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Controlled Supramolecular Assembly of Peptides via Chemical Reactions

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Abstract

Peptide-based bioresponsive systems have gained significant attention in supramolecular chemistry and biomaterials. These systems have enabled the development of synthetic platforms within living cells, providing valuable insights into how structural formation influences cellular processes. This thesis investigates the design, synthesis, and characterization of different stimulus-responsive peptide systems for the in situ formation of intracellular nanostructures, and examines their consequent effects on cellular processes.

In the first chapter, a platinum (II)-containing tripeptide was engineered to aggregate into intracellular fibrillar nanostructures, disrupting metabolic functions such as aerobic glycolysis and oxidative phosphorylation, consequently instigating apoptosis systematically. This system offers insights into novel building blocks for the construction of peptide-assembling platforms and elucidates the biological mechanisms of action and biochemical profiles associated with nanostructure formation, which implicates system-level effects, such as the regulation of energy and redox homeostasis.

In the second chapter, the development of pH/oxidative stress-responsive isopeptide system was described, including the real-time tracking of the assembling processes and illustration of formed nanostructures, hollow spherical nanofibers, in epithelial cells (MDA-MB 231 cells). Phasor-fluorescence lifetime imaging (FLIM) was utilized to map the transformation from monomers to supramolecular assemblies and correlative light-electron microscopy/tomography (CLEM) revealed formation of nanofibers and their fusion with endosomes to finally form hollow fiber clusters. Spatiotemporal splicing of the assembly events shows time-correlated metabolic dysfunction. This study paves the way to understand and visualize the supramolecular processes of nanostructure formation in biology to ultimately address aggregation-based dysfunction in diseases.

Finally, the construction of two amphiphilic pro-assembling peptide sequences protected by a visible light-sensitive cage group was presents. The peptides underwent a cascade of visible light-induced molecular and supramolecular transformations to form nanofibers in living cells. The irradiation with light enable full control over the reaction cascade where the monomer generation and concentration in turn regulates

the assembly kinetics. Phasor-FLIM traced the formation of various assembly states in cells and revealed subsequent out-of-equilibrium dynamics associated with monomer activation and consumption. This study facilitates precise control over supramolecular events at discrete time points, and the new imaging technologies offer deeper insights into the dynamic assembly processes with native cells.

In summary, this thesis introduces a range of methodologies for controlling and imaging intracellular peptide assembly processes and their associated cellular effects. These findings provide valuable insights into supramolecular assembly mechanisms within complex cellular environments and the role of aggregation-based dysfunction in disease.

Zusammenfassung

Peptidbasierte bioresponsive Systeme haben in der supramolekularen Chemie und bei Biomaterialien große Aufmerksamkeit erlangt. Diese Systeme haben die Entwicklung von synthetischen Plattformen in lebenden Zellen ermöglicht und wertvolle Einblicke in die Art und Weise geliefert, wie die Strukturbildung zelluläre Prozesse beeinflusst. In dieser Arbeit werden das Design, die Synthese und die Charakterisierung verschiedener auf Stimuli reagierender Peptidsysteme für die in situ Bildung von intrazellulären Nanostrukturen untersucht und ihre Auswirkungen auf zelluläre Prozesse erforscht.

Im ersten Kapitel wurde ein platin(II)-haltiges Tripeptid so konstruiert, dass es zu intrazellulären fibrillären Nanostrukturen assembliert und Stoffwechselfunktionen wie die aerobe Glykolyse und die oxidative Phosphorylierung stört, wodurch systematisch Apoptose ausgelöst wird. Dieses System bietet Einblicke in neuartige Bausteine für die Konstruktion von Peptid-Assemblierungsplattformen und klärt die biologischen Wirkmechanismen und biochemischen Profile im Zusammenhang mit der Bildung von Nanostrukturen auf, was Auswirkungen auf Systemebene, wie die Regulierung der Energie- und Redox-Homöostase, impliziert.

Im zweiten Kapitel wurde die Entwicklung eines auf pH-Wert und oxidativen Stress reagierenden Isopeptidsystems beschrieben, einschließlich der Echtzeitverfolgung der Assemblierungsprozesse und der Darstellung der gebildeten Nanostrukturen, hohlkugelförmiger Nanofasern, in Epithelzellen (MDA-MB 231 Zellen). Phasor-Fluoreszenz-Lebensdauer-Imaging (FLIM) wurde eingesetzt, um die Umwandlung von Monomeren in supramolekulare Assemblies abzubilden, und korrelative Licht-Elektronen-Mikroskopie/Tomographie (CLEM) zeigte die Bildung von Nanofasern und ihre Fusion mit Endosomen, um schließlich hohle Fasercluster zu bilden. Die räumlich-zeitliche Aufteilung der Assemblierungsereignisse zeigt eine zeitkorrelierte metabolische Dysfunktion. Diese Studie ebnet den Weg zum Verständnis und zur Visualisierung der supramolekularen Prozesse der Nanostrukturbildung in der Biologie, um letztlich die auf Aggregation beruhenden Funktionsstörungen bei Krankheiten anzugehen.

Schließlich wurde die Konstruktion von zwei amphiphilen pro-assemblierenden Peptidsequenzen vorgestellt, die durch eine für sichtbares Licht empfindliche

Schutzgruppe geschützt sind. Die Peptide durchliefen eine Kaskade von durch sichtbares Licht ausgelösten molekularen und supramolekularen Umwandlungen, um in lebenden Zellen Nanofasern zu bilden. Die Bestrahlung mit Licht ermöglicht eine vollständige Kontrolle über die Reaktionskaskade, bei der die Monomererzeugung und -konzentration wiederum die Assemblierungskinetik steuert. Durch Phasor-FLIM wurde die Bildung verschiedener Assemblierungszustände in Zellen verfolgt und zeigte die anschließende Dynamik außerhalb des Gleichgewichts, die mit der Aktivierung und dem Verbrauch von Monomeren verbunden ist. Diese Studie ermöglicht eine präzise Kontrolle supramolekularer Ereignisse zu diskreten Zeitpunkten, und die neuen Bildgebungstechnologien bieten tiefere Einblicke in die dynamischen Assemblierungsprozesse in nativen Zellen.

Zusammenfassend lässt sich sagen, dass in dieser Arbeit eine Reihe von Methoden zur Kontrolle und Darstellung intrazellulärer Peptid-Assemblierungsprozesse und der damit verbundenen zellulären Auswirkungen vorgestellt werden. Diese Erkenntnisse liefern wertvolle Einblicke in die Mechanismen der supramolekularen Assemblierung in komplexen zellulären Umgebungen und in die Rolle von aggregationsbedingten Funktionsstörungen bei Krankheiten.

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1 Introduction

1.1 Assembly of peptides

1.1.1 Supramolecular assembly in Nature

In nature, supramolecular assembly plays an indispensable role in cellular life, where it ubiquitously pervades all biological pathways¹. From DNA replication/transcription, RNA translation to protein maturation/expression, non-covalent forces drive these biomolecular interactions and specific self-assembling nanostructure formation at a fundamental, molecular level (Figure 1.1)². Among them, a particularly diverse class of self-assembling materials are formed from proteins³. From instance, cellular motility and traction to surfaces are largely controlled through the self-assembly of cytoskeletal proteins⁴. These proteins reversibly self-assemble to enable highly regulated extension and contraction of cells, thus allowing their movement. Moreover, networks of such protein assemblies generate force for a wide range of active processes, including cell migration, movement of endocytic vesicles and other membrane-bound organelles within cells, along with intercellular transport of certain bacterial and viral pathogens⁵⁻⁷. As such, supramolecular assembly regulates various biological processes in cells (Figure 1.1).

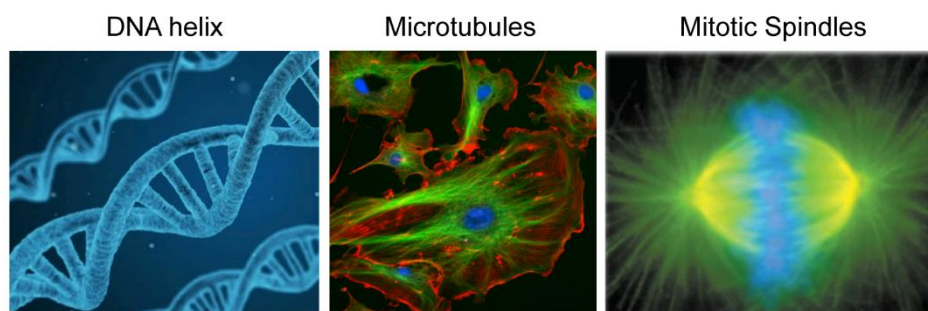


Figure 1.1 Schematic illustration of the natural self-assembly behavior in living systems. Copyright © 2025 The Royal Society

Much progress has been made in understanding the fundamental principles that govern native-protein self-assembly processes by studying naturally occurring building blocks⁸⁻¹¹. Mimicking these naturally assembled structures by integrating synthetic self-assembled nanostructures in a biological context is an important milestone in

supramolecular chemistry, as these systems expand the boundaries of both nanomedicine and synthetic biology. Moreover, the bottom-up approach of developing non-natural assemblies in complex, dynamics intracellular environment could help elucidate molecular mechanisms of naturally occurring cellular processes and provide the foundation of unravel the origin of life.

Even though nature commonly uses protein sequences of up to several hundred amino-acid residues as building blocks of functional materials, substantially shorter sequences can also exhibit highly sophisticated self-assembly behavior^{12, 13}. As such, biomimetic peptide-based motifs, including peptide amphiphiles, lipopeptides and conjugates with other organic and inorganic molecules, exemplify how design and chemistry can be successfully employed to generate multifunctional molecules that assemble predictably and interact with specific biological ligands. The recent emergence of the field of peptide biomimetics, which combines principles from disciplines including biology, chemistry and engineering, allows the preparation of synthetic materials with functions similar to or surpassing those of natural products¹⁴⁻¹⁶. Bio-inspired peptides, especially short peptide building blocks, are minimal recognition modules used to mediate and facilitate processes of molecular recognition and self-assembly¹⁷. The synthesis and chemical modification of these building blocks are facile and they can assemble with remarkable efficiency into biocompatible and controllably biodegradable materials. Because of these advantages, over the past three decades, this progress has generated substantial interest in developing peptide-based nanostructures for novel materials application in biomedicine, drug delivery and tissue engineering^{18, 19}. In particular, peptide-based biomaterials demonstrate significant promise for precision therapy and the discovery of macromolecular drugs²⁰⁻²³. Further discussion on this topic is included in the subsequent chapters.

1.1.2 Supramolecular interactions between peptides

Peptide self-assembly refers to the formation of molecular aggregates with a specific arrangement, driven by various non-covalent interactions^{24, 25}. These non-covalent forces primarily include hydrogen bonding, hydrophobic interactions, π - π interactions, electrostatic interactions, and enabling peptide assemblies to form unique and ordered nanostructures (Figure 1.2)⁵.

Hydrogen bonds is one of the important forces determining the peptide assembly structure of self-assembly process^{24, 26}. Hydrogen bonds can be formed between the amino and carboxyl groups on the main and side chains of peptides. For example, amino acids which contain hydrophilic side chain functional groups like serine, threonine, glutamine and asparagine can interact with each other by forming hydrogen bonds. Meanwhile, hydrogen bonds can also be formed in the peptide backbone by C=O and N-H residues of amide bonds (Figure 1.2). In general, the β -sheet secondary structure is formed through hydrogen bonds perpendicular to adjacent peptide chains. Conversely, the α -helix structure arises from hydrogen bonds between amide-based amino acids within the same peptide chain. Moreover, hydrogen bonds are essential for the formation and stabilization of peptide secondary structures, facilitating the assembly of peptides into various nanostructures.

Hydrophobic interactions are another significant driving force for peptide self-assembly, primarily resulting from the hydrophobic regions on the peptides. When amphiphilic molecules are introduced into water, their hydrophobic segments tend to aggregate in an effort to reduce their contact with water, consequently exposing the hydrophilic segments to the aqueous environment (Figure 1.2). In general, Hydrophobic interactions occur between amino acids with alkyl and aryl side chains, such as alanine (Ala, A), leucine (Leu, L), isoleucine (Ile, I), valine (Val, V), and methionine (Met, M).

Another kind of weak non-covalent interaction for peptide assembly is π - π interactions, which are particularly important for peptides with aromatic groups. Generally, π - π stacking induces specific directional growth in the assemblies by providing driving force and directionality to the self-assembly processes of peptides. Peptides containing aromatic groups typically exhibit poor solubility, leading to the formation of more robust nanostructures in aqueous solutions.

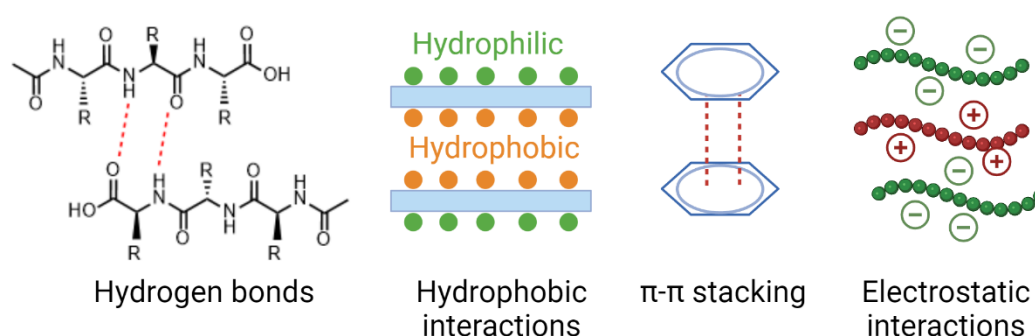


Figure 1.2. Supramolecular interactions between peptides.

In addition to the aforementioned forces, the self-assembly of peptides is also driven by electrostatic interactions between oppositely charged peptide molecules. These interactions influence the self-assembly process through the charged groups of amino acids, leading to either electrostatic attraction or repulsion.

1.1.3 Peptide assembly inside living cells

As discussed before, peptide and protein assembly play an important role in cellular life that can regulate a variety of biological processes. Inspired by these, the assembly of peptides into specific nanostructures within living cells has been extensively investigated as a platform for regulating cellular metabolism and modulating cell behavior, offering a novel approach to the development of synthetic biomaterials for cancer therapy and tissue engineering. However, the living cell is a non-equilibrium system characterized by compartmentalization and the energy-dependent maintenance of concentration gradients that are essential for proliferation and function. As cellular compartments fulfil different tasks within the cell, they often provide distinct (bio)chemical environments, such as a certain pH²⁷⁻³¹, redox environment^{22, 32-35}, level of molecular crowding or the presence of specific enzymes³⁶⁻⁴². Ideally, the assembly process should be controlled by a specific trigger that can be selectively activated at the target site, ensuring the formation of peptide nanostructures at the desired location⁴³. In this context, various examples of peptide assemblies responsive to different stimuli, such as pH, reactive oxygen species (ROS), enzyme, glutathione, light, are discussed below.

The physiological pH in tissues and inside cellular organelles depends on a variety of factors and represents an important parameter for biological processes. Compared with healthy tissue and cells, the tumor microenvironment is characterized by a slightly acidic pH (6.7 - 7.1), which is a result from the enhanced glycolytic rate of cancer cells that leads to hypoxia and acidification of the immediate extracellular matrix⁴⁴. At cellular level, the endocytic pathway is accompanied by a pH gradient ranging from 6.3 in the early endosomes to 5.5 in the late endosomes and 4.7 in the lysosome⁴⁵. These changes in pH during the entry of endosomes can serve as an endogenous trigger for the self-assembly or the morphological transformation of a pH-responsive

nanomaterials^{28, 30, 31, 46}. Based on this, Maruyama et al, reported a construction of pH-induced self-assembly of a peptide amphiphile for selective cell death (Figure 1.3)⁴⁷. They designed a palmitoylated hexapeptide sequence, C₁₆-VVAEEE, containing a Glu-Glu-Glu sequence that acts as a pH-responsive part due to the carboxyl groups of the side chains and the C-terminus. C₁₆-VVAEEE formed a hydrogel below pH 7. The formation of the nanofibrous self-assembly was responsive to a small pH change around pH 7, which was correlated with the cytotoxicity. This study demonstrated the distinct microenvironment inside living cells can be used as a trigger for the intracellular self-assembly of a peptide amphiphile, which provide a new tool to drug discovery.

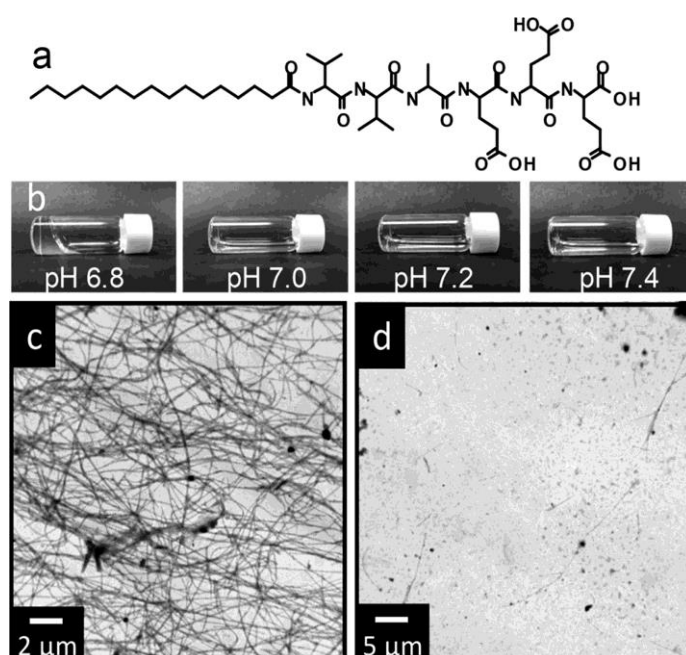


Figure 1.3 (a) Molecular structure of N-palmitoyl-Val-Val-Ala-Glu-Glu-Glu (C₁₆-VVAEEE). (b) Gelation tests of PBS after 24 h at various pH. The concentration of C₁₆-VVAEEE was 0.15 wt %. (c,d) TEM images of the freeze-dried hydrogel and PBS solution of 0.15 wt % C₁₆-VVAEEE. (c) pH 6.8 and (d) pH 7.4. Copyright © 2021 American Chemical Society

Reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), peroxynitrite (ONOO⁻), and the hydroxyl radical (OH[•]), play a critical role as secondary messengers in cellular processes such as cell growth, apoptosis, and migration⁴⁸⁻⁵⁰. ROS are generated during mitochondrial oxidative metabolism, as well as in response to xenobiotics, cytokines, and bacterial invasion. The controlled production of ROS within cells serves essential biological functions, including the regulation of signal transduction pathways involved in processes such as angiogenesis and insulin

metabolism. However, excessive ROS production leads to oxidative stress, which is associated with several pathological conditions, in particular abnormal cell growth that can drive cancer initiation and progression. Therefore, ROS have often been targeted to induce self-assembly, facilitating the formation of supramolecular architectures within cells. For example, our group developed a H_2O_2 -responsive assembly system that underwent a molecular and supramolecular transformation cascade that leading to the formation of nanofibers inside living cells (Figure 1.4)²². Endogenous H_2O_2 facilitates the cleavage of the boronic acid masking group and the intracellular pH-controlled O, N-acyl rearrangement to generate the self-assembling peptide sequence. Nanostructure-driven apoptosis that is characterized by Annexin V binding, demonstrates that programmed cell death can be promoted by the peptide assembly. By combining the supramolecular chemistry and biological results, we demonstrate that the in situ construction of architectures within cells will broaden the community's perspective on how structure formation can impact biological systems.

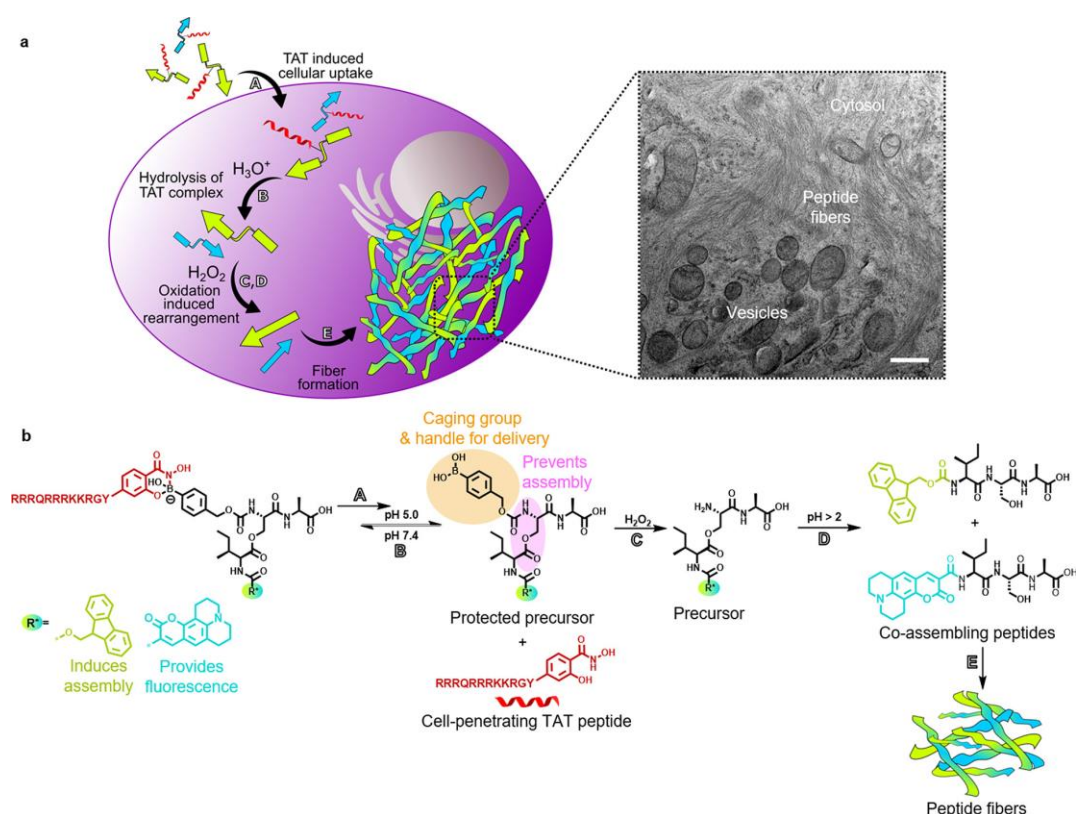


Figure 1.4 Intracellular coassembly of peptides. **a**, Depsipeptides (kinked arrows) are uptaken by cells due to dynamic covalently bound salicylhydroxamate-TAT (SHA-TAT, red coil, step A). After hydrolysis of the complex in an acidic environment (step B), the boronic acid headgroup of the depsipeptides is cleaved by intracellular hydrogen

peroxide (step C). Subsequent O, N-acyl shift forms the linear coassembling peptides (step D). Linear peptides (straight arrows) form fibrillar networks inside A549 cells (step E), which are visible by transmission electron microscopy (TEM, scale bar 500 nm). **b**, Chemical reactions that lead to cellular uptake, peptide linearization, and peptide coassembly of Fmoc (green) and coumarin 343 (blue) functionalized ISA. Copyright © 2020 American Chemical Society.

Enzymes are essential to cellular metabolism, owing to their remarkable specificity and catalytic efficiency. They play an integral role in facilitating almost all biochemical reactions within cells. In cancer cells, specific enzymes are frequently overexpressed or demonstrate altered activities compared to those in normal cells. Based on this, enzyme-specific recognition motifs have been extensively utilized in the chemical design of synthetic precursors to generate corresponding assembly monomers, which, subsequently facilitate the formation of nanostructures within cells. For example, various types of enzymes, including alkaline phosphatases (ALPs), furin and esterases, have been used to activate the structure formation processes in living cells. Xu et al, reported a concept of inserting phosphorylated tyrosine (pY) into amphiphilic peptide motifs and showed that the polarity shift upon dephosphorylation by ALP produces nanofibers on the surface of cancer cells (Saos2)⁵¹. The formed assemblies can enter the cells via endocytosis and the TPP group on the molecule facilitates the transport to the mitochondria, therefore resulting in cell death (Figure 1.5a). Bulte et al, also designed an intracellular self-assembly platform through furin-triggered cleavage and GSH-induced activation to form olsalazine nanoparticles for enhanced magnetic resonance imaging and tumor therapy (Figure 1.6a, b)⁵². Liu et al, described an intracellular polymerization strategy via the cathepsin B and GSH-triggered cleavage that can simultaneously promote the accumulation and retention of the AIEgen in the tumor for prolonged imaging and enhanced tumor growth inhibition (Figure 1.6c)⁵³. In addition, Sun et al, presented a novel strategy to program supramolecular self-assembly specifically in mitochondria by leveraging on a unique enzyme SIRT5 (Figure 1.5b, c, d)⁵⁴. The SIRT5-induced cleavage activated the precursors and facilitated the subsequent self-assembly process, thereby inducing cellular apoptosis.

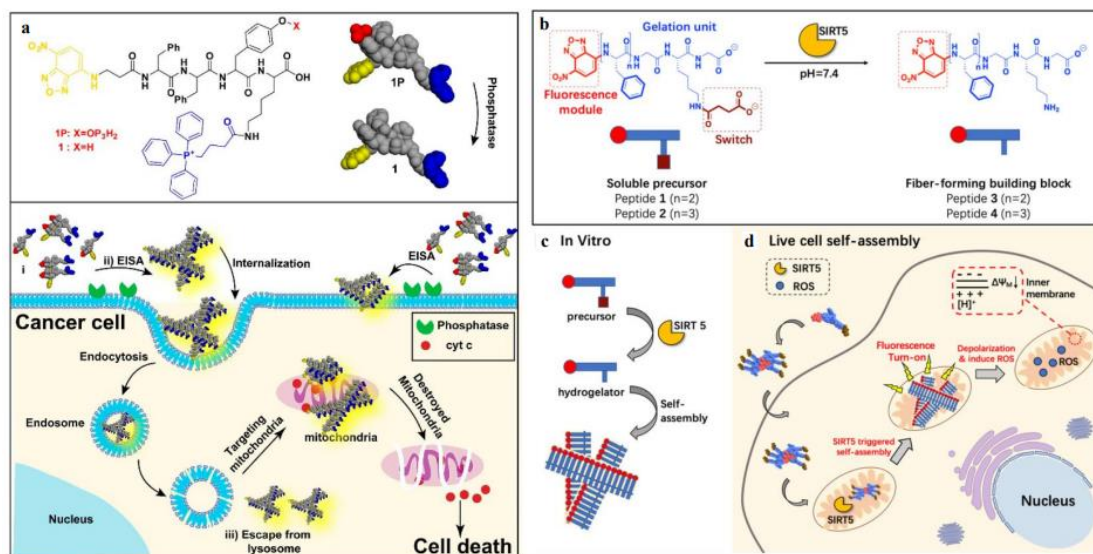
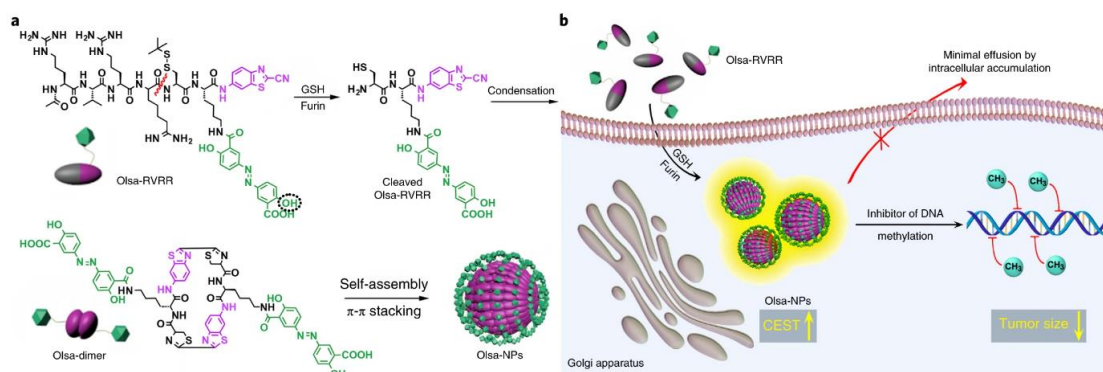


Figure 1.5 a, Schematic representation of intracellular nanofiber formation on the surface of cancer cells and subsequent localization on mitochondria, inducing cell death. (b-d) Mitochondria-restricted self-assembly of peptide nanofibers catalyzed by SIRT5. b, Molecular structures of precursors and fiber-forming building blocks. Peptide precursors 1 and 2 can be desuccinylated by SIRT5 to produce fiber-forming building blocks 3 and 4, respectively. c, Schematic representation of the self-assembly process triggered by SIRT5 in vitro. d, Schematic representation of intracellular fiber formation in mitochondria via specific interaction of internalized peptide precursors with SIRT5 enzyme. Copyright © 2020 American Chemical Society. Copyright © 2016 American Chemical Society.



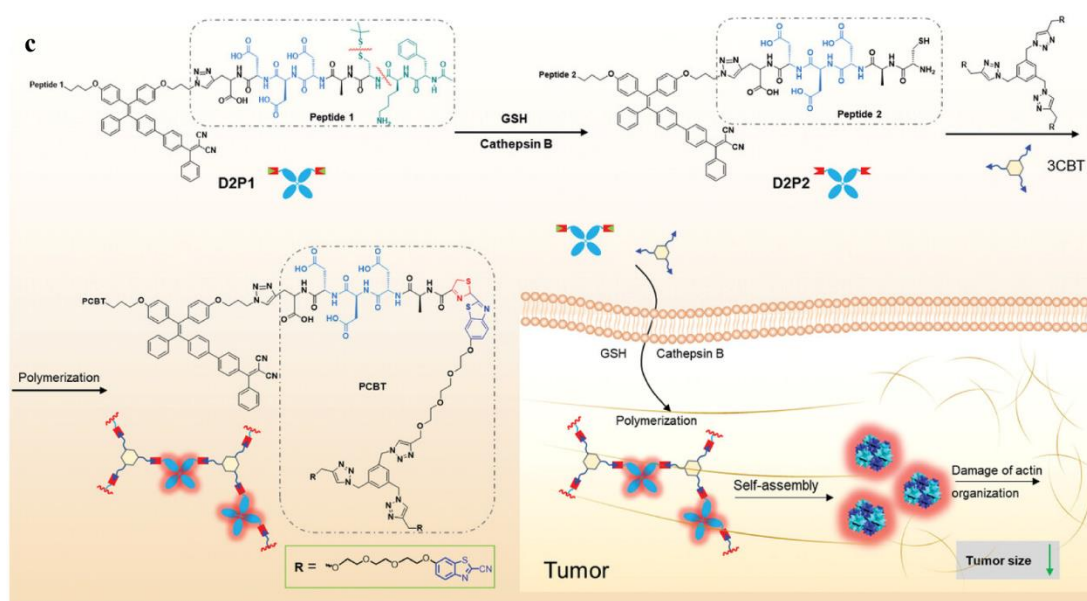


Figure 1.6 Schematic illustration of a furin-mediated and cathepsin B-mediated intracellular self-assembly platform to form olsalazine nanoparticles for enhanced magnetic resonance imaging and tumor therapy. **a**, Chemical design and **b**, schematic illustration of furin-mediated self-assembly platform. **c**, Schematic illustration for the formation of AIEgen-based nanostructures by enzyme-mediated intracellular reduction and condensation of D2P1 and 3CBT, resulting in enhanced fluorescence signal and tumor treatment efficacy. Copyright © 2021 Wiley-VCH GmbH.

Glutathione (GSH), composed of three amino acids: glutamate, cysteine and glycine, is the most prevalent non-protein thiol in mammalian cells and the most abundant low molecular-weight peptide present in eukaryotic cells^{55, 56}. Acting as a reducing agent, a nucleophile and a cell-protective antioxidant, GSH plays an important role in maintaining redox homeostasis⁵⁷. Compared to healthy cells, many cancer cells exhibit elevated levels of intracellular GSH that correlates with higher cellular proliferation and metastatic activity, as well as multidrug resistance caused by an increased efflux of glutathionylated chemotherapeutics^{58, 59}. Therefore, due to its ubiquity, GSH has been frequently exploited as endogenous stimulus for controlled intracellular self-assembly^{32, 35, 60, 61}. For instance, Yu et al. designed a GSH-responsive self-sorting system that promotes the formation of isolated nanofibrils through the cleavage of disulfide-linked hydrophilic domains or the reduction of selenoxide groups (Figure 1.7)⁶². Incorporation of thiol and ER-targeting moieties into these self-sorted assemblies enables simultaneous targeting of both the endoplasmic reticulum and the Golgi apparatus, resulting in combinatorial organelle dysfunction and subsequent cell death. These

findings demonstrate the in situ formation of self-sorting peptide assemblies within living cells, offering a novel platform for targeted drug delivery and presenting an alternative strategy for modulating biological processes.

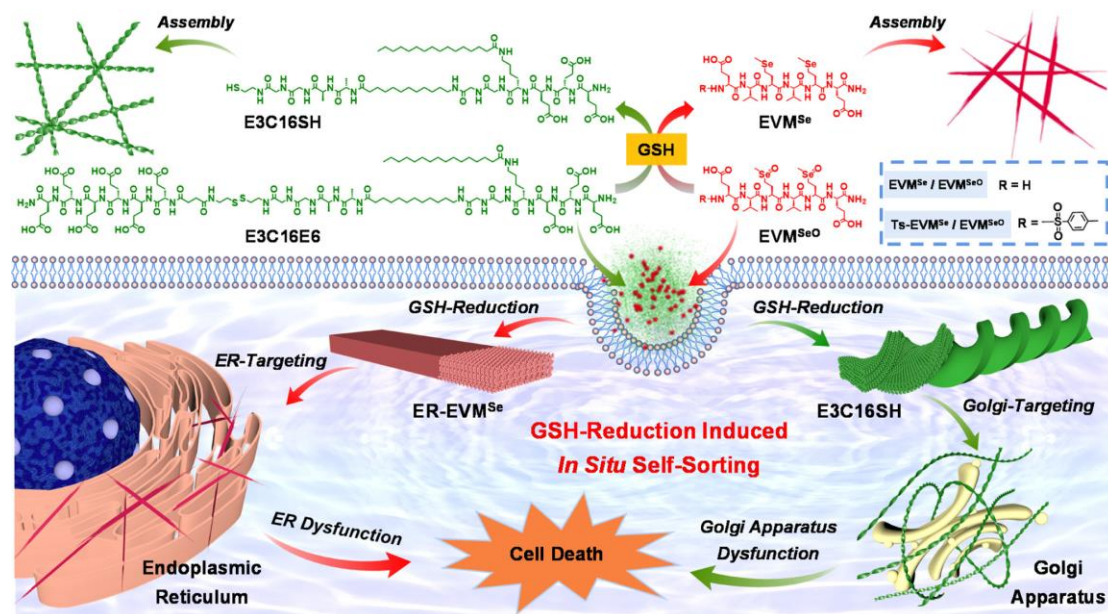


Figure 1.7 Schematic illustration of the concept of self-sorting peptide assembly system. Top: Chemical structures of peptides E3C16E6 and ER-EVMSeO and their corresponding GSH-reduced counterparts assembling into twisted nanofibrils and flat nanoribbons, respectively. Bottom: In situ self-sorting of the internalized peptides E3C16E6 and ER-EVMSeO induced by GSH reduction for the formation of peptide assemblies simultaneously targeting the ER and Golgi apparatus to promote organelle dysfunction and subsequent cell death. Copyright © 2022 American Chemical Society

In addition to the aforementioned endogenous stimuli, light, as an exogenous stimulus, provides energy to initiate photoreactions without significantly interacting with most cellular biomolecules, allowing precise spatiotemporal control over self-assembly processes within living cells. Wang et al. reported a light-triggered molecule that enabled spatiotemporal control of self-assembly morphology within living cells (Figure 1.8)⁶³. The platform consisted of a light-controlled unit, 3-methylene-2- (quinolin-8-yl) isoindolin-1-one, which could alter the hydrophilic/lipophilic balance by regulating molecular conformation from nanoparticles to nanofibers, and a peptide capable of rapid reassembly through internal hydrogen bonding interactions. Nanoparticles containing this light-responsive unit underwent morphological transformation on the cell surface and within lysosomes upon irradiation. The transformation disrupted the

lysosomal membrane, induced cell apoptosis, thereby enhanced cisplatin sensitization.

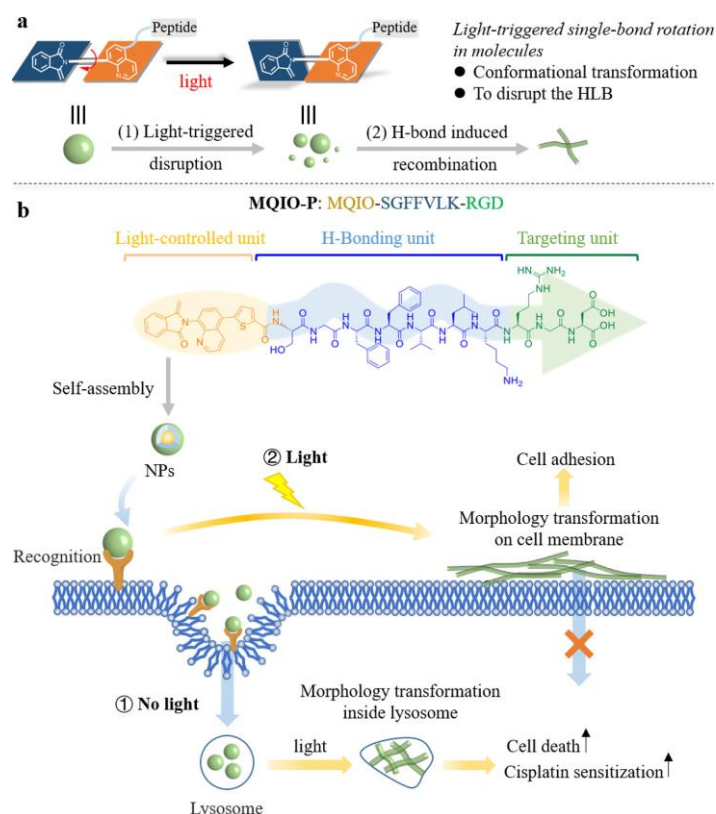


Figure 1.8 Schematic illustration diagram of light-triggered self-assembly and transformation by molecular conformation changes and H-bond interactions. (a) Illustration diagram for single-bond rotation in molecules and morphology transformation from nanoparticles to nanofibers induced by light. (b) Molecular structure of MQIO-P and its self-assembly and transformation process formed by light on the cell surface and inside lysosome. Reproduced with permission. Copyright 2022, American Chemical Society.

In summary, peptide-based molecules have demonstrated immense potential in the design of controlled supramolecular platforms for intracellular nanostructure formation. However, several critical challenges and knowledge gaps remain to be addressed. For instance, achieving more precise control over assembly behavior and regulating the diverse assembly species is essential for gaining a deeper understanding of the relationship between assembly behavior and their biological effects. Besides, regulating more complex dynamic assembly processes, such as controlling nanostructure formation with varying morphologies or enabling reversible, rather than unidirectional assembly processes, is important for understanding the dynamic

processes in Nature, such as the assembly and disassembly of proteins and DNA. Furthermore, the development of assembly systems targeting different subcellular compartments will enhance our understanding of assembly-driven biological functions.

1.2 Photoactivatable systems

1.2.1 Photoactivatable groups

Photoactivatable protecting groups (PPGs), also referred to as photoremovable, photoreleasable, or photocleavable groups, are widely employed to enable precise, non-invasive spatiotemporal control over the release of various target molecules, including biologically active compounds, synthetic precursors, fluorescent probes, polymerization initiators, fragrances, and gasotransmitters^{64, 65}. As such, they represent a significant and widely utilized application of photochemistry across various fields of research. The first reported PPGs were introduced in the early works of Barltrop⁶⁶, Barton^{67, 68}, Woodward⁶⁹, and Sheehan⁷⁰, and their first biological applications were presented by Engels and Schlaeger⁷¹ and Kaplan⁷² and co-workers. Since then, a myriad of photoactivatable molecules and systems in the fields of chemistry, biology, medicine and their related disciplines have been developed. Among them, many PPGs including *o*-nitrobenzyl, arylmethyl, coumarin, pyrenylene, xanthene, BODIPY and their derivatives, have been designed and developed as phototriggers with chemical reactivity and photosensitivity (Figure 1.9).

The key criteria for the design and application of PPGs, are often tailored to specific use cases. However, in general, a PPG must meet the following requirements: (a) it should exhibit sufficient absorption of the irradiated light, ensuring that no other molecules in the system absorb the light or cause undesired photochemical reactions; (b) it should release the protected species within a time-frame suitable for the intended application; (c) it should not generate reactive or toxic by-products upon irradiation; (d) it must be soluble and stable in the target medium or environment, such as aqueous solutions typically used in biological or medical contexts; and (e) it should be detectable in the medium, for instance, through light emission⁷³.

In this section, several typical PPGs, including nitroaryl groups, coumarin groups, arylmethyl groups, xanthene groups, BODIPY groups, as well as their photolysis

mechanisms, will be described, respectively.

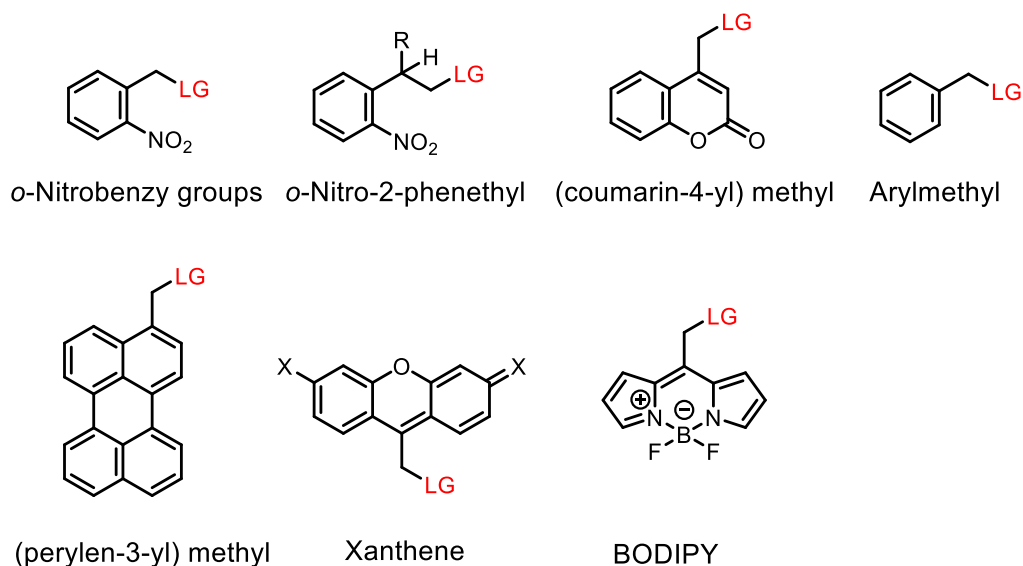


Figure 1.9 Chemical structures of representative organic photoactivatable compounds. LG: leaving groups.

1.2.1.1 Nitroaryl groups

The nitroaryl group has demonstrated its versatility as a valuable scaffold in the design of photoremovable protecting groups (PPGs), giving rise to multiple structural families such as *o*-nitrobenzyl, *o*-nitro-2-phenethyl, and *o*-nitroanilide derivatives. To improve its efficiency, biocompatibility, and practical applications of this molecule in various fields such as photopharmacology, biological imaging, and material science, many efforts have been focused to bathochromically shift the absorption spectra of *o*-nitrobenzyl and *o*-nitro-2-phenethyl PPGs toward the visible part of the spectrum⁷⁴⁻⁷⁶. The absorption spectra of some representative nitroaryl PPGs (Figure 1.10)⁷³.

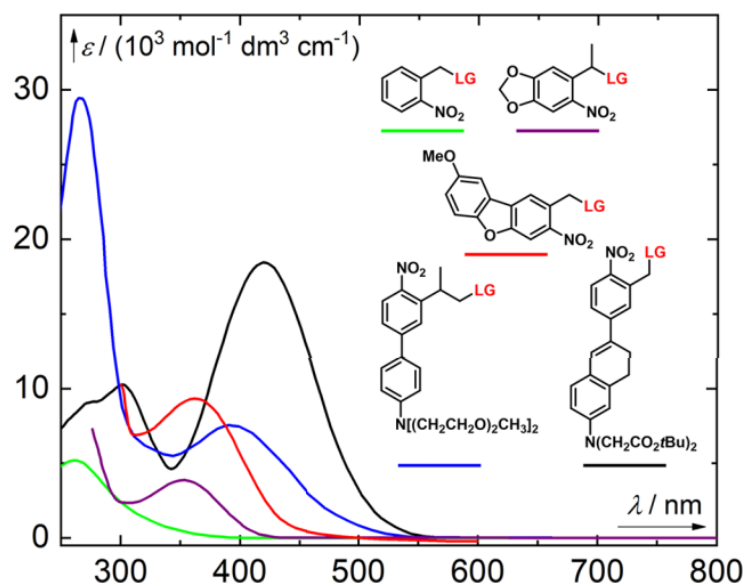


Figure 1.10 Absorption spectra of selected nitroaryl PPGs. Copyright © 2012 American Chemical Society.

The *o*-Nitrobenzyl derivative (*o*NB) make up a library of general-purpose PPGs that have been developed since the 1960s and are still widely utilized currently^{69, 77}. Their photolysis mechanism has been studied extensively (Figure 1.11)⁷⁸⁻⁸³. In general, the excitation of the ground state of an *o*NB derivative (**1**) is followed by intramolecular hydrogen abstraction by the nitro group to form an *aci*-nitro intermediate (**2**). The decay rate constant of the *aci*-nitro intermediate depends on the substitution of the *o*NB group, the solvent, and the pH. An irreversible cyclization of the *aci*-nitro intermediate the result in the formation of a 1,3-dihydrobenz[*c*]isoxazol-1-ol (**3**). The subsequent ring-opening leads to a hemiacetal intermediate that hydrolyzes to release the leaving group (LG) and form an *o*-nitrosobenzaldehyde byproduct (**4**). It has proven that the photorelease of many functional groups including carboxylic acids, phosphates, thiols, alcohols, and amines are feasible, even though alcohols and amines are typically attached as a carbonic acid derivative^{71, 76, 84, 85}.

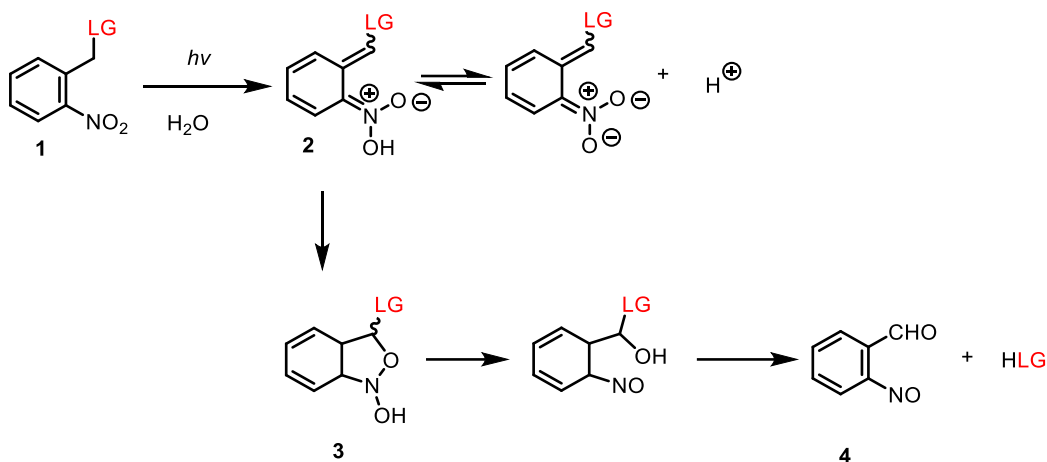


Figure 1.11 Proposed photoreaction mechanism of *o*-Nitrobenzyl PPGs.

In addition to the *o*-Nitrobenzyl (*o*NB) groups, nitroaryl PPGs have another separate typical class, including the 1-(2-nitrophenyl) ethyloxycarbonyl (NPEOC) group⁸⁶ and its α -methyl analog (NPPOC; the “OC” stands for the $-\text{OC}(=\text{O})$ group, which is typically a part of the LG)⁸⁷. Even though this class is similar to *o*NB in chemical structure, the proposed photolysis mechanism of *o*-nitro-2-phenethyl derivatives is significantly different, referring to a photoinduced elimination step (Figure 1.12)⁸⁶.

Compared to the *o*NB analogues, this class possess much higher quantum yields, which makes it a good candidate in automated light-mediated oligonucleotide synthesis (DNA-chips)^{88, 89}, the preparation of peptide and RNA microarrays⁹⁰⁻⁹⁴, the synthesis of aptamers and carbohydrates, and gene assembly⁹⁵⁻⁹⁷.

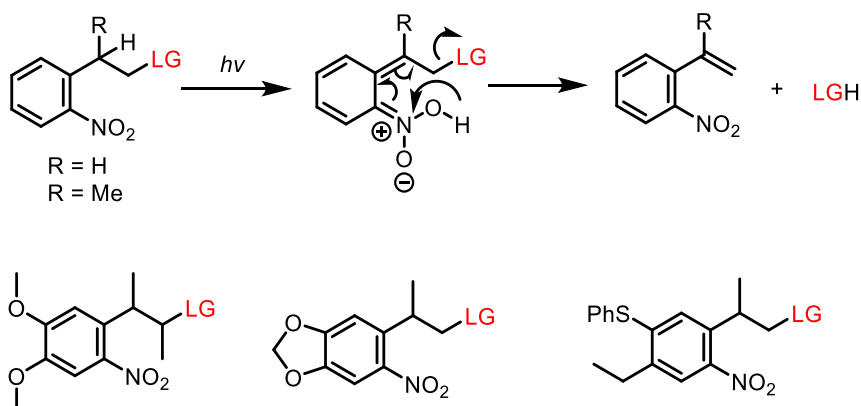


Figure 1.12 Photolysis mechanism of *o*-Nitro-2-phenethyl PPGs and representative modified *o*-Nitro-2-phenethyl PPGs.

1.2.1.2 The (coumarin-4-yl) methyl groups

Coumarin (2*H*-chromen-2-on) is a secondary metabolite found in many plants that was first isolated from the Tonka bean, known in French as coumarou, in 1820⁹⁸⁻¹⁰⁰. The development of coumarins as a new class of photoresponsive protecting groups start with the discovery of Givens and Matuszewski that the (coumarin-4-yl) methyl group presents photoreactivity, enabling the controllable release of phosphate esters (Figure 1.13)¹⁰¹.

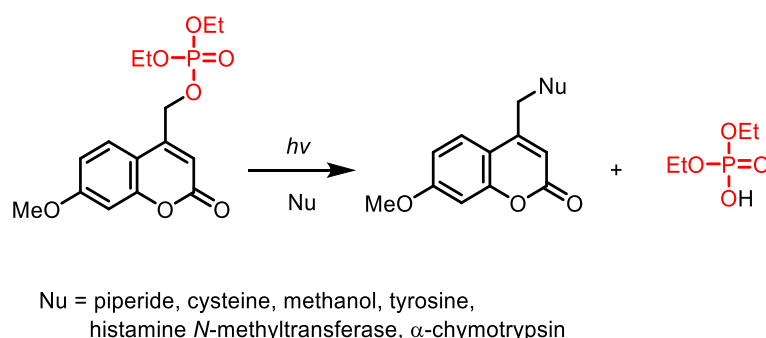


Figure 1.13 Release of phosphate from 7-Methoxycoumarin

The mechanism of photolysis from (coumarin-4-yl) methyl derivatives has been comprehensively explored¹⁰²⁻¹⁰⁵. In general, the cleavage of a heterolytic C–X bond happens from the lowest $^1\pi, \pi^*$ singlet excited state, while competing processes such as non-radiative decay and fluorescence emission reduce its efficiency (Figure 1.14). A tight ion pair (TIP) was proposed to be the key intermediate in this process; the (coumarin-4-yl) methyl cation in this pair could react directly with potential nucleophiles or solvents to result in the formation of a new stable (coumarin-4-yl) methyl product. Recombination of the TIP to regenerate the ground-state caged derivative would be a competing non-radiative pathway in this mechanism.

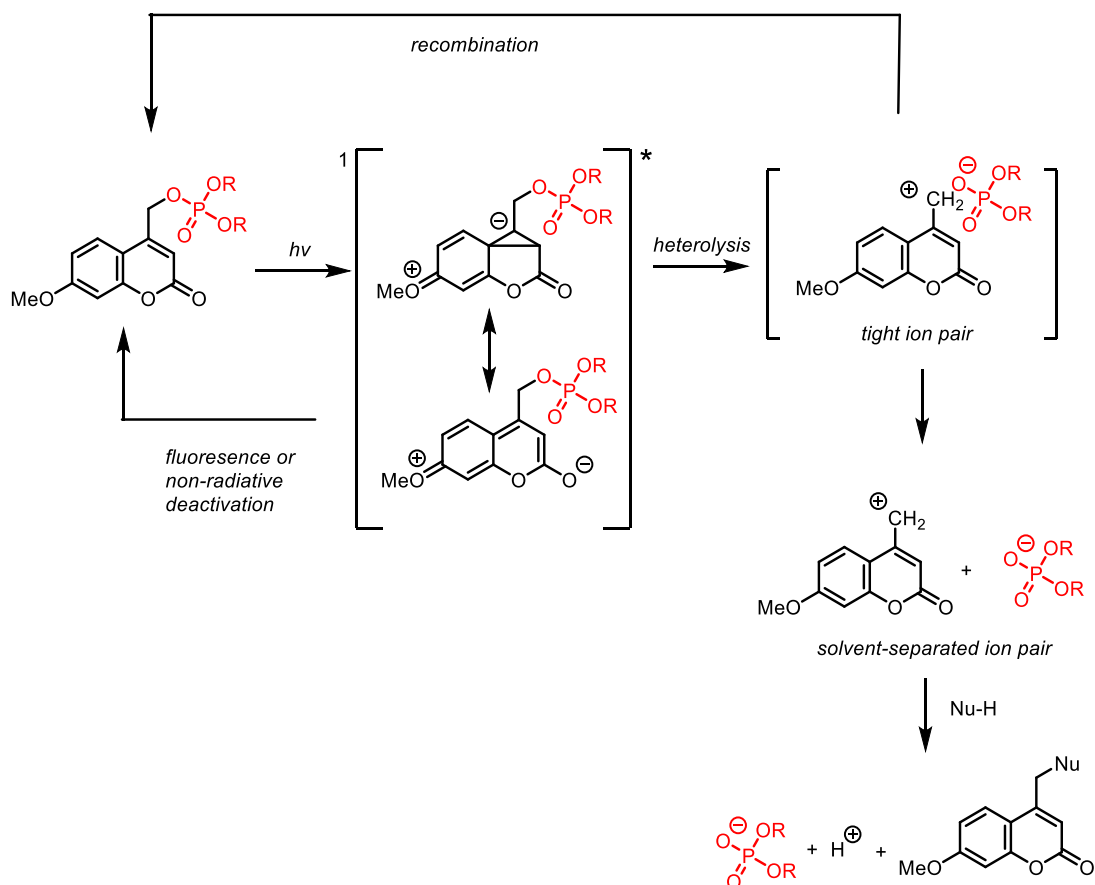


Figure 1.14 Photocleavage mechanism of (coumarin-4-yl) methyl-caged photocages

Compared to other PPGs, in general, coumarin-based PPGs provide various advantages: (1) high molar absorption coefficients at wavelengths above 350 nm, (2) high photolysis efficiencies, (3) comparable stabilities in the dark, (4) adjustable photolysis kinetics, and (5) practically useful 2-photon excitation cross-sections. Furthermore, from the perspective of the molecular structure, the coumarin scaffold could be easily modified by varying the substituents on the coumarin ring and therefore, their spectroscopic, photochemical, and other relevant properties (e.g., solubility and conjugation) can easily be tuned (Figure 1.16). For example, the modifications of the substituents at C6 and C7 in the coumaryl group can address low water solubility, typical of the first generation 7-alkoxycoumaryl PPG, and also help to extend the absorption maximum of the group into the biologically benign range (≥ 350 nm) (Figure 1.15). Therefore, the introduction of an additional alkoxy group at C6 in the structure of parent of the parent 7-alkoxycoumarinylmethanol leads to a shift of the absorption maximum by 30 nm. For the second generation, the introduction of 7-amino substituents further improved the spectroscopic and photochemical properties of the compounds, pushing the absorption maxima to 350-400 nm and presenting the highest

quantum yields among these analogues. Moreover, the water solubility can be further improved by installing polar groups such as carboxylates to the aniline moiety. Polyaromatic analogues were also synthesized to improve fluorescent properties of the cage for use both as fluorescent tags and as PPGs.

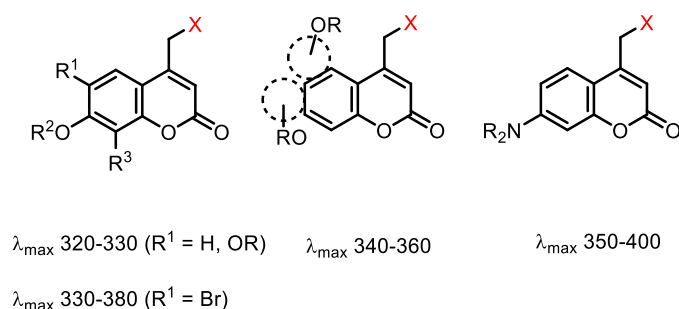


Figure 1.15 Modification strategies for improve the water solubility and spectroscopic properties of coumarin-based PPGs.

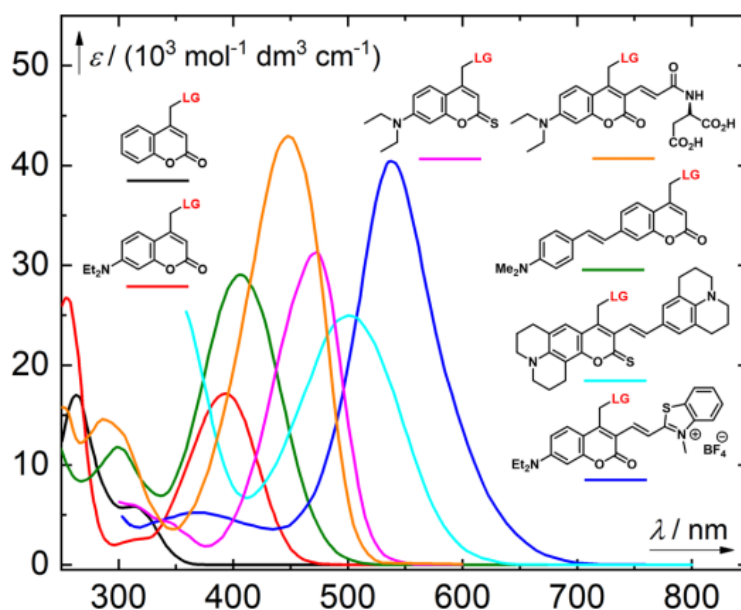


Figure 1.16 Absorption spectra of selected (coumarin-4-yl) methyl PPGs. LG: leaving group. Copyright © 2012 American Chemical Society.

1.2.1.3 Arylmethyl groups

Polyaromatic cores provide chemical platforms for engineering π -extended arylmethyl PPGs. This family of photo-responsive protecting groups is based on the photochemically induced hydrolysis (or less commonly alcoholysis) of benzyl or

heterobenzyl esters or ethers (Figure 1.17).

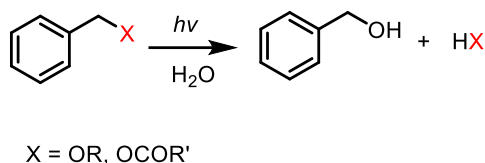


Figure 1.17 Photochemical cleavage mechanism of benzyl protection.

From the perspective of the photolysis reaction mechanism, this process can proceed via initial light-induced homolysis of the C-O bond, followed by rapid electron transfer to produce an ion pair (Figure 1.18). The ion pair is then trapped by the solvent to yield the final products. The mechanism above is the predominant pathway in the photolysis reaction of simple benzyl derivatives¹⁰⁶. However, photolysis of benzyl esters is often accompanied by the formation of radical products¹⁰⁷⁻¹⁰⁹.

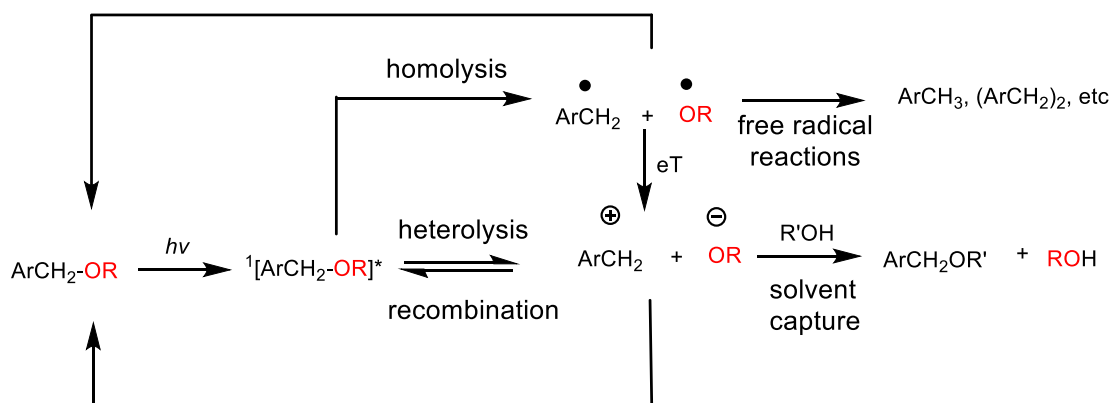


Figure 1.18 Photochemistry of arylmethyl ethers.

For example, the (scridin-9-yl) methyl group (**1**) was introduced by Zhang and co-workers as a UV- activatable PPG for alcohols¹¹⁰ and was later used with carboxylic acids (Figure 1.19)¹¹¹⁻¹¹³. The photolysis mechanism was proposed to proceed through an ion-pair intermediate. Singh et al. also developed a similar system, the π -extended [benzo(a)acridin-12-yl] methyl derivative (**2**), which exhibits a bathochromic shift of ~ 20 nm ($\lambda_{\text{max}} \approx 374$ nm, $\epsilon_{\text{max}} \approx 5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) and extended absorption up to ~ 425 nm¹¹⁴.

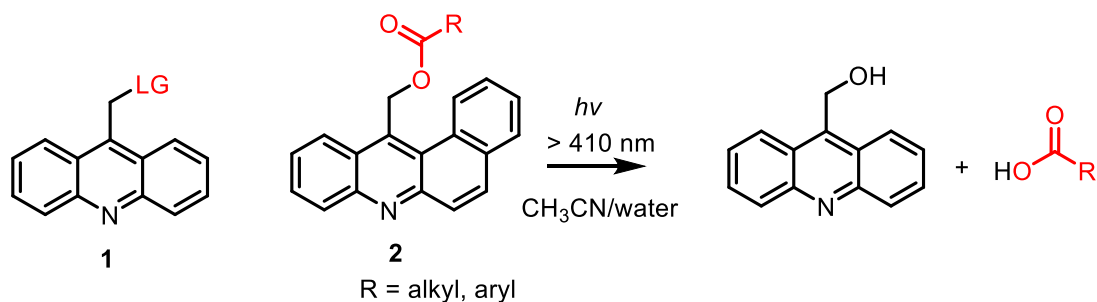


Figure 1.19 Photochemistry of (Acridin-9-yl) methyl PPGs.

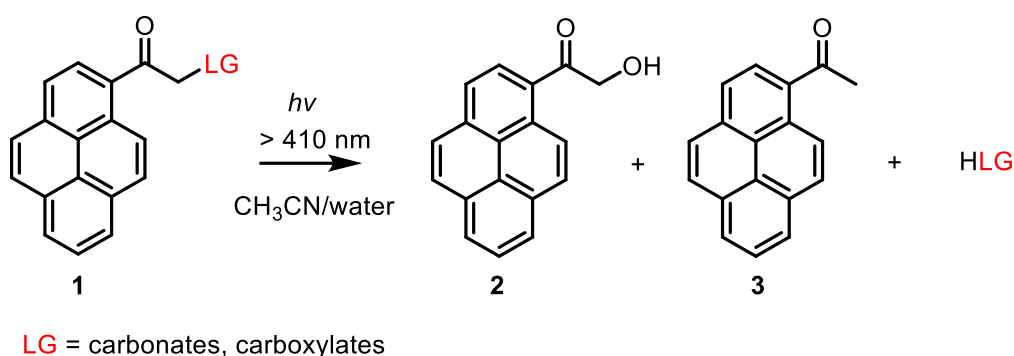


Figure 1.20 Photochemistry of 1- (Hydroxyacetyl) pyrene PPG

Furthermore, 1-(hydroxyacetyl) - pyrene was introduced by Singh and co-workers as a variant of the well-established (pyren-1-yl) methyl PPG. In contrast to (pyren-1-yl) methyl, which absorbs only in the UV region, the addition of a hydroxyacetyl group in (**1**) (Figure 1.20) caused a bathochromic shift of λ_{abs} (~ 355 nm), resulting in sufficient absorption above 400 nm ($\epsilon_{410} = 2.7\text{--}3.9 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) to enable visible light-induced photolysis¹¹⁵. The photorelease ($\lambda_{\text{irr}} \geq 410$ nm) of carboxylic acids^{115, 116} and alcohols¹¹⁷ (as carbonates) in a 1:1 acetonitrile/ H_2O solution proceeded with near-quantitative chemical yields ($>94\%$) and high quantum efficiencies ($\Phi_r = 0.30\text{--}0.41$ and $0.17\text{--}0.20$, respectively) (Figure 1.20). These results can be compared to those achieved with a (pyren-1-yl) methyl group, which released carboxylic acids and alcohols with (solvent dependent) $\Phi_r = 0.0029\text{--}0.139$ upon excitation at 350 nm¹¹⁸⁻¹²⁰.

Another arylmethyl- type PPG studied by Singh group, which absorbs in the visible range ($\lambda_{\text{abs}} = 438$ nm, $\epsilon_{\text{max}} = 2.4\text{--}3.5 \times 10^4$) and presents characteristic fluorescence ($\lambda_{\text{max}} = 445$ nm, $\Phi_F = 0.9$), is the (perylene-3-yl) methyl group (**1**) (Figure 1.21)¹²¹. In an acetonitrile/ H_2O (3:1) solution, carboxylic acids and alcohols (attached as carbonates) can be successfully photoreleased from (**1**) with high chemical yields ($>89\%$) and moderate quantum efficiencies ($\Phi_r = 7.7\text{--}9.3 \times 10^{-2}$).

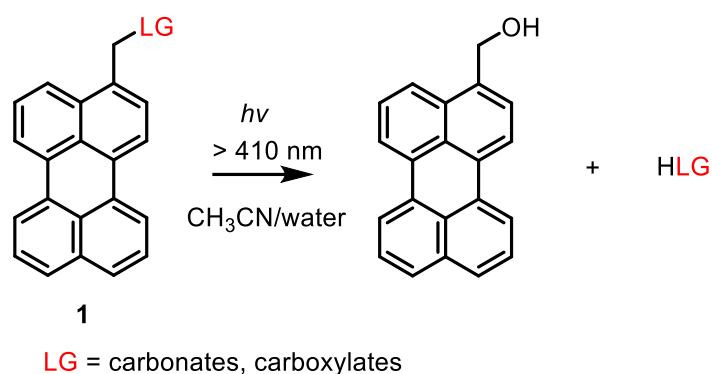


Figure 1.21 Photochemistry of the (Perylen-3-yl) methyl PPG

1.2.1.4 Xanthene and pyronin groups

Xanthene is the basic skeleton of a large class of fluorescent substances, dyes, synthetic drugs¹²². Wirz and co-workers have demonstrated that acetate and diethyl phosphate can be released from a 1:1 (molar ratio) complex of (**1**) with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) upon irradiation with visible light (over 500 nm) (Figure 1.22)¹²³. The potential photolysis mechanism showed that the primary photoproduct in aqueous solutions, a *meso*-methylhydroxy derivative formed from the corresponding cationic intermediate, is further oxidized by DDQ to release 6-hydroxy-3-oxo-3H-xanthene-9-carboxylic acid (**3**) as the major photoproduct. Hückel's Molecular Orbital (HMO) calculations of the xanthene frontier molecular orbitals presented that the HOMO and LUMO are disjoint at the *meso*-position, leading to an increase in its negative charge upon HOMO-LUMO excitation. The phenomena were linked to specific photoreactivity such as the extrusion of the leaving groups¹²⁴.

Another novel class of pyronin analogue (**1**) (Figure 1.23), placing a 1,3-dithian-2-yl substituent at the *meso*-position of pyronin, gave a stable photoproduct that absorbed at 382 nm and emitted at 448 nm¹²⁵. It has been proved that the transformation of (**1**) is a multi-photon, multi-step process. Specific intermediates in this process were identified and their structural relationships were defined using steady-state and time-resolved optical spectroscopy, mass spectrometry, and NMR spectroscopy. The simplified mechanism for this complex reaction demonstrated that the process involves at least three light-initiated steps (Figure 1.23)^{126, 127}.

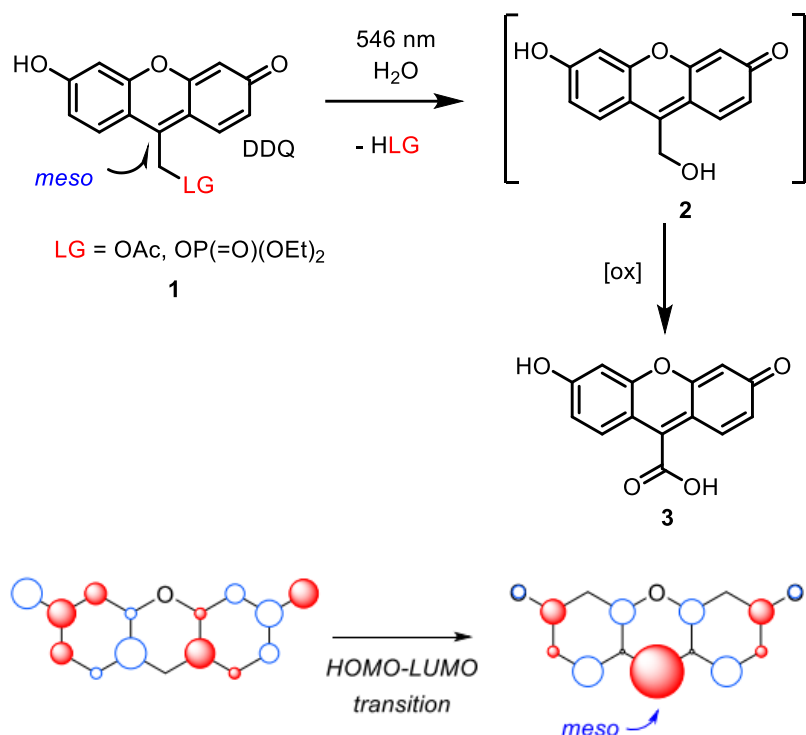


Figure 1.22 Photochemistry of Xanthene PPG and Schematic Representation of the HOMO and LUMO of its π -system. Copyright © 2012 American Chemical Society.

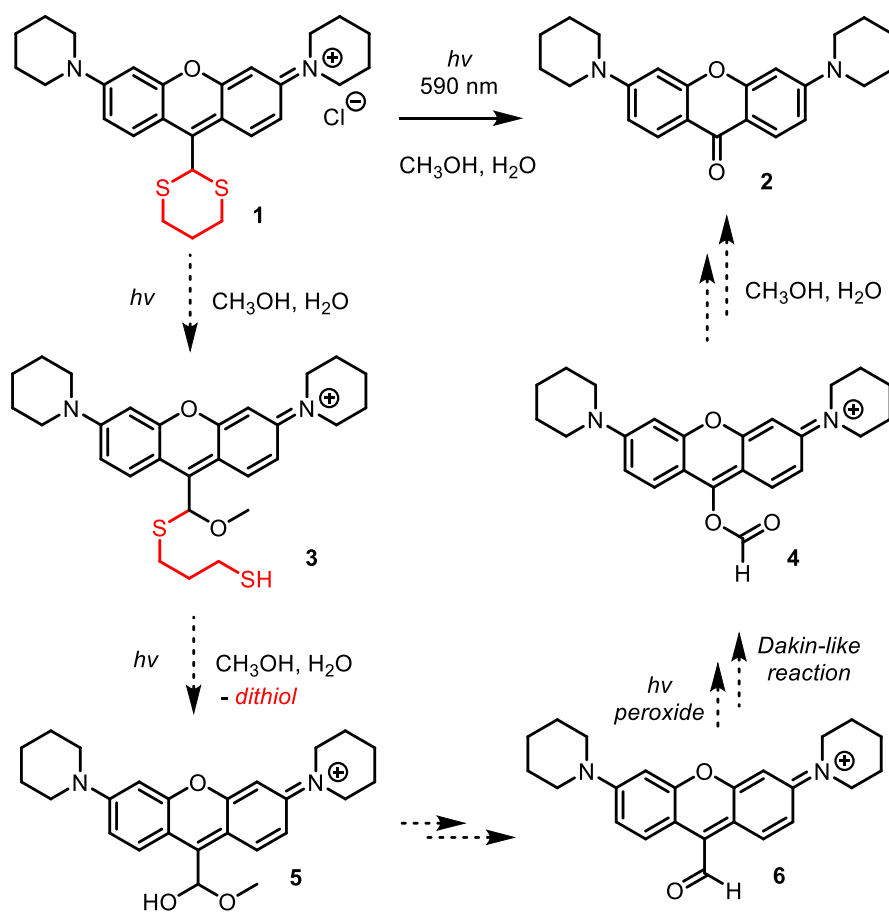


Figure 1.23 Proposed photochemistry mechanism of Pyronin 1.

1.2.1.5 BODIPY groups

4, 4-Difluoro-4-bora-3a, 4a-diaza-s-indacene (BODIPY) and its derivatives are highly fluorescent dyes and are widely used in chemistry¹²⁸⁻¹³² and chemical biology¹³³⁻¹³⁷. This molecule provides advantages of strong absorption, high photostability, low phototoxicity, tunability, and versatility in biological systems, making it an attractive option for controlled release applications in both chemical and biological research. Their favorable spectroscopic properties are mainly due to their rigid and planar chemical structures, which make the potential energy surfaces of their S_0 and S_1 states quite similar. Consequently, narrow Gaussian-shaped absorption and emission bands are typically observed for their lowest energy transition^{138, 139}. These families have high Φ_{HF} values (typically >0.5) because of inefficient non-radiative decay and intersystem crossing (ISC)¹³⁹⁻¹⁴¹. Simple BODIPY chromophores typically possess two narrow bands in the visible range ($\lambda_{max} \approx 490-540$ nm, $\epsilon \approx 3-9 \times 10^4$ M⁻¹ cm⁻¹) and an intense 0-0 band due to S_0-S_1 the transition, and a shoulder attributed to the 0-1 vibrational transition¹⁴²⁻¹⁴⁶. These favorable properties make this chromophore an effective photoactivatable group.

In general, the BODIPY chromophore can be used as a photorelease system with different strategies by introducing LGs at different positions (Figure 1.24). Among them, *meso*-methyl BODIPY derivatives are most widely used due to its facile synthesis and tunable spectral properties^{147, 148}, therefore, this chapter will primarily focus on the discussion of *meso*-methyl BODIPY as a PPG.

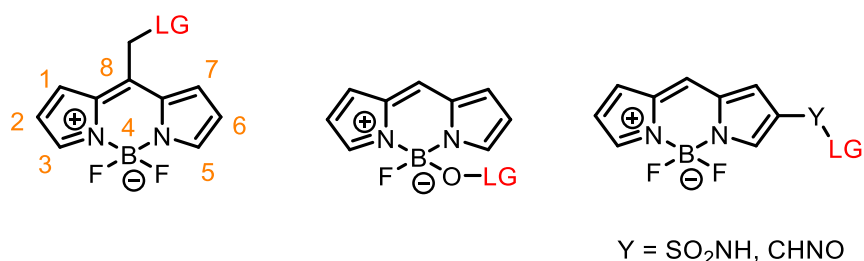


Figure 1.24 Structures of BODIPY PPGs with LGs at different positions.

The release of leaving groups from *meso*-methyl BODIPY derivatives upon

photoexcitation with green light has been well studied by the groups of Winter and Weinstain¹⁴⁸ (a general structure is shown in Figure 1.25). The basis for this photoreactivity can be traced to the properties of the chromophore's *meso* position. Heterolytic photodissociation to give a *meso*-methyl carbocation is favored when electronic excitation to the lowest singlet-excited state involves a substantial transfer of electron density to the sp^2 carbon atom of a chromophore bearing a LG in the α -position such that the antibonding σ^* orbital is mixed with the chromophore's LUMO¹²⁴. In such cases, the promotion of an electron from the HOMO to the LUMO weakens the C–LG bond, facilitating heterolytic dissociation of the leaving group. This mechanism is responsible for, among other things, Zimmerman's *meta*-effect in the photosolvolysis of methoxybenzyl acetates¹⁴⁹ and the photochemical reactivity of coumarinyl-4-methyl PPGs. HMO calculations revealed that BODIPY also reacts in this way because its HOMO and LUMO are disjoint at the *meso* position, and the negative charge at this position increases upon excitation¹³⁹ (Figure 1.25), as also predicted for xanthene chromophores. The *meso*-Methyl carbocations were reported to have a low-lying excited state, suggesting a near degenerate diradical configuration¹⁴⁷. Such low-energy diradicals can have a close-lying conical intersection between the closed-shell singlet and singlet diradical forms¹⁵⁰. The photoheterolysis of C–LG bonds to generate ion pairs was shown to be favored when the ion pair has access to a nearby productive conical intersection that provides an efficient channel for the excited state of the precursor to decay to the ground-state ion pair^{150, 151}.

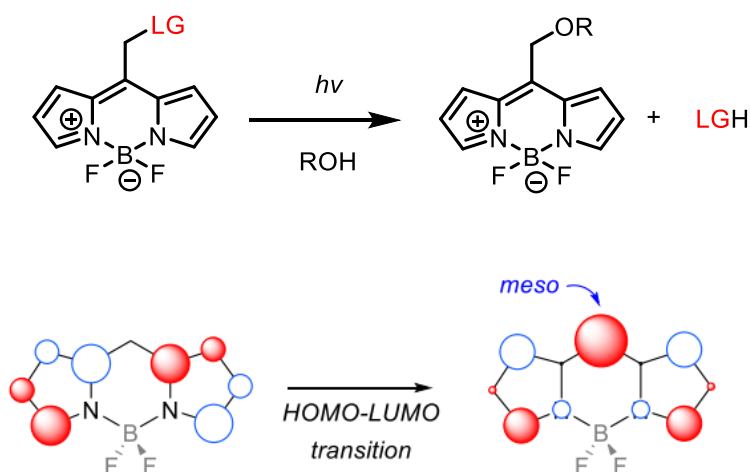


Figure 1.25 Photorelease of LG from *meso*-Methyl BODIPY Derivatives, and Schematic Representation of the HOMO and LUMO of the BODIPY π -System (BF_2 Is Omitted). Copyright © 2012 American Chemical Society.

The *meso*-Methyl BODIPY PPGs are a large family with absorption bands from 490–700 nm. For example, (1) (2) (3) typically have absorption bands with $\lambda_{\max}$ at 490–545 nm, while π -extended *meso*-Methyl BODIPYs with $\lambda_{\max}$ at 586–693 nm have been also reported (4) (Figure 1.26)^{152, 153}. The absorption spectra of representative *meso*-methyl BODIPY PPGs discussed in this section are shown in Figure 1.27¹⁵⁴⁻¹⁵⁶.

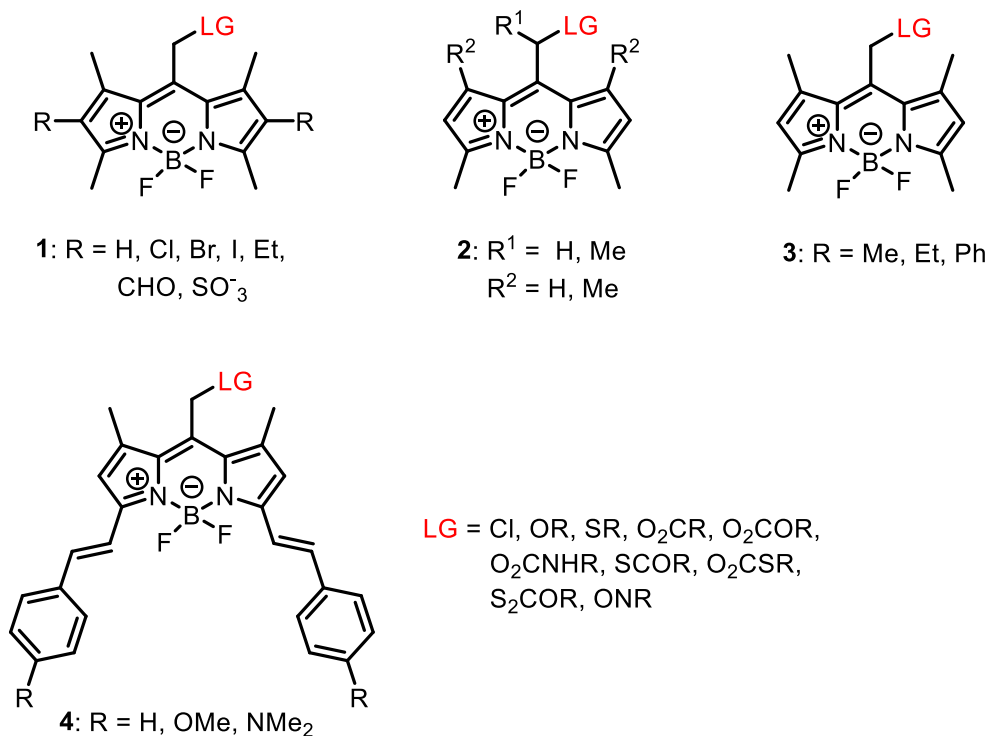


Figure 1.26 Chemical structures of *meso*-methyl BODIPY PPGs.

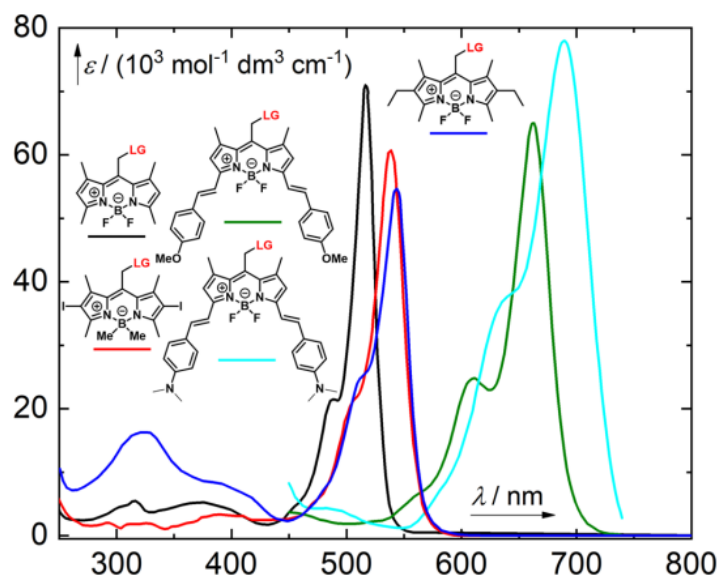


Figure 1.27 Absorption spectra of selected *meso*-methyl BODIPY PPGs. Black line, 1,3,5,7-tetramethyl derivative (LG = acetate); blue line, a 2,6-diethyl-1,3,5,7-tetramethyl derivative (LG = (4 - nitrophenyl) carbamate); red line, a 2,6-diiodo-B-dimethyl derivative (LG = acetate); green line, a bis (p - methoxystyryl) derivative (LG = benzoate); cyan line, a bis (p - dimethylaminostyryl) derivative (LG = benzoate). Copyright © 2012 American Chemical Society.

In conclusion, this section highlights several widely used photocages with broad absorption ranges, including nitroaryl groups, (coumarin-4-yl) methyl groups, arylmethyl groups, xanthene groups, and BODIPY-based photoactivatable protecting groups (PPGs). Their chemical structures, photophysical properties as well as photolysis mechanisms have been introduced.

1.2.2 Application of photoactivatable systems in biological systems

Light-control systems in living cells offer a valuable method for precisely regulating photolysis reactions within complex cellular environments. By using light as a trigger, researchers can manipulate specific proteins, genes, or cellular functions with high spatial and temporal precision¹⁵⁷⁻¹⁵⁹. This innovative technology has been extensively explored for applications such as controlling gene expression, regulating cell behavior, facilitating targeted drug release, and guiding the formation of nanostructures within living cells¹⁶⁰⁻¹⁶². The following section will include discussions on these applications.

Optogenetic techniques have been developed to allow control over the activity of selected cells within a highly heterogeneous tissue, using a combination of genetic engineering and light¹⁶³⁻¹⁶⁷. Research in the field mainly focus on the regulation of gene expression and cell behavior. For example, Deiters et al, described a light-activated T7RNAP gene expression system in mammalian cells engineered with additional protein biosynthetic machinery for genetically encoded protein modification with a caged lysine amino acid (PCK) (Figure 1.28)¹⁶⁸. The photocaging led to full inactivation of RNA polymerization. Light irradiation at 365 nm removed the caging group and enabled the expression of transcripts that were placed under the control of the T7 promoter, which was successfully demonstrated for two reporter genes, luciferase and EGFP.

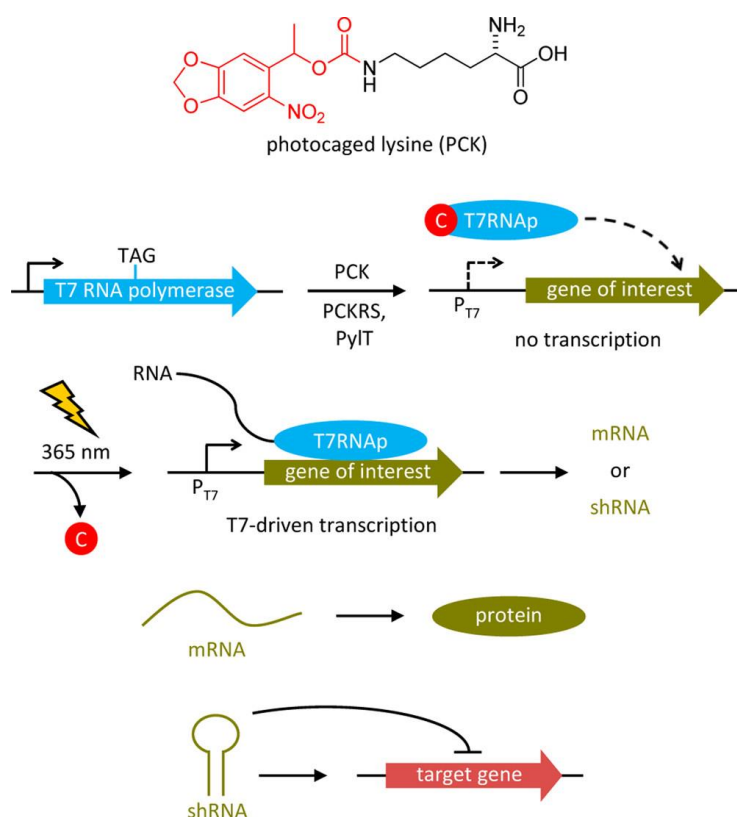


Figure 1.28 Light-activated T7RNAp is expressed in mammalian cells through site-specific incorporation of a photocaged lysine (PCK) into its active site in response to an amber stop codon (TAG) via an engineered tRNA synthetase (PCKRS) that misacylates an amber-suppressor tRNA (PylT) with PCK. The caged T7RNAp is completely inactive until irradiation with 365 nm UV light induces decaging and activation of T7-driven transcription. Depending on the function of the transcribed RNA, light-induced protein expression from mRNA or light-induced gene silencing via RNA interference from shRNA is achieved. Copyright © 2013 American Chemical Society.

Zanin et al. reported a photocaging strategy to reversibly arrest the cell cycle at metaphase or induce apoptosis using blue-light irradiation (Figure 1.29)¹⁶⁹. Upon irradiation, reversible cell-cycle arrest in the metaphase is clearly proven in Hela cells. The light-control system enabled precise spatiotemporal control of the cell cycle with high modulation. Moreover, the regulation of proteasome activity using light was studied, revealing that the uncaging processes enabled precise control over proteasome inhibition, thus inducing apoptosis. These findings underscore the value of this light-responsive tool for biological and medical applications that require high-precision targeting of the proteasome, as well as the regulation of the cell cycle and apoptosis.

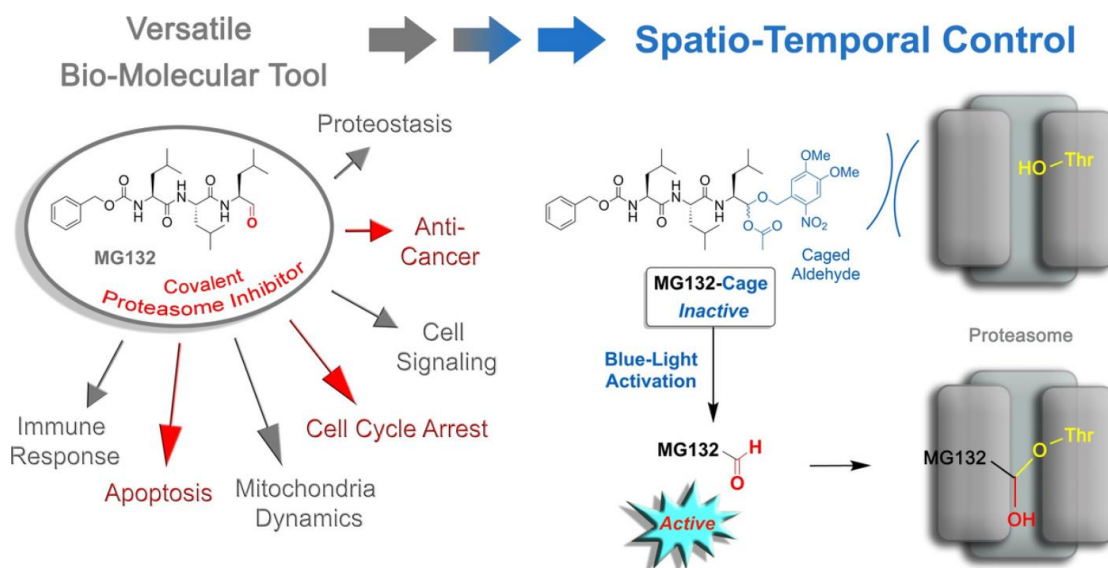


Figure 1.29 Transformation of the versatile covalent proteasome inhibitor MG132 into a blue-light-controlled biomolecular tool MG132-Cage (1) enabling spatio-temporal control of various fundamental biological phenomena. Upon blue-light irradiation the aldehyde function is restored and covalent proteasome binding and inhibition is triggered. Copyright © 2020 Wiley-VCH GmbH.

Beside, light-control systems have also been used to enable precise control of drug release to improve drug accumulation and therapeutic efficiency. Wang et al. presented a visible light-responsive drug delivery system, self-assembled from photocleavable trigonal small molecules, designed to achieve light-triggered intraocular drug accumulation (Figure 1.30)¹⁷⁰. In vivo results demonstrated the system's efficacy in controlling drug release and accumulation, effectively suppressing retinoblastoma growth.

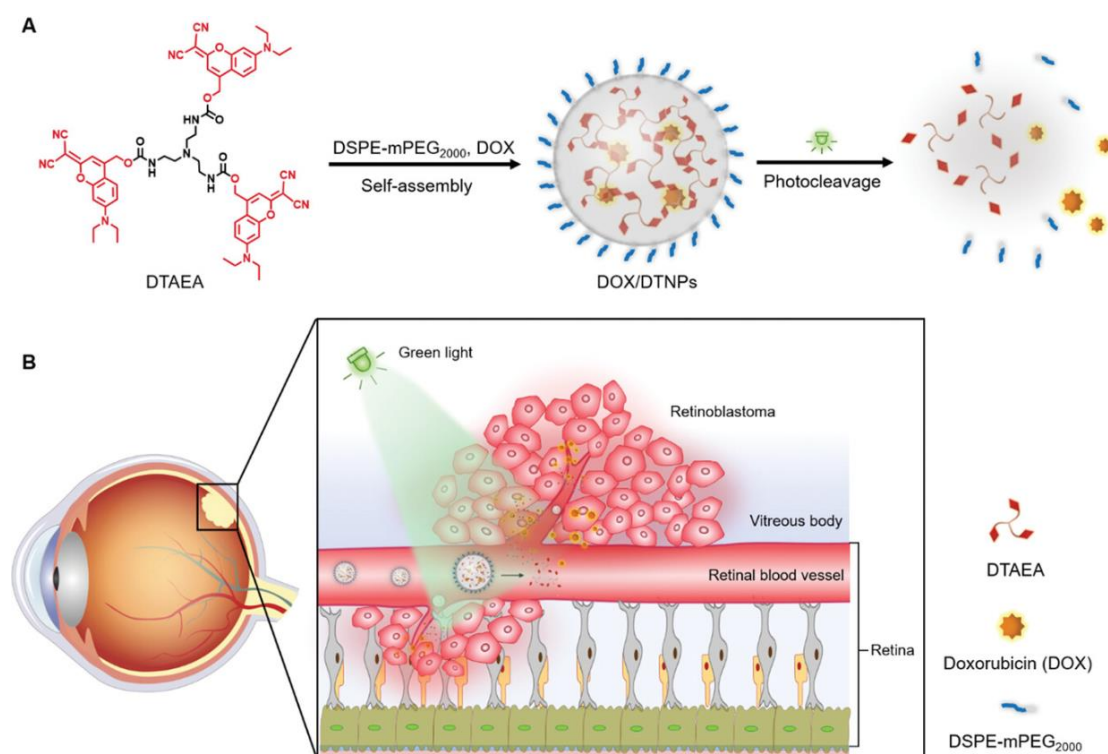


Figure 1.30 Schematic illustration of photo-triggered chemotherapy of retinoblastoma. A, Self-assembly, photo-induced disassembly, and drug release of the DOX-loaded (DEAdcCM) 3-TAEA (DTAEA) nanocarrier; B, Proposed mechanism of photo-enhanced drug accumulation in the eye.

Moreover, this technique also shows great potential for regulating nanostructure formation within cells. Wang et al. designed a light-triggered platform that enables spatiotemporal control of self-assembly from nanoparticles into nanofibers in living cells through subtle molecular conformational changes and internal H-bonding interactions (Figure 1.8)⁶³. The conformational changes from nanoparticles into nanofibers disrupted the hydrophilic/lipophilic balance and subsequently facilitated the formation of nanofibers, thereby inducing cell apoptosis. This study demonstrated that light-triggered assembly can be applied for regulating cellular behaviors in living systems.

In conclusion, the results presented here demonstrate that light-controlled systems within living cells have emerged as powerful tools for regulating cellular behavior, drug release, and gene expression, among other applications. Inspired by this knowledge, it is envisioned that the light-controlled system can be utilized to accurately control the generation and concentration of assembly monomers within living cells, which in turn regulates the kinetics of supramolecular assembly. As such, the generation of various

assembling species, the supramolecular assembly pathway and corresponding biological functions can be precisely characterized, which can be a significant advancement in understanding the assembly-driven therapeutic applications.

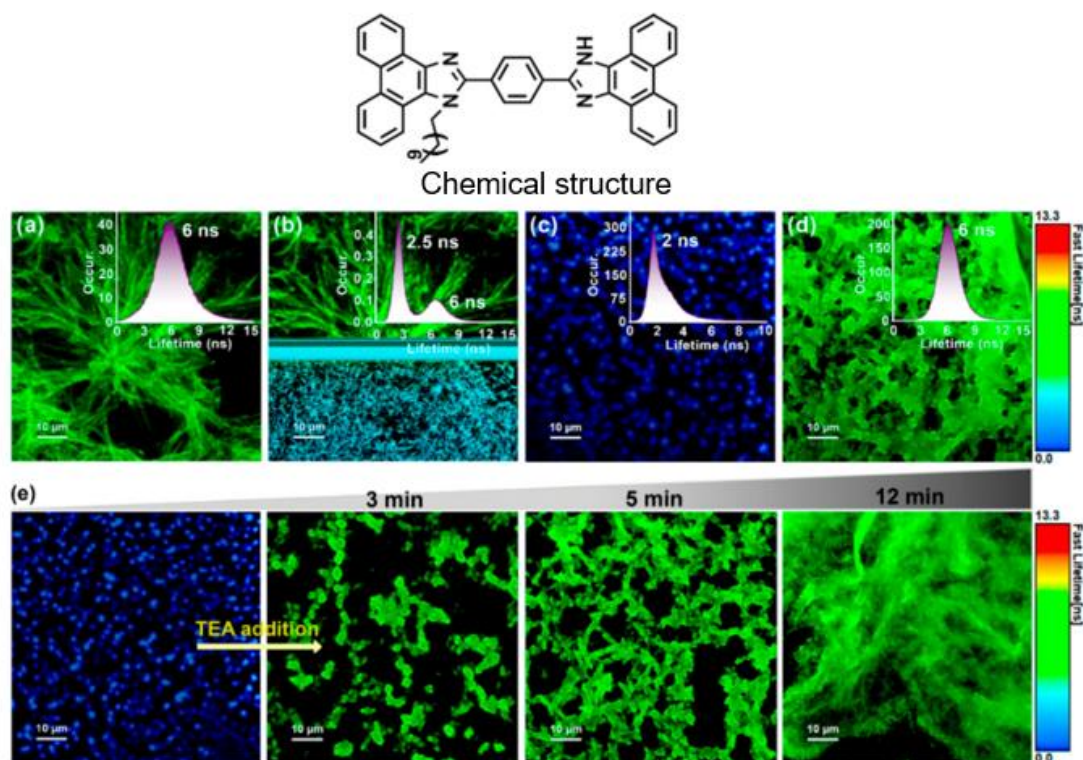


Figure 1.31 Chemical structure and Fluorescence lifetime imaging microscopy (FLIM) images of (a) the pristine BPIB1 gel, (b) the BPIB1 gel with the addition of trifluoroacetic acid (TFA, 1 μ L), (c) complete formation of the BPIB1 sol, and (d) the BPIB1 gel after the addition of triethylamine (TEA, 1 μ L) to the acidified sol; inset: corresponding fluorescence lifetime histograms demonstrating the reversible gel–sol–gel transformation; the average lifetime of the gel ($\sim$ 6 ns), the sol ($\sim$ 2 ns), and the mixture of the sol–gel is indicated. (e) Morphological transformation during the sol-to-gel transformation upon adding 1 μ L of TEA in an acidified sol of BPIB1 elucidated through real-time FLIM images. The green and blue colors in the images refer to fluorescence lifetime distribution; scale = 10 μ m.

Although light-control technology provides the availability to precisely control the supramolecular assembly process inside living cells, however, another obstacle lies in the difficulty in tracking the assembly processes and their dynamics within complex cellular environment. Recently, Patra et. al unraveled the real-time, early stage

environment-sensitive dynamic molecular self-assembly processes and stimuli-induced reversible morphological transformation between supramolecular fibers and spherical nanoaggregates of alkyl chain-substituted phenanthroimidazole-based luminogen (BPIB1) through fluorescence lifetime imaging microscopy (FLIM) (Figure 1.31)¹⁷¹. Inspired by this result, we propose that the formation processes of peptide-based nanostructures can be tracked using FLIM. This approach has the potential to enhance our understanding of the relationship between supramolecular chemistry and biological function.

1.3 Fluorescence lifetime

1.3.1 Fluorescence lifetime

Fluorescence lifetime is the average time that a molecule spends in its excited state before returning to the ground state, typically with the emission of a photon¹⁷²⁻¹⁷⁴. The fluorescence lifetime can vary from picoseconds to hundreds of nanoseconds, and even up to microseconds or milliseconds, depending on the fluorophore (Figure 1.32)¹⁷². During the excited state lifetime, a fluorophore can undergo conformational changes, interact with other molecules, and rotate and diffuse through the local environment.

When a sample containing a fluorophore is excited using a pulse of light, an initial population (n_0) of fluorophores in the excited state is created. This population decays with time due to fluorescence emission and non-radiative processes, a process described by the differential equation:

$$\frac{dn(t)}{dt} = - (k_r + k_{nr})n(t) \quad [1]$$

where $n(t)$ is the number of molecules in the excited state at time t following the excitation. k_r and k_{nr} are the radiative and non-radiative rate constants respectively. This results in an exponential decay of the excited state, given by

$$n(t) = n_0 \exp(-t/\tau) \quad [2]$$

τ is the fluorescence lifetime of the molecule and is expressed as the reciprocal of the total decay rate,

$$\tau = (k_r + k_{nr})^{-1} \quad [3]$$

Fluorescence lifetime is an intrinsic property of a fluorophore and therefore does not depend on the method of measurement¹⁷⁴. Fluorescence lifetime can be considered as a state function because it also does not depend on initial perturbation conditions, such as excitation wavelength, duration of light exposure, single- or multi-photon excitation, and is not affected by photobleaching. In addition, fluorescence lifetime is a

parameter that is largely independent of the fluorescence intensity and fluorophore concentration¹⁷⁵⁻¹⁸¹. Since this process is affiliated with an energetically unstable state, fluorescence lifetime can be sensitive to a great variety of internal factors defined by the fluorophore structure and external factors that include temperature, polarity, and the presence of fluorescence quenchers. The combination of environmental sensitivity and parametric independence mentioned above renders fluorescence lifetime a separate yet complementary method to traditional fluorescence intensity measurements.

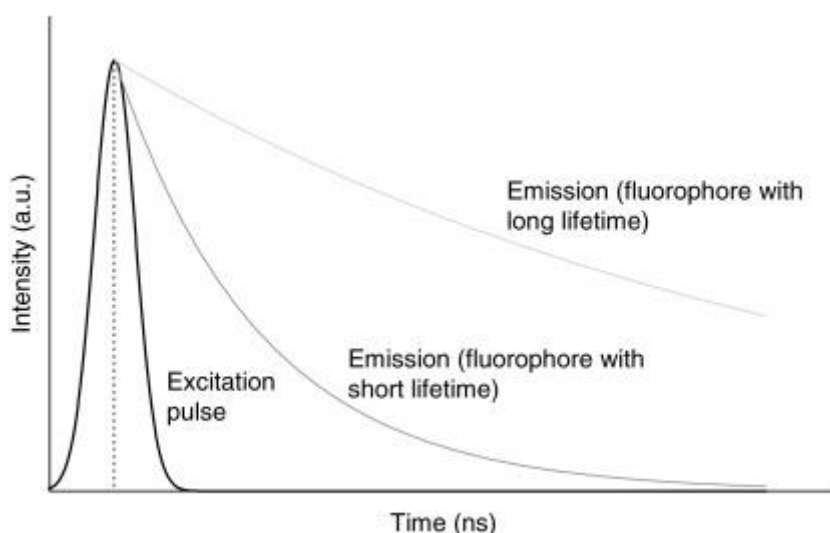


Figure 1.32 Schematic of fluorescence decay upon pulsed excitation. Fluorophores are excited using a short pulse of light. The emitted fluorescence is measured in a time-resolved manner, forming the basis for time-domain lifetime measurement.

1.3.2 Fluorescence lifetime imaging

Typical FLIM applications are not primarily aiming at separating signals of different fluorophores. Instead, FLIM is used to distinguish different fractions of the same fluorophore in different states of interaction with its environment. This is due to the basic property of the fluorescence lifetime: the fluorescence lifetime of a fluorophore depends on its molecular environment but, within reasonable limits, not on its concentration. In general, the fluorescence lifetime of a fluorophore can be influenced by many factors, such as fluorescence quencher, pH, viscosity, FRET process, conformation of a fluorophore and so on (Figure 1.33).

A variety of fluorescence quenchers, including certain ions, especially Ca^{2+} and Cl^- , Cu^+ , can significantly quench fluorescence or phosphorescence. Additionally, oxygen acts as an efficient quencher for many fluorophores. Strong quenching by oxygen has been demonstrated in the phosphorescence of organic complexes involving ruthenium, europium, platinum, and palladium. Upon quenching, the decay dynamics are markedly altered, resulting in significant changes in the fluorescence lifetime. For example, Vilar et al. reported a novel fluorescent probe with specific binding affinity for copper(I), resulting in marked changes in fluorescence lifetime¹⁸². This approach demonstrated the feasibility of visualizing copper(I) pools in the lysosomes of living cells using fluorescence lifetime imaging (Figure 1.33a).

The pH is another critical factor that can influence fluorescence lifetime, as many fluorescent molecules exist in both protonated and deprotonated forms. When these two forms exhibit different fluorescence lifetimes, the apparent lifetime can serve as an indicator of the local pH. For example, Ruedas-Rama et al. investigated that the photoluminescence (PL) lifetime of mercaptopropionic acid-capped QDs (MPA-QDs) collected from FLIM images can be used to determine intracellular pH¹⁸³. They performed the successful detection of changes in the intracellular pH of different cell types by examining the PL decay time of QDs (Figure 1.33b).

For fluorophores with a high degree of internal flexibility, the surrounding viscosity can significantly influence their internal rotation, leading to variations in non-radiative decay rates. Consequently, changes in viscosity can modulate the fluorophore's lifetime. For example, Yan et al. developed a fluorescent probe, incorporating an aldehyde group (CHO), enabling dual mode fluorescence imaging, including ratiometry imaging and fluorescence lifetime imaging, of intracellular viscosity (Figure 1.33c)¹⁸⁴. The rotation of the CHO group gave rise to internal conversion by a non-radiative process. The viscosity changes inside cells, showing some regional differences, which can be clearly observed by both ratiometry imaging and fluorescence lifetime imaging (FLIM). These findings highlight the enhanced reliability and accuracy that FLIM provides for measuring intracellular viscosity, offering capabilities previously unavailable with conventional techniques.

The fluorescence lifetime can be influenced by Förster resonance energy transfer

(FRET). FRET is a process in which energy is transferred from one molecule, the donor, to another molecule, the acceptor. This technique is widely used to study protein interactions and conformational changes in proteins. Among its applications, FRET measurement is the most common use of fluorescence lifetime imaging microscopy (FLIM). For example, Lamond et al. presented the detection of protein-protein interactions in vivo based on the measurement of FRET using the multiphoton fluorescence lifetime imaging microscopy (FLIM)¹⁸⁵. This FLIM-FRET approach allows the precise spatial mapping and quantification of these interactions within living cells (Figure 1.33d).

The fluorescence lifetime of a fluorophore can vary upon binding to a biological target. This change may result from an alteration in the fluorophore's conformation, which subsequently affects the rate of non-radiative decay. Kuimova et al. developed a novel thiazole orange derivative, TOR-G4, that exhibits a unique fluorescence lifetime (3.0 ns) when bound to G4s compared to other structures, allowing G4 binding to be sensitively distinguished from non-G4 binding¹⁸⁶. The probe also colocalizes with RNA in the cytoplasm and nucleoli of cells, making it the first lifetime-based probe validated for exploring the emerging role of RNA G4s in cellulo (Figure 1.33e).

Based on the aforementioned results, FLIM proves to be a versatile tool for measuring various parameters within living cells.

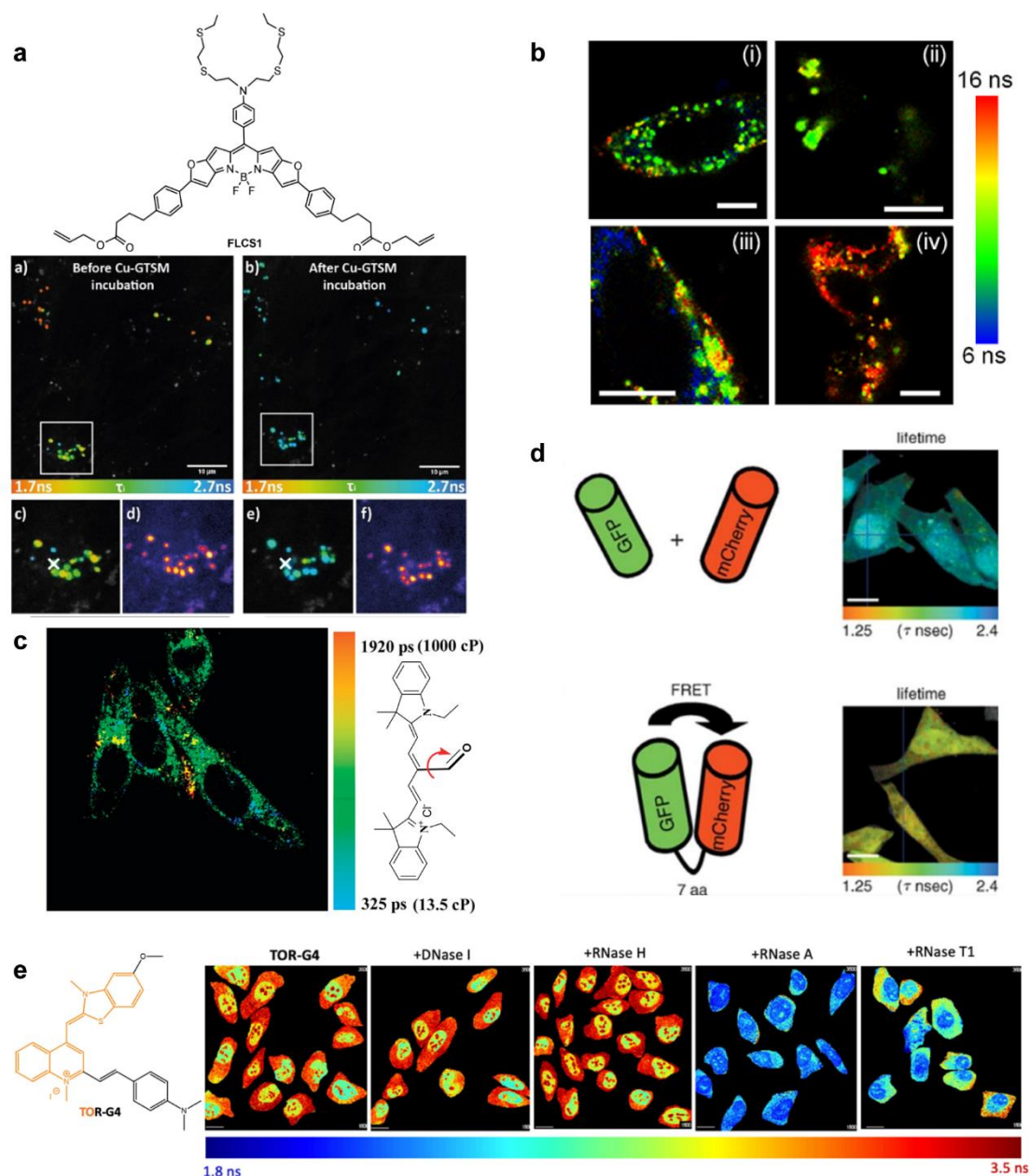


Figure 1.33 Various Applications of FLIM. a, chemical structure and FLIM imaging recorded before and after incubation with 5 μ M Cu-CTSM for 20 min; b, FLIM images of MPA-QDs in the cytoplasm of CHO-k1 cells after incubation with nigericin in extracellular buffers (EBs) at different pH values: 5.70 (i), 6.50 (ii), 6.73 (iii), and 8.19; c, chemical structure and FLIM image of probe in MCF-7 cells; d, schematic illustration of FLIM-FRET method and corresponding FLIM images; e, chemical structure and FLIM images of TOR-G4 in cells following treatment with nucleases.

1.3.3 Phasor-Fluorescence lifetime imaging (Phasor-FLIM)

As described above, fluorescence lifetime imaging microscopy (FLIM) is used in diverse disciplines, including biology, chemistry and biophysics¹⁸⁷⁻¹⁹³, but its use has been limited by the complexity of the data analysis. The phasor approach to FLIM has the potential to markedly reduce this complexity and at the same time provide a powerful visualization of the data content^{194, 195}. Phasor plots for fluorescence lifetime analysis were originally developed as a graphical representation of excited-state fluorescence lifetimes for in vitro systems. The method's simple mathematics and specific rules avoid errors and confusion common in the study of complex and heterogeneous fluorescence¹⁹⁴. In the case of FLIM, the phasor approach has become a powerful method for simple and fit-free analyses of the information contained in the many thousands of pixels constituting an image^{188, 196}.

The phasor approach to FLIM transforms the decay data from each pixel of an image into the phasor plot (Figure 1.34)^{194, 197, 198}. The transformation is based on the mathematics originally described by Weber¹⁹⁵ to resolve heterogeneous systems, and the graphical representation as a phasor plot was introduced by Jameson et al¹⁹⁹. The phasor transformation can use data collected in either the time or the frequency domain (FD)¹⁹⁹⁻²⁰². In the time-correlated single-photon counting (TCSPC) technique, the histogram of the photon arrival times at each pixel of the image is transformed into Fourier space, and the phasor coordinates are calculated using the following relations:

$$g_{i,j}(\omega) = \frac{\int_0^T I(t) \cdot \cos(n\omega t) dt}{\int_0^T I(t) dt} \quad [4]$$

$$s_{i,j}(\omega) = \frac{\int_0^T I(t) \cdot \sin(n\omega t) dt}{\int_0^T I(t) dt} \quad [5]$$

in which $g_{i,j}(\omega)$ and $s_{i,j}(\omega)$ are the x and y coordinates of the phasor plot, n and ω are the harmonic frequency and the angular frequency of excitation, respectively, and T is the repeat frequency of the acquisition. Frequency domain data acquisition from each pixel can be converted to phasor points using the following transformations:

$$g_{i,j}(\omega) = m_{i,j} \cdot \cos(\phi_{i,j}) \quad [6]$$

$$s_{i,j}(\omega) = m_{i,j} \cdot \sin(\phi_{i,j}) \quad [7]$$

in which $m_{i,j}$ and $\phi_{i,j}$ are the modulation and phase shift, respectively, of the frequency domain measurement at pixel i,j . The decay from each pixel can therefore be translated to a point in the phasor plot. By taking these transformations and solving the integrals, the following relationships between the phasor and the lifetime can be derived:

$$g = \sum_{i=1}^N \frac{f_i}{1 + \omega^2 \tau_i^2} \quad \text{and} \quad s = \sum_{i=1}^N \frac{f_i \omega \tau_i}{1 + \omega^2 \tau_i^2} \quad (8)$$

N is the number of the fluorescent species, and f_i is the fractional contribution of the i th species of the fluorescence lifetime T_i to the total intensity.

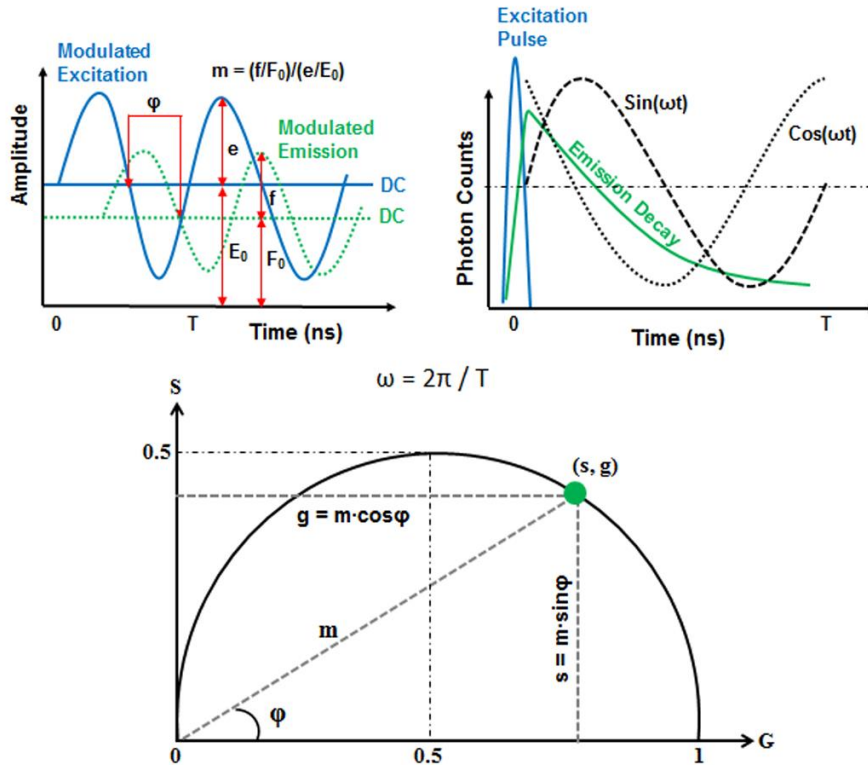


Figure 1.34 Mapping FD and TCSPC FLIM data to the phasor plot.

For a single-lifetime species ($N = 1$), the Eq. [8] is reduced to Eq. [9] and the lifetime can be directly determined by the coordinate values of a phasor. It is further derived that the two coordinates of a phasor representing a single-lifetime species must have the

relationship given by Eq. [10]. The relationship is drawn as a semicircle curve centered at ($G = 0.5$, $S = 0$) with a radius of 0.5 in the phasor plot, and this semicircle curve is often called the universal semicircle or the universal trajectory (Figure 1.34)^{195, 203}. The universal semicircle defined by Eq. [10] becomes an explicit lifetime ruler for single-lifetime species in the phasor plot. In the semicircle, the hypothetical zero lifetime lies in (1, 0) and the infinite lifetime lies in (0, 0) (Figure 1.34).

$$g = \frac{1}{1 + \omega^2 \tau^2} \quad s = \frac{\omega \tau}{1 + \omega^2 \tau^2} \quad \tau = \frac{1}{\omega} \left(\frac{s}{g} \right) \quad [9]$$

$$(g - 0.5)^2 + s^2 = 0.25 \quad [10]$$

Based on this, in the phasor transformation, the decays that correspond to a mono-exponential appear on the universal semicircle (Figure 1.35a). The decays that arise from multi-exponentials appear inside the semicircle (Figure 1.35b). Phasors of decay profiles which result from the convolution of several processes end outside the semicircle (Figure 1.35c). An example is the emission of a FRET-excited acceptor, which is the convolution of the decay function of the interacting donor with the fluorescence decay of the acceptor. The values of g (the cosine transform) are between 0 and 1, and s (the sine transform) has values between 0 and 0.5. In the phasor plot, the coordinates are shown in polar coordinates, where M is the modulus of the phasor and ϕ is the phase.

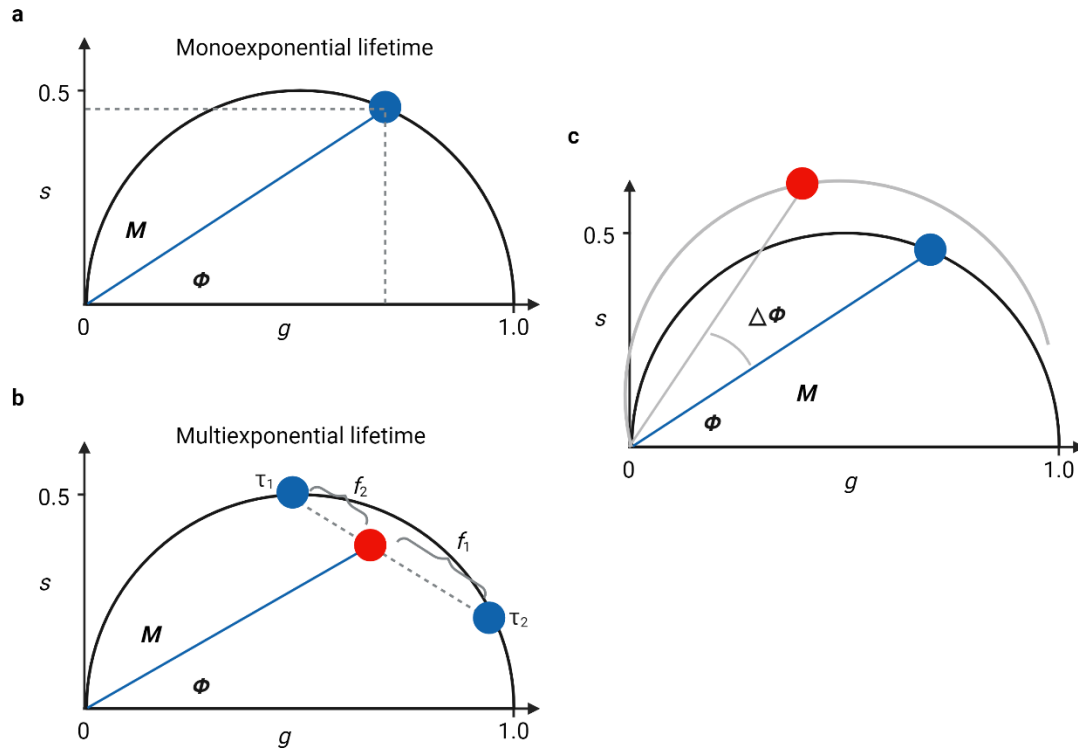


Figure 1.35 Different examples of phasor plots. **a**, Single-component phasor example. **b**, Linear combination in phasor space. **c**, Reactions in the excited state result in a phasor moving outside of the universal semicircle. ϕ , phase; f_x , fraction contribution; M , modulation; τ_x , lifetime.

The goal of FLIM acquisition is to generate a color-mapped images, where different colors correspond to different fluorescence decay profiles. In the phasor approach to FLIM, the fluorescence decay is directly transformed to a phasor plot by simple mathematics in which phasor points originating from different pixels in the image are represented by their location in the phasor plot. In the phasor plot, similar fluorescence decays can be easily recognized and the points in the image where they originate can be painted using color cursors. For example, in Figure 1.36 (top right), blue- and red-colored cursors select the two major clusters of phasor distribution originating from the cytoplasm and nucleus, respectively, and the image is colored accordingly (phasor-mapped FLIM image in Figure 1.36, bottom right). A feature of phasor analysis is that any region of the phasor plot (which represent one specific fluorescence decay value) can be selected in order to directly characterize the pixels in the image with the given fluorescence decay. Conversely, selecting a region of the image (Figure 1.36, top left) allows to observe the parts of the phasor plot that represent the decay of the region (Figure 1.36, bottom left). This reciprocity (indicated by the red two-headed arrow)

provides the phasor approach to FLIM with the ability to identify the related components, even if they are spatially separated and are present in only very few pixels. The red two-headed arrow is representative of the reciprocity principle of the phasor approach, which shows that the phasor-mapped FLIM image and the phasor plot are strictly correlated. Selecting a cluster in the phasor plot results in selection of the parts of the image from which those phasor points originate, and, similarly, a part of the FLIM image can be selected (Figure 1.36, top left), and the distribution of the corresponding phasor points (Figure 1.36, bottom left) can be captured.

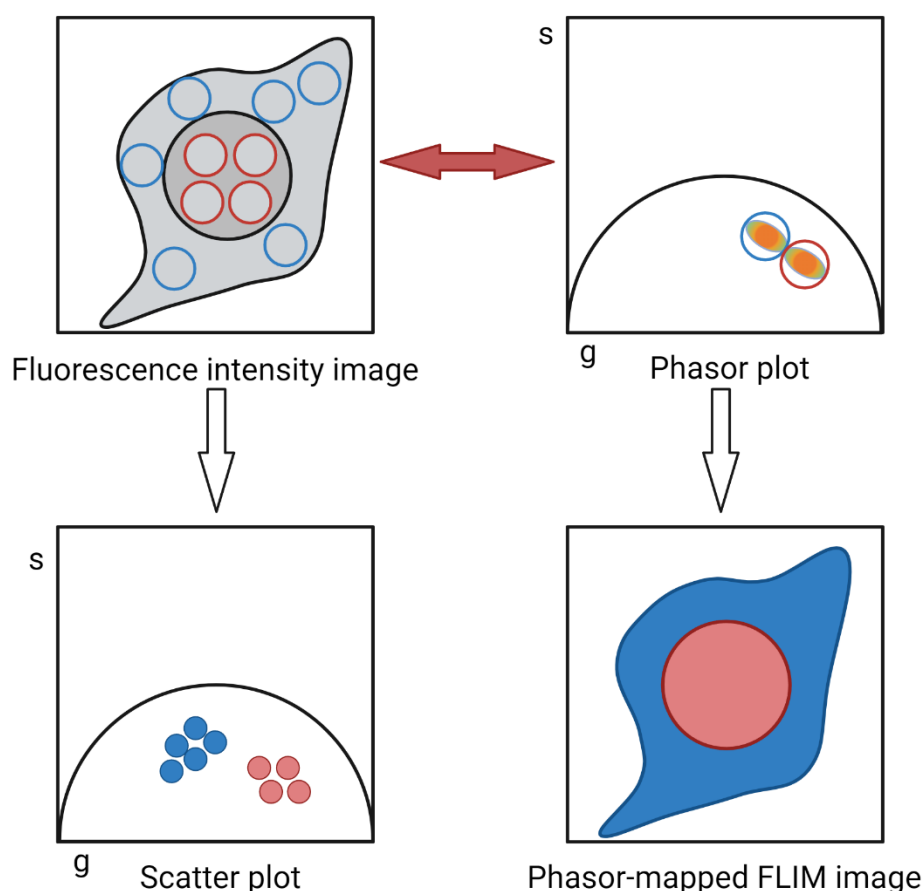


Figure 1.36 The reciprocity principle using the phasor approach. (Top) The phasor approach converts the fluorescence lifetime information of the FLIM/intensity image (top left) to a phasor plot (top right). Selecting different regions of the phasor plot lead to the selection of different areas of the image and a phasor color-mapped FLIM image (bottom right). The reciprocity principle (red arrow) dictates that the process can be reversed, and different areas of the FLIM images can be selected to obtain the corresponding phasor distribution (bottom left). $g = M\cos\phi$; $s = M\sin\phi$, where M is the modulation and ϕ is the phase shift in frequency domain measurements.

Based on the aforementioned knowledge, the phasor approach to fluorescence lifetime imaging microscopy (FLIM) offers a significantly more efficient method than conventional FLIM for reducing complexity while providing enhanced visualization of data. Its straightforward mathematical framework and well-defined principles minimize the errors and confusion often encountered in studies involving complex and heterogeneous fluorescence. Especially for those fluorophores with similar excitation and emission spectra but different lifetimes, this approach enables the unique characterization, facilitating the detection of subtle changes and dynamic processes within living cells. Furthermore, phasor-FLIM offers superior spatial resolution, as its lifetime imaging capability enables the generation of spatial maps, in contrast to other techniques such as Fluorescence Correlation Spectroscopy (FCS), which provides only point-based measurements.

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2 Motivation

Self-assembly in biological systems allows individual macromolecules to assemble into a wide set of supramolecular structures and architectures. In this manner, nature capitalizes on self-assembly to diversify chemically simple building blocks into sophisticated materials and structures that function cooperatively in living systems. Among them, peptides/amino acids have been widely utilized as building blocks to construct bioactive materials to facilitate desired functions in living systems due to their facile synthesis via solid-phase peptide synthesis (SPPS), remarkable biocompatibility, and adjustable properties. Although numerous significant research endeavors have been undertaken within the field, several persistent issues continue to impede the advancement of knowledge.

Over the past decades, the directed assembly of nanostructures within cells using natural or synthetic polypeptides has been shown to culminate in various end-effect cellular responses, including causing mechanical stress and metabolic alterations, thus inducing cytotoxicity. As mentioned, the biological significance of the assembly pathway and intermediates remains poorly understood. The main obstacle lies in the spatiotemporal tracking of monomers, oligomers, and polymers within a dynamic cellular environment so that snapshots of these components with time-dependent biological effects can be mapped. However, conventional intensity-based microscopy provides limited insight into the self-assembly process within cells. To overcome this limitation, phasor-FLIM enables the precise resolution of self-assembly dynamics by mapping distinct assembly species according to their unique fluorescence lifetimes. By integrating relevant biological effects, this approach offers a deeper understanding of the intricate interplay between supramolecular chemistry and cellular processes.

Besides, although numerous intracellular assembly systems have been designed and studied to regulate cellular metabolism and behavior, most of these systems are responsive to endogenous stimuli, limiting precise control over assembly dynamics within cells. Additionally, the formation of diverse assembly states, the supramolecular assembly dynamics inside cells, and their correlation with biological effects remain largely unexplored. Achieving breakthroughs in this area could not only provide a more comprehensive understanding of supramolecular assembly dynamics within cells but also clarify the relationship between distinct assembly species and their corresponding

biological effects. Therefore, there is an urgent need to develop novel systems that enable precise control over assembly states and dynamics within the complex intracellular environment. This could be crucial for gaining a deeper understanding of the mechanisms underlying assembly-driven diseases.

Moreover, existing studies in this field focus primarily on native peptide sequences and the end-effect of nanostructures or nanoaggregates formation, which leads to cell death. However, the mechanisms of action and biochemical profiles associated with these assemblies remain poorly understood. Thus, the development of novel building blocks to study assembly behavior and the elucidation of the biological mechanisms underlying supramolecular chemistry are crucial to enhancing our understanding of assembly-driven biological effects and their potential biomedical applications.

To address the aforementioned challenges, this thesis primarily focused on three key objectives: first, to expand the diversity of molecular building blocks that drive self-assembly in living cells and to study the biological mechanisms of action and biochemical profiles associated with nanostructure formation, thereby establishing the chemical foundation for assembly-driven biomedical research; second, to design a novel peptide assembly system capable of tracking the process of supramolecular assembly within living cells and elucidating its corresponding biological effects; and third, to develop a innovative platform enabling spatiotemporal control of assembly behavior in cells, facilitating the study of the correlation between distinct assembly species and their biological impact (Figure 2.1).

In the first work (Chapter 3.1), to broaden the diversity of building blocks to drive self-assembly and study the mechanisms of action and biochemical profiles associated with these assemblies, platinum (II) terpyridine (Pt-tpy) was conjugated with tripeptide to construct an architecture, aligning the Pt-tpy complex with its β -amyloid-like sequence, thus fostering the propagation of supramolecular order through π - π and metal-metal interactions. Meanwhile, the Pt-tpy can also present near-infrared (NIR) fluorescence which allows intracellular imaging. In collaboration with Zhixuan Zhou et al., we present a platinum metallo-iso-tripeptide that undergoes a cascade of molecular and supramolecular transformations under specific intracellular environments to form near-infrared (NIR) emitting nanofibers. By combining the features of platinum(II) complexes and peptides together with boronic acid-

salicylhydroxamate (SHA) chemistry that can be triggered by both physiological pH and reactive oxygen species, the progression from a dormant assembling precursor to the final superstructure can be controlled and imaged within cells. With a meticulously crafted sequence of chemical transformations in situ, the biological activity on aerobic glycolysis (AGlyc) and oxidative phosphorylation (OxPhos) is demonstrated on A549 lung cancer cells and MDA-MB-231 metastatic breast cancer cells. The correlation between chemical transformations and biological activity enabled a deeper insight into the mechanism of action of the nanofiber formation.

The peptide assembly system presented in the second work (Section 3.2) is based on a pro-assembly isopeptide with a core-modified diethoxynaphthalene diimide (NDI) moiety that promotes the controlled formation of synthetic fibrillar superstructures inside cells. To enable real-time monitoring of intracellular self-assembly via phasor-FLIM - an approach unattainable with conventional intensity-based microscopy - the NDI fluorophore was integrated into the peptide as a fluorescence lifetime reporter. Its fluorescence lifetime dynamically shifts in response to intracellular assembly events. This platform enables real-time tracking of nanofiber formation and their subsequent fusion with endosomes, leading to the emergence of hollow fiber clusters. Utilizing phasor-FLIM in epithelial cells (L929, A549, MDA-MB-231), along with correlative light-electron microscopy and tomography (CLEM), we reveal the spatiotemporal progression of assembly events and their correlation with metabolic dysfunction. The biological impact is initiated by assembly-induced endosomal disruption, which impairs glucose transport into the cell and consequently inhibits mitochondrial respiration.

To improve the precision of peptide assembly regulation within living cells, a novel system that can be activated via visible light (505 nm wavelength), is presented in the third project (Chapter 3.3). Inspired by the groundbreaking advancements in optogenetic technologies, we propose a light-induced self-assembly system that enables precise spatiotemporal control of the self-assembly process within cells. In contrast to endogenous stimulus-responsive self-assembly systems, light irradiation allows full control over the reaction cascade where the monomer generation and concentration in turn regulates the assembly kinetics. Herein, we select a coumarin-based photocage due to its compatibility with biocompatible wavelengths, which can be cleaved by visible light at 505 nm, and a relatively slow photolysis rate (30 minutes for complete cleavage), providing an extended detection window for studying assembly

kinetics. However, in living cells, the selection of a photochemical system is only one of many aspects that dictates the assembly of supramolecular monomers. Depending on the internalization, transport and localization of the molecules, the crowded intracellular environment imposed by the high density of bio-macromolecules also influences reaction and assembly dynamics. As these parameters only exist in living cells and cannot be studied in model systems, in-situ technologies such as Phasor-FLIM are invaluable for tracking the formation of various assembly states in cells and revealing subsequent out-of-equilibrium dynamics associated with monomer activation and consumption. These temporally resolved assemblies show that the emergence of cytotoxicity is correlated with the accumulation of oligomers beyond the cellular efflux threshold.

This thesis introduces innovative methods for creating peptide-based nanomaterials that can form functional supramolecular structures within living cells. By designing peptide platforms that are responsive to various stimuli and employing advanced imaging techniques, such as phasor-FLIM and CLEM, this research bridges the gap between biomaterials and cell biology. These advances enhance our understanding of how programmed materials interact with cells.

The findings from the aforementioned projects have been documented in three publications (the third paper, submitted to *Nature Synthesis*, has now been accepted). These results are presented in the following chapter.

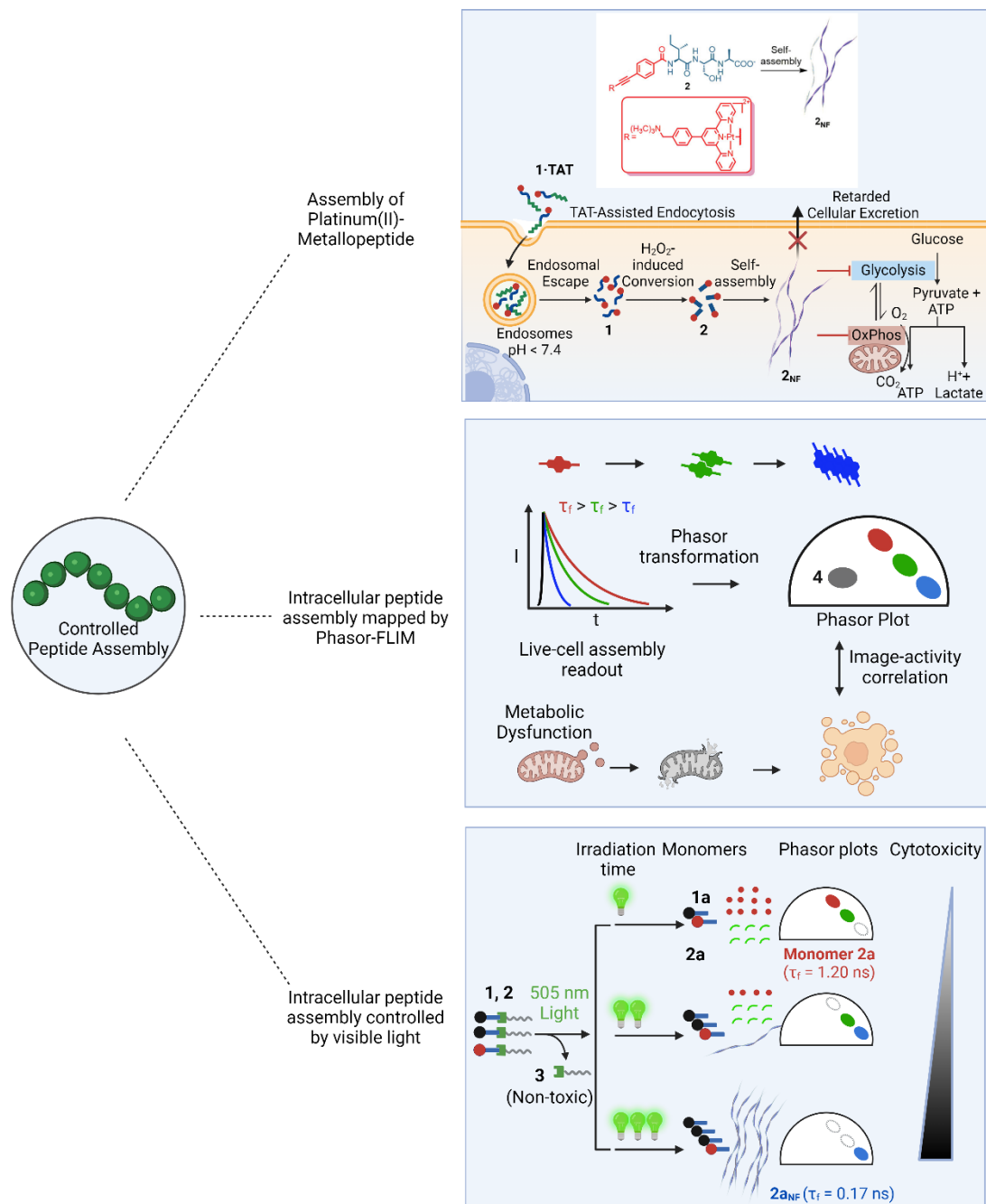


Figure 2.1 Scope of this thesis and overview of the three main goals.

3 Results

3.1 In Situ Assembly of Platinum(II)-Metallopeptide Nanostructures Disrupts Energy Homeostasis and Cellular Metabolism

Zhixuan Zhou, Konrad Maxeiner, Pierpaolo Moscariello, Siyuan Xiang, Yingke Wu, Yong Ren, Colette J Whitfield, Lujuan Xu, Anke Kaltbeitzel, Shen Han, David Mücke, Haoyuan Qi, Manfred Wagner, Ute Kaiser, Katharina Landfester, Ingo Lieberwirth, David YW Ng*, Tanja Weil*

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Abstract

Nanostructure-based functions are omnipresent in nature and essential for the diversity of life. Unlike small molecules, which are often inhibitors of enzymes or biomimetics with established methods of elucidation, we show that functions of nanoscale structures in cells are complex and can implicate system-level effects such as the regulation of energy and redox homeostasis. Herein, we design a platinum(II)-containing tripeptide that assembles into intracellular fibrillar nanostructures upon molecular rearrangement in the presence of endogenous H₂O₂. The formed nanostructures blocked metabolic functions, including aerobic glycolysis and oxidative

phosphorylation, thereby shutting down ATP production. As a consequence, ATP-dependent actin formation and glucose metabolite-dependent histone deacetylase activity are downregulated. We demonstrate that assembly-driven nanomaterials offer a rich avenue to achieve broad-spectrum bioactivities that could provide new opportunities in drug discovery.

Contributions

Zhixuan Zhou: Conceptualization, molecular design of peptides. Synthesis and characterization of peptides and intermediates. LC-MS kinetics analysis, CD measurement and NMR analysis. Data analysis and interpretation of results. Writing of the manuscript.

Konrad Maxeiner: Cell experiment, metabolism assay study and analysis.

Pierpaolo Moscariello: Metabolism assay study and analysis.

Siyuan Xiang: TEM measurement and analysis.

Yingke Wu: TEM measurement and analysis.

Yong Ren: TEM measurement and analysis, synthesis, purification and structural characterization of intermediates, NMR data analysis and interpretation.

Colette J Whitfield: AFM measurement and analysis.

Lujuan Xu: Assistance in compound purification.

Anke Kaltbeitzel: CLEM measurement and analysis.

Shen Han: Cryo-TEM measurement and analysis.

David Mücke: Cryo-TEM measurement and analysis.

Haoyuan Qi: Cryo-TEM measurement and analysis.

Manfred Wagner: NMR measurement and analysis.

Ute Kaiser: Cryo-TEM measurement and analysis.

Katharina Landfester: CLEM measurement and analysis.

Ingo Lieberwirth: CLEM measurement and analysis.

David YW Ng: Design and discussion on the concept and results analysis. Writing and correction of the manuscript.

Tanja Weil: Acquiring funding for the project. Design and discussion on the concept and results analysis. Correction of the manuscript.

In Situ Assembly of Platinum(II)-Metallopeptide Nanostructures Disrupts Energy Homeostasis and Cellular Metabolism

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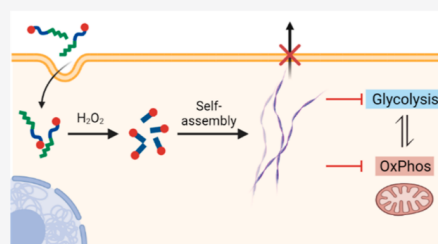


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ABSTRACT: Nanostructure-based functions are omnipresent in nature and essential for the diversity of life. Unlike small molecules, which are often inhibitors of enzymes or biomimetics with established methods of elucidation, we show that functions of nanoscale structures in cells are complex and can implicate system-level effects such as the regulation of energy and redox homeostasis. Herein, we design a platinum(II)-containing tripeptide that assembles into intracellular fibrillar nanostructures upon molecular rearrangement in the presence of endogenous H_2O_2 . The formed nanostructures blocked metabolic functions, including aerobic glycolysis and oxidative phosphorylation, thereby shutting down ATP production. As a consequence, ATP-dependent actin formation and glucose metabolite-dependent histone deacetylase activity are downregulated. We demonstrate that assembly-driven



nanomaterials offer a rich avenue to achieve broad-spectrum bioactivities that could provide new opportunities in drug discovery.

INTRODUCTION

In cells, protein nanostructures are abundant, serving as cellular scaffolds, multi-domain catalysts, and transport highways.¹ Among them, ordered protein assemblies consisting of β -sheet structures, the so-called β -amyloids, play both functional and pathological roles. They serve as biotemplates within melanocytes for melanin biosynthesis^{2,3} or as nucleating centers toward the amyloidosis in Type 2 diabetes.^{4,5} Mechanistically, the monomers of these ordered nanostructures do not possess intrinsic functions of their own but rely on the propagation of long-range hierarchical structures to feature their rich biological activities. Several strategies have since been attempted to re-engineer these nanostructures using simplified peptide-based monomers to elicit biomedical functions.^{6–13} Pharmacologically, self-assembling nanostructures in cells combine features of small molecules such as deep cell/tissue penetration with properties from larger superstructures like enzymatic stability and retardation of cellular efflux.^{10,14,15} Existing studies, with a major focus on native peptide sequences, found that the formation of nanostructures or nanoaggregates stimulated via physiological means (pH, enzymes, and concentration) has commonly led to cell death, also among in vivo models.^{6–13} However, very little is known in terms of their mechanism of action and biochemical profile as the assembly of intracellular structures relies

intricately on both supramolecular dynamics and cellular processes.

Nonetheless, from the biological features of β -amyloids, it is implied that the nanostructures plausibly exhibit broad bioactivity on a systemic level that impacts multiple pathways that ultimately leads to cell death. As such, we rationalized that an investigation into process families accompanied by precise chemical tools will enable a greater understanding to the cellular dynamics. Among these processes, metabolism is a critical facet that defines cellular life, and through it, the basic unit of energy in the form of adenosine triphosphate (ATP) is produced.¹⁶ Hence, metabolic interference that overwhelms the cell's capability to adapt between glucose fermentation (glycolysis) and oxygen conversion oxidative phosphorylation (OxPhos) leads to the impairment of ATP-dependent pathways.^{17,18} In this regard, various supramolecular strategies have recently been developed and shown to be effective to target the accelerated metabolism of cancer.^{19,20} By coupling an in-depth metabolic study with a switchable self-assembling

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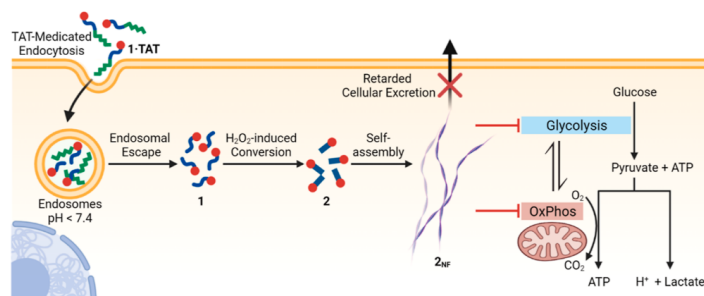


Figure 1. Schematic illustration of the intracellular H_2O_2 -induced formation of linear platinum(II) complexes that self-assembles into platinum(II)-containing nanofibers, which affects energy homeostasis and disrupts cellular metabolism. Created with BioRender.com.

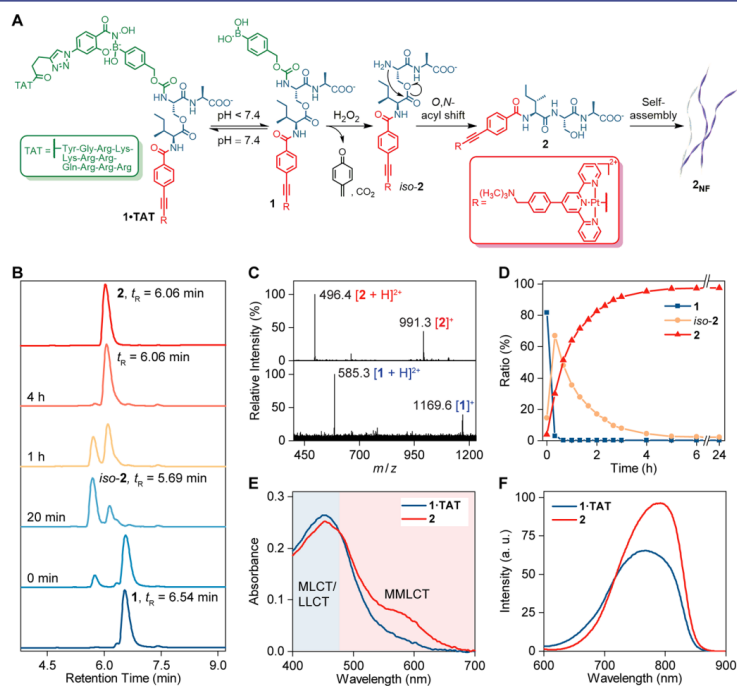


Figure 2. (A) Chemical design and reaction scheme for all chemical transformations of the ISA-platinum(II) complex 1-TAT into nanofibers 2_{NF}. (B) LC-MS kinetic analysis over the H_2O_2 (0.5 mM)-induced linearization of 1 (50 μM) in a mixture of NH_4HCO_3 buffer (pH 7.4, 20 mM) and CH_3OH (9/1, vol %). (C) Convolved MS spectra in the LC-MS analysis for a reaction time of 0 min and $t_R = 6.54$ –6.69 min (identified as complex 1) and a reaction time of 4 h and $t_R = 6.06$ –6.21 min (identified as complex 2). (D) Molar ratio of 1, iso-2, and 2 after addition of H_2O_2 based on the peak integration at 254 nm. (E) UV-vis absorption spectra of 1-TAT and 2 (50 μM) in PB (pH 7.4, 50 mM). (F) Luminescence emission spectra of 1-TAT and 2 (50 μM) in PB (pH 7.4, 50 mM). Excitation wavelength = 488 nm.

platform in living cells, we aim to establish a correlation of bioactive functions with superstructure formation.

Herein, we present a platinum metallo-*iso*-tripeptide that undergoes a cascade of molecular and supramolecular

transformations under specific intracellular environments to form near-infrared (NIR) emitting nanofibers (Figure 1). The isomerization of the *iso*-tripeptide forms the backbone, aligning the platinum(II) terpyridine (Pt-tpy) complex with its β -

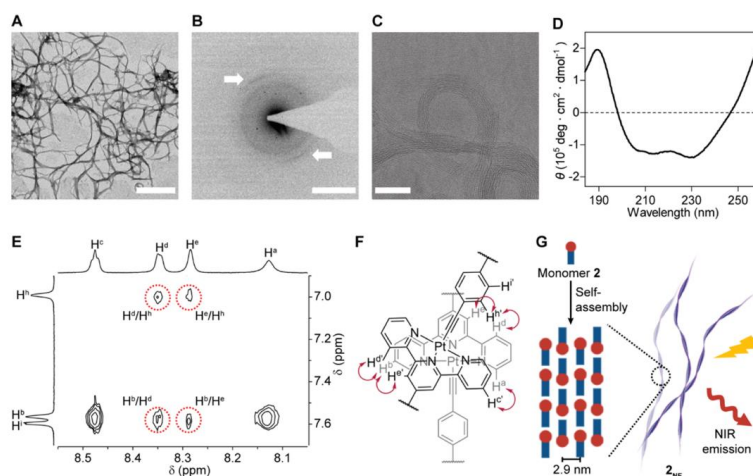


Figure 3. Nanofiber 2_{NF} with a high degree of molecular order formed by self-assembly of **2**. (A) TEM image of the nanofibers 2_{NF} (25 μ M) in PB (pH 7.4, 10 mM). Scale bar, 200 nm. (B) SAED pattern of the nanofiber 2_{NF} (100 μ M) in H_2O . The white arrows indicate diffraction arcs at 3.0 nm^{-1} , that is, $3.3\text{ \AA}$. Scale bar, 3 nm^{-1} . (C) Cryogenic high-resolution TEM image of the nanofiber 2_{NF} (25 μ M) in PB (pH 7.4, 50 mM). Scale bar, 50 nm. (D) CD spectra of 2_{NF} in a mixture of PB (pH 7.4, 10 mM) and CH_3CN (98/2, vol %). (E) Partial 1H , 1H NOESY NMR spectrum (850 MHz, 343 K) of **2** (1 mg/mL) in PB (pH 7.4, 50 mM). Intermolecular NOE cross-peaks are circled in the NMR spectrum. (F) Proposed molecular stacking mode of the Pt-tpy group in 2_{NF} based on the NOESY NMR study. The red arrows indicate intermolecular NOE interactions between protons. (G) Proposed arrangement of monomer **2** in 2_{NF} based on microscopy and NMR studies. The nanofibers showed a mean interfiber distance of 2.9 nm. Nanofiber formation also promotes NIR emission originated from the Pt-tpy groups in 2_{NF} . Intracellular formation of 2_{NF} induces actin restructuring.

amyloid-like sequence, thus fostering the propagation of supramolecular order through π - π and metal-metal interactions. Pt complexes enable rich molecular topologies and diverse self-assembly structures in both the solid state and solutions.^{21–25} The synergy between platinum(II) complexes with other self-assembling motifs are well-known, often producing assemblies with emerging morphological and photoluminescence properties.^{12,25–32} By merging the features of platinum(II) complexes and peptides together with boronic acid-salicylhydroxamate (SHA) chemistry that responds to both physiological pH and reactive oxygen species,^{11,33} the progression from a dormant assembling precursor to the final superstructure can be controlled and imaged within cells. With the robust sequence of chemical transformation in place, the biological activity on aerobic glycolysis (AGlyc) and OxPhos is demonstrated on A549 lung cancer cells and MDA-MB-231 metastatic breast cancer cells. These cancer cell lines are known to be metabolically adaptive,^{18,34} thus enabling a deeper insight into the mechanism of action of the formed nanofiber. Additionally, pathways including histone deacetylase (HDAC) activity³⁵ and actin restructuring^{36,37} that are dependent on ATP or metabolites will be assessed to evaluate the extent of the cellular impact.

RESULTS AND DISCUSSION

Design and Synthesis of the Pro-Assembling Metallo-Isopeptide 1-TAT. The metallo-isotriptide **1-TAT** is composed of three functionalities (Figure 2A): (1) a pro-assembling isopeptide (ISA) caged by an immolative boronic acid group,¹¹ (2) a transporter peptide, trans-activator of

transcription (TAT), that interacts with cellular membranes, enabling cellular uptake and endosomal release.³⁸ This peptide carries a SHA group that binds to the boronic acid via a pH responsive dynamic covalent bond.^{11,33} (3) a Pt-tpy complex coordinated to the alkynyl group on the N-terminus of the *iso*-ISA peptide. The minimalistic design allows each peptide segment to perform their specified roles in a controlled, sequential manner. Hence, the expected mechanism of how the complex interacts with cells would first begin with the TAT-mediated endocytosis and intracellular endosomal escape of the complex.¹¹ Once released to the near-neutral (pH 7.4) cytosol, endogenous H_2O_2 possesses sufficient oxidative strength to immolate the boronic acid cage generating the serine residue with a primary amino group. Next, an *O,N*-acyl shift occurs, causing the peptide to rapidly isomerize into the monomeric self-assembling peptide **2**, which assembles into the nanofibers 2_{NF} (Figure 2A).

The synthesis of the Pt-isotriptide **1** and the TAT-SHA is performed separately using a combination of solution and solid-phase peptide syntheses (Figure S1). The complexation of the Pt-tpy unit to the alkynyl *iso*-ISA **S1** is performed in solution catalyzed by CuI, to afford the target Pt-isotriptide **1** characterized by one-dimensional/two-dimensional nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography (LC)-mass spectrometry (MS) (Figures S8–S13). As a control and reference for subsequent mechanistic studies, the expected product **2** that will be formed at the end of the transformation cascade was also separately synthesized (Figures S14–S25). The dynamic covalent boronic acid-SHA bond between the Pt-isotriptide **1** and TAT-SHA was

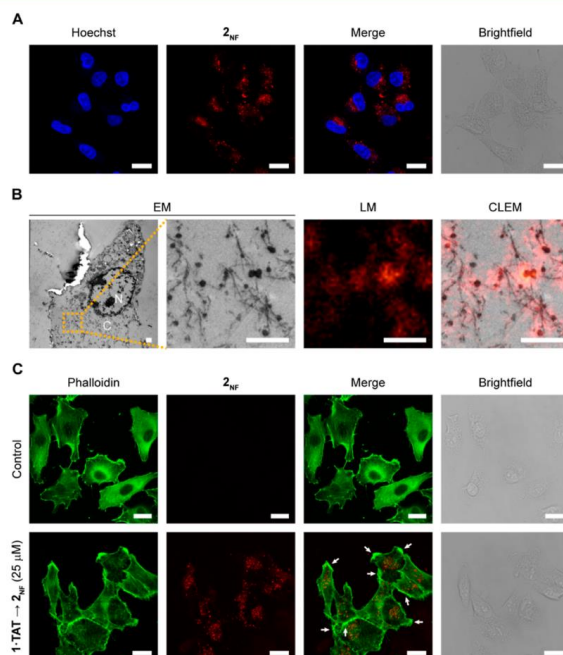


Figure 4. Internalization and assembly into nanofibers $1\text{-TAT} \rightarrow 2_{\text{NF}}$ in the cytoplasm restructure actin filaments. (A) Confocal laser scanning micrographs of A549 cells treated for 4 h with 1-TAT (25 μM) and Hoechst 33342 dye. Scale bar, 20 μm . (B) CLEM of A549 cells treated for 6 h with 1-TAT (50 μM). N, nucleus. C, cytosol. Scale bar, 1 μm . (C) Confocal laser scanning micrographs of A549 cells treated for 4 h with 1-TAT (25 μM) and Phalloidin-iFluor 405. White arrows indicate the fluorescent loci along the cell membrane. Scale bar, 20 μm . Nanofibers 2_{NF} disrupt AGlyc and OxPhos metabolic pathways.

formed in PBS (pH 7.4) to yield the cell-penetrating 1-TAT , characterized by matrix-assisted laser desorption/ionization–MS (Figure S26).

Formation of the Assembly Precursor Peptide 2 by Chemical Triggers. The first chemical transformation occurs under mildly acidic pH, where the boronic acid-SHA bond is cleaved to release **1** into the cytosol.¹¹ The increase to near-neutral pH within the cytosol empowers the oxidation of the boronic acid by endogenous H_2O_2 , resulting in its immolation, thus converting **1** to *iso*-**2** and initiating the rearrangement into **2**. The kinetics of the H_2O_2 -triggered formation of **2** in solution was investigated by LC–MS (Figures 2B–D and S28). In aqueous solution, incubation with H_2O_2 for 20 min resulted in the complete disappearance of **1** and the emergence of two new peaks with retention times (t_R) of 5.69 and 6.06 min, respectively (Figure 2B), both showing m/z values in agreement with the chemical formula of **2** ($m/z = 496.4$ for $[\mathbf{2} + \text{H}]^{2+}$ and 991.3 for $[\mathbf{2}]^+$) (Figure 2C and Figure S29). In comparison with **2** obtained via direct synthesis ($t_R = 6.06$ min), the former was assigned to be the intermediate formed (*iso*-**2**) upon the immolation of the phenylboronic acid prior to isomerization. Further incubation with H_2O_2 led to an increase of **2**, with a 95% conversion at 4 h (Figure 2D). In contrast, less than 5% conversion was observed after 24 h incubation of

1 without H_2O_2 (Figure S30). Similar conversion kinetics is observed when **1** was incubated with H_2O_2 at cellular concentrations of 2.1 μM (Figures S31–S32 and S69), indicating that the intracellular oxidative stress is sufficient for initiating the conversion of **1**.

The square-planar platinum(II) center in the complexes endows the complex with optical properties associated with the nature of self-assembly. Complexes 1-TAT and **2** displayed absorption bands centered at 460 nm in PB (Figure 2E), which can be assigned to a combination of $d\pi(\text{Pt}) \rightarrow \pi^*(\text{tpy})$ metal-to-ligand charge-transfer (MLCT) and alkynyl-to-tpy ligand-to-ligand charge-transfer (LLCT) transitions. A lower energy absorption shoulder at ca. 570 nm is observed for both complexes, which can be attributed to a metal MLCT (MMLCT) transition.^{25,39} Complex **2** exhibited weaker MLCT/LLCT transition but markedly stronger MMLCT absorption than 1-TAT . This indicates a higher extent of intermolecular d_π interactions between platinum(II) centers in neighboring molecules of **2**, suggesting a proclivity for self-assembly in PB forming 2_{NF} .^{25,39} Both solutions showed NIR emission originating from the MMLCT excited states upon excitation at 488 nm (Figure 2F). Complex 1-TAT exhibited emission centered at 770 nm, whereas complex **2** displayed red-shifted emission ($\lambda_{\text{max}} = 792$ nm) with a 1.5-fold higher

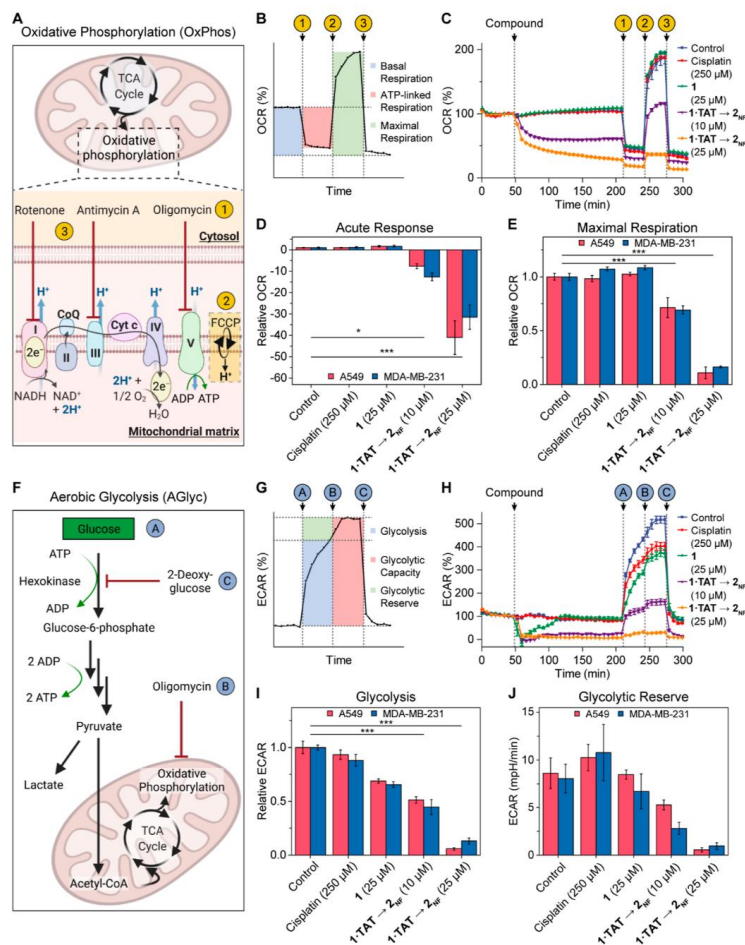


Figure 5. Transformation 1-TAT $\rightarrow$ 2-NF disrupts both AGlyc and OxPhos metabolism of A549 and MDA-MB-231 cells. (A) Scheme of a mitochondrion with focus on the ETC, the site of OxPhos. The compounds of the Mito stress test modulate the ETC when added in the indicated injection sequence. (B) Profile of the OCR during the Mito stress test. Numbers represent the compounds of the Mito stress test shown in Figure 5A. (C) Effect of 2-NF on the OCR of MDA-MB-231. The last measurement before treatment injection is set as 100%. (D,E) Acute response (D) and maximal respiration (E) of A549 and MDA-MB-231 cells. (F) Schematic representation of glycolysis and cell respiration, which are affected by the compounds of the glycolysis stress test. Each target of the modulators of the glycolysis and OxPhos used in the assay is indicated with respect to the injection sequence. (G) Profile of the ECAR during the glycolysis stress test. Characters represent the compounds of the glycolysis stress test shown in Figure 5F. (H) Effect of 2-NF on the ECAR of MDA-MB-231. (I,J) Effect of 2-NF on the glycolysis (I) and glycolytic reserve (J) of A549 and MDA-MB-231 cells. For (D,E,I), the data are expressed as relative values to untreated control cells. Data are presented as mean $\pm$ s.e.m., $n \geq 5$. Statistical significance was calculated by ANOVA with a Tukey post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

intensity, which can be ascribed to the enhanced MMLCT transition as a result of self-assembly. The difference in the photophysical profile between the solutions of the complexes facilitates the tracking of their H_2O_2 -induced conversion and subsequent assembly of 2 to 2-NF in PB. Treatment of 1-TAT with H_2O_2 (0.5 mM) led to a red shift in the λ_{max} with a

concomitant increase in emission intensity (Figure S33). The conversion is completed in 280 min with the final emission profile resembling 2-NF, in agreement with the LC-MS study.

Amyloid-Like Nanofibers 2-NF Form with High Molecular Order. The self-assembly of the complexes was visualized using transmission electron microscopy (TEM). Nanofibers

were observed for 2_{NF} at concentrations ranging from 5 to 100 μM (Figures 3A and S34), whereas **1** and **1-TAT** showed no defined nanostructures (Figure S35). Upon oxidation of **1-TAT** by H_2O_2 , the transformation into **2** and subsequent self-assembly produced fibrillar morphology similar to the control (Figure S36). Selected-area electron diffraction (SAED) on 2_{NF} showed diffraction arcs with a 3.3 Å lattice spacing, in agreement with the intermolecular distance of π - π interactions within the nanofibers.⁴⁰ The diffraction arcs are perpendicular to the long axis of the fibers (Figures 3B and S37). Nanofiber formation is further supported by cryogenic high-resolution TEM studies on 2_{NF} in PB, where the growth axis suggested an end-to-end molecular arrangement of the complexes (Figures 3C and S38). Each fiber was observed to be bundled with a mean interfiber distance of 2.9 nm.

Circular dichroism (CD) spectroscopy analysis on 2_{NF} revealed a maximum at 190 nm corresponding to the $\pi \rightarrow \pi^*$ of the carbonyl group, depicting H-bond interactions centered on the peptidic backbone. This band is coupled with the red-shifted $n \rightarrow \pi^*$ transition visible at the 229 nm minimum.⁴¹ Exciton coupling parallel to the peptide backbone also exists for 2_{NF} at 210 nm (Figure 3D).⁴¹ Additionally, a strong positive signal beyond 250 nm corresponding to the $\pi \rightarrow \pi^*$ transition of the Pt-tpy group²⁵ is also observed for 2_{NF} , demonstrating the importance of the Pt-tpy group in the self-assembly. In contrast, **1** only exhibited a minimum at 208 nm (Figure S39). The self-assembly behavior of 2_{NF} at the molecular level was further examined using NMR. At 298 K, 2_{NF} showed broad signals in the ^1H NMR spectrum. Increasing the temperature led to sharpening of the signals accompanied by significant downfield shifts (Figure S41), which indicates self-assembly at 298 K. Nuclear Overhauser effect spectroscopy (NOESY) of 2_{NF} revealed NOE cross-peaks between the protons on the tpy group and the phenyl ring of the alkynyl ligand ($H^{\text{d}}/H^{\text{b}}$ and $H^{\text{c}}/H^{\text{b}}$) and between the non-neighboring protons on the tpy moiety ($H^{\text{a}}/H^{\text{c}}$, $H^{\text{b}}/H^{\text{d}}$, and $H^{\text{b}}/H^{\text{e}}$) (Figures 3E and S47), implying that the Pt-tpy group adopts a twisted head-to-tail stacking upon self-assembly (Figure 3F), which is characteristic for Pt-tpy complexes.³⁹ The microscopy and spectroscopy experiments suggest that 2_{NF} possesses a high degree of molecular order, which is highly favorable for studies in a biological context because it enhances the proteolytic stability of the nanofibers and promotes NIR emission originated from the Pt-tpy group (Figure 3G).

The growth of the nanofibers **1-TAT** $\rightarrow$ 2_{NF} was visualized in A549 lung alveolar adenocarcinoma cells and MDA-MB-231 metastatic breast cancer cells using confocal laser scanning microscopy (Figures 4A, S48, and S49). Within 4 h, TAT-dependent internalization was observed (Figure 4A). Assisted by the NIR emission of the platinum(II) center, correlative light-electron microscopy (CLEM) observed that newly formed luminescent nanofibers within the cytoplasm occur after escaping from the endosomal vesicles (Figures 4B, S50, and S51). In line with the pH dependence of endogenous H_2O_2 activity on the boronic acid, the TEM micrographs revealed that the entry into the near-neutral (pH 7.4) cytosol from the acidic (pH 6.0) endosomes initiates the nanofiber formation. At the onset of nanofiber growth, the first metabolic consequence of newly formed 2_{NF} can be detected through early changes in the cytoskeleton, whose formation is tightly coupled to glycolytic pathways.^{36,37} An investigation using cytoskeleton stain Phalloidin-iFluor 405 demonstrated a restructuring of actin filaments toward the cellular membrane

(Figures 4C and S54). Using both 10 and 25 μM , the appearance of fluorescent loci along the cell membrane was only observed at 25 μM (Figure 4C, lower panel), suggesting that a critical concentration threshold of 2_{NF} has to be reached for actin restructuring. Actin filaments in cells are regulated by Rho-GTPases,⁴² which are accumulated on the lateral membranes in cultured mammalian epithelial cells.⁴³ Disruption can occur at the Rho-GTPase level or by the disassembly of F-actin, each leading to different observations through phalloidin stain. F-actin fragmentation generates phalloidin signals aggregating around the remnants of the actin filaments.⁴⁴ In contrast, interference toward Rho-GTPases accumulates phalloidin signals to the membrane because F-actin cannot propagate without a functioning Rho-GTPase or its substrate, GTP.⁴⁵ As the equilibrium of GTP and ATP is typically maintained by nucleoside-diphosphate kinases,⁴⁶ we hypothesize that broader, systemic effects may have been the origin of the observations since the nanofibers are unlikely to inhibit specific enzymes.

We investigate the impact of 2_{NF} on the main mechanisms of cellular metabolism, AGlyc and OxPhos pathways, which are core upstream processes involving at least 10% of the human proteome.⁴⁷ The consumption of oxygen to fuel mainly OxPhos, and the extracellular acidification as a function of AGlyc were analyzed in A549 and MDA-MB-231 cells using a Seahorse extracellular flux analyzer (Figure 5). Treatment of both cell lines with **1-TAT** that transforms into 2_{NF} demonstrated a reduction in the oxygen consumption rate (OCR) within 4 h (Figure 5C), with 25 μM inducing 41-fold and a 32-fold decrease, respectively, in A549 and MDA-MB-231 cells (Figure 5D). The OCR, expressed as pmol/min, is a first measure of OxPhos (Figure 5A–C) activity, which takes place primarily in the mitochondria.

Key parameters of mitochondrial respiration were investigated to unravel the impact of 2_{NF} on OxPhos by the sequential use of modulators of the electron transport chain (ETC): rotenone (Complex I), antimycin A (Complex III), oligomycin (ATP synthase), and FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) to disrupt the mitochondrial membrane potential.^{48,49} Treated cells exhibit less than 10% of the FCCP-induced maximal respiration compared to untreated cells, highlighting the inability of cells to sustain energy demand even when stimulated to operate at maximum capacity (Figure 5E). Thus, in addition to the impact on OxPhos depicted by the acute response, 2_{NF} induces a massive and persistent effect on mitochondrial respiration that prevents cells from adapting to stress. This phenomenon is further confirmed by a reduction of most of the OxPhos-related parameters, such as ATP production and spare respiratory capacity, which is a potential indicator of mitochondrial damage (Figures S58–60). Based on the burden of **1-TAT** $\rightarrow$ 2_{NF} on mitochondrial respiration, we subsequently evaluated parameters related to AGlyc where cancer cell types are known for being able to rewire their cell metabolism between OxPhos and AGlyc, upregulating accordingly one of the two metabolic pathways to satisfy their energetic needs.^{18,34}

Therefore, the AGlyc of A549 and MDA-MB-231 cells was measured using their extracellular acidification rate ($\text{ECAR}_{\text{glyc}}$), expressed as mpH/min (Figure 5F–H). Extracellular acidification occurs when glucose is converted to pyruvate and lactate, accompanied by the extrusion of protons into extracellular space⁵⁰ (Figure 5F). The cells first underwent glucose starvation to account for the basal non-glycolytic

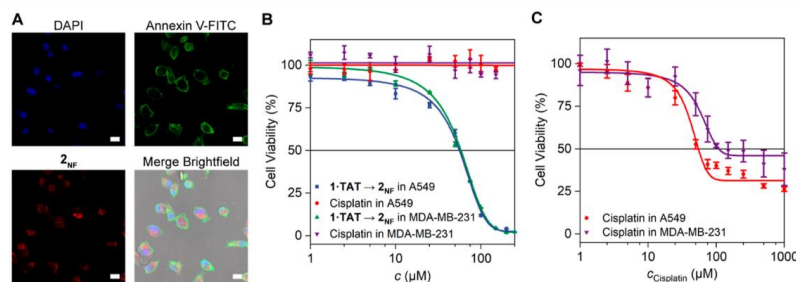


Figure 6. Toxicity of 1-TAT $\rightarrow$ 2NF toward A549 and MDA-MB-231 cells. (A) Apoptosis was investigated for A549 cells using Annexin V assay. Cells were incubated 4 h with 1-TAT (red) and treated with Annexin V (green) afterward. Nuclei were stained using DAPI (blue). Annexin V binds to the cell membrane upon an inversion of phosphatidylserine motifs toward the extracellular space, indicating apoptosis. Scale bar, 50 μ m. (B) Cell viability assay of A549 and MDA-MB-231 cells treated with 1-TAT and cisplatin for 4 h, revealing viable cells after cisplatin treatment but significantly reduced cell viability after transformation into 2NF. (C) Cell viability assay of cisplatin at 24 h. The cell viability was determined using the CellTiter-Glo Luminescence Cell Viability Assay Kit.

acidification, followed by the addition of saturating amounts of glucose to trigger glycolysis. The formation of the nanofibers at 25 μ M shuts down glycolysis of A549 and MDA-MB-231 to 6 ± 1 and $13 \pm 3\%$, respectively (Figure S1). The capability of cells rewiring their metabolism⁵¹ was demonstrated by adding oligomycin, the ATP synthase inhibitor,⁴⁸ to prevent OxPhos and thereby inducing the switch to glycolysis. In the untreated control cells, the incubation with oligomycin raised the glycolytic activity by $+8.6 \pm 1.6$ mpH/min (A549) as well as $+8.0 \pm 1.5$ mpH/min (MDA-MB-231) (Figure S1). This adaptation is termed the glycolytic reserve, which is activated upon mitochondrial stress/dysfunction. On the contrary, inducing the metabolic switch in cells where 2NF I is formed only increases the glycolytic acidification by only 0.6 ± 0.2 mpH/min for A549 cells and 0.9 ± 0.4 mpH/min for MDA-MB-231 cells (Figure S1). Hence, even under severe stress conditions, aerobic glycolysis remained low, suggesting that the capabilities of the cells to compensate for this disruption were limited. To further ascertain that the extracellular acidification is the consequence of glucose metabolism, 2-deoxy-glucose, a competitive inhibitor toward glucose hexokinase,⁴⁸ was added. By inhibiting the first enzyme of glycolysis and thus the suppression of ECAR, we demonstrated that the observed extracellular acidification is an accurate measure of their glycolytic activities. In summary, the transformation of 1-TAT $\rightarrow$ 2NF has shown the ability to impair cancer cell fitness by interfering with both OxPhos and AGlyc and preventing metabolic adaptation, one of the most prominent features of cancer cells to react and address stress stimuli.⁵²

Nanofibers 2NF Induce Early Apoptosis. With the formation of 2NF disrupting both AGlyc and OxPhos processes, the production of metabolites such as glucose-6-phosphate (G6P) is investigated. Hence, G6P-related pathways such as the downstream production of NADPH by G6P dehydrogenase will be impeded, leading to the downregulation of HDAC activity.³⁵ Indeed, treated A549 and MDA-MB-231 cells showed concentration dependent reduction in HDAC activity at 4 h, with 25 μ M showing a 25 and 10% decrease, respectively (Figure S66). With the impairment of metabolic pathways and HDAC activity, apoptosis of the cells was evaluated using Annexin V-FITC and cell viability assays. At 25 μ M, 2NF induced the presence of phosphatidylserine on the

external leaflet of the cell membrane, which was detected by the binding of Annexin V-FITC. This characteristic observation of early apoptosis suggests that the population of cells were undergoing the hallmark changes associated with programmed cell death (Figures 6A and S67).⁵³ Importantly, the formation of 2NF is effective at 4 h in both A549 cells and MDA-MB-231 cells, whereas cisplatin is ineffective at this timeframe. Even with 24 h treatment of cisplatin at 1 mM, >25% of the cells survived, demonstrating, at first glance, the mechanistical difference between small molecules and nanostructures in terms of cellular impact (Figure 6B,C).⁵⁴ The disparity in time-dependent efficacies of cisplatin and 2NF is likewise apparent in the metabolic studies, where a 4 h cisplatin treatment did not produce observable effects on AGlyc and OxPhos even at a 10-fold higher (250 μ M) concentration (Figure S5). It is interesting to note that even though the formation of nanofibers demonstrates a more significant impact on oxygen-dependent ATP production on A549 cells compared to MDA-MB-231 cells, the IC₅₀ values of 1-TAT $\rightarrow$ 2NF on both cell lines (60.6 μ M/A549; 58.5 μ M/MDA-MB-231) are not significantly different. We hypothesize that upon the critical assembly concentration of the nanofibers, the damage threshold toward cellular metabolism has reached a point of no return. At this stage, any further damage on cells that are already undergoing apoptosis would not provide additional statistical weight on the overall cell viability. In comparison, 1-TAT $\rightarrow$ 2NF showed similar cytotoxicity toward non-cancerous CHO and HEK cell lines (IC₅₀ = 49.8 and 72.3 μ M against CHO and HEK cells, respectively, Figure S68) compared to cancerous cells lines because a similar H₂O₂ concentration was detected (Figure S69). With these results, we highlight the broad system-level impact that is cell line independent as these structures affect the fundamental pathways of cell vitality.

CONCLUSIONS

In conclusion, we have designed a pro-assembling Pt(II) metallo-peptide that undergoes a step-wise transformation into the NIR emitting nanofibers within A549 and MDA-MB-231 cells. The Pt-tpy complex directs the supramolecular order and directionality of the packing within the fiber axis, while providing NIR photoluminescence. The formation of the

nanofibers rapidly damages energy homeostasis and essential metabolic pathways AGlyc and OxPhos, preventing the cells from mounting adaptive strategies that are known, particularly in cancer, to resist specific small molecule inhibitors, increasing metastasis. Pathways that are intrinsically linked to ATP production (cytoskeleton) and glucose metabolites (HDAC) are impaired, confirming the mechanistic origin of the formed 2_{NF} nanofiber. Rapid apoptosis is induced within 4 h compared to cisplatin and found to leverage a similar potency on both cell types. Collectively, assembly-driven strategies to design metabolically active materials within cancer cells can be exploited to induce systemic level effects and compensate limitations of small molecules and biologics. By demonstrating that complex cellular functions can be addressed purely by nanostructure formation through controlled cascade reactions and self-assembly, we showcase a new avenue to address grand challenges in drug discovery and synthetic biology.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.2c03215>.

Details of instruments, synthesis and characterization of the complexes, and experimental procedures for cellular assays (PDF)

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SUPPLEMENTARY INFORMATION

In Situ Assembly of Platinum (II)-Metallopeptide Nanostructures Disrupts Energy Homeostasis and Cellular Metabolism

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1 Supplementary methods

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 CH₃CN in HPLC grade and H₂O for HPLC and reactions was obtained from a Millipore purification system.

1.2 Synthesis of the complexes

Solid-phase peptide synthesis was conducted to prepare the alkynyl-functionalized peptidic precursors. Dehydrohalogenation reaction between the alkynyl group and a chloroplatinum (II) complex under an inert atmosphere led to the coordination of the alkynyl ligand to the Pt-tpy moiety to afford **1**, the precursor for **1·TAT** with a free boronic acid group. Linear conjugate **2** was synthesized, as a control, using a similar strategy. SHA-functionalized TAT peptide motif (**SHA-TAT**) was synthesized using a reported method.² Complexation of **1** and **SHA-TAT** via SHA-boronic acid dynamic covalent bond was achieved by mixing them in a 1:1 molar ratio in a mixture of DMSO and phosphate buffer (PB, pH 7.4, 50 mM) (DMSO/PB, 2/98, vol%), leading to the formation of **1·TAT**. The compounds were identified by NMR spectroscopy, high-performance liquid chromatography, and mass spectrometry. A detailed procedure for the synthesis and characterization of the complexes can be found in Section S2.

1.3 Self-assembly formation of **2_{NF}**

Monomeric complex **2** was dissolved in DMSO at a high concentration (10 mM) before diluted to different concentrations using PB (pH 7.4, 50 mM). Self-assembly was induced by agitating the solution in an Eppendorf Thermomixer for 2 h at 37 °C at 500 r.p.m.

1.4 Spectroscopy analysis

The UV/Vis absorption spectra for the solutions of the compounds were recorded on a Thermo Scientific™ NanoDrop 2000/2000c spectrophotometer in a Hellma high precision quartz cell, 10 × 10 mm light path. The luminescence emission spectra for the solutions of the compounds were recorded on a Cary Eclipse fluorescence/luminescence spectrophotometer in a Hellma high precision quartz cell, 10 × 10 mm light path. For tracking of H₂O₂-induced conversion from **1** to **2_{NF}** by luminescence, Compound **1** was dissolved in DMSO at a high concentration (10 mM) before diluted to desired concentrations using PB (pH 7.4, 50 mM) containing H₂O₂ (0.5 mM). The luminescence emission profile of the mixture was recorded over 360 min with 10 min intervals. CD spectra were recorded on a JASCO J-1500 spectrometer in a Hellma high precision cell with 1 mm light path.

1.5 HPLC-MS analysis of H₂O₂-induced conversion

Complex **1** was dissolved in a mixture of NH₄HCO₃ buffer (pH 7.4, 20 mM) and CH₃OH (90/10, vol%) at a concentration of 100 μ M. Equal volume of H₂O₂ (1 mM) solution in a mixture of NH₄HCO₃ buffer (pH 7.4, 20 mM) and CH₃OH (90/10, vol%) was added to initiate the conversion. HPLC-MS analysis of the mixture was performed on a Shimadzu LC-MS 2020 equipped with a Kinetex EVO C18 column (50 $\times$ 2.1 mm, 2.6 μ m, 100 Å), an SPD-20A UV-Vis detector, and an electrospray ionization source. 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, and then the CH₃CN content was linearly increased to 95% over 14 min. The molar ratio of the compounds was calculated using peak areas at 254 nm. For analysis of lower concentration of complex **1** (final concentration of 2.0 μ M) and H₂O₂ (final concentration of 2.1 μ M), the solution mixture was prepared using the same protocol and the conversion was investigated using a ZORBAX Eclipse XDB-C18 HPLC column (9.4 $\times$ 250 mm, 5 μ m).

1.6 TEM analysis

TEM samples were prepared by dropping a solution of **2_{NF}** onto a carbon-coated copper grid. After 5 min, the excess solution was removed by a filter paper. The sample was then stained inverting the TEM grid (sample side down) on a droplet (10 μ L) of 2% uranyl acetate solution for 30 seconds. After removing the staining solution with a filter paper, the sample was washed with Milli-Q water for three times (shaken in water for 6 seconds for each time). After drying in air, the measurement was conducted on a JEOL JEM-1400 TEM operating at an accelerating voltage of 120 kV.

1.7 SAED Analysis

Sample for SAED analysis was prepared by drop-casting a solution of **2_{NF}** in H₂O on an ultrathin carbon grid. SAED analysis was performed on an image-side aberration-corrected FEI Titan 80-300 operated at 300 kV. The microscope is equipped with a CEOS hexapole aberration-corrector which corrects the geometrical axial aberrations up to the 3rd-order. Diffraction data acquisition was conducted on a Gatan UltraScan CCD camera. For SAED acquisition, we used a selected-area aperture with a physical diameter of 50 μ m, corresponding to a diameter of 700 nm in the image plane. In order to avoid substantial electron radiation damage on the specimen, a low-dose technique has been applied. The total electron dose for SAED acquisition was 0.8 e-/Å² (dose rate: 0.04 e-/Å²s, acquisition time: 20 s).

1.8 Cryo-TEM analysis

For cryo-TEM examinations the sample was prepared using a Mark V Vitrobot® (Thermo Fisher Scientific) cryo plunge device. Samples were plunged from a climate chamber operated at room temperature with 100 % relative humidity. The sample dispersion was applied either to a Quantifoil® or lacey holey carbon grid and plunged into liquid ethane. Subsequently, the specimen grid was transferred to a Titan Krios G4 TEM (Thermo Fisher Scientific). Images were acquired using a Gatan K3 direct detection camera.

1.9 ¹H, ¹H NOESY NMR experiments

The NOESY NMR measurements were performed with a 5 mm QXI ¹H/¹³C/¹⁵N/³¹P probe equipped with a z-gradient on the 850 MHz Bruker AVANCE III system. The spectroscopic widths of the ¹H, ¹H NOESY experiments were 13500 Hz (850 MHz; 16 ppm) in both dimension (f1 and f2) and the relaxation delay is 2 s. The mixing time was kept at 300ms. A detailed analysis for the NOESY NMR experiments can be found in Section S5.

1.10 Cell culture

A549 and 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% FBS. Both cell lines were cultured in T75 culture flask and subcultivated two to three times per week.

1.11 Cellular uptake studies

A549 and MDA-MB-231 cells were seeded at a density of 25,000 cells/well in an 8-well confocal plate. After adhering for 24 h, cells were treated with the sample for 4 h at 37 °C. Before adding to the cells, samples were pre-incubated to form the **1·TAT** by dissolving **1** in DMSO and mixing with an equimolar amount of the TAT peptide, which was dissolved in Dulbecco's PBS. The sample was further diluted with DPBS to a final volume of 40 µL (DMSO/DPBS, 10/90, vol%). Sample solutions were further diluted 1:4 with DMEM and added to the cells (total DMSO content = 2%), after removing the existing medium from the wells. After the incubation time was over, the cell nucleus was stained with Hoechst 33342 dye for 20 min at 37 °C. A459 cells were additionally treated with Phalloidin-iFluor™ 405 instead of the Hoechst 33342 dye to stain the cytoskeleton. The staining solution was removed, and fresh FluoroBrite DMEM Medium was added to the cells before they were imaged by confocal laser scanning microscopy. Confocal images of cells were taken on a Leica TCS SP5 and Visitron Spinning Disc microscope. To monitor the Hoechst, DAPI and Phalloidin dyes, a 405 nm excitation diode laser was used with an emission filter from 415-500 nm. For Annexin-FITC, an Argon laser was used for excitation at 488 nm with an emission filter from 500-600 nm. To study the uptake of the compound **1·TAT** and subsequent formation of **2_{NF}**, a DPSS laser was used at 561 nm excitation and an emission range from 600-800 nm.

1.12 CLEM studies

One day before treatment, cells were seeded and grown on carbon pre-coated sapphire disks (3 mm; M. Wohlwend GmbH), which were sterilized and oxygen plasma cleaned before use (30 s, 0.2 mbar, 20% power; Femto, diener electronic). The disks were placed in a 24-well plate with 50,000 cells/well, which was incubated at 37°C, 5% CO₂ overnight. After incubation with **1·TAT** (50 µM, 6h), each sapphire disk was collected and slightly immersed into 1-hexadecene before placing them between two aluminum plates (3 mm, Plano). The aluminum plates with the sample were placed into a specimen holder for high pressure freezing in a Wohlwend HPF Compact 01 high-pressure freezer with a pressure of 2100 bar for 2–3 s. The specimen holder was withdrawn from the freezer and immersed into liquid nitrogen to release the sample. The frozen sample was then stored in a container filled with liquid nitrogen. Subsequently, freeze substitution of the sample was carried out in a 0.5 mL Eppendorf tube using an AFS2 freeze substitution unit (Leica). Each tube contained freeze

substitution solution, consisting of 0.2wt/vol% osmium tetroxide, 0.1wt/vol% uranyl acetate, and 5% distilled water in acetone. The tubes were firstly kept at -90°C and automatically warmed up to 0°C in 24 h. After keeping at room temperature for 1 h, the substitution solution was removed, and the samples were washed three times with acetone. Each sample was infiltrated in an ascending epoxy resin series (30%, 50%, and 75% in acetone) for 1 h before final infiltration in 100% epoxy resin overnight. Subsequently, each sample was transferred into a new Eppendorf tube containing freshly prepared pure epoxy resin for polymerization at 60°C for 72 h. After polymerization, sample blocks were kept at room temperature until their sectioning. Sample blocks for each time point were trimmed and sectioned into 100 nm sections by a 35° diamond knife (Diatome) in EM UC6 ultramicrotome (Leica). Sections were then carefully placed onto H6 copper finder grids (Plano) and imaged on a confocal light microscope (Leica SP5) using 561 nm excitation and a 610/60 ET bandpass. For later registration to TEM images, transmitted light and reflected light images were simultaneously recorded. Electron microscopy images were collected on a Jeol 1400 120kV using a 2K bottom mount CCD camera and a Tecnai F20 200 kV TEM by FEI using a K2 Gatan camera. The TEM images were stitched to show the entire cell. Registration of light and electron microscopy images was done by manual landmark registration on entire cells using ImageJ. Landmark selection was based on features identified in the reflected light and transmitted light images.

1.13 Cell metabolism assay

The effect of the compounds on the metabolism of A549 and MDA-MB-231 cells was investigated using the Seahorse XFe96 Analyzer (Agilent Technologies). Seahorse XF Glycolysis Stress Test Kit and Seahorse XF Cell Mito Stress Test Kit were used following the manufacture's protocol to determine the glycolytic and respiratory activity of the cells. Cells were seeded one day before the assay and incubated overnight at 37°C , 5% CO_2 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- CO_2 incubator. On the day of the assay, Seahorse XF DMEM, pH 7.4 was supplemented with glutamine, glucose and pyruvate regarding to the protocols for the specific assay kits. For the Mito Stress Assay the XF DMEM contained pyruvate (1 mM), glutamine (2 mM), and glucose (10 mM). For the Glycolysis Stress Assay the XF DMEM contained 2 mM glutamine. Cells were washed once with XF DMEM (200 μL) and the medium was substituted with fresh XF DMEM (180 μL) and placed in a 37°C non- CO_2 incubator for 45 to 60 minutes. The cartridge was prepared by loading the samples (20 μL) in Port A of each well and the components of the performed assay in Ports B-D. 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 pH, to monitor the extracellular acidification rate (ECAR) and the oxygen concentration, to monitor the oxygen consumption rate (OCR) were constantly measured throughout the entire time of the assay. Both, Glycolysis Stress Assay and Mito Stress Assay were performed in two independent experiments with $n=4$. Bar graphs are shown for the mean $\pm$ standard error of the mean (s.e.m.) for raw and processed data. For the processed data, all values were set to 100% OCR/ECAR 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 * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

For the Mito Stress Assay, Ports B-D were loaded with Oligomycin (22 μ L), carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) (25 μ L) and a mixture of Rotenone and Antimycin A (Rot/AA) (27 μ L). After loading the cell plate eight measurement cycles were performed as baseline control. After addition of the compound, 25 measurement cycles were performed before the components of the Mito Stress Assay were added. For each component five measurement cycles were performed. These cycles resulted in an overall incubation time of the cells with the compounds of 4 h.

For the Glycolysis Stress Assay, Ports B-D were loaded with glucose (22 μ L), oligomycin (25 μ L) and 2-Deoxyglucose (27 μ L). After loading the cell plate eight measurement cycles were performed as baseline control. After addition of the compound 25 measurement cycles were performed before the components of the Glycolysis Stress Assay were added. For each component five measurement cycles were performed. These cycles resulted in an overall incubation time of the cells with the compounds of 4 h.

1.14 Histone deacetylase (HDAC) activity assay

Cells were seeded at a density 5000 cells/well (DMEM) in a 96-well half-area white plate (Greiner Bio-One) and left to adhere overnight at 37°C, 5% CO₂. The DMEM was aspirated and **1•TAT** was introduced into each well in quadruplicates. The treated cells were incubated for 4 h at 37°C, 5% CO₂. The HDAC activity assay was conducted using Trichostatin A as inhibitor control, Jurkat Cell Lysate as positive control and referenced against calibration standards according to the *In-Situ* HDAC Activity Fluorometric Assay Kit (Sigma Aldrich). The fluorescence emission was measured using TECAN microplate reader 20 M (λ_{ex} = 368 nm, λ_{em} = 442 nm).

1.15 Annexin V staining assay

The apoptotic nature of cell death was investigated using Annexin V staining after incubation of A549 cells with **1•TAT** at 25 μ M. Cells were seeded the day before the assay in an 8-well Ibidi plate at a density of 25,000 cells/well and were incubated overnight at 37 °C to allow them to adhere on the surface. After treatment with **1•TAT** for 4 h, cells were incubated with Annexin-V FITC from the Apoptosis Detection Kit FITC by Invitrogen. After washing the cells once with binding buffer (200 μ L), cells in each well were incubated with Annexin V (5 μ L) in binding buffer (200 μ L) for 15 minutes in the dark. Cells were washed with binding buffer and additionally the cell nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI) in binding buffer, incubating the cells for 5 minutes in the dark. The staining solution was removed and the cells were washed twice with binding buffer. The washing solution was replaced with fresh binding buffer (200 μ L) before imaging.

1.16 Cell viability assay

A549, MDA-MB-231, CHO, and HEK cells were both 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. On the next day, different concentrations of the **1•TAT** or cisplatin were prepared in PBS at a 10x concentration of the final concentration, and this solution (20 μ L) was added to a fresh medium (180 μ L). The old medium of the cells was removed and substituted with a fresh medium (50 μ L) containing different

concentrations of sample, prepared as described before. For each condition, triplicates were performed. Cells were incubated with the sample for 4 h or 24 h. After the treatment, CellTiter-Glo® Assay solution (50 µL) 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 manufacturer's protocol.

1.17 Determination of intracellular H₂O₂ concentration

The intracellular H₂O₂ concentration in A549, MDA-MB-231, CHO, and HEK cell lines were determined using the Intracellular Hydrogen Peroxide Assay Kit by Sigma-Aldrich. Cells were seeded in a black cell culture 96 well plate with clear bottom at a density of 10000 cells per well and were left to adhere overnight. Three wells of cells were prepared for each sample (PMA treated and non-treated control cells). To enhance the intracellular hydrogen peroxide concentration cells were treated with 100 nM PMA for 4 h. In order to measure the H₂O₂ concentration the fluorescent peroxide sensor was prepared following the protocol of the vendor and added to the cells after washing them with PBS. After incubation in the dark for 30 min, cells were washed with PBS and fluorescence intensity was recorded using a well plate reader at 525 nm after excitation at 490 nm (bandwidth 10 nm each). To determine the concentration of H₂O₂ inside cells, a standard curve was generated by measuring the fluorescence intensity of a dilution series of hydrogen peroxide at the concentrations of 5, 2.5, 1.25, 0.625, 0.3125, 0.15625 and 0 µM (background blank) after incubation with the fluorescent sensor for 30 min in the dark following the vendor's protocol. All measurements for the standard curve and cells were performed in triplicates, and the background blank was subtracted from all values.

2. Synthesis and characterization details

2.1 Instruments

Nuclear Magnetic Resonance Spectroscopy (NMR). The NMR experiments were measured with a 5 mm QXI ¹H/¹³C/¹⁵N/¹⁹F probe equipped with a z-gradient on the 700.4 MHz and a 5 mm QXI ¹H/¹³C/¹⁵N/³¹P probe equipped with a z-gradient on the 850 MHz Bruker AVANCE III system.

For a quantitative ¹H NMR (850 MHz) measurements at different temperature 64 transients were used with an 10,8 µs long 90° pulse and a 34000 Hz (40 ppm) spectral width together with a recycling delay of 15 s. The temperatures were calibrated with a standard ¹H methanol or ethylene glycol NMR samples using the Topspin 3.2 software (Bruker). The control of the temperature was realized with a VTU (BCU II; variable temperature unit) and an accuracy of +/- 0,1K.

For the proton spectra in the 700 MHz system, 64 transients were used with a 14 µs long 90° pulse and a 12600 Hz (18 ppm) spectral width together with a recycling delay of 8 s, comparable to the 850 MHz system (17000 Hz; 20 ppm, relaxation delay 15 s). The spectroscopic widths of the homonuclear ¹H, ¹H COSY and ¹H, ¹H NOESY experiments were typically 10000 Hz (700 MHz; 15 ppm) or 13500 Hz (850 MHz; 16 ppm) in both dimension (f1 and f2) and the relaxation delay is 2 s. The spectroscopic widths of the homo-nuclear ¹H, ¹H NOESY experiment was typically 13500 Hz (850 MHz; 16 ppm) in both dimension (f1 and f2) and the relaxation delay 2 s. The mixing time used in the ¹H, ¹H NOESY was kept at 300ms.

Additionally, carbon NMR were measured with a sequence using power gated decoupling (zgpg30) with 12288 number (236 ppm spectral width) of scans and a relaxation delay of 2 s and a 90° pulse of 14 μ s on a 700 MHz-magnet system.

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 ppm and $\text{DMSO}-d_6$ = 2.50 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. For ^{13}C NMR, the spectra were recorded are reported in parts per million (ppm) from high to low frequency using the residual solvent peak as the internal reference ($\text{DMSO}-d_6$ = 39.5 ppm). All ^{13}C resonances are reported to the nearest 0.1 ppm. Chemical shifts for protons of the compounds were assigned on the basis of COSY and HSQC correlations. The data were processed in MestReNova.

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

High-Performance Liquid Chromatography (HPLC). The conjugates were purified by preparative HPLC using a setup by Shimadzu. For purification either a ZORBAX Eclipse XDB-C18 HPLC column (9.4 $\times$ 250 mm, 5 μ m) was used at a flowrate of 4 mL/min or a Phenomenex Gemini 5 μ m NX-C18 110 Å 150 $\times$ 30 mm was used at a flowrate of 25 mL/min.

High-Performance 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 μ m EVO C18 100 Å LC 50 $\times$ 2.1 mm column. MilliQ water, acidified with 0.1% formic acid and CH_3CN were used as solvents for all measurements. The solvent gradient started with 5% CH_3CN and 95% water. This solvent ratio was kept constant for 2 min, then the CH_3CN content was linearly increased to 95% over 14 min.

Matrix-Assisted Laser Desorption/Ionisation - Time of Flight Mass Spectrometry (MALDI-TOF). All MALDI-TOF spectra were recorded on either a rapifleX MALDI-TOF/TOF from Bruker or MALDI Synapt G2-SI from Waters. Samples were mixed with a saturated solution of the matrix S7 α -cyano-4-hydroxycinnamic acid (CHCA) in $\text{H}_2\text{O}/\text{CH}_3\text{CN}$ 1/1 + 0.1% CF_3COOH .

2.2 Reaction scheme for the synthesis of the compounds

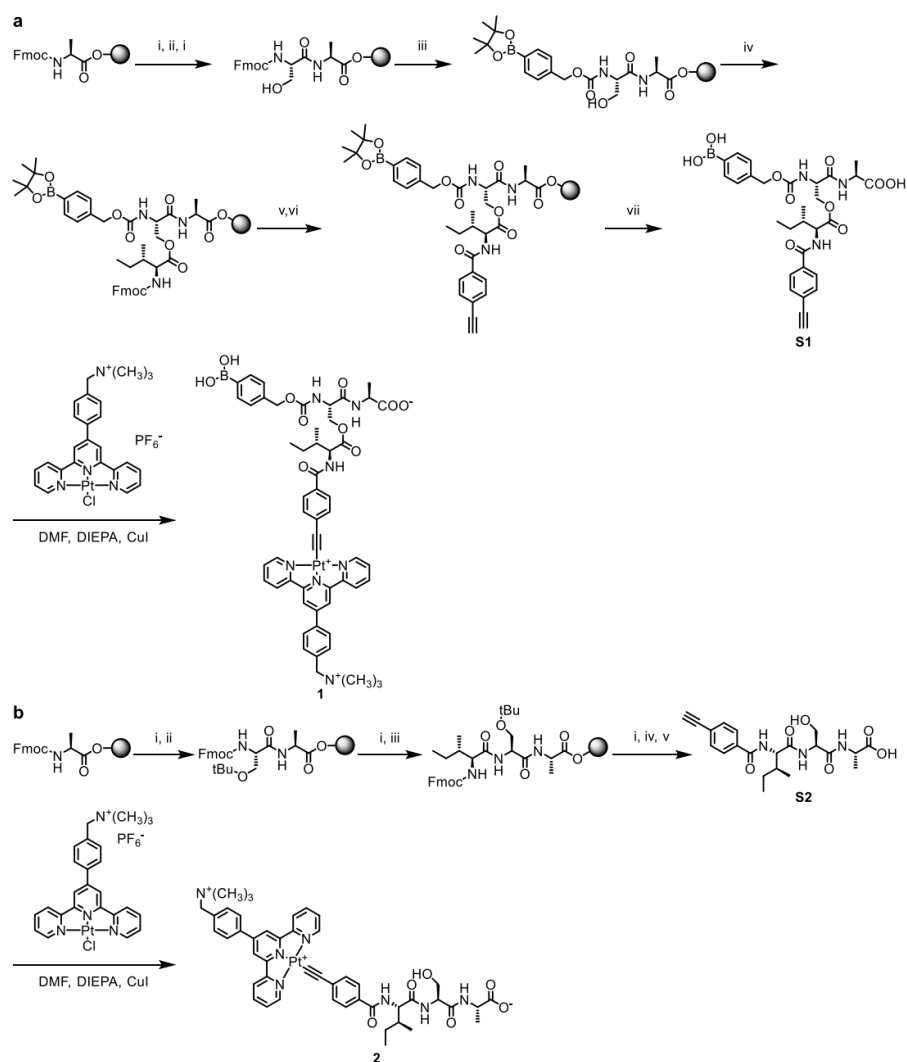
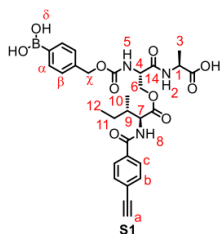


Figure S1. Synthesis of compounds **1** and **2**. **a**, Synthesis of **1**. (i) 20% piperidine in DMF. (ii) Fmoc-serine, PyBOP, DIEPA, DMF, 75 °C, 20 min. (iii) 4-nitrophenyl (4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl), DIEPA, DMF, RT. (iv) Fmoc-Ile, DIC, 4-DMAP, RT. (v) 20% piperidine in DMF. (vi) 4-Ethynylbenzoic acid, PyBOP, DIEPA, DMF, 75 °C, 20 min. (vii) 95% TFA, 2.5% TIPS, 2.5% H₂O. **b**, Synthesis of **2**. (i) 20% piperidine in DMF. (ii) Fmoc-Ser(tBu), PyBOP, DIEPA, DMF,

75 °C, 20 min. (iii) Fmoc-Ile, PyBOP, DIEPA, DMF, 75 °C, 20 min. (iv) 4-Ethynylbenzoic acid, PyBOP, DIEPA, DMF, 75 °C, 20 min. (v) 95% TFA, 2.5% TIPS, 2.5% H₂O.

2.3 Synthesis of compound S1



Synthesis: Compound **S1** was synthesized using the Fmoc solid phase peptide synthesis strategy by Merrifield,² synthesizing the peptide from C to N-terminus in a microwave assisted peptide synthesizer. Fmoc-Ala preloaded Wang resin (0.5 mmol) was swollen in DMF for 1 h before use. First, the Fmoc group was removed by two consecutive deprotection steps (2 and 5 min) with 20% piperidine in DMF (10 mL) at 75 °C (i). After deprotection, the resin was washed four times with DMF (7 mL). Fmoc-serine (5 equiv in 10 mL DMF) was coupled to the N-terminus by using the activator PyBOP (5 equiv in 4 mL DMF) and activator base DIPEA (10 equiv in 2 mL DMF) at 75 °C for 20 min (ii). After deprotection of the Fmoc protecting group (i), the N terminus was modified using 4-nitrophenyl (4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl) carbonate (1.25 equiv) in 10 mL DMF and DIPEA (5 equiv) overnight at room temperature (iii). After the resin was washed with DMF and DCM, Fmoc-Ile (10 equiv in 5 mL DMF), DIC (10 equiv) and 4-DMAP (1 equiv) were added to form the ester bond in the serine side chain. After the reaction mixture was stirred for 12 h at room temperature, the resin was washed with DMF and DCM, dried (iv). The Fmoc group was then removed in two deprotection steps using 20% piperidine in DMF (10 min each, 5 mL) at room temperature (v). 4-Ethynylbenzoic acid (1.2 equiv) was coupled onto the N-terminus overnight at room temperature, using PyBOP (2 equiv) and DIPEA (4 equiv) as the coupling reagents. The resin was washed with DMF and DCM, dried (vi). The product was cleaved from the resin by using 2.5 mL of a cleavage cocktail (95% TFA, 2.5% TIPS, 2.5% H₂O). This step also removed the pinacol protecting group of the PBA. After 2 hours, the cleavage cocktail was removed in vacuo (vii). The product was purified by HPLC using the Zorbax Eclipse column at a flowrate of 4 mL/min. The gradient started with 20% CH₃CN in H₂O (+0.1% CF₃COOH) and this solvent ratio was kept for 1 min, after which the CH₃CN content was increased to 100% over 15 min. The product **S1** eluted from the column after 11.8 min and was received as a white powder after lyophilization (18.2 mg, 6.1% overall yield).

¹H NMR (700 MHz, DMSO-*d*₆, 298 K): δ 8.63 (*H*^δ, d, *J* = 7.9 Hz, 1H), 8.37 (*H*^ε, d, *J* = 7.3 Hz, 1H), 7.87 (*H*^γ, d, *J* = 8.1 Hz, 2H), 7.78 (*H*^α, d, *J* = 7.6 Hz, 2H), 7.61 (*H*^δ, d, *J* = 8.6 Hz, 1H), 7.56 (*H*^β, d, *J* = 8.0 Hz, 2H), 7.31 (*H*^β, d, *J* = 7.6 Hz, 2H), 5.04 (*H*^κ, s, 2H), 4.32 – 4.35 (*H*^δ and *H*^γ, m, 2H), 4.37 (*H*^β, s, 1H), 4.32 (*H*^δ, dd, *J* = 11.2, 4.4 Hz, 1H), 4.24 – 4.17 (*H*^γ, m, 1H), 4.11 (*H*^ε, dd, *J* = 11.0, 8.0 Hz, 1H), 1.94 (*H*^δ, dq, *J* = 11.5, 6.9 Hz, 1H), 1.48 (*H*^γ, dtd, *J* = 13.7, 7.4, 4.4 Hz, 1H), 1.30 – 1.20 (*H*^β and *H*^γ, m, 4H), 0.92 – 0.86 (*H*^γ, m, 3H), 0.84 (*H*^γ, t, *J* = 7.5 Hz, 3H).

¹³C NMR (176 MHz, DMSO-*d*₆, 298 K): δ 173.7, 171.1, 168.4, 166.2, 155.9, 138.6, 134.2, 134.0, 131.5, 128.0, 128.0, 126.5, 124.6, 82.9, 65.6, 63.9, 57.4, 53.6, 47.7, 40.0, 35.8, 25.1, 17.1, 15.4, 10.9.

LCMS: LC retention time (min): 8.14. MS (ESI⁺, *m/z*): 596.3 [M + H]⁺, 618.3 [M + Na]⁺.

HRMS (ESI⁺, *m/z*): Calculated for [M + H]⁺ (C₂₉H₃₅BN₃O₁₀⁺): 596.2421, found 596.2460. Calculated for [M + Na]⁺ (C₂₉H₃₄BN₃O₁₀Na⁺): 618.2240, found 618.2284.

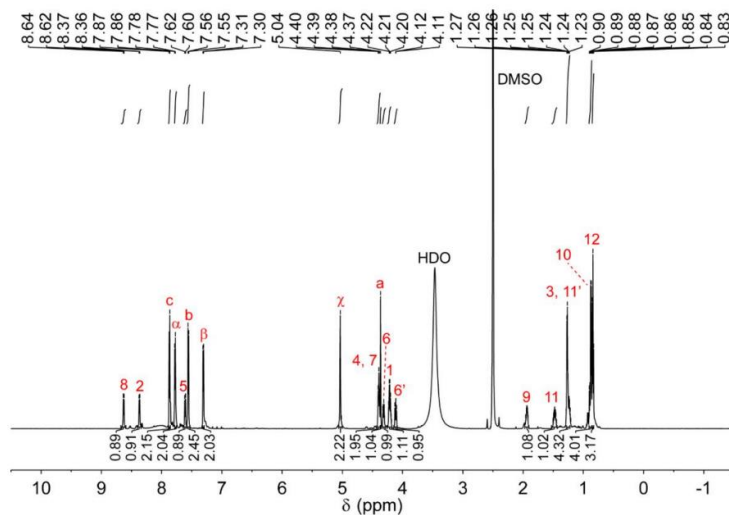
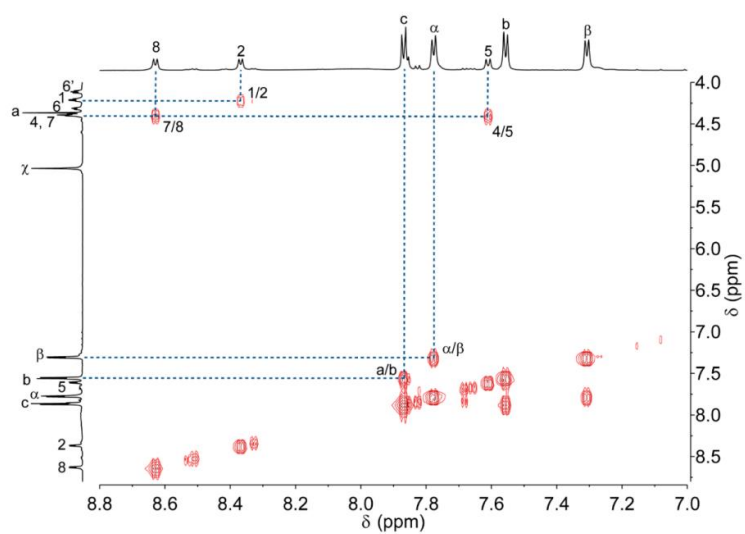


Figure S2. ¹H NMR spectrum (700 MHz, DMSO-*d*₆, 298 K) of compound **S1**.



S12

Figure S3. Partial ^1H NMR and $^1\text{H}, ^1\text{H}$ COSY NMR spectra (700 MHz, $\text{DMSO}-d_6$, 298 K) of compound **S1** (aromatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectrum.

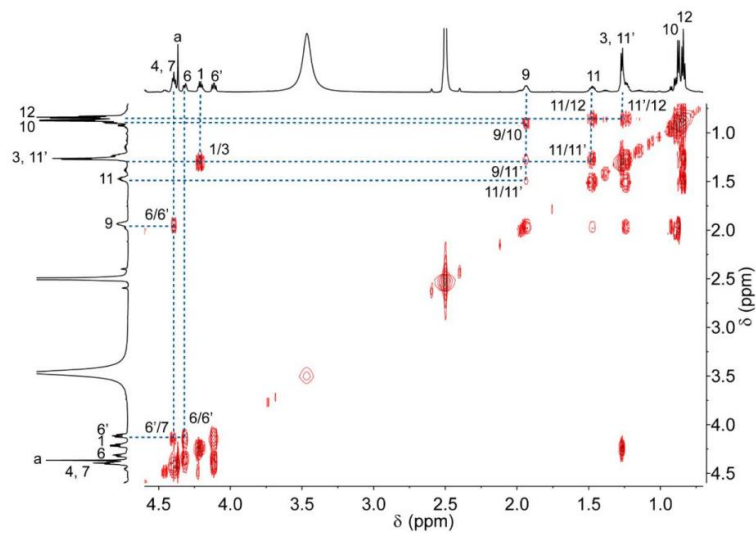
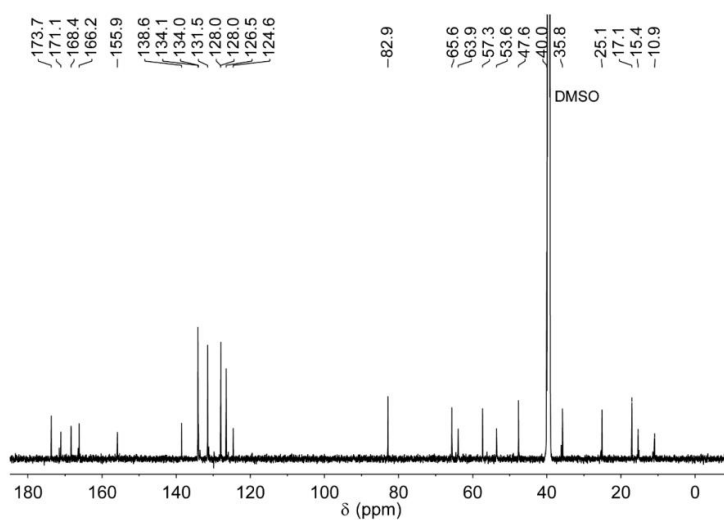


Figure S4. Partial ^1H NMR and $^1\text{H}, ^1\text{H}$ COSY NMR spectra (700 MHz, $\text{DMSO}-d_6$, 298 K) of compound **S1** (aliphatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectra.



S13

Figure S5. ^{13}C NMR spectrum (175 MHz, $\text{DMSO-}d_6$, 298 K) of compound **S1**.

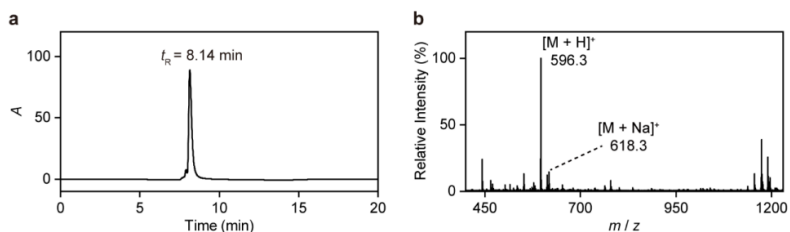


Figure S6. LC-MS study of compound **S1**. **a**, HPLC trace of **S1**. Retention time (t_R) = 8.14 min. **b**, Convoluted MS spectrum for $t_R = 8.10$ -8.30 min, showing peaks of $[\text{M} + \text{H}]^+$ and $[\text{M} + \text{Na}]^+$.

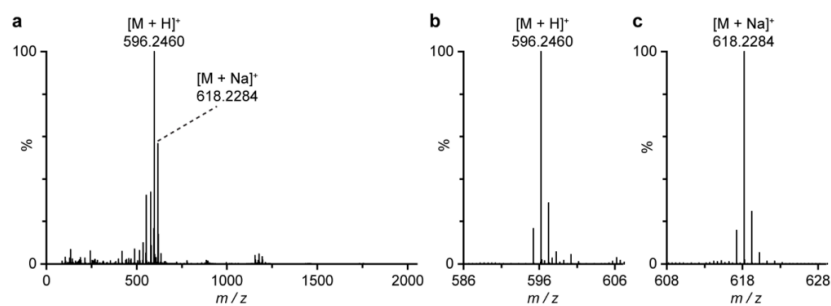
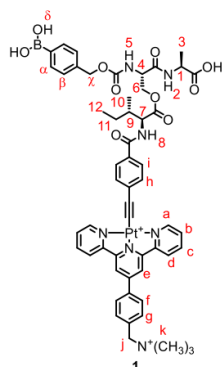


Figure S7. High-resolution MS spectrum of **S1**. **a**, Full MS spectrum. **b**, partial MS spectrum showing $[\text{M} + \text{H}]^+$ and $[\text{M} + \text{Na}]^+$.

2.4 Synthesis of compound 1



Synthesis: Compound **S1** (11.9 mg, 0.02 mmol), the Pt(tpy)Cl complex (15.1 mg, 0.02 mmol), DIEPA (12.9 mg, 0.10 mmol), DMF (5 mL), and a catalytic amount of CuI were placed in a Schlenk flask. After filling the flask with Ar gas using the freeze-pump-thaw method, the mixture was heated to 50 °C for 24 h. The mixture was cooled and Et₂O was added to the mixture. The precipitate was collected, and the product was purified by HPLC using the Zorbax Eclipse column at a flowrate of 4 mL/min. The gradient started with 20% CH₃CN in H₂O (+0.1% CF₃COOH) and this solvent ratio was kept for 1 min, after which the CH₃CN content was increased to 100% over 15 min. The compound **1** (with CF₃COO⁻ counterion) eluted from the column after 10.2 min and was received as a dark brown powder after lyophilization (8.1 mg, 29.0% yield).

¹H NMR δ (700 MHz, DMSO-*d*₆, 298 K): 9.08 (*H*^a and *H*^b, s, 4H), 8.86 (*H*^c, d, *J* = 7.5 Hz, 2H), 8.59 – 8.51 (*H*^d and *H*^e, m, 3H), 8.39 (*H*^f, d, *J* = 6.2 Hz, 1H), 8.29 (*H*^g, d, *J* = 7.5 Hz, 2H), 7.95 – 7.89 (*H*^h, m, 2H), 7.85 (*H*ⁱ, d, *J* = 7.8 Hz, 2H), 7.82 (*H*^j, d, *J* = 7.6 Hz, 2H), 7.79 (*H*^k, d, *J* = 7.2 Hz, 2H), 7.66 (*H*^l, d, *J* = 8.4 Hz, 1H), 7.53 (*H*^m, d, *J* = 7.7 Hz, 2H), 7.33 (*H*ⁿ, d, *J* = 7.0 Hz, 2H), 5.06 (*H*^o, s, 2H), 4.66 (*H*^p, s, 2H), 4.48 – 4.40 (*H*^q and *H*^r, m, 3H), 4.39 – 4.33 (*H*^s, m, 1H), 4.25 – 4.20 (*H*^t, m, 1H), 4.17 – 4.11 (*H*^u, m, 1H), 3.12 (*H*^v, s, 9H), 2.06 – 1.91 (*H*^w, m, 1H), 1.73 (*H*^x, s, 1H), 1.57 – 1.46 (*H*^y and *H*^z, m, 4H), 0.96 – 0.90 (*H*^{aa}, m, 3H), 0.88 (*H*^{ab}, t, *J* = 9.6 Hz, 3H).

¹³C NMR (176 MHz, DMSO-*d*₆, 298 K): δ 173.8, 171.4, 168.4, 166.4, 158.6, 157.8, 157.6, 155.9, 154.3, 154.2, 151.8, 147.6, 142.1, 138.6, 136.9, 134.2, 133.7, 131.4, 131.3, 129.9, 128.3, 127.7, 126.5, 126.1, 121.6, 67.2, 65.7, 57.3, 53.6, 52.1, 47.7, 45.9, 40.0, 35.8, 25.9, 25.2, 17.1, 15.5, 10.9.

LCMS: LC retention time (min): 6.54 MS (ESI⁺, *m/z*): 585.3 [M + H]²⁺, 1169.6 [M]⁺.

HRMS (ESI⁺, *m/z*): Calculated for [M + H]²⁺ (C₅₄H₅₈BN₇O₁₀Pt⁺): 585.7039, found 585.7054. Calculated for [M]⁺: (C₅₄H₅₇BN₇O₁₀Pt⁺): 1169.3921, found 1169.3925.

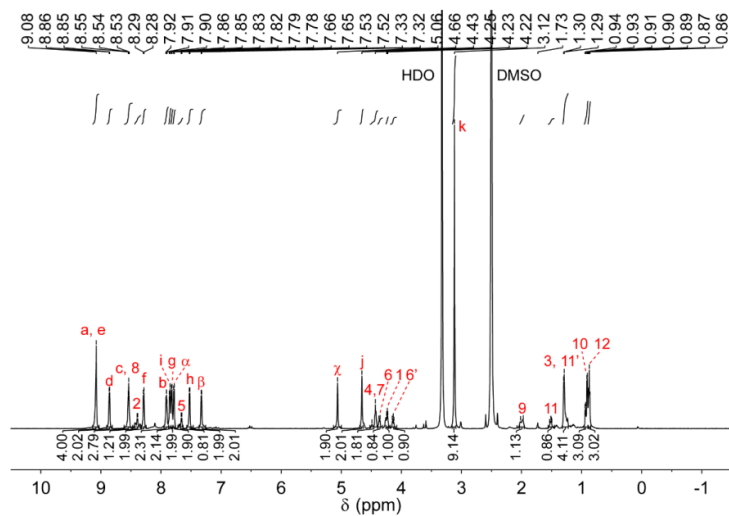


Figure S8. ^1H NMR spectrum (700 MHz, $\text{DMSO}-d_6$, 298 K) of compound **1**.

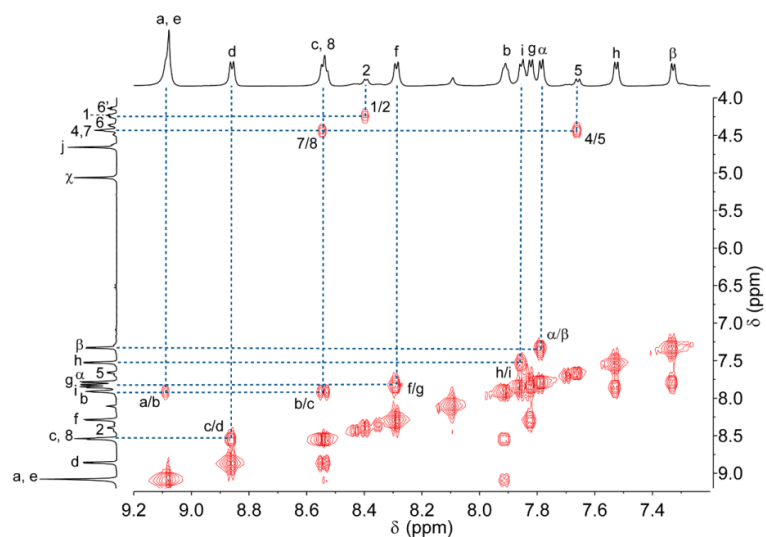


Figure S9. Partial ^1H NMR and $^1\text{H}, ^1\text{H}$ COSY NMR spectra (700 MHz, $\text{DMSO}-d_6$, 298 K) of compound **1** (aromatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectra.

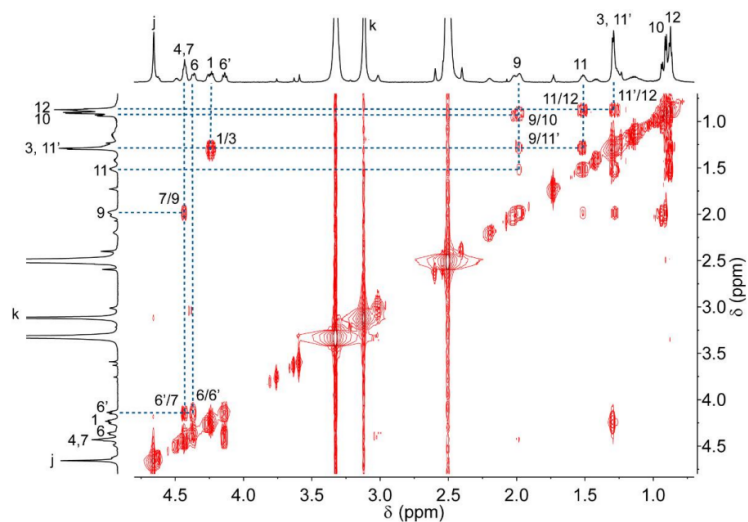


Figure S10. Partial ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra (700 MHz, $\text{DMSO}-d_6$, 298 K) of compound **1** (aliphatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectra.

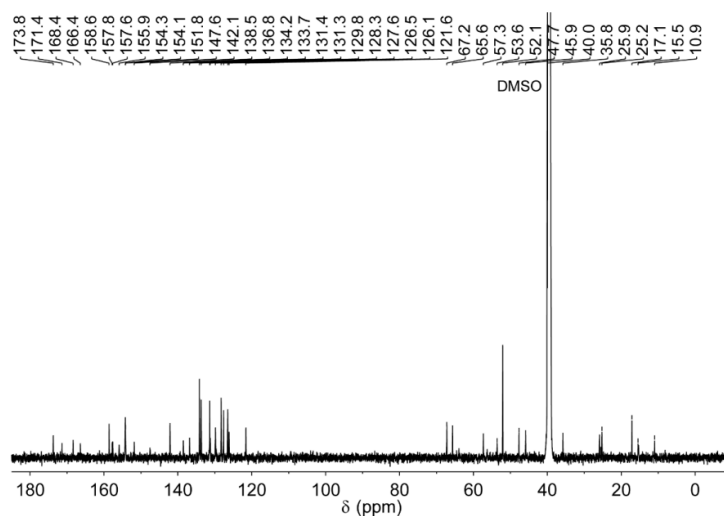


Figure S11. ^{13}C NMR spectrum (175 MHz, $\text{DMSO}-d_6$, 298 K) of compound **1**.

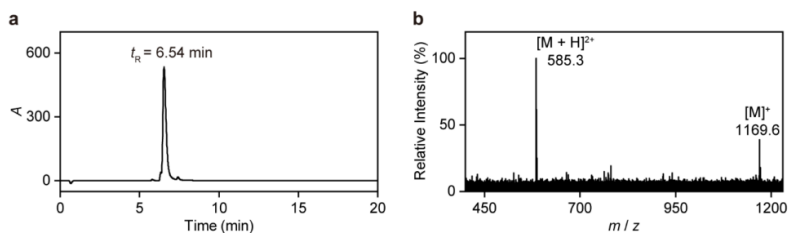


Figure S12. LC-MS study of compound **1**. **a**, HPLC trace of **1**. Retention time (t_R) = 6.54 min. **b**, Convoluted MS spectrum for t_R = 6.52-6.60 min, showing peaks of $[M + H]^{2+}$ and $[M]^+$.

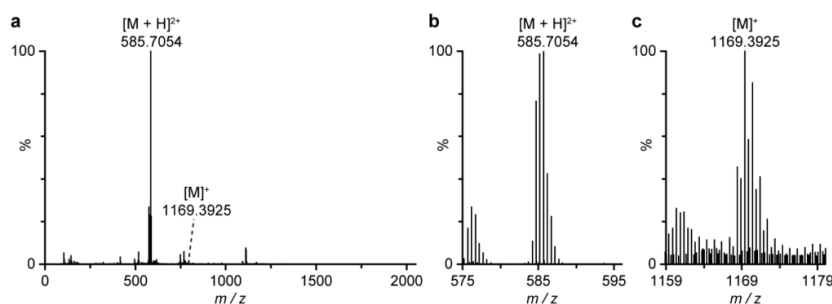
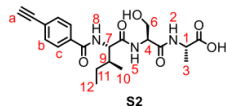


Figure S13. High-resolution MS spectrum of **1**. **a**, Full MS spectrum. **b**, partial MS spectrum showing $[M + H]^{2+}$ and $[M]^+$.

2.5 Synthesis of compound S2



Synthesis: Compound **S2** was synthesized using the Fmoc solid phase peptide synthesis strategy by Merrifield,² synthesizing the peptide from C to N-terminus in a microwave assisted peptide synthesizer at a 0.25 mmol scale. Fmoc-Ala preloaded Wang resin (0.5 mmol) was swollen in DMF for 1 h before use. After deprotection of the Fmoc group (3 mL of 20% piperidine in DMF, 2 and 5 min at 75 °C) (i), Fmoc-Ser(tBu) (ii), Fmoc-Ile (iii), or 4-ethynylbenzoic acid (iv) were double coupled onto the peptide at 75 °C for 20 min. 5 equiv of the compounds were used in 2.5 mL DMF. PyBOP (5 equiv in 1 mL DMF) and DIPEA (10 equiv in 0.5 mL) were used for the coupling reaction. The product was cleaved from the resin by using 2.5 mL of a cleavage cocktail (95% TFA, 2.5% TIPS, 2.5% H₂O). This step also removed the pinacol protecting group of the PBA. After 2 h, the cleavage cocktail was removed in vacuo (v). The product was purified by HPLC using the Zorbax Eclipse column at a flowrate of 4 mL/min. The gradient started with 20% CH₃CN in H₂O (+0.1% CF₃COOH) and this solvent ratio was kept for 1 min, after which the CH₃CN content was increased

to 100% over 15 min. The compound **S2** eluted from the column after 9.1 min and was received as a white powder after lyophilization (68.0 mg, 65.2% overall yield).

¹H NMR (700 MHz, DMSO-*d*₆, 298 K): δ 8.43 (*H*⁸, d, *J* = 8.5 Hz, 1H), 8.06 (*H*⁶, d, *J* = 7.8 Hz, 1H), 7.99 (*H*², d, *J* = 7.2 Hz, 1H), 7.88 (*H*⁷, d, *J* = 8.0 Hz, 2H), 7.57 (*H*⁵, d, *J* = 8.0 Hz, 2H), 4.41 – 4.36 (*H*³ and *H*⁷, m, 2H), 4.33 (*H*⁴, q, *J* = 6.2 Hz, 1H), 4.20 (*H*¹, p, *J* = 7.2 Hz, 1H), 3.64 – 3.53 (*H*⁶, m, 2H), 1.96 – 1.87 (*H*⁹, m, 1H), 1.54 – 1.46 (*H*¹¹, m, 1H), 1.25 (*H*⁹, d, *J* = 7.2 Hz, 3H), 1.17 (*H*¹¹, dp, *J* = 14.9, 7.3 Hz, 1H), 0.91 (*H*¹⁰, d, *J* = 6.7 Hz, 3H), 0.83 (*H*¹², t, *J* = 7.4 Hz, 3H).

¹³C NMR (176 MHz, DMSO-*d*₆, 298 K): δ 173.9, 171.0, 169.5, 165.7, 134.3, 131.5, 127.8, 124.5, 82.8, 61.6, 58.0, 54.9, 47.5, 40.0, 36.0, 24.8, 17.3, 15.4, 10.8.

LCMS: LC retention time (min): 6.97. MS (ESI⁺, *m/z*): 418.2 [M + H]⁺, 440.2 [M + Na]⁺.

HRMS (ESI⁺, *m/z*): Calculated for [M + H]⁺ (C₂₁H₂₈N₃O₆⁺): 418.1978, found 418.2006. Calculated for [M + Na]⁺ (C₂₁H₂₇N₃O₆Na⁺): 440.1797, found 440.1817.

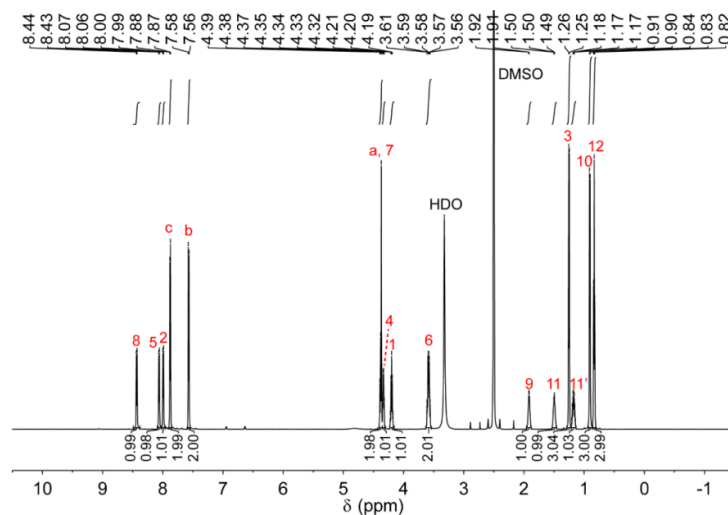


Figure S14. ¹H NMR spectrum (700 MHz, DMSO-*d*₆, 298 K) of compound **S2**.

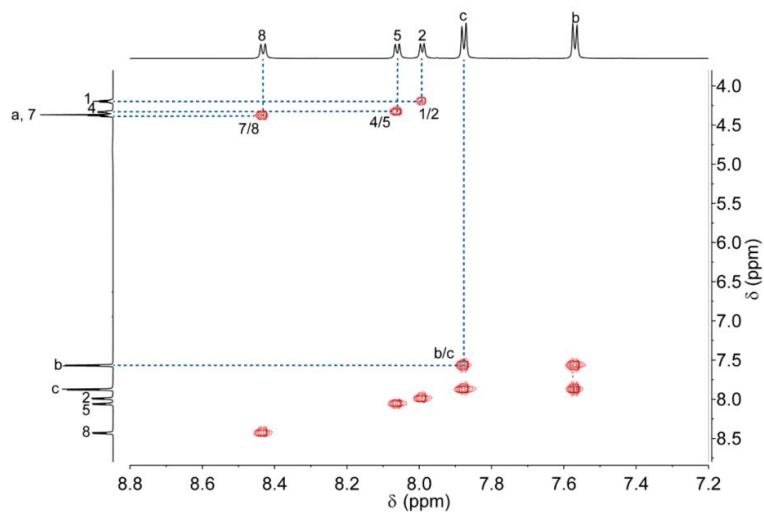


Figure S15. Partial ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra (700 MHz, $\text{DMSO}-d_6$, 298 K) of compound **S2** (aromatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectra.

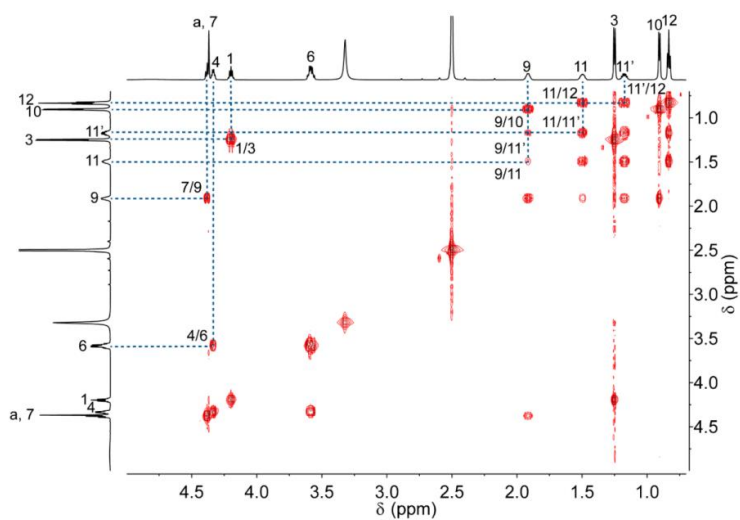


Figure S16. Partial ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra (700 MHz, $\text{DMSO}-d_6$, 298 K) of compound **S2** (aliphatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectra.

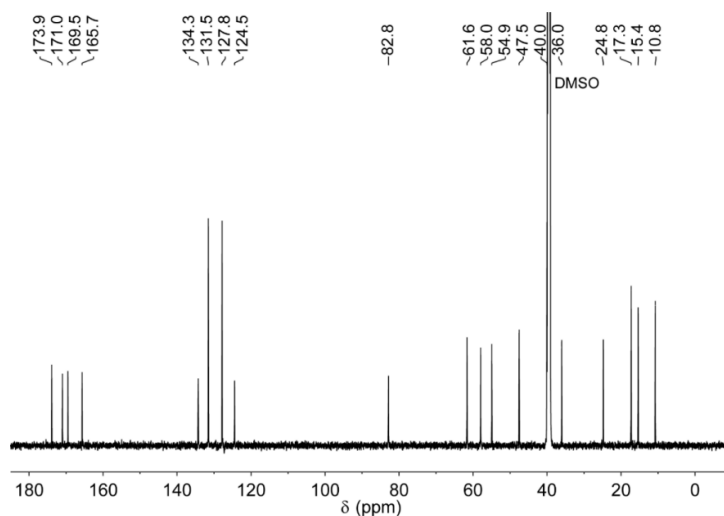


Figure S17. ^{13}C NMR spectrum (175 MHz, $\text{DMSO-}d_6$, 298 K) of compound **S2**.

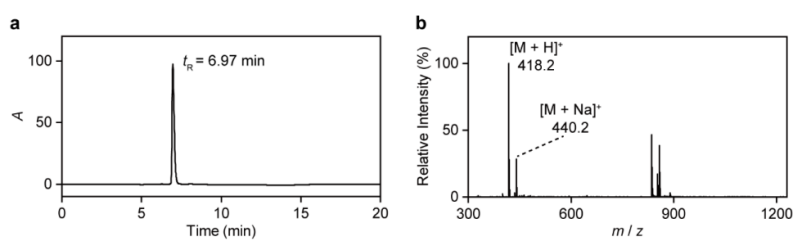
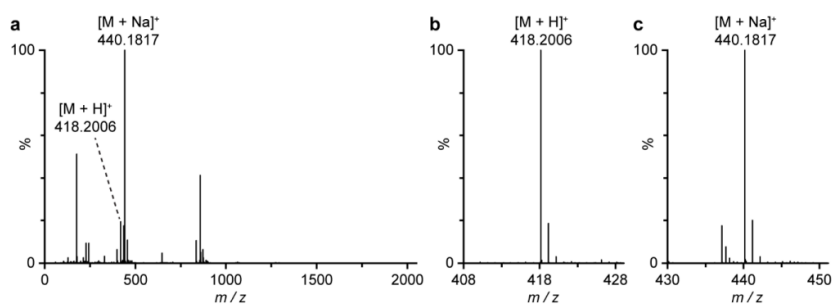


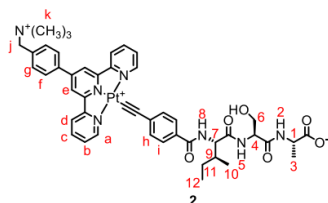
Figure S18. LC-MS study of compound **S2**. **a**, HPLC trace of **S2**. Retention time (t_R) = 6.97 min. **b**, Convoluted MS spectrum for t_R = 6.95-7.10 min, showing peaks of $[\text{M} + \text{H}]^+$ and $[\text{M} + \text{Na}]^+$.



S21

Figure S19. High-resolution MS spectrum of **S2**. **a**, Full MS spectrum. **b**, partial MS spectrum showing $[M + H]^+$ and $[M + Na]^+$.

2.6 Synthesis of compound 2



Synthesis: Compound **S2** (20.9 mg, 0.05 mmol), the Pt(tpy)Cl complex (37.8 mg, 0.05 mmol), DIEPA (32.3 mg, 0.25 mmol), DMF (10 mL), and a catalytic amount of CuI were placed in a Schlenk flask. After filling the flask with Ar gas using the freeze-pump-thaw method, the mixture was heated to 50 °C for 24 h. The mixture was cooled and Et₂O was added to the mixture. The precipitate was collected, and the product was purified by HPLC using the Zorbax Eclipse column at a flowrate of 4 mL/min. The gradient started with 10% CH₃CN in H₂O (+0.1% TFA) and this solvent ratio was kept for 1 min, after which the CH₃CN content was increased to 100% over 15 min. The compound **2** eluted from the column after 8.5 min and was received as a dark brown powder (27.1 mg, 51.6% yield) after lyophilization in a NH₄HCO₃ buffer solution.

¹H NMR (850 MHz, DMSO-*d*₆, 323 K): δ 9.26 (*H^a*, d, *J* = 5.6 Hz, 2H), 9.10 (*H^b*, s, 2H), 8.89 (*H^c*, d, *J* = 7.9 Hz, 2H), 8.59 (*H^d*, t, *J* = 8.5 Hz, 2H), 8.34 (*H^e*, d, *J* = 8.3 Hz, 2H), 8.20 (*H^f*, d, *J* = 8.4 Hz, 1H), 8.02 – 7.98 (*H^g*, m, 2H), 7.96 (*H^h*, d, *J* = 7.9 Hz, 1H), 7.90 – 7.88 (*Hⁱ* and *H^j*, m, 3H), 7.86 (*H^k*, d, *J* = 8.4 Hz, 2H), 7.60 (*H^l*, d, *J* = 8.2 Hz, 2H), 4.67 (*H^m*, s, 2H), 4.43 (*Hⁿ*, t, *J* = 8.4 Hz, 1H), 4.40 – 4.35 (*H^o*, m, 1H), 4.25 (*H^p*, p, *J* = 7.3 Hz, 1H), 3.63 (*H^q*, d, *J* = 6.1 Hz, 2H), 3.13 (*H^r*, s, 9H), 2.03 – 1.93 (*H^s*, m, 1H), 1.61 – 1.51 (*H^t*, m, 1H), 1.28 (*H^u*, d, *J* = 7.1 Hz, 3H), 1.27 – 1.15 (*H^v*, m, 1H), 0.95 (*H^w*, d, *J* = 6.7 Hz, 3H), 0.88 (*H^x*, t, *J* = 7.5 Hz, 3H).

¹³C NMR (175 MHz, DMSO-*d*₆, 323 K): δ 170.9, 169.2, 165.7, 158.3, 157.3, 153.9, 153.6, 151.6, 141.7, 136.5, 133.3, 131.6, 131.1, 130.9, 129.4, 129.2, 128.0, 127.1, 125.9, 121.4, 67.3, 61.7, 58.1, 54.9, 52.1, 48.5, 45.9, 39.8, 36.0, 24.6, 17.6, 15.3, 10.5.

LCMS: LC retention time (min): 6.06. MS (ESI⁺, *m/z*): 496.4 $[M + H]^{2+}$, 991.4 $[M]^+$.

HRMS (ESI⁺, *m/z*): Calculated for $[M + H]^{2+}$ (C₄₆H₅₁N₇O₆Pt²⁺): 496.1776, found 496.1817. Calculated for $[M]^+$ (C₄₆H₅₀N₇O₆Pt⁺): 991.3474, found 991.3532.

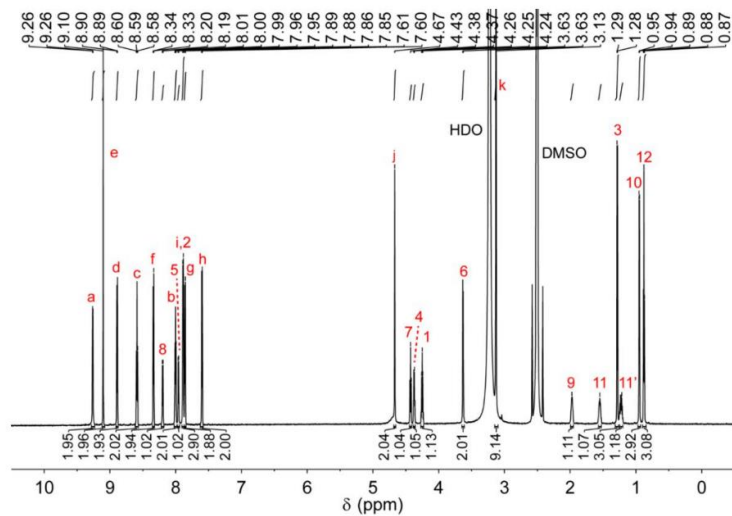


Figure S20. ^1H NMR spectrum (850 MHz, $\text{DMSO}-d_6$, 298 K) of compound **2**.

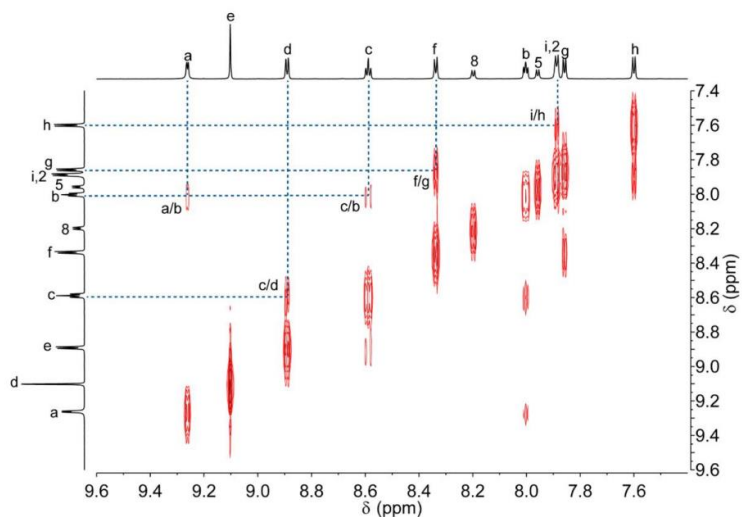


Figure S21. Partial ^1H NMR and ^1H , ^1H COSY NMR spectra (850 MHz, $\text{DMSO}-d_6$, 298 K) of compound **2** (aromatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectra.

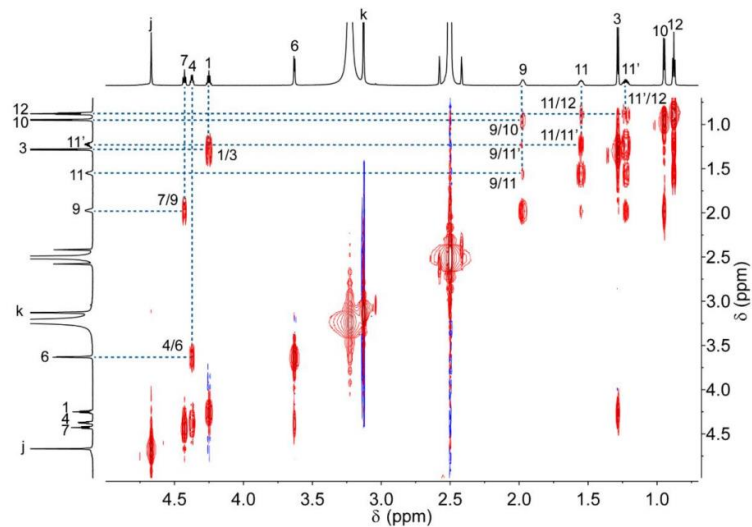


Figure S22. Partial ^1H NMR and ^1H , ^1H COSY NMR spectra (850 MHz, $\text{DMSO}-d_6$, 298 K) of compound **2** (aliphatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectra.

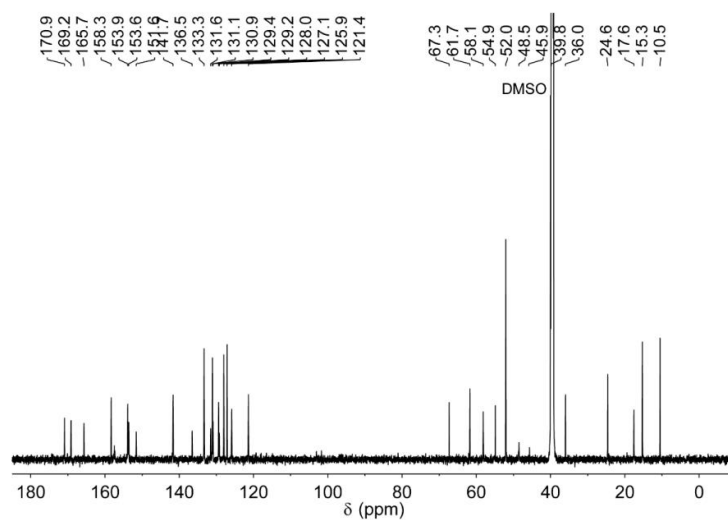


Figure S23. ^{13}C NMR spectrum (213 MHz, $\text{DMSO}-d_6$, 298 K) of compound **2**.

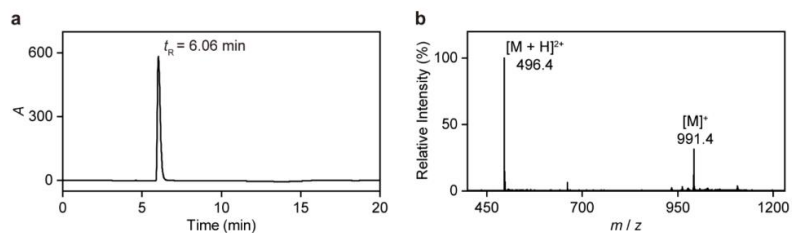


Figure S24. LC-MS study of compound **2**. **a**, HPLC trace of **2**. Retention time (t_R) = 6.97 min. **b**, Convoluted MS spectrum for t_R = 6.95-7.10 min, showing peaks of $[M + H]^{2+}$ and $[M]^+$.

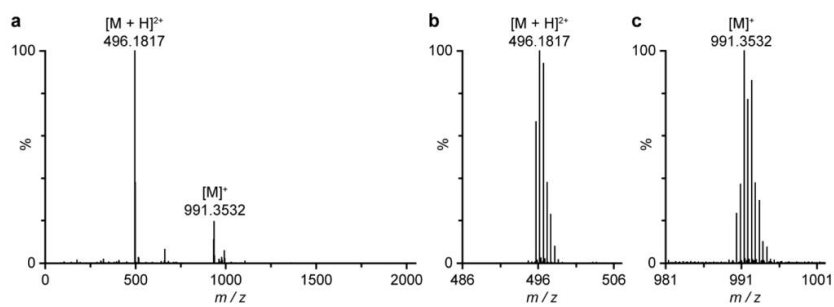


Figure S25. High-resolution MS spectrum of **2**. **a**, Full MS spectrum. **b**, partial MS spectrum showing $[M + H]^{2+}$ and $[M]^+$.

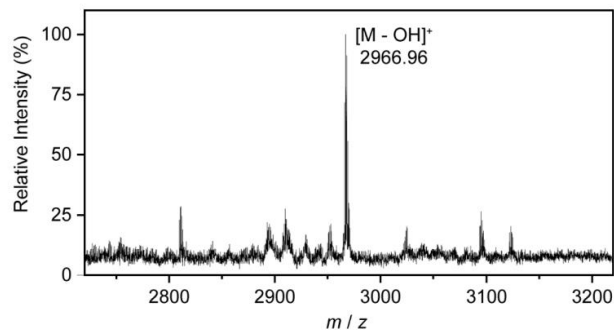


Figure S26. MALDI-TOF MS spectrum for **1·TAT**.

3 H₂O₂ cascade transformation

Figure S28. HPLC spectra for H_2O_2 (0.5 mM) induced-conversion of **1** (50 μM) to **2** in a mixture of NH_4HCO_3 buffer (pH 7.4, 20 mM) and methanol (v:v = 9:1).

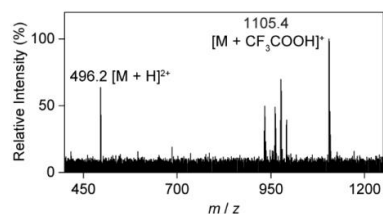


Figure S29. Convolutd MS spectrum for a reaction time of 20 min and $t_R = 5.60\text{-}5.80$ min (identified as *iso-2*).

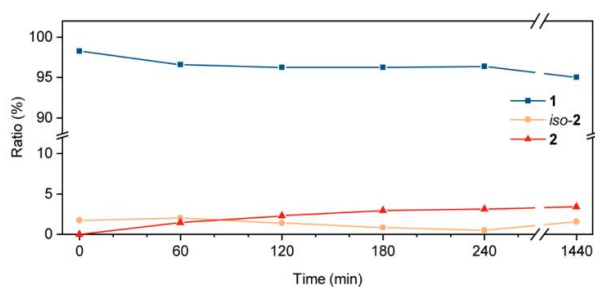


Figure S30. Molar ratio of compounds **1**, *iso-2* and **2** in LC-MS kinetic analysis when **1** (50 μM) was incubated in a mixture of NH_4HCO_3 buffer (pH 7.4, 20 mM) and methanol (v:v = 9:1).

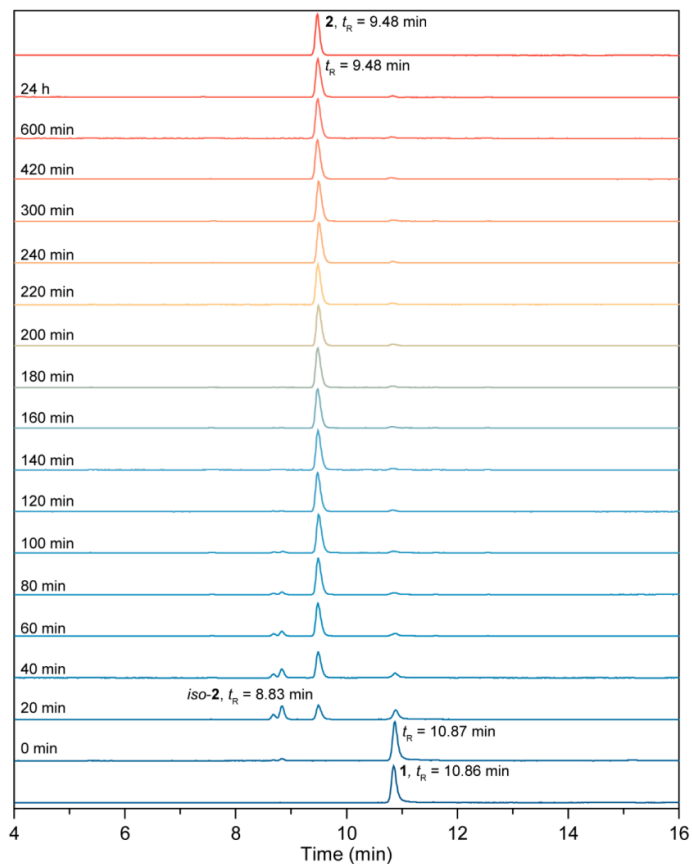
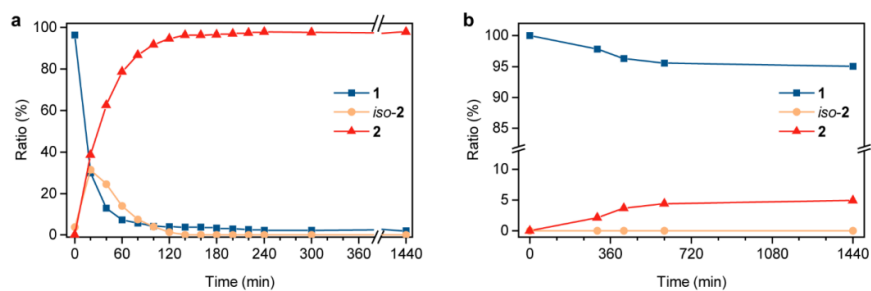


Figure S31. HPLC spectra for H_2O_2 ($2.1 \mu\text{M}$) induced-conversion of **1** ($2.0 \mu\text{M}$) to **2** in a mixture of NH_4HCO_3 buffer (pH 7.4, 20 mM) and methanol (v:v = 9:1). The retention times for the compounds are different from Figure 2B and S28 due the use of a different column.



S28

Figure S32. Molar ratio of compounds **1**, *iso-2* and **2** in LC-MS kinetic analysis when **1** (2.0 μM) was incubated in a mixture of NH_4HCO_3 buffer (pH 7.4, 20 mM) and methanol (v:v = 9:1) with (a) or without (b) H_2O_2 (2.1 μM).

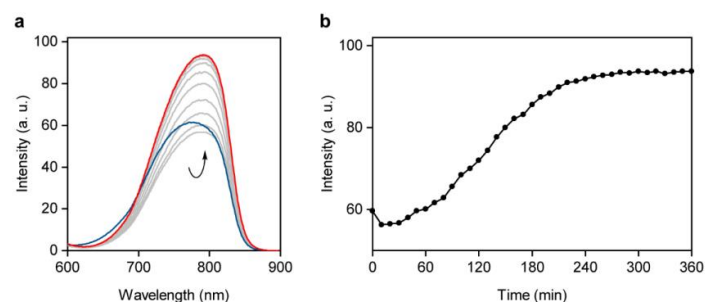


Figure S33. Luminescence analysis of H_2O_2 -induced conversion from **1** to **2_{NF}**. **a**, Time-dependent luminescence emission spectra of **1** (50 μM) in PB (pH 7.4, 50 mM) with H_2O_2 (0.5 mM). Blue line: incubation time = 0 min. Red line: incubation time = 300 min. Excitation wavelength = 488 nm. **b**, Time-dependent luminescence emission intensity for **1** (50 μM) in PB (pH 7.4, 50 mM) with H_2O_2 (0.5 mM) at 792 nm.

4 Self-assembly profiles of **2_{NF}**.

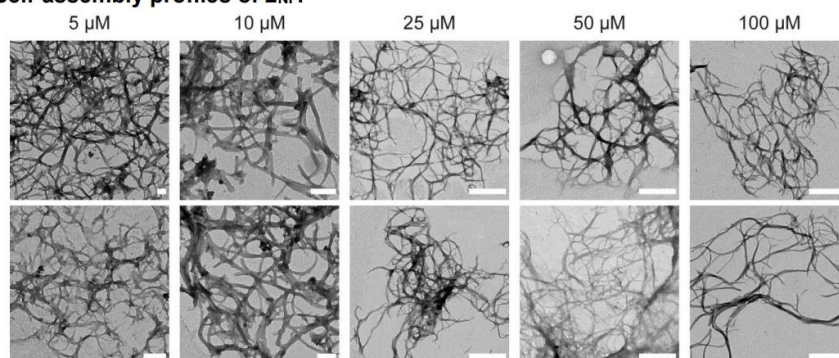


Figure S34. TEM image of the nanofiber **2_{NF}** at different concentrations in PB (pH 7.4, 50 mM). Scale bars, 200 nm.

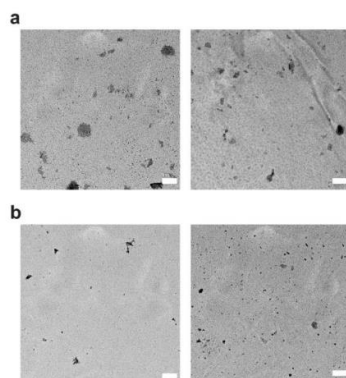


Figure S35. TEM image of **1** (a) and **1·TAT** (b) in PB (pH 7.4, 50 mM). Scale bars, 200 nm.

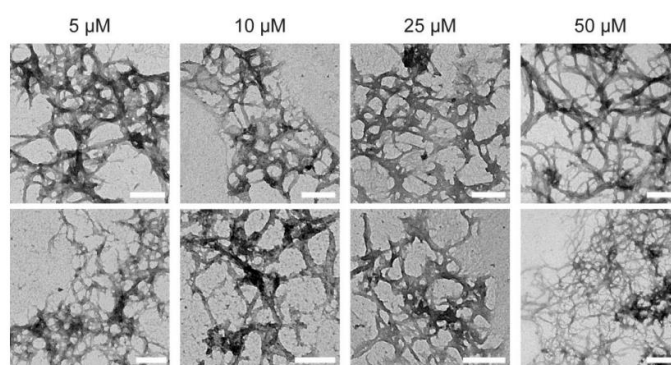


Figure S36. TEM image of the nanofiber form upon H_2O_2 -induced conversion of **1·TAT**. Complex **1·TAT** and H_2O_2 (10 equiv) at various concentrations were incubated in PB (50 mM, pH 7.4) for 10 h. The reaction mixture was then drop-casted on a carbon grid and imaged using TEM. Scale bars, 200 nm.

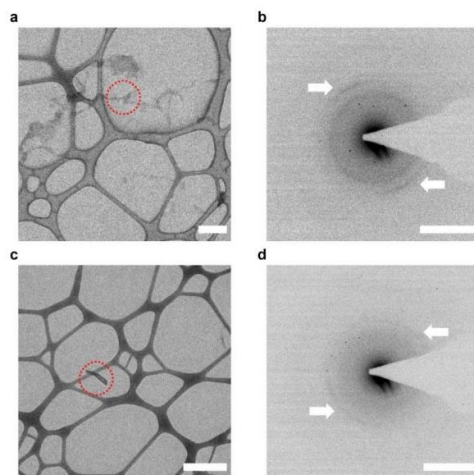


Figure S37. Selected-area electron diffraction analysis of the nanofiber. (a,c) Bright-field TEM images of the nanofiber. The red circles mark the position of the selected-area aperture. Scale bars, 500 nm. (b,d) Selected-area electron diffraction patterns obtained from the circular regions in (a) and (c), respectively. Scale bars, 3 nm^{-1} . White arrows indicate the diffraction arcs.

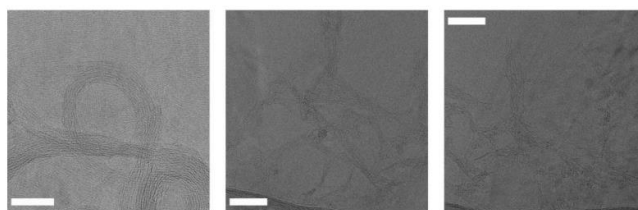


Figure S38. Cryogenic high-resolution TEM image of 2_{NF} in PB (pH 7.4, 50 mM). Scale bars, 50 nm.

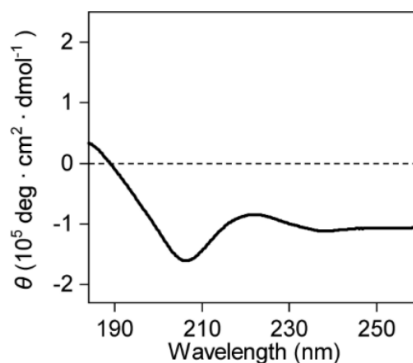


Figure S39. Circular dichroism (CD) spectra of **1** in a mixture of PB (pH 7.4, 10 mM) and CH₃CN (v:v = 98:2).

5 NMR analysis on the self-assembly profile of **2_{NF}**.

At room temperature (298K), sharp, well-defined signals were observed for **2** (1 mg/mL) in the ¹H NMR spectrum, and increasing the temperature resulted in no significant change in the chemical shifts for the signals, except for the protons of the amide group (Figure S38), likely due to faster exchange with H₂O in elevated temperature. These observations suggested that **2** remain as monomers in DMSO. In contrast, a solution of **2** at the same concentration in PB exhibited broad, overlapping signals at 298 K (Figure S39), in both aromatic and aliphatic region, suggesting strong self-assembly tendency of **2** in PB driven by a combination of π–π interactions and Pt–Pt interactions originating from the Pt-tpy group, and the hydrophobic interactions and hydrogen bond interactions originated from the peptide, forming nanofibers **2_{NF}**. Increasing the temperature of the measurement led to sharpening of the signals, accompanied by downfield shifts, indicative of reduced shielding effect due to disassembly. To further investigate the self-assembly profile of **2** in PB, NMR studies were conducted at 353 K, at which the proton NMR signals are sharp and well-resolved.

¹H NMR was first conducted at 353K (Figure S42) and the peaks were assigned based on the ¹H NMR of **2** in DMSO, as well as ¹H,¹³C HSQC NMR and ¹H, ¹H COSY NMR studies. In detail, the ¹H,¹³C HSQC NMR spectrum of **2** in DMSO revealed carbon atoms proximity to the nitrogen atom of the tpy group showing highest chemical shifts in ¹³C signals (Figure S43, 152.5 ppm for carbon directly bond to H^a and 141.2 ppm for carbon directly bond to H^c), whereas no other carbons that bond to a proton have chemical shifts above 135 ppm. Based on this observation, ¹H signals for **2** in PB can be identified using a combination of the ¹H,¹³C HSQC spectrum (Figure S44, 152.3 ppm for carbon directly bond to H^a and 142.8 ppm for carbon directly bond to H^c, no other carbons that bond to a proton have chemical shifts above 135 ppm) and ¹H, ¹H COSY NMR spectrum (Figures S45 and S46, showing correlations between neighboring protons).

¹H,¹H NOESY NMR spectrum for **2** in PB was then collected (Figure S47) to probe signals arise from protons that are close to each other in space even if they are not bonded, hence providing information on the self-assembly character. Similar to the COSY study discussed previously, strong off-diagonal

cross peaks can be observed for bonded neighboring protons. In addition, five sets of cross peaks from non-neighboring protons, H^a/H^c , H^d/H^b , H^e/H^b , H^b/H^d , and H^b/H^e were identified. These signals suggest that **2** adopt a head-to-tail conformation upon self-assembly in PB, forming **2_{NF}**.

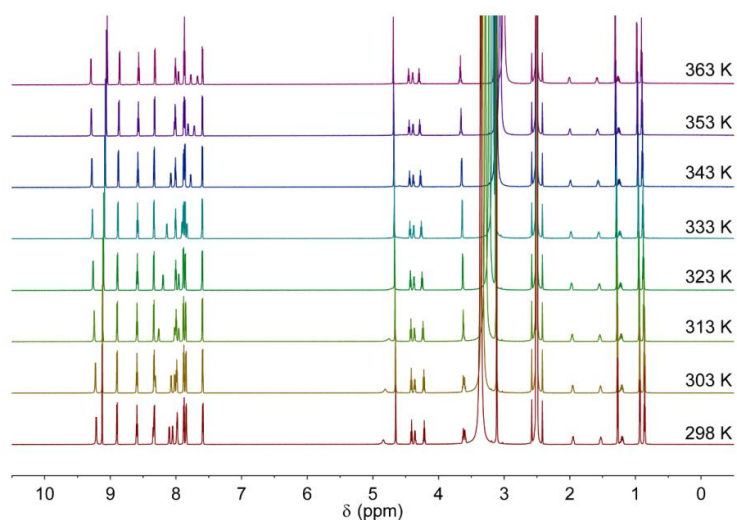
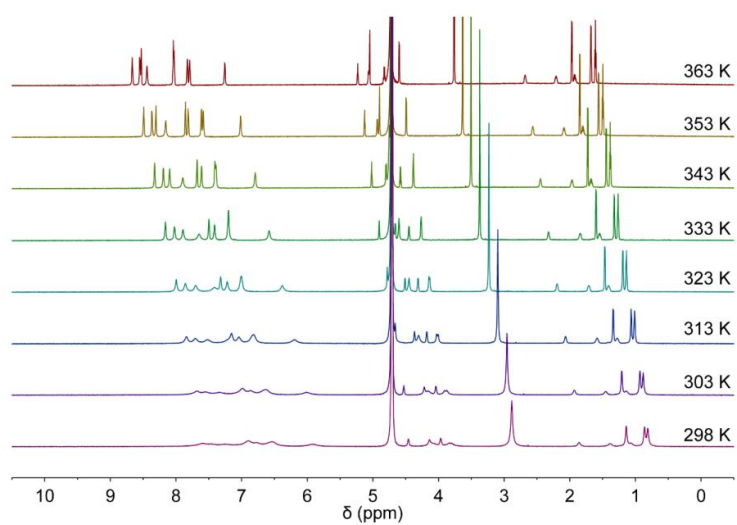


Figure S40. ^1H NMR spectra (850 MHz, $\text{DMSO}-d_6$) of compound **2** at various temperature.



S33

Figure S41. ^1H NMR spectra [850 MHz, PB (pH 7.4, 50 mM) in D_2O] of compound **2** at various temperature.

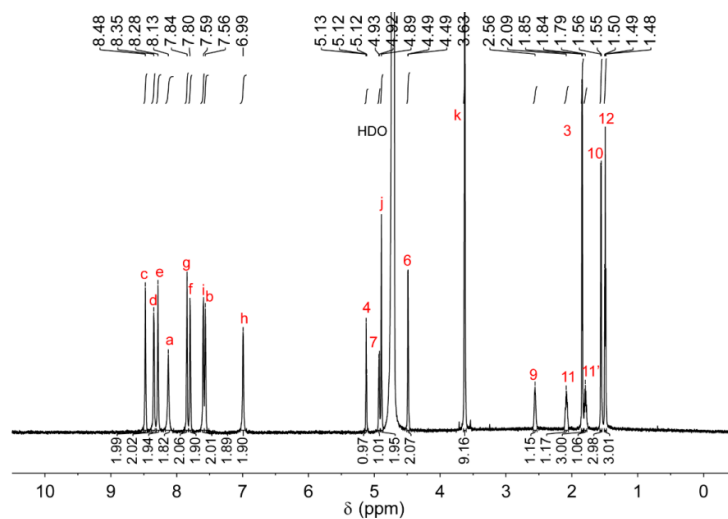


Figure S42. ^1H NMR spectrum [850 MHz, PB (pH 7.4, 50 mM) in D_2O , 353K] of compound **2**.

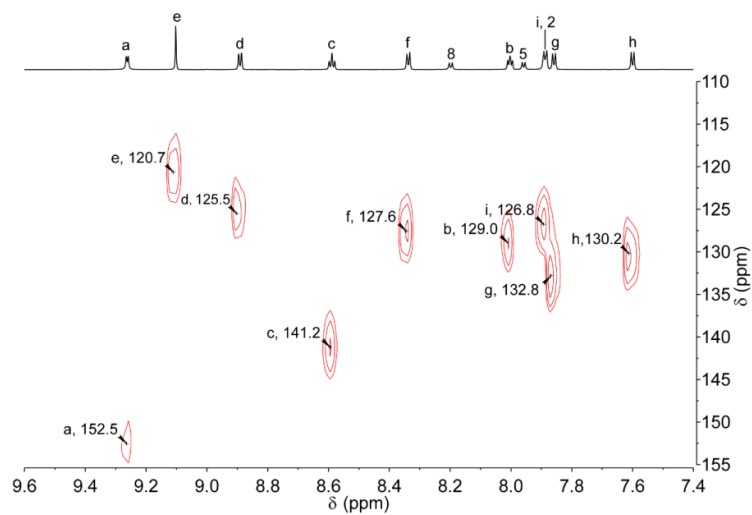


Figure S43. ^1H , ^{13}C HSQC NMR spectra (850 MHz, $\text{DMSO}-d_6$, 298K) of compound **2**.

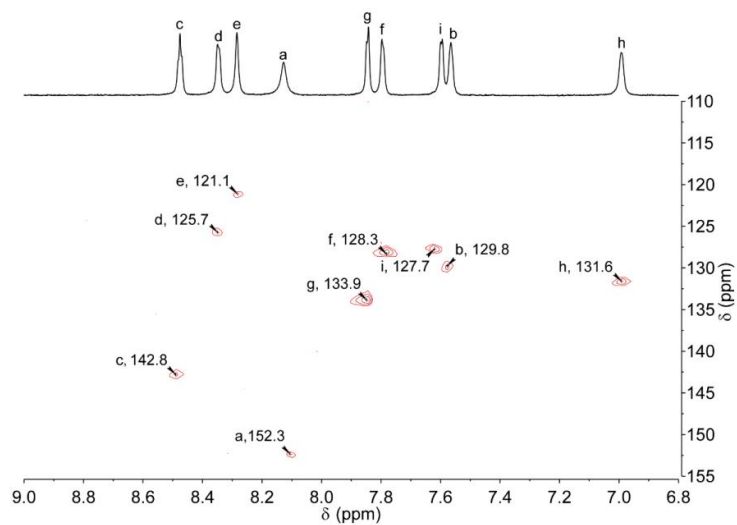


Figure S44. ^1H , ^{13}C HSQC NMR spectra [850 MHz, PB (pH 7.4, 50 mM) in D_2O , 353K] of compound **2**.

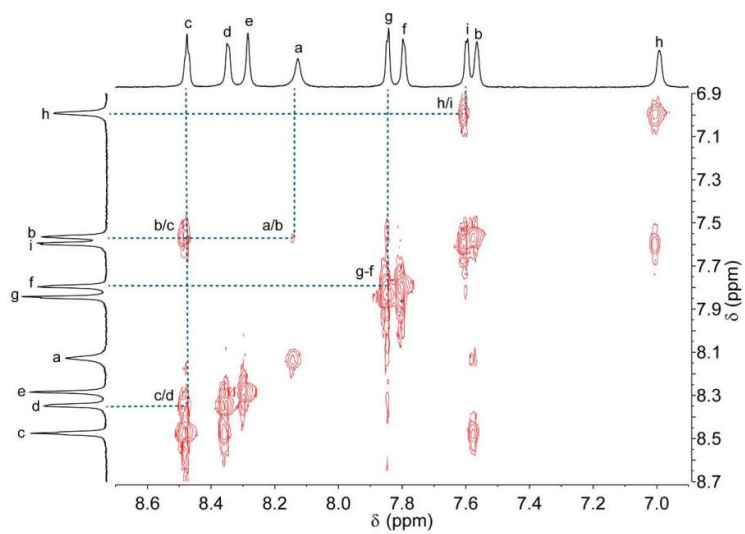


Figure S45. Partial ^1H NMR and ^1H , ^1H COSY NMR spectra [850 MHz, PB (pH 7.4, 50 mM) in D_2O , 353K] of compound **2** (aromatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectrum.

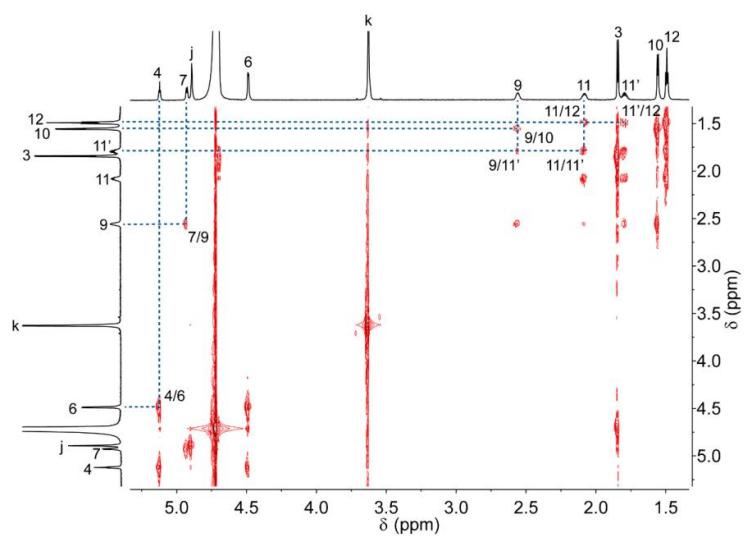
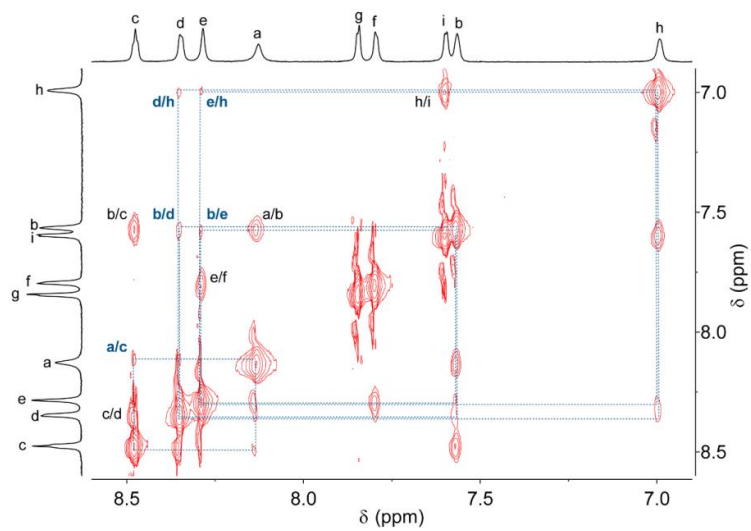


Figure S46. Partial ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra [850 MHz, PB (pH 7.4, 50 mM) in D_2O , 353K] of compound **2** (aliphatic region). The correlations between ^1H signals are highlighted with dashed lines in the COSY spectrum.



S36

Figure S47. $^1\text{H}, ^{13}\text{H}$ NOESY NMR spectrum [850 MHz, PB (pH 7.4, 50 mM) in D_2O , 353K] of compound **2**. Off-diagonal cross peaks that corresponds to intermolecular NOE signals were highlighted in blue.

6 Cellular uptake and intracellular self-assembly

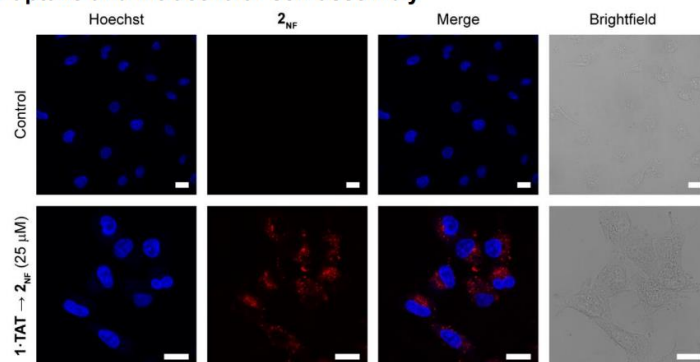


Figure S48. Confocal laser scanning micrographs of A549 cells treated for 4 h with **1-TAT** (25 μM) and Hoechst 33342 dye. Scale bars, 20 μm .

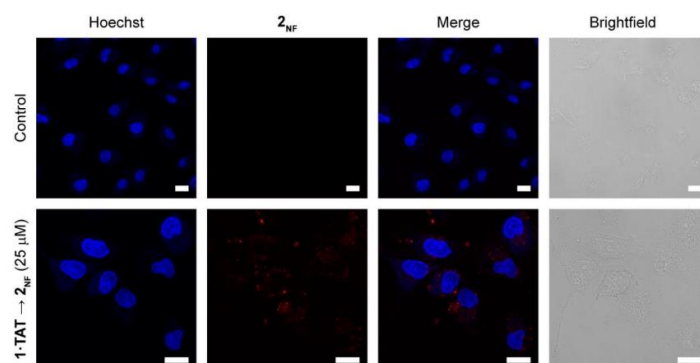


Figure S49. Confocal laser scanning micrographs of MDA-MB-231 cells treated for 4 h with **1-TAT** (25 μM) and Hoechst 33342 dye. Scale bars, 20 μm .

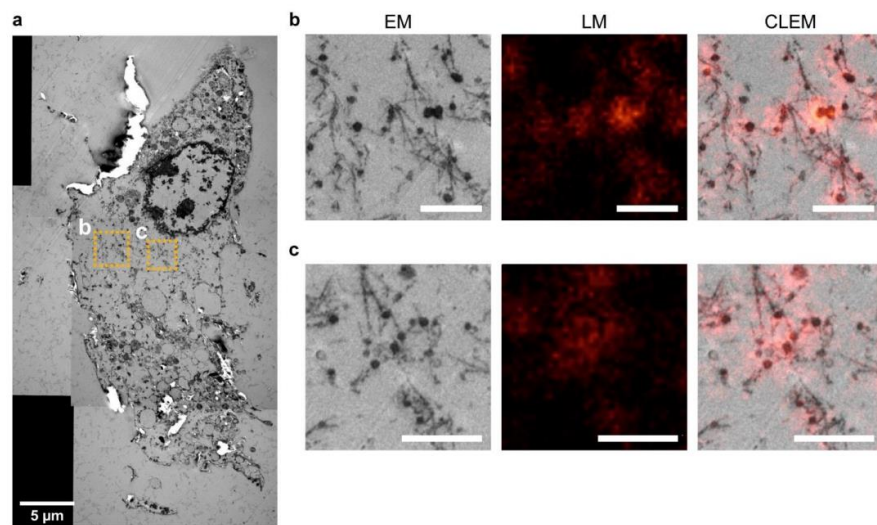


Figure S50. CLEM analysis of A549 cells treated with **1-TAT** (50 μ M, 6 h). **a**, Stitched TEM image of an A549 cell showing intracellular peptide fiber formation. Scale bars, 5 μ M. **b-c**, TEM, LM, and CLEM image of the box area showing the intracellular formation of luminescent nanofibers. Scale bars, 1 μ m.

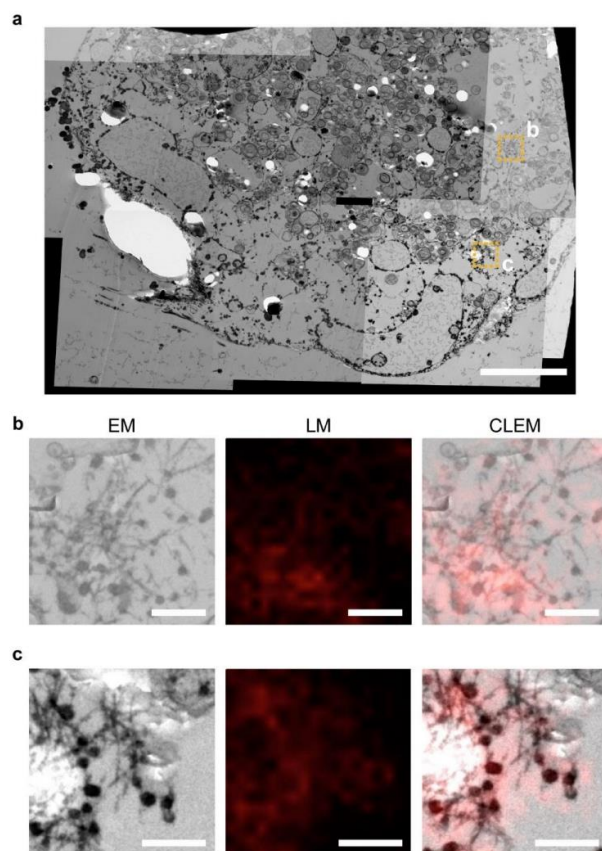


Figure S51. CLEM analysis of A549 cells treated with **1-TAT** (50 μ M, 6 h). **a**, Stitched TEM image of an A549 cell showing intracellular peptide fiber formation. Scale bars, 1 μ m. **b-c**, TEM, LM, and CLEM image of the box area showing intracellular formation of luminescent nanofibers. Scale bars, 500 nm.

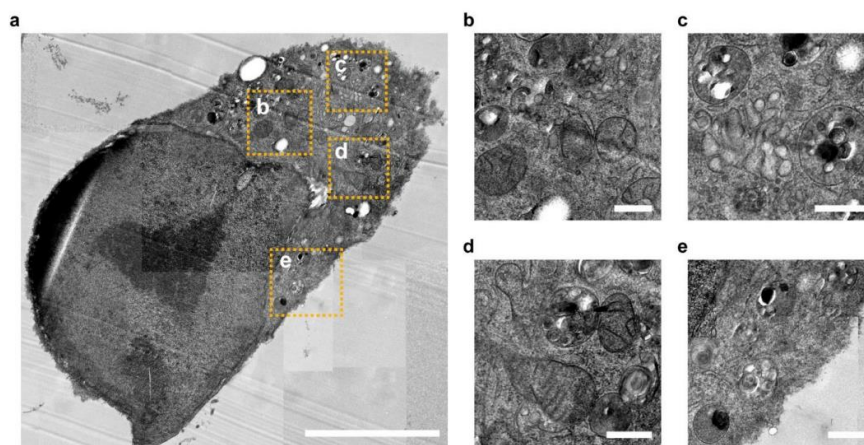


Figure S52. TEM image of a control A549 cell that was not treated with **1·TAT** after stitching TEM micrographs. **a**, Full view of the A549 cell after stitching TEM micrographs. Scale bars, 5 μm . **b-e**, Zoom in TEM images of the A594 cells. Scale bars, 500 nm. No nanofibers were observed in the control cells.

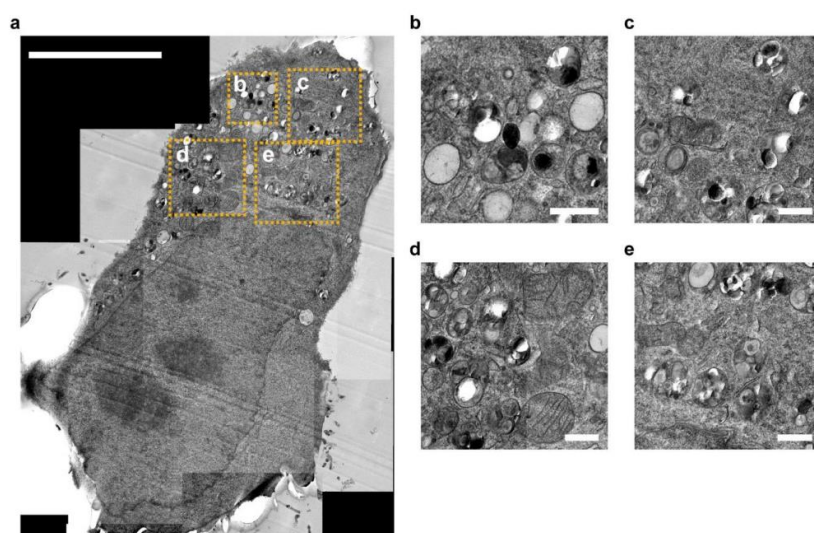


Figure S53. TEM image of a control A549 cell that was not treated with **1·TAT** after stitching TEM micrographs. **a**, Full view of the A549 cell after stitching TEM micrographs. Scale bars, 5 μm . **b-e**, Zoom in TEM images of the A594 cells. Scale bars, 500 nm. No nanofibers were observed in the control cells.

S40

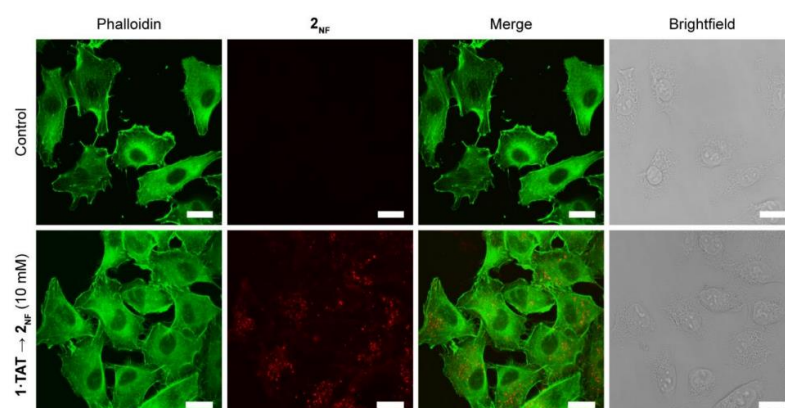


Figure S54. Confocal laser scanning micrographs of A549 cells treated for 4 h with **1-TAT** (10 μ M) and Phalloidin-iFluor™ 405 dye. Scale bars, 20 μ m.

7 Metabolic inhibitions by the **1-TAT**→**2NF**

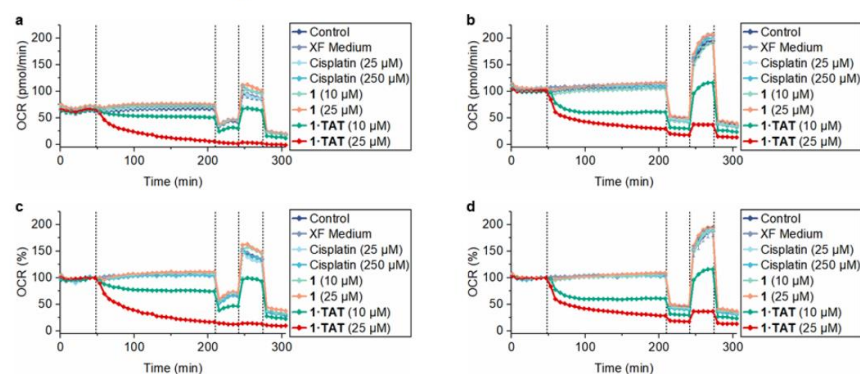


Figure S55. Results of the Mito Stress Assay for A549 and MDA-MB-231 cells. Graphs are shown for the raw data and after setting the last rate measurement before sample addition to 100% baseline. Dashed vertical lines indicate the time points of compound addition. The first time point indicates the addition of sample, followed by oligomycin, FCCP and lastly Rotenone and Antimycin A as a combination. **a** and **c** show the data before and after baseline correction respectively, for A549 cells. **b** and **d** show the data before and after baseline correction respectively, for MDA-MB-231 cells.

Acute Response

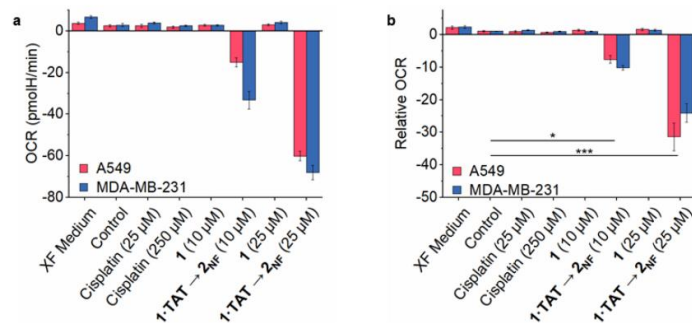


Figure S56. Acute Response of A549 and MDA-MB-231 cells during the Mito Stress Assay upon treatment with **1**, **1-TAT** as well as XF Medium and Cisplatin controls. Data are shown for the raw data (a) and for a baseline set to 100% before the sample addition (b). In the latter case, each cell line was normalized to the DMSO control. (b) $n \geq 6$.

Maximal Respiration

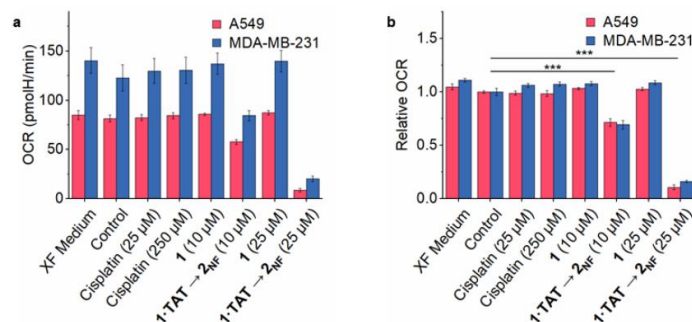


Figure S57. Maximal Respiratory Capacity of A549 and MDA-MB-231 cells during the Mito Stress Assay upon treatment with **1**, **1-TAT** as well as XF Medium and Cisplatin controls. Data are shown for the raw data (a) and for a baseline set to 100% before the sample addition (b). In the latter case, each cell line was normalized to the DMSO control. (b) $n \geq 6$.

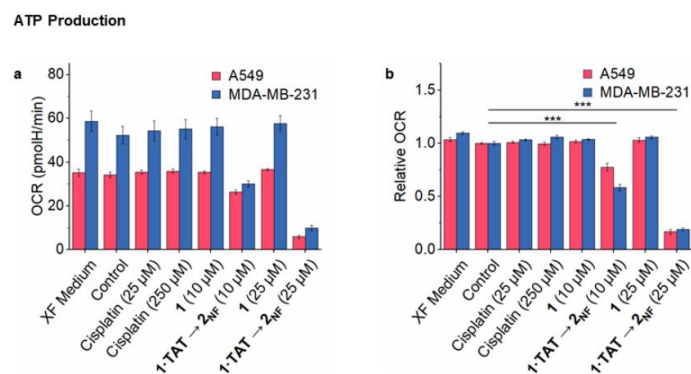


Figure S58. ATP Production of A549 and MDA-MB-231 cells during the Mito Stress Assay upon treatment with **1**, **1-TAT** as well as XF Medium and Cisplatin controls. Data are shown for the raw data (**a**) and for a baseline set to 100% before the sample addition (**b**). In the latter case, each cell line was normalized to the DMSO control. (**b**) $n \geq 6$.

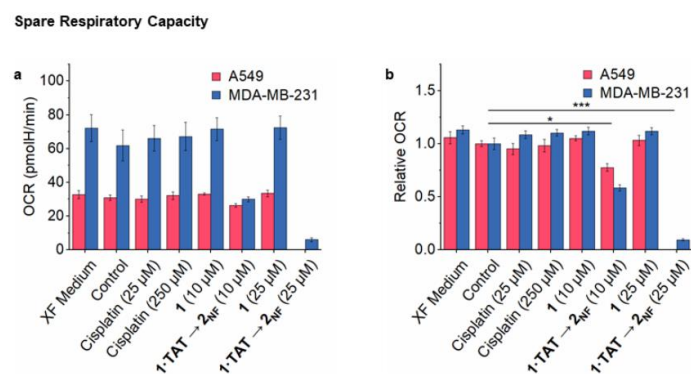


Figure S59. Spare Respiratory Capacity of A549 and MDA-MB-231 cells during the Mito Stress Assay upon treatment with **1**, **1-TAT** as well as XF Medium and Cisplatin controls. Data are shown for the raw data (**a**) and for a baseline set to 100% before the sample addition (**b**). In the latter case, each cell line was normalized to the DMSO control. (**b**) $n \geq 6$.

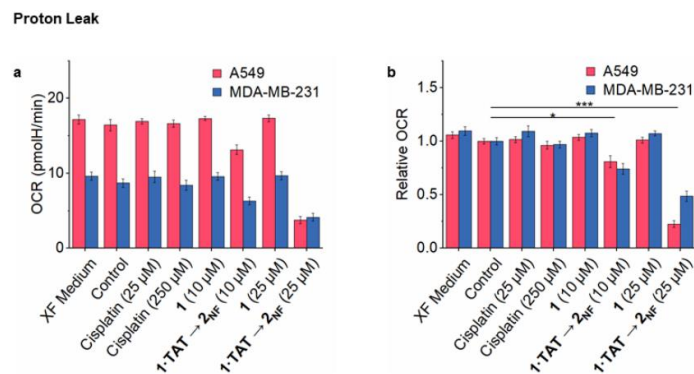


Figure S60. Proton Leak of A549 and MDA-MB-231 cells during the Mito Stress Assay upon treatment with **1**, **1-TAT** as well as XF Medium and Cisplatin controls. Data are shown for the raw data (a) and for a baseline set to 100% before the sample addition (b). In the latter case, each cell line was normalized to the DMSO control. (b) n $\geq$ 6.

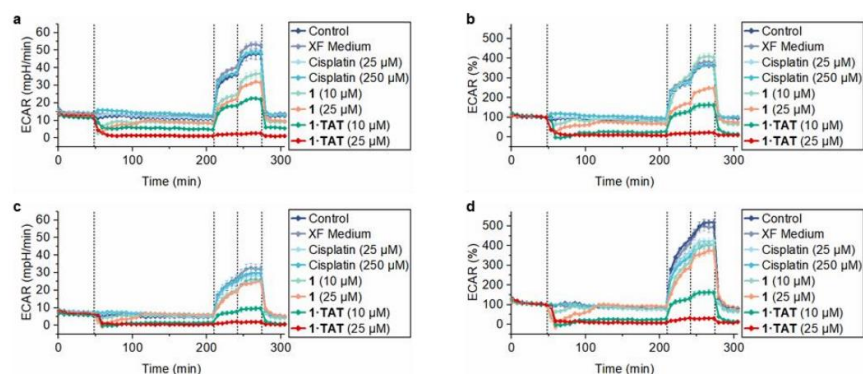


Figure S61. Results of the Glycolysis Stress Assay for A549 and MDA-MB-231 cells. Graphs are shown for the raw data and after setting the last rate measurement before sample addition to 100% baseline. Dashed vertical lines indicate the time points of compound addition. The first time point indicates the addition of the sample, followed by glucose, oligomycin and 2-deoxyglucose respectively. **a** and **c** show the data before and after baseline correction respectively, for A549 cells. **b** and **d** show the data before and after baseline correction respectively, for MDA-MB-231 cells.

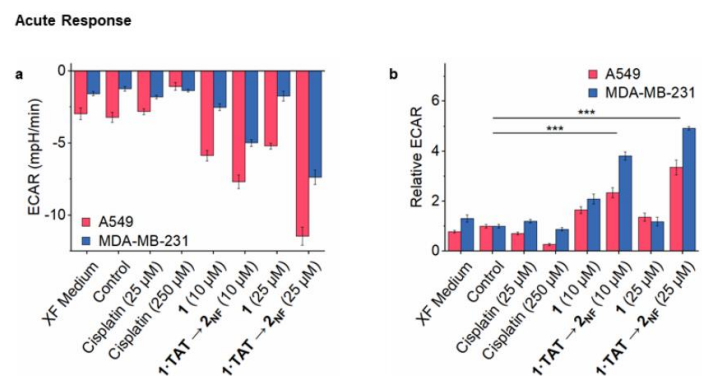


Figure S62. Acute Response of A549 and MDA-MB-231 cells during the Glycolysis Stress Assay upon treatment with **1**, **1**:TAT as well as XF Medium and Cisplatin controls. Data are shown for the raw data (a) and for a baseline set to 100% before the sample addition (b). In the latter case, each cell line was normalized to the DMSO control. (b) $n \geq 6$.

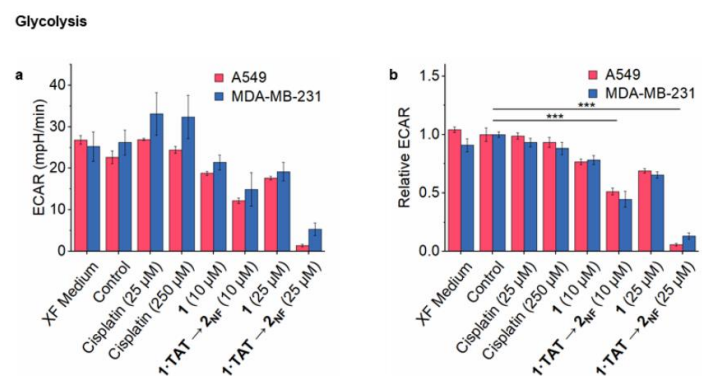


Figure S63. Glycolysis of A549 and MDA-MB-231 cells during the Glycolysis Stress Assay upon treatment with **1**, **1**:TAT as well as XF Medium and Cisplatin controls. Data are shown for the raw data (a) and for a baseline set to 100% before the sample addition (b). In the latter case, each cell line was normalized to the DMSO control. (b) $n \geq 6$.

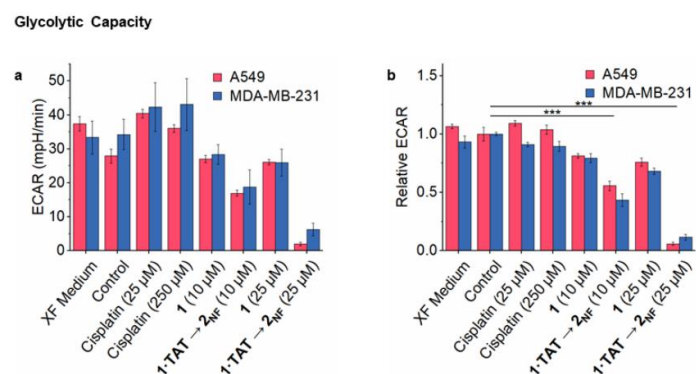


Figure S64. Glycolytic Capacity of A549 and MDA-MB-231 cells during the Glycolysis Stress Assay upon treatment with **1**, **1-TAT** as well as XF Medium and Cisplatin controls. Data are shown for the raw data (**a**) and for a baseline set to 100% before the sample addition (**b**). In the latter case, each cell line was normalized to the DMSO control. (**b**) $n \geq 5$.

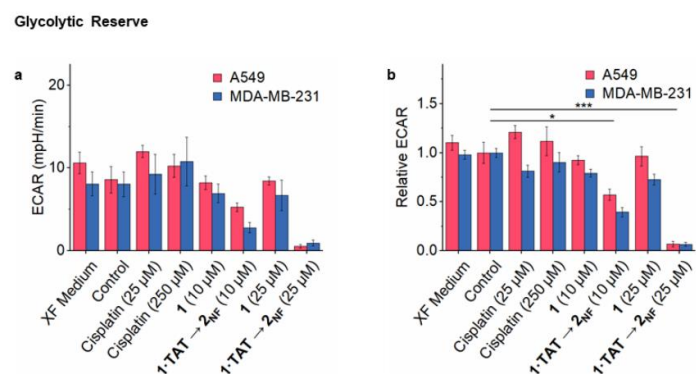


Figure S65. Glycolytic Reserve of A549 and MDA-MB-231 cells during the Glycolysis Stress Assay upon treatment with **1**, **1-TAT** as well as XF Medium and Cisplatin controls. Data are shown for the raw data (**a**) and for a baseline set to 100% before the sample addition (**b**). In the latter case, each cell line was normalized to the DMSO control. (**b**) $n \geq 5$.

8 Cytotoxicity of 1·TAT→2_{NF}

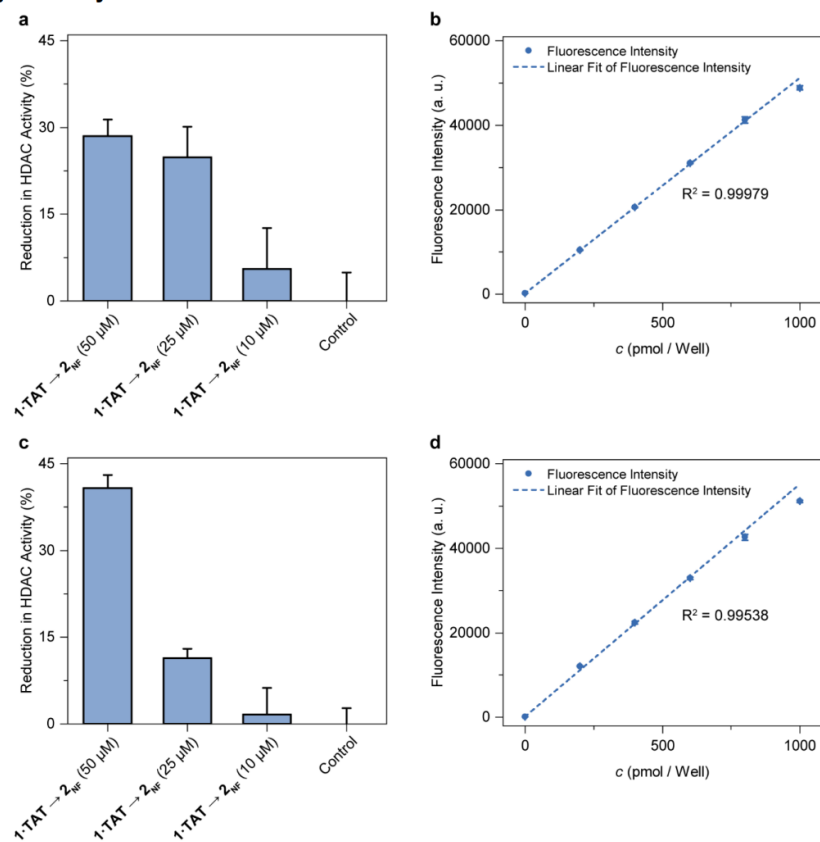


Figure S66. Examination of cellular histone deacetylase (HDAC) activity after incubation with 1·TAT for 4 h. **a**, Reaction in HDAC activity by 1·TAT at various concentrations against A549 cells. **b**, Calibration curve for the assay in **a**. **c**, Reaction in HDAC activity by 1·TAT at various concentrations against MDA-MD-231 cells. **d**, Calibration curve for the assay in **c**.

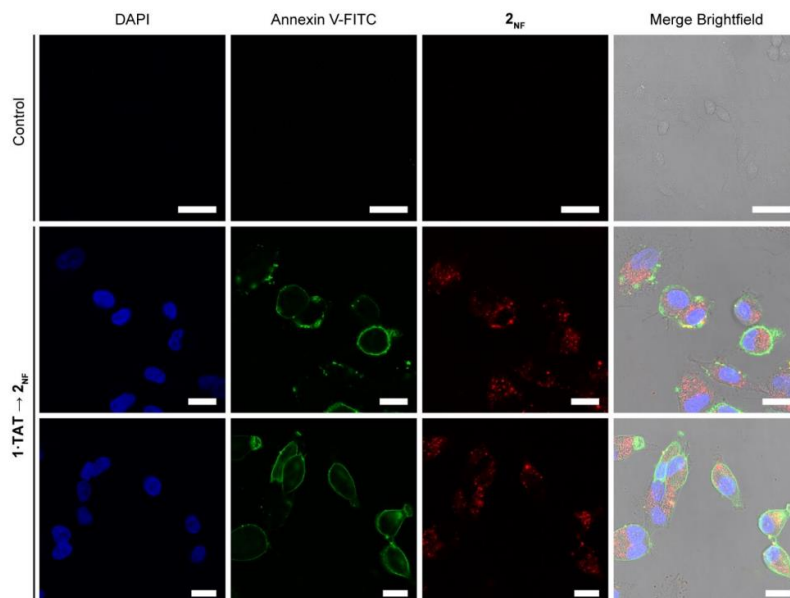


Figure S67. Examination of the apoptotic nature of cell death by using Annexin V assay. Cells were incubated 4 h with **1-TAT** (25 μ M) (red) and treated with Annexin V (green) afterwards. Nuclei were stained using DAPI (blue). Annexin V binds to the cell membrane upon an inversion of phosphatidylserine motifs towards the extracellular space, indicating apoptosis. Each panel represent results from replicate of the experiment. Scale bars, 50 μ m.

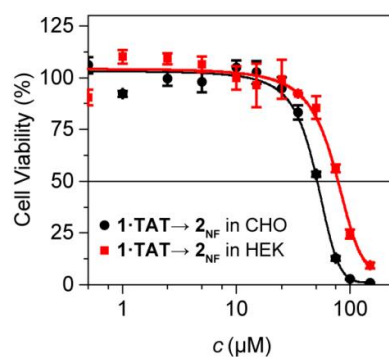


Figure S68. Cell viability assay of CHO and MDA-MB-231 cells treated with **1-TAT** and cisplatin for 4 h. The IC_{50} values for **1-TAT** $\rightarrow$ **2_{NF}** are determined to be 49.8 μ M and 72.3 μ M against CHO and HEK cells, respectively.

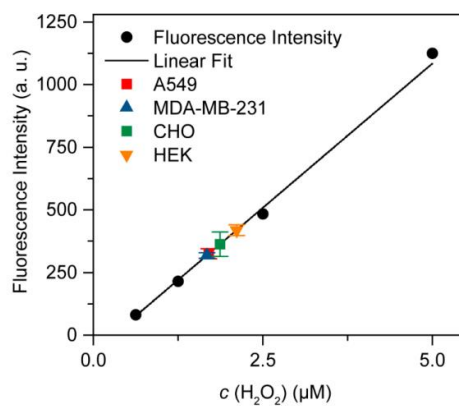


Figure S69. Standard curve for determination of the intracellular H_2O_2 concentration in A549, MDA-MB-231, CHO, and HEK cell lines. The intracellular H_2O_2 concentration is determined to be 1.70 μM in A549 cells, 1.67 μM in MDA cells, 1.87 μM in CHO cells, and 2.11 μM in HEK cells.

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3.2 Supramolecular Assembly in Live Cells Mapped by Real-Time Phasor-Fluorescence Lifetime Imaging

Yong Ren, Zhixuan Zhou, Konrad Maxeiner, Anke Kaltbeitzel, Iain Harley, Jiaqi Xing, Yingke Wu, Manfred Wagner, Katharina Landfester, Ingo Lieberwirth, Tanja Weil, *, David Y.W. Ng, *

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Abstract

The complex dynamics and transience of assembly pathways in living systems complicate the understanding of these molecular to nanoscale processes. Current technologies are unable to track the molecular events leading to the onset of assembly, where real-time information is imperative to correlate their rich biology. Using a chemically designed pro-assembling molecule, we map its transformation into nanofibers and their fusion with endosomes to form hollow fiber clusters. Tracked by phasor-fluorescence lifetime imaging (phasor-FLIM) in epithelial cells (L929, A549, MDA-MB 231) and correlative light-electron microscopy and tomography (CLEM), spatiotemporal splicing of the assembly events shows time-correlated metabolic dysfunction. The biological impact begins with assembly-induced endosomal disruption that reduces glucose transport into the cells, which, in turn, stymies mitochondrial respiration.

Contributions

Yong Ren: Conceptualization, molecular design of peptides. Synthesis and characterization of peptides and intermediates. LC-MS kinetics analysis, CD and TEM

measurement and NMR analysis. Phasor-FLIM imaging and cellular assay study. Data analysis and interpretation of results. Writing of the manuscript.

Zhixuan Zhou: Conceptualization, molecular design of peptides. Synthesis and characterization of peptides and intermediates. LC-MS kinetics analysis. Data analysis and interpretation of results. Writing of the manuscript.

Konrad Maxeiner: Metabolism assay study and analysis. Writing of the manuscript.

Anke Kaltbeitzel: CLEM measurement and analysis.

Iain Harley: Cryo-TEM measurement and analysis. Tomography measurement and analysis.

Jiaqi Xing: DLS measurement and analysis.

Yingke Wu: TEM measurement.

Manfred Wagner: NMR measurement and analysis.

Katharina Landfester: Cryo-TEM measurement and analysis. CLEM measurement and analysis.

Ingo Lieberwirth: Cryo-TEM measurement and analysis. CLEM measurement and analysis.

Tanja Weil: Acquiring funding for the project. Design and discussion on the concept and results analysis. Correction of the manuscript.

David Y.W. Ng: Design and discussion on the concept and results analysis. Phasor-FLIM imaging, analysis and data processes. Writing and correction of the manuscript.

Supramolecular Assembly in Live Cells Mapped by Real-Time Phasor-Fluorescence Lifetime Imaging

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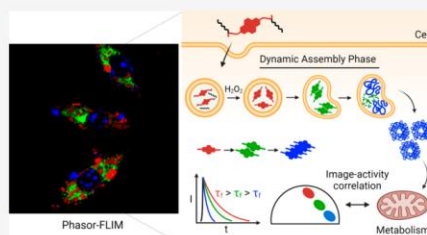
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ABSTRACT: The complex dynamics and transience of assembly pathways in living systems complicate the understanding of these molecular to nanoscale processes. Current technologies are unable to track the molecular events leading to the onset of assembly, where real-time information is imperative to correlate their rich biology. Using a chemically designed pro-assembling molecule, we map its transformation into nanofibers and their fusion with endosomes to form hollow fiber clusters. Tracked by phasor-fluorescence lifetime imaging (phasor-FLIM) in epithelial cells (L929, A549, MDA-MB 231) and correlative light-electron microscopy and tomography (CLEM), spatiotemporal splicing of the assembly events shows time-correlated metabolic dysfunction. The biological impact begins with assembly-induced endosomal disruption that reduces glucose transport into the cells, which, in turn, stymies mitochondrial respiration.



INTRODUCTION

The autonomous assembly of molecules to form nanoscale structures and aggregates in biology is a complex multiscale challenge that is poorly understood. Every structural precursor molecule within the human cell is chemically processed at different time points, and their subsequent accumulation creates a variety of supramolecular intermediates that evolve over time.^{1–4} As a result, many intermediate species exist only transiently, and their biological consequences observed at a later time point are no longer traceable. This lack of knowledge limits progress in understanding assembly-driven biological functions and the development of targeted medical solutions. Therefore, a platform that captures molecular-level assembly dynamics and localization in real time is urgently needed.

Synthetic molecules serve as important tools to gain fundamental insights of cellular responses and biological pathways without the need for genetic engineering.^{5–7} However, when crossing from discrete oligomers to the formation of higher ordered structures and aggregates, it is usually not possible to resolve the dynamics within the early phase of molecular assembly.^{8–11} To unravel this elusive phase, we have integrated chemical switches, real-time imaging technologies, and biological readouts into a short peptide amphiphile that is programmed to undergo intracellular assembly (Figure 1). The assembly into nanostructures is reported live by monitoring fluorescence lifetime (τ_f) changes of the diethoxynaphthyl diimide (NDI) moiety on the peptide. Unlike intensity- or wavelength-based sensors that require specific chromo-

phores,^{12–14} the fluorescence lifetime response as a function of molecular aggregation is a universal property. We combine phasor plot analysis with fluorescence lifetime imaging (phasor-FLIM),^{15–17} to map assembly progression within the cell over space and time. Based on these dynamic insights, critical aggregation time points are easily identified for high-resolution correlative light-electron microscopy/tomography (CLEM) to visualize nanoscale morphologies and endosomal interactions.

By coupling imaging technologies with time-resolved extracellular flux analysis, we found that the onset of structure formation impaired endosomal functions, signaling the depletion of glucose-dependent metabolic pathways and subsequent mitochondrial dysfunction. Over time, the production of nanofibers fused with the endosomes, maturing into hollow, spherical clusters that are ejected from the apoptotic cell. We show that our integrative approach provides the breadth of technologies required to track the complex supramolecular dynamics in situ, enabling a comprehensive understanding of bioassembly processes at the molecular level.

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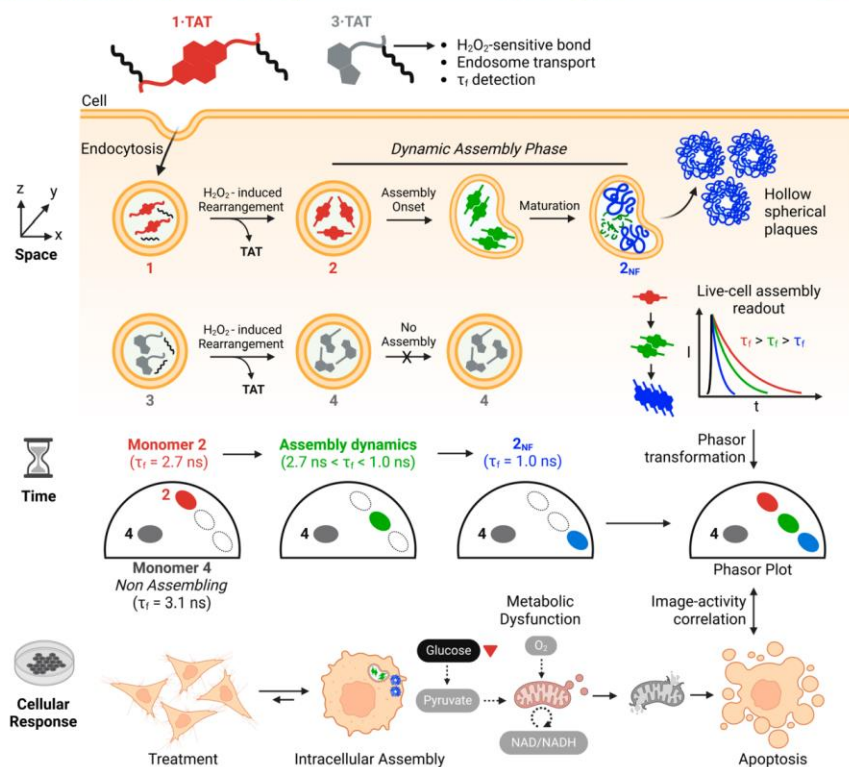


Figure 1. Schematic illustration of endosomal assembly of peptides traced via phasor-FLIM and real-time bioactivity correlation. Pro-assembling peptide 1-TAT ($\tau_f = 2.7$ ns) entered epithelial cells via TAT-mediated endocytosis and was converted to the supramolecular monomer 2 by an endogenous H₂O₂ switch. Endosomal confinement directs the assembly of nanofibers to fuse and form discrete hollow clusters 2_{NF} ($\tau_f = 1.0$ ns). The assembly dynamics are observed in real time as photons move between 2.7 ns and $\tau_f > 1.0$ ns, correlating to the extent of metabolic dysfunction.

RESULTS AND DISCUSSION

Design, Synthesis, and H₂O₂-Induced Transformation of Pro-assembling Peptide. To enable the NDI for τ_f observation in the visible range, we modify the NDI core with two ethoxy groups occupying the 2,6-bay positions. The diimide positions are covalently linked to tripeptide Ile-Ser-Ala, where the design is inspired from short self-assembling peptides known in the literature.^{18–21} The serine residue, caged by a phenylboronic acid (R²), serves as an O → N intramolecular acyl migration switch that can be turned on by intracellular H₂O₂. Structural propagation occurs upon the triggered alignment of the molecular backbone between the NDI core (π interactions)²² and the tripeptides (van der Waals, H-bonds).^{23–25} Compound 1 and its H₂O₂-induced, rearranged product 2 (as a control) were synthesized using Fmoc solid-phase peptide synthesis to install the NDI moiety (Figure 2A, Figures S1–S8). HPLC-MS investigation on the conversion of compound 1 demonstrated the kinetics of the chemical switch, which is eventually transformed into 2 with 97% conversion (Figure 2B,C, Figure S21). In the absence of H₂O₂, 1 remained intact

after 24 h (Figure S22). NBD-functionalized compound 3 and its H₂O₂-induced conversion product 4 were also synthesized as non-assembling control compounds (Figure 2A, Figures S1 and S9–S16). The H₂O₂-induced conversion of 3 to 4 was consistent with that of 1 to 2 (Figure S27). To transport peptides 1 and 3 into cells via endosomal uptake, the transactivator of transcription (TAT) derived from HIV,²⁶ functionalized with a salicylhydroxamic acid (SHA) motif (SHA-TAT), was designed. TAT-functionalized complexes (1-TAT, 3-TAT) were obtained through a dynamic covalent bond between the SHA moiety and the phenylboronic acid (PBA) group present in compounds 1 and 3 at pH 7.4 (Figure 2A, Figures S17–S20).²⁷ These SHA-PBA bound forms (1-TAT, 3-TAT) show similar responsiveness to H₂O₂ as their parent compounds (1, 3) (Figures S23–S25, S27–S31).^{21,28}

Optical properties of 1 and 2 revealed similar absorbance and fluorescence fingerprints, implying that the chemical transformations do not affect their basic photophysical behavior. In detail, 1 displayed a low-energy absorption band centered at 468 nm, assigned to the n- π^* transition of the NDI group.²⁹ A

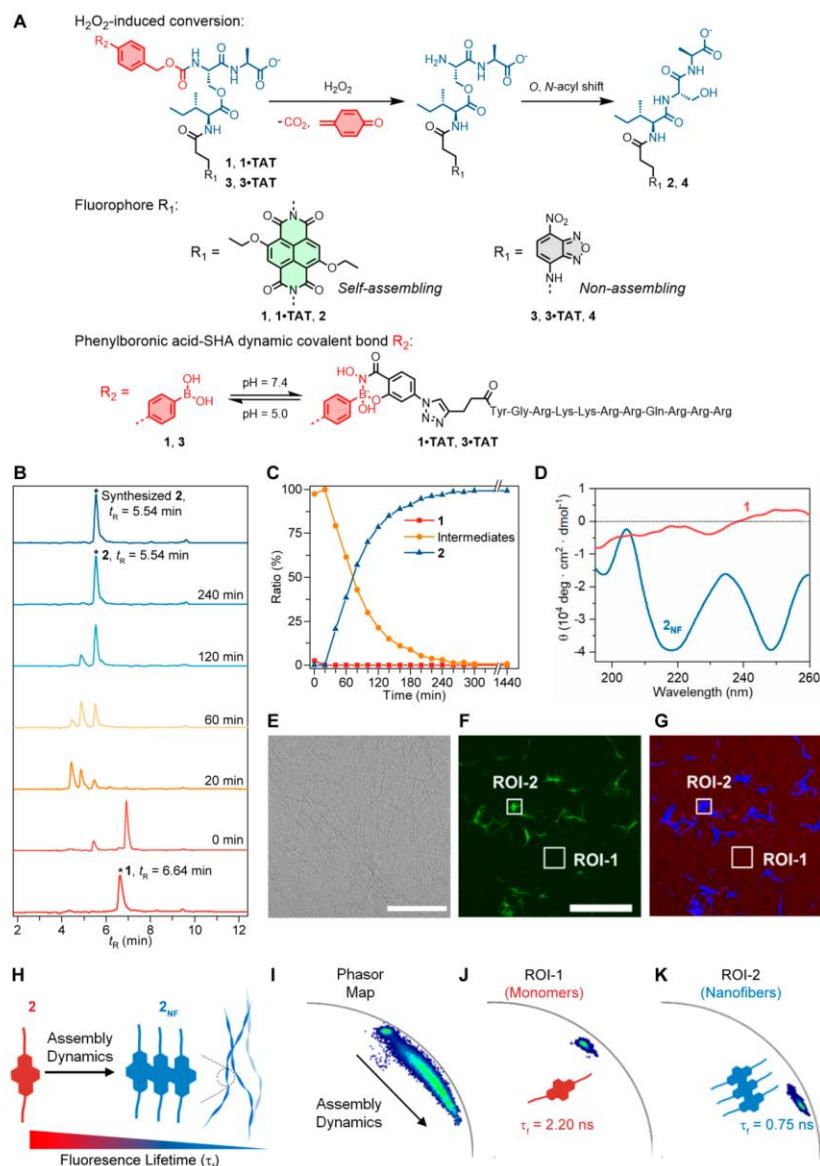


Figure 2. Self-assembly analysis of nanofiber 2_{NF}. (A) Structure of the compounds 1 and 3, their TAT-functionalized complexes 1-TAT and 3-TAT obtained by salicylhydroxamic acid (SHA)-PBA dynamic covalent interaction with SHA-TAT, and their conversion products 2 and 4. The formation of the SHA-PBA bond exhibited no apparent influence on the conversion properties of the parent compounds. (B) Kinetic analysis over the H_2O_2 (1.0 mM)-induced linearization of 1 (50 μM) to 2 in a mixture of NH_4HCO_3 buffer (pH 7.4, 10 mM) and THF (v:v = 1:1) by using HPLC-MS. (C) Molar ratio of precursor 1, deboronation and rearrangement intermediates, and 2 after incubating with H_2O_2 based on the peak integration at 254 nm. (D)

Figure 2. continued

Circular dichroism (CD) spectra of **1** and **2_{NF}** in a mixture of PB (pH 7.4, 100 mM) and CH₃CN (0.5 vol %). (E) Cryo-TEM micrograph of the nanofibers **2_{NF}** (100 μ M) in DPBS. Scale bar: 100 nm. (F) Intensity-based confocal image for **2_{NF}** in a cell-free condition. λ_{ex} = 469 nm, λ_{em} = 500–520 nm. Scale bar: 20 μ m. (G) Corresponding fluorescence lifetime image for **2_{NF}**. λ_{ex} = 469 nm. The red channel indicates species with τ_f = 2.20 ns, and the blue channel indicates species with τ_f = 0.75 ns. Scale bar, 20 μ m. (H) Schematic illustration of the assembly progression from monomers to assemblies, resulting in decreased fluorescence lifetime (2.20 ns $\rightarrow$ 0.75 ns) upon formation of nanofiber morphology. (I) Fluorescence lifetime analysis of assembly progression in a cell-free system using phasor analysis. Two major species showing τ_f values of 2.20 and 0.75 ns can be identified. (J) Phasor plots of ROI-1 (monomers) in Dulbecco's modified Eagle medium (DMEM), τ_f = 2.20 ns. (K) Phasor plots of ROI-2 (assemblies) in DMEM, τ_f = 0.75 ns.

higher-energy absorption band was observed at 340 nm, representing the π - π^* transition (Figure S32).²⁹ Upon excitation of the n - π^* transition at 468 nm, both **1** and **2** exhibited a broad emission band centered at around 510 nm, characteristic of NDI chromophores (Figure S32).³⁰ As peptide **2** is the active self-assembling species, it exhibited a slightly lower absorption intensity and a minor hypsochromic shift in the absorption and fluorescence emission relative to **1**. These observations can be attributed to the enhanced exciton migration in the closely packed NDI cores,^{29,31} providing first hints toward assembly. Control compounds **3** and **4** exhibited nearly identical absorption bands centered at 475 nm and emission centered at 540 nm, suggesting no assembly behavior for either compound (Figure S32).

Assembly Profile and Phasor-FLIM Calibration. At concentrations ranging from 10 to 100 μ M in Dulbecco's phosphate-buffered saline (DPBS, pH 7.4), peptide **2** assembled into nanofibers (Figure 2E and Figures S33, S35). The assembled form of **2** is henceforth labeled as **2_{NF}**. The critical assembly concentration was assessed by Nile Red assay to be around 9 μ M (Figure S36). In situ H₂O₂-triggered transformation of **1** (10–100 μ M) further confirmed the formation of **2_{NF}** as the final state (Figure S34). The conversion and assembly process were supported by dynamic light scattering (DLS) analysis (Figures S37, S38). In the absence of H₂O₂, compounds **1**, **1**·TAT, **3**, **3**·TAT, and **4** exhibited no assembly behavior (Figure S37). Circular dichroism (CD) spectroscopy analysis on **2_{NF}** revealed a strong negative signal at 250 nm corresponding to the $\pi \rightarrow \pi^*$ transition of the aromatic core,³⁰ reflecting the importance of the NDI core during assembly. Additionally, a strong negative $n \rightarrow \pi^*$ transition of the carbonyl group was observed at around 220 nm, indicating the H-bond interactions of the peptide backbone (Figure 2D).³² As a control, **1** showed no obvious signal between 200 and 260 nm. Variable-temperature (293–353 K) ¹H NMR studies support the formation of intermolecular bonds as the basis of the structural assembly of **2_{NF}** (Figures S40, S41).

The fluorescence lifetime profile toward the assembly of **2** is calibrated under cell-free conditions using Dulbecco's modified Eagle medium (DMEM) and imaged using phasor-FLIM. Intensity-based fluorescence shows large regions of nanofibers upon the assembly of **2** into **2_{NF}** (Figure 2F). By separating the lifetimes of emitted photons on a phasor plot, the population of photons corresponding to their spatial distribution was gathered (Figure 2F–I, Figures S42, S43). Regions containing the nanofibers (ROI-2) show a distinct locus on the phasor plot, with an average lifetime of 0.75 ns (Figure 2K, Figures S42e, S43e), whereas free molecules of **2** (ROI-1) in solution emit photons with a longer lifetime of 2.2 ns (Figure 2J, Figures S42d, S43d). The image pixels corresponding to the assembly dynamics are linearly distributed across these two phasors (Figure 2I, Figures S42c, S43c). Pixels found closer to the 0.75

ns locus imply a larger photon population corresponding to the fiber assembly. Using these parameters, live observation of the transformation of **1**·TAT to **2_{NF}** in the presence of H₂O₂ showed increasing population of photons with shorter lifetimes and their spatial correlation toward the assemblies (Figure S44, Video 1).

Spatial Visualization of **2_{NF} Using CLSM and CLEM Tomography in Cells.** The TAT-dependent endocytosis into MDA-MB-231 metastatic breast cancer cells was visualized using confocal laser scanning microscopy (CLSM) (Figure 3A, Videos 2, 3). At 4 h of treatment using 25 and 50 μ M **1**·TAT, concentration-dependent assembly into aggregates within the cells occurs along with apparent cell shrinkage and blebbing (Figure S45). To overcome the limitation of intensity-based fluorescence microscopy in resolving assembly states, we performed corresponding imaging in the lifetime domain. Phasor plot analysis shows the assembly profile (molecules, intermediates, aggregates) and lifetime mapped to their spatial localization at this time point (4 h; Figure 3A). Attempts to control aggregate formation were subsequently performed with lower concentrations (5 and 10 μ M of **1**·TAT) and for longer incubation times of 12 and 24 h. At 12 h, photons that are emitted intracellularly exhibit fluorescence lifetimes of 2.7 ns $>$ τ_f $>$ 2.0 ns, correlating to the initial phase of self-assembly (Figure S46). Upon further incubation to 24 h, cells remained healthy, and phasor plots reveal that further progression into fibrillar aggregates was not observed. Photon population analysis implies that at these low concentrations the cells are able to maintain homeostasis in a way that assembly intermediates do not accumulate enough to propagate into nanofibers. Control experiments with **3**·TAT, which correspondingly forms non-assembling **4**, did not produce aggregates and therefore showed a single-photon population with a τ_f of 3.1 ns (Figures S47, S48). In alternative cell lines such as A549 lung adenocarcinoma cells and L929 murine fibroblasts (Figure 3A), applying the sensitive phasor-FLIM technique reveals differences in the variety and distribution of assembled species within each cell type.

High spatial resolution provided by CLEM and tomography revealed the morphology of the nanostructured assemblies as discrete hollow spheres, each surrounded by a corona of fibers (Figure 3B–E, J–K). In detail, fluorescence signals from the NDI core were correlated to electron micrographs of the MDA-MB-231 cells, capturing endosomal vesicles at different stages of structure formation (Figure 3B–I and Figures S49–S52). In intact healthy cells, heavily distorted vesicles with signs of intravesicular phase separation were observed, showing the initial stages of chemical transformation of **1**·TAT (Figure 3F–I and Figures S49, S50). In contrast, dying cells show the formation of iconic fibrillar spheres, and when the cell boundary contracts, these structures are extruded to the periphery, similar to what is observed in live CLSM (Figure 3B–E and Figures S51, S52, Video 2). Characterization by electron tomography demonstrated that these fibrillar spheres contain a hollow core

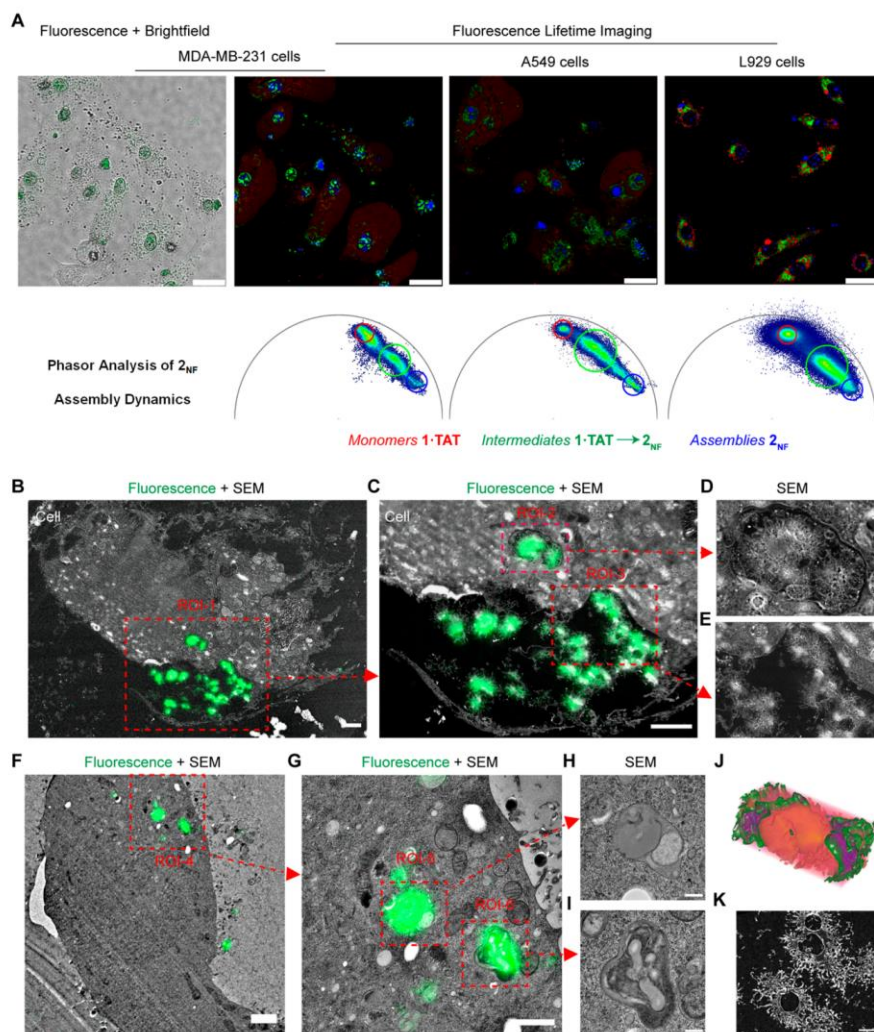


Figure 3. Phasor-FLIM and CLEM analysis of the formation of hollow nanofiber clusters of 2_{NF} . (A) Live-cell fluorescence lifetime images of MDA-MB-231 cells, A549 cells, and L929 cells treated for 4 h with $1 \cdot TAT$ ($25 \mu M$) and corresponding phasor analysis of assembly progression. Scale bars: $20 \mu m$. (B) CLEM-SEM micrographs of MDA-MB-231 cells treated for 1 h with $1 \cdot TAT$ ($25 \mu M$), showing the nanostructures of the fiber clusters as the cell shrinks. Scale bars: $2 \mu m$. (C) Magnified CLEM-SEM micrographs correspond to ROI-1, scale bar: $2 \mu m$. (D, E) Magnified SEM micrographs corresponding to ROI-2 and ROI-3. (F) CLEM-SEM micrographs of MDA-MB-231 cells with developing nanostructures within distorted endosomes. Scale bars: $1 \mu m$. (G) Magnified CLEM-SEM micrographs correspond to ROI-4, scale bar: $1 \mu m$. (H, I) Magnified SEM micrographs corresponding to ROI-5 and ROI-6, showing the phase separation and endosomal distortion brought about by the assembly dynamics. Scale bar: $200 nm$. (J) 3D volume EM tomogram of the hollow nanofiber clusters, where green and red represent peptide structures and less dense areas, respectively. Purple represents spatially overlapping regions of green and red. (K) SEM micrographs of the nanofiber clusters featuring the hollow core. Scale bar: $500 nm$.

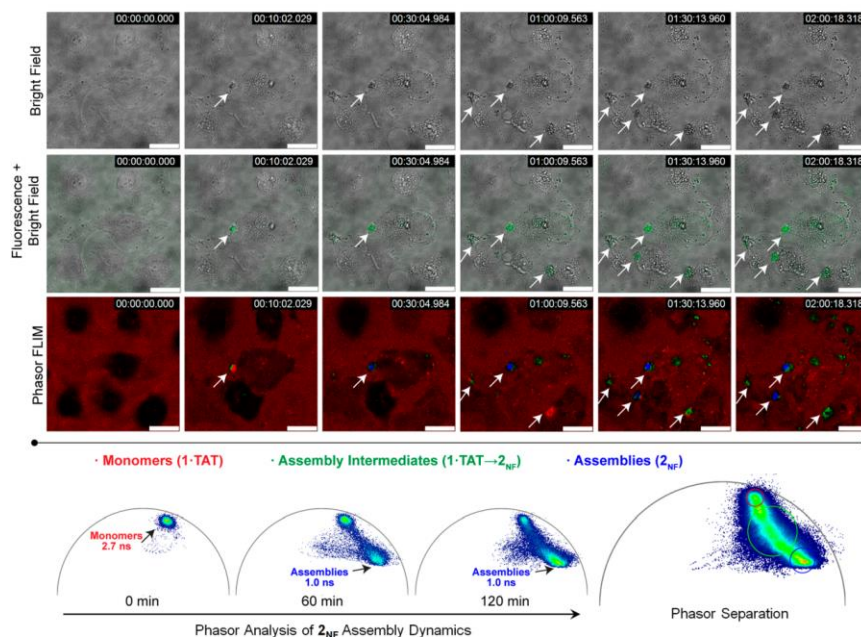


Figure 4. Time-lapsed phasor-FLIM analysis of 1-TAT internalization and assembly into 2-NF clusters. Time-dependent brightfield, fluorescence + brightfield, and phasor-FLIM images of MDA-MB-231 cells treated with 1-TAT (25 μ M) (0–120 min). Bottom row: Phasor maps of MDA-MB-231 cells treated with 1-TAT (25 μ M) at various time points as well as the total cumulative phasor distribution used to perform phasor separation. Arrows indicate the dynamics leading to nanofiber formation and growth with a concomitant change in the phasor-FLIM. Scale bars = 20 μ m.

(Figure 3J,K, Video 4, Figure S53), showing that the endosomal vesicles provided a discrete volume that spatially controls the assembly and growth of 2-NF nanostructures.

Temporal Analysis of Nanofiber Clusters Using Phasor-FLIM. The formation of the hollow clusters was tracked using time-lapsed live cell phasor-FLIM imaging. The extent of aggregation over time can be followed as the photon population shifts between the two characteristic phasors (representing molecular 2 and assembled 2-NF) on the phasor plot. Control experiments using lysates of MDA-MB-231 cells rule out nonspecific interactions of fluorescence lifetime between 1-TAT and cellular components (Figure S57). Immediately upon treatment of MDA-MB 231 cells with 1-TAT (t_0), emitted photons exhibit a homogeneous, single-component fluorescence decay with $\tau_f = 2.7$ ns, corresponding to free molecules. With time, the cellular uptake of 1-TAT initiated a shift of photon population toward the aggregated phasor $\tau_f = 1.0$ ns (Figure 4, Video 5). The nanofiber clusters form and enlarge within 1 h, and the corresponding population of photons shifting to $\tau_f = 1.0$ ns increases. From 2 h onward, the phasor plot exhibits minimal changes, suggesting that the aggregation process has been completed. Several observations, including cellular blebbing and nuclear shrinkage, which are hallmarks of cell death, were detected as aggregation progresses. In live-cell imaging, these morphological changes clearly occurred before the enlargement of the nanofiber clusters, implying that the observed biological

responses were initiated at an earlier time point. Individual cells that survived treatment showed no intracellular aggregation, suggesting that these cells plausibly had higher efflux rates that prevented the accumulation of assembling precursor 2. Hence, for each cell, the time point of cell death depends on how long it can maintain efflux to continuously expel the peptides (Video 6).

Real-Time Biological Response upon Intracellular Assembly. The imaging studies by CLEM and phasor-FLIM showed that 1-TAT entered the cell's endosomes where exposure to endogenous H_2O_2 oxidatively cleaves the boronic acid moiety to release the assembling precursor 2 over time. Subsequent assembly and phase separation of 2 leads to the formation of hollow nanofiber clusters of 2-NF. Due to the extensive endosome distortion observed, we hypothesized that the assembly process could disable native endosomal activities.

Among their plethora of functions, endosomes are crucial in transporting extracellular materials, including essential nutrients such as glucose.^{33–36} Using glucose to probe for endosomal transport, time-lapsed quantification of glucose uptake showed that the MDA-MB-231 cells treated with 25 μ M of 1-TAT showed >65% reduction in intracellular glucose levels within 30 min, whereas controls with nonassembling 3-TAT retain normal function even after 4 h (Figure 5A). Within the cell, glucose is metabolized through glycolysis to form pyruvate, NADH, and ATP, with a corresponding release of protons into the

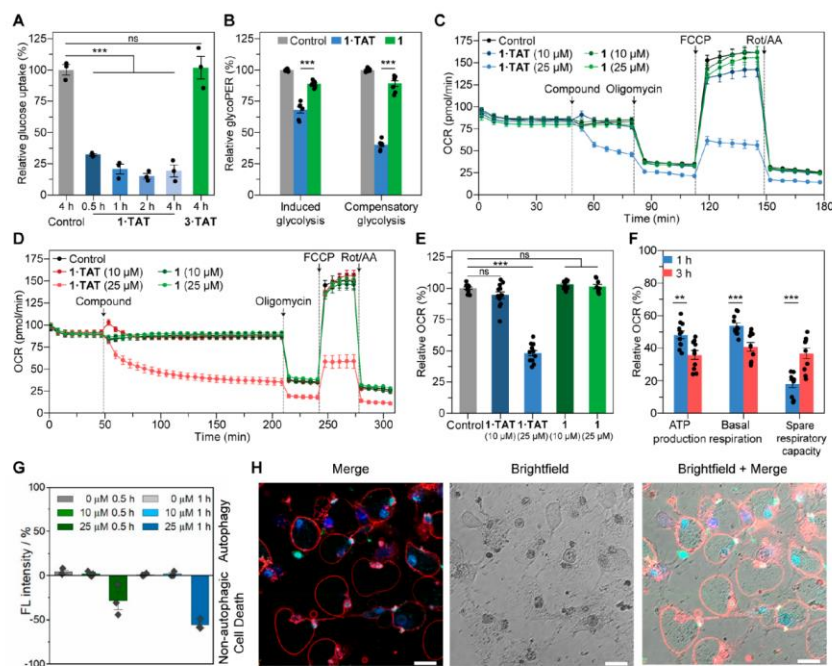


Figure 5. Inhibition of oxidative phosphorylation by intracellular transformation of 1-TAT into 2NF. (A) Glucose uptake assay based on bioluminescence detection of 2-deoxyglucose-6-phosphate upon the treatment of 2-deoxyglucose. MDA-MB-231 cells were incubated with 1-TAT (25 μ M) for various durations (0.5, 1, 2, and 4 h) and 3-TAT (25 μ M) for 4 h as a control. Analysis was performed by a luminescence read-out. $n = 3$. (B) Real-time glycolytic flux assay and analysis of MDA-MB-231 cells treated with 1-TAT (25 μ M). The glycolytic proton efflux rate (glycoPER) obtained was used to quantify glycolytic activity. Glycolytic activity with functional mitochondria is termed induced glycolysis, whereas glycolytic activity with suppressed mitochondria is termed compensatory glycolysis. $n = 7$. All data are presented as mean $\pm$ s.e.m. Statistical significance was calculated by ANOVA with a Tukey post hoc test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (C, D) Effect of 1-TAT and 1 on the oxygen consumption rate (OCR) of MDA-MB-231 cells after incubation for 1 h (C) and 3 h (D). FCCP: Carbonyl cyanide-4 (trifluoromethoxy)phenylhydrazone, Rot/AA: Rotenone and antimycin. (E) Comparison of the effects of 1-TAT (10 and 25 μ M) and 1 (10 and 25 μ M) on cellular ATP production (OCR) after incubation for 1 h. $n \geq 9$. (F) Effect of 1-TAT (25 μ M) on ATP production, basal respiration, and spare capacity of the OCR after incubation for 1 and 3 h. Values are normalized toward the untreated control group. The last measurement before the compound injection is set as 100%. $n \geq 9$. (G) Fluorescence labeling assay of autophagosomes with relative fluorescence is presented against untreated controls. An increase in fluorescence intensity indicates formation of autophagosomes, whereas a decrease in fluorescence suggests non-autophagic cell death. Data presented as s.e.m. $n = 3$. (H) Annexin V/DAPI assay on MDA-MB-231 cells treated with 1-TAT (25 μ M). Scale bar: 20 μ m.

extracellular medium. The reduction in glucose uptake would impair glycolysis and stymie the supply of pyruvate entering the citric acid cycle in the mitochondria, thereby destabilizing the redox activity within the electron transport chain. Real-time quantification of the glycolytic proton efflux rate (glycoPER) shows a decrease of $68.1 \pm 2.4\%$ in basal glycolytic activity (Figure 5B). The impact is magnified to a decrease of $40.1 \pm 1.4\%$ when compensatory activity from the mitochondria is artificially suppressed with rotenone and antimycin A (Figure 5B).

Without sufficient pyruvate produced by glycolysis, the NAD⁺/NADH homeostasis maintained by the citric acid cycle and electron transport chain would be disrupted.³⁷ Analyses using treated cells with 25 μ M 1-TAT show a time-dependent impact on NAD⁺, with a $51.1 \pm 3.3\%$ reduction in NAD⁺ content at 4 h (Figure S59). The impact on the electron transport chain within

the mitochondria was quantified by monitoring oxidative phosphorylation (OxPhos) where ATP is produced through the consumption of oxygen. Real-time monitoring of the cells upon treatment with 1-TAT (25 μ M) reveals an acutely rapid decrease of the oxygen consumption rate (OCR), corroborating with the assembly onset observed in phasor-FLIM. Inhibitors of the protein complexes within the electron transport chain were used to evaluate ATP production and mitochondrial function. Two start points for the inhibitors were selected, at 30 min and 2 h 30 min, corresponding to the dynamic assembly phase and when the formation of nanofiber clusters 2NF is mostly completed. Accounting for the assay time, the first impact toward mitochondria respiration was received at 1 and 3 h, respectively (Figure 5C,D). Cells treated with 1-TAT showed concentration dependency, with 25 μ M demonstrating a marked decrease in ATP production to $48.1 \pm 2.3\%$ relative to that of

untreated control cells (Figure S5E). Other mitochondrial functions associated with basal ($53.8 \pm 1.7\%$, Figures S61a, S62b) and spare respiratory capacities ($17.9 \pm 2.2\%$, Figures S61b, S62c) were similarly reduced by the formation of 2_{NF} . No significant inhibitory effect was detected for cells treated with 1_{NF} , supporting the idea that the metabolic disruption is exerted by intracellular aggregation of 2_{NF} . Between the two phases of assembly (Figure S5F), the reduction in ATP production was consistent, suggesting that the size of the nanofiber clusters seems to be irrelevant in impacting mitochondrial function. In contrast, an elevated level of spare respiratory capacity ($+105.6\%$ relative to 1 h stage, $36.8 \pm 3.3\%$ relative to untreated cells) was observed at 3 h compared to treated cells at 1 h (Figure S5F). The respiratory spare capacity is defined as the difference between basal ATP production and its maximal activity, which is switched on to counter mitochondrial stress. These responses prevent the amplification of initial stress, which may otherwise further impair the respiratory chain or promote mutations in mitochondrial DNA.^{38,39}

The collective impact of the assembly of 2_{NF} on the cellular respiratory functions led to cell death, where treatment with 1_{NF} revealed an IC_{50} value of $69 \pm 5 \mu M$ at 4 h (Figure S63). In contrast, nonassembling 3_{NF} is nontoxic up to $100 \mu M$ (Figure S64). Labeling experiments confirmed the absence of autophagosomes, ruling out mitophagy as a plausible mechanism for respiratory dysfunction (Figure S5G). Corresponding positive binding of annexin-V suggests apoptosis as the primary mechanism of cell death (Figure S5H, Figure S65).

CONCLUSION

By tracking the assembly dynamics of nanostructure formation in living cells, we have demonstrated the importance of establishing a temporally and spatially resolved profile of biological activities. Within this highly transient window, when molecules begin to assemble, the phasor-FLIM technique was invaluable in resolving assembly progression in real time. The time-lapse study, in conjunction with CLEM, provided a precise rationalization of the assembly process from the molecular to the nanoscale, where precursor peptides developed into hollow nanofiber clusters under endosomal fusion. The onset of assembly was critical in the disruption of endosomal function, severely impairing the associated glucose-dependent metabolism and cellular respiration. In contrast, the growth of mature assemblies and accumulation did not induce additional biological responses. Our study paves the way to understand and visualize the supramolecular chemistry of nanostructure formation in biology to ultimately address aggregation-based dysfunction in diseases.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/jacs.4c01279>.

Details of instruments, materials, and methods (NMR, MS, HPLC, UV-vis), cell assays, sample preparation workflow for microscopy, microscopy parameters, setup, and analysis (PDF)

Phasor-FLIM imaging of the cell-free transformation of 1_{NF} to 2_{NF} in the presence of H_2O_2 (MP4)

CLSM imaging (zoomed) of TAT-dependent endocytosis into MDA-MB-231 metastatic breast cancer cells (MP4)

CLSM imaging of TAT-dependent endocytosis into MDA-MB-231 metastatic breast cancer cells (MP4)

Video of electron tomography of fibrillar spheres (MP4)

Phasor-FLIM imaging (zoomed) of the intracellular assembly of 1_{NF} to 2_{NF} (MP4)

Phasor-FLIM imaging of the intracellular assembly of (MP4)

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Notes

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Supramolecular Assembly in Live Cells mapped by Real-time Phasor-Fluorescence Lifetime Imaging

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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 CH₃CN in HPLC grade (containing 0.1% CF₃COOH) and H₂O for HPLC (containing 0.1% CF₃COOH) and reactions were obtained from a Millipore purification system.

1.2 Instruments

1.2.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR spectra for all compounds and intermediates were recorded on a Bruker Avance III 700 MHz NMR spectrometer. Temperature control was achieved using a VTU (BCU II; variable temperature unit) with an accuracy of ± 0.1 K. The samples were stabilized at the desired temperature for 20 minutes prior to the measurements. 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 (D₂O δ = 4.79 ppm and DMSO-d₆ = 2.50 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. Chemical shifts for protons of the compounds were assigned on the basis of COSY and HSQC correlations. The data were processed in MestReNova.

1.2.2 Microwave Peptide Synthesizer

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

1.2.3 Preparative High-Performance Liquid Chromatography (HPLC)

The compounds were purified by preparative HPLC using a setup by Shimadzu. For purification either a ZORBAX Eclipse XDB-C18 HPLC column (9.4 $\times$ 250 mm, 5 μ m) was used at a flowrate of 4 mL/min or a Phenomenex Gemini 5 μ m NX-C18 110 Å 150 $\times$ 30 mm was used at a flowrate of 25 mL/min. Experiments were performed using CH₃CN in HPLC grade (containing 0.1% CF₃COOH) and MilliQ water (containing 0.1% CF₃COOH).

1.2.4 Analytical High-Performance Liquid Chromatography (HPLC)

The samples were analyzed by analytical HPLC using a setup by Shimadzu. An Atlantis T4 column (4.6 $\times$ 100 mm, 5 μ m) was used at a flowrate of 1 mL/min. Experiments were performed using CH₃CN in HPLC grade (containing 0.1% CF₃COOH) and MilliQ water (containing 0.1% CF₃COOH). The solvent gradient started with 100% water, then the CH₃CN content was linearly increased to 100% in 20 min. The molar ratio of the compounds was calculated using peak areas at 254 nm. Data were processed in LabSolutions and Origin.

1.2.5 High-Performance Liquid Chromatography - Mass Spectrometry (HPLC-MS)

The 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 $\times$ 2.1 mm column. MilliQ water, acidified with 0.1% formic acid and CH_3CN were used as solvents for all measurements. The solvent gradient started with 5% CH_3CN and 95% water. This solvent ratio was kept constant for 2 min, then the CH_3CN content was linearly increased to 95% in 14 min. The molar ratio of the compounds was calculated using peak areas at 254 nm. Data were processed in LabSolutions and Origin.

1.2.6 Matrix-Assisted Laser Desorption/Ionisation - Time of Flight Mass Spectrometry (MALDI-TOF-MS)

All MALDI-TOF spectra were recorded on either a rapifleX MALDI-TOF/TOF from Bruker or MALDI Synapt G2-SI from Waters. Samples were mixed with a saturated solution of the matrix S7 α -cyano-4-hydroxycinnamic acid (CHCA) in NH_4HCO_3 buffer (20 mM)/ CH_3CN 1/1. Data processing was performed in mMass.

1.2.7 Spectroscopy

The UV/Vis absorption spectra for the solutions of the compounds were recorded on a Thermo Scientific™ NanoDrop 2000/2000c spectrophotometer in a Hellma high precision quartz cell, 10 x 10 mm light path. The luminescence emission spectra for the solutions of the compounds were recorded on a Cary Eclipse fluorescence/luminescence spectrophotometer in a Hellma high precision quartz cell, 10 x 10 mm light path. For the CAC analysis using Nile Red, the luminescence spectra were recorded using a Tecan Spark 20M microplate reader. CD spectra were recorded on a JASCO J-1500 spectrometer in a Hellma high precision cell with 1 mm light path. Data were processed in Spectra Analysis by JASCO and Excel.

1.2.8 Dynamic Light Scattering (DLS)

Single-angle DLS measurements were performed at 25 °C using a Malvern ZetaSizer Nano S purchased from Malvern Instruments Ltd. (Malvern, Great Britain) with a He/Ne Laser ($\lambda = 633 \text{ nm}$) at a fixed scattering angle of 173°. All measurements were performed in triplicate. The obtained data was processed by cumulant fitting for D_h , or by CONTIN fitting for intensity weighted particle size distribution. Samples were prepared at 50 μM . Dust was removed prior to each measurement by filtration through GHP syringe filters (0.2 μm pore size, Acrodisc).

1.2.9 Transmission Electron Microscopy (TEM)

TEM images of the conjugate solutions were taken on a JEOL 1400 transmission electron microscope at a voltage of 120 kV. Samples were prepared on Formvar/carbon-film coated copper grids (300 mesh) by Plano GmbH. In order to prepare the TEM grids, 4 μL of the sample solution were put on freshly glow discharged (30 s at 40 W in a 6:1 oxygen: hydrogen plasma) Formvar coated copper grids. After 5 min the solution was removed using a filter paper and grids were stained with 4% uranyl

acetate for 2.5 min. The grids were washed three times with MilliQ water and dried before measuring. TEM images were processed in ImageJ.

1.2.10 Cryogenic Transmission Electron Microscopy

For cryo-TEM examination the samples were vitrified using a Vitrobot Mark V (Thermo Fisher, Hillsboro Oregon) plunging device. 3 μ l of the sample dispersion was applied to a Quantifoil or a lacey carbon coated TEM grid that has been glow discharged in a 6:1 oxygen: hydrogen plasma (Diener Nano[®], Diener electronic, Germany) shortly before. After removing excess sample solution with a filter paper, the grid is immediately plunged into liquid ethane. For the subsequent examination the specimen is transferred to a TEM (FEI Titan Krios G4) keeping cryogenic conditions.

Conventional TEM imaging was done using an acceleration voltage of 300 kV. Micrographs were acquired with a 4k Direct Electron Detection Camera (Gatan K3) under low dose conditions.

1.2.11 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% FBS. Both cell lines were cultured in T75 culture flask and subcultivated two to three times per week.

1.2.12 Luminescence Detection of Cell Viability

After performing the CellTiter-Glo[®] Luminescent Cell Viability Assay in a half area, white 96-well plate, luminescence intensity was measured on a Promega GloMax[®]-Multi Detection System using the protocol for the CellTiter-Glo[®] Luminescent Cell Viability Assay preinstalled on the instrument. Data were processed in Excel and graphs were generated in Origin.

1.2.13 Confocal Laser Scanning Microscopy & Fluorescence Lifetime Imaging Microscopy

CLSM-FLIM imaging was performed on a STELLARIS 8 Leica DMI8 microscope (Leica Microsystems, S/N: 8300000313). The live cells imaging was conducted in a microscope Incubator (okolab).

Cells are cultured in an IBIDI 8-well glass bottom chamber at a density of 25,000 cells per well in DMEM and allowed to adhere overnight at 37°C, 5% CO₂. The medium was aspirated and samples pre-dissolved in DMEM at the respective concentrations were introduced into each well. Incubation was performed at 37°C, 5% CO₂.

Cells were imaged live using an incubator-equipped Leica Stellaris[®] 8 microscope (40x glycerol immersion objective) with fast lifetime contrast (FALCON) module. Samples were excited using a 40 MHz pulsed white light laser tuned to 469 nm for both intensity and fluorescence lifetime measurements. Emitted photons were detected using HyD[®] X (GaAsP hybrid photocathode) detector with a filter window at 480 nm – 650 nm.

Fluorescence lifetime imaging (FLIM) was conducted with living cells at 2.5 min intervals, with scanning resolution of 1024 x 1024 pixels at 200 Hz, 15 frame accumulation equipped with adaptive

focusing on each frame and well position. Acquisition was performed for 100 frames. Photons were counted based on FALCON-modified time correlated single photon counting (TCSPC) method. Each pixel is transformed into a phasor plot according to the following equation:

$$g_{ij}(\omega) = \int_0^T I(t) \cdot \cos(n\omega t) dt / \int_0^T I(t) dt$$

$$s_{ij}(\omega) = \int_0^T I(t) \cdot \sin(n\omega t) dt / \int_0^T I(t) dt$$

in which $g_{ij}(\omega)$ and $s_{ij}(\omega)$ are the x and y coordinates of the phasor plot, n and ω are the harmonic frequency and the angular frequency of excitation, respectively, and T is the repeat frequency of the acquisition. Frequency domain data acquisition from each pixel can be converted to phasor points using the following transformations:

$$g_{ij}(\omega) = m_{ij} \cdot \cos(\phi_{ij})$$

$$s_{ij}(\omega) = m_{ij} \cdot \sin(\phi_{ij})$$

in which m_{ij} and ϕ_{ij} are the modulation and phase shift, respectively, of the frequency domain measurement at pixel ij . The decay from each pixel can hence be translated to a point in the phasor plot.¹

Phasor components were identified and separated using LAS X software. Photons that lie beyond the defined phasor are excluded from the images.

1.2.14 Seahorse XFe96 Analyzer

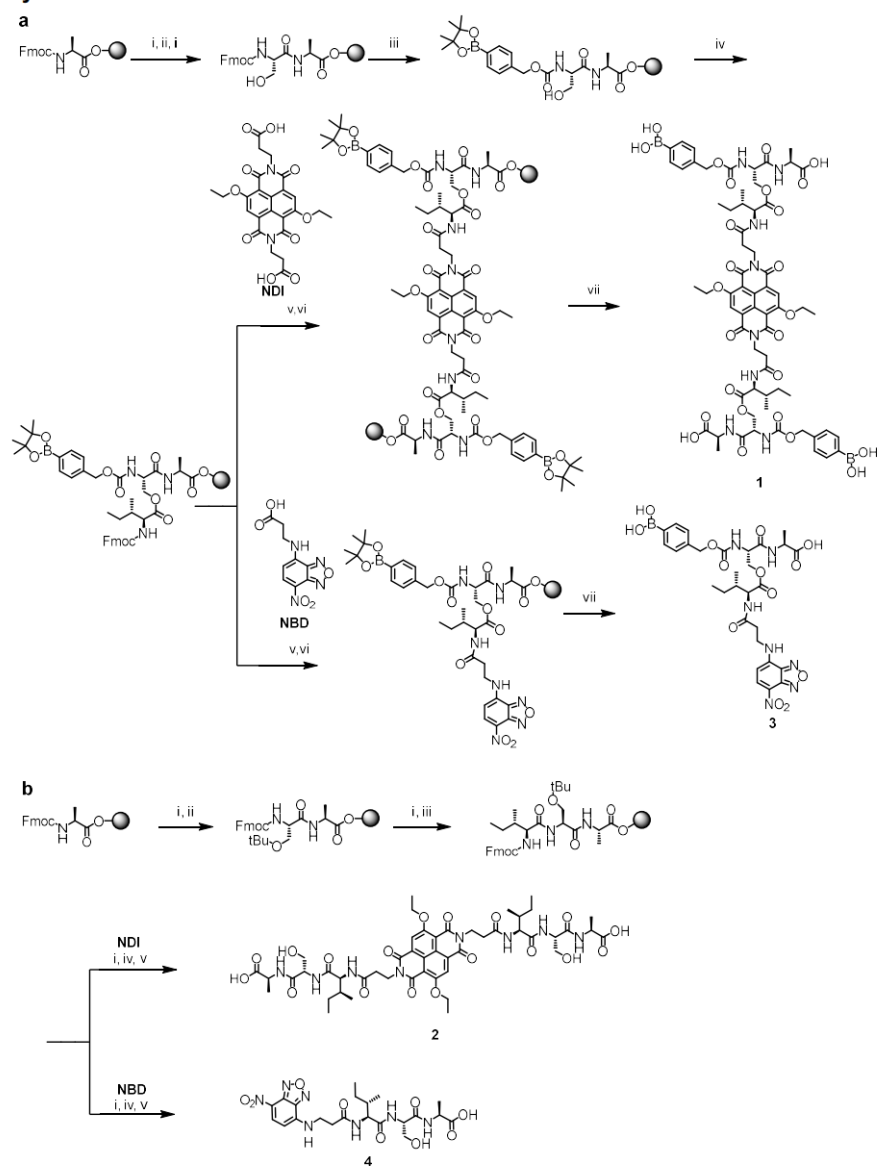
Effects of the compound on the cell metabolism were investigated using the Seahorse XFe96 Analyzer (Agilent Technologies) measuring the Extracellular Acidification Rate (ECAR) and Oxygen Consumption Rate (OCR). Seahorse XF Glycolysis Stress Test Kit and Seahorse XF Cell Mito Stress Test Kit were used following the manufacture's protocol to determine the glycolytic and respiratory activity of the cells. Cells were seeded at various densities on an XFe 96 well plate one day prior to the assay. On the day of the assay cells were treated with different concentrations of **1** and **1·TAT**. ECAR and OCR were measured in real-time. Data were processed in Excel and graphs were generated in Origin.

1.2.15 Fluorescence Microscopy

Images of the cells after Seahorse measurement were taken using a Leica DMI8 microscope using a 10x objective by Leica. Cells were stained using NucBlue® Live ReadyProbes® Reagent and fluorescence was imaged with 350/50 nm excitation and 460/50 nm emission filter.

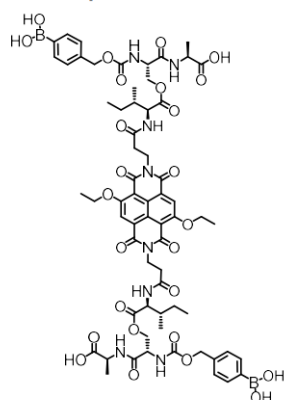
S6

2 Synthesis and structural characterization



core-substituted NDI/3-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)propanoic acid, HATU, DIEA, DMF, RT. The c-NDI molecule was synthesized according to a previous report.² **b**, Synthesis of **2** and **4**. (i) 20% piperidine in DMF. (ii) Fmoc-Ser(tBu), PyBOP, DIPEA, DMF, 75 °C, 20 min. (iii) Fmoc-Ile, PyBOP, DIPEA, DMF, 75 °C, 20 min. (iv) core-substituted NDI/3-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)propanoic acid, HATU, DIPEA, DMF, RT, overnight. (v) 95% CF₃COOH, 2.5% TIPS, 2.5% H₂O.

2.1 Compound 1



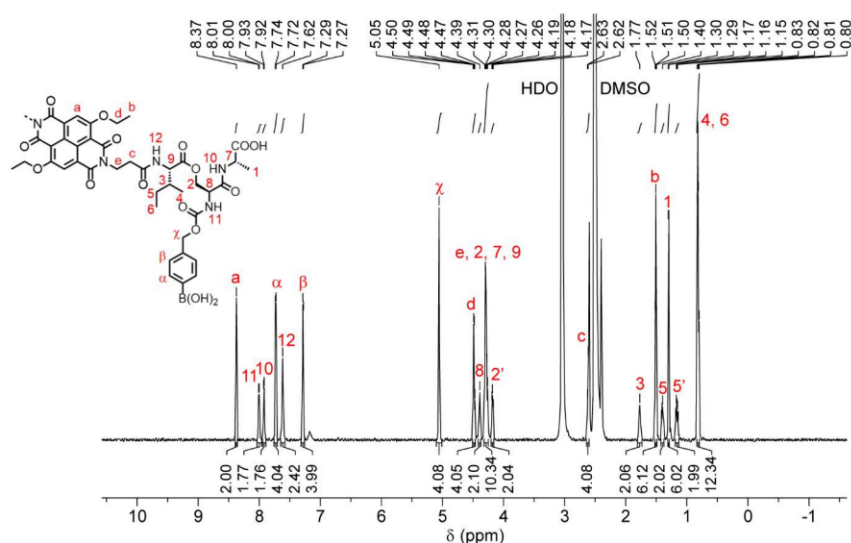
Synthesis: Compound **1** was synthesized using the Fmoc solid phase peptide synthesis strategy by Merrifield, synthesizing the peptide from C to N-terminus in a microwave assisted peptide synthesizer. Fmoc-Ala preloaded Wang resin (0.5 mmol) was swollen in DMF for 1 h before use. First, the Fmoc group was removed by two consecutive deprotection steps (2 and 5 min) with 20% piperidine in DMF (10 mL) at 75 °C (i). After deprotection, the resin was washed four times with DMF (7 mL). Fmoc-serine (5 equiv. in 10 mL DMF) was coupled to the N-terminus by using the activator PyBOP (5 equiv. in 4 mL DMF) and activator base DIPEA (10 equiv. in 2 mL DMF) at 75 °C for 20 min (ii). After deprotection of the Fmoc protecting group (i), the N terminus was modified using 4-nitrophenyl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl carbonate (1.25 equiv.) in 10 mL DMF and DIPEA (5 equiv.) overnight at room temperature (iii). After the resin was washed with DMF and DCM, Fmoc-Ile (10 equiv. in 5 mL DMF), DIC (10 equiv.) and 4-DMAP (1 equiv.) were added to form the ester bond in the serine side chain. After the reaction mixture was stirred for 12 h at room temperature, the resin was washed with DMF and DCM, dried (iv). The Fmoc group was then removed in two deprotection steps using 20% piperidine in DMF (10 min each, 5 mL) at room temperature (v). 3,3'-(4,9-diethoxy-1,3,6,8-tetraoxo-1,3,6,8-tetrahydrobenzo[imn][3,8]phenanthroline-2,7-diyl)dipropionic acid (0.2 equiv.) was coupled onto the N-terminus overnight at room temperature, using HATU (5 equiv.) and DIPEA (5 equiv.) as the coupling reagents. The resin was washed with DMF and DCM, dried (vi). The product was cleaved from the resin by using 2.5 mL of a cleavage cocktail (95% CF₃COOH, 2.5% TIPS, 2.5% H₂O). This step also removed the pinacol protecting group of the PBA. After 2 hours, the cleavage cocktail was removed in vacuo (vii). The product was purified by HPLC using the Zorbax Eclipse column at a flowrate of 4 mL/min. The gradient started with 20% CH₃CN in H₂O (+0.1% CF₃COOH) and this solvent ratio was kept for 1 min, after which the CH₃CN content was

increased to 100% in 15 min. The product **S1** eluted from the column after 11.8 min and was received as a yellow powder after lyophilization (15.2 mg, 5.1% overall yield).

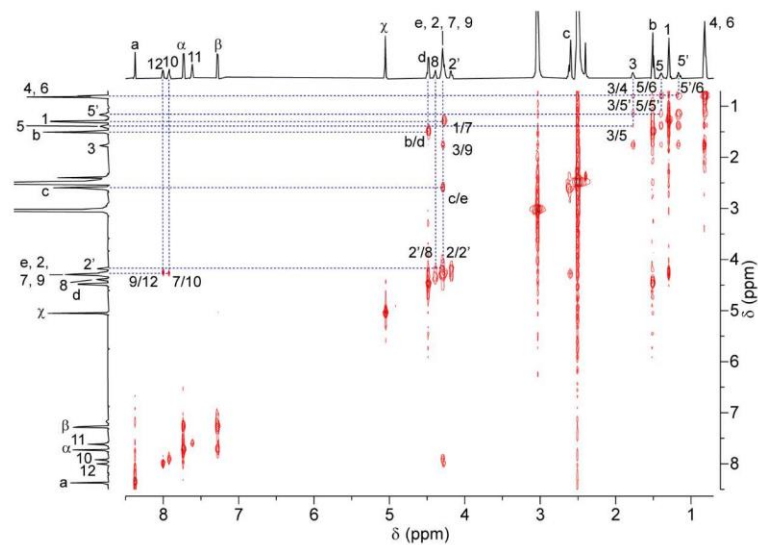
¹H NMR (700 MHz, DMSO-*d*₆): δ 8.37 (s, 2H), 8.00 (d, *J* = 6.9 Hz, 2H), 7.92 (d, *J* = 7.2 Hz, 2H), 7.73 (d, *J* = 7.6 Hz, 4H), 7.62 (s, 2H), 7.28 (d, *J* = 7.9 Hz, 4H), 5.05 (s, 4H), 4.48 (q, *J* = 6.9 Hz, 4H), 4.42 – 4.36 (m, 2H), 4.33 – 4.25 (m, 10H), 4.21 – 4.14 (m, 2H), 2.64 – 2.61 (m, 4H), 1.81 – 1.72 (m, 2H), 1.51 (t, *J* = 6.9 Hz, 6H), 1.43 – 1.36 (m, 2H), 1.30 (d, *J* = 7.2 Hz, 6H), 1.16 (dt, *J* = 15.8, 7.7 Hz, 2H), 0.87 – 0.77 (m, 12H).

LCMS: LC retention time (min): 6.64. MS (ESI⁺, *m/z*): 1396.9 [M + H]⁺, 1379.6 [M + H - H₂O]⁺, 1361.6 [M + H - 2H₂O]⁺.

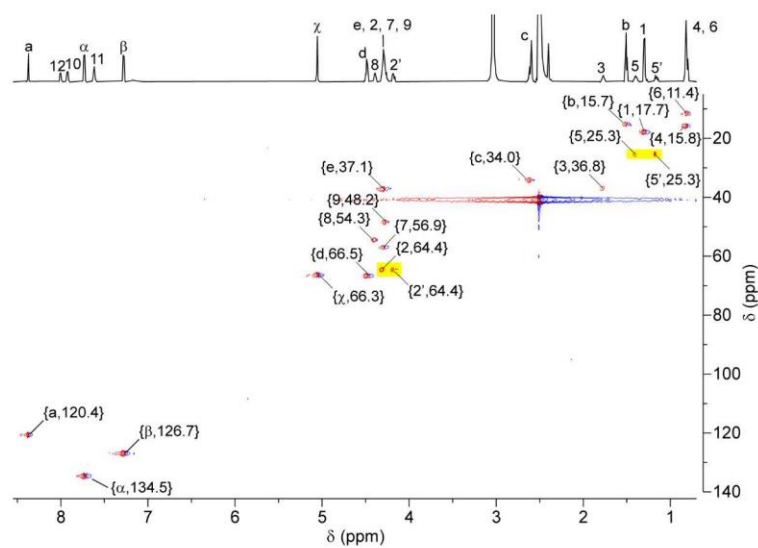
HRMS (ESI⁺, *m/z*): Calculated for [M - H₂O + H]⁺ (C₆₄H₇₇B₂N₈O₂₅⁺): 1379.5180, found 1379.5317.



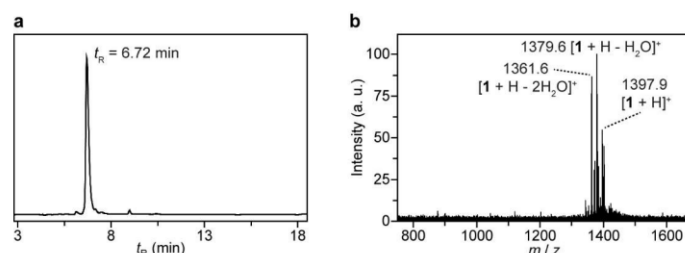
Supplementary Fig. 2| ¹H NMR spectrum (700 MHz, DMSO-*d*₆, 353 K) of compound **1**.



Supplementary Fig. 3| ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra (700 MHz, $\text{DMSO}-d_6$, 353 K) of compound **1**. The correlations between ^1H signals are highlighted with blue dashed lines in the COSY spectra.

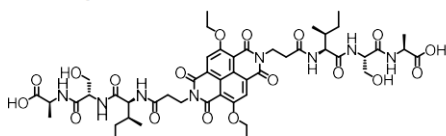


Supplementary Fig. 4| ^1H NMR and $^1\text{H},^{13}\text{C}$ HSQC NMR spectra (700 MHz, $\text{DMSO}-d_6$, 353 K) of compound **1**. Splitting of H_2 and H_5 traces are highlighted in the HSQC spectra.



Supplementary Fig. 5] LC-MS study of compound **1**. **a**, HPLC trace of **1**. Retention time (t_R) = 6.72 min. **b**, Convolved MS spectrum for t_R = 6.15-6.90 min, showing peaks of $[M + H]^+$, $[M + H - 2H_2O]^+$ and $[M + H - H_2O]^+$

2.2 Compound 2

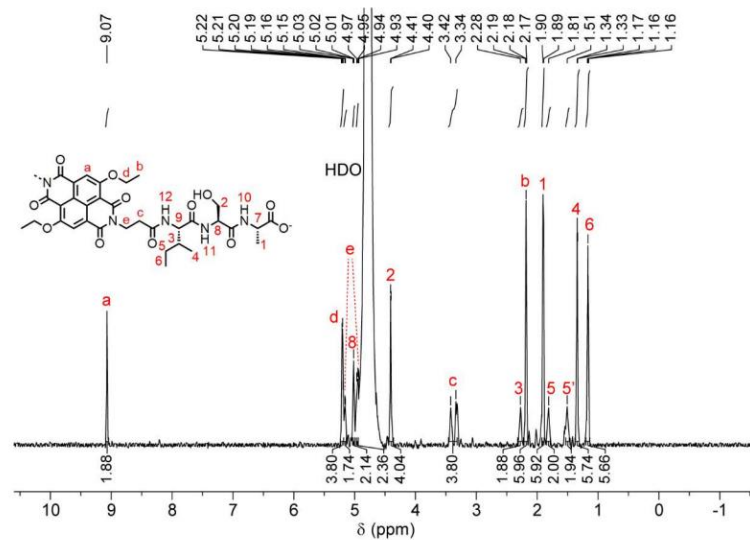


Synthesis: Compound **2** was synthesized using Merrifield's Fmoc solid-phase peptide synthesis strategy, constructing the peptide from the C-terminus to the N-terminus in a microwave-assisted peptide synthesizer at a 0.25 mmol scale. Fmoc-Ala preloaded Wang resin (0.5 mmol) was swollen in DMF for 1 h before use. After deprotection of the Fmoc group (3 mL of 20% piperidine in DMF, 2 and 5 min at 75 °C) (i), Fmoc-Ser(tBu) (ii), Fmoc-Ile (iii) were double coupled onto the peptide at 75 °C for 20 min. 5 equiv. of the compounds were used in 2.5 mL DMF. PyBOP (5 equiv. in 1 mL DMF) and DIPEA (10 equiv. in 0.5 mL) were used for the coupling reaction. After deprotection of the Fmoc group (3 mL of 20% piperidine in DMF, 2 and 5 min at 75 °C) (i), 0.2 equiv. of 3,3'-(4,9-diethoxy-1,3,6,8-tetraoxo-1,3,6,8-tetrahydrobenzo[Imn][3,8]phenanthroline-2,7-diyl)dipropionic acid were coupled onto the peptide at room temperature overnight (iv). PyBOP (5 equiv. in 1 mL DMF) and DIPEA (10 equiv. in 0.5 mL) were used for the coupling reaction. The product was cleaved from the resin by using 2.5 mL of a cleavage cocktail (95% CF₃COOH, 2.5% TIPS, 2.5% H₂O). The product was purified by HPLC using the Zorbax Eclipse column at a flowrate of 4 mL/min. The gradient started with 20% CH₃CN in H₂O (+0.1% CF₃COOH) and this solvent ratio was kept for 1 min, after which the CH₃CN content was increased to 100% in 15 min. The compound eluted from the column after 5.5 min and was received as a yellow powder after lyophilization (10.3 mg, 3.5% overall yield).

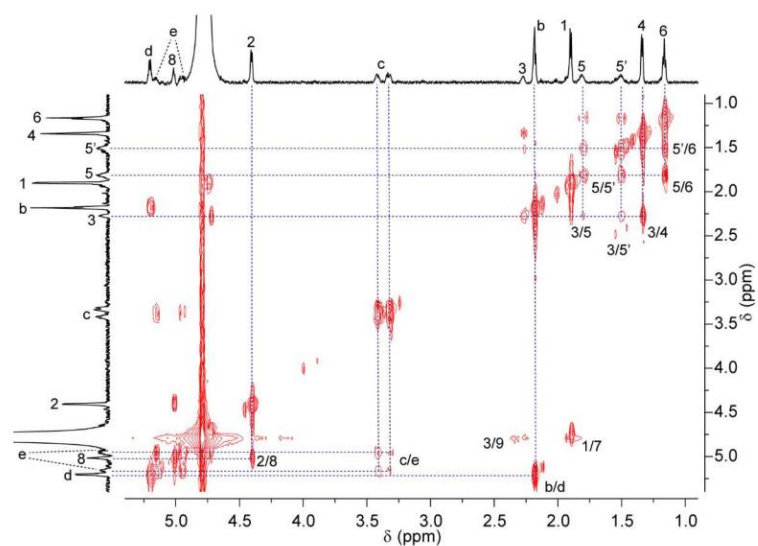
¹H NMR [700 MHz, PB (pH = 7.4, 5 mM) in D₂O, 353 K]: δ 9.07 (s, 2H), 5.20 (q, J = 6.4 Hz, 4H), 5.18 – 5.12 (m, 2H), 5.02 (t, J = 5.6 Hz, 2H), 4.98 – 4.92 (m, 2H), 4.41 (d, J = 5.7 Hz, 4H), 3.48 – 3.31 (m, 4H), 2.28 (s, 2H), 2.18 (t, J = 6.6 Hz, 6H), 1.90 (d, J = 7.3 Hz, 6H), 1.81 (s, 2H), 1.51 (s, 2H), 1.34 (d, J = 5.9 Hz, 6H), 1.16 (t, J = 6.9 Hz, 6H).

LCMS: LC retention time (min): 5.54. MS (ESI⁺, m/z): 1041.4 $[M + H]^+$, 1063.4 $[M + Na]^+$.

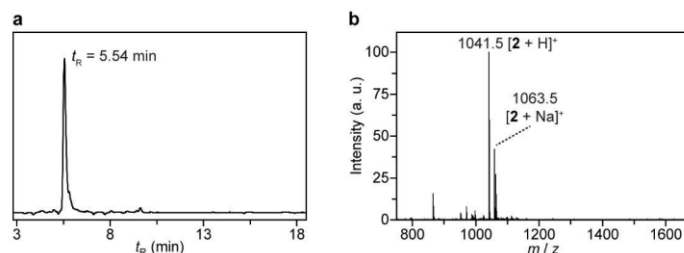
HRMS (ESI⁺, m/z): Calculated for $[M + H]^+$ (C₄₈H₆₄N₈O₁₈⁺): 1041.4411, found 1041.4440.



Supplementary Fig. 6 ^1H NMR spectrum [700 MHz, PB (pH = 7.4, 5 mM) in D_2O , 353 K] of compound **2**.

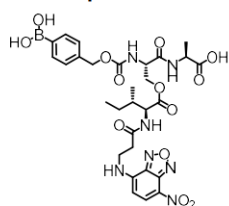


Supplementary Fig. 7 ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra [700 MHz, PB (pH = 7.4, 5 mM) in D_2O , 353 K] of compound **2**. The correlations between ^1H signals are highlighted with blue dashed lines in the COSY spectra.



Supplementary Fig. 8 LC-MS study of compound **2**. **a**, HPLC trace of **2**. Retention time (t_R) = 5.54 min. **b**, Convoluted MS spectrum for t_R = 5.26-5.90 min, showing peaks of $[M + H]^+$ and $[M + Na]^+$.

2.3 Compound 3



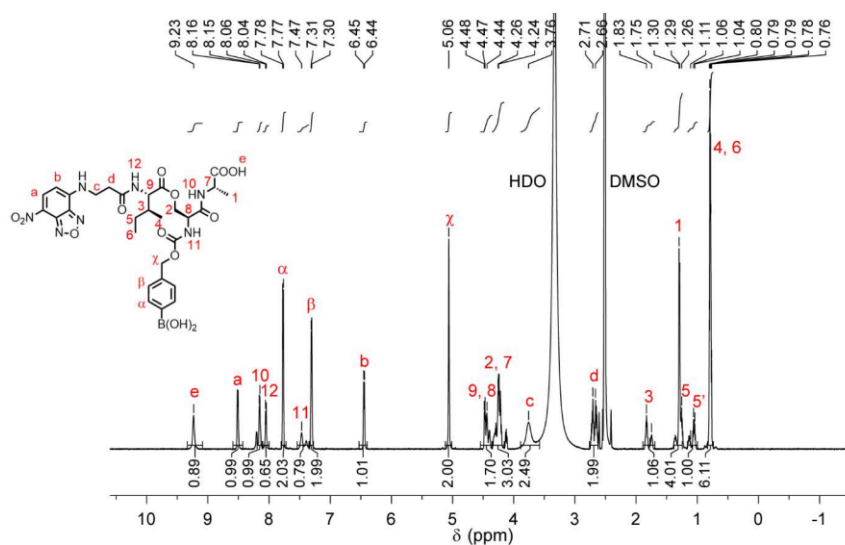
Synthesis: Compound **3** was synthesized using Merrifield's Fmoc solid-phase peptide synthesis strategy, constructing the peptide from the C-terminus to the N-terminus in a microwave-assisted peptide synthesizer at a 0.25 mmol scale. Fmoc-Ala preloaded Wang resin (0.5 mmol) was swollen in DMF for 1 h before use. First, the Fmoc group was removed by two consecutive deprotection steps (2 and 5 min) with 20% piperidine in DMF (10 mL) at 75 °C (i). After deprotection, the resin was washed four times with DMF (7 mL). Fmoc-serine (5 equiv. in 10 mL DMF) was coupled to the N-terminus by using the activator PyBOP (5 equiv. in 4 mL DMF) and activator base DIPEA (10 equiv. in 2 mL DMF) at 75 °C for 20 min (ii). After deprotection of the Fmoc protecting group (i), the N terminus was modified using 4-nitrophenyl (4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl) carbonate (1.25 equiv.) in 10 mL DMF and DIPEA (5 equiv.) overnight at room temperature (iii). After the resin was washed with DMF and DCM, Fmoc-Ile (10 equiv. in 5 mL DMF), DIC (10 equiv.) and 4-DMAP (1 equiv.) were added to form the ester bond in the serine side chain. After the reaction mixture was stirred for 12 h at room temperature, the resin was washed with DMF and DCM, dried (iv). The Fmoc group was then removed in two deprotection steps using 20% piperidine in DMF (10 min each, 5 mL) at room temperature (v). 3-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)propanoic acid (0.2 equiv.) was coupled onto the N-terminus overnight at room temperature, using HATU (5 equiv.) and DIPEA (5 equiv.) as the coupling reagents. The resin was washed with DMF and DCM, dried (vi). The product was cleaved from the resin by using 2.5 mL of a cleavage cocktail (95% CF₃COOH, 2.5% TIPS, 2.5% H₂O). This step also removed the pinacol protecting group of the PBA. After 2 hours, the cleavage cocktail was removed in vacuo (vii). The product was purified by HPLC using the Zorbax Eclipse column at a flowrate of 4 mL/min. The gradient started with 20% CH₃CN in H₂O (+0.1% CF₃COOH) and this solvent ratio was kept for 1 min, after which the CH₃CN content was increased

to 100% in 15 min. The product **3** eluted from the column after 11.8 min and was received as an orange powder after lyophilization (8 mg, 11.3% overall yield).

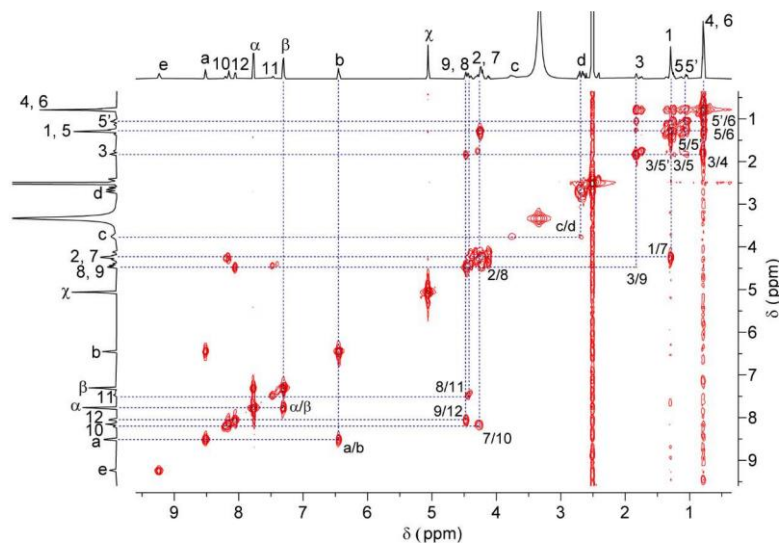
¹H NMR (700 MHz, DMSO-*d*₆, 293 K): δ 9.23 (s, 1H), 8.51 (d, *J* = 8.9 Hz, 1H), 8.15 (d, *J* = 6.3 Hz, 1H), 8.05 (d, *J* = 9.3 Hz, 1H), 7.77 (d, *J* = 8.1 Hz, 2H), 7.55 – 7.36 (m, 1H), 7.31 (d, *J* = 7.8 Hz, 2H), 6.45 (d, *J* = 9.0 Hz, 1H), 5.06 (s, 2H), 4.57 – 4.37 (m, 2H), 4.34 – 4.17 (m, 3H), 3.88 – 3.65 (m, 2H), 2.84 – 2.58 (m, 2H), 1.91 – 1.69 (m, 1H), 1.45 – 1.20 (m, 4H), 1.18 – 0.96 (m, 1H), 0.85 – 0.73 (m, 6H).

LCMS: LC retention time (min): 5.94. MS (ESI⁺, *m/z*): 702.3 [M + H]⁺, 724.3 [M + Na]⁺.

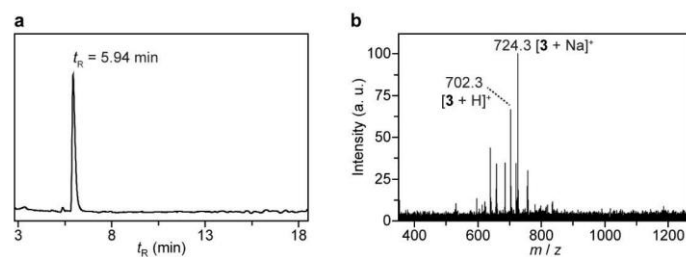
HRMS (ESI⁺, *m/z*): Calculated for [M + Na]⁺ (C₂₉H₃₆BN₇NaO₁₃)⁺: 724.2362, found 724.2328.



Supplementary Fig. 9| ¹H NMR spectrum (700 MHz, DMSO-*d*₆, 293 K) of compound **3**.

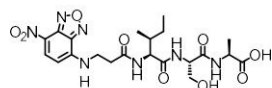


Supplementary Fig. 10 | ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra (700 MHz, $\text{DMSO}-d_6$, 293 K) of compound **3**. The correlations between ^1H signals are highlighted with blue dashed lines in the COSY spectra.



Supplementary Fig. 11 | LC-MS study of compound **3**. **a**, HPLC trace of **3**. Retention time (t_R) = 5.94 min. **b**, Convolved MS spectrum for t_R = 5.90-6.15 min, showing peaks of $[\text{M} + \text{H}]^+$ and $[\text{M} + \text{Na}]^+$.

2.4 Compound 4



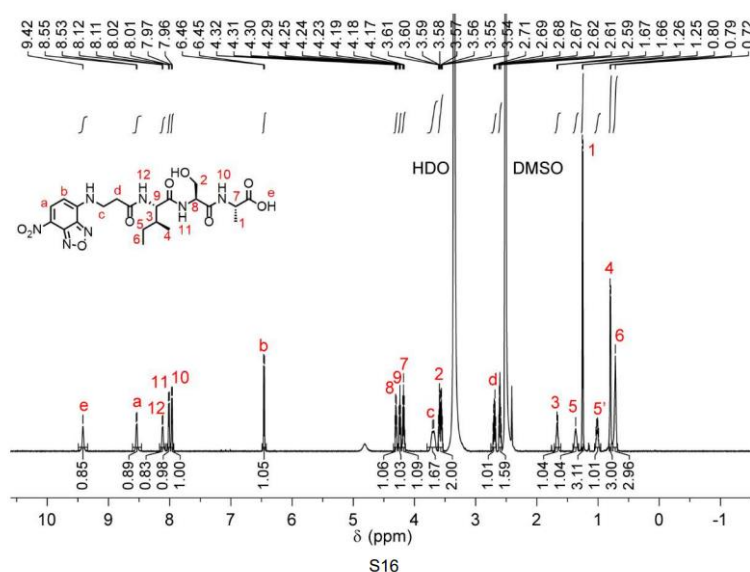
Synthesis: Compound **4** was synthesized using the Fmoc solid phase peptide synthesis strategy by Merrifield, synthesizing the peptide from C to N-terminus in a microwave assisted peptide synthesizer at a 0.25 mmol scale. Fmoc-Ala preloaded Wang resin (0.25 mmol) was swollen in DMF for 1 h before

use. After deprotection of the Fmoc group (3 mL of 20% piperidine in DMF, 2 and 5 min at 75 °C) (i), Fmoc-Ser(tBu) (ii), Fmoc-Ile (iii) were double coupled onto the peptide at 75 °C for 20 min. 5 equiv. of the compounds were used in 2.5 mL DMF. PyBOP (5 equiv. in 1 mL DMF) and DIPEA (10 equiv. in 0.5 mL) were used for the coupling reaction. After deprotection of the Fmoc group (3 mL of 20% piperidine in DMF, 2 and 5 min at 75 °C) (i), 0.2 equiv. of 3-((7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino)propanoic acid were coupled onto the peptide at room temperature overnight (iv). PyBOP (5 equiv. in 1 mL DMF) and DIPEA (10 equiv. in 0.5 mL) were used for the coupling reaction. The product was cleaved from the resin by using 2.5 mL of a cleavage cocktail (95% CF₃COOH, 2.5% TIPS, 2.5% H₂O). The product was purified by HPLC using the Zorbax Eclipse column at a flowrate of 4 mL/min. The gradient started with 20% CH₃CN in H₂O (+0.1% CF₃COOH) and this solvent ratio was kept for 1 min, after which the CH₃CN content was increased to 100% in 15 min. The compound eluted from the column after 5.5 min and was received as an orange powder after lyophilization (8.3 mg, 31.7% overall yield).

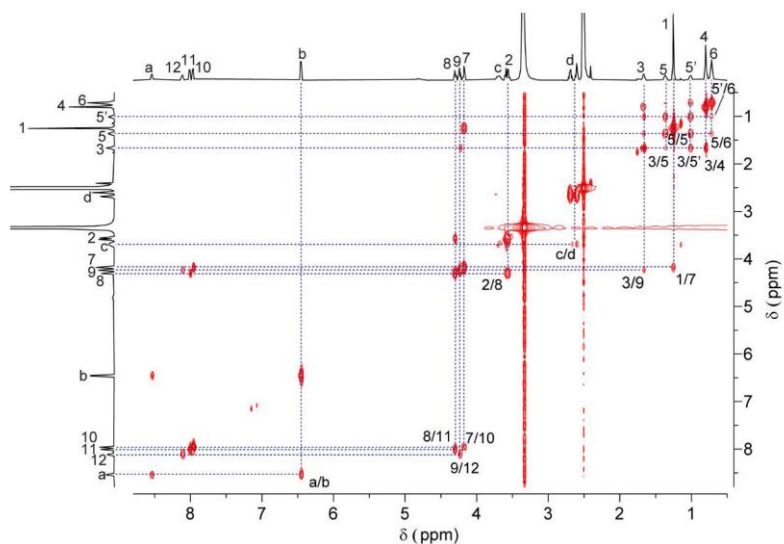
¹H NMR (700 MHz, DMSO-*d*₆, 293 K): δ 9.42 (s, 1H), 8.54 (d, *J* = 8.2 Hz, 1H), 8.12 (d, *J* = 7.5 Hz, 1H), 8.01 (d, *J* = 8.0 Hz, 1H), 7.96 (d, *J* = 6.9 Hz, 1H), 6.46 (d, *J* = 9.1 Hz, 1H), 4.30 (q, *J* = 6.6 Hz, 1H), 4.24 (t, *J* = 7.9 Hz, 1H), 4.18 (p, *J* = 7.3 Hz, 1H), 3.72 – 3.67 (m, 2H), 3.62 – 3.53 (m, 2H), 2.69 – 2.57 (m, 2H), 1.70 – 1.64 (m, 1H), 1.42 – 1.32 (m, 1H), 1.25 (d, *J* = 7.2 Hz, 3H), 1.05 – 0.98 (m, 1H), 0.80 (d, *J* = 6.8 Hz, 3H), 0.74 – 0.69 (m, 3H). [700 MHz, PB (pH = 7.4, 5 mM) in D₂O, 353 K]: δ 8.58 (d, *J* = 9.1 Hz, 1H), 6.44 (d, *J* = 9.2 Hz, 1H), 4.34 (t, *J* = 5.8 Hz, 1H), 4.12 – 4.06 (m, 2H), 3.98 – 3.83 (m, 2H), 3.78 (d, *J* = 5.6 Hz, 2H), 2.84 – 2.69 (m, 2H), 1.72 – 1.66 (m, 1H), 1.29 (d, *J* = 7.2 Hz, 3H), 1.27 – 1.20 (m, 1H), 1.00 – 0.89 (m, 1H), 0.77 (d, *J* = 6.8 Hz, 3H), 0.66 (t, *J* = 7.5 Hz, 3H).

LCMS: LC retention time (min): 4.78. MS (ESI⁺, *m/z*): 524.2 [M + H]⁺, 1047.5 [2M + H]⁺.

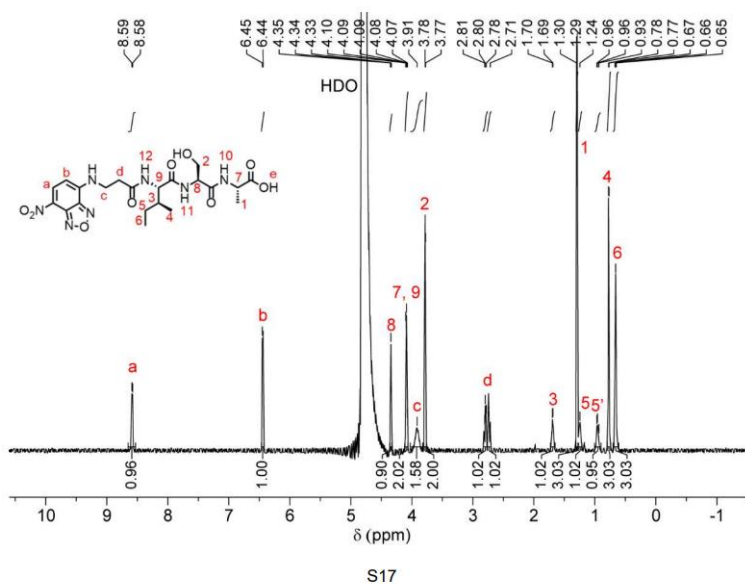
HRMS (ESI⁺, *m/z*): Calculated for [M + H]⁺ (C₂₁H₃₀N₇O₉): 524.2105, found 524.2106.



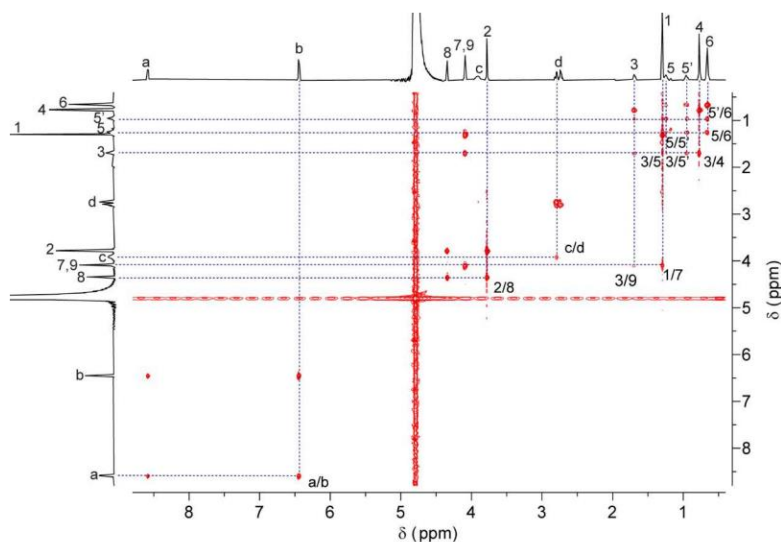
Supplementary Fig. 12 | ^1H NMR spectrum (700 MHz, $\text{DMSO}-d_6$, 293 K) of compound **4**.



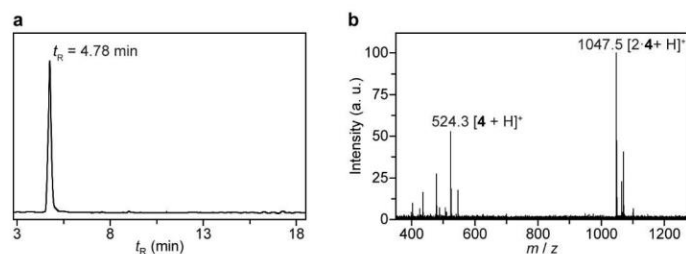
Supplementary Fig. 13 | ^1H NMR and ^1H , ^1H COSY NMR spectra (700 MHz, $\text{DMSO}-d_6$, 293 K) of compound **4**. The correlations between ^1H signals are highlighted with blue dashed lines in the COSY spectra.



Supplementary Fig. 14 ^1H NMR spectrum [700 MHz, PB (pH = 7.4, 50 mM) in D_2O , 293 K] of compound **4** (500 μM). Well-defined signals with high intensity were obtained, suggesting no aggregation of compound **4** under this condition.



Supplementary Fig. 15 ^1H NMR and $^1\text{H},^1\text{H}$ COSY NMR spectra [700 MHz, PB (pH = 7.4, 50 mM) in D_2O , 293 K] of compound **4** (500 μM). The correlations between ^1H signals are highlighted with blue dashed lines in the COSY spectra. Well-defined signals with high intensity were obtained, suggesting no aggregation of compound **4** under this condition.

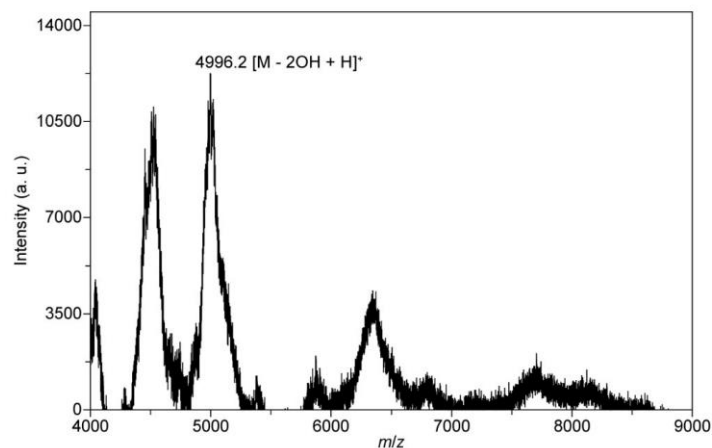


Supplementary Fig. 16 LC-MS study of compound **4**. **a**, HPLC trace of **4**. Retention time (t_R) = 4.78 min. **b**, Convoluted MS spectrum for t_R = 4.75-4.95 min, showing peaks of $[\text{M} + \text{H}]^+$ and $[2\text{M} + \text{H}]^+$.

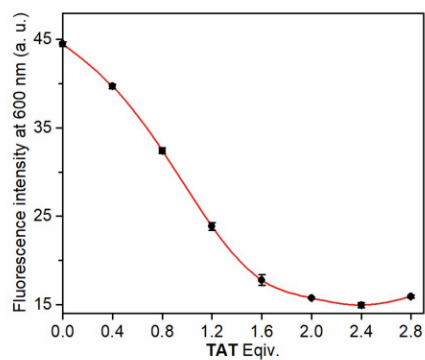
2.5 Compound 1-TAT/3TAT

Compounds **1-TAT**/ **3-TAT** were obtained by mixing compound **1/3** and SHA-TAT in DPBS solution at a 1:2 volume ratio for **1-TAT** and a 1:1 volume ratio for **3-TAT**, respectively. The resulting compounds were used directly in subsequent experiments without further purification.

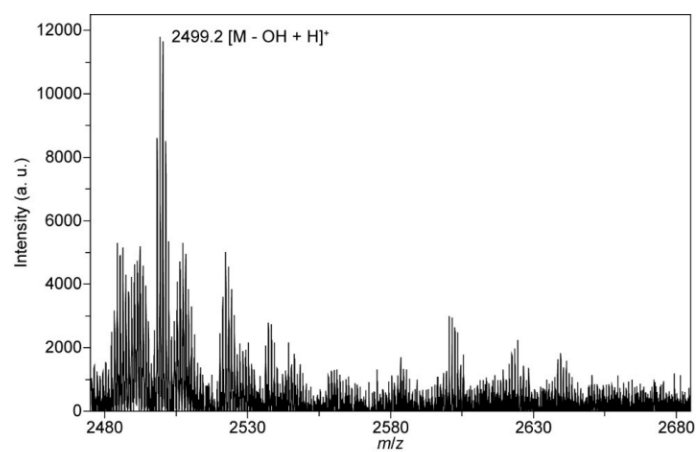
To confirm the formation of the TAT-functionalized compounds by PBA-SHA dynamic covalent interaction, an Alizarin Red S assay was conducted in addition to the MALDI-TOF-MS analysis. A mixture containing phenylboronic acid compounds (50 μM for **1** and 100 μM for **3**, respectively), TAT-SHA (0 to 140 μM , 20 μM per increment), and Alizarin Red S (250 μM) was prepared in DPBS and incubated in an orbital shaker for 30 minutes. The fluorescence of the mixture (λ_{ex} = 495 nm, λ_{em} = 600 nm) was recorded and plotted as a function of the stoichiometric ratio between TAT-SHA and the phenylboronic acid-containing compound. The endpoint of the titration is indicated when no further changes in emission are observed upon the addition of more **SHA-TAT**.



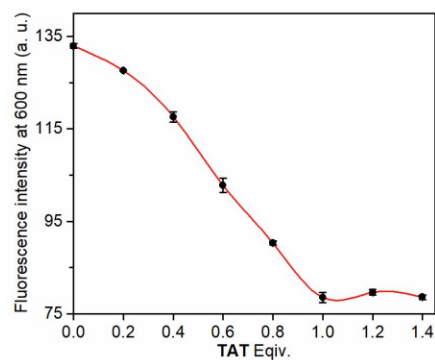
Supplementary Fig. 17| MALDI-TOF-MS spectrum for 1-TAT. Peak at m/z = 4996.2 can be identified, correspond to $[M - 2OH + H]^+$.



Supplementary Fig. 18| Fluorescence titration curves displaying the stoichiometric substitution of the fluorogenic Alizarin Red S/boronic acid complex on compound **1** by **SHA-TAT**. The endpoint of the titration (2.0 equiv.) corresponds to the results obtained through MALDI-TOF-MS analysis (Supplementary Fig. 17).



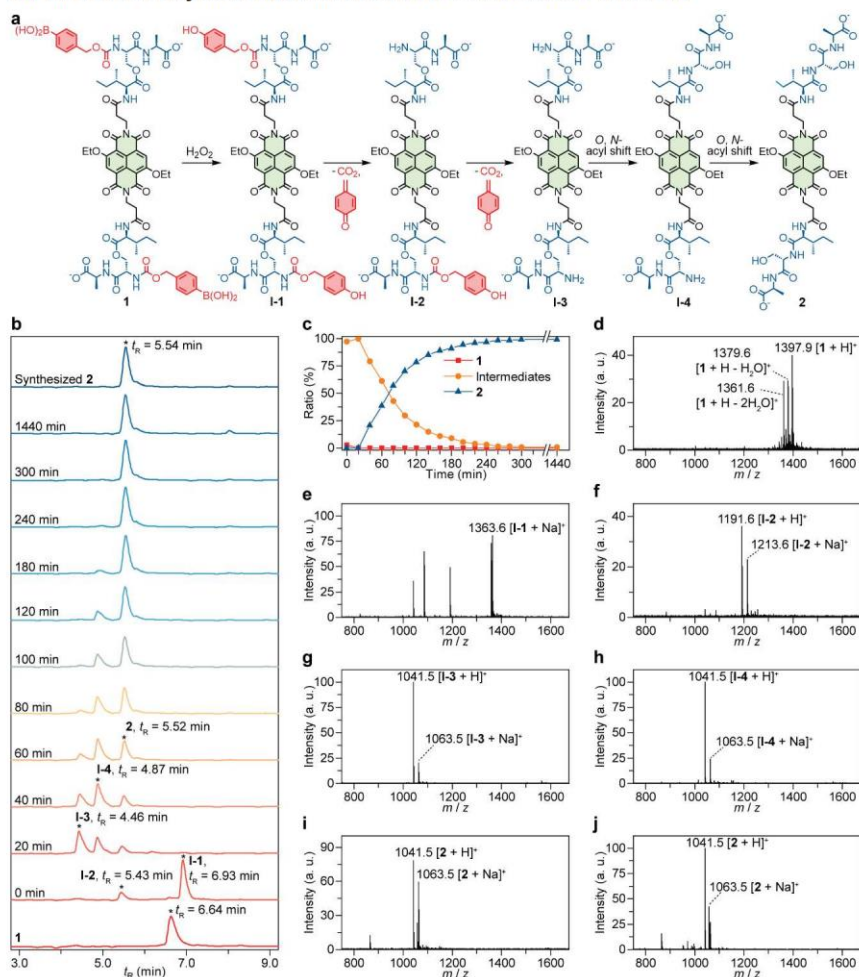
Supplementary Fig. 19| MALDI-TOF-MS spectrum for **3-TAT**. Peak at $m/z = 2499.2$ can be identified, correspond to $[M - OH + H]^+$.



Supplementary Fig. 20] Fluorescence titration curves displaying the stoichiometric substitution of the fluorogenic Alizarin Red S/boronic acid complex on compound **3** by **SHA-TAT**. The endpoint of the titration (1.0 equiv.) corresponds to the results obtained through MALDI-TOF-MS analysis (Supplementary Fig. 19).

3 H₂O₂-induced formation of 2

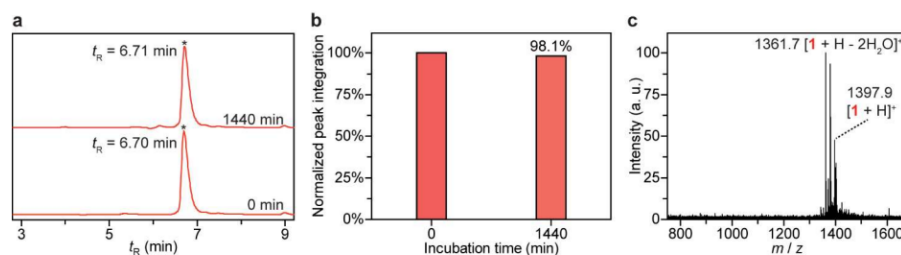
3.1 HPLC-MS analysis of H₂O₂-induced conversion from 1/1-TAT to 2.



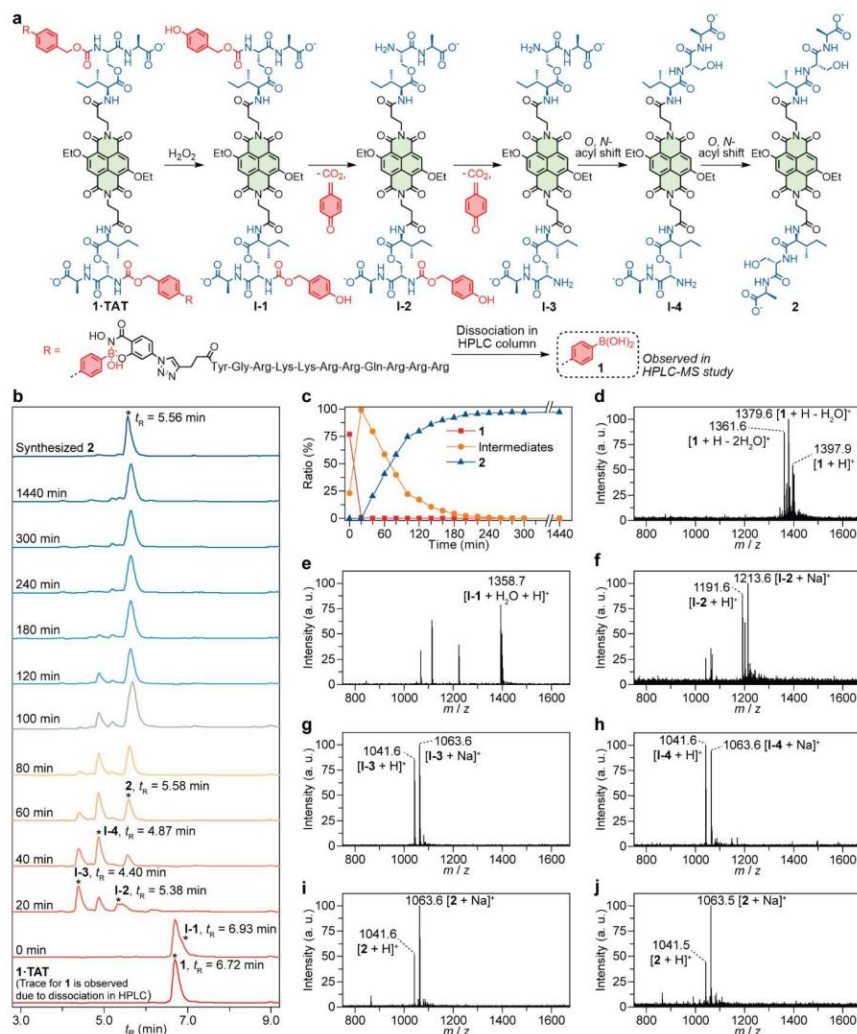
Supplementary Fig. 21| H₂O₂-induced conversion of 1 to 2. **a**, Reaction scheme for the conversion. The oxidation of the phenylboronic acid by H₂O₂ leads to the formation of intermediate **I-1**, followed by the self-immolative cleavage of the carbamate linker to generate **I-2** and **I-3**. The isopeptide bond then undergoes *O*, *N*-acyl shift to afford **I-4** and finally compound **2**. **b**, HPLC spectra for H₂O₂ (1.0 mM) induced-conversion of **1** (50 μ M) to **2** in a mixture of NH₄HCO₃ buffer (pH 7.4, 10 mM) and THF (v:v = 1:1). **c**, Molar ratio of precursor **1**, deboronation and rearrangement intermediates, and final product **2** after incubating with H₂O₂ based on the peak integration at 254 nm. Deboronation reaction was completed within 20 min of incubation, followed by *O*, *N*-acyl shift that afford **2** in >95% conversion rate within 240 min of incubation. **d-j**, Convolved MS spectra for compound **1** ($t_R = 6.64$ min) before the addition of H₂O₂ (**d**), a reaction time of 0 min and $t_R = 6.93$ min (**e**, identified as compound

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I-1), a reaction time of 0 min and $t_R = 5.43$ min (**f**, identified as compound **I-2**), a reaction time of 20 min and $t_R = 4.46$ min (**g**, identified as compound **I-3**), a reaction time of 40 min and $t_R = 4.87$ min (**h**, identified as compound **I-4**), a reaction time of 60 min and $t_R = 5.52$ min (**i**, identified as compound **2**), and compound **2** obtained by synthesis ($t_R = 5.54$ min) (**j**). The key information of this figure is presented in the main manuscript as Figs. 2b-d.



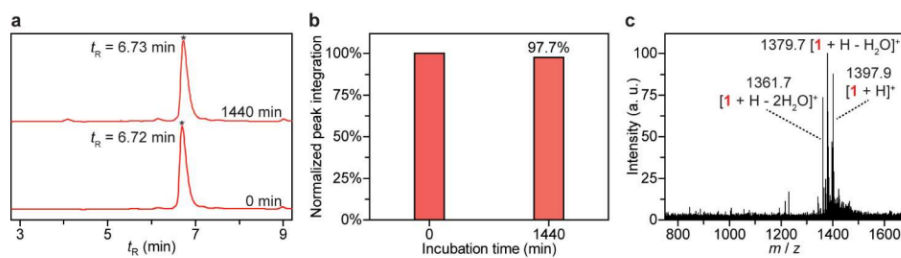
Supplementary Fig. 22| Stability of compound 1. **a**, Comparison of HPLC spectra of compound **1** (50 μ M) incubated in a mixture of NH_4HCO_3 buffer (pH 7.4, 20 mM) and tetrahydrofuran ($v:v = 1:1$) for 0 and 1440 min. **b**, Normalized peak integration of the HPLC traces of compound **1** before and after the incubation for 1440 min, which revealed that 98.1% of the compound **1** remained in the mixture. **c**, Convoluted MS spectrum of $t_R = 6.71$ min after incubation for 1440 min. The peak was identified as compound **1**.



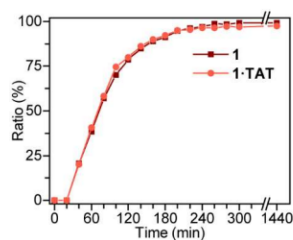
Supplementary Fig. 23] H_2O_2 -induced conversion of 1-TAT to 2. **a**, Reaction scheme for the conversion. The oxidation of the phenylboronic acid by H_2O_2 leads to the formation of intermediate I-1, followed by the self-immolation of the carbamate linker to generate I-2 and I-3. The isopeptide bond then undergoes O, N-acyl shift to afford I-4 and finally compound 2. **b**, HPLC spectra for H_2O_2 (1.0 mM) induced-conversion of 1-TAT (50 μM) to 2 in a mixture of NH_4HCO_3 buffer (pH 7.4, 20 mM) and THF ($v:v = 1:1$). **c**, Molar ratio of precursor 1, deboronation and rearrangement intermediates, and 2 after incubating with H_2O_2 based on the peak integration at 254 nm. Deboronation reaction was completed within 20 min of incubation, followed by O, N-acyl shift that afford 2 in >95% conversion rate within 240 min of incubation. **d-j**, Convolved MS spectra in the HPLC-MS analysis for compound 1 ($t_R = 6.72$ min) before addition of H_2O_2 (d), a reaction time of 0 min and $t_R =$

S24

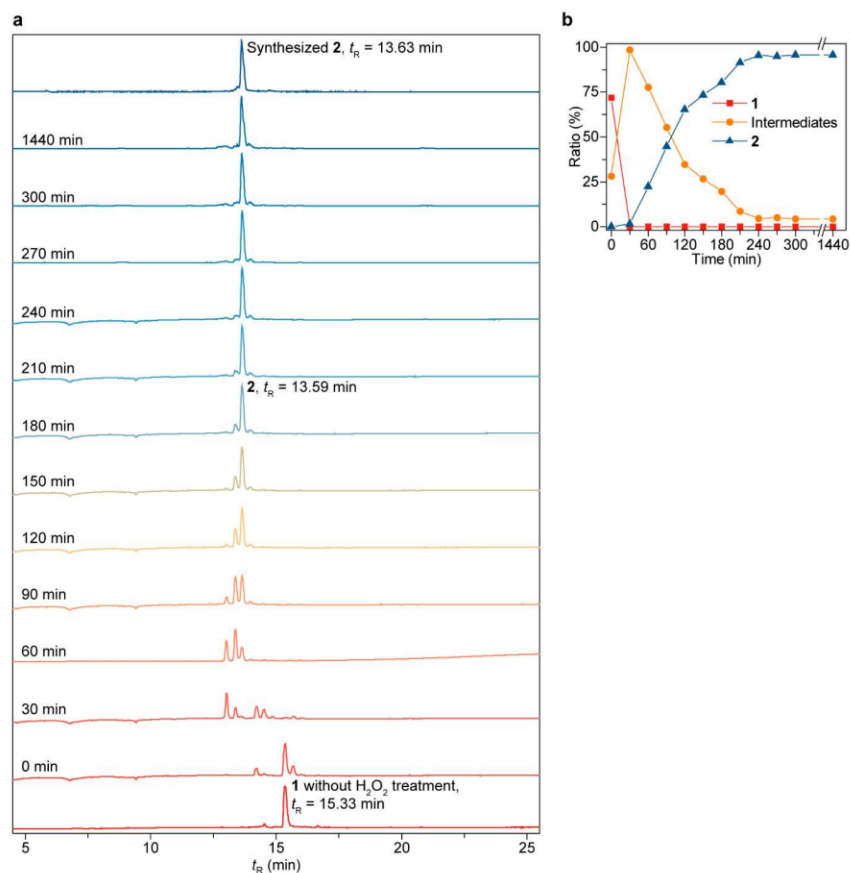
6.93 min (e, identified as compound **I-1**), a reaction time of 20 min and $t_R = 5.38$ min (f, identified as compound **I-2**), a reaction time of 20 min and $t_R = 4.40$ min (g, identified as compound **I-3**), a reaction time of 40 min and $t_R = 4.87$ min (h, identified as compound **I-4**), a reaction time of 60 min and $t_R = 5.58$ min (i), identified as compound **2**), and compound **2** obtained by synthesis ($t_R = 5.56$ min) (j). As the boronic acid-SHA dynamic covalent interaction dissociates in the acidic mobile phase of the HPLC, only trace correspond to **1** can be observed after the injection of **1**·TAT to the HPLC.



Supplementary Fig. 24| Stability of compound **1·TAT.** a, Comparison of HPLC spectra of compound **1**·TAT (50 μ M) incubated in a mixture of NH_4HCO_3 buffer (pH 7.4, 20 mM) and tetrahydrofuran ($v:v = 1:1$) for 0 and 1440 min. b, Normalized peak integration of the HPLC traces of compound **1** before and after the incubation for 1440 min, which revealed that 97.7% of the compound **1**·TAT remained in the mixture. c, Convolved MS spectrum of $t_R = 6.73$ min after incubation for 1440 min. The peak was identified as compound **1**. As the boronic acid-SHA dynamic covalent interaction dissociates in the acidic mobile phase of the HPLC, only trace correspond to **1** can be observed after the injection of **1**·TAT to the HPLC.

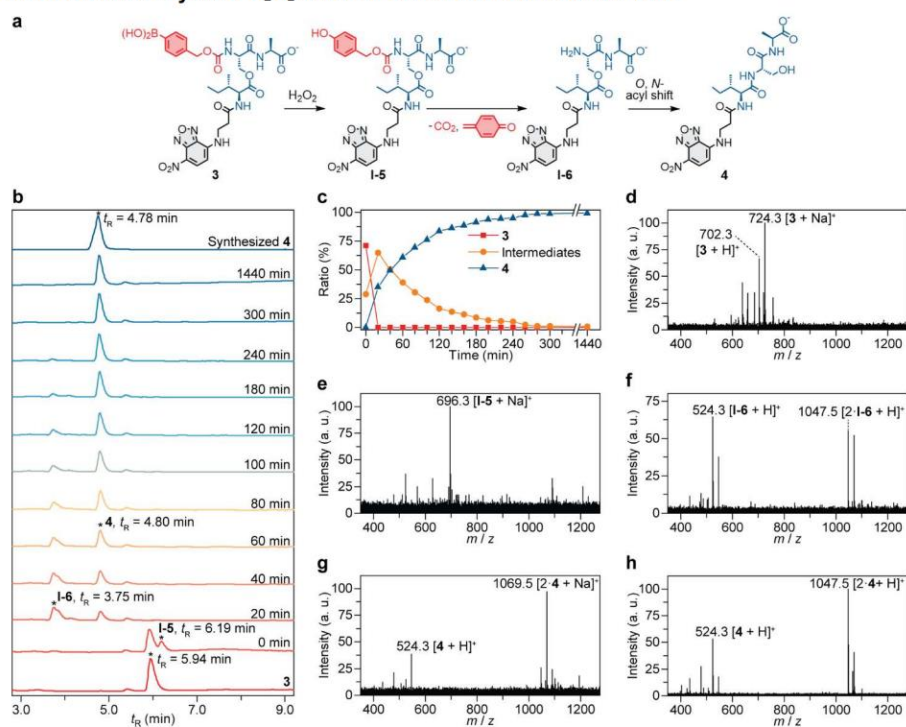


Supplementary Fig. 25| Comparison of conversion rates of final compound **2 generated by H_2O_2 -induced conversion of **1** and **1**·TAT based on the peak integration at 254 nm in the corresponding HPLC spectra.**

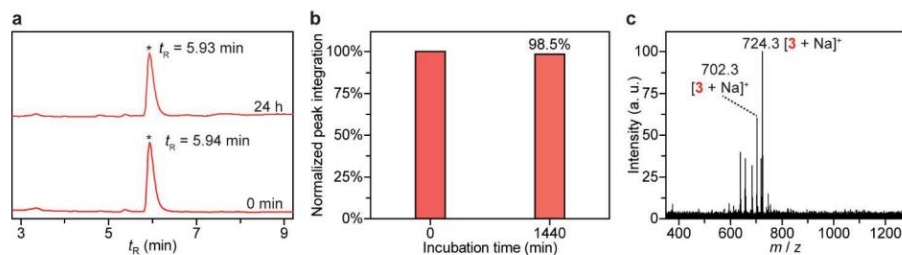


Supplementary Fig. 26| HPLC kinetics analysis of conversion from 1 to 2 at intracellular H_2O_2 concentration. **a**, HPLC spectra for H_2O_2 (2.0 μM) induced-conversion of **1** (1.8 μM) to **2** in a mixture of NH_4HCO_3 buffer (pH 7.4, 20 mM) and tetrahydrofuran ($v:v = 1:1$). **b**, Molar ratio of precursor **1**, deboronation and rearrangement intermediates intermediates, and final linearized compound **2** after incubating with H_2O_2 based on the peak integration at 254 nm. Deboronation reaction was completed within 20 min of incubation, followed by *O*, *N*-acyl shift that afford **2** in >95% conversion rate within 240 min of incubation, in agreement with the HPLC-MS analysis on conversion of **1** at higher concentrations.

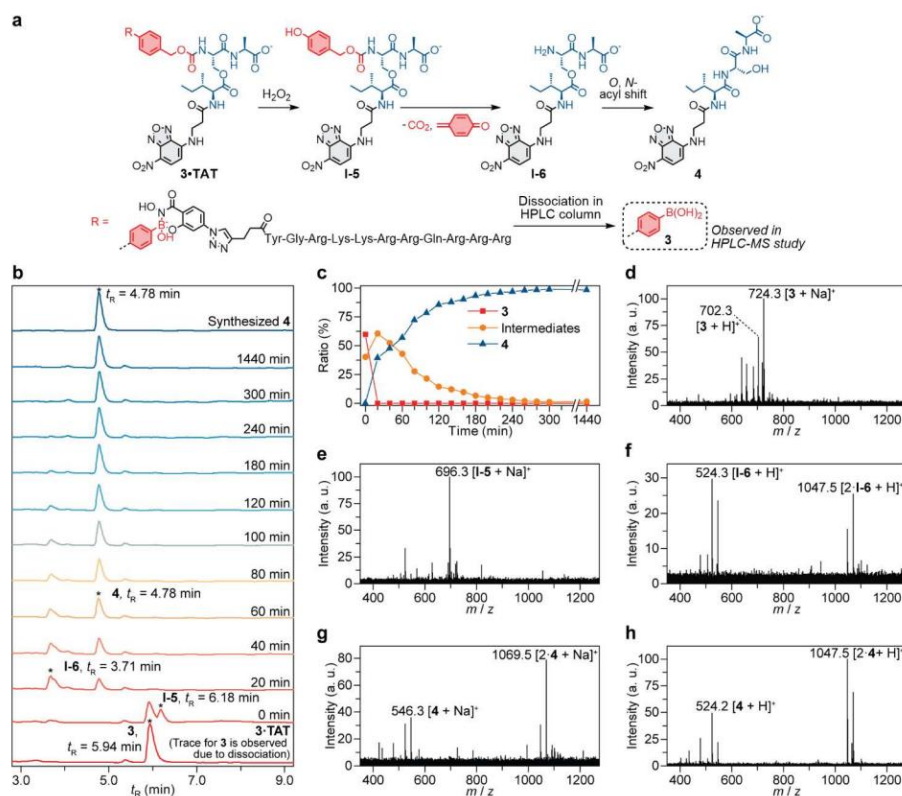
3.2 HPLC-MS analysis of H₂O₂-induced conversion from 3/3-TAT to 4.



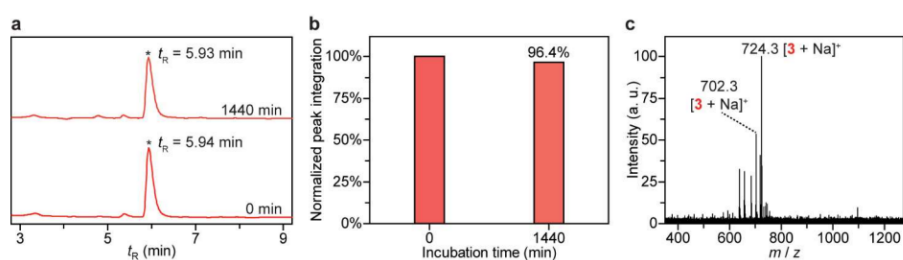
Supplementary Fig. 27| HPLC-MS kinetics analysis for H₂O₂ induced conversion from 3 to 4. **a**, Reaction scheme for the conversion. The oxidation of the phenylboronic acid by H₂O₂ leads to the formation of intermediate **I-5**, followed by the self-immolation of the carbamate linker to generate **I-6**. The isopeptide bond then undergoes *O, N*-acyl shift to afford compound **4**. **b**, HPLC spectra for H₂O₂ (1.0 mM) induced-conversion of **3** (100 μ M) to **4** in a mixture of NH₄HCO₃ buffer (pH 7.4, 20 mM) and tetrahydrofuran (v:v = 1:1). **c**, Molar ratio of precursor **3**, intermediates, and final product **4** after incubating with H₂O₂ based on the peak integration at 254 nm. Deboronation reaction was completed within 20 min of incubation, followed by *O, N*-acyl shift that afford **4** in >95% conversion rate within 240 min of incubation. **d–h**, Convolved MS spectra in the HPLC-MS analysis for compound **3** (t_R = 5.94 min) before addition of H₂O₂ (**d**), a reaction time of 20 min and t_R = 6.19 min (**e**, identified as compound **I-5**), a reaction time of 20 min and t_R = 3.75 min (**f**, identified as compound **I-6**), a reaction time of 40 min and t_R = 4.80 min (**g**, identified as compound **4**), and compound **4** obtained by synthesis (t_R = 4.78 min) (**h**).



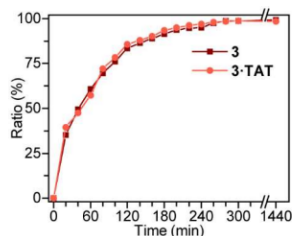
Supplementary Fig. 28 | Stability of compound 3. **a**, Comparison of HPLC spectra of compound **3** (100 μ M) incubated in a mixture of NH_4HCO_3 buffer (pH 7.4, 20 mM) and tetrahydrofuran ($v:v = 1:1$) for 0 and 1440 min. **b**, Normalized peak integration of the HPLC traces of compound **3** before and after the incubation for 1440 min, which revealed that 98.5% of the compound **3** remained in the mixture. **c**, Convoluted MS spectrum of $t_R = 5.93$ min after incubation for 1440 min. The peak was identified as compound **3**.



Supplementary Fig. 29| HPLC-MS kinetics analysis for H₂O₂ induced conversion from 3·TAT to 4. **a**, Reaction scheme for the conversion. The oxidation of the phenylboronic acid by H₂O₂ leads to the formation of intermediate **I-5**, followed by the self-immolation of the carbamate linker to generate **I-6**. The isopeptide bond then undergo *O*, *N*-acyl shift to afford compound **4**. **b**, HPLC spectra for H₂O₂ (1.0 mM) induced-conversion of **3·TAT** (100 μM) to **4** in a mixture of NH₄HCO₃ buffer (pH 7.4, 20 mM) and tetrahydrofuran (v:v = 1:1). **c**, Molar ratio of precursor **3**, intermediates and final product **4** after incubating with H₂O₂ based on the peak integration at 254 nm. Deboronation reaction was completed within 20 min of incubation, followed by *O*, *N*-acyl shift that afford **4** in >95% conversion rate within 240 min of incubation. **d–h**, Convolved MS spectra in the HPLC-MS analysis for compound **3** (*t_R* = 5.94 min) before addition of H₂O₂ (**d**), a reaction time of 20 min and *t_R* = 6.18 min (**e**, identified as compound **I-5**), a reaction time of 20 min and *t_R* = 3.71 min (**f**, identified as compound **I-6**), a reaction time of 40 min and *t_R* = 4.78 min (**g**, identified as compound **4**), and compound **4** obtained by synthesis (*t_R* = 4.78 min) (**h**). As the boronic acid-SHA dynamic covalent interaction dissociates in the acidic mobile phase of the HPLC, only trace correspond to **3** can be observed after the injection of **3·TAT** to the HPLC.

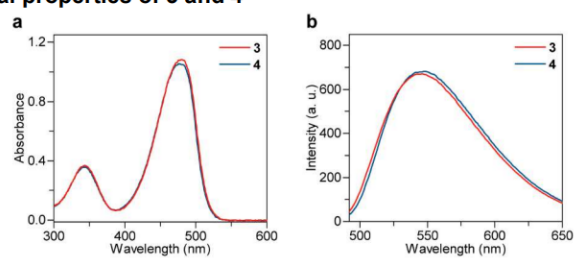


Supplementary Fig. 30| Stability of compound 3·TAT. **a**, Comparison of HPLC spectra of compound **3·TAT** (100 μM) incubated in a mixture of NH₄HCO₃ buffer (pH 7.4, 20 mM) and tetrahydrofuran (v:v = 1:1) for 0 and 1440 min. **b**, Normalized peak integration of the HPLC traces of compound **3** before and after the incubation for 1440 min, which revealed that 96.4% of the compound **3·TAT** remained in the mixture. **c**, Convolved MS spectrum of *t_R* = 5.93 min after incubation for 1440 min. The peak was identified as compound **3**. As the boronic acid-SHA dynamic covalent interaction dissociates in the acidic mobile phase of the HPLC, only trace correspond to **3** can be observed after the injection of **3·TAT** to the HPLC.



Supplementary Fig. 31| Comparison of conversion rates of final compound 4 generated by H₂O₂-induced conversion of **3 and **3·TAT** based on the peak integration at 254 nm in the corresponding HPLC spectra.**

4. Photophysical properties of **3** and **4**

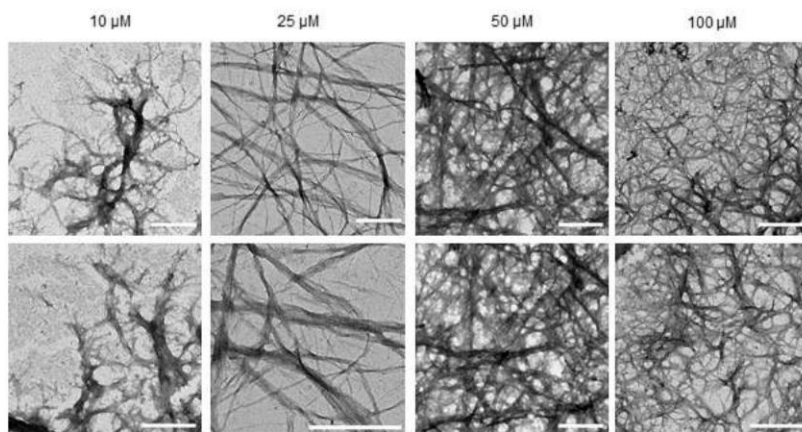


Supplementary Fig. 32| Absorption and fluorescence emission profiles of compounds **3 and **4**.** **a**, UV-Vis absorption spectra of **3** and **4** (25 μ M) in phosphate buffer (PB) (pH 7.4, 50 mM). **b**, Fluorescence emission spectra of **3** and **4** (25 μ M) in PB (pH 7.4, 50 mM). Excitation wavelength = 468 nm.

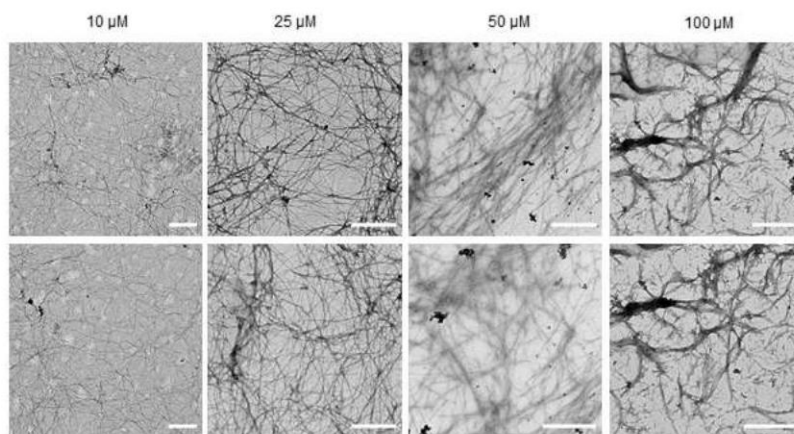
5. Self-assembly profiles of **2_{NF}**

Monomeric complex **2** was dissolved in DMSO at a high concentration (10 mM) before diluted to different concentrations using PB (pH 7.4, 50 mM). Self-assembly formation of **2_{NF}** was induced by agitating the solution in an Eppendorf Thermomixer for 2 h at 37 °C at 500 r.p.m.

5.1 TEM analysis of the self-assemblies

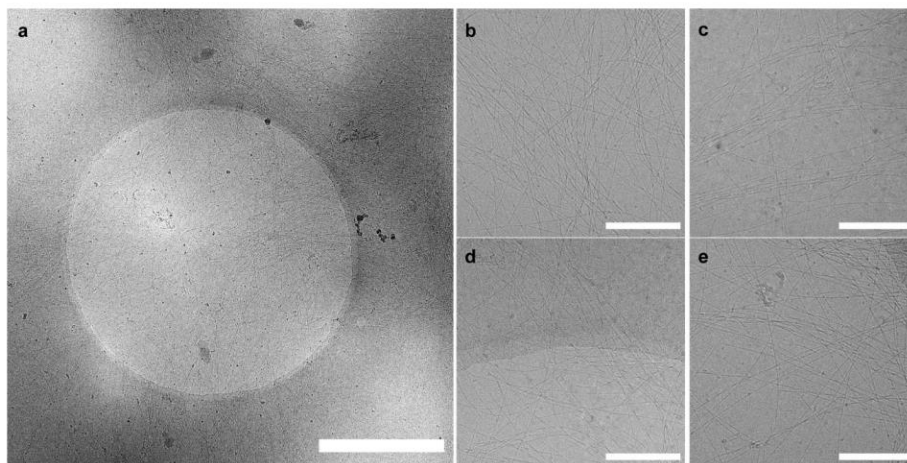


Supplementary Fig. 33 | TEM micrographs of the nanofiber. Compound **2** at various concentrations was dissolved in DPBS and incubated for 10 h, before drop-casted on a carbon grid and imaged using TEM. Scale bars, 500 nm.



S31

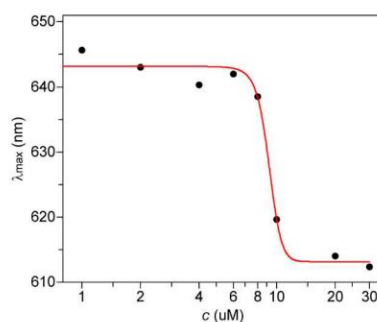
Supplementary Fig. 34 TEM micrographs of the nanofiber form upon H₂O₂-induced conversion of **1**. **1** and H₂O₂ (10 equiv.) at various concentrations were incubated in DPBS for 10 hours. The reaction mixture was then drop-casted on a carbon grid and imaged using TEM. Scale bars, 500 nm.



Supplementary Fig. 35 Cryo TEM micrographs of the nanofibers. Complex **2** at 100 μ M was dissolved in DPBS and incubated for 1 h, Scale bars, **a**, 500 nm; **b-e**, 100 nm.

5.2 Determination of critical aggregation concentration (CAC)

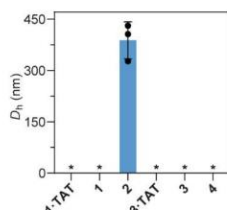
The critical aggregation of compound **2** was determined using Nile Red dye (9-diethylamino-5-benzo[α]-phenoxazinone), a well-known fluorogenic sensor for peptide aggregation.³ Nile Red and compound **2** were mixed in DPBS. The concentration of Nile Red was maintained at 250 nM, while the concentrations of compound **2** were varied. The maximum emission wavelengths (λ_{max}) of the mixture, originating from the Nile Red dye ($\lambda_{\text{ex}} = 550$ nm), were recorded. The λ_{max} values were plotted as a function of the concentration of compound **2**, and the CAC values corresponded to the lowest concentration at which a blueshift was observed.



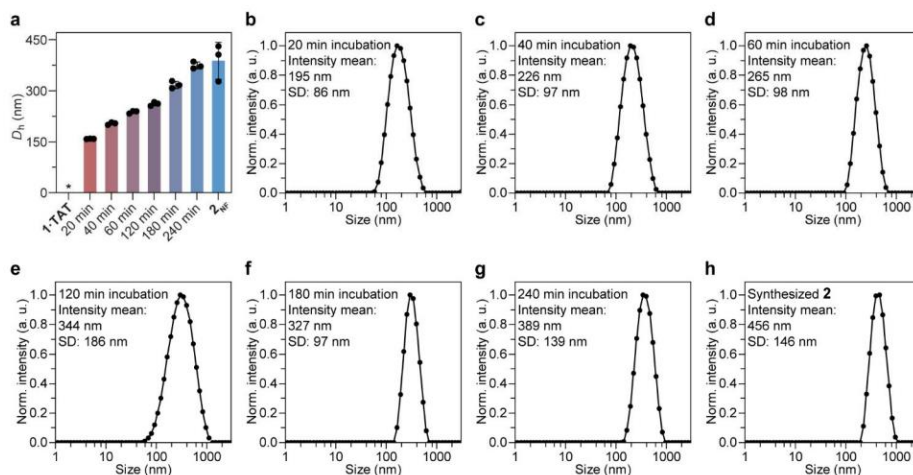
S32

Supplementary Fig. 36| Critical aggregation concentration (CAC) study of compound 2. The shifts in maximum emission wavelength (λ_{max}) as a function of concentration of **2** were plotted, and the CAC value ($\sim 9 \mu\text{M}$) represented the lowest concentrations at which a redshift was observed. The maximum represents the average of three measurements.

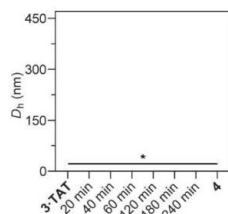
5.3 DLS analysis



Supplementary Fig. 37| DLS analysis of the synthesised compounds at $25 \mu\text{M}$ in DPBS. a. 1-TAT. b. 1. c. 3-TAT. d. 3. Data are presented as mean $\pm$ s.d., $n = 3$. * The count rate was below detection limits for analysis, suggesting that there were no nanosized aggregates present in the solution.

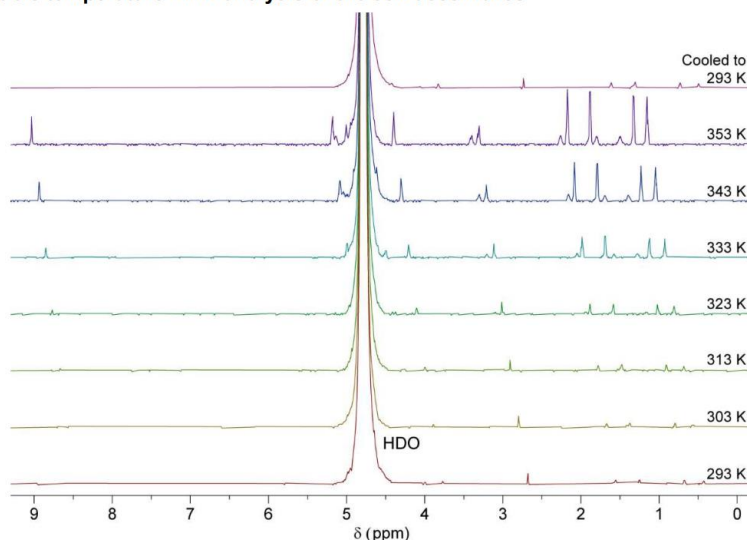


Supplementary Fig. 38| Time-dependent DLS assay of 1-TAT ($25 \mu\text{M}$) with H_2O_2 (0.5 mM) in DPBS. a. Hydrodynamic diameter (D_h) of the solution of 1-TAT before and after incubation with H_2O_2 for varying amount of time, and solution of synthesized compound **2**. Data are presented as mean $\pm$ s.d., $n = 3$. *The count rate was too low for analysis, suggesting that there were no nanosized aggregates present in the solution. b-h, Intensity mean results obtained through the DLS analysis.

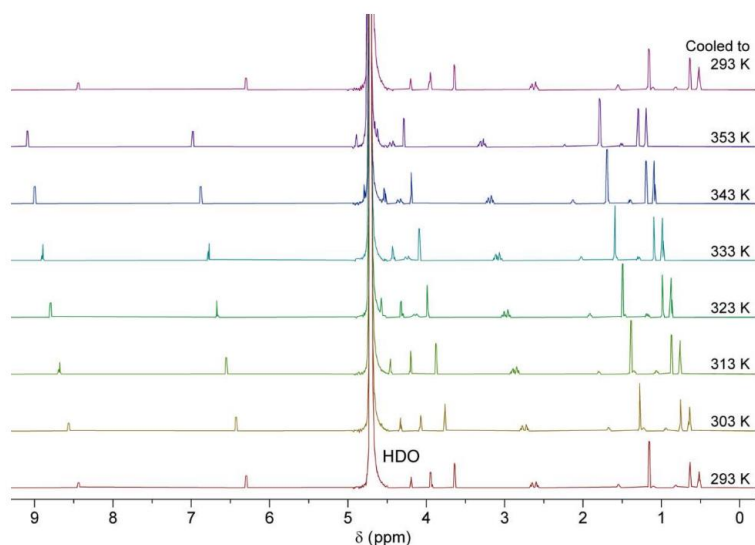


Supplementary Fig. 39| Time-dependent DLS assay of 3-TAT (25 μ M) with H_2O_2 (0.5 mM) in DPBS. Hydrodynamic diameter (D_h) of the solution of 3-TAT before and after incubation with H_2O_2 for varying amount of time, and solution of synthesized compound 4. *The count rate was too low for analysis, suggesting that there were no nanosized aggregates present in the solution.

5.4 Variable temperature NMR analysis of the self-assemblies



Supplementary Fig. 40| Variable temperature 1H NMR spectra [700 MHz, PB (pH = 7.4, 50 mM) in D_2O] of compound 2 (250 μ M). At 293 K, broad overlapping signals are observed in both aromatic and aliphatic regions, suggesting a strong self-assembly tendency of compound 2. This phenomenon is driven by a combination of π - π interactions originating from the cNDI core, as well as hydrophobic interactions and hydrogen bond interactions arising from the peptide, resulting in the formation of nanofibers 2_{NF}. Increasing the temperature of the measurement results in the sharpening of the signals and increased signal intensity, which is indicative of disassembly of the nanostructures. Cooling the sample back to 293 K led to the recovery of broad overlapping signals, suggesting the reversible nature of the self-assembly.

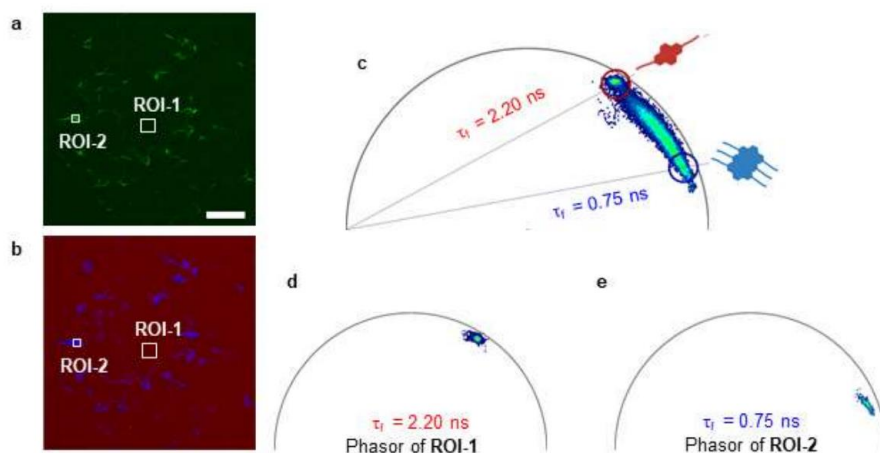


Supplementary Fig. 41| Variable temperature ^1H NMR spectra [700 MHz, PB (pH = 7.4, 50 mM) in D_2O] of compound **4 (500 μM).** At 293 K, well-resolved peaks are observed for the sample that allowed for detailed peak assignment. Heating the sample up to 353K resulted in minimal changes in peak intensity, indicating no self-assembly at 293K.

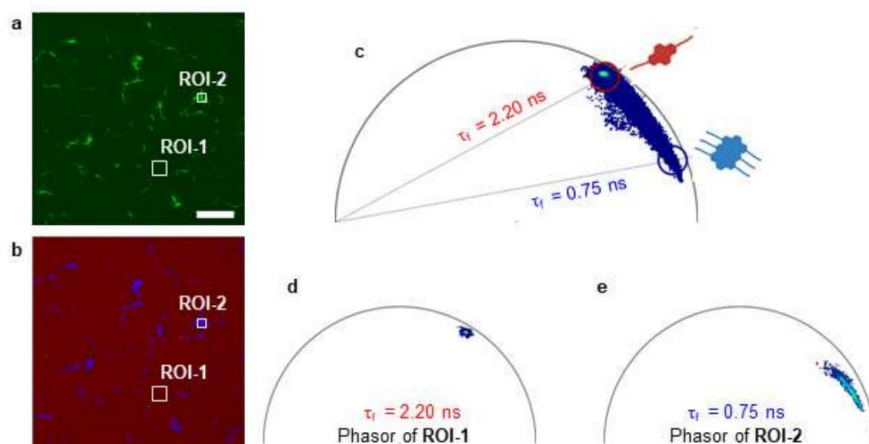
5.5 Fluorescence lifetime imaging of assembled fibers in a Cell-Free system

After **2** was dissolved in DMSO at a concentration of 10 mM, the stock solution was diluted to 120 μM with Dulbecco's Modified Eagle Medium (DMEM). After 2 h incubation with shaking at room temperature, the solution was added into an 8-well confocal plate. The samples were imaged using phasor-resolved fluorescence lifetime imaging microscopy (phasor-FLIM). Three different regions were imaged and analyzed (Fig. 3f-k and Supplementary Figs. 42-43).

For the kinetics analysis for the H_2O_2 -induced conversion of **1-TAT** to **2_{NF}**. A mixture of **1-TAT** (120 μM) and H_2O_2 (1.2 mM) was prepared using DMEM. The mixture was added into an 8-well confocal plate and imaged using phasor-resolved fluorescence lifetime imaging microscopy (phasor-FLIM). Imaging parameters are identical to those found in point **1.2.13**.

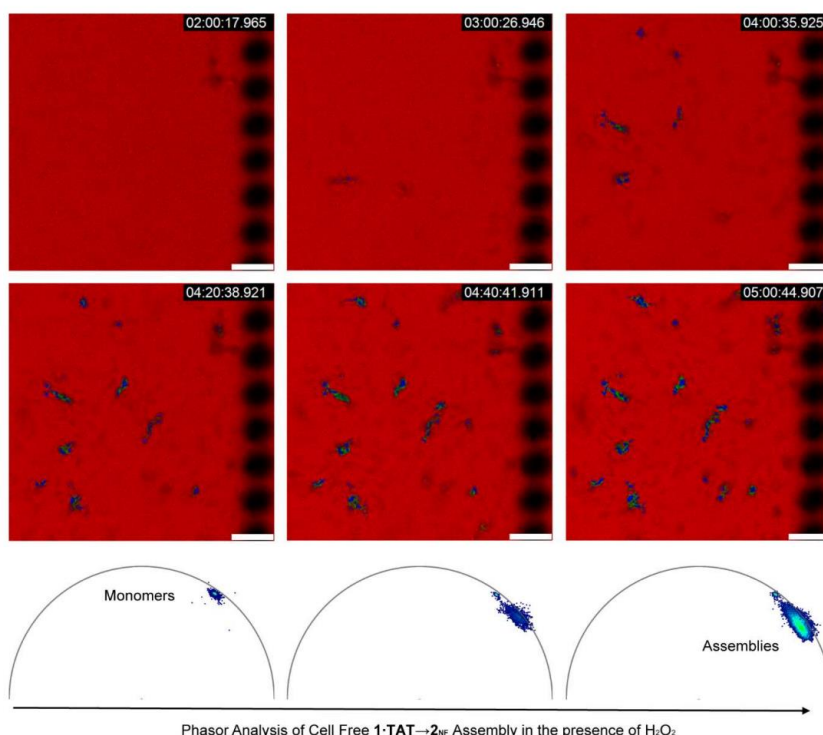


Supplementary Fig. 42| **a**, Intensity-based confocal image for 2_{NF} in a cell-free condition. $\lambda_{ex} = 469$ nm, $\lambda_{em} = 500-520$ nm. Scale bar, 20 μ m. **b**, Corresponding fluorescence lifetime image for 2_{NF} . Red channel indicates species with a $\tau_f = 2.20$ ns, and blue channel indicates species with a $\tau_f = 0.75$ ns. **c**, Phasor plots of ROI-1 (monomers) in DMEM, $\tau_f = 2.20$ ns. **d**, Phasor plots of ROI-2 (assemblies) in DMEM, $\tau_f = 0.75$ ns. **h**, Fluorescence lifetime analysis of monomers and assemblies in cell free system using phasor analysis. Two major species showing $\tau_f = 2.20$ and 0.75 ns, respectively, can be identified.



Supplementary Fig. 43| **a**, Intensity-based confocal image for 2_{NF} in a cell-free condition. $\lambda_{ex} = 469$ nm, $\lambda_{em} = 500-520$ nm. Scale bar, 20 μ m. **b**, Corresponding fluorescence lifetime image for 2_{NF} . Red channel indicates species with a $\tau_f = 2.20$ ns, and blue channel indicates species with a $\tau_f = 0.75$ ns. **c**, Phasor plots of ROI-1 (monomers) in DMEM, $\tau_f = 2.20$ ns. **d**, Phasor plots of ROI-2 (assemblies) in DMEM, $\tau_f = 0.75$ ns. **h**,

Fluorescence lifetime analysis of monomers and assemblies in cell free system using phasor analysis. Two major species showing $\tau_f = 2.20$ and 0.75 ns, respectively, can be identified.



Supplementary Fig. 44 Time lapsed snapshots and phasor analysis of **1-TAT**→**2-NF** in the presence of H_2O_2 in cell free system. **Red**: monomers, **Blue**: assemblies. Nanofibers **2-NF** can be identified after 3h incubation with H_2O_2 . Scale bars: 20 μm .

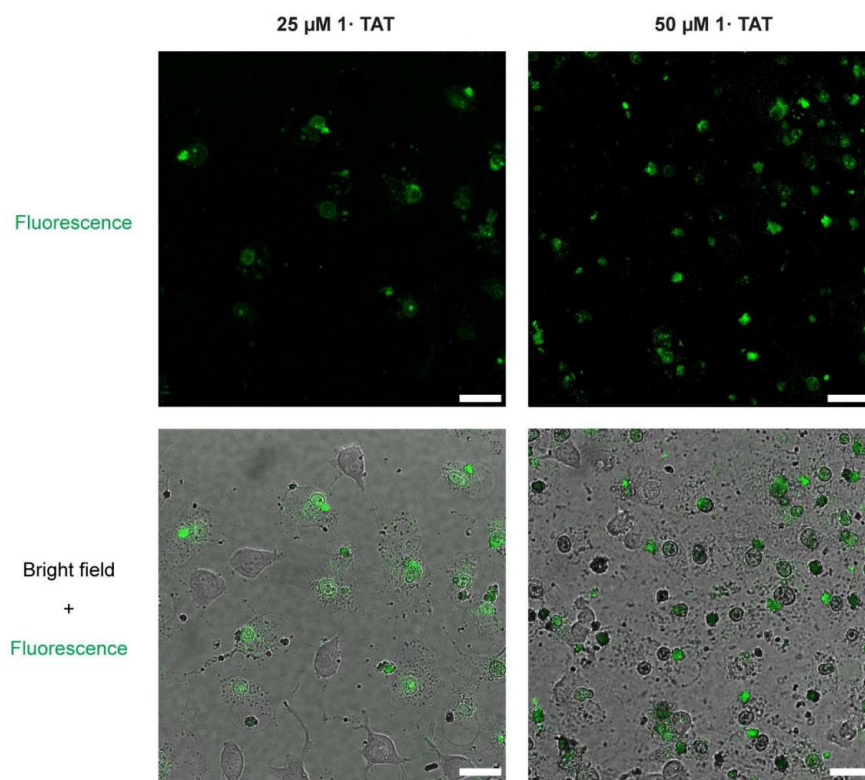
6 Cellular uptake of **1-TAT** and **3-TAT** and intracellular self-assembly of **1-TAT**

6.1 Cellular uptake of **1-TAT**

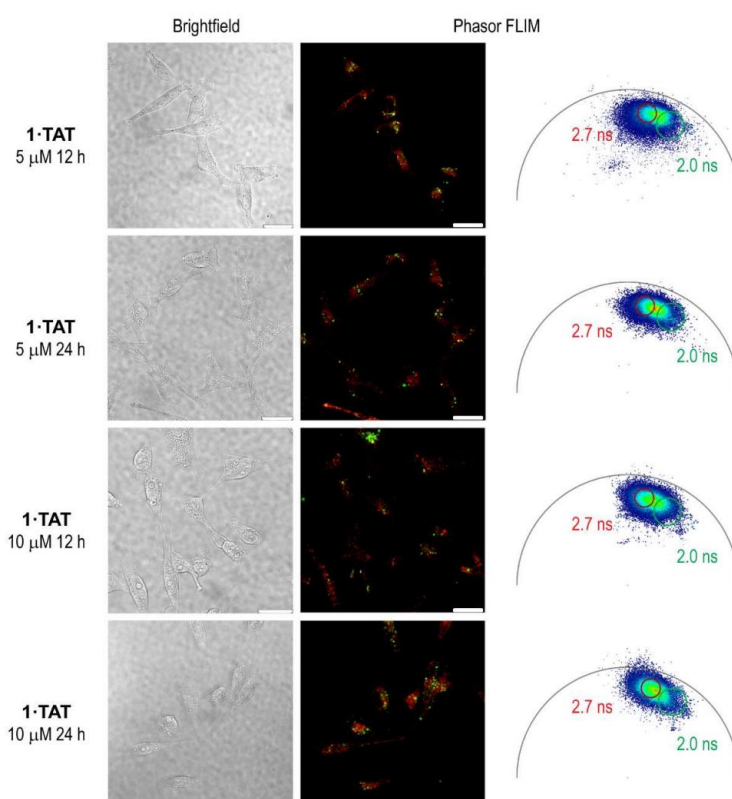
MDA-MB-231 cells were seeded at a density of 30,000 cells/well in an 8-well confocal plate. After adhering for 24 h, cells were treated with the sample for 4 h at 37 °C. Before adding to the cells, samples were pre-incubated to form the **1-TAT** by dissolving **1** in DMSO and mixing with two equimolar amount of **TAT** peptide, which was dissolved in Dulbecco's PBS. The sample was further diluted with DPBS to a final volume of 40 μL (DPBS:DMSO 9:1). Sample solutions were further diluted 1:4 with DMEM and added to the cells (total DMSO content = 2%), after removing the existing medium from the wells. After the incubation time was over, the cell mitochondria was stained with MitoTracker[®] Deep Red for 30 min at 37 °C and the cell nucleus was stained with HCS NuclearMask[™] Stains for

S37

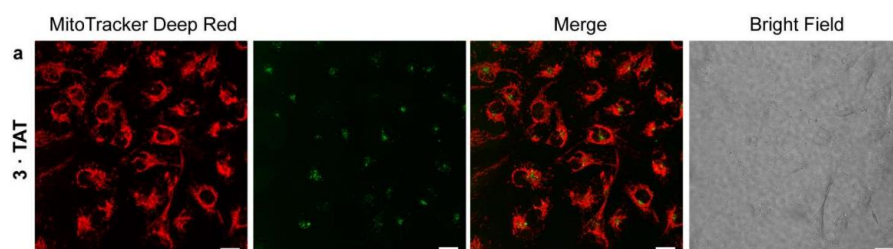
20 min at 37 °C. The staining solution was removed, and fresh FluoroBrite DMEM Medium was added to the cells before they were imaged by confocal laser scanning microscopy.



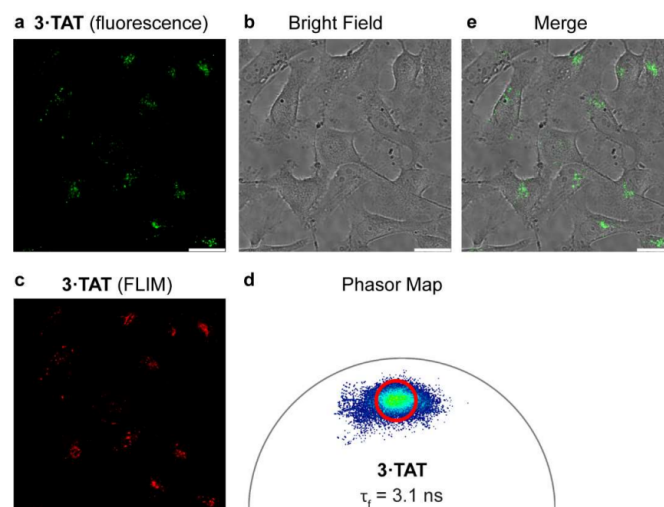
Supplementary Fig. 45 CLSM images of MDA-MB-231 cells treated for 4 h with 1-TAT (25 μM and 50 μM). Scale bars, 20 μm.



Supplementary Fig. 46| Phasor-FLIM analysis of MDA-MB-231 cells treated with **1-TAT** (5 μ M and 10 μ M) for 12 h and 24 h. Scale bars, 20 μ m.



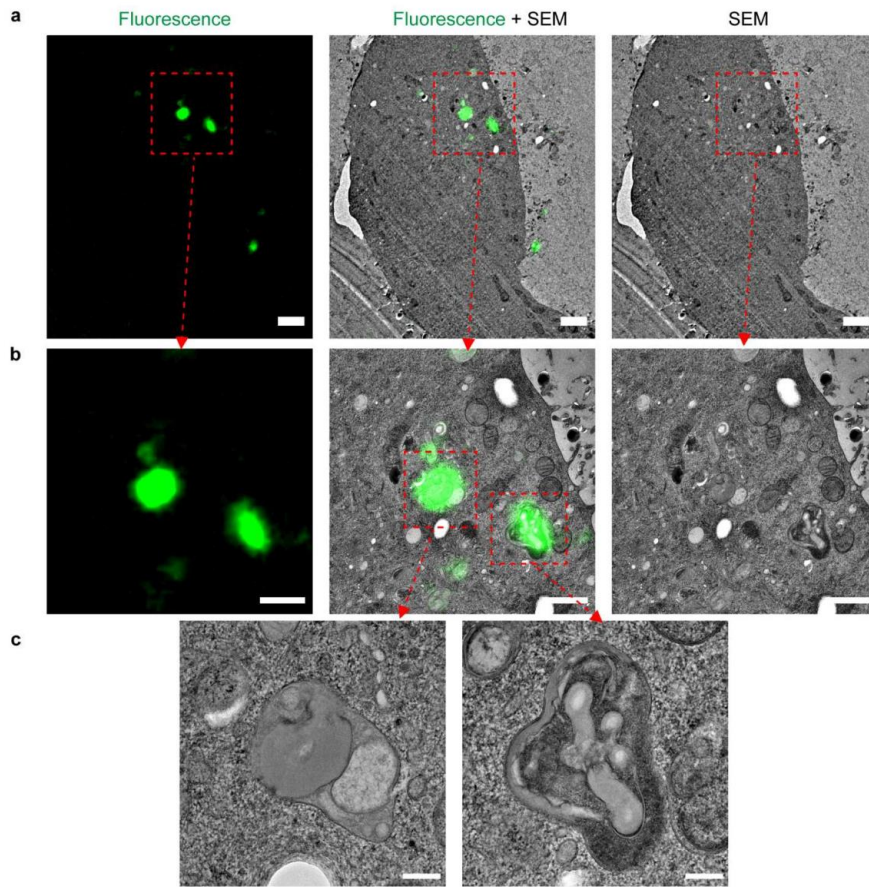
Supplementary Fig. 47| a CLSM images of MDA-MB-231 cells treated for 4 h with **3-TAT** (25 μ M) and MitoTracker® Deep Red, Scale bars, 20 μ m.



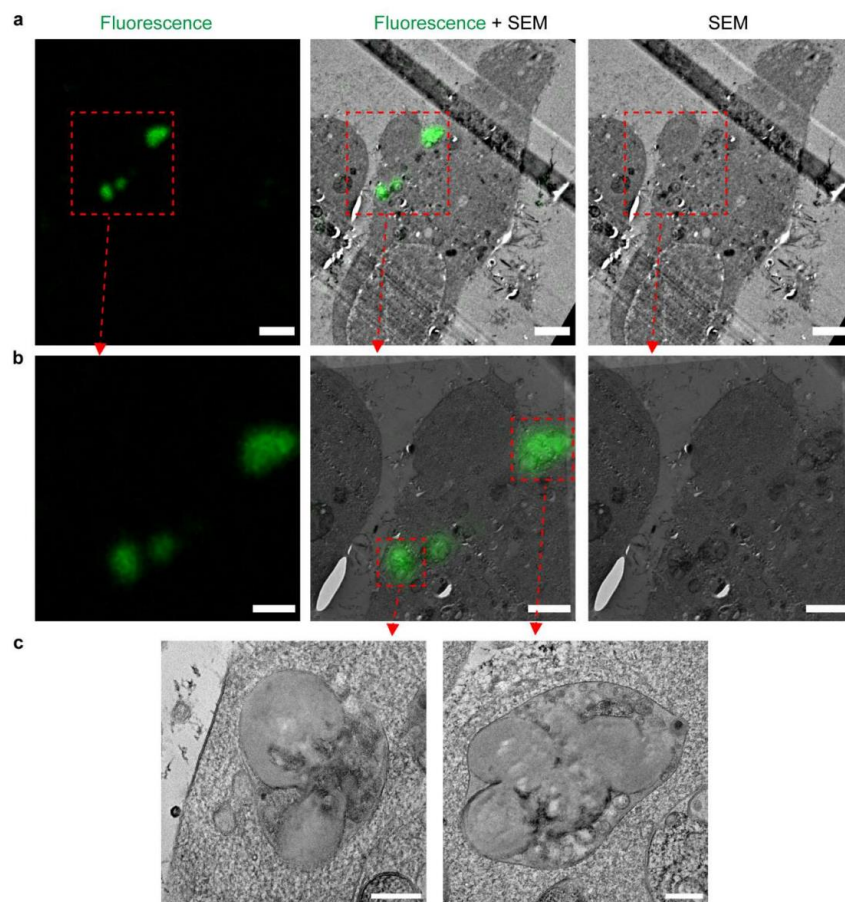
Supplementary Fig. 48 | Phasor-FLIM analysis of MDA-MB-231 cells treated with 3-TAT (25 μ M) for 4 h. Scale bars, 20 μ m.

6.2 Electron microscopy and correlative light- and electronmicroscopy of cells

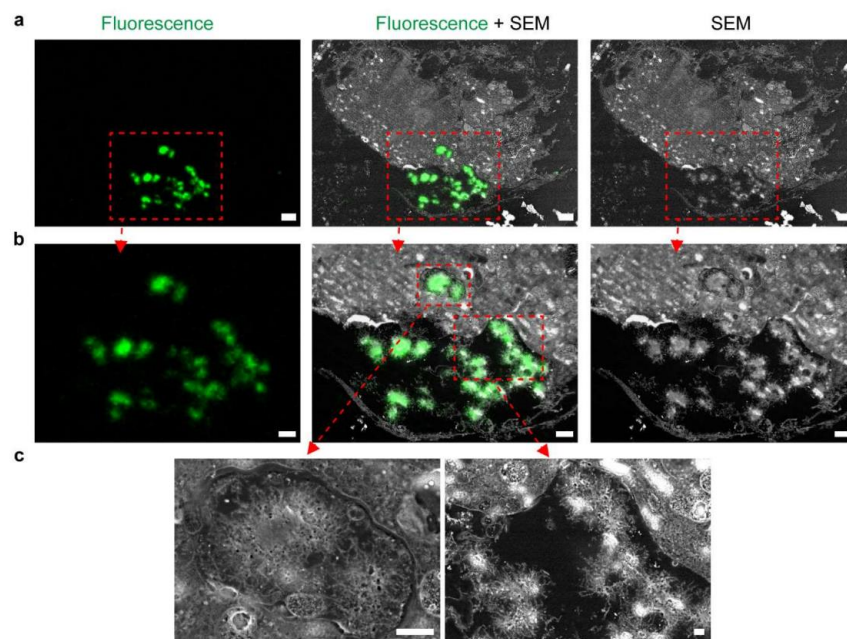
For electron microscopy (EM) studies and correlation with confocal light microscopy, MDA-MB-231 cells were seeded at a density of approximately 30000 cells/well on sapphire disks and incubated with **1-TAT** as described above. Staining with NucBlue® Live ReadyProbes® was not performed. After incubation, cells on the sapphire discs were frozen using high-pressure freezing (Wohlwendt HPF 01). This was followed by freeze substitution, in which the cells were stained at low temperatures with OsO₄ and uranyl acetate for electron microscopic examination. Then, the cells were embedded in epoxy resin (EPON) and finally, ultrathin sections were prepared in an ultramicrotome (Leica FC7) using a diamond knife (Diatome). The thickness of the thin sections and the substrate onto which the sections were transferred were adjusted to meet the requirements of the subsequent EM investigation. For TEM and TEM tomography, the sections were transferred to Cu grids. The nominal section thickness for TEM investigations was 100 nm and for TEM tomography 300 nm. For SEM investigations, the nominal section thickness was 100 nm, and the sections were transferred to ITO-coated glass coverslips for this purpose. To identify the **1-TAT** component in the EM images, we correlated its fluorescence with the EM images (correlative light and electron microscopy - CLEM). For this purpose, the fluorescence of the **1-TAT** was first measured in a confocal laser scanning microscope (Leica SP5) at a wavelength of 500-550 nm. Then, the same thin section was examined in TEM (FEI Tecnai F20) or SEM (Hitachi SU8000), and the fluorescence image was precisely superimposed on the electron microscopy image using ImageJ plugin 1.53e.



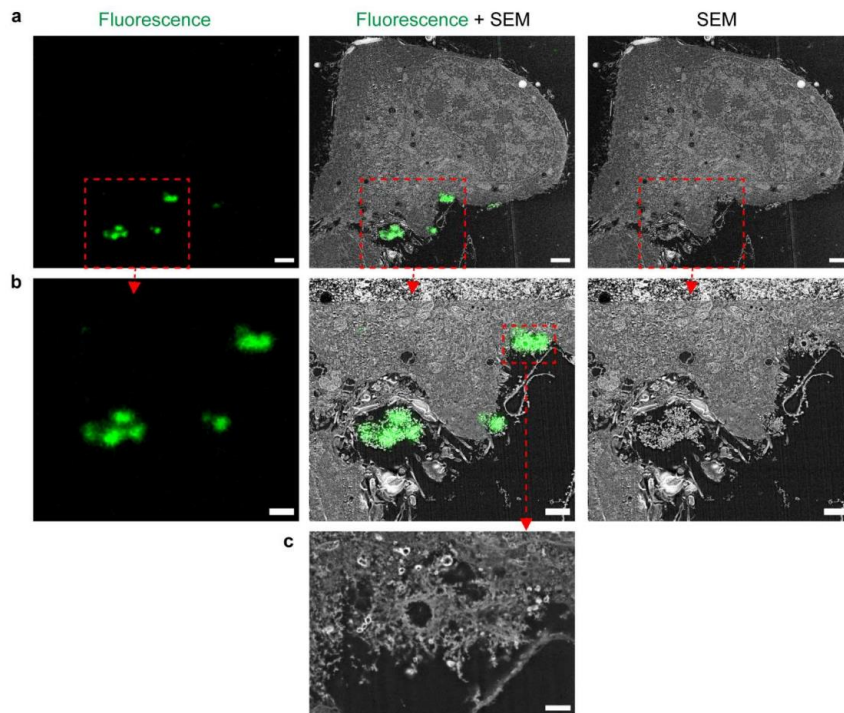
Supplementary Fig. 49| SEM-CLEM micrographs of intracellular localization within the endosomes of 1-TAT. Endosomes containing 1-TAT are significantly distorted compared to surrounding vesicles. Magnified images show the presence of multiple phase separated sub-compartments and protofibrils. **a**, Scale bars, 2 μm . **b**, Scale bars, 1 μm . **c**, Scale bars, 200 nm.



Supplementary Fig. 50 SEM – CLEM micrographs of intracellular localization within the endosomes of 1-TAT. Endosomes containing 1-TAT are significantly distorted compared to surrounding vesicles. Magnified images show the presence of multiple phase separated sub-compartments and protofibrils. **a**, Scale bars, 2 µm. **b**, Scale bars, 1 µm. **c**, Scale bars, 200 nm.



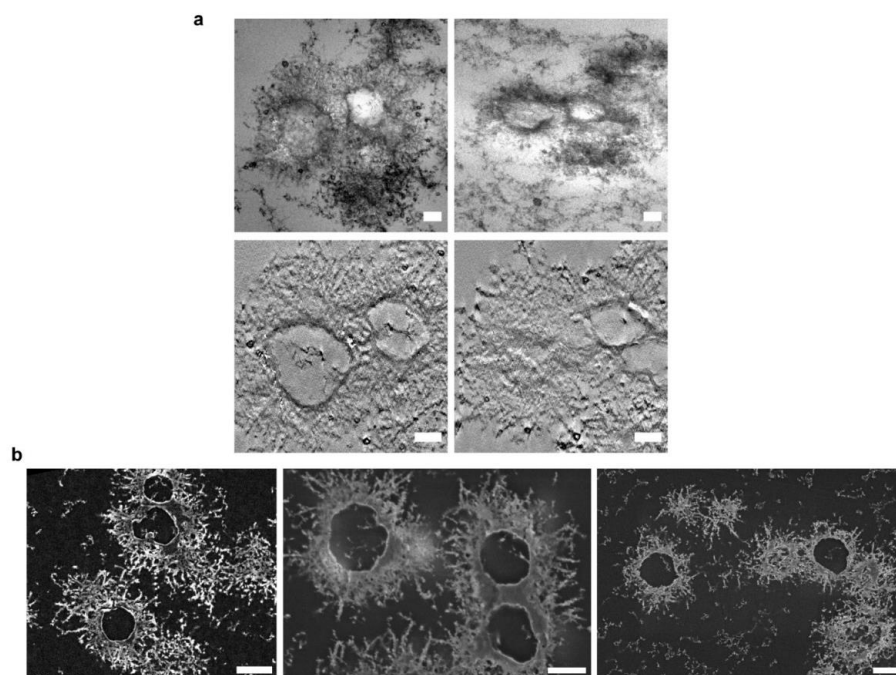
Supplementary Fig. 51 SEM – CLEM micrographs of MDA-MB-231 cells treated for 1 h with **1·TAT** (25 μ M) showing the nanostructures of the characteristic dark nodules as the cell shrinks. **a**, Scale bars, 2 μ m. **b**, Scale bars, 1 μ m. **c**, Scale bars, 200 nm.



Supplementary Fig. 52 SEM – CLEM micrographs of MDA-MB-231 cells treated for 1 h with **1-TAT** (25 μ M) showing the nanostructures of the characteristic dark nodules as the cell shrinks. **a**, Scale bars, 2 μ m. **b**, Scale bars, 1 μ m. **c**, Scale bars, 200 nm.

6.3 Tomography

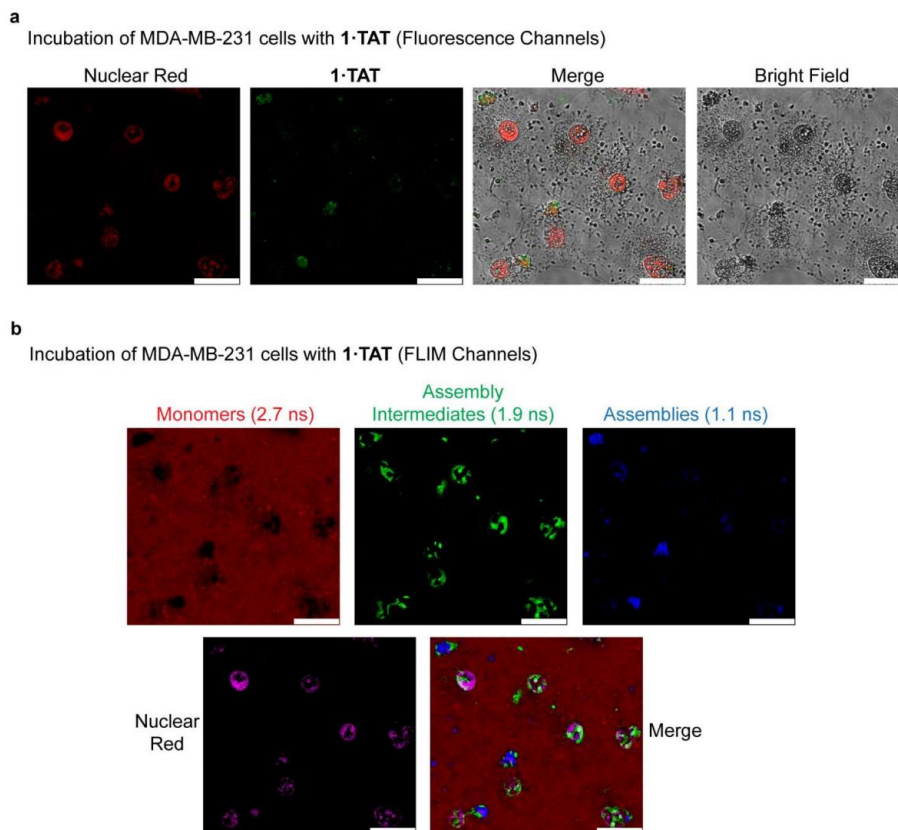
Tomographic 3D reconstruction of the volume was performed using cryo-TEM. For this, 300 nm thick sections on TEM Cu grids were used as described above. Tilt series were acquired on a Thermo-Fischer Titan Krios G4 using SerialEM software (the University of Colorado Boulder). Tomographic reconstruction was performed with imod (the University of Colorado Boulder) and Tomoviz (Cornell University) was used for 3D volume visualization.



Supplementary Fig. 53| Tomography and EM micrographs of hollow spherical objects induced by 1-TAT. **a**, magnified slice of TEM tomogram, scale bars: 200 nm; **b**, SEM images of the spherical objects taken under using different parameters. Scale bars, 500 nm.

6.4 Mapping intracellular formation of 2_{NF} for live cells using Phasor-FLIM

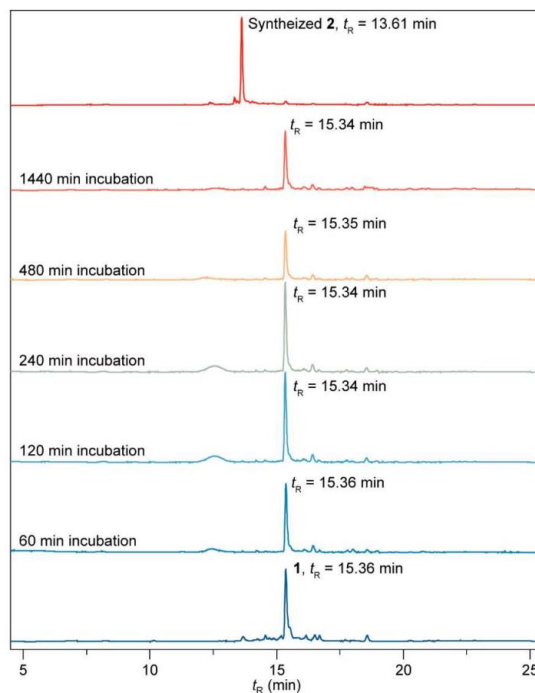
MDA-MB-231 cells were seeded at a density of 30,000 cells/well in an 8-well confocal plate. After adhering for 24 h, cells were treated with the sample for 4 h at 37 °C. The sample was dissolved in DMSO at a concentration of 10 mM, then the sample was further diluted with DPBS to a final volume of 40 μ L (DPBS: DMSO = 9:1). Sample solutions were further diluted 1:4 with DMEM and added to the cells (total DMSO content = 2%), at the same time the 8-well confocal plate was placed in the incubation box of the phasor-resolved fluorescence lifetime imaging microscopy (phasor-FLIM) and the cells were imaged in real time.



Supplementary Fig. 54 | Separated channels for fluorescence lifetime imaging of MDA-MB-231 cells treated with **1**·TAT (25 μ M) for 30 min. **a**, Fluorescence channels. **b**, FLIM channels. Different species including monomers, assembly intermediates, assemblies, and their merge were shown in separated images, Scale bars, 20 μ m.

6.5 Stability of compound **1** in cell lysate

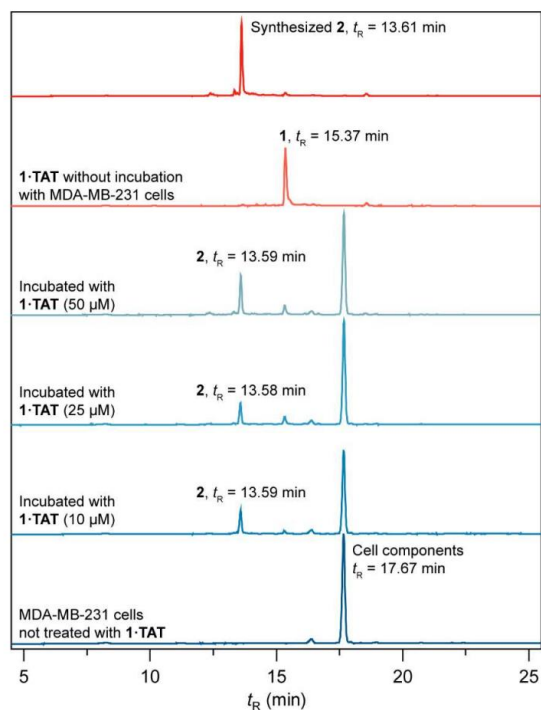
MDA-MB-231 cells were seeded at 15,000 cells/well in an 8-well confocal plate and subsequently washed with DPBS for three times. The cells were then lysed using of radioimmunoprecipitation assay (RIPA) lysis buffer (100 μ L) on ice for 5 min. Cells were detached using a cell scraper, and the cell lysate was collected. A solution of compound **1** (1.8 μ M) was prepared using the cell lysate as the solvent. To analyze the mixture, CH₃OH (400 μ L) was added, and the resulting mixture was centrifuged at 13,000 rpm at 4 °C for 20 minutes to remove proteins and cellular debris. The supernatant was then analyzed using analytical HPLC (Supplementary Fig. 55).



Supplementary Fig. 55| HPLC analysis for the stability of 1 in lysates of MDA-MB-231 cells. Compound **1** (1.8 μ M) was incubated with cell lysate of MDA-MB-231 cells and subsequently analyzed over 24 hours. No degradation is observed during the experiment, confirming that **1** is solely responsive to H_2O_2 .

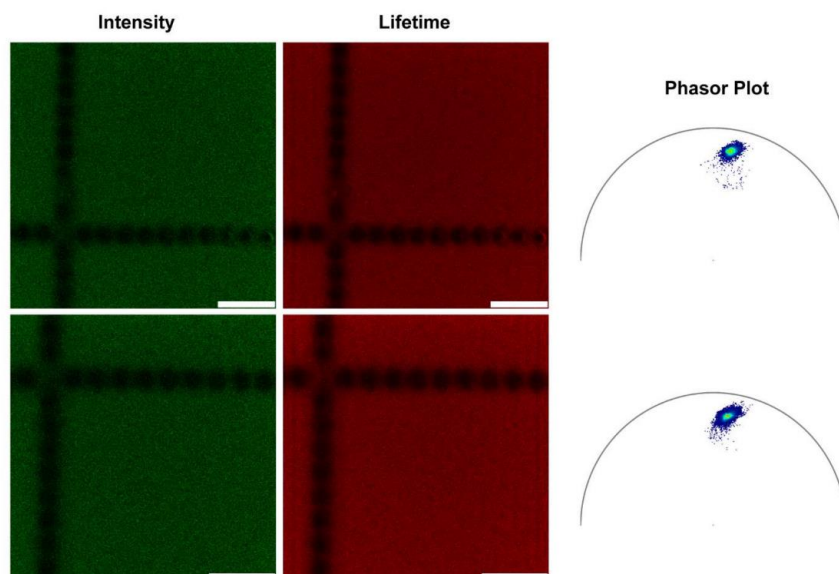
6.6 HPLC analysis of intracellular transformation of 1·TAT into 2_{NF}

MDA-MB-231 cells were seeded at 15,000 cells/well in an 8-well confocal plate. The cells were incubated with **1·TAT** at various concentrations for 4 hours. After incubation, the cells were washed with DPBS three times and lysed using of RIPA lysis buffer (100 μ L) on ice for 5 minutes. Cells were detached using a cell scraper, and the cell lysate was transferred into a PCR tube. To analyze the lysate, CH_3OH (400 μ L) was added, and the resulting mixture was centrifuged at 13,000 rpm at 4 °C for 20 minutes to remove proteins and cellular debris. The resulting supernatant was collected and analyzed using analytical HPLC (Supplementary Fig. 56).



Supplementary Fig. 56| HPLC assay on the lysate of MDA-MB-231 cells treated with **1-TAT.** In the control group which cells are not treated with **1-TAT**, trace with retention time (t_R) = 17.67 min can be identified. This trace can be attributed to cell components. For cells treated with **1-TAT**, peaks corresponds to compound **2** (t_R = 13.59 min), the final product for the intracellular H_2O_2 -induced conversion of **1-TAT** can be observed, suggesting endogenous H_2O_2 is sufficient for decomposing the phenylboronic acid cage and generate **2** as the final conversion product.

6.7 Phasor-FLIM analysis of 1-TAT incubated in cell lysates of MDA-MB-231 cells



Supplementary Fig. 57 | Phasor FLIM studies performed with **1-TAT** (25 μ M) incubated in cell lysates of MDA-MB-231 cells for 4 h. Analysis of two different regions shows no presence of aggregation or non-specific interactions that changes the fluorescence lifetime of **1-TAT**. Phasor plots are presented with a single locus with $\tau = 2.9$ ns. Scale bar = 50 μ m.

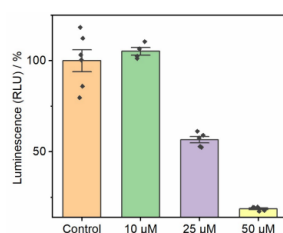
7. Biological response of intracellular superstructure formation

7.1 Glucose Uptake-Glo™ Assay

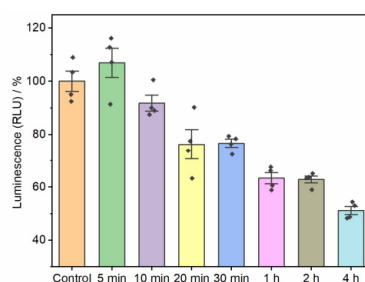
MDA-MB-231 cells were seeded in a clear-bottom 96-well plate at a density of 5,000 cells/well. They were then incubated at 37°C overnight to facilitate adherence. The following day, the cells were incubated with **3-TAT** (25 μ M) for 4 h, or with **1-TAT** (25 μ M) for intervals of 0.5 h, 1 h, 2 h, and 4 h. Once the incubation was completed, the medium was aspirated from the wells, and the cells were washed with DPBS (100 μ L). Subsequently, 1 mM 2-deoxyglucose (2-DG) (50 μ L) was added to each well. After a brief shake, the plate was allowed to incubate for 10 minutes at room temperature. Stop Buffer (25 μ L) and Neutralization Buffer (25 μ L) were then added to the wells, followed by a brief shake to ensure even distribution of the buffers. To complete the preparation, 2DG6P Detection Reagent (100 μ L) was added to each well. After a short shake, the plate was incubated for an additional 0.5 h at room temperature. Luminescence measurements were taken using the Promega GloMax®-Multi Detection System, adhering to the manufacturer's protocol specified for luminescence readout. The result was presented in Fig. 5A in the main text.

7.2 NAD/NADH assay

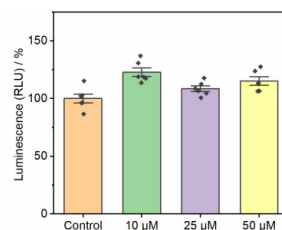
MDA-MB-231 cells were both seeded at a density of 5000 cells/well in a white half area 96-well plate and incubated at 37°C overnight to allow them to adhere. On the next day, 250 μ M 1-TAT were prepared in PBS at a 10x concentration of the final concentration and of this solution (20 μ L) were added to fresh medium (180 μ L). The old medium of the cells was removed and substituted with fresh medium (50 μ L) containing different concentrations of sample, prepared as described before. For each condition triplicates were performed. Cells were incubated with the sample for 5 min, 10 min, 20 min, 30 min, 1 h, 2 h and 4 h. After the treatment, NAD/NADH-Glo™ Assay solution (50 μ L) were 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 the Promega GloMax®-Multi Detection System with using the provided protocol for luminescence readout.



Supplementary Fig. 58| NAD/NADH assay MDA-MB-231 cells treated with different concentrations of 1-TAT for 4 h. A concentration-dependent effect of 1-TAT on NAD/NADH pathway is observed. Values are normalized towards the untreated control group. Data are presented as mean $\pm$ s.e.m., n = 6.



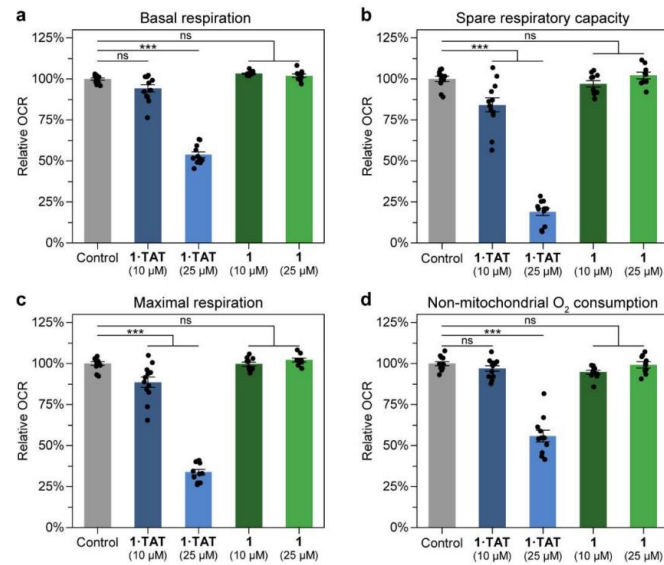
Supplementary Fig. 59| NAD/NADH assay MDA-MB-231 cells incubated with 1-TAT (25 μ M) for 5 min, 10 min, 20 min, 30 min, 1 h, 2 h, 4 h. A time-dependent effect of 1-TAT on the NAD/NADH pathway is observed. Values are normalized towards the untreated control group. Data are presented as mean $\pm$ s.e.m., n = 4.



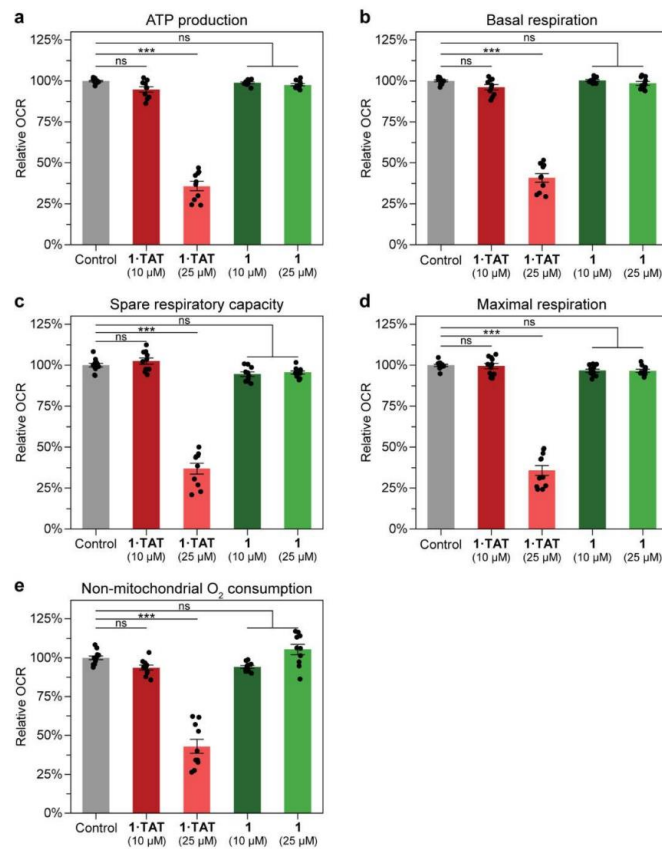
Supplementary Fig. 60| NAD/NADH assay MDA-MB-231 cells treated with different concentrations of 3-TAT for 4 h. 3-TAT shows no significant influence on NAD/NADH pathway at 10 - 50 µM. Values are normalized towards the untreated control group. Data are presented as mean $\pm$ s.e.m., n = 6.

7.3 Effect on cellular oxidative phosphorylation

The effect of **1** and **1-TAT** on cellular oxidative phosphorylation of MDA-MB-231 was investigated using the Agilent Seahorse XFe96 Analyzer. Cells were seeded one day before the assay and incubated overnight at 37°C, 5% CO₂. 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, XF DMEM, pH 7.4 was supplemented with glutamine, glucose and pyruvate regarding to the protocols. Cells were washed once with XF DMEM (200 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. The cartridge was prepared by loading the samples (20 µL) in Port A of each well and the components of the performed assay in Ports B-D. The cartridge and 96-well plate with the cells were loaded in the instrument and the components of the ports were added to the cell medium, while the pH, respectively the extracellular acidification rate (ECAR) and the oxygen concentration, respectively the oxygen consumption rate (OCR) was measured.



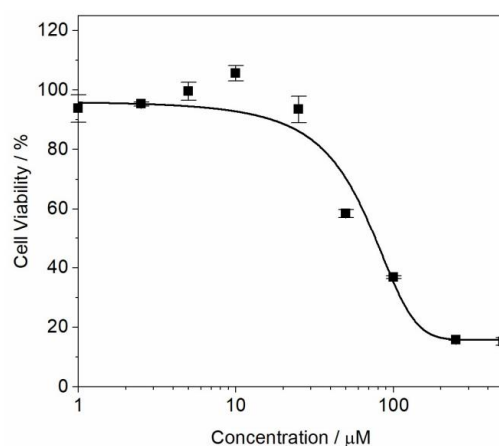
Supplementary Fig. 61| Comparison of 1-TAT (10 μ M and 25 μ M) and 1 (10 μ M and 25 μ M) incubation on key cellular Oxphos parameters after incubation for 1 h. a, Basal respiration. b, Spare respiratory capacity. c, Maximal respiration. d, Non-mitochondrial oxygen consumption. Values are normalized towards the untreated control group. The last measurement before the compound injection is set as 100%. Data are presented as mean $\pm$ s.e.m., $n \geq 9$. Statistical significance was calculated by ANOVA with a Tukey post hoc test. *p < 0.05, **p < 0.01, *p < 0.001.**



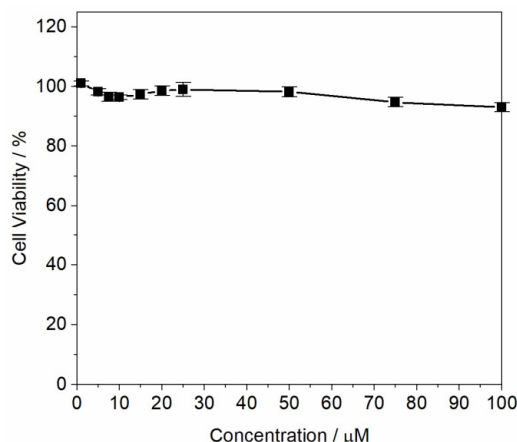
Supplementary Fig. 62| Comparison of 1-TAT (10 μM and 25 μM) and 1 (10 μM and 25 μM) on key cellular Oxphos parameters after incubation for 3 h. a, ATP production. b, Basal respiration. c, Spare respiratory capacity. d, Maximal respiration. e, Non-mitochondrial oxygen consumption. Values are normalized towards the untreated control group. The last measurement before the compound injection is set as 100%. Data are presented as mean ± s.e.m., n ≥ 9. Statistical significance was calculated by ANOVA with a Tukey post hoc test. *p < 0.05, **p < 0.01, *p < 0.001.**

7.4 Cell viability assay

MDA-MB-231 cells were both 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. On the next day, different concentrations of the **1·TAT** or **3·TAT** were prepared in PBS at a 10x concentration of the final concentration and of this solution (20 µL) were added to fresh medium (180 µL). The old medium of the cells was removed and substituted with fresh medium (50 µL) containing different concentrations of sample, prepared as described before. For each condition triplicates were performed. Cells were incubated with the sample for 4 h or 24 h. After the treatment, CellTiter-Glo Assay solution (50 µL) were 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 the Promega GloMax®-Multi Detection System with using the provided protocol for luminescence readout.



Supplementary Fig. 63 Cytotoxicity assay for MDA-MB-231 cells treated with different concentrations of **1·TAT** for 4 h. The IC₅₀ value is determined to be 69 ± 5 µM. Values are normalized towards the untreated control group. Data are presented as mean ± s.e.m., n ≥ 4.



Supplementary Fig. 64 | Cytotoxicity assay for MDA-MB-231 cells treated with different concentrations of **3-TAT** for 4 h. **3-TAT** shows no cytotoxicity up to 100 μM . Values are normalized towards the untreated control group. Data are presented as mean $\pm$ s.e.m., $n \geq 4$.

7.5 Autophagy assay

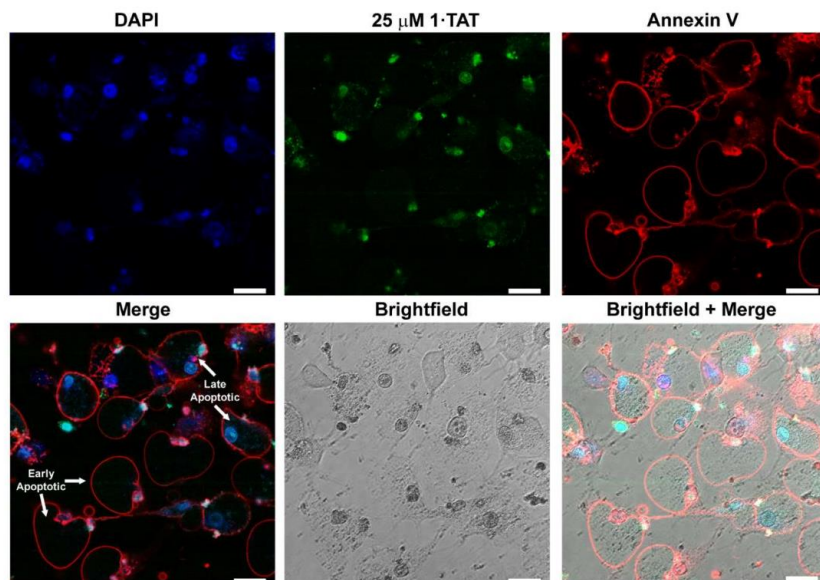
MDA-MB-231 cells were seeded at a density of 10,000 cells/well in a flat-bottom 96-well plate and incubated at 37°C overnight to allow for adherence. The following day, the cells were treated with **1-TAT** at concentrations of 10 μM and 25 μM for durations of 0.5 h and 1 h. After the incubation period, the medium was aspirated from the wells, and 100 μL of the Autophagosome Detection Reagent working solution was added to each well. The cells were then incubated at 37°C in an atmosphere containing 5% CO_2 for 30 minutes. Subsequent to this incubation, the cells were washed three times with Wash Buffer by gently adding 100 μL of the buffer to each well. The fluorescence intensity, with an excitation wavelength (λ_{ex}) of 360 nm and an emission wavelength (λ_{em}) of 520 nm, was measured using a microplate reader.

7.6 Annexin V/DAPI apoptosis assay

The apoptotic nature of cell death was investigated using Annexin V staining after incubation of MDA-MB-231 cells with **1-TAT** at 25 μM . MDA-MB-231 cells were seeded in an 8-well Ibidi plate at a density of 30,000 cells/well the day before the assay. They were then incubated overnight at 37°C to allow for adherence to the surface. The following day, the cells were treated with **1-TAT** for 4 hours. Post-treatment, they were incubated with Annexin-V FITC, obtained from the Apoptosis Detection Kit FITC by Invitrogen. After this step, the cells were washed once with binding buffer (200 μL). Then, cells in each well were exposed to Annexin V red (5 μL) in binding buffer (200 μL) and incubated for 15 minutes in the dark. Once the Annexin V staining was complete, the cells were washed with binding buffer (200 μL). The cell nuclei were subsequently stained using 4',6-diamidino-2-phenylindole (DAPI) in binding buffer (200 μL), incubating the cells for 5 minutes in the dark. After the staining, the solution was removed, and the cells were washed twice with binding buffer (200 μL each time). The washing

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solution was replaced with fresh binding buffer (200 μ L) before imaging using confocal laser scanning microscopy (CLSM).



Supplementary Fig. 65| Annexin V/DAPI assay on MDA-MB-231 cells treated with 1-TAT (25 μ M). The cells were treated with Annexin V red and DAPI after the incubation. Early apoptosis is characterized by annexin V binding but are not yet permeable towards DAPI, whereas late apoptosis/early necrotic cells show both positive annexin V binding and DAPI permeability. Representative early apoptotic and late apoptotic cells were identified by white arrows. Scale bar = 20 μ m.

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2.3 Visible Light-Controlled Intracellular Synthesis of Supramolecular Peptide Nanostructures

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Abstract

The complex dynamics and transience of supramolecular pathways in living cellular environments impede the correlation between diverse hierarchical species and their biological functions. The necessary breakthrough requires the precise control of supramolecular events at discrete time points via synthetic chemistry and their real-time visualization in native cells. Herein, we designed two peptide sequences that undergo a cascade of visible light-induced molecular and supramolecular transformations to form various assembly species in cells. In contrast to endogenous stimulus-responsive self-assembling systems, the irradiation with light enable full control over the reaction cascade where the monomer generation and concentration in turn regulates the assembly kinetics. Phasor-fluorescence lifetime imaging (phasor-FLIM) traced the formation of various assembly states in cells and revealed subsequent out-of-equilibrium dynamics associated with monomer activation and consumption. These temporally resolved assemblies show that the emergence of cytotoxicity is correlated to the accumulation of oligomers beyond the cellular efflux threshold.

Contributions

Yong Ren: Conceptualization, molecular design of peptides. Synthesis and

characterization of peptides and intermediates. LC-MS kinetics analysis, CD and TEM measurement and NMR analysis. Phasor-FLIM imaging and cellular assay study. Data analysis and interpretation of results. Writing of the manuscript.

Zhixuan Zhou: LC-MS kinetics measurement and analysis.

Iain Harley: Cryo-TEM measurement and analysis.

Özlem Aydin: Synthesis of intermediates.

Jiaqi Xing: DLS measurement and analysis.

Konrad Maxeiner: Cellular assay study.

Ingo Lieberwirth: Cryo-TEM measurement and analysis.

Katharina Landfester: Cryo-TEM measurement and analysis.

David Y.W. Ng: Design and discussion on the concept and results analysis. Writing and correction of the manuscript.

Tanja Weil: Acquiring funding for the project. Design and discussion on the concept and results analysis. Correction of the manuscript.

Visible Light-Controlled Intracellular Synthesis of Supramolecular Peptide Nanostructures

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Abstract

The complex dynamics and transience of supramolecular pathways in living cellular environments impede the correlation between diverse hierarchical species and their biological functions. The necessary breakthrough requires the precise control of supramolecular events at discrete time points via synthetic chemistry and their real-time visualization in native cells. Herein, we designed two peptide sequences that undergo a cascade of visible light-induced molecular and supramolecular transformations to form various assembly species in cells. In contrast to endogenous stimulus-responsive self-assembling systems, the irradiation with light enable full control over the reaction cascade where the monomer generation and concentration in turn regulates the assembly kinetics. Phasor-fluorescence lifetime imaging (phasor-FLIM) traced the formation of various assembly states in cells and revealed subsequent out-of-equilibrium dynamics associated with monomer activation and consumption. These temporally resolved assemblies show that the emergence of cytotoxicity is correlated to the accumulation of oligomers beyond the cellular efflux threshold.

Introduction

Optogenetic techniques have been transformative in cell biology, enabling the use of light as a trigger to initiate biochemical processes in living cells with high spatiotemporal resolution¹⁻³. This enables the manipulation of neuronal function, gene expression and signaling in heterogeneous tissues using a combination of genetic engineering and light in a minimally invasive manner⁴⁻⁶. The technique relies on the expression of light-sensitive ion channels, enzymes or transcription factors to precisely

1

control biochemical signaling pathways by external light activation. More recently, the expression and light-activated assembly of aggregation-prone proteins, such as amyloids, succeeded in elucidating the framework involved in amyloid formation and its biochemical impact on living cells⁷⁻⁹. However, the drawback of expressing light-activated proteins is the inability to control local concentrations, i.e. of self-assembling amyloid proteins, which would be necessary to unravel the transformation dynamics, assembly kinetics and the impact of supramolecular defects on their biochemical properties. It would therefore be essential to control supramolecular assembly at discrete time points to characterize the formation of transient intermediates and stable assemblies.

The molecular pathways of peptide and protein assembly in cells and their impact on cellular function are being subject of intense research¹⁰⁻¹⁹. The classical model of amyloid self-assembly is based on monomer-by-monomer addition. Recently, it has been proposed that amyloid-like self-assembling peptides can undergo liquid-liquid phase separation prior to their self-assembly²⁰⁻²². In this case, peptide-rich liquid droplets serve as nucleation precursors while intermediate species trigger nucleation, assembly and growth²³. Challenges arise from the fact that many parameters direct the self-assembly pathways and the subsequent growth of the formed aggregates such as the stability and lifetime of the monomers and intermediates, their local concentrations, the nucleation barrier as well as temperature²⁴⁻²⁹. The heterogeneity of aggregates is further amplified by the formation of kinetically-driven metastable structures upon encountering different intracellular environments. As such, synthetic approaches that control the supply of active monomers to produce different assembly states and dynamics is necessary to provide the critical breakthrough in correlating aggregation-based function in living cells.

However, controlling monomer concentrations and supramolecular assembly in the living cellular environment is challenging due to the intricate processes of internalization, transport and localization³⁰. Depending on the spatial localization, the crowded intracellular medium imposed by the high density of bio-macromolecules and organelles influences reaction kinetics^{31,32}, while supramolecular interactions depend on how active molecules are transported intracellularly and locally enriched. As these parameters only exist in living cells and cannot be studied in model systems, in-situ technologies such as the use of phasor- fluorescence lifetime imaging (phasor-FLIM)

are invaluable for tracking dynamic self-assembly processes in real time using time-resolved fluorescence³³. By combining photocleavable self-assembling peptides with phasor-FLIM imaging technology, we aim to understand how assembly is controlled, promoted and propagates in living cells.

In this study, we present the construction of two amphiphilic pro-assembling peptide sequences (**1**, **2**) protected by a visible light-sensitive cage group. The peptides are designed to undergo a cascade of visible light-induced molecular and supramolecular transformations to form nanofibers in the crowded environment of living cells (Fig. 1). Cell internalization of the caged peptides is facilitated by a cell membrane-penetrating oligo-arginine peptide sequence. The photolysis reaction and subsequent conversion of the stable photocaged monomers (**1**, **2**) into the "active" self-assembling peptides (**1a**, **2a**) was realized inside living cells upon irradiation with 505 nm visible light. In contrast to previously reported caged peptides that respond to endogenous stimuli such as pH or reactive oxygen species, the irradiation time now controls the uncaging reaction and thus monomer generation and concentration, which in turn regulates the assembly kinetics. A near-infrared (NIR) fluorophore has been attached to peptide **2** to allow tracking of the assembly dynamics, in particular the transition of different molecular and supramolecular species, using the recently reported phasor-FLIM technology. We report an intracellular synthesis strategy that encodes the molecular information for light sensitivity and fibril formation into the molecular structure of peptides to control and image different assembly states and dynamics of self-assembling peptide monomers, intermediates and nanofibers in living cells using visible light. Direct imaging of the complex assembly processes inside cells is correlated with their impact on cell viability.

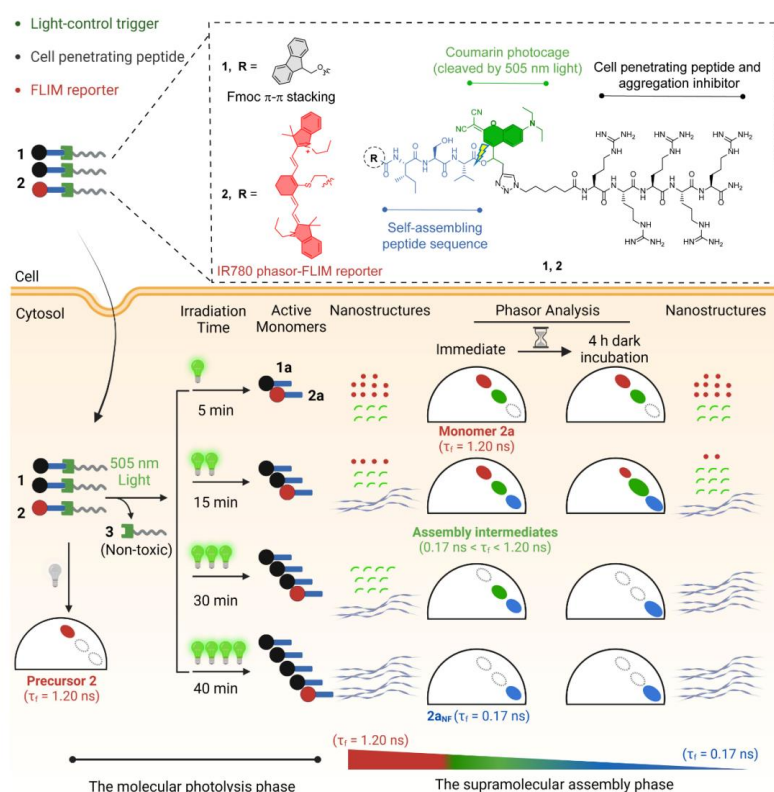


Fig. 1. Design of the photocaged pro-assembling peptide **1** and **2** encoded with the molecular information to form aggregates by light ("optoaggregates") inside cells and image the assembly process beyond the background emission of cells and the photocage. Pro-assembling peptide **1** and **2** (τ_f = 1.20 ns) entered A549 cells via "R₅"-mediated endocytosis. Visible light serves as an external stimulus to transform **1** and **2** to self-assembling peptide **1a** and **2a** upon irradiation. The assembly dynamics are monitored as photons change between τ_f = 1.20 ns and τ_f = 0.17 ns during assembly.

Design and synthesis of the photocaged pro-assembling peptides (**1** and **2**)

The photocleavable pro-assembling peptides (**1** and **2**) have been designed to contain four main functionalities: (1) the self-assembling peptide sequence (Fmoc-Ile-Ser-Val,

Fmoc-ISV) carrying (2) the IR780 chromophore as a phasor-FLIM reporter for intracellular imaging of the aggregation process; (3) the redshifted coumarin photocage that can be cleaved by 505 nm visible light and (4) a transporter peptide comprising five arginine (R_5) residues, whose polarity simultaneously prevents assembly and facilitates cellular uptake prior to irradiation. The proposed mechanism involves the initial internalization of the pro-assembling precursors (**1**, **2**) into cells supported by the R_5 sequence. Here, a co-assembly strategy is essential because **2a**, the photolysis product of precursor **2**, is unable to self-assemble into nanofibers, even at a high concentration of 100 μ M (Fig. S24). After exposure to 505 nm light, the photocage is cleaved, leading to the release of the active self-assembling monomers (**1a**, **2a**), which co-assemble into NIR emitting nanofibers. The irradiation time allows controlling the generation of the self-assembling monomers (**1a**, **2a**) and their respective local concentrations. In this way, different assembly states as well as nanostructure formation can be regulated within cells.

The synthesis of precursors **1** and **2** involves a combination of solid-phase peptide synthesis (SPPS) and solution-phase methods. The photocage is synthesized in solution through multiple reaction steps (Fig. S1a), while the peptide components, including **Fmoc-ISV** and the R_5 peptide, are synthesized using SPPS (Fig. S1b and S1c). The conjugate of the photocage and R_5 are synthesized in solution via a copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction (Fig. S1d), followed by coupling with **Fmoc-ISV** using diisopropylcarbodiimide and 4-(dimethylamino)pyridine (Fig. S1e). Finally, all protecting groups are cleaved in the presence of 95% trifluoroacetic acid (TFA) to obtain precursors **1** and **2**, characterized by liquid chromatography-mass spectrometry (LC-MS) and matrix-assisted laser desorption ionization (MALDI)-MS (Fig. S20-23). The synthesis details are included in the Supporting Information (Fig. S1).

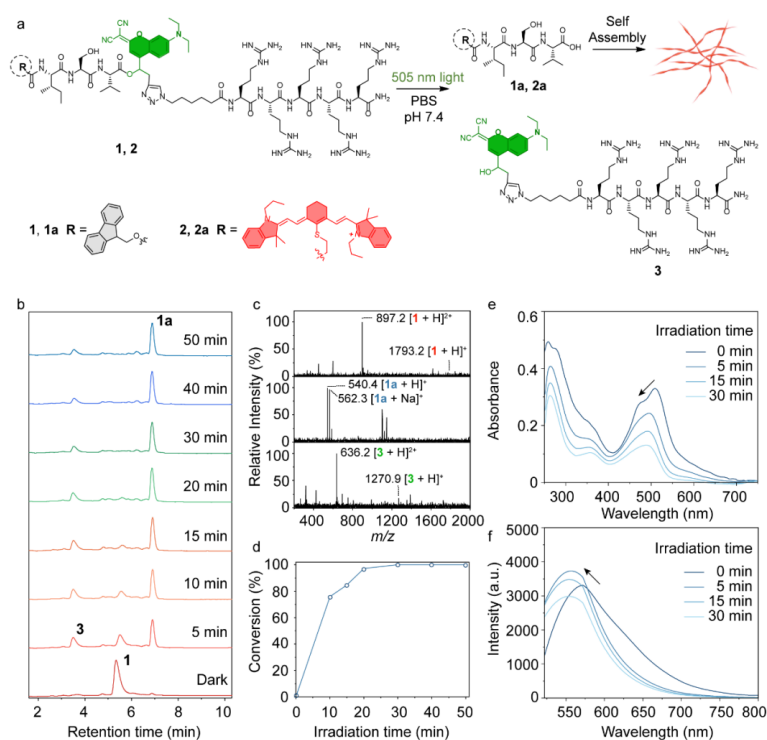


Fig. 2. (a) Molecular design and reaction scheme for all chemical transformations of photocaged peptides **1** and **2** (25 μ M, ratio of **1**:**2** = 9:1) that assemble into nanofibers ("optoaggregates"). (b) LC-MS kinetic analysis for the photolysis reaction of **1** (25 μ M) in NH_4HCO_3 buffer (pH 7.4, 20mM) upon irradiation with 505 nm visible light. (c) Convolutional MS spectra in the LC-MS analysis for compound **1** (t_R = 5.12-5.73 min) and corresponding products **1a** (t_R = 6.73-7.11 min) and **3** (t_R = 3.23-3.75 min) after photolysis. (d) The correlation between light-irradiation time and conversion. The connecting line is drawn only to aid visualization of the trend. (e) The changes of UV-vis absorption spectra of **1** (25 μ M) in PB (pH 7.4, 50 mM) after irradiation for 5 min, 15 min, 30 min; (f) Changes in the fluorescence spectra of **1** (25 μ M) in PB (pH 7.4, 50 mM) after irradiation for 5 min, 15 min, 30 min.

The photolysis reaction was optimized by LC-MS (Fig. 2b). In NH_4HCO_3 buffer (pH 7.4, 20 mM), precursor **1** remained stable for 24 h in the absence of light (Fig. S25). Upon light irradiation at 505 nm, the ester bond of **1** (25 μ M) was hydrolyzed in aqueous

buffer (NH_4HCO_3 buffer, pH 7.4, 20 mM), resulting in the formation of self-assembling peptide **1a** and the released photocage-R₅ **3**. After 5 min, there was a significant consumption of **1**, accompanied by the emergence of two new peaks with retention times (t_R) of 6.91 min and 3.52 min, respectively, both exhibiting m/z values consistent with the chemical formula of **1a** ($m/z = 540.4$ for $[\mathbf{1a} + \text{H}]^+$ and 562.3 for $[\mathbf{1a} + \text{Na}]^+$) and **3** ($m/z = 636.2$ for $[\mathbf{3} + \text{H}]^{2+}$ and 1270.9 for $[\mathbf{3} + \text{H}]^+$) (Fig. 2c). Extending the irradiation time to 20 minutes leads to a 98% conversion from **1** to **1a** while an irradiation of 30 minutes resulted in the complete disappearance of **1** (Fig. 2b, 2c and 2d). No further discernible effect on the photolysis process was observed if irradiation times were prolonged to 40 and 50 mins (Fig. 2b, 2d). To demonstrate that precursor **2** can also be cleaved by 505 nm light, the corresponding photolysis reaction kinetics were measured. The results indicated that the ester bond of **2** ($2.5 \mu\text{M}$) was completely hydrolyzed within 10 minutes of irradiation at 505 nm, resulting in the formation of peptide **2a** (Fig. S26).

The optical properties associated with the photolysis and self-assembly processes were then investigated. Precursor **1** exhibited an absorption band centered at 505 nm, characteristic of the coumarin chromophore. With increasing irradiation time, the absorption of the coumarin chromophore gradually decreased, accompanied by a slight hypsochromic shift (Fig. 2e). This shift can be attributed to the self-assembly behavior of **1a** in solution. The emission of the coumarin chromophore on the peptide initially exhibited a slight increase upon irradiation, probably due to its release from the peptide³⁴. However, this was followed by a gradual decrease, which can be attributed to the self-assembly of **1a** in solution (Fig. 2f).

Light-induced Assembly Profile and Phasor-FLIM Calibration

The light-induced self-assembly and optoaggregate formation was initially studied by dynamic light scattering (DLS) analysis, which revealed a gradual increase in the sizes of the formed nanostructures over the irradiation times (Fig. 3d). Without irradiation, a $25 \mu\text{M}$ solution of **1** in PB (pH 7.4, 50 mM) remained soluble and exhibited no aggregation (Fig. 3d). After 1 min, nanometer sized aggregates were already formed that steadily increased and during 10 min of irradiation leading to the formation of micrometer sized aggregates ("optoaggregates"). The formation of the optoaggregates was imaged by time-dependent transmission electron microscopy (TEM) for similar irradiation times. Distinct nanofiber structures with increasing fiber densities occurred proportional to the irradiation time. Initially, a small number of fiber fragments were

observed after 5 min of irradiation. This was followed by a significant increase in fiber quantity after 15 min of irradiation (Fig. 3a, 3b). With prolonged irradiation to 30 min, the formation of denser nanofiber networks became apparent. Time-dependent cryogenic high-resolution TEM images confirmed the formation of similar nanostructures in solution upon light exposure (Fig. 3c). Interestingly, the image at 5 min presented the existence of discrete liquid droplets, potentially formed as a result of a liquid-liquid phase separation (LLPS) process. These droplets further convert to thermodynamically favorable nanofibers when more monomers were generated after longer irradiation time. Time-dependent circular dichroism (CD) analysis revealed an increasing signal at 203 nm (Fig. 3e), indicating the formation of ordered β -sheet secondary structures, which increased during irradiation. Additionally, the characteristic $\pi \rightarrow \pi^*$ transition of the Fmoc group at 265 and 274 nm correlated positively with irradiation time, suggesting that the Fmoc group participates in the assembly. However, in the absence of irradiation, precursor **1** showed no obvious signal between 180 and 280 nm (Fig. S27). To validate the co-assembly behavior, co-assembled nanofibers (**1a** + **2a**, mol. ratio = 9:1) were prepared in DMEM and imaged using confocal laser scanning microscopy, revealing the presence of NIR emitting fibers, confirming the successful co-assembly process (Fig. S28). To calibrate the fluorescence lifetime profile under physiological conditions, the nanofibers were assembled in DMEM and transferred onto a culture of A549 lung adenocarcinoma cells. Herein, 0.1 eq. of peptide **2** with an IR780 dye as a phasor-FLIM reporter were used for co-assembly. The free molecule control was accomplished using non-irradiated **1+2** (mol. ratio = 9:1) in A549 cells. Phasor-FLIM analysis revealed that free molecules exhibited a fluorescence lifetime (τ_f) of 1.20 ns prior to irradiation (Fig. S29), whereas the assembled nanofibers revealed a τ_f ranging from 0.75 ns to 0.17 ns, representing different assembly states (Fig. 3f, 3g).

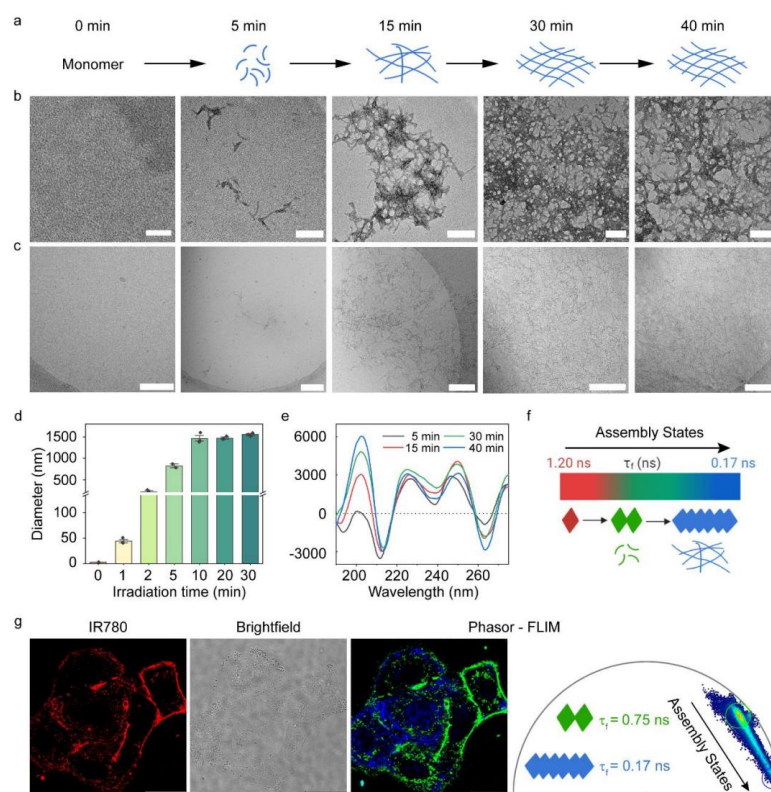


Fig. 3. Nanofibers formation observed in the light-induced self-assembly over time. (a) Scheme of light-induced peptide assembly and (b) corresponding TEM images of co-assembled (25 μ M, ratio of 1:2 = 9:1) nanofibers formed at different irradiation time. Scale bar: 200 nm. (c) Corresponding cryogenic high-resolution TEM images of the co-assembled nanofibers (25 μ M) in PB (pH 7.4, 50 mM) over time. Scale bar, 200 nm. (d) Dynamic light scattering kinetics analysis for the light-induced nanostructure formation of **1** (25 μ M) in PB (pH 7.4, 50 mM) over time. Changes in diameter were measured. (e) CD kinetics analysis of **1** in a mixture of PB (pH 7.4, 100 mM) and MeOH (0.5 vol %) over irradiation time. (f) Schematic illustration of the assembly dynamics from monomers to assemblies, leading to decreased fluorescence lifetimes (1.20 ns to 0.75 ns to 0.17 ns) upon oligomerization and formation of the optoaggregates. (g) Fluorescence lifetime analysis of assembly progression out of A549 cells using phasor analysis. Two major species can be identified showing τ_f values of 0.75 ns (oligomers) and 0.17 ns

(larger assemblies, optoaggregates).

Visualization of the Light-induced Nanofiber Formation Process in Cells Using CLSM and Phasor-FLIM

The R_S -induced endocytosis into A549 lung alveolar adenocarcinoma cells was visualized using confocal laser scanning microscopy (CLSM) (Fig. S30). Different incubation times with 25 μ M **1+2** (mol. ratio = 9:1), ranging from 2 h to 6 h, were tested to determine the optimal conditions for cellular uptake. After 4 h of incubation, peak cellular uptake was observed, as evidenced by both the green fluorescence from the coumarin dye and the NIR emission of IR780 (Fig. S30). To acquire an understanding of the assembly progression, we performed corresponding imaging using Phasor-FLIM. Cells were pre-treated with **1+2** for 4 h, followed by washing off the excess extracellular precursors. Subsequently, the cells were subjected to irradiation for 5 min, 10 min, 15 min, 30 min and 40 min, followed by immediate phasor-FLIM imaging (Fig. 4d-4i). The progression of aggregation over time can be tracked by the shifting of photon populations between two characteristic phasors on the phasor plot, representing monomers and assemblies (Fig. 4e-4i). In the absence of light, the precursors exhibited a homogenous, single-component fluorescence decay with $\tau_f = 1.20$ ns (Fig. S31). Upon irradiation, the photocaged peptides (**1** and **2**) generated the corresponding active monomers (**1a** and **2a**), which subsequently self-assembled into nanofibers exhibiting a decreased τ_f . After 5 min of irradiation, the phasor analysis showed intracellular photons exhibiting fluorescence lifetimes of $1.20 \text{ ns} > \tau_f > 0.75 \text{ ns}$ indicating that the major component were still monomers and first traces of assembled species were observed (Fig. 4e). Supported by TEM and cryo-TEM images, after 5 min of irradiation, only monomers were produced at a low concentration, and the formation of oligomers occurred but no further progression into mature nanofibers was observed (Fig. 3b, 3c). When increasing the irradiation time to 10 min, morphological changes of the cells were detected (Fig. 4c) along with the emergence of a new photon population with $\tau_f = 0.17$ ns, indicative of the formation of nanofibers (Fig. 4f). Extending the irradiation time to 15 minutes lead to a further consumption of the generated monomers and the concomitant formation of oligomers and assemblies (Fig. 4g). Upon 30-min of irradiation, the pool of intracellular precursors was fully consumed and corresponding population of photons shifting to $\tau_f = 0.17$ ns increased significantly, implying the progression of most supramolecular species into assemblies (Fig. 4h). Finally, irradiation for 40 min resulted in the complete transformation of the photocaged

peptides into assemblies (Fig. 4i). As irradiation time increased, notable morphological changes in the cells were observed (Fig. 4c). Therefore, a time-dependent cell viability assay was conducted, revealing that 30 min of irradiation in the presence of **1** and **2** reduced cell viability to approximately 37% under these conditions (Fig. S32). This suggests that the assembly formation need to reach a critical point to induce significant cytotoxicity.

To rule out the possibility that the cytotoxicity observed was due to the phototoxicity of 505 nm light, the phototoxic effect of IR780 dye or the byproduct of photolysis **3**, pertinent experiments were conducted. Initially, phototoxicity from the light source was assessed by conducting cell viability assays and confocal imaging on untreated cells (Fig. S33, S36), revealing that light (505 nm wavelengths) induced no significant toxicity to A549 cells. Furthermore, to investigate the potential cytotoxic effects induced by both the phototoxic effect of IR780 dye and **3**, cell viability was studied on A549 cells treated with **2** at a concentration of 2.5 μ M used for co-assembly, and **3** at 25 μ M (Fig. S36), with and without 30-minute irradiation. Additionally, confocal imaging was performed for each corresponding treatment (Fig. S34, S35). The results clearly indicated that both compounds did not induce damage to A549 cells (Fig. S34-36). Therefore, it can be concluded that the observed cytotoxicity solely resulted from the formation of nanostructures induced by light.

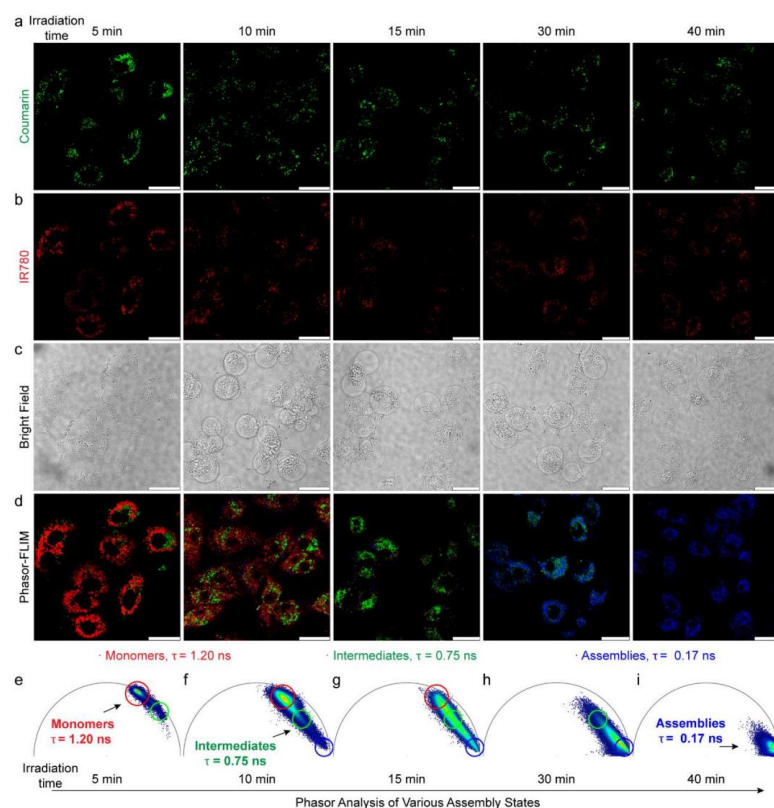


Fig. 4. Time-lapsed phasor-FLIM analysis of **1** and **2** internalization and assembly states upon different irradiation times. Time-dependent fluorescence of coumarin (a) and IR780 (b), brightfield (c), and phasor-FLIM images (d) of A549 cells treated with **1** and **2** (25 μ M, ratio of **1**:**2** = 9:1). Bottom row: Phasor maps of A549 cells treated with **1** and **2** (25 μ M, ratio of **1**:**2** = 9:1) upon different irradiation times, including 5 min (e), 10 min (f), 15 min (g), 30 min (h) and 40 min (i). Arrows indicate that the irradiation results in chemical photolysis and the nanostructure formation and growth with a concomitant change in the phasor-FLIM. Scale bars, 25 μ m.

Temporal Analysis of Precisely Controlled Nanofiber Formation in Cells Using Phasor-FLIM

In contrast to endogenous stimulus-responsive self-assembling systems, the light-triggered assembly offers a unique opportunity to control the assembly kinetics within

cells by modulating the intracellular concentration of the active monomers. The kinetics involved consist of two distinct phases: the molecular photolysis phase, generating the “active” self-assembling monomers, and the supramolecular assembly phase, during which nuclei are formed that progress to oligomers and mature fibers³⁵. At the molecular level, the photolysis reaction regulates the generation and local concentration of the active monomers that proceed into the self-assembly phase. During assembly, active monomers are consumed relative to their local concentration, which determines the nanostructure formation process. When the local concentration is below the critical aggregation concentration (CAC), assembly does not start and no nanostructures are formed. Conversely, when the concentration of active monomers exceeds the CAC, the production and consumption rates determine the extent of nanostructure formation. If the photolysis production rate of active monomers exceeds their assembly consumption rate, the excess of the active monomers will continue to undergo assembly even after the light has been switched off, causing a loss in temporal precision of the supramolecular polymerization process. To demonstrate these limits, we conduct an extensive analysis of assembly processes and subsequent biological effects on A549 cells (Fig. 5). Following identical 4 h peak internalization, the cells were exposed to light for 5 min, 10 min, 15 min and 30 min, respectively, corresponding to the photolysis conversion of the photocaged precursors (**1** and **2**) in cell-free conditions (Fig. 2d), to control the amount of active monomers that then progressed into the subsequent assembly process. This was followed by a dark incubation period of 1 to 4 hours, during which we examined the assembly progression and the associated biological effects using phasor analysis and cell viability assays (Fig. 5a-h).

With an irradiation time of 5 min, subsequent incubation in the dark for 1-4 h did not show further progression on the phasor plot (Fig. 5a, Fig. S38). Phasor analysis revealed that the population of photons remained $1.20 \text{ ns} > \tau_{\text{f}} > 0.75 \text{ ns}$, indicating that the concentration of active monomers surpassed the critical aggregation concentration (CAC), therefore leading to nucleation. However, it did not reach a critical concentration threshold for propagation. After irradiation for 10 min, 1 h incubation in the dark led to increase in nucleation (Fig. 5b), whereas after 4 h, most monomers were consumed (Fig. 5b, Fig. S39). The results indicated that 10 min of irradiation is sufficient to produce the active monomers in excess that continued to fuel the assembly process even during the dark phase when the light was switched off. Extending the irradiation to 15 min yielded even more noteworthy changes of phasor plots (Fig. 5c, Fig. S40).

Incubation for 4 h resulted in the increase of the population of photons shifting to 0.75 ns $> \tau_f > 0.17$ ns, indicative of a higher consumption of monomers, further progression into oligomers and the subsequent propagation into a larger number of assemblies compared to the 10 min irradiation experiment (Fig. S40). Compared to 10 min irradiation, 15 min irradiation generated a considerably higher light-induced uncaging, producing higher monomer concentrations, which further promoted the supramolecular nucleation process and subsequently propagated to nanofibers within the same timeframe.

Furthermore, 30 min irradiation led to complete consumption of the active monomers, with photon populations of 0.75 ns $> \tau_f > 0.17$ ns, corresponding to the formation of a substantial number of assemblies and the presence of a small number of oligomers. 2 h of incubation led to significant increase in assembly propagation and 3 h incubation resulted in the complete transformation of the active monomers and oligomers into assemblies (Fig. 5d, Fig. S41). The observation indicated that the formation of a substantial amount of assemblies facilitated the propagation of all remaining oligomers, thereby driving the assembly process to completion. Overall, these findings demonstrated that during assembly, when the photolysis production rate of monomers exceeds their assembly consumption rate, the excess monomers or unconsumed oligomers continue to contribute towards the assembly process even after the cessation of light exposure. In total, our strategy enables to effectively govern the molecular photolysis phase to generate accurate concentrations of active monomers that contributes towards the self-assembly phase, which remains unattainable with assembly systems induced by endogenous stimuli. Additionally, the photocaged pro-assembling peptides offer a unique opportunity to visualize and unravel supramolecular assembly processes inside living cells, which will be crucial for the formation of artificial nanostructures, compartments or synthetic organelles, an area of emergent importance.

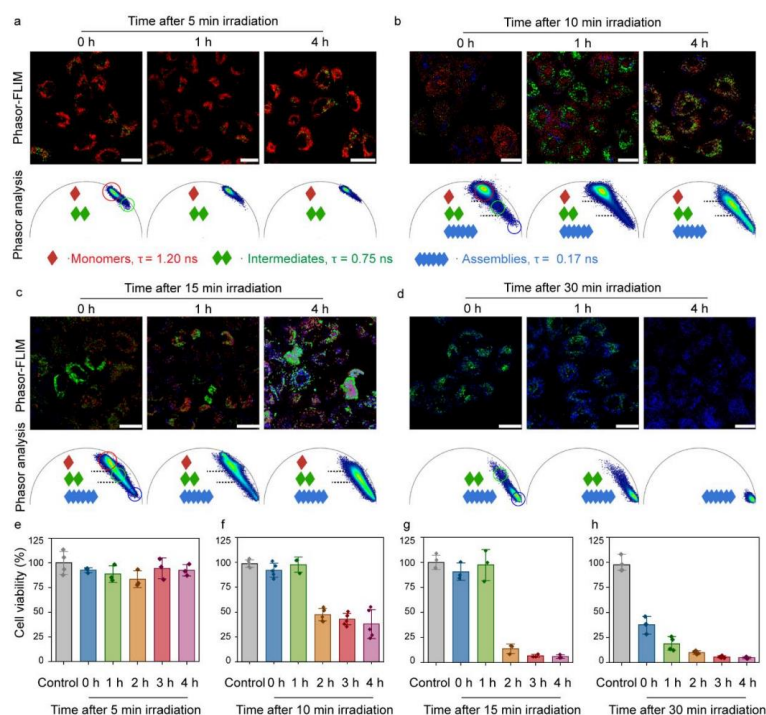


Fig. 5. Phasor-FLIM analysis and cell viability study of various assembly states and dynamics for 1-4 h incubation after different irradiation times. Phasor-FLIM analysis of A549 cells for 1-4 h incubation after irradiation for 5 min (a), 10 min (b), 15 min (c), 30 min (d). Cell viability study for A549 cells for 1-4 h incubation after irradiation for 5 min (e), 10 min (f), 15 min (g), 30 min (h). Scale bars, 25 μ m.

Inspired by the finding that the assembly phase would be continuously fueled by the over-production of active monomers, even after the light has been switched off, we hypothesized that associated cytotoxicity due to the accumulation of oligomers will see a delayed response. To investigate whether the species that were dynamically formed during supramolecular assembly correlate with the corresponding biological effects, cell viability was examined as a function of post-illumination assembly. Interestingly, 5 min of irradiation did not result in significant toxicity towards A549 cells even after 4 h of incubation (Fig. 5e).

However, 10 min of irradiation did not induce significant toxicity within the first hour of incubation, but resulted in an approximately 50% decrease in cell viability between 2 h and 4 h (Fig. 5f). In addition, 15 min of irradiation resulted in higher toxicity, reducing cell viability to 10% after 2 h (Fig. 5g). After 30 min of light exposure, cell viability immediately decreased to approximately 37%, ultimately dropping to less than 5% after 4 h (Fig. 5h).

By integrating the cellular effects with phasor analysis and the LLPS-mediated supramolecular pathway (Fig. 6), it is evident that during the early stages of assembly, the formation of peptide-rich liquid droplets and oligomers does not induce significant cytotoxicity. However, as these oligomers accumulated and further propagated into larger assemblies, notable cytotoxic effects emerge. These effects are correlated with the quantity of assemblies; specifically, greater numbers of assemblies result in more pronounced cellular toxicity.

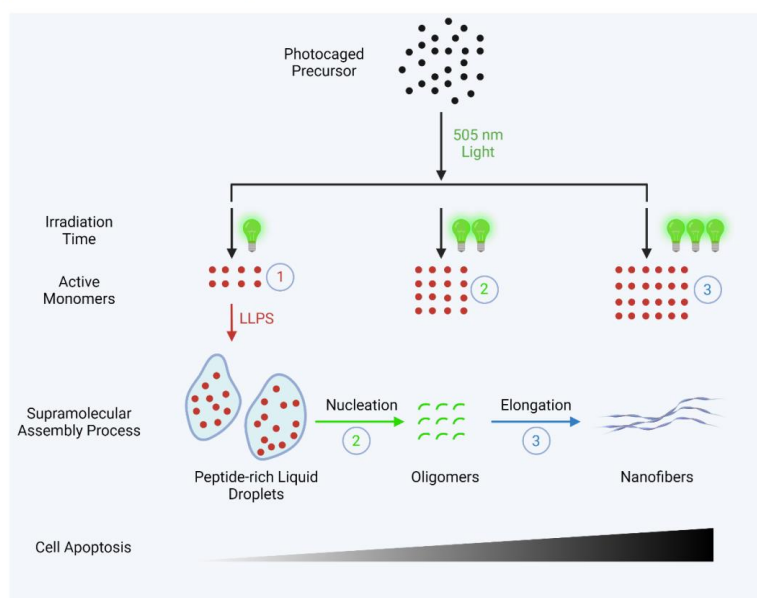


Fig. 6. Illustration of LLPS-mediated supramolecular assembly pathway and its correlation with cellular effects. The liquid-liquid phase separation (LLPS) into peptide-rich liquid droplets is a critical step prior to the nucleation of supramolecular nanofibers. The cellular toxicity results show the formation of liquid droplets and oligomers did not induce significant cell toxicity. After further elongation into nanofibers, observable cytotoxic effects emerge.

Conclusion

In conclusion, we present photocaged pro-assembling peptides to control and image visible light-induced supramolecular assembly inside complex cellular environments. In combination with advanced imaging technologies, precise control over the generation of diverse assembly states and real-time imaging of these processes was achieved. By integrating imaging and relevant biological assays, we discovered that during the initial stage of peptide assembly, the formation of oligomers did not lead to observable cytotoxic effects. Significant cytotoxicity was observed only upon the formation of sufficient quantities of the supramolecular assemblies. Furthermore, by performing phasor analysis on A549 cells post-irradiation, we have elucidated the intricate dynamics of assembly in greater detail in cells. The observation highlighted that the concentration, production rate, and consumption rate of monomers significantly influence assembly dynamics inside cells. While precise control over the molecular phase of photolysis is achievable, managing the supramolecular phase becomes challenging when the production rates of monomers exceed their consumption rates. Time-dependent cell viability analysis revealed that the cytotoxic effects are closely correlated with the quantities of nanostructures formed intracellularly. Our study facilitates precise control over supramolecular events at discrete time points, and the new imaging technologies offer deeper insights into the dynamic assembly processes with native cells. Our results will pave the way to address grand challenges in supramolecular drug discovery and relevant biomedical research.

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Visible Light-Controlled Intracellular Synthesis of Supramolecular Peptide Nanostructures

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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 CH₃CN in HPLC grade (containing 0.1% CF₃COOH) and H₂O for HPLC (containing 0.1% CF₃COOH) and reactions was obtained from a Millipore purification system.

1.2 Instruments

1.2.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR spectra for all compounds and intermediates were recorded on a Bruker Avance III 700 MHz NMR spectrometer. Temperature control was achieved using a VTU (BCU II; variable temperature unit) with an accuracy of +/- 0.1K. The samples were stabilized at the desired temperature for 20 minutes prior to the measurements. 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 (D₂O δ = 4.79 ppm and DMSO-d₆ = 2.50 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. Chemical shifts for protons of the compounds were assigned on the basis of COSY and HSQC correlations. The data were processed in MestReNova.

1.2.2 Microwave Peptide Synthesizer

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

1.2.3 Preparative High-Performance Liquid Chromatography (HPLC)

The compounds were purified by preparative HPLC using a setup by Shimadzu. For purification either a ZORBAX Eclipse XDB-C18 HPLC column (9.4 × 250 mm, 5 μm) was used at a flowrate of 4 mL/min or a Phenomenex Gemini 5 μm NX-C18 110 Å 150 × 30 mm was used at a flowrate of 25 mL/min. Experiments were performed using CH₃CN in HPLC grade (containing 0.1% CF₃COOH) and MiliQ water (containing 0.1% CF₃COOH).

1.2.4 Analytical High-Performance Liquid Chromatography (HPLC)

The samples were analyzed by analytical HPLC using a setup by Shimadzu. An Atlantis T4 column (4.6 × 100 mm, 5 μm) was used at a flowrate of 1 mL/min. Experiments were performed using CH₃CN in HPLC grade (containing 0.1% CF₃COOH) and MiliQ water (containing 0.1% CF₃COOH). The solvent gradient started

with 100% water, then the CH₃CN content was linearly increased to 100% in 20 min. The molar ratio of the compounds was calculated using peak areas at 254 nm. Data were processed in LabSolutions and Origin.

1.2.5 High-Performance Liquid Chromatography - Mass Spectrometry (HPLC-MS)

The 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. The molar ratio of the compounds was calculated using peak areas at 254 nm. Data were processed in LabSolutions and Origin.

1.2.6 Matrix-Assisted Laser Desorption/Ionisation - Time of Flight Mass Spectrometry (MALDI-TOF-MS)

All MALDI-TOF spectra were recorded on either a rapifleX MALDI-TOF/TOF from Bruker or MALDI Synapt G2-SI from Waters. Samples were mixed with a saturated solution of the matrix S7 α-cyano-4-hydroxycinnamic acid (CHCA) in NH₄HCO₃ buffer (20 mM)/CH₃CN 1/1. Data processing was performed in mMass.

1.2.7 Spectroscopy

The UV/Vis absorption spectra for the solutions of the compounds were recorded on a Thermo Scientific™ NanoDrop 2000/2000c spectrophotometer in a Hellma high precision quartz cell, 10 x 10 mm light path. The luminescence emission spectra for the solutions of the compounds were recorded on a Cary Eclipse fluorescence/luminescence spectrophotometer in a Hellma high precision quartz cell, 10 x 10 mm light path. For the CAC analysis using Nile Red, the luminescence spectra were recorded using a Tecan Spark 20M microplate reader. CD spectra were recorded on a JASCO J-1500 spectrometer in a Hellma high precision cell with 1 mm light path. Data were processed in Spectra Analysis by JASCO and Excel.

1.2.8 Dynamic Light Scattering (DLS)

Single-angle DLS measurements were performed at 25 °C using a Malvern ZetaSizer Nano S purchased from Malvern Instruments Ltd. (Malvern, Great Britain) with a He/Ne Laser (λ = 633 nm) at a fixed scattering angle of 173°. All measurements were performed in triplicate. The obtained data was processed by cumulant fitting for D_h , or by CONTIN fitting for intensity weighted particle size distribution. Samples were prepared at 25 µM. Dust was removed prior to each measurement by filtration through GHP syringe filters (0.2 µm pore size, Acrodisc).

1.2.9 Transmission Electron Microscopy (TEM)

TEM images of the conjugate solutions were taken on a JEOL 1400 transmission electron microscope at a voltage of 120 kV. Samples were prepared on Formvar/carbon-film coated copper grids (300 mesh) by Plano GmbH. In order to prepare the TEM grids, 4 µL of the sample solution were put on freshly glow discharged (30 s at 40 W in a 6:1 oxygen: hydrogen plasma) Formvar coated copper grids. After 5 min the solution was removed using a filter paper and grids were stained with 4% uranyl acetate for 2.5 min. The grids were washed three times with MilliQ water and dried before measuring. TEM images were processed in ImageJ.

1.2.10 Cryogenic Transmission Electron Microscopy

For cryo-TEM examination the samples were vitrified using a Vitrobot Mark V (Thermo Fisher, Hillsboro Oregon) plunging device. 3 µl of the sample dispersion was applied to a Quantifoil or a lacey carbon coated TEM grid that has been glow discharged in a 6:1 oxygen: hydrogen plasma (Diener Nano[®], Diener electronic, Germany) shortly before. After removing excess sample solution with a filter paper, the grid is immediately plunged into liquid ethane. For the subsequent examination the specimen is transferred to a TEM (FEI Titan Krios G4) keeping cryogenic conditions. Conventional TEM imaging was done using an acceleration voltage of 300 kV. Micrographs were acquired with a 4k Direct Electron Detection Camera (Gatan K3) under low dose conditions.

1.2.11 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% FBS. Both cell lines were cultured in T75 culture flask and subcultivated two to three times per week.

1.2.12 Luminescence Detection of Cell Viability

After performing the CellTiter-Glo[®] Luminescent Cell Viability Assay in a half area, white 96-well plate, luminescence intensity was measured on a Promega GloMax[®]-Multi Detection System using the protocol for the CellTiter-Glo[®] Luminescent Cell Viability Assay preinstalled on the instrument. Data were processed in Excel and graphs were generated in Origin.

1.2.13 Confocal Laser Scanning Microscopy & Fluorescence Lifetime Imaging Microscopy

CLSM-FLIM imaging was performed on a STELLARIS 8 Leica DMI8 microscope (Leica Microsystems, S/N: 8300000313).

Cells are cultured in an IBIDI 8-well glass bottom chamber at a density of 25,000 cells per well in DMEM and allowed to adhere overnight at 37°C, 5% CO₂. The medium was aspirated and samples pre-dissolved in DMEM at the respective concentrations were introduced into each well. Incubation was performed at 37°C, 5% CO₂.

Samples were excited using an 80 MHz pulsed white light laser tuned to 752 nm for both intensity and fluorescence lifetime measurements. Emitted photons were detected using HyD® X (GaAsP hybrid photocathode) detector with a filter window at 763 nm – 835 nm.

Fluorescence lifetime imaging (FLIM) was conducted with fixed cells or living cells, with scanning resolution of 1024 x 1024 pixels at 100 Hz, 2 frame accumulation equipped with adaptive focusing on each frame and well position. Photons were counted based on FALCON-modified time correlated single photon counting (TCSPC) method. Each pixel is transformed into a phasor plot according to the following equation:

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

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

in which $g_{ij}(\omega)$ and $s_{ij}(\omega)$ are the x and y coordinates of the phasor plot, n and ω are the harmonic frequency and the angular frequency of excitation, respectively, and T is the repeat frequency of the acquisition. Frequency domain data acquisition from each pixel can be converted to phasor points using the following transformations:

$$g_{ij}(\omega) = m_{ij} \cdot \cos(\phi_{ij})$$

$$s_{ij}(\omega) = m_{ij} \cdot \sin(\phi_{ij})$$

in which m_{ij} and ϕ_{ij} are the modulation and phase shift, respectively, of the frequency domain measurement at pixel ij . The decay from each pixel can hence be translated to a point in the phasor plot.¹

Phasor components were identified and separated using LAS X software. Photons that lie beyond the defined phasor are excluded from the images.

2 Synthesis and structural characterization

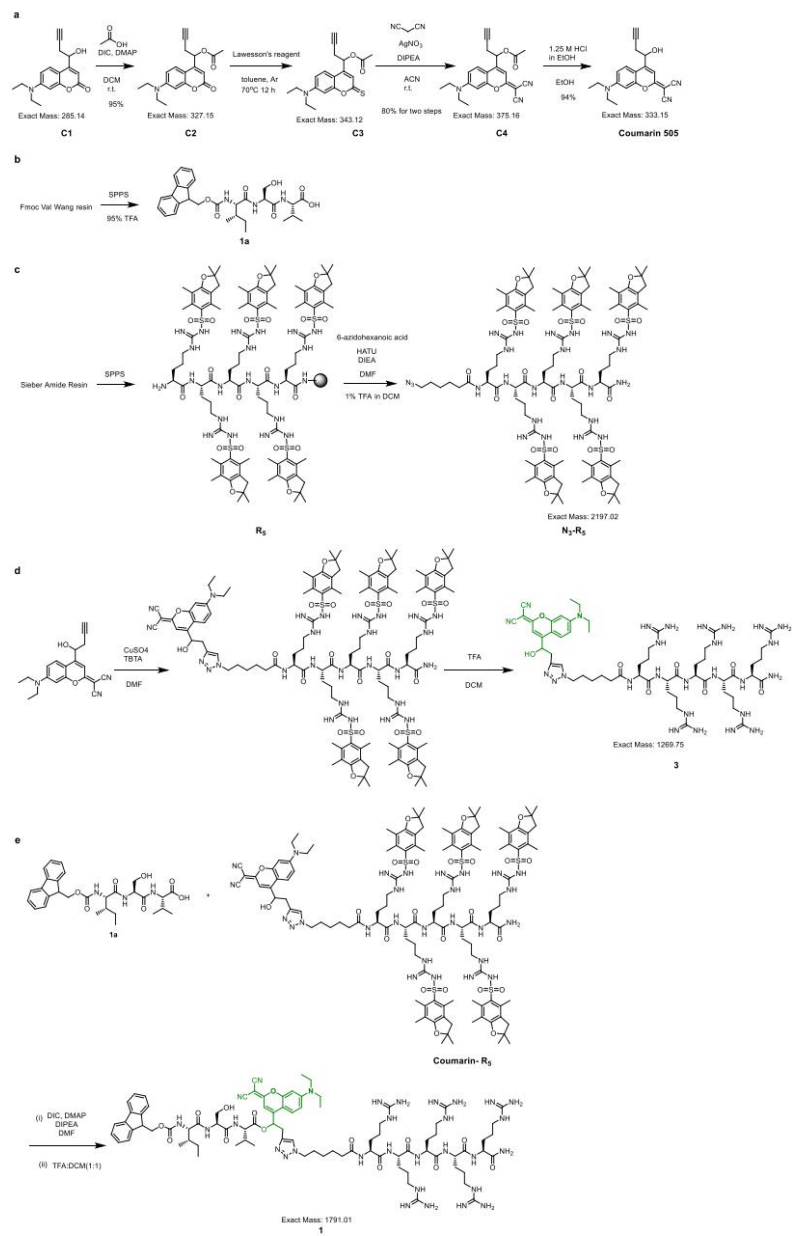
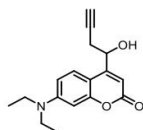


Fig. S1. Synthesis route of compound **1**. **a**, synthesis route of compound **coumarin 505**; **b**, synthesis route of peptide **1a**; **c**, synthesis route of peptide **N₃-R₅**; **d**, synthesis route of compound **3**; **e**, synthesis route of compound **1**.

2.1 Synthesis of compound C1:



Synthesis: The compound was synthesized according to a reported literature².

¹H NMR (400 MHz, DMSO-*d*₆, 298 K): δ 7.61 (d, *J* = 9.3 Hz, 1H), 6.70 (dd, *J* = 9.1, 2.6 Hz, 1H), 6.55 (d, *J* = 2.6 Hz, 1H), 6.12 (s, 1H), 5.87 (d, *J* = 4.6 Hz, 1H), 5.07 (t, *J* = 5.5 Hz, 1H), 3.45 (q, *J* = 7.0 Hz, 4H), 2.85 (t, *J* = 2.6 Hz, 1H), 2.75 – 2.56 (m, 2H), 1.14 (t, *J* = 7.0 Hz, 6H).

¹³C NMR (101 MHz, DMSO-*d*₆, 298 K): δ 161.58, 158.04, 156.48, 150.56, 126.21, 109.06, 106.00, 105.61, 97.46, 81.23, 73.94, 67.03, 44.41, 27.50, 12.81.

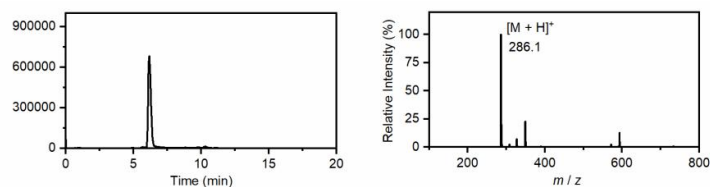


Fig. S2. LC-MS study of compound **C1**. HPLC trace (left) and corresponding MS spectrum (right), showing peaks of $[M + H]^+ = 286.1$.

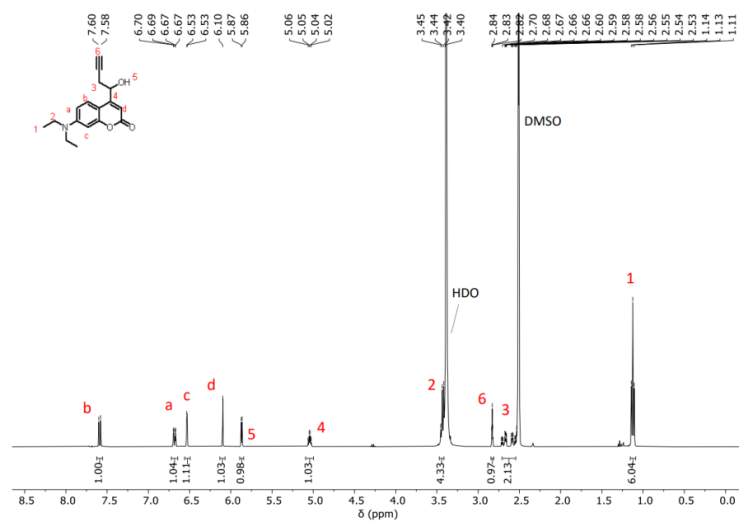


Fig. S3. ¹H NMR spectrum (400 MHz, DMSO-*d*₆, 298 K) of compound **C1**.

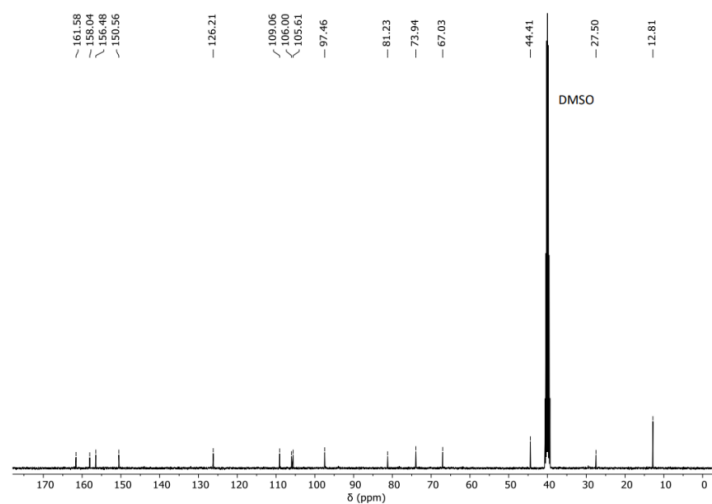
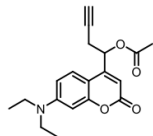


Fig. S4. ¹³C NMR spectrum (101 MHz, DMSO-*d*₆, 298 K) of compound **C1**.

2.2 Synthesis of compound C2:



Synthesis: To a solution of Compound **C1** in anhydrous DCM, acetic acid, DIC and a catalytic amount of DMAP were added and resulting solution was stirred at room temperature for 4 hours. The mixture was quenched with water and extracted with DCM. Combined organic phase was concentrated in vacuum and the mixture was purified by column chromatography. **C2** was obtained as a yellow powder (1.2 g, yield 95%).

¹H NMR (400 MHz, DMSO-*d*₆, 298 K): δ 7.62 (d, J = 9.3 Hz, 1H), 6.72 (dd, J = 9.1, 2.6 Hz, 1H), 6.56 (d, J = 2.6 Hz, 1H), 6.06 (t, J = 5.8 Hz, 1H), 6.02 (s, 1H), 3.45 (t, J = 7.1 Hz, 4H), 2.97 (t, J = 2.6 Hz, 1H), 2.84 (qdd, J = 17.4, 5.7, 2.6 Hz, 2H), 2.16 (s, 3H), 1.13 (t, J = 7.1 Hz, 6H).

¹³C NMR (101 MHz, DMSO-*d*₆, 298 K): δ 169.96, 161.07, 156.49, 153.40, 150.90, 125.92, 109.38, 105.39, 97.56, 89.78, 79.47, 74.77, 68.76, 44.47, 24.60, 21.09, 12.79.

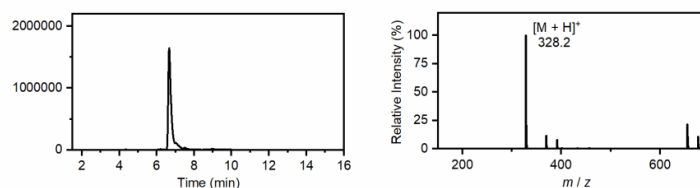


Fig. S5. LC-MS study of compound **C2**. HPLC trace (left) and corresponding MS spectrum (right), showing peaks of $[M + H]^+ = 328.2$.

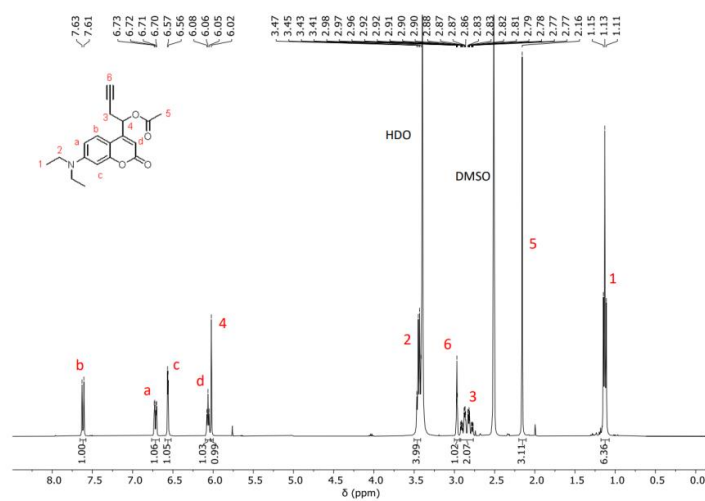


Fig. S6. ¹H NMR spectrum (400 MHz, DMSO-*d*₆, 298 K) of compound **C2**.

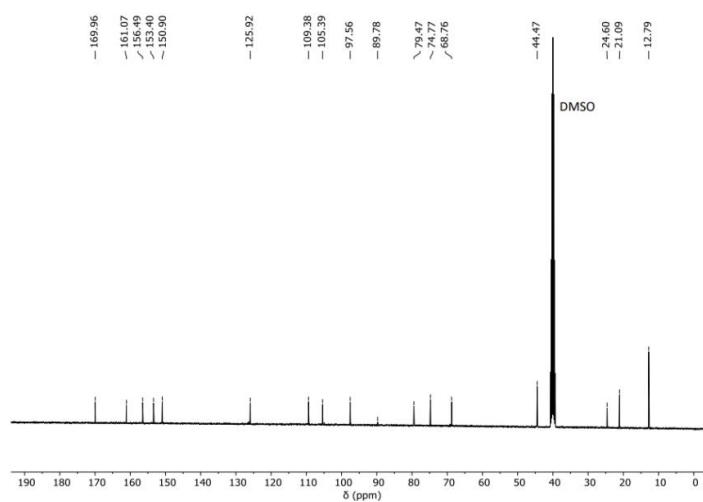
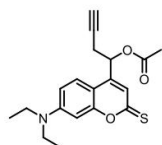


Fig. S7. ¹³C NMR spectrum (101 MHz, DMSO-*d*₆, 298 K) of compound **C2**.

2.3 Synthesis of compound C3:



Synthesis: Lawesson's reagent was added to a solution of compound and refluxed in dry toluene under inert gas and dark overnight. The evaporation of the solvent and subsequently mixture was used in the next step without further purification.

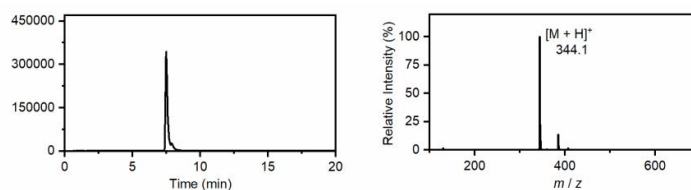
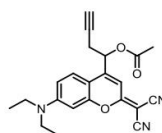


Fig. S8. LC-MS study of compound **C3**. HPLC trace (left) and corresponding MS spectrum (right), showing peaks of $[M + H]^+ = 344.1$.

2.4 Synthesis of compound C4:



Synthesis: Silver nitrate was added to a solution of **C3**, malononitrile, and DIPEA in dry CAN under an argon atmosphere. The reaction mixture was stirred for 3 h in the dark at room temperature and then concentrated under reduced pressure. The crude was purified by column chromatography to give a 500 mg (yield 80 % for two steps) of an orange solid.

^1H NMR (400 MHz, $\text{DMSO}-d_6$, 298 K): δ 7.82 (d, $J = 9.4$ Hz, 1H), 6.93 (dd, $J = 9.3, 2.6$ Hz, 1H), 6.71 (d, $J = 2.6$ Hz, 1H), 6.66 (s, 1H), 6.22 (t, $J = 5.6$ Hz, 1H), 3.55 (q, $J = 7.1$ Hz, 4H), 3.04 (t, $J = 2.6$ Hz, 1H), 3.00 – 2.82 (m, 2H), 2.18 (s, 3H), 1.19 (t, $J = 7.0$ Hz, 6H).

^{13}C NMR (101 MHz, $\text{DMSO}-d_6$, 298 K): δ 171.61, 169.81, 155.40, 152.20, 151.07, 126.84, 112.57, 111.87, 106.82, 104.63, 97.04, 79.13, 75.20, 68.81, 53.08, 44.75, 24.85, 21.07, 12.78, 8.84.

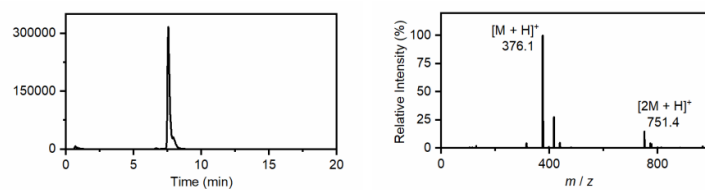


Fig. S9. LC-MS study of compound **C4**. HPLC trace (left) and corresponding MS spectrum (right), showing peaks of $[M + H]^+ = 376.1$ and $[2M + H]^+ = 751.4$.

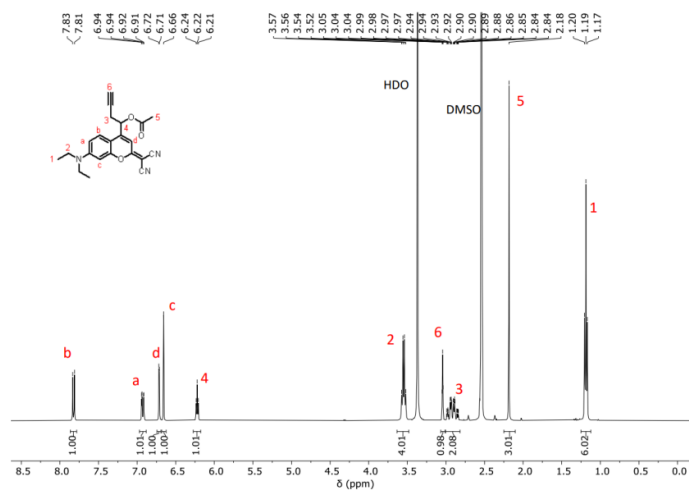


Fig. S10. ¹H NMR spectrum (400 MHz, DMSO-*d*₆, 298 K) of compound **C4**.

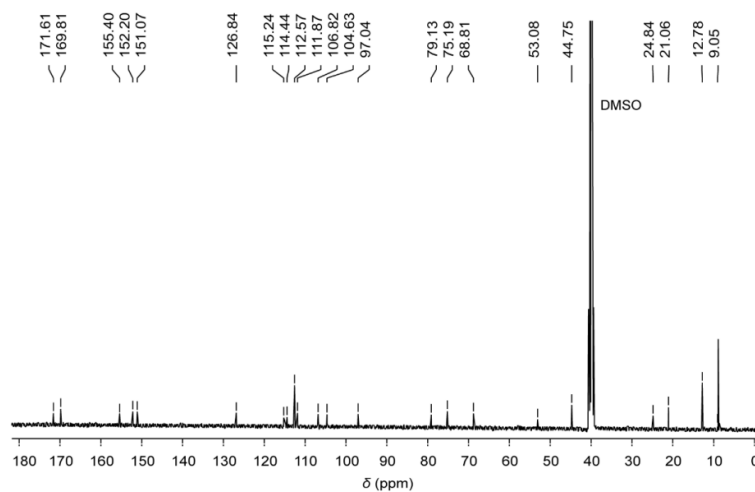
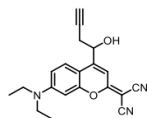


Fig. S11. ^{13}C NMR spectrum (101 MHz, $\text{DMSO}-d_6$, 298 K) of compound **C4**.

2.5 Synthesis of compound Coumarin 505:



Synthesis: **C4** was solved in 20 mL 1.2 M HCl and heated for 5 h at 60°C. Water was removed under reduced pressure and mixture was purified using a column chromatography on silica gel with EA/cyclohexane 1:5 to give an orange solid (180 mg, yield 94%).

^1H NMR (400 MHz, CDCl_3-d_6 , 298 K): δ 7.42 (d, J = 9.3 Hz, 1H), 6.93 (d, J = 0.9 Hz, 1H), 6.66 (dd, J = 9.1, 2.6 Hz, 1H), 6.60 (d, J = 2.6 Hz, 1H), 5.17 (q, 1H), 3.45 (q, J = 7.1 Hz, 4H), 2.82 (ddd, J = 17.0, 4.3, 2.6 Hz, 1H), 2.67 (s, 1H), 2.66 – 2.57 (m, 1H), 2.21 (t, J = 2.7 Hz, 1H), 1.24 (t, J = 7.1 Hz, 6H).

^{13}C NMR (101 MHz, $\text{DMSO}-d_6$, 298 K): δ 171.98, 156.62, 155.25, 151.88, 126.88, 115.65, 114.76, 111.62, 107.30, 104.93, 96.83, 80.77, 74.39, 66.42, 52.00, 44.68, 27.99, 12.79.

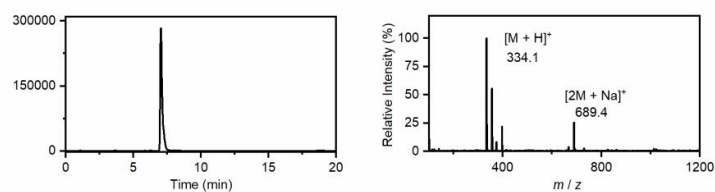


Fig. S12. LC-MS study of compound **Coumarin 505**. HPLC trace (left) and corresponding MS spectrum (right), showing peaks of $[M + H]^+ = 334.1$ and $[2M + Na]^+ = 689.4$.

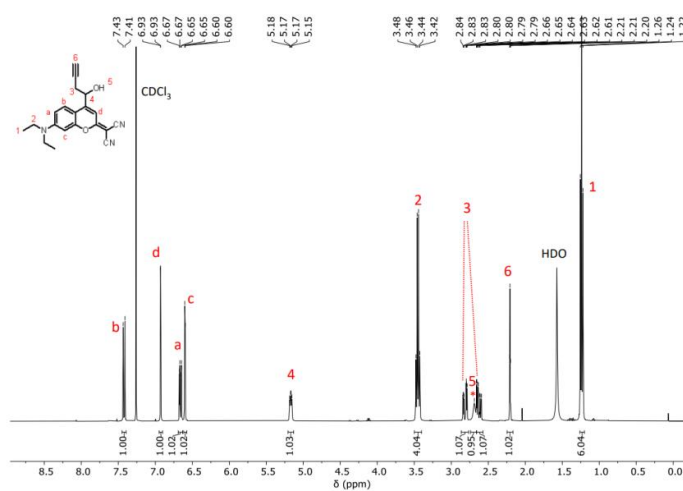


Fig. S13. ^1H NMR spectrum (400 MHz, CDCl_3 - d_6 , 298 K) of **Coumarin 505**.

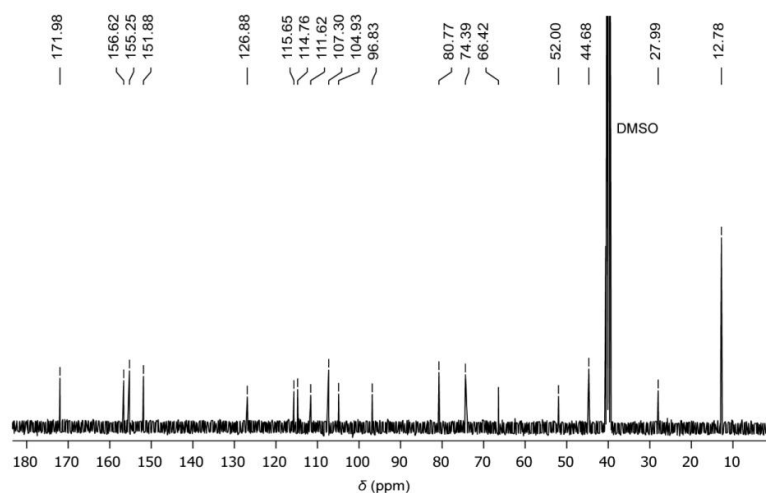
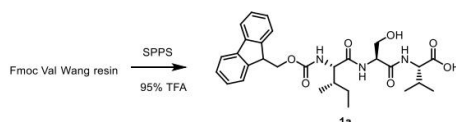


Fig. S14. ^{13}C NMR spectrum (101 MHz, $\text{DMSO}-d_6$, 298 K) of **Coumarin 505**.

2.6 Synthesis of **1a**:



Synthesis of 1a: Compound **1a** was synthesized using Merrifield's Fmoc solid-phase peptide synthesis strategy, constructing the peptide from the C-terminus to the N-terminus in a microwave-assisted peptide synthesizer at a 0.25 mmol scale. Fmoc-Val preloaded Wang resin (0.5 mmol) was swollen in DMF for 1 h before use. After deprotection of the Fmoc group (3 mL of 20% piperidine in DMF, 2 and 5 min at 75 °C) (i), Fmoc-Ser(tBu) (ii), Fmoc-Ile (iii) were double coupled onto the peptide at 75 °C for 20 min. 5 equiv. of the compounds were used in 2.5 mL DMF. PyBOP (5 equiv. in 1 mL DMF) and DIPEA (10 equiv. in 0.5 mL) were used for the coupling reaction. The product was cleaved from the resin by using 2.5 mL of a cleavage cocktail (95% CF_3COOH , 2.5% TIPS, 2.5% H_2O). The product was purified by HPLC using the Zorbax Eclipse column at a flowrate of 4 mL/min. The gradient started with 20% CH_3CN in H_2O (+0.1% CF_3COOH) and this solvent ratio was kept for 1 min, after which the CH_3CN content was increased to 100% in 15 min. The compound eluted from the column after 5.5 min and was received as a yellow powder after lyophilization (15 mg, 11.2 % overall yield).

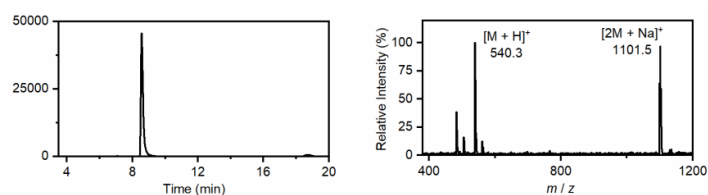
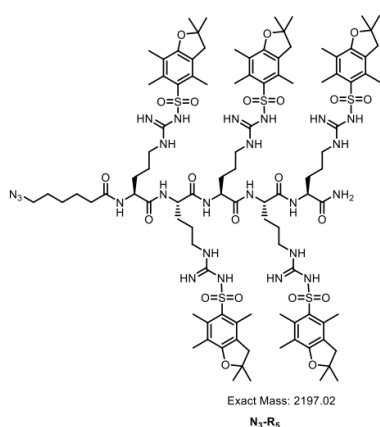


Fig. S15. LC-MS study of compound **1a**. HPLC trace (left) and corresponding MS spectrum (right), showing peaks of $[M + H]^+ = 540.3$, $[2M + Na]^+ = 1101.5$.

2.7 Synthesis of **N₃-R₅**:



Compound **N₃-R₅** was synthesized using the Fmoc solid phase peptide synthesis strategy by Merrifield, synthesizing the peptide from C to N-terminus in a microwave assisted peptide synthesizer. Sieber amide resin was used to prepare the peptide N3-5R on resin. Next, 6-azidohexanoic acid (1.2 eq), HATU (2.0 eq) and DIPEA (4.0 eq) were added into **R₅** on resin in 2 mL DMF solution. After overnight reaction, 1% trifluoroacetic acid (TFA) in dichloromethane (DCM) was used for cleavage for a duration of 30 minutes. Subsequently, the resulting product underwent purification through preparative high-performance liquid chromatography (prep-HPLC) to get pure compound **N₃-R₅** (60 mg, 26 % overall yield).

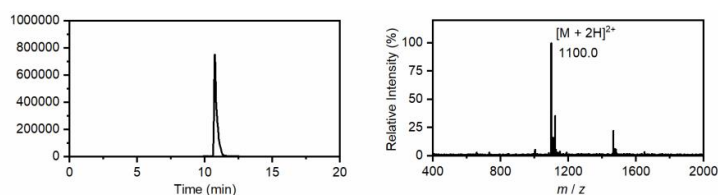


Fig. S16. LC-MS study of compound **N₃-R₅**. HPLC trace (left) and corresponding MS spectrum (right), showing peaks of $[M + 2H]^{2+} = 1100.0$.

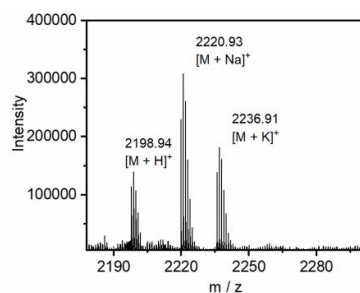
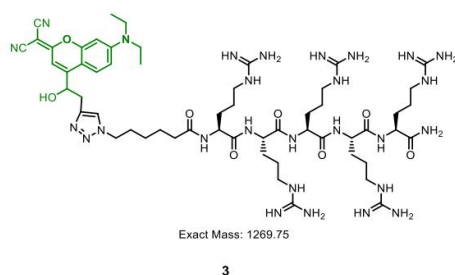


Fig. S17. MALDI-TOF-MS spectrum for **N₃-R₅**. Peaks at $m/z = 2198.94$, 2220.93 , 2236.91 can be identified, correspond to $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$.

2.8 Synthesis of 3:



Synthesis of **3**: **Coumarin 505** (1.0 eq), CuSO_4 (0.76 eq), $\text{Tris}((1\text{-benzyl-4-triazolyl})\text{methyl})\text{amine}$ (TBTA) (0.76 eq) and Sodium ascorbate (4.0 eq) were added into **N₃-R₅** in 5 mL DMF solution. After 2 h reaction at 50 °C in the microwave, resulting crude product was purified using prep-HPLC. Then the product was cleaved in the presence of 95% TFA, after which compound **3** was purified using prep-HPLC to get pure compound **3** (9 mg, 24 % overall yield).

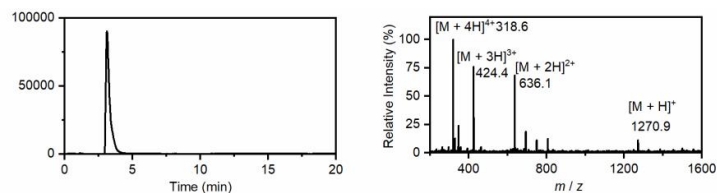


Fig. S18. LC-MS study of compound **3**. HPLC trace (left) and corresponding MS spectrum (right), showing peaks of $[M + H]^+ = 1270.9$, $[M + 2H]^{2+} = 636.1$, $[M + 3H]^{3+} = 424.4$ and $[M + 4H]^{4+} = 318.6$.

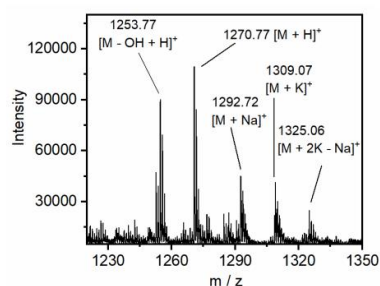
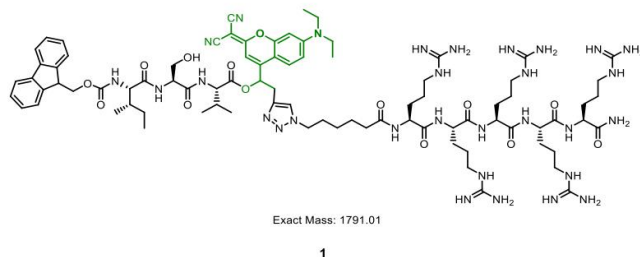


Fig. S19. MALDI-TOF-MS spectrum for **3**. Peaks at $m/z = 1253.77$, 1270.77 , 1292.72 , 1309.07 , 1325.06 can be identified, correspond to $[M - OH + H]^+$, $[M + H]^+$, $[M + Na]^+$, $[M + K]^+$ and $[M + 2K - Na]^+$.

2.9 Synthesis of **1**:



Synthesis of **1**: **1a** (1.0 eq) and **Coumarin-R₅** (1.0 eq) were added into 5 ml DMF, then DIC (2.0 eq), DMAP (0.2 eq) were added into the mixture. After overnight reaction at room temperature, the crude product was purified by prep-HPLC. The resulting product was deal with TFA/DCM (1:1) for 2 h to remove the protection group. After the reaction is finished, the crude product was purified by prep-HPLC to get pure compound **1** (13 mg, 38 % yield).

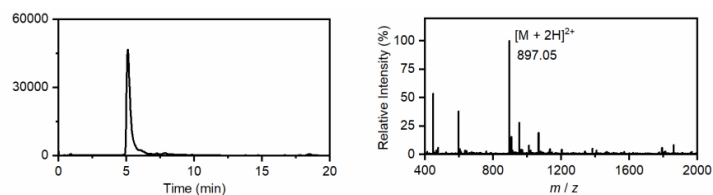


Fig. S20. LC-MS study of compound **1**. HPLC trace (left) and corresponding MS spectrum (right), showing peaks of $[M + 2H]^{2+} = 897.05$.

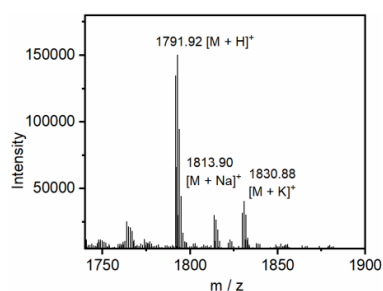
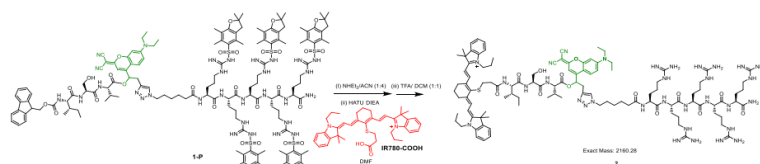


Fig. S21. MALDI-TOF-MS spectrum for **1**. Peaks at $m/z = 1791.92$, 1813.90 , 1830.88 can be identified, correspond to $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$.

2.10 Synthesis of **2**:



Synthesis of **2**: **1-P** (1.0 eq) was added in Acetonitrile/ Diethylamine (v:v=4:1) at room temperature for 30 min to remove the Fmoc group, after which the mixture was washed three times with water. The organic phase was dried and then concentrated under reduced pressure, the resulting oil was added into 5 mL DMF, then **IR780-COOH** (1.0 eq), DIC (2.0 eq), DMAP (0.2 eq) were added into the mixture. After overnight reaction at room temperature, the crude product was purified by prep-HPLC. The resulting product was deal with TFA/DCM (1:1) for 2 h to remove the protection group. After the reaction is complete, the crude product was purified by prep-HPLC to get pure compound **1** (8 mg, 28% yield).

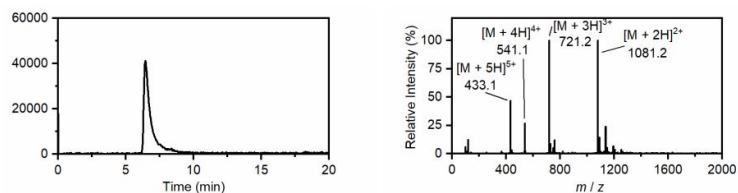


Fig. S22. LC-MS study of compound **2**. HPLC trace (left) and corresponding MS spectrum (right), showing peaks of $[M + 5H]^{5+} = 433.1$, $[M + 4H]^{4+} = 541.1$, $[M + 3H]^{3+} = 721.2$, $[M + 2H]^{2+} = 1081.2$.

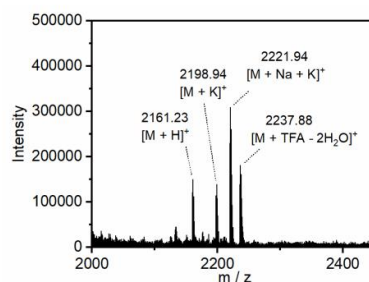


Fig. S23. MALDI-TOF-MS spectrum for **2**. Peaks at $m/z = 2161.23$, 2198.94 , 2221.94 , 2237.88 can be identified, correspond to $[M + H]^+$, $[M + K]^+$, $[M + Na + K]^+$ and $[M + TFA - 2H_2O]^+$.

3 Self-assembly profiles

3.1 TEM analysis of the assemblies

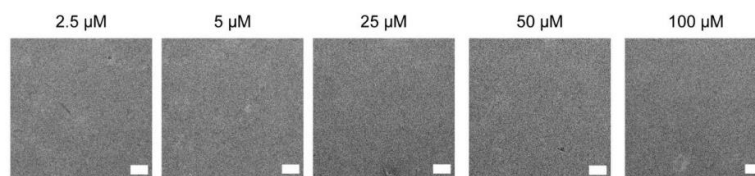


Fig. S24. TEM micrographs of the nanostructures formation upon visible light-induced conversion of **2** at various concentrations. The reaction mixture was then drop-casted on a carbon grid and imaged using TEM. Scale bars, 200 nm. The results indicate that the conversion from precursor **2** into **2a** cannot result in the formation of nanofibers. So co-assembly of **1a** and **2a** is necessary to form nanofibers.

3.2 Stability and kinetics study of precursor

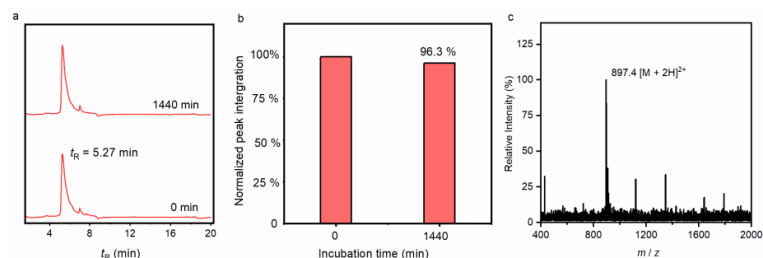


Fig. S25. Stability study of precursor **1** in the absence of light. The aqueous solution of **1** (25 μ M) was kept in room temperature for 24 h in the dark. **a**, Comparison of HPLC spectra of compound **1** (25 μ M) for 0 and 1440 min. **b**, Normalized peak integration of the HPLC traces of compound **1** before and after the incubation for 1440 min, which revealed that 96.3 % of the compound **1** remained in the mixture. **c**, Convolved MS spectrum of $t_R = 5.27$ min after incubation for 1440 min. The peak was identified as compound **1**.

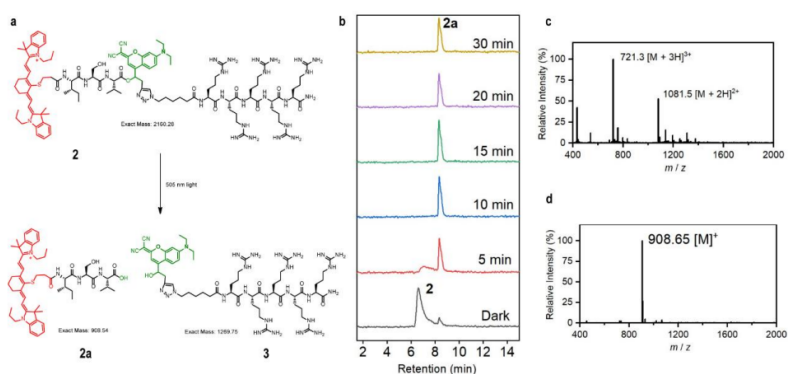


Fig. S26. LC-MS kinetic analysis over photolysis reaction of **2** (2.5 μ M), same concentration of intracellular co-assembly upon irradiation of 505 nm light. **a**, reaction scheme for the light-induced conversion. The photolysis reaction leads to the formation of compound **2a** and **3**. **b**, HPLC spectra for light-induced conversion of **2** to **2a** in NH_4HCO_3 buffer (pH 7.4, 20mM). **c**, convolved MS spectra for compound **2** in the presence of light. **d**, convolved MS spectra for compound **2a**.

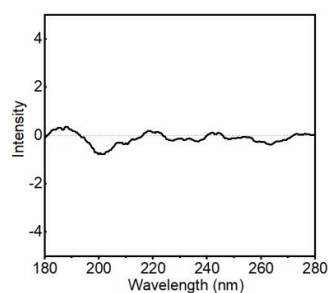


Fig. S27. CD analysis of **1** in a mixture of PB (pH 7.4, 100 mM) and MeOH (0.5 vol %) in the absence of irradiation. The figure indicated the precursor **1** showed no obvious signal between 180 and 280nm, therefore did not form any secondary structures.

3.3 Confocal imaging of co-assembled nanofibers in a cell-free system

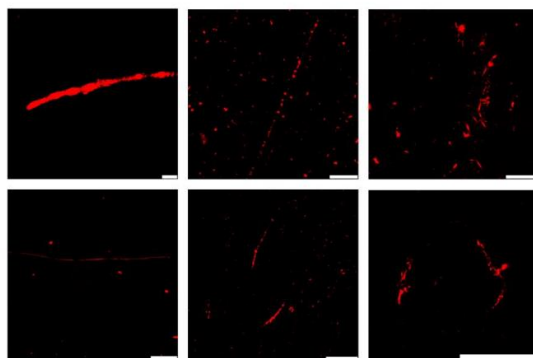


Fig. S28. Fluorescence microscope images of **1a** + **2a** (ratio = 9:1) co-assembled nanofibers. Scale bars, 20 μ M. Co-assembled nanofibers were prepared in DMEM and then imaged using confocal microscopy, revealing the presence of NIR emitting fibers. The results demonstrated that the co-assembly strategy is feasible.

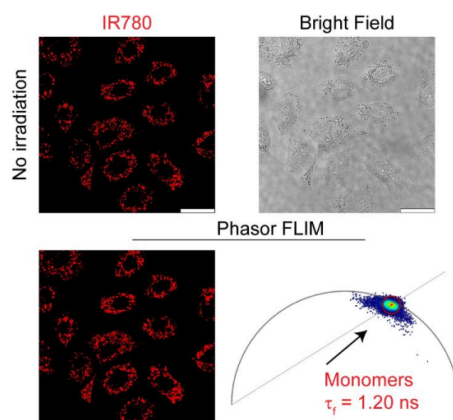


Fig. S29. Phasor-FLIM analysis of free molecules in the absence of irradiation. The free molecule control was accomplished using non-irradiated **1+2** (mol. ratio = 9:1) in A549 cells. Scale bars, 25 μ m.

4 Cellular uptake and intracellular self-assembly of **1** and **2**

A549 cells were seeded at a density of 25,000 cells/well in an 8-well confocal plate. After adhering for 24 h, cells were treated with the sample for 4 h at 37 °C. The sample was dissolved in DMSO at a concentration of 10 mM, then the sample was further diluted with DPBS to a final volume of 40 μ L (DPBS: DMSO = 9:1). Sample solutions were further diluted 1:4 with DMEM and added to the cells (total DMSO content = 2%), after removing the existing medium from the wells. After the incubation time was over, the cells were washed three times using fresh medium. Then the cells were imaged by the phasor-resolved fluorescence lifetime imaging microscopy (phasor-FLIM). For irradiation groups, after 4 h incubation, the cells were washed three times to remove the excess extracellular precursors. After washing, irradiation was performed using a 505 nm light source for the required duration.

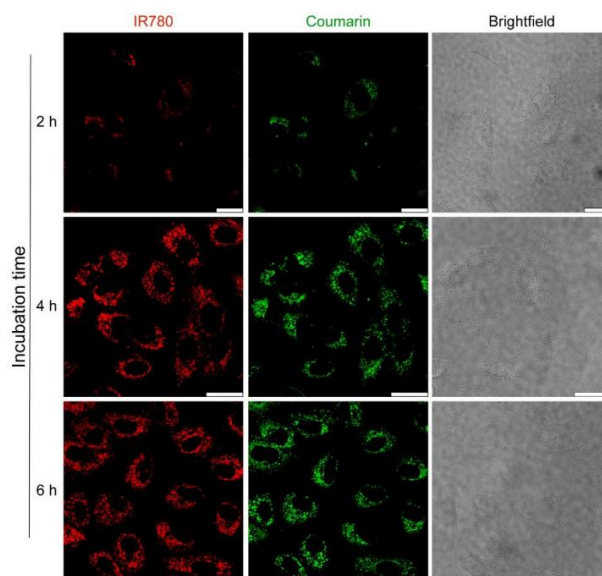


Fig. S30. Confocal laser scanning micrographs of A549 cells treated with 1 and 2 (25 μ M, ratio of 1:2 = 9:1) for different times, including 2 h, 4 h, 6 h. Cellular uptake was tested according to the NIR fluorescence and green fluorescence. Incubation for 4 h is an ideal time point for imaging experiment. Scale bars, 25 μ m.

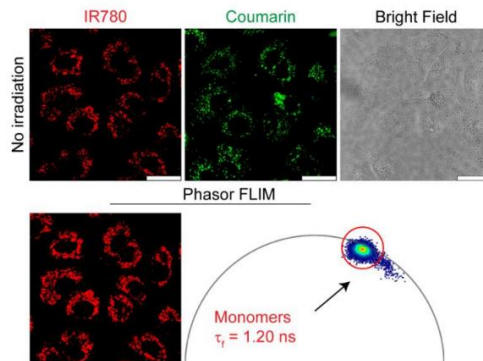


Fig. S31. Phasor-FLIM analysis of A549 cells treated for 4 h with 1 and 2 (25 μ M, ratio of 1:2 = 9:1) without irradiation. Scale bars, 25 μ m. The exclusive existence of a single-photon population with a τ_r of 1.20 ns indicated the presence of solely free molecules in the absence of irradiation.

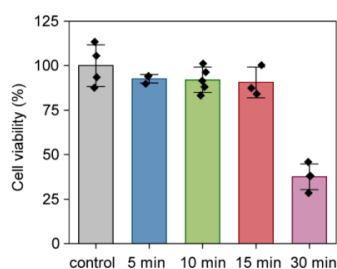


Fig. S32. Cell viability study of A549 cells treated for 4 h with 25 μ M of **1** with different irradiation times. After irradiation, immediate cell viability studies were conducted, revealing that 30 min irradiation reduced the cell viability to 37%.

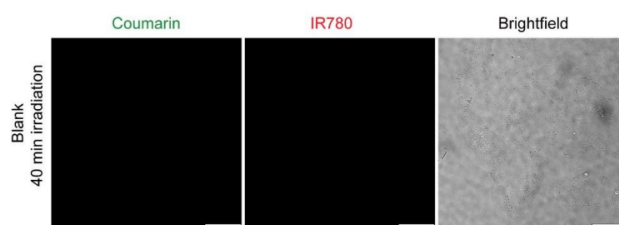


Fig. S33. Confocal laser scanning micrographs of untreated A549 cells after 40 min of irradiation. Scale bar, 20 μ m. The morphology of cells indicates a healthy state after irradiation for 40 min using a light with 505 nm wavelength.

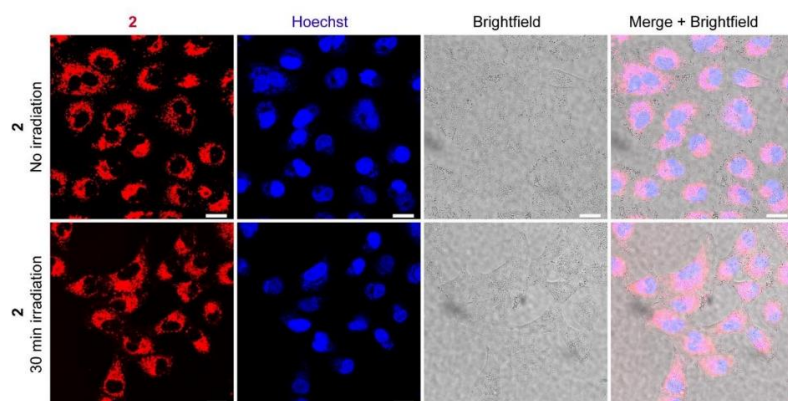


Fig. S34. Confocal laser scanning micrographs of A549 cells treated for 4 h with **2** (2.5 μ M, consistent with the concentration for co-assembly in cells) and Hoechst 33342 dye with and without 30 min irradiation. Scale bar, 20 μ m.

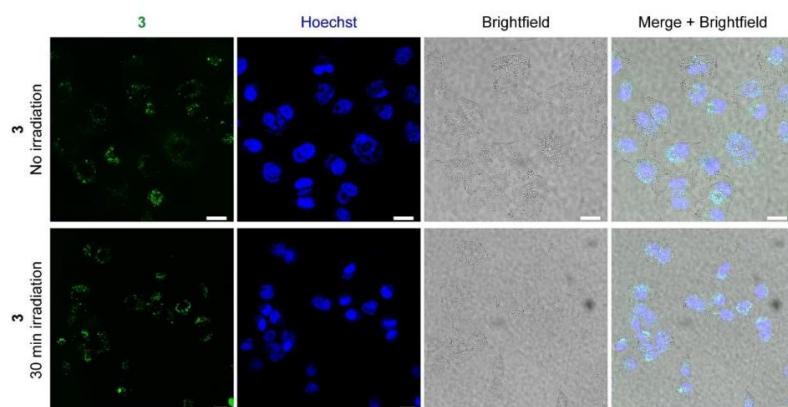


Fig. S35. Confocal laser scanning micrographs of A549 cells treated for 4 h with synthetic **3** (25 μ M) and Hoechst 33342 dye with and without 30 min irradiation. Scale bar, 20 μ m.

5 Cell viability assay

A549 cells were both 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. On the next day, different concentrations of **2** or **3** were prepared in PBS at a 10x concentration of the

final concentration and of this solution (20 μL) were added to fresh medium (180 μL). The old medium of the cells was removed and substituted with fresh medium (50 μL) containing different concentrations of sample, prepared as described before. For each condition triplicates were performed. Cells were incubated with the sample for 4h. Then the 30-min irradiation was performed using light with 505 nm wavelength. After the irradiation, CellTiter-Glo Assay solution (50 μL) were 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 the Promega GloMax® - Multi Detection System with using the provided protocol for luminescence readout.

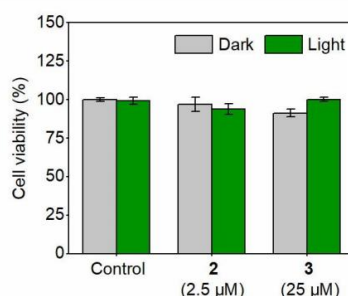


Fig. S36. Cell viability study of A549 cells treated for 4 h with blank, **2** (2.5 μM) and synthetic **3** (25 μM) with and without 30 min irradiation. Dark: the corresponding cells were incubated for 4 h without irradiation. Green: the corresponding cells were incubated with **2** or **3** for 4 h and then irradiated for 30 min.

A549 cells were both 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. On the next day, different concentrations of **1** were prepared in PBS at a 10x concentration of the final concentration and of this solution (20 μL) were added to fresh medium (180 μL). The old medium of the cells was removed and substituted with fresh medium (50 μL) containing different concentrations of sample, prepared as described before. For each condition triplicates were performed. Cells were incubated with the sample for 4h. After 4 h incubation, the cells were washed to remove the excess extracellular precursors. After that, the 30-min irradiation was performed using light with 505 nm wavelength. After the irradiation, CellTiter-Glo Assay solution (50 μL) were 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 the Promega GloMax® -Multi Detection System with using the provided protocol for luminescence readout.

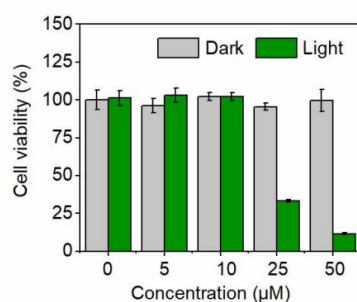


Fig. S37. Cell viability study of A549 cells treated for 4 h with different concentrations of **1** (5, 10, 25, 50 μM) with and without 30 min irradiation. Dark: the corresponding cells were incubated with different concentrations of **1** for 4 h without irradiation. Green: the corresponding cells were incubated with different concentrations of **1** for 4 h and then irradiated for 30 min.

6 Phasor-FLIM analysis of assembly dynamics in A549 cells for 1-4 h incubation after irradiation for 5 min, 10 min, 15 min or 30 min

A549 cells were seeded at a density of 25,000 cells/well in an 8-well confocal plate. After adhering for 24 h, cells were treated with the sample for 4 h at 37 °C. The sample was dissolved in DMSO at a concentration of 10 mM, then the sample was further diluted with DPBS to a final volume of 40 μL (DPBS: DMSO = 9:1). Sample solutions were further diluted 1:4 with DMEM and added to the cells (total DMSO content = 2%), after removing the existing medium from the wells. After the incubation time was over, the cells were washed three times using fresh medium. Afterwards the cells were irradiated for 5 min, 10 min, 15 min and 30 min using 505 nm wavelength light. Then the cells were imaged by the phasor-resolved fluorescence lifetime imaging microscopy (phasor-FLIM) over 1-4 h incubation.

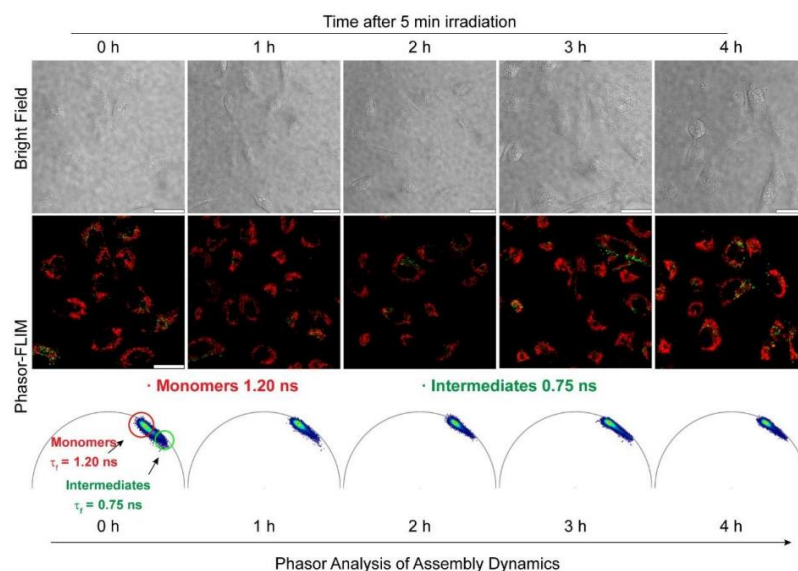


Fig. S38. Brightfield and phasor-FLIM images of A549 cells for 1-4 h incubation with **1** and **2** (25 μ M, ratio of **1:2** = 9:1) after irradiation for **5 min**. Scale bars, 25 μ m. Arrows indicates over 4 h of incubation, only monomers and intermediates exist and no further assembly progression occurred.

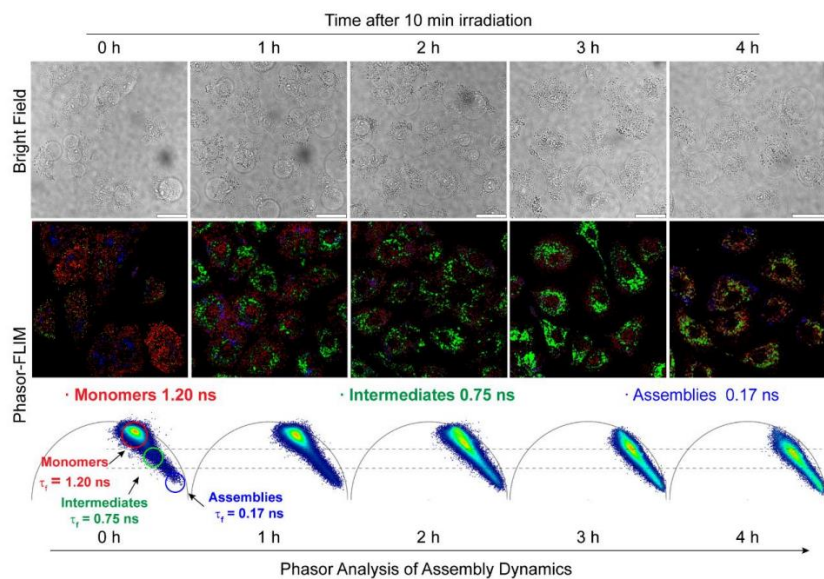


Fig. S39. Brightfield and phasor-FLIM images of A549 cells for 1-4 h incubation with **1** and **2** (25 μ M, ratio of **1:2** = 9:1) after irradiation for **10 min**. Scale bars, 25 μ m.

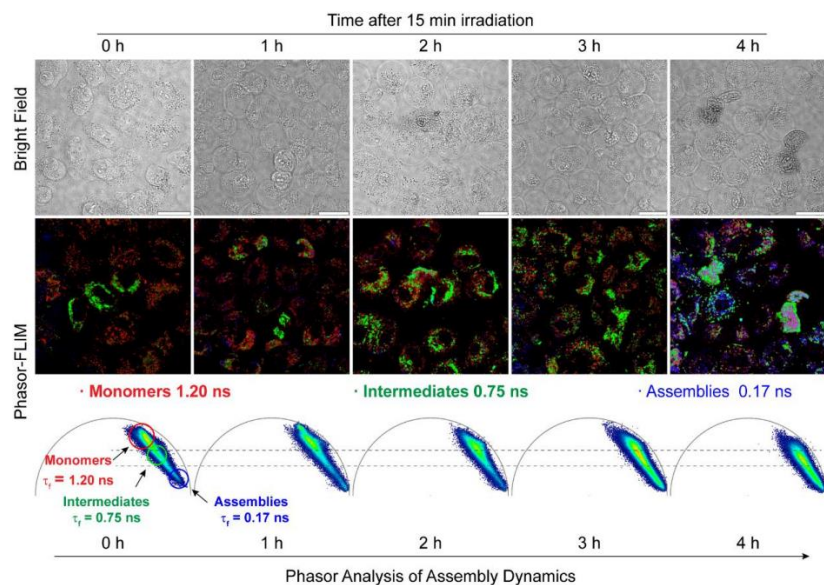


Fig. S40. Brightfield and phasor-FLIM images of A549 cells for 1-4 h incubation with **1** and **2** (25 μ M, ratio of **1:2** = 9:1) after irradiation for **15 min**. Scale bars, 25 μ m.

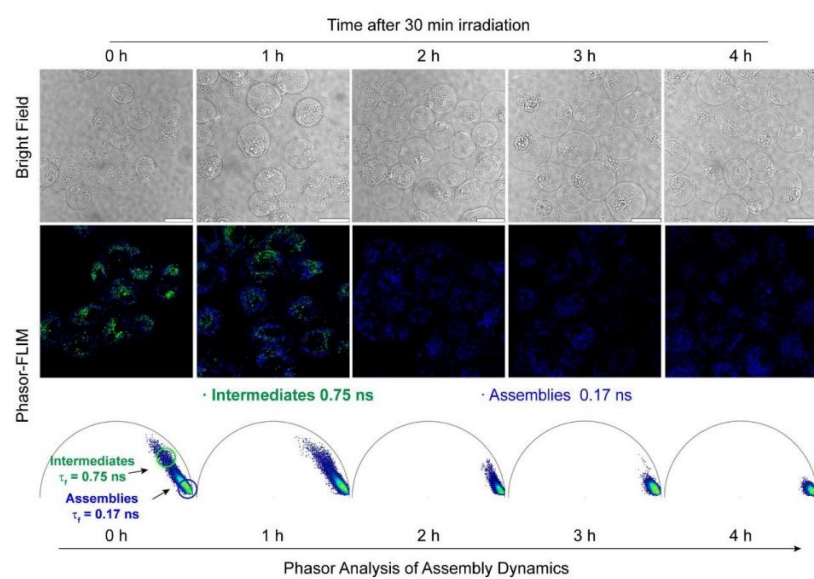


Fig. S41. Brightfield and phasor-FLIM images of A549 cells for 1-4 h incubation with **1** and **2** (25 μ M, ratio of **1:2** = 9:1) after irradiation for **30 min**. Scale bars, 25 μ m.

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<https://doi.org/10.1038/s41596-018-0026-5>
- 2 Seyfried, P., Eiden, L., Grebenovsky, N., Mayer, G. & Heckel, A. Photo-Tethers for the (Multi-)Cyclic, Conformational Caging of Long Oligonucleotides. *Angewandte Chemie International Edition* **56**, 359-363 (2017).
<https://doi.org/https://doi.org/10.1002/anie.201610025>

4 Conclusion

The investigation of bioresponsive peptide nanostructures for intracellular assembly focuses on the use of endogenous or exogenous stimuli to drive the programmed formation of synthetic nanostructures within living cells. Although numerous systems have been developed as intracellular assembly platforms for cancer therapy, several challenges in this field remain unexplored. These challenges include a comprehensive understanding of the kinetics of supramolecular assembly processes within living cells, the precise control of these assembly processes, and the mechanisms behind how structure formation affects cellular metabolism. To address these issues, this thesis investigated the molecular design, synthesis and characterization of three different assembly systems and their application to intracellular structure formation in living cells.

In the first work, we aimed to expand the diversity of building blocks that drive self-assembly and to investigate the underlying mechanisms and biochemical profiles of supramolecular chemistry. To achieve this, we focused on designing and constructing a platinum(II)-containing tripeptide capable of controlled nanostructure formation within living cells. The molecular design, synthesis, characterization of the assembly dynamics in buffer and inside cells were described. The findings demonstrated that the endogenous pH/H₂O₂-induced nanofiber formation can disrupt metabolic functions such as aerobic glycolysis and oxidative phosphorylation, and consequently systematically lead to apoptosis. The correlation between chemical transformations and biological activity enabled a deeper understanding of the mechanism of action of nanofiber formation.

In the second project, aiming to track assembly events in situ within the complex intracellular environment and correlate them with associated biological effects, we employed a chemically designed pro-assembling molecule, an NDI-containing peptide, to visualize its transformation into nanofibers using phasor-FLIM. This approach enabled us to map the dynamic assembly process and, through correlative light and electron microscopy (CLEM), reveal the fusion of these nanofibers with endosomes, leading to the formation of hollow fiber clusters. Unlike conventional intensity-based microscopy, phasor-FLIM allowed real-time resolution of the assembly process by detecting shifts in the fluorescence lifetime of the peptide's fluorophore. The time-lapse study, in conjunction with CLEM, provided a precise rationalization of the assembly

process from the molecular to the nanoscale, where precursor peptides developed into hollow nanofiber clusters under endosomal fusion. The onset of assembly was critical in the disruption of endosomal function, severely impairing the associated glucose-dependent metabolism and cellular respiration. In contrast, the growth of mature assemblies and accumulation did not induce additional biological responses.

In the third work, to improve the precision of peptide assembly regulation within living cells, we investigated the use of a visible-light controlled system for intracellular structure formation inside living cells and tracked distinct assembly states and their subsequent assembly dynamics using phasor-FLIM. In contrast to other endogenous stimulus-responsive assembly systems, the irradiation with light enable full control over the reaction cascade where the monomer generation and concentration in turn regulates the assembly kinetics. To achieve this, a coumarin-based photocage was utilized due to its compatibility with biocompatible wavelengths, which can be cleaved by visible light at 505 nm, and a relatively slow photolysis rates (30 minutes for complete cleavage), which provides an extended detection window for studying assembly kinetics. Furthermore, the complex intracellular environment imposed by the high density of bio-macromolecules implicates the reaction and assembly dynamics. To address these challenges, in-situ technologies such as Phasor-FLIM was used to track the formation of various assembly states within cells and reveal subsequent dynamics associated with monomer activation and consumption. These temporally resolved assemblies show that the emergence of cytotoxicity is correlated with the accumulation of oligomers beyond the cellular efflux threshold.

In summary, this thesis presented the three different strategies for controlled assembly platforms for intracellular structure formation and investigated their biological effects in living cells. Despite the progress made, several challenges and unresolved issues remain in this research, necessitating further research. Factors such as the complex and transient nature of the assembly pathways, as well as the dynamic and crowded nature of the intracellular environment, continue to impede a comprehensive understanding of these molecular-to-nanoscale processes. Greater focus is needed on improving targeting efficiency between cancer and healthy cells, mimicking natural dynamic assembly and disassembly processes, and controlling assembly within distinct subcellular compartments. Addressing these challenges will provide deeper insights, thereby facilitating assembly-driven macromolecular drug discovery and

advancing cancer treatment.

5 Publications

Yong Ren, Zhixuan Zhou, Iain Harley, Özlem Aydin, Leon Driehaus-Ortiz, Anke Kaltbeitzel, Jiaqi Xing, Konrad Maxeiner, Ingo Lieberwirth, Katharina Landfester, David YW Ng*, Tanja Weil* Visible Light-Controlled Intracellular Synthesis of Supramolecular Peptide Nanostructures. *Nat. Synth* (2025). *Accepted*. (*ChemRxiv*, doi.org/10.26434/chemrxiv-2024-2w2cs)

Yong Ren, Zhixuan Zhou, Konrad Maxeiner, Anke Kaltbeitzel, Iain Harley, Jiaqi Xing, Yingke Wu, Manfred Wagner, Katharina Landfester, Ingo Lieberwirth, Tanja Weil*, and David Y. W. Ng* Supramolecular Assembly in Live Cells Mapped by Real-Time Phasor-Fluorescence Lifetime Imaging. *J. Am. Chem. Soc.* 2024, 146, 17, 11991–11999

Zhixuan Zhou, Konrad Maxeiner, Pierpaolo Moscariello, Siyuan Xiang, Yingke Wu, **Yong Ren**, Colette J Whitfield, Lujuan Xu, Anke Kaltbeitzel, Shen Han, David Mücke, Haoyuan Qi, Manfred Wagner, Ute Kaiser, Katharina Landfester, Ingo Lieberwirth, David YW Ng*, Tanja Weil* In situ assembly of platinum (II)-metallopeptide nanostructures disrupts energy homeostasis and cellular metabolism. *Journal of the American Chemical Society* 144 (27), 12219-12228

Giulia Giubertoni, Sarah Chagri, Pablo G Argudo, Leon Prädel, Daria Maltseva, Alessandro Greco, Federico Caporaletti, Alberto Pavan, Ioana M Ilie, **Yong Ren**, David YW Ng, Mischa Bonn, Tanja Weil, Sander Woutersen* Structural adaptability and surface activity of peptides derived from tardigrade proteins. *Protein Science* 33 (9), e5135

Sarah Chagri, Anna Burgstaller, Claudia Schirra, Julian Link, Zhixuan Zhou, Patrick Roth, Raphael Meyer, Jana Fetzer, **Yong Ren**, Shutian Si, Francesca Mazzotta, Manfred Wagner, Ingo Lieberwirth, Katharina Landfester, David Y.W. Ng*, Oskar Staufer*, Tanja Weil* Synthetic intracellular nanostructures enhance cytotoxic T cell function via assembly-driven chemical engineering. *ChemRxiv*, doi: 10.26434/chemrxiv-2024-3lxgk

6 Curriculum Vitae

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7 Acknowledgement

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I am also deeply appreciative of my group leader, Dr. David Y. W. Ng, for his mentorship throughout my PhD. I am especially grateful for his time, support, and the many inspiring scientific discussions—always available, except during lunchtime, of course. I have particularly cherished our spontaneous conversations around his office door, where he continuously shared thought-provoking ideas that fueled my curiosity and creativity. His keen scientific insight and high standards have greatly influenced my approach to research, helping me refine my skills and broaden my perspective. His expertise and guidance have played a pivotal role in my development as a researcher.

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