compound **4a**



The isopeptide **4a** was synthesized via solid phase supported peptide synthesis using Fmoc-Ala Wang resin at a scale of 0.5 mmol. The resin was swollen in DMF for 1 h before the synthesis. Before the coupling step and as the final deprotection, the *N*-terminal Fmoc group was cleaved by two deprotection steps using 20% piperidine in DMF (10 ml) for 25 s and 65 s at 70 °C and 90 °C respectively. Fmoc-serine without side chain protecting group (4 eq. in 10 ml) was coupled for 30 s and 120 s at 70 °C and 90 °C respectively using DIC (0.5 M) and Oxyma (1 M) in a total of 16 ml DMF. Afterwards, the resin was washed with DCM and DMF and suspended in dry DMF (5 ml) under N₂. To the suspension dry DIPEA (323 mg, 0.44 ml, 2.5 mmol, 5 eq.) was added. A solution of compound **12** (289 mg, 686 μmol, 1.4 eq.) in dry DMF (5 ml) was added dropwise to the suspended resin. The suspension was shaken at room temperature overnight and subsequently washed repeatedly with DMF. In a second step, the resin was suspended again in dry DMF (3 ml) and Fmoc-Ile (530 mg, 1.5 mmol, 3 eq.), DMAP (61 mg, 0.5 mmol, 1 eq.) and DIC (189 mg, 0.22 ml, 1.5 mmol, 3 eq.) in dry DMF (5 ml) was added dropwise. After two hours the solution was drained and the same reactants in dry DMF (5 ml) were added again. After shaking at room temperature overnight, the solution was drained and the resin was washed repeatedly with DMF and DCM. Afterwards, the cleavage solution (5 ml of 95% TFA, 2.5% TIPS, 2.5% H₂O) was added and the mixture was shaken for 2 h at room temperature. The peptide solution was collected by filtering out the resin and the resin was additionally washed with TFA (1 ml). The solvent was removed in vacuo and flash chromatography (EA:cHex 3:1 + 1% formic acid) yielded compound **4a** as a colorless solid (83 mg, 524 μmol, 21%).

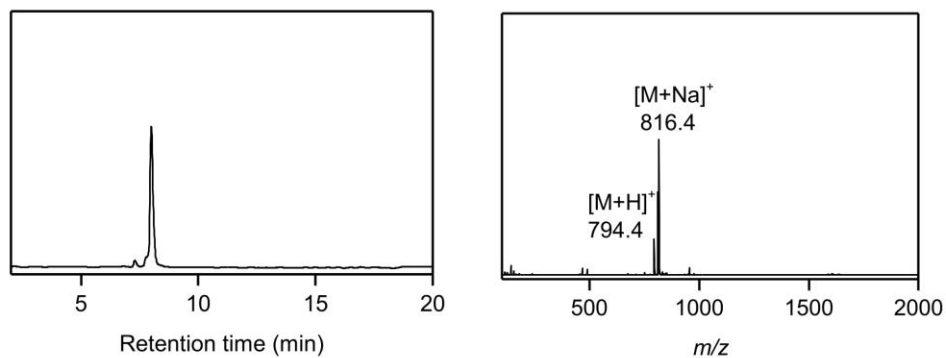
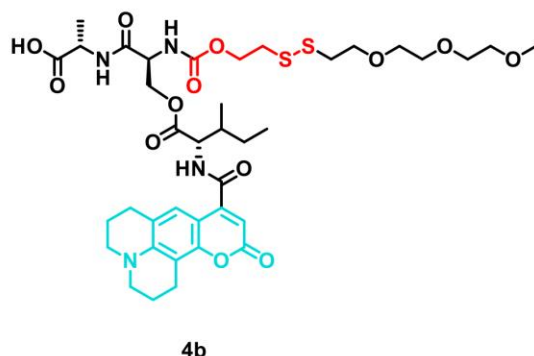


Figure S16 LC-MS data of compound **4a**. Left: LC trace (214 nm) with t_R (**4a**) = 7.99 min. Right: Convolved ESI-MS spectrum showing peaks of $[M+H]^+$ (calculated: 794.30) and $[M+Na]^+$ (calculated: 816.28).

2.2.1.3. Synthesis of compound **4b**



In a round-bottom flask compound **8b** (55 mg, 67 μmol , 1 eq.) was dissolved in a 1:1-mixture of TFA and DCM (4 ml:4ml) and stirred for 4 h. The solvents were removed in vacuo. The remaining bright green oil was dissolved in EtOH:AcOH (1:20, 10 ml) under N_2 . Afterwards, 2-(2-(2-methoxyethoxy)ethoxy)ethane-1-thiol (46 mg, 255 μmol , 3.8 eq.) was dissolved in EtOH:AcOH (5:2, 3.5 ml) under N_2 and the mixture was added dropwise to the solution of **8b**. The resulting reaction mixture was stirred for 24 h at room temperature. The solvents were removed in vacuo and subsequent flash column chromatography (DCM: MeOH 10:1) yielded compound **4b** as a bright green solid (27 mg, 524 μmol , 47%).

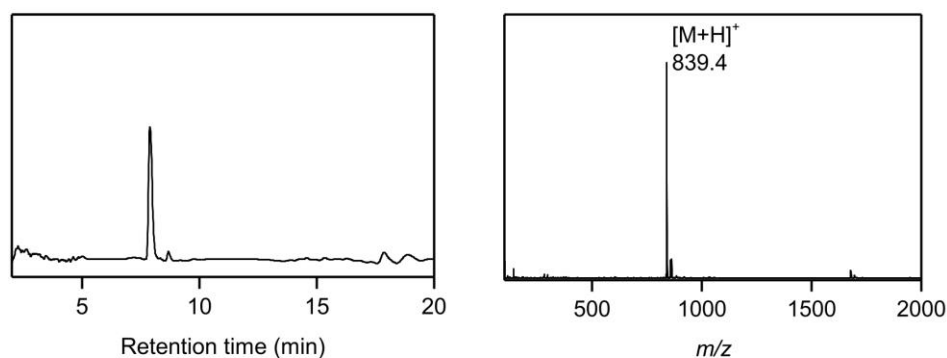


Figure S17 LC-MS data of compound **4b**. Left: LC trace (214 nm) with t_R (**4b**) = 8.68 min. Right: Convolved ESI-MS spectrum showing peaks of $[\text{M}+\text{H}]^+$ (calculated: 839.32).

2.2.2. Analytical data of linear peptides

2.2.2.1. Analysis of compound **3a**

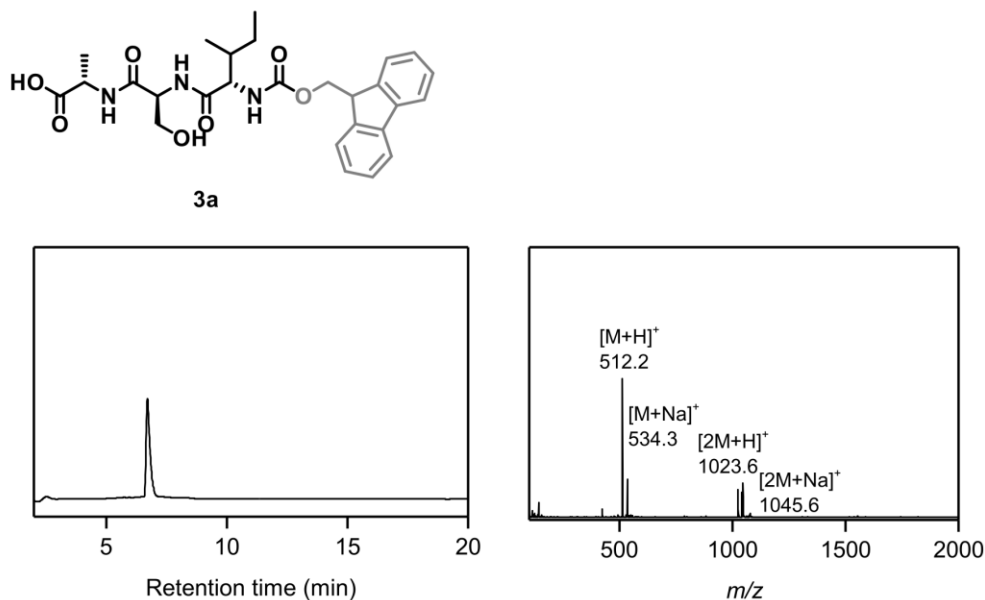


Figure S18 LC-MS data of compound **3a**. Left: LC trace (214 nm) with t_r (**3a**) = 6.71 min. Right: Convolved ESI-MS spectrum the showing peaks of $[M+H]^+$ (calculated: 512.24), $[M+Na]^+$ (calculated: 534.22), $[2M+H]^+$ (calculated: 1023.47) and $[2M+Na]^+$ (calculated: 1045.45).

2.2.2.2. Analysis of compound **3b**

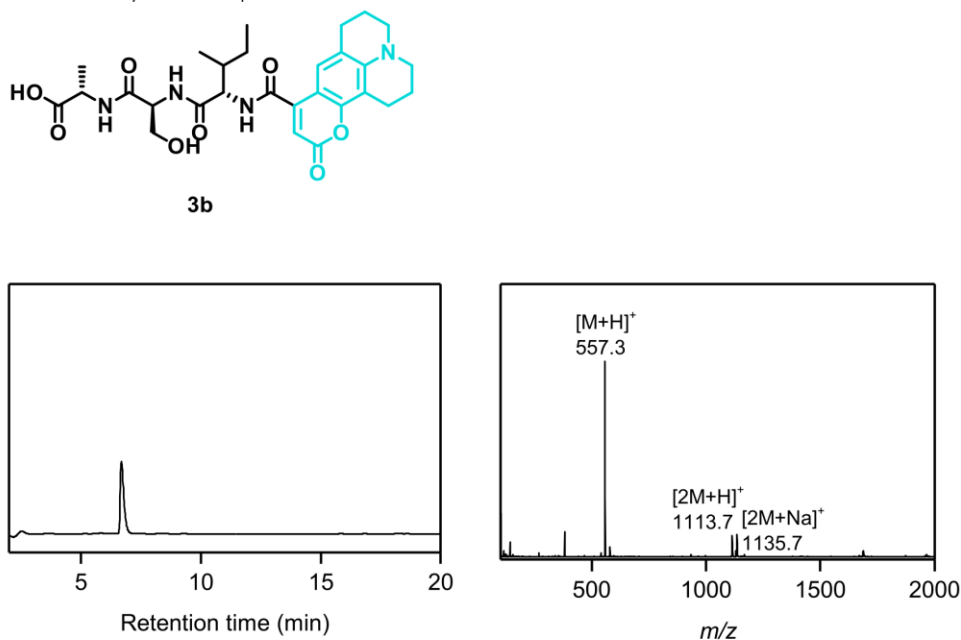


Figure S19 LC-MS data of compound **3b**. Left: LC trace (214 nm) with t_r (**3b**) = 6.68 min. Right: Convolved ESI-MS spectrum the showing peaks of $[M+H]^+$ (calculated: 557.26), $[2M+H]^+$ (calculated: 1113.51) and $[2M+Na]^+$ (calculated: 1135.50).

2.2.2.2. Analysis of compound **3c**

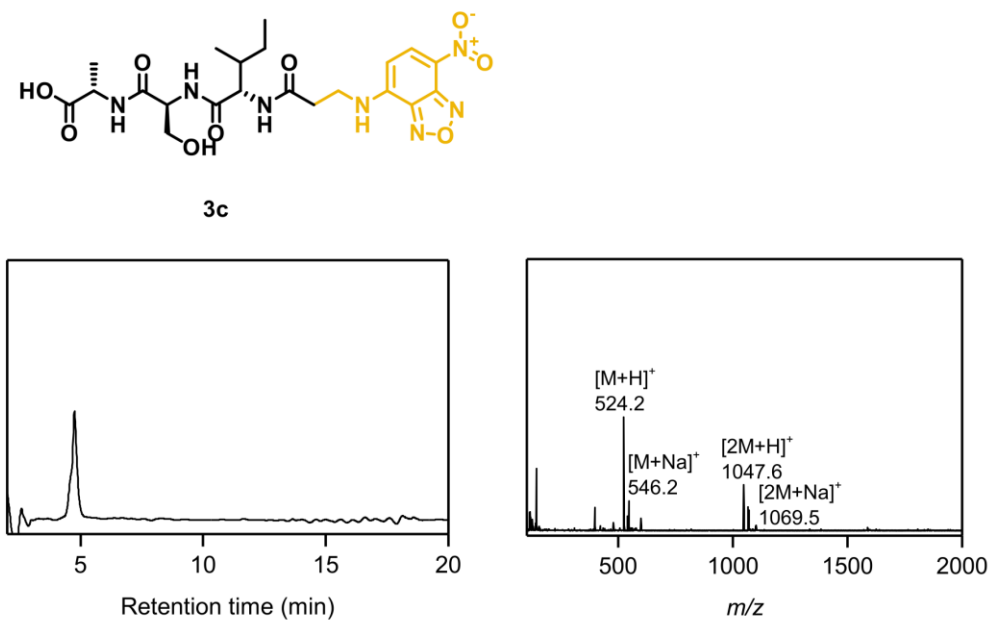


Figure S20 LC-MS data of compound **3c**. Left: LC trace (214 nm) with t_r (**3c**) = 4.75 min. Right: Convoluted ESI-MS spectrum the showing peaks of [M+H]⁺ (calculated: 524.21), [M+Na]⁺ (calculated: 546.19), [2M+H]⁺ (calculated: 1047.41) and [2M+Na]⁺ (calculated: 1069.39).

3. Analysis of secondary structure and assembly behavior

3.1. TEM analysis

The linear peptides **3a**, **3b** and **3c** were each dissolved in DMSO to achieve stock solution with 10 mM or 50 mM concentration. The respective DMSO stock solutions were mixed at a 5:1 ratio of Fmoc-ISA **3a** and Coumarin 343-ISA **3b** yielding the stock solutions of the mixed linear peptides. The same was done for the kinked assembly precursors **1a** and **1b**. The DMSO stock solutions of the mixed linear peptides **3a** and **3b** and the mixed isopeptides **1a** and **1b** were diluted with DPBS at a 1:99 ratio to yield 500 μM or 100 μM peptide solutions. The same was done for the stock solutions of the linear NBD-ISA **3c** and the control isopeptide **1c**. The TEM samples were prepared as described in section 1.2.5.

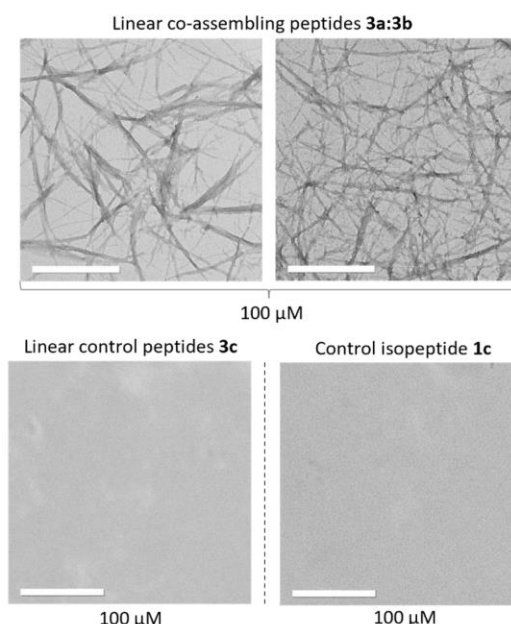


Figure S21 TEM images of nanofibers formed by co-assembling peptides **3a** and **3b** (5:1 ratio at 100 μM) in DPBS (pH 7.4) and DMSO (99:1). TEM images of linear control peptide **3c** (100 μM) and TEM images of control isopeptide **1c** (100 μM) in DPBS (pH 7.4) and DMSO (99:1). Scale bar 500 nm.

We conducted additional TEM analyses across a range of **3a:3b** ratios (50:1, 9:1, and 2:1) to assess the impact on assembly propensity and structural morphology. At a total peptide concentration of 500 μM , fibrillar nanostructures were observed for all tested ratios. However, at 100 μM , the 2:1 ratio of **3a:3b** resulted in shorter, less defined, and more irregular structures, whereas the 50:1 and 9:1 ratios maintained well-organized assemblies (Fig. S22). This difference likely stems from the higher proportion of Coumarin343-labeled peptide **3b** in the 2:1 ratio, which is less conducive to ordered structure formation compared to the non-labeled Fmoc-ISA **3a**.

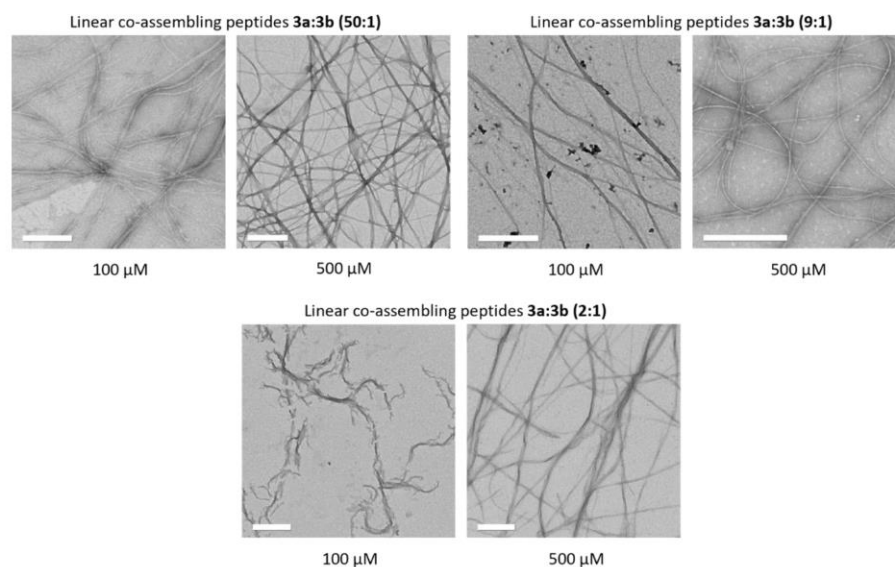


Figure S22 TEM images of nanofibers formed by co-assembling peptides **3a** and **3b** at different ratios (50:1, 9:1, 2:1) at concentrations of 100 and 500 μM in DPBS (pH 7.4) and DMSO (99:1). Scale bar 500 nm.

3.2. Proteostat assay for the determination of the critical aggregation concentration

For the analysis of the critical aggregation concentration of the linear peptides **3a:3b** (5:1) the commercial Proteostat protein aggregation assay kit by *Enzo Life Sciences, Inc.* was used as previously shown in a study by our group.¹ The peptide samples were prepared by diluting DMSO stock solutions of the linear peptides **3a:3b** (5:1) in various concentrations (1 to 100 μM) with DPBS in a 1:99 ratio. The resulting peptide samples were incubated for 24 h at room temperature while shaking. The Proteostat working solution was prepared by mixing 0.52 μL of the Proteostat stock solution with 98.48 μL MilliQ water and 1 μL assay buffer. After the incubation, 27 μL of each peptide solution were mixed with 3 μL of the Proteostat working solution. 9 μL of each solution were pipetted into a well of a Greiner 384 flat black well plate (3 wells per sample of each peptide concentration). The plate was incubated in the dark for 15 min while shaking. The fluorescence intensity of the Proteostat dye was subsequently measured with an excitation and emission bandwidth of 20 nm and an emission wavelength of 600 nm after excitation at 550 nm.

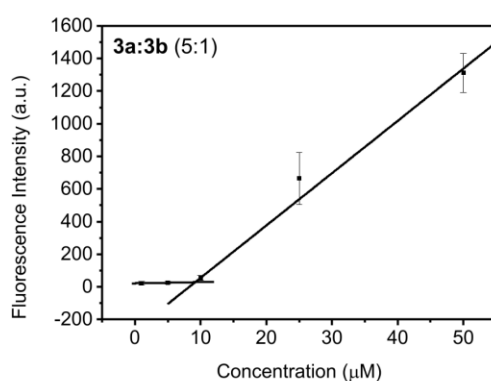


Figure S23 Proteostat aggregation assay of the mixed linear peptides Fmoc-ISA **3a** and C343-ISA **3b** (5:1) in DPBS:DMSO (99:1). The samples were incubated at room temperature for 24 h before the analysis. By calculating the intersection of the linear fits, the critical aggregation concentration of the mixed linear peptides **3a:3b** was determined to be 9.2 μM .

3.3. Stability analysis of isopeptide 1b in cell culture medium

To evaluate the stability of isopeptide **1b** in cell culture medium, **1b** was dissolved in DMEM (1% DMSO) at a concentration of 100 μM , with Fmoc-tryptophan included as an internal standard at 25 μM . The samples were incubated, and at different time points, aliquots of the mixture were collected, centrifuged (13,000 rpm, 10 min) and the supernatant was injected into an analytical HPLC system to monitor the stability of **1b** in DMEM. The results indicate no degradation of **1b** during incubation in the cell culture medium.

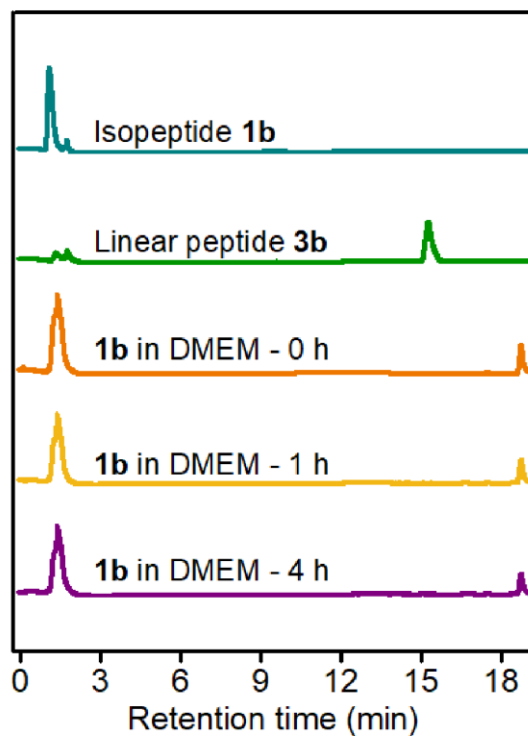


Figure S24 Stability analysis of isopeptide 1b (100 μ M) in DMEM cell culture medium (1% DMSO) over time. Representative elugrams of isopeptide **1b** (t_R = 1.43 min) and its degradation product, linear peptide **3b** (t_R = 15.32 min), are shown for comparison. Fmoc-tryptophan (25 μ M, t_R = 18.78 min) was used as an internal standard.

4. Cell Experiments in 2D cell culture

4.1. Cell uptake analysis

MDA-MB-231 cells were seeded at a density of 20,000 cells/well in an 8-well confocal plate. After adhering for 24 h, cells were treated with the samples containing either **1a:1b** or the control compounds **1c**, **3a:3b** or **4a:4b** at different concentrations and incubated for 4 h at 37 °C. The sample solutions were prepared by dissolving the respective compounds in DMSO to prepare different stock solutions (100x) containing **1a:1b**, **3a:3b** or **4a:4b** in a 5:1 molar ratio or **1c**. These stocks were each diluted with DPBS in a 9:1 ratio yielding the DPBS:DMSO stocks (10x). Another dilution with DMEM (9:1) gave the final samples (total DMSO content: 1%) which were added to the cells after removing the old medium. After the incubation time of 4 h was over, the cells were fixed for 15 min at room temperature using 4% paraformaldehyde solution. The nucleus was stained with HCS Nuclear Mask Deep Red Stain for 20 min at room temperature. The staining solution was removed, and fresh DPBS was added to the cells before they were imaged by confocal laser scanning microscopy. Confocal images of cells were taken on a Leica Stellaris® 8 microscope.

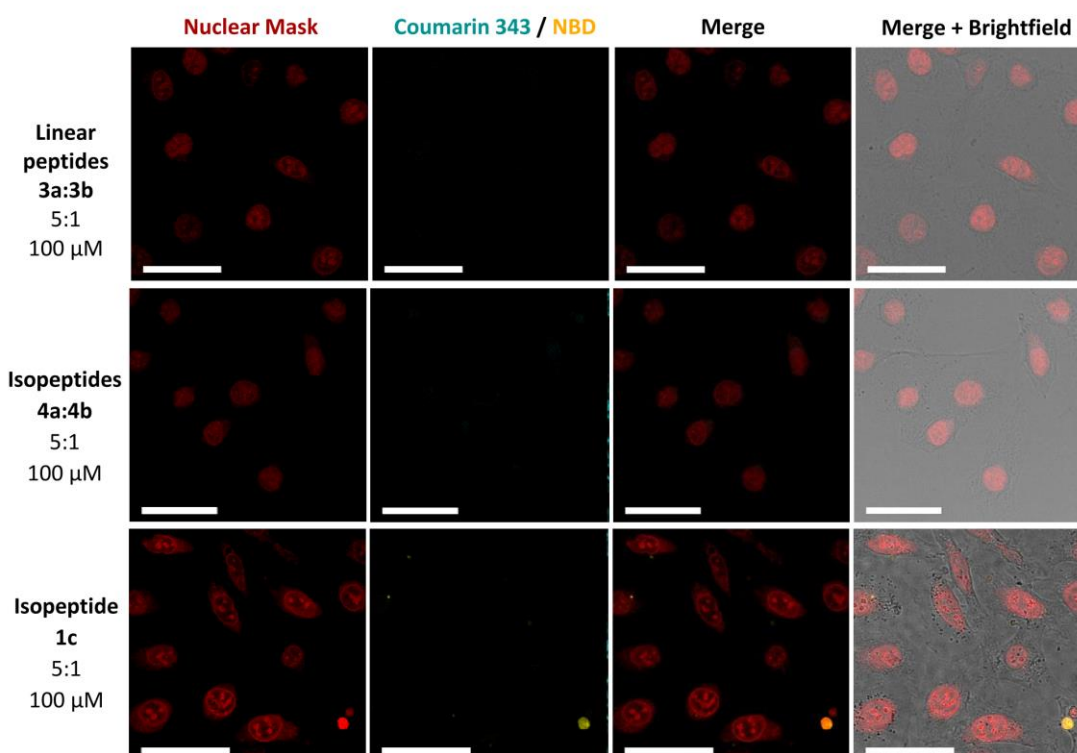


Figure S25 Cell uptake in cancer cells. Confocal laser scanning microscopy (CLSM) images of MDA-MB-231 breast cancer cells after 4 h of incubation with control compounds **3a:3b**, **4a:4b** (5:1) or **1c** at 100 μM. The nucleus was stained with HCS Nuclear Mask Deep Red (red). Scale bars 50 μm.

4.2. Live-cell imaging

MDA-MB-231 cells were cultured in an 8-well confocal plate at a density of 20,000 cells per well in DMEM and allowed to adhere overnight at 37°C, 5% CO₂. The medium was removed and the sample of **1a:1b** in DMEM/DPBS/DMSO (90%/9%/1%) was introduced into a well. The cells were imaged live using an incubator-equipped Leica Stellaris® 8 microscope (40x glycerol immersion objective) with fast lifetime contrast (FALCON) module (Leica Microsystems GmbH). The incubator (okolab) is set and held constant at 37 °C, 5% CO₂ and a relative humidity of 90% throughout all measurements.

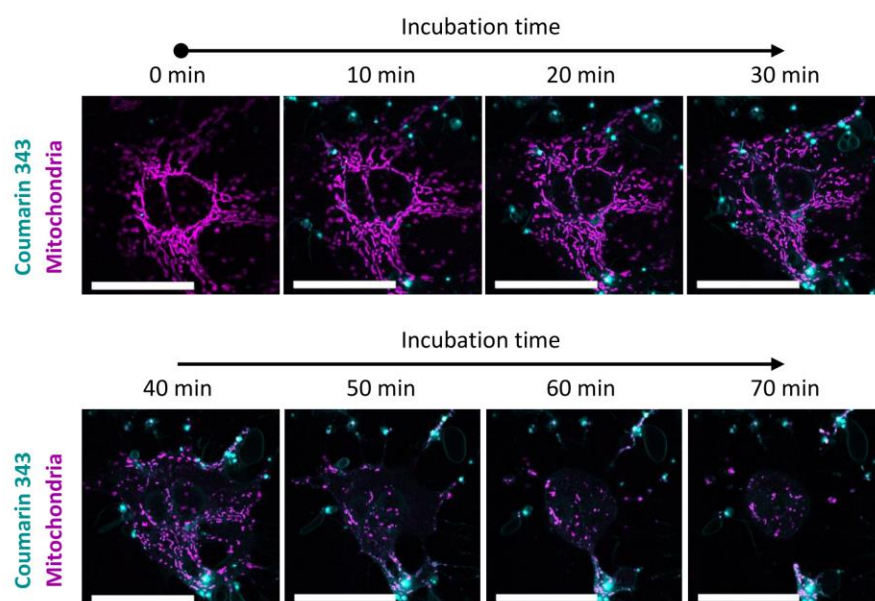


Figure S26 Live cell imaging of mitochondria-stained cancer cells. CLSM images of live-cell imaging of MDA-MB-231 cells treated with 50 μM of **1a:1b** (cyan). Cells were previously transfected with CellLight™ Mitochondria-RFP (pink). Scale bars 50 μm.

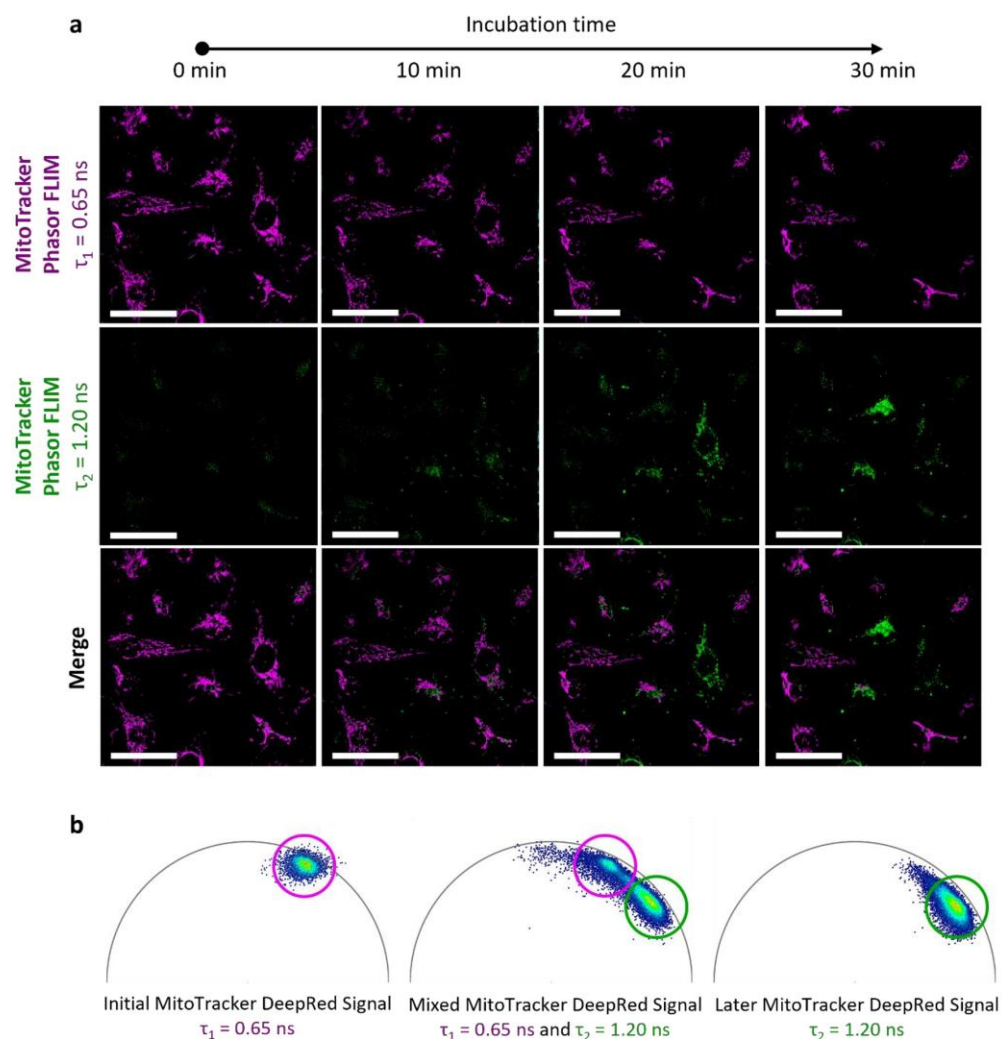


Figure S27 Phasor plots of MitoTracker DeepRed FLIM measurements. Live-cell imaging of MDA-MB-231 cells treated with 50 μ M of **1a:1b**. Cells were previously stained with MitoTracker DeepRed. Fluorescence lifetime measurements of the stain were conducted concurrently with fluorescence imaging, revealing two distinct fluorescence lifetime signals for MitoTracker DeepRed over time. Scale bars 50 μ m.

4.3 Correlative light- and electron microscopy (CLEM) of cells

For electron microscopy (EM) studies correlated with confocal light microscopy, MDA-MB-231 cells were seeded at a density of 20,000 cells per well on sapphire disks and incubated with 100 μ M of **1a:1b** for 4 h. After incubation, the cells on the sapphire disks were frozen using high-pressure freezing (Wohlwendt HPF 01). Freeze substitution followed, with the cells stained at low temperatures using OsO_4 and uranyl acetate for EM examination. The cells were then embedded in epoxy resin (EPON), and ultrathin sections were prepared with an ultramicrotome (Leica FC7) using a diamond knife (Diatome). The section thickness and substrate were adjusted for subsequent EM investigation: for TEM, sections (100 nm thick) were transferred to Cu grids; Fluorescence of the co-assembling peptides was first measured in a confocal laser scanning microscope (Leica SP5) (458nm excitation, 470-500 nm emission detection bandwidth). The same thin section was then examined in TEM (FEI Tecnai F20). To identify the fluorescent peptide assemblies, we correlated fluorescence with EM images by superimposing the images using the “Landmark correspondence” plugin in ImageJ 1.53e.

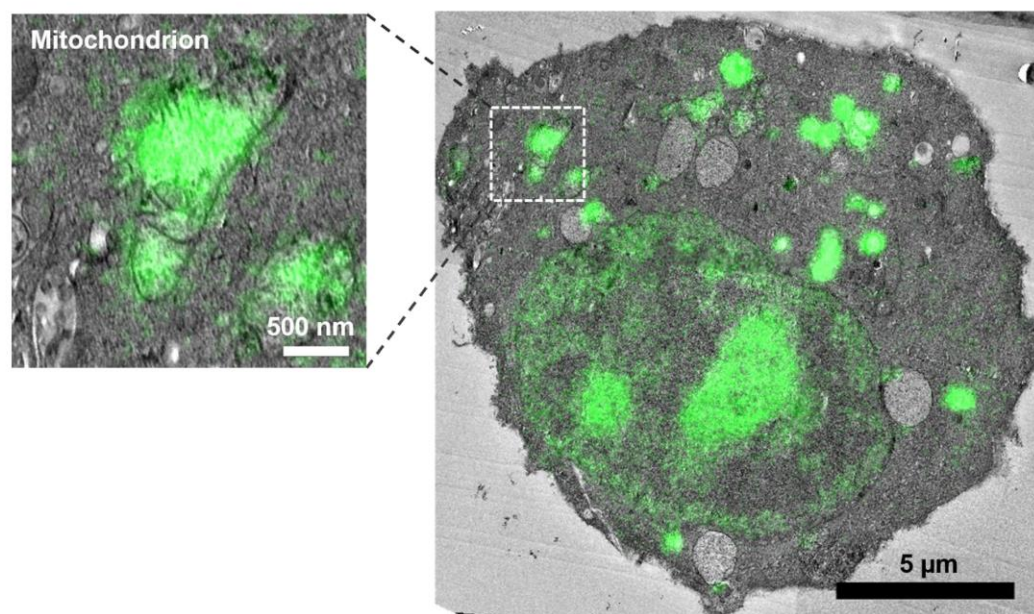


Figure S28 Correlative light- and electron microscopy (CLEM) image of MDA-MB-231 cell incubated with **1a:1b** (100 μ M) (green) for 4 h.

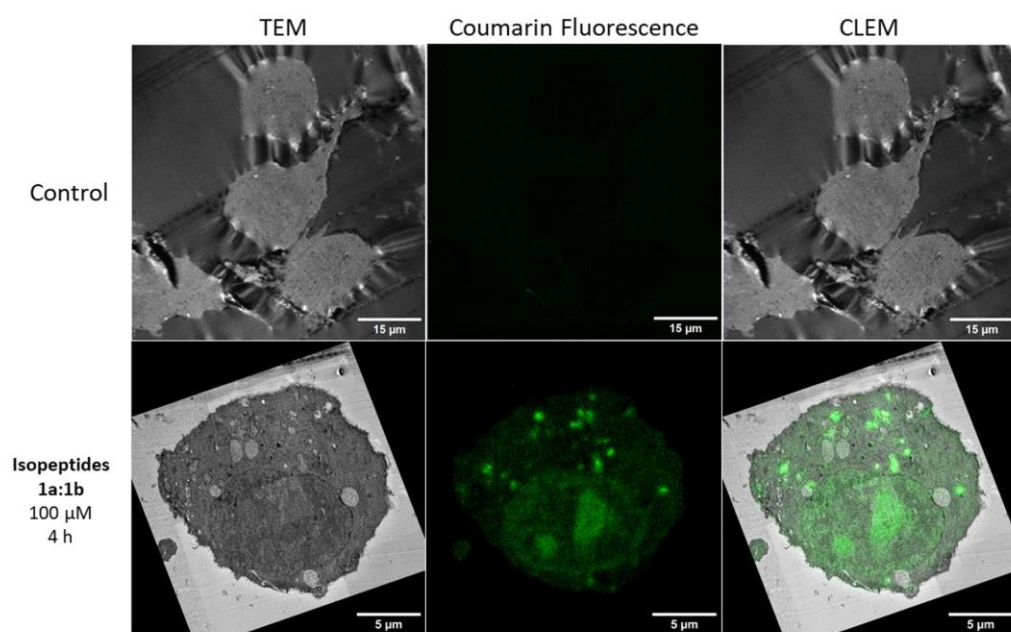


Figure S29 Correlative light- and electron microscopy (CLEM) image of an untreated MDA-MB-231 cell as control (first row) compared to a cell incubated with **1a:1b** (100 μM) for 4 h (second row).

4.4. Co-staining of actin filaments

For the co-staining of actin filaments MDA-MB-231 cells were seeded at a density of 20,000 cells/well in an 8-well confocal plate. After adhering for 24 h, cells were treated with the samples containing **1a:1b** at different concentrations and incubated for 4 h at 37 °C. The sample preparation is described in section 3.1. After the incubation the medium was removed and the cells were washed with DPBS and fixed for 15 min at room temperature using 4% paraformaldehyde solution. After rinsing with DPBS three times, the actin filament stain Alexa Fluor™ 555 Phalloidin was added to the cells (diluted 1:20 in DPBS) and incubated for 15 min at room temperature. Afterwards the cells were washed once with DPBS and fresh DPBS was added before imaging.

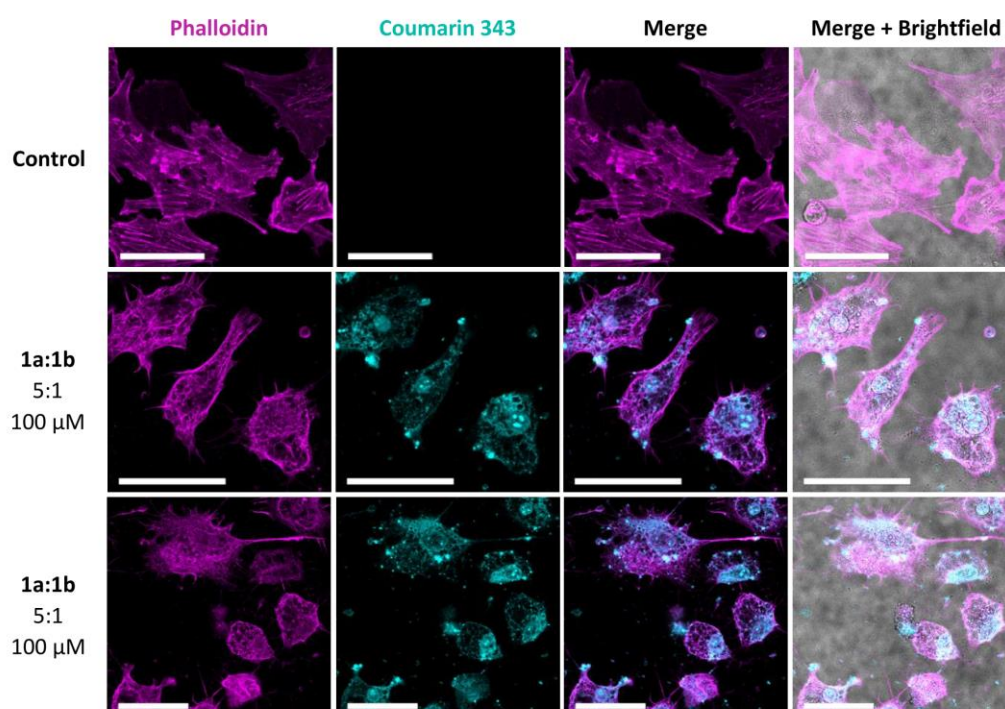


Figure S30 Disruption of actin filaments by in-situ formed nanostructures. Confocal laser scanning microscopy (CLSM) images of MDA-MB-231 cells after 4 h of incubation with glutathione-responsive isopeptides **1a** and **1b** (5:1) (cyan) and additional staining with Alexa Fluor™ 555 Phalloidin (pink). Scale bars 50 μm.

To assess the time-dependent effects on cytoskeletal integrity, we also performed phalloidin staining on cells treated with the assembly-inducing isopeptides **1a:1b** (at 100 and 10 μM) and the linear peptides **3a:3b** (at 100 μM) as a control. Imaging at 1, 2, 4, and 24 hours revealed that structural disruption of the cytoskeleton occurs rapidly, with pronounced effects already evident within 1 hour of incubation (Fig. S29-S31). The extent of cytoskeletal damage remained consistent at later time points, indicating that the effect is an early and sustained response to peptide treatment.

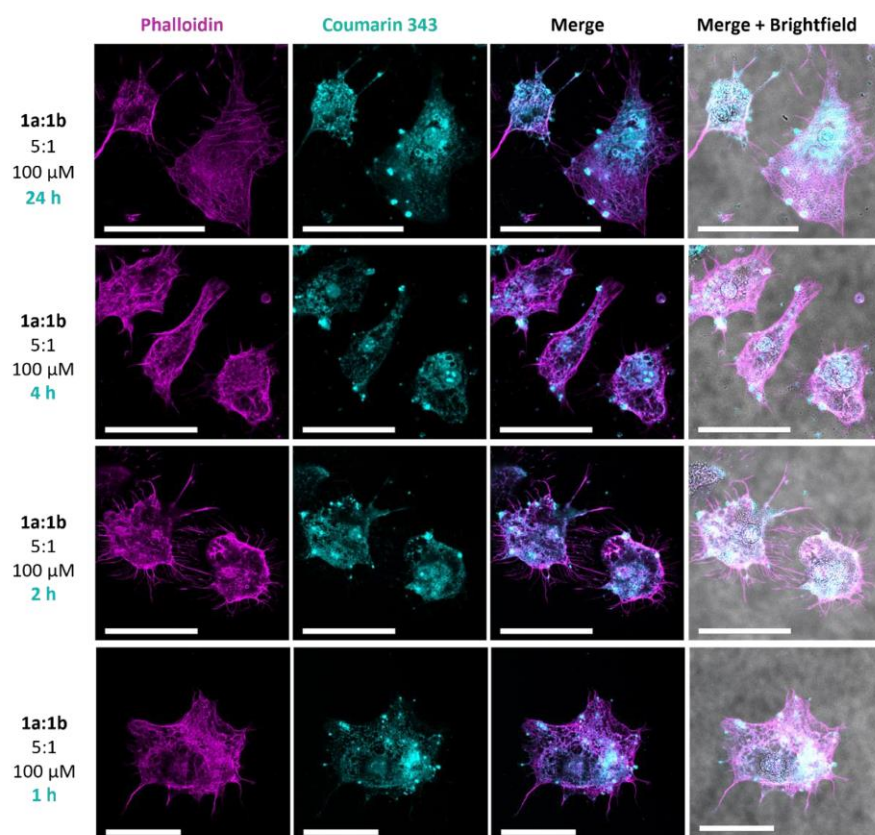


Figure S31 Confocal laser scanning microscopy (CLSM) images of MDA-MB-231 cells after 1, 2, 4 and 24 h of incubation with glutathione-responsive isopeptides **1a** and **1b** (5:1) (cyan) at 100 μM and additional staining with Alexa Fluor™ 555 Phalloidin (pink). Scale bars 50 μm.

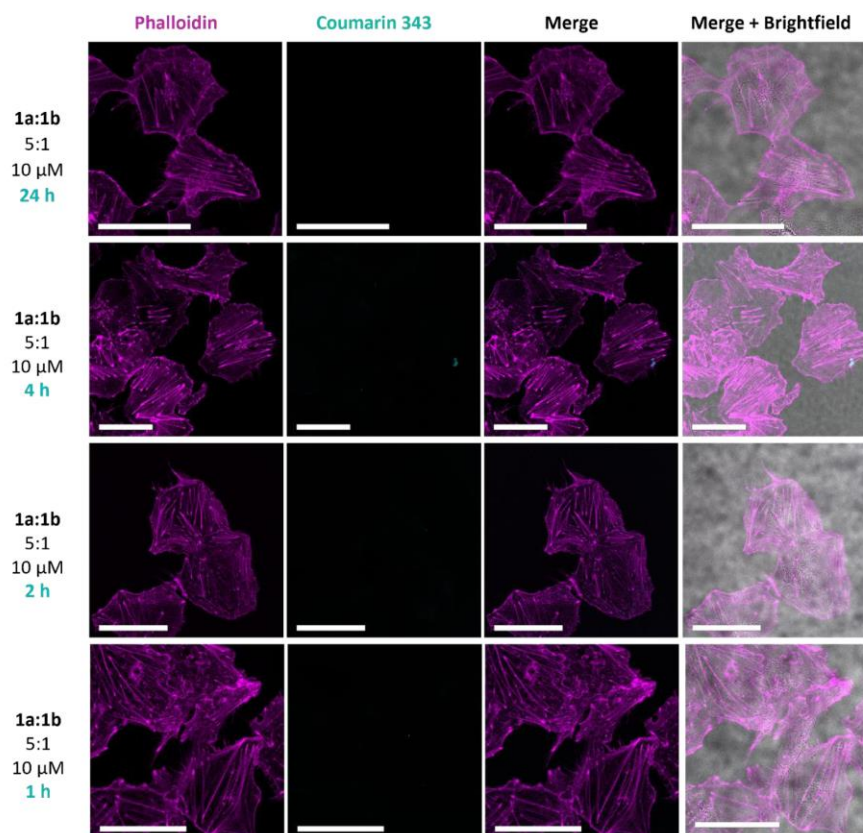


Figure S32 Confocal laser scanning microscopy (CLSM) images of MDA-MB-231 cells after 1, 2, 4 and 24 h of incubation with glutathione-responsive isopeptides **1a** and **1b** (5:1) (cyan) at 10 μM and additional staining with Alexa Fluor™ 555 Phalloidin (pink). Scale bars 50 μm.

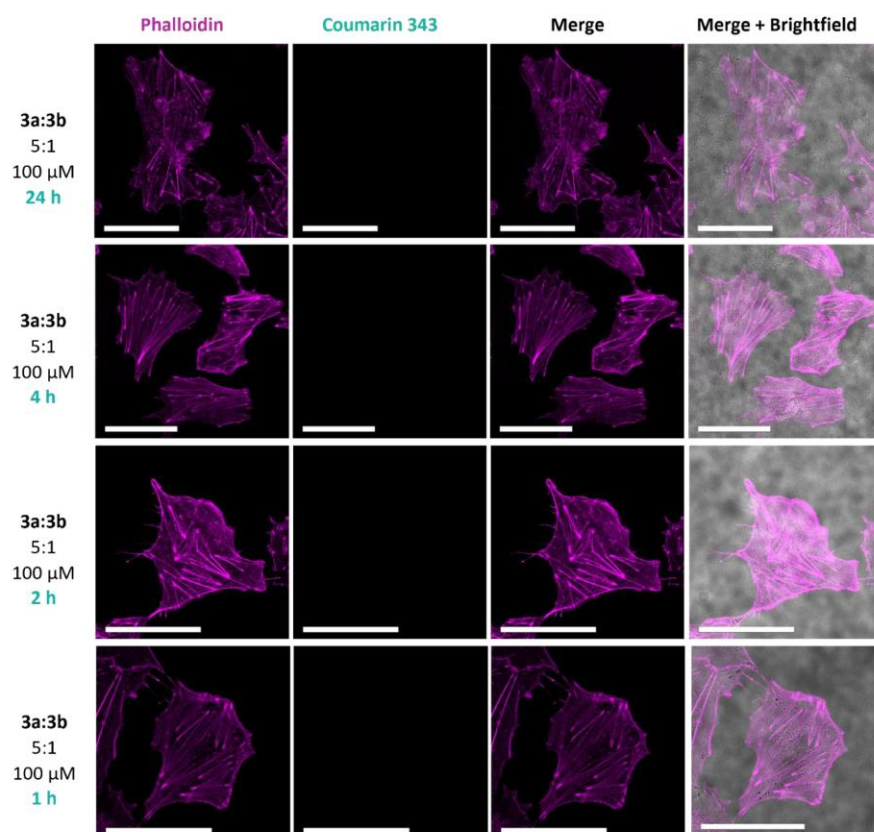


Figure S33 Confocal laser scanning microscopy (CLSM) images of MDA-MB-231 cells after 1, 2, 4 and 24 h of incubation with linear peptides **3a** and **3b** (5:1) (cyan) at 100 μ M and additional staining with Alexa Fluor™ 555 Phalloidin (pink). Scale bars 50 μ m.

4.5. Analysis of G-/F-actin ratio

MDA-MB-231 cells were seeded at a density of 200,000 cells/well in a 12-well plate and incubated at 37°C overnight to allow them to adhere. On the next day cells were incubated with the *isopeptide* **1a:1b** (5:1) and the linear peptide **3a:3b** (5:1) at 50 μ M each for 2 h. Two wells were used as controls, where the old medium was substituted with fresh DMEM/1% DMSO. After the incubation time the G-actin /F-actin assay was performed as described in the manufacturer's protocol. Briefly, cells were lysed by adding 125 μ L warm LAS02 buffer to each well (Lysis and F-actin Stabilization Buffer) and harvested using a cell scraper. Samples were homogenized using a Dounce homogenizer before incubating them at 37°C for 10 minutes. Prior to the incubation one of the controls was used for a positive control by adding 1.25 μ L of a 100x F-actin Enhancing Solution, which is included in the assay kit, to the cell lysate. After the incubation the samples were centrifuged at 350 x g for 5 minutes to remove cell debris and the supernatant was transferred into ultracentrifugation tubes. To separate F-actin and G-actin the samples were centrifuged at 100,000 x g for 1 h at 37°C. After removing the supernatant and collecting it in fresh tubes, the F-actin in the pellet were depolymerized by adding 100 μ L F-actin depolymerization buffer and incubate the samples on ice for 1 h. After incubation 25 μ L of a 5x SDS sample buffer were added and samples were stored at -20°C overnight.

On the next day SDS-PAGE was performed using a 4–20% Mini-PROTEAN® TGX™ Precast Protein Gel loading 10 μ L sample per well. In addition to the samples, 4 μ L Protein Marker VI (10 - 245) as well as G-actin standards corresponding to 10, 20 and 50 ng G-actin, were loaded. The gel was run at constant

voltage of 125 V for 90 minutes. After running the gel the proteins were transferred to a PVDF-membrane using methanol free Pierce Western Blot Transfer Buffer at a constant current of 350 mA for 60 minutes. The membrane was washed in TBST buffer (20 mM Tris, 150 mM NaCl, 0.1 % (w/v) Tween 20) before blocking with TBST/ 5% non-fat milk powder at 4°C overnight. The next day, the membrane was washed 3x with TBST before adding the primary antibody anti-actin mouse monoclonal antibody and incubating for 1 h at room temperature. After washing 3x with TBST the secondary antibody Rabbit Anti-Mouse IgG H&L (HRP) was added and the membrane was incubated again for 1 h. After washing 5x with TBST the G-actin on the membrane was visualized using HRP substrate and imaged using BioRad ChemiDoc Imaging System. For calculations of the G-/F-actin ratio the intensities of the G-actin bands were calculated using ImageJ.

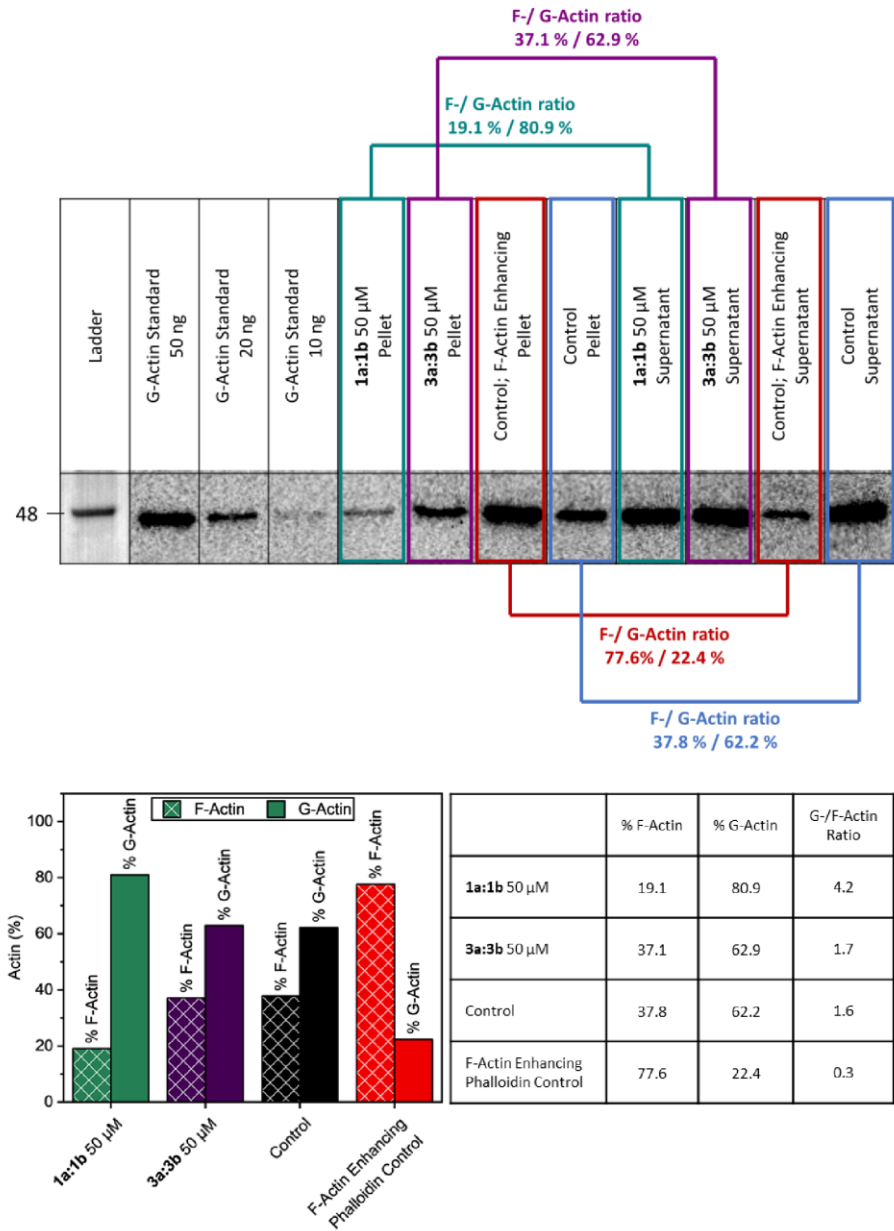


Figure S34 Western blot analysis of G-/F-actin fractionation of MDA-MB-231 cells. **a)** Representative western blot showing G-actin and F-actin distribution after fractionation. G-actin standards (10 ng, 20 ng and 50 ng) are included for reference. Samples include an untreated control, F-actin-enhancing condition, and treatments with isopeptides **1a:1b** and linear peptides **3a:3b** (50 μ M each). Pelleted fractions correspond to F-actin content, while supernatant fractions represent G-actin content. The G-/F-actin ratio for cells treated with **1a:1b** is 4.2 and for cells treated with the linear control peptides **3a:3b** is 1.7. Untreated control cells have a G-/F-Actin ratio of 1.6. The molecular weight marker (ladder) is shown on the left. Actin (40 kDa) bands were detected slightly below the 48 kDa band. **b)** Ratio of G- and F-actin for each treatment.

4.6. Cell viability assay

MDA-MB-231 or A549 cells were seeded at a density of 2,500 cells/well in a white half area 96-well plate and incubated at 37°C overnight to allow them to adhere. The following day, different concentrations of the glutathione-responsive isopeptides **1a:1b**, the glutathione-responsive non-assembly-inducing isopeptide **1c** or the other control compounds. For each condition, quadruplicates were performed. Cells were incubated with 50 μ L of the sample for 4 h. After the treatment, CellTiter-Glo® Assay solution (50 μ L) was added to each well, and the plate was placed on an orbital shaker for 2 minutes and subsequently incubated 10 minutes at room temperature. Luminescence was measured using a Promega GloMax®-Multi Detection System using the manufacturer's protocol.

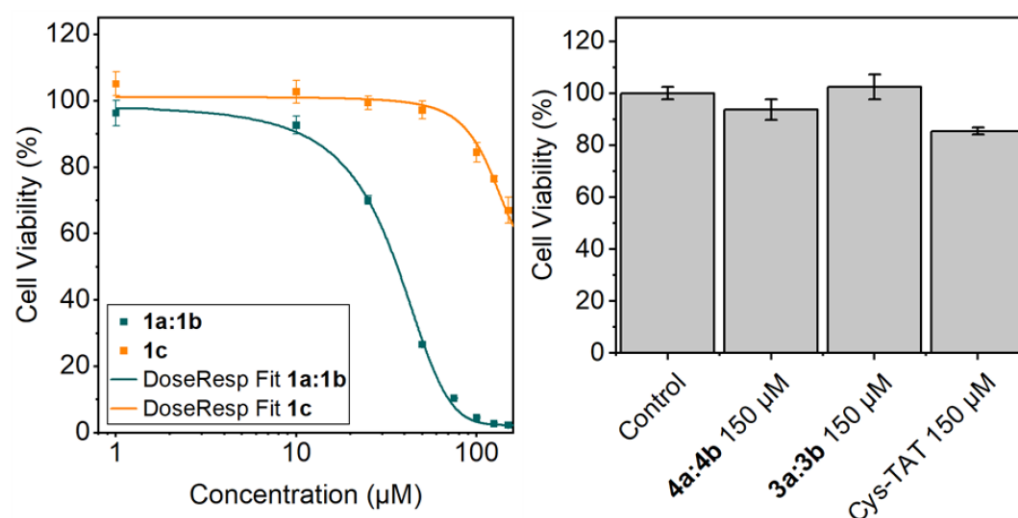


Figure S35 Cell viability of MDA-MB-231 cells incubated with **1a:1b** (green curve) or **1c** (orange curve) for 4 h (left) and with the other control compounds (right). The IC_{50} value of **1a:1b** for MDA-MB-231 cells is 36.4 ± 3.1 μ M.

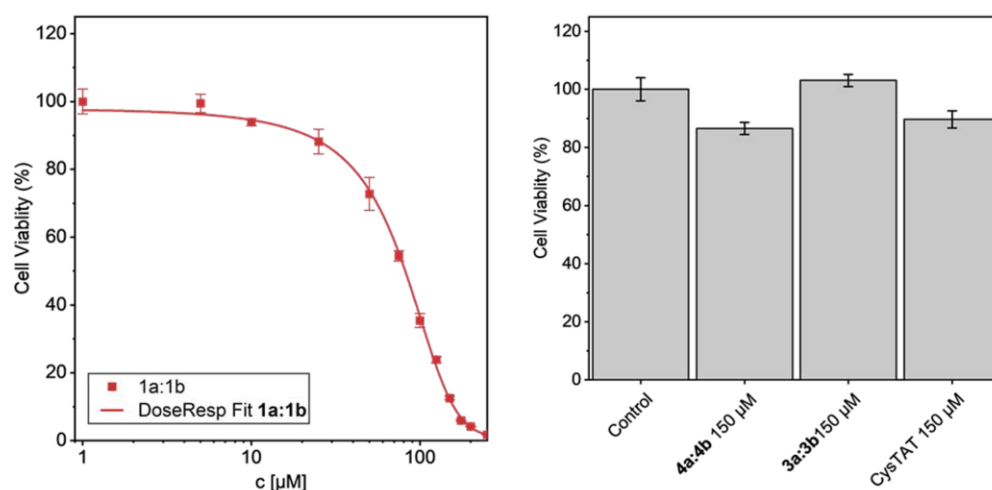


Figure S36 Cell viability of A549 cells incubated with **1a:1b** (red curve) for 4 h (left) and with the control compounds (right). The IC_{50} value of **1a:1b** for A549 cells is 81.7 ± 7.1 μ M.

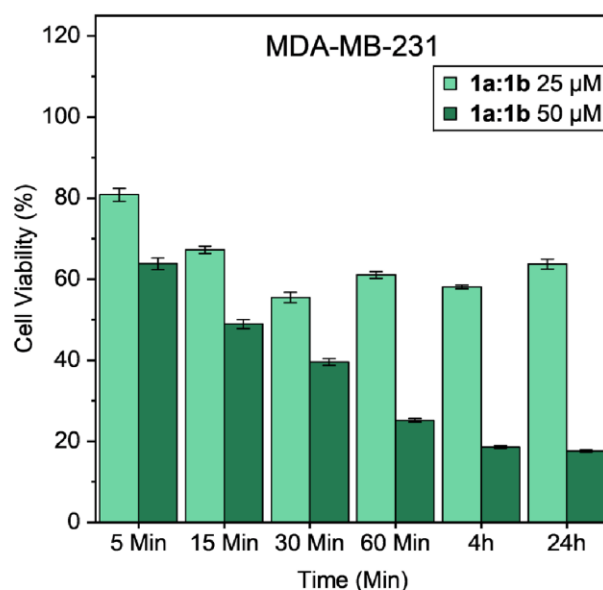


Figure S37 Cell viability of MDA-MB-231 cells incubated with **1a:1b** at 25 or 50 μM for 5 min, 15 min, 30 min, 60 min, 4 and 24 hours.

4.7. Analysis of apoptosis

4.7.1. Caspase-3/7 Glo Assay

Caspase-3/7 Glo Assay is used to observe the apoptotic nature of cell death of MDA-MB-231 cells upon treatment with isopeptide **1a:1b**. MDA-MB-231 cells were seeded at a density of 10,000 cells/well in a 96-well half-area white plate and incubated at 37°C, 5% CO₂ overnight to allow them to adhere. The next day cells were incubated with isopeptides **1a:1b** (5:1) in DMEM/DPBS/DMSO (90%/9%/1%) at 25 and 100 μM for different incubation times (5, 10, 15, 30, 45 minutes). As control cells were incubated with **3a:3b** (5:1) in DMEM/DPBS/DMSO (90%/9%/1%) at 100 μM for different incubation times (15 and 45 minutes). After the designated incubation time the 96 well plate was removed from the incubator and equilibrated to room temperature before adding 50 μL Caspase-Glo 3/7 reagent to each well. After shaking the plate gently on a plate shaker for 30 seconds, the samples were incubated at room temperature for 60 minutes before measuring luminescence intensity using the luminescence intensity readout at a Tecan Spark.

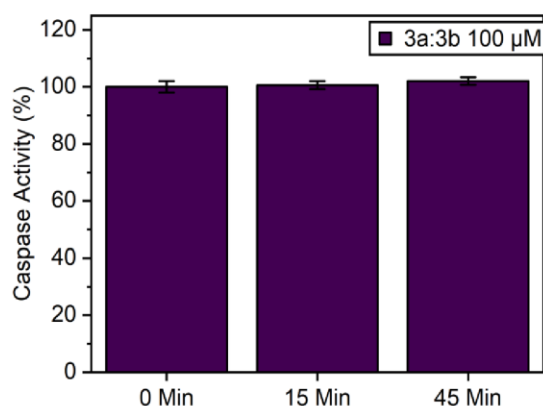


Figure S38 Caspase-Glo Assay results showing caspase activation in MDA-MB-231 cells after incubation with linear peptides **3a:3b** at 100 μ M at different time points. No change in caspase-3/7 activity compared to untreated control cells was detected. Data represent mean $\pm$ s.e.m. (n = 4).

4.7.2. Annexin V staining

Annexin V staining is used to observe apoptotic cell death upon treatment of MDA-MB-231 cells with *isopeptide* **1a:1b** at 50 μ M. MDA-MB-231 cells were seeded at a density of 20,000 cells/well in an 8-well Ibidi plate and incubated at 37°C, 5% CO₂ overnight to allow them to adhere. The next day cells were incubated with *isopeptides* **1a:1b** (5:1) in DMEM/DPBS/DMSO (90%/9%/1%) at 50 μ M for 1 h. After washing with 1x binding buffer cells were then incubated with Annexin V FITC (25 μ L/mL) and propidium iodide (25 μ L/mL), both obtained from Apoptosis Detection Kit by Invitrogen. Cells were kept at room temperature for 20 minutes before removing the staining solution and washing them 1x with binding buffer. The washing solution was replaced with fresh 1x binding buffer (200 μ L) and cells were imaged using a Leica Stellaris® 8 microscope (40x glycerol immersion objective).

4.7.3. Caspase-3/7 live cell imaging

MDA-MB-231 cells were cultured in an 8-well confocal plate at a density of 20,000 cells per well in DMEM and allowed to adhere overnight at 37°C, 5% CO₂. The medium was removed and the cells were stained using BioTracker NucView® 530 Red Caspase-3 Dye according the manufacturer's protocol. Immediately before imaging, the sample of **1a:1b** at 50 μ M in DMEM/DPBS/DMSO (90%/9%/1%) was introduced into a well. The cells were imaged live using an incubator-equipped Leica Stellaris® 8 microscope (40x glycerol immersion objective) with fast lifetime contrast (FALCON) module (Leica Microsystems GmbH). The incubator (okolab) is set and held constant at 37 °C, 5% CO₂ and a relative humidity of 90% throughout all measurements.

5. Analysis of Metabolic Disruption

5.1. Mito Stress test

The effect of the compounds on the metabolism of MDA-MB-231 cells or A549 cells was investigated using the Seahorse XFe96 Analyzer (Agilent Technologies). The Seahorse XF Cell Mito Stress Test Kit was used following the manufacture's protocol to determine the respiratory activity of the cells. Cells were seeded at a density of 20,000 cell/well one day before the assay and incubated overnight at 37°C, 5% CO₂ in DMEM, high glucose supplemented with 10% FBS. The cartridge for sample loading was loaded with calibration solution (200 µL per well) and incubated at 37°C in a non-CO₂ incubator. On the day of the assay, Seahorse XF DMEM, pH 7.4 was supplemented with pyruvate (1 mM), glutamine (2 mM), and glucose (10 mM). Cells were washed twice with XF DMEM (2x 100 µL) and the medium was substituted with fresh XF DMEM (180 µL) and placed in a 37°C non-CO₂ incubator for 45 to 60 minutes before loading the plate into the instrument. Assay components of the Mito Stress Test Assay were prepared as described in the manufacturer's protocol. Briefly, Oligomycin was resuspended in 630 µL, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) in 720 µL and Rotenone/Antimycin A (Rot/AA) in 540 µL supplemented XF DMEM and vortexed vigorously to ensure complete dissolution. Stocks were diluted to 3 ml total; Oligomycin: 450 µL stock in 2,550 µL XF DMEM (final concentration 1.5 µM); FCCP: 300 µL in 2,700 µL XF DMEM (final concentration 1 µM); Rot/AA: 300 µL in 2,700 µL XF DMEM (final concentration 0.5 µM). Samples were prepared at 10x stocks with 10% DMSO in XF DMEM. The cartridge was prepared by loading the samples (20 µL) in Port A of the designated wells and the components of the Mito Stress Test Assay in Ports B-D of each well. The cartridge and 96-well plate with the cells were loaded in the instrument and the content of the ports was injected sequentially according to the specific assay. The oxygen concentration, to monitor the oxygen consumption rate (OCR), was constantly measured throughout the entire time of the assay. The Mito Stress Assay were performed in two independent experiments with n=4. Bar graphs are shown for the mean ± standard error of the mean (s.e.m.) for raw and processed data. For the processed data, all values were set to 100% OCR for the last measurement prior to sample addition before calculating the results. Additionally, the values were normalized to the control for each independent experiment, before calculating the mean value and SEM. Statistical significance was calculated by ANOVA with a Tukey post hoc test and was defined as *p < 0.05, **p < 0.01, ***p < 0.001.

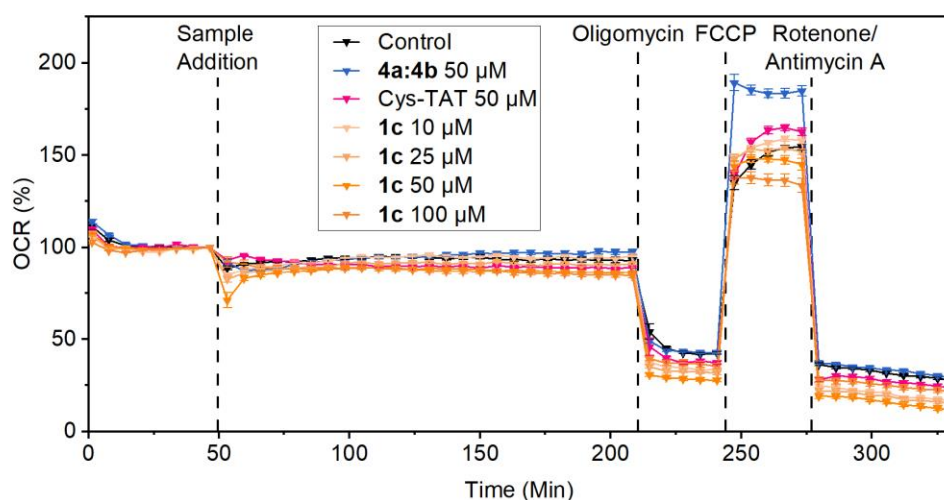


Figure S39 Effect on the oxygen consumption rate (OCR) of MDA-MB-231 cells treated with the control compounds **1c**, **4a:4b** and Cys-TAT for 4 hours total. During the last 1.5 hours Mito Stress Test was performed. At different time points specific modulators of the electron transport chain (ETC) were added to investigate the influence of the samples on cellular respiration in more detail: (1) oligomycin inhibits ATP synthase, (2) FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) disrupts the mitochondrial membrane potential, (3) rotenone inhibits complex I and antimycin A inhibits complex III of the ETC.²⁷ The last measurement before treatment injection of the compound is set as 100%. Data are presented as mean $\pm$ s.e.m., $n \geq 8$.

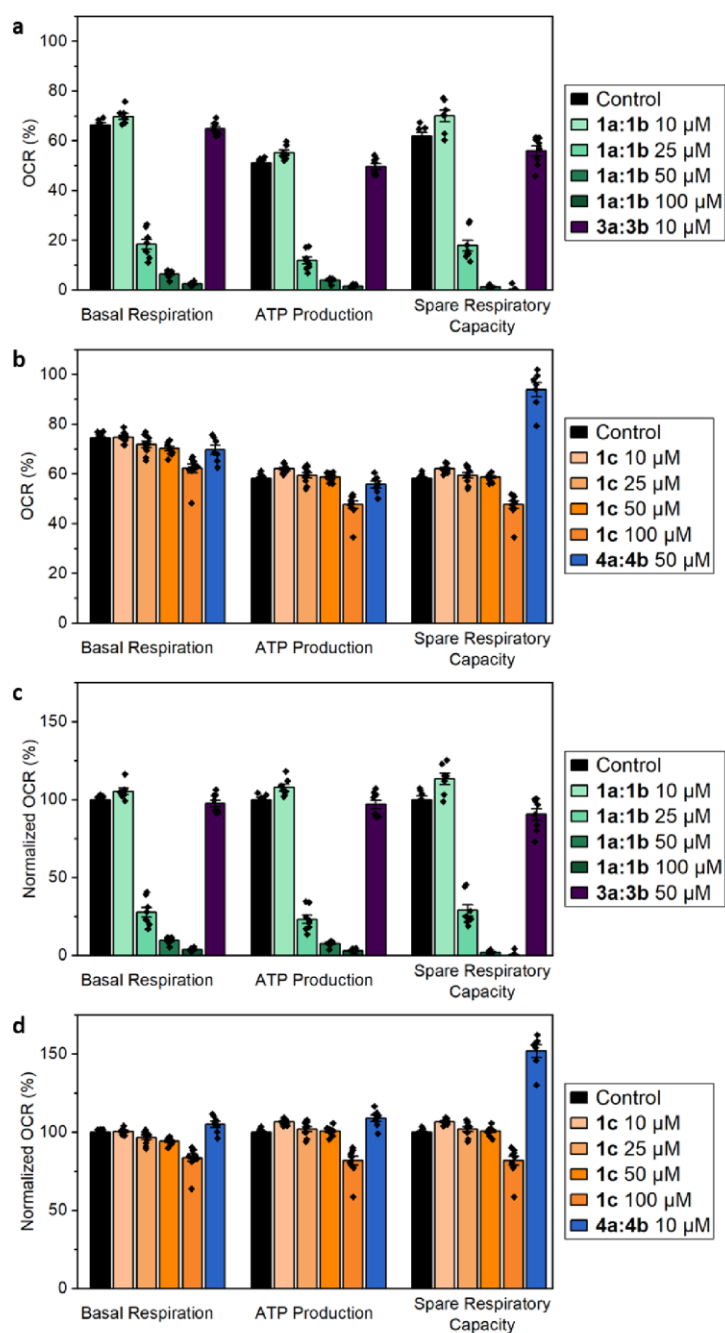


Figure S40 Comparison of the effect of **1a:1b**, **1c**, **3a:3b** and **4a:4b** on characteristic values of mitochondrial function after 4 h total incubation. Basal Respiration, ATP Production and Spare Respiratory Capacity are shown. Values were calculated using the data from graphs with the last measurement before sample addition set to 100% OCR and are presented as OCR (%) (**a** and **b**). Additionally, the data was normalized to the control of each experiment and data are shown as Normalized OCR (%) (**c** and **d**). Data are presented as mean $\pm$ s.e.m., $n \geq 6$.

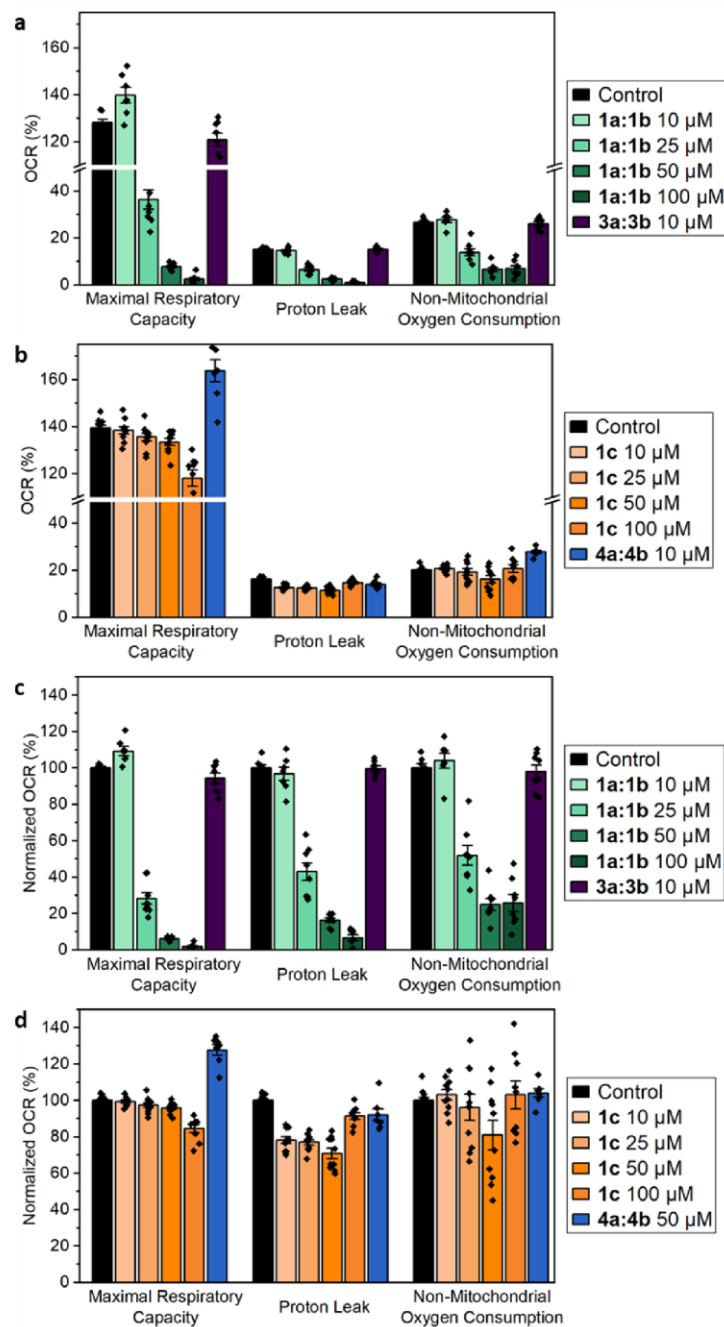


Figure S41 Comparison of the effect of **1a:1b**, **1c**, **3a:3b** and **4a:4b** on characteristic values of mitochondrial function after 4 h total incubation. Maximal Respiratory Capacity, Proton Leak and Non-Mitochondrial Oxygen Consumption are shown. Values were calculated using the data from graphs with the last measurement before sample addition set to 100% OCR and are presented as OCR (%) (**a** and **b**). Additionally, the data was normalized to the control of each experiment and data are shown as Normalized OCR (%) (**c** and **d**). Data are presented as mean $\pm$ s.e.m., $n \geq 6$.

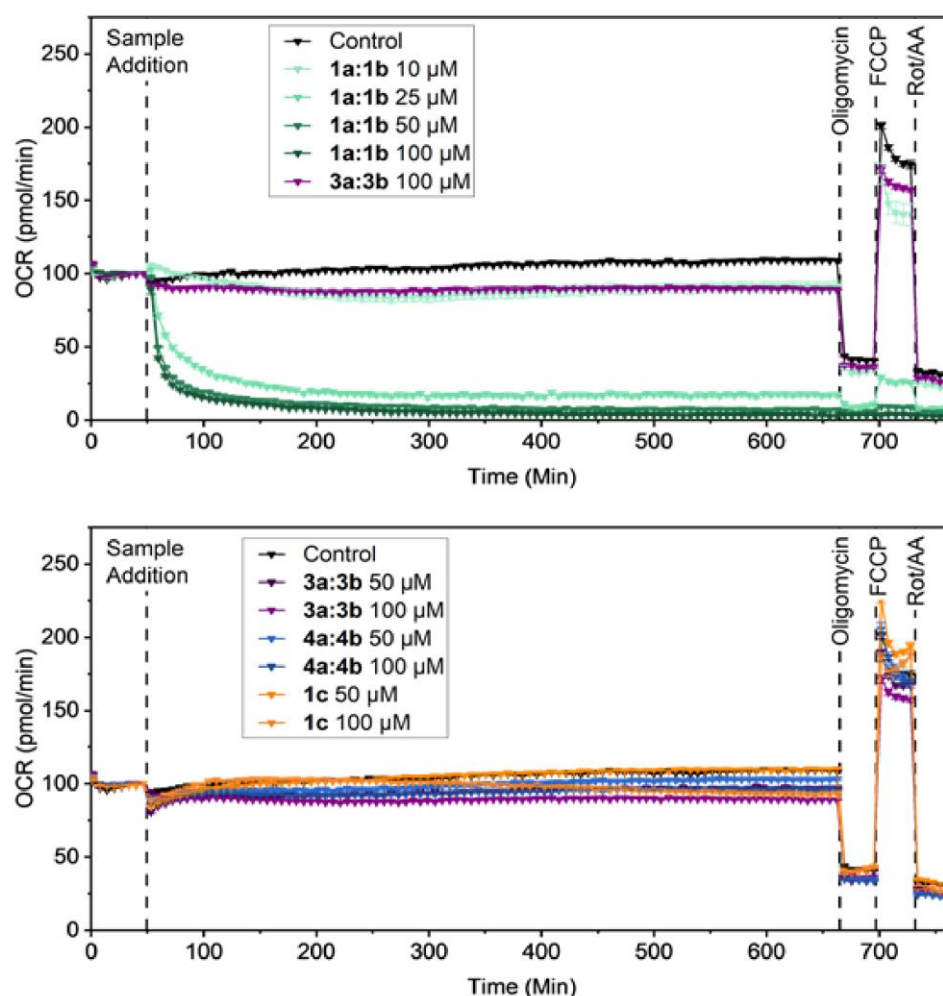


Figure S42 Effect on the oxygen consumption rate (OCR) of MDA-MB-231 cells treated with the isopeptides **1a:1b** or the linear peptides **3a:3b** (upper graph) or the control compounds **1c**, **4a:4b** and additional concentrations of **3a:3b** (lower graph) for 12 hours total. During the last 90 minutes the Mito Stress Test was performed. At different time points specific modulators of the electron transport chain (ETC) were added to investigate the influence of the samples on cellular respiration in more detail: (1) oligomycin inhibits ATP synthase, (2) FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) disrupts the mitochondrial membrane potential, (3) rotenone inhibits complex I and antimycin A inhibits complex III of the ETC. The last measurement before treatment injection of the compound is set as 100%. Data are presented as mean $\pm$ s.e.m., $n \geq 4$.

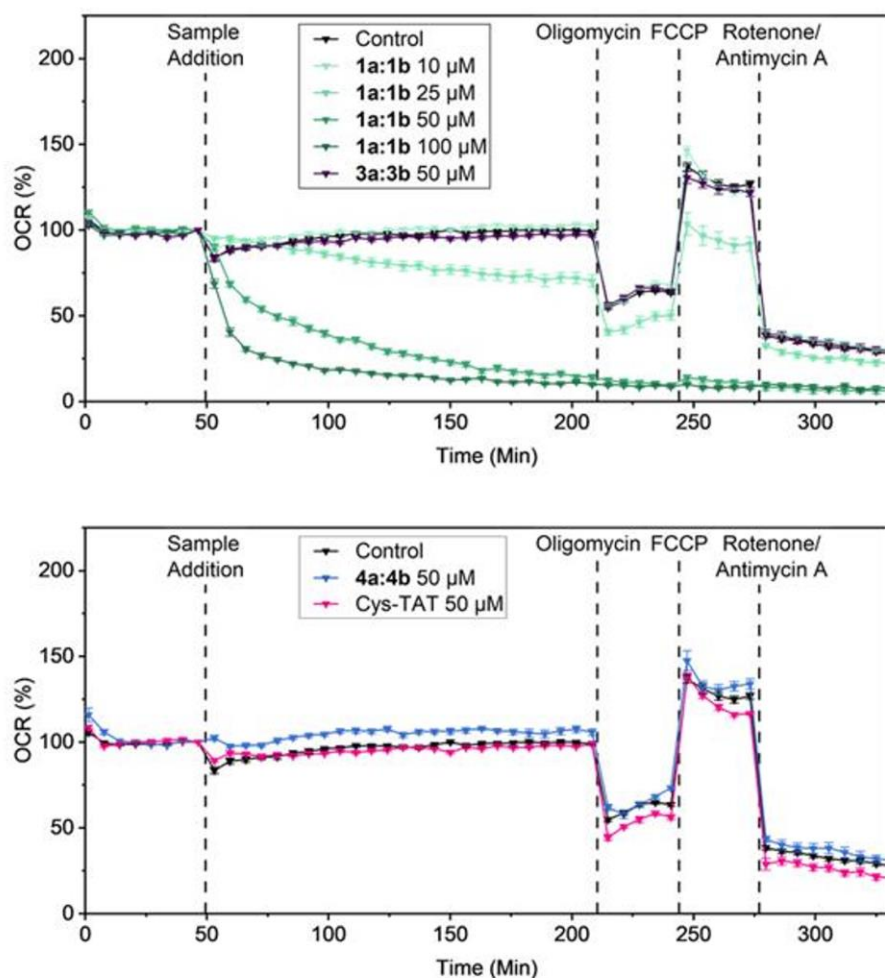


Figure S43 Effect on the oxygen consumption rate (OCR) of A549 cells treated with the isopeptides **1a:1b** or control compounds **1c**, **4a:4b** and Cys-TAT for 4 hours total. During the last 1.5 hours Mito Stress Test was performed. At different time points specific modulators of the electron transport chain (ETC) were added to investigate the influence of the samples on cellular respiration in more detail: (1) oligomycin inhibits ATP synthase, (2) FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) disrupts the mitochondrial membrane potential, (3) rotenone inhibits complex I and antimycin A inhibits complex III of the ETC. The last measurement before treatment injection of the compound is set as 100%. Data are presented as mean $\pm$ s.e.m., $n \geq 7$.

6. Tumor Spheroid Experiments

6.1. Evaluation of uptake and toxicity on MDA-MB-231 spheroids

Prior to treatment, fully developed MDA-MB-231 spheroids were imaged using a Keyence BZ-X810 fluorescence microscope. Subsequently, spheroids were treated with the synthesized and previously characterized compounds (**1a:1a** (100 and 50 μ M), **1c** (100 μ M), **3a:3b** (100 μ M) and **4a:4b** (100 μ M) in a final volume of 100 μ L/well and incubated at 37°C, 5% CO₂. Untreated spheroids were used as experimental control where the medium was substituted with fresh DMEM containing 1% DMSO. Cellular uptake after 4 h incubation was observed using the GFP filter of a Keyence BZ-X810 microscope. Growth and morphological changes were monitored by brightfield imaging using a Keyence BZ-X810 microscope after 4 h incubation. At the end of the incubation time spheroids were washed with DPBS to remove treatment residuals and incubated with Propidium Iodide (8 μ g/ml; Sigma-Aldrich, P4864). After incubation with the staining solutions spheroids were washed with DMEM FluoroBrite and imaged using either a Keyence BZ-X810 or a Leica Stellaris® 8 microscope. Experiments were carried out on two independent cultures with internal replicates (n $\geq$ 5).

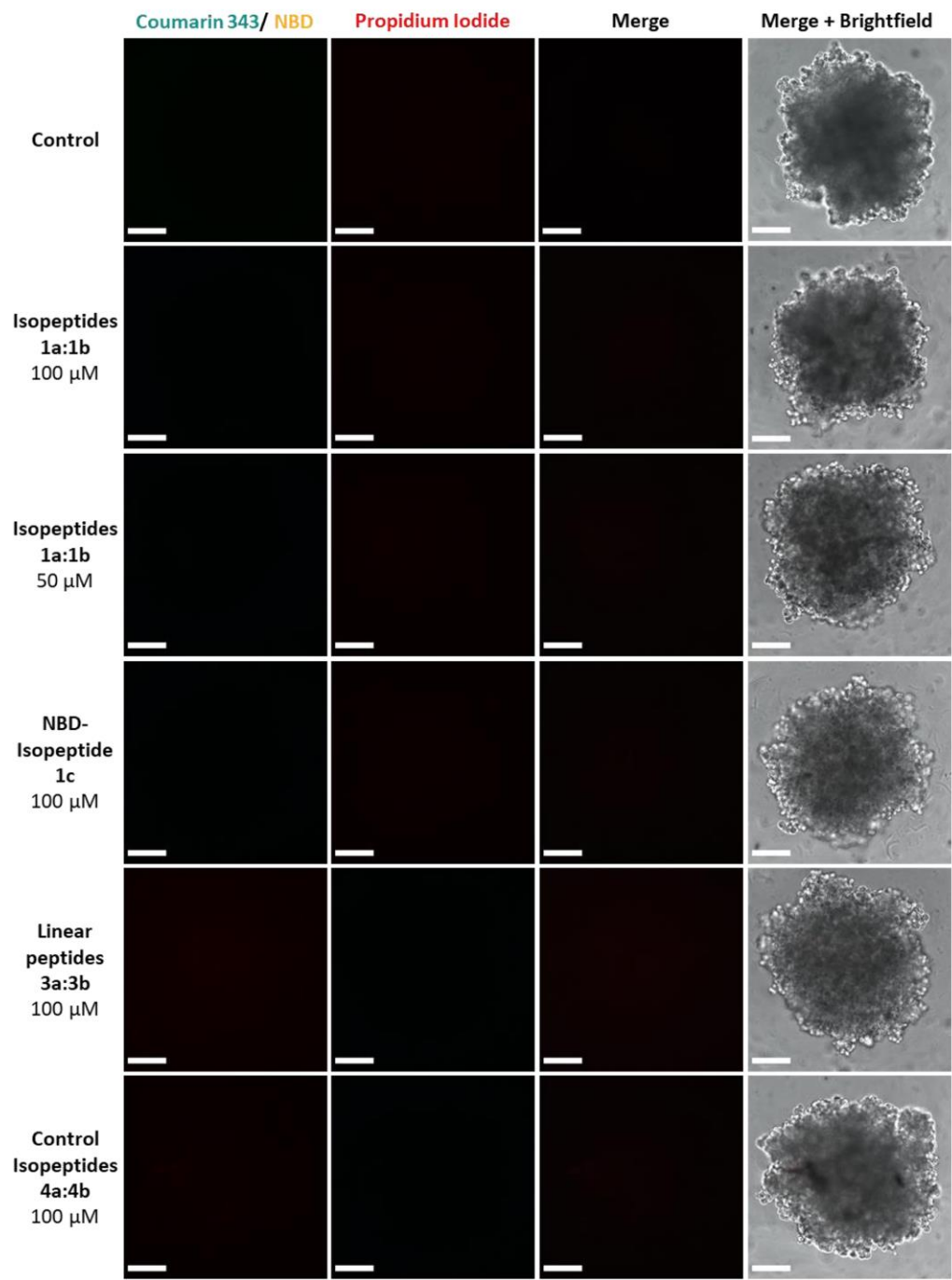


Figure S44 Fluorescence microscopy images of MDA-MB-231 tumor spheroids before treatment. Coumarin 343 (cyan) and propidium iodide (red) fluorescence were imaged using GFP and Cy5 filter settings. Scale bars 100 μm.

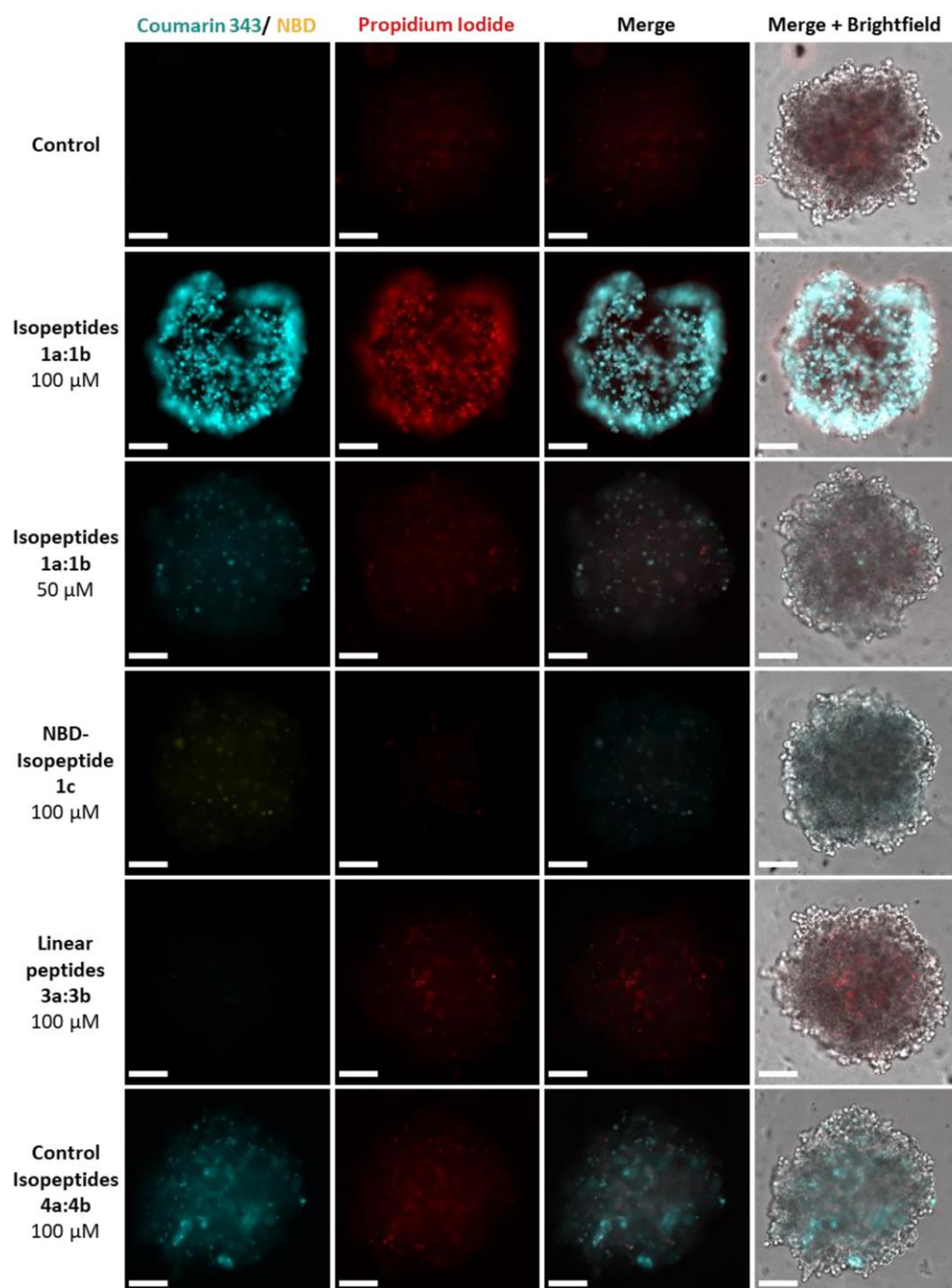


Figure S45 Fluorescence microscopy images of MDA-MB-231 tumor spheroids after 4 h treatment with **1a:1b** at 100 and 50 μ M and **1c**, **3a:3b** and **4a:4b** at 100 μ M. Coumarin 343 (cyan) or NBD (yellow) and propidium iodide (red) fluorescence were imaged using GFP and Cy5 filter settings. Scale bars 100 μ m.

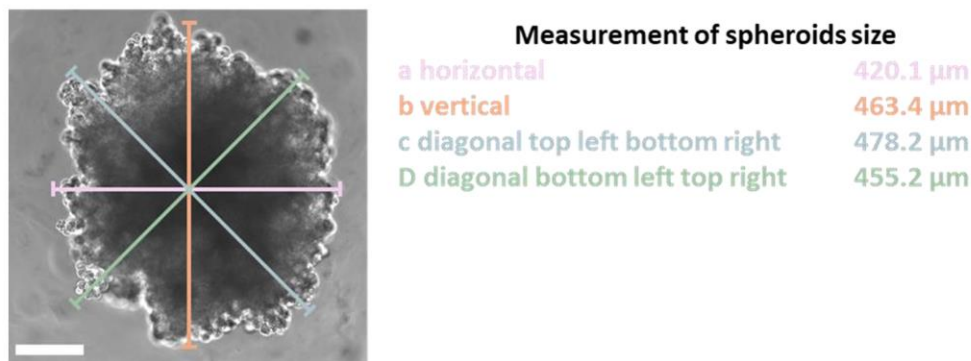


Figure S46 Measurement of spheroid size shown for the example of an untreated control spheroid at t_0 . For each spheroid the size was measured at four different positions at t_0 and after 4 h incubation with compounds. To ensure comparability, the size of each spheroid was measured horizontally, vertically and diagonally. The mean of the four measurements was used to evaluate the change in size within the 4 h incubation time. Images were processed using ImageJ. Scale bar 100 μm .

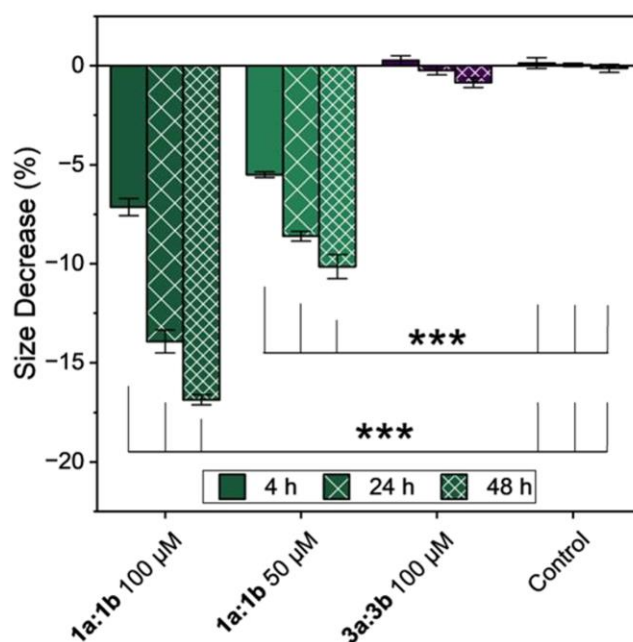


Figure S47 Size decrease of MDA-MB-231 tumor spheroids treated with isopeptides **1a:1b** or linear peptides **3a:3b** for 4, 24 or 48 hours. Spheroid size was measured before and after incubation with glutathione-responsive isopeptides using brightfield images in ImageJ. Diameter of each spheroid was measured at 4 different places (Figure S31). Data are presented as mean $\pm$ s.e.m., $n=5$.

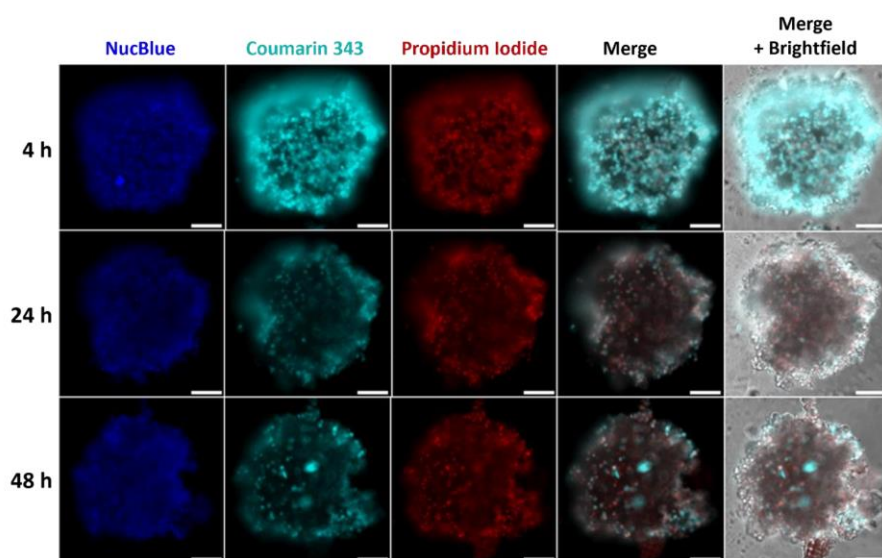


Figure S48 Fluorescence microscopy images of MDA-Mb-231 tumor spheroids after treatment with **1a:1b** at 100 μ M for 4, 24 or 48 hours. NucBlue fluorescence (blue), Coumarin 343 (cyan) and propidium iodide (red) fluorescence were imaged using DAPI, GFP and Cy5 filter settings, respectively. Scale bars 100 μ m.

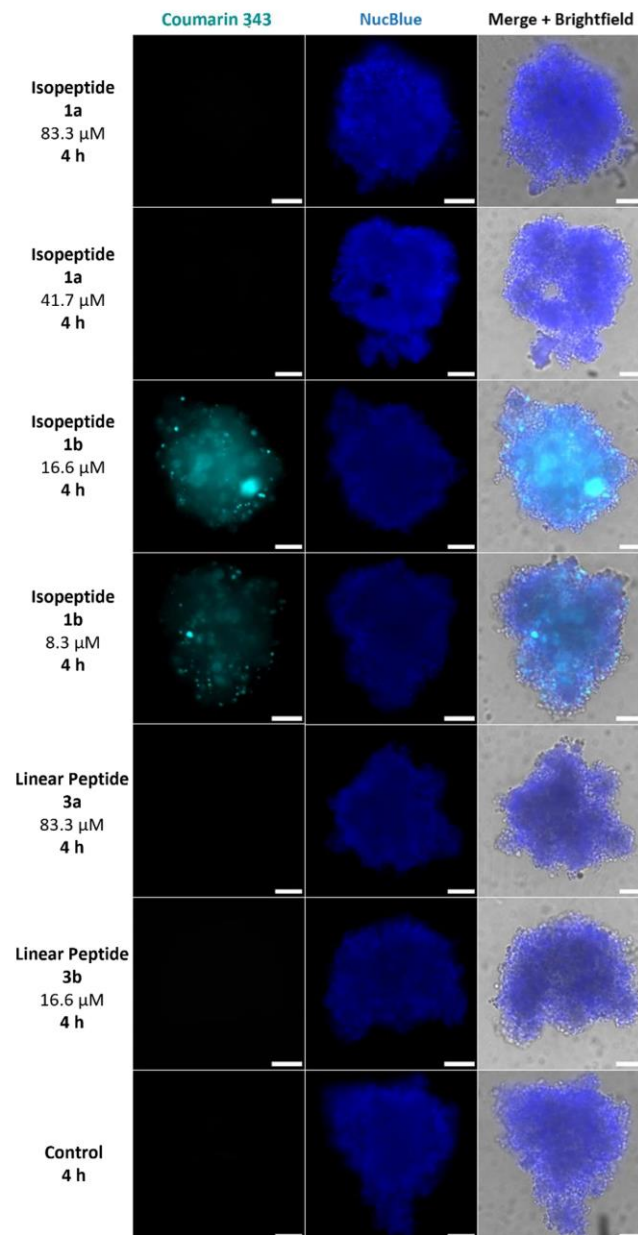


Figure S49 Fluorescence microscopy images of MDA-MB-231 tumor spheroids after treatment with **1a**, **1b**, **3a** or **3b** individually for 4 h. Coumarin 343 (cyan) and NucBlue (blue) fluorescence were imaged using GFP and DAPI filter settings. Scale bars 100 μ m.

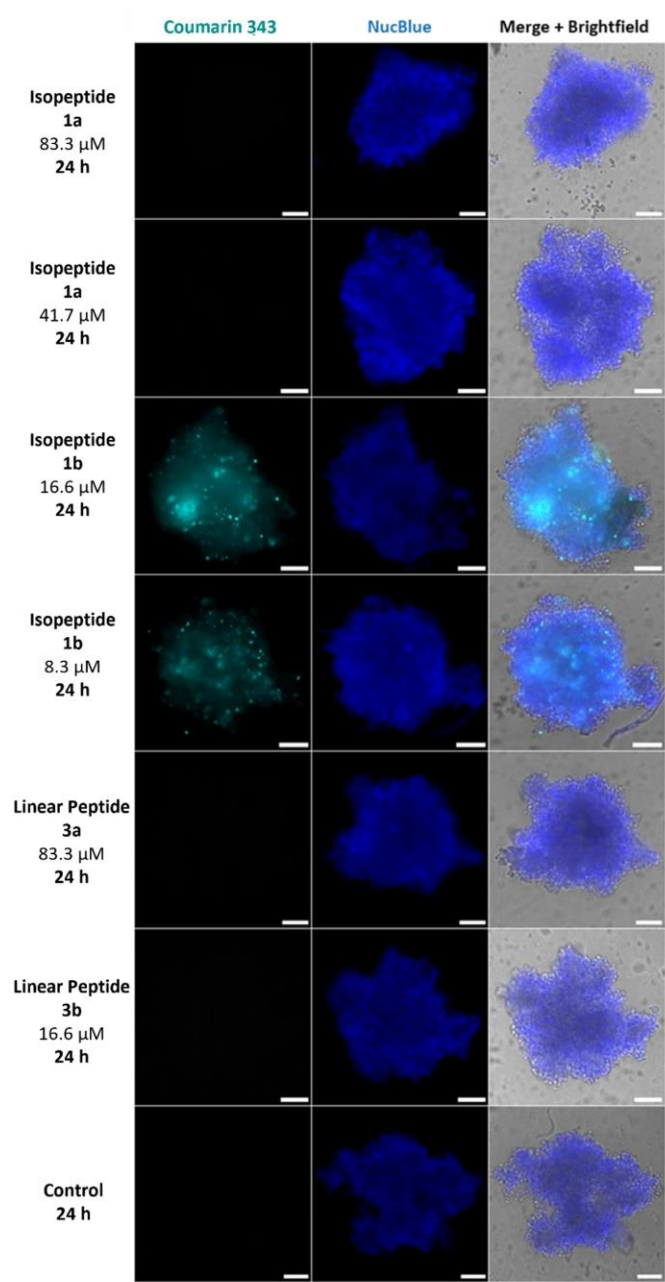


Figure S50 Fluorescence microscopy images of MDA-MB-231 tumor spheroids after treatment with **1a**, **1b**, **3a** or **3b** individually for 24 h. Coumarin 343 (cyan) and NucBlue (blue) fluorescence were imaged using GFP and DAPI filter settings. Scale bars 100 μm.

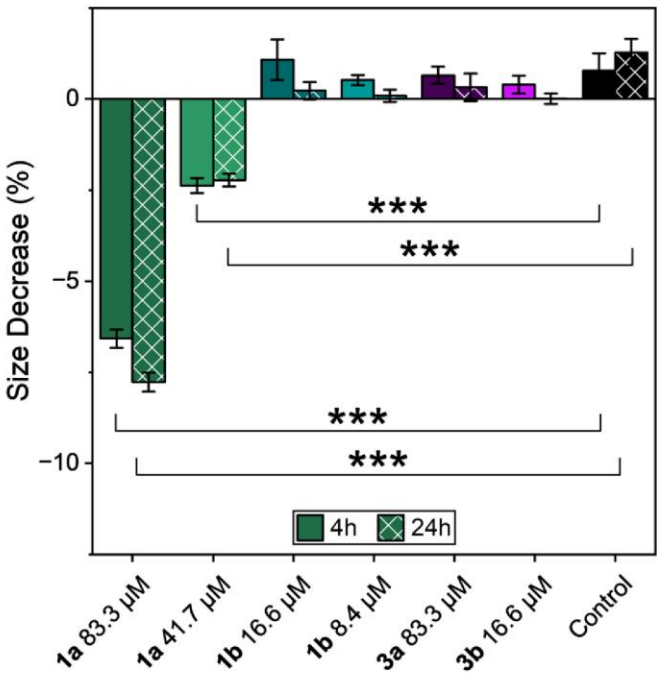


Figure S51 Size decrease of MDA-MB-231 tumor spheroids treated with isopeptide **1a** or **1b** alone, as well as linear peptide **3a** or **3b** alone for 4 or 24 hours. The concentrations of the individual peptides reflect their respective concentration in the previously investigated 5:1 ratios of either isopeptides **1a:1b** or linear peptides **3a:3b**. Spheroid size was measured before and after incubation with glutathione-responsive isopeptides using brightfield images in ImageJ. Diameter of each spheroid was measured at 4 different places (Figure S31). Data are presented as mean ± s.e.m., n=4.

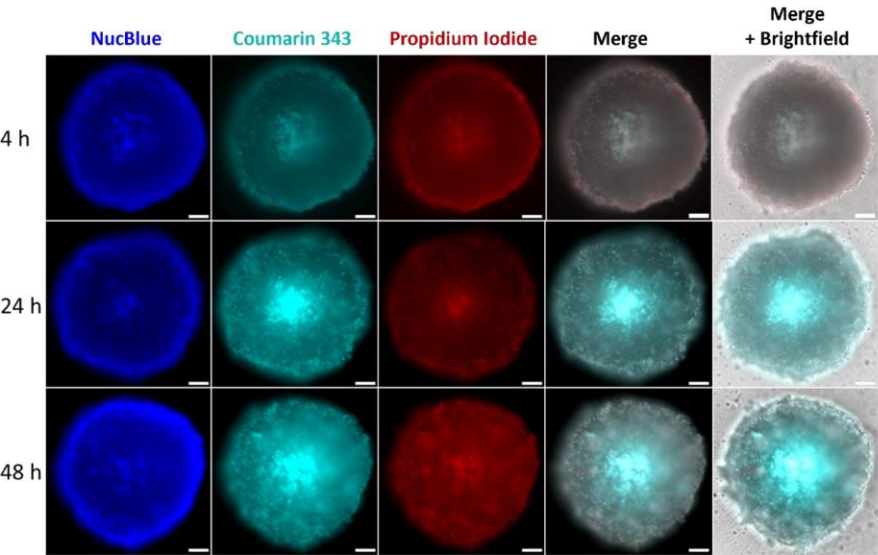


Figure S52 Fluorescence microscopy images of A549 tumor spheroids after treatment with **1a:1b** at 100 μ M for 4, 24 or 48 hours. NucBlue fluorescence (blue), Coumarin 343 (cyan) and propidium iodide (red) fluorescence were imaged using DAPI, GFP and Cy5 filter settings, respectively. Scale bars 100 μ m.

6.2. Slicing of spheroids

Spheroids were prepared and treated as described in 6.1. Before transferring the spheroids gelatin was dissolved in warm water (175 mg/mL) and transferred into a small plastic container, where it was allowed to solidify. After incubation spheroids were washed with DPBS and transferred on top of solidified gelatin and excess buffer was carefully removed. Spheroids were immobilized by carefully adding a warm drop of gelatin on top and allowing it to solidify. On top of the spheroids a second layer of gelatin was added before the plastic box was stored at -80°C overnight. The next day the gelatin cubes were sliced using a Cryostat Leica CS3050 S and slices of $16\text{ }\mu\text{m}$ were collected. Slices of treated spheroids of MDA-MB-231 and A549 cells were then imaged using a Keyence BZ-X810 fluorescence microscope and a 20x LD PH lens.

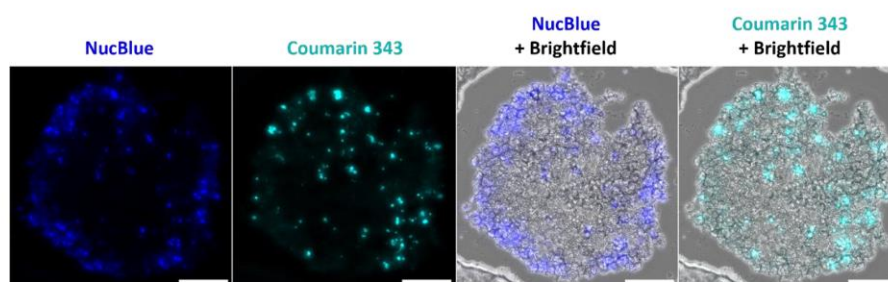


Figure S53 Slices of MDA-MB-231 tumor spheroid. Upon incubation with 1a:1b at $100\text{ }\mu\text{M}$ for 4 h, MDA-MB-231 spheroids were embedded in gelatin. Slices of $16\text{ }\mu\text{m}$ thickness were obtained from the gelatin blocks which were then imaged using Keyence BZ-X810. Scale bar $100\text{ }\mu\text{m}$.

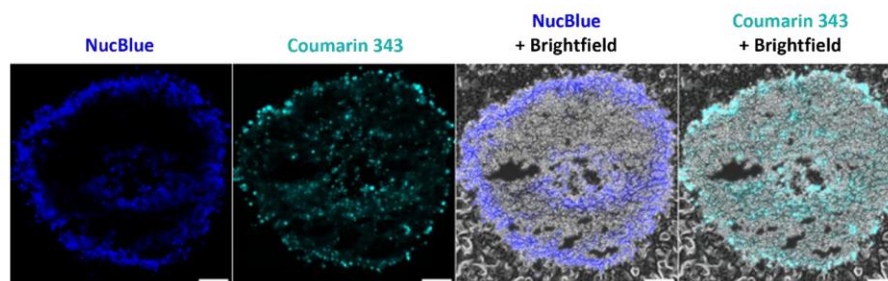


Figure S54 Slices of A549 tumor spheroid. Upon incubation with 1a:1b at $100\text{ }\mu\text{M}$ for 4 h, A549 spheroids were embedded in gelatin. Slices of $16\text{ }\mu\text{m}$ thickness were obtained from the gelatin blocks which were then imaged using Keyence BZ-X810. Scale bar $100\text{ }\mu\text{m}$.

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6 Conclusion and Outlook

This thesis demonstrated the modulation of assembly and disassembly processes, while also introducing pathway selectivity to a system. Furthermore the controlled assembly of nanostructures was also investigated in the complex environment of the cell.

In chapter 3, the light-responsive peptides Anthracene-GISVK and Anthracene-GISVKAE were introduced that perform a [4+4] cycloaddition upon irradiation with UV-light. These peptides assembled into two distinct morphologies, 2D-sheets or twisted ribbons and exhibited disparate secondary structure interactions between the anthracene moieties. Moreover, despite employing analogous structural motifs, only Ant-GISVK exhibited a divergent structure upon increasing the pH, going from sheets to unordered aggregates. UV-irradiation with subsequent photodimerization led to a distortion of the previously flat aromatic groups, thereby impeding the process of π - π stacking. This alteration in intramolecular interactions, in conjunction with the introduction of strain within the system, resulted in either disassembly or the formation of unordered aggregates from ordered nanostructures. Furthermore, kinetic differences were observed in the conversion of the anthracene moiety when comparing the assembled state to the reaction in solution. This discrepancy was attributed to preorganization within the nanostructure, which was enhancing or impeding the conversion depending on the morphology of the assembly. This demonstrated the influence of supramolecular morphology on the chemistry that governs disassembly behavior. However, the assembly process is subject to thermodynamic control, and the irradiation process generates a new thermodynamic minimum that induces disassembly. To achieve complex, multi-step assembly systems, it is necessary to move the system out of equilibrium and introduce pathway selectivity, where for example one pathway leads to disassembly and another pathway results in a change in morphology. Moreover, the reversibility of the [4+4] cycloaddition of anthracene has not been examined in this study, as it is reported to be initiated by lower wavelength UV light, which could potentially enable the recovery of the nanostructure and, by cycling between the states, the emergence of a new structure.

Chapter 4 described the introduction of a photolytic reaction cascade that demonstrated pathway selectivity of the assembled nanostructures. For this purpose, a pro-assembling iso-peptide was caged with the light-responsive Nvoc-group. Upon UV-irradiation, the Nvoc group was cleaved and subsequently, the linear Fmoc-ICA was released, which was able to form thin nanofibers. However, within the cascade, the nitroso moiety of the cleaved Nvoc-group oxidizes the Fmoc-ICA, resulting in the formation of disulfide DiFmocICA. This disulfide assembles into short fibers, exhibiting a distinct morphology from the structure achieved by dissolution of the dimer, thus exhibiting pathway selectivity. In this cascade, the transience, interconversion, and production rates of the peptide in the respective oxidation state were modulated with dithiothreitol (DTT) as a reducing agent. Depending on the equivalents of DTT, the cascade was either halted at the thiol level or the disulfide formation was delayed through redox cycling until the reducing agent was depleted. It is noteworthy that this modulation was only observed in the presence of DTT, as benzyl mercaptan completely impeded the oxidation of Fmoc-ICA. Moreover, the cycling between the redox states caused the emergence of new secondary structures and chirality as well as the elongation of the fibrillar assembly. The precise mechanisms underlying the three distinct morphologies of DiFmoc-ICA remain to be elucidated. The discrepancy between the straight fibers from the cascade assembly and the twisted fibers from the direct assembly by dissolution may be attributed to a templating effect by Fmoc-ICA. The dimer could assemble on nuclei formed by Fmoc-ICA, thereby acquiring a similar morphology. However, given the structural deviation between the monomer and the dimer, the extension of the fibers could lead to increased geometric frustration, thereby limiting the fiber length. Furthermore, oxidation could occur in an already formed straight, thin fiber of the monomer, resulting in its fragmentation due to geometric frustration. By cycling between these states, a more suitable conformation could be achieved, enabling the assembly into longer fibers. Another potential factor influencing this process is the coexistence of monomer and dimer in conjunction with the byproducts of the Nvoc group, which modifies the assembly process. The formation of twisted fibers could be attributed to the presence of high local concentrations of dimer upon dissolution, leading to a kinetic trap within the system. To address these inquiries, further experimental research, in conjunction with computational simulations, is required.

In Chapter 5, the iso-peptide strategy was employed to develop a pro-assembling peptide caged with a bifunctional glutathione-responsive linker. Upon cellular uptake, facilitated by the cell-penetrating TAT peptide, the increased glutathione levels in the cytosol of cancer cells cleaved the linker and initiated a cascade reaction that released the linear peptide. This process led to the assembly of nanofibers, which disrupted the mitochondrial integrity and inhibited the energy homeostasis of multidrug-resistant MDA-MB-231 triple negative breast cancer cells. This process was observed in real-time by live-cell and fluorescent lifetime imaging, as well as by measuring the oxygen consumption rate. Subsequently, the metabolic impact of the nanostructures resulted in cell death. This phenomenon was examined in both two-dimensional cell cultures and three-dimensional tumor spheroid models, where peptide nanostructures were observed to induce a reduction in tumor size.

In summary, this thesis presented several methods for modulating precise nanostructures with different morphologies by controlling their assembly and disassembly. This was achieved by introducing stimuli-responsive groups that respond to light, redox, or pH changes.

Several challenges remain in this field that must be addressed in future research. For instance, the development of systems exhibiting complex pathway selectivity or far-from-equilibrium thermodynamics remains a formidable challenge. Achieving this objective necessitates a more profound comprehension of the underlying dynamics and interactions within these systems, as well as an exploration of the underlying mechanisms that govern pathway selectivity. The integration of multiple, orthogonal stimulus-responsive groups into a peptide system has the potential to facilitate a multi-step noncovalent synthesis of supramolecular nanostructures. Moreover, the simultaneous assembly and disassembly of synthetic nanostructures at opposite ends, analogous to the treadmilling of actin filaments, poses significant scientific challenges. The development of complex systems capable of responding to minor alterations in the cellular environment could potentially pave the way for the creation of smart biomaterials. This integration would facilitate the incorporation of responsive nanostructures into functional biomaterials. Achieving this objective necessitates a more profound comprehension of the impact of the chemical milieu on the nanostructure. This enhanced understanding can possibly be attained by initially assembling the architecture in a specified crowded medium with increasing levels of

complexity and subsequently analyzing the consequent alterations. Furthermore, the implementation of a disassembly trigger would facilitate the regulation of the biomaterial's lifespan, thereby enabling a more targeted application.

7 Bibliography

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Appendix

Curriculum Vitae

Personal Details:

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Education:

- | | |
|-------------------|--|
| Since 06.2020 | PhD student at Max Planck Institute für Polymer research in the group of Prof. Weil
„Controlling reaction pathways to modulate assembly processes” |
| 07.2018 – 04.2020 | Master of Science in Chemistry, Johannes Gutenberg-Universität Mainz |
| 09.2019 – 04.2020 | Master thesis at Max Planck Institute für Polymer research in the group of Prof. Weil about „Reductive depolymerizable and decationizable Polycarbonates“ |
| 04.2014 – 07.2018 | Bachelor of Science in Chemie, Johannes Gutenberg-Universität Mainz |
| 09.2017 – 03.2018 | DAAD-ISAP Student at the University of Massachusetts, Amherst, USA in the group of Prof. Todd Emrick about „Synthesis of Aggregation-Induced Emission conjugated Polymers“ |
| 04.2017 – 08.2017 | Bachelor thesis in the group of Prof. Waldvogel about „Elektrochemische Generierung von Nitril-N-Oxiden und deren Verwendung in der Cycloaddition“ |
| 07.2013 | Abitur, Gutenbergschule, Wiesbaden |

List of Publications

Roth, P.*, **Meyer, R.***, Harley, I. *et al.* Supramolecular assembly guided by photolytic redox cycling. *Nat. Synth* **2**, 980–988 (2023). doi.org/10.1038/s44160-023-00343-1

C. S. Krevert, L. Gunkel, J. Sutter, **R. Meyer**, P. Schneider, Y. Nagata, J. Hunger, J. *Phys. Chem. B* **2024**, 128, 10688–10698. doi.org/10.1021/acs.jpccb.4c04760

S. Chagri*, K. Maxeiner*, M. J. S. A. Silva, L. Förch, J. Link, P. Roth, **R. Meyer**, J. Fetzer, A. Kaltbeitzel, I. Lieberwirth, K. Landfester, M. Wagner, D. Y. Ng, T. Weil, Intracellular Formation of Synthetic Peptide Nanostructures Causes Mitochondrial Disruption and Cell Death in Tumor Spheroids. *Adv. Sci.* 2025, 2412606.

Chagri S, Burgstaller A, Schirra C, Link J, Zhou Z, Roth P, **Meyer R**, et al. Synthetic intracellular nanostructures enhance cytotoxic T cell function via assembly-driven chemical engineering. ChemRxiv. 2024; doi.org/10.26434/chemrxiv-2024-3lxgk