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

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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. 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 or as nucleating centers toward the amyloidosis in Type 2 diabetes. 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. 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. 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. 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. 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. In this regard, various supramolecular strategies have recently been developed and shown to be effective to target the accelerated metabolism of cancer. By coupling an in-depth metabolic study with a switchable self-assembling...
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-
amphiphilic character and allows them to stack in an hydrophobic environment, thus forming a nanofiber. The nanofibers are characterized by a high degree of molecular order, as evidenced by the TEM image in Figure 3A. The cryogenic high-resolution TEM image (Figure 3B) reveals a nanofiber formation with a high degree of molecular order, forming a nanofiber with a diameter of 2.9 nm. Nanoinsight into the mechanism of action of the formed nanofiber is provided by the cryogenic high-resolution TEM image (Figure 3B). The SAED pattern (Figure 3C) of the nanofiber shows the progression from a dormant assembling precursor to the nanofiber formation, providing insights into the nanofiber's structural properties.

The nanofiber is composed of three functionalities (Figure 2A): (1) a pro-assembling isopeptide (ISA) caged by an immolative boronic acid group, (2) a transporter peptide, trans-activator of transcription (TAT), that interacts with cellular membranes, enabling cellular uptake and endosomal release. This peptide carries a SHA group that binds to the boronic acid via a pH responsive dynamic covalent bond. (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 its specific tasks 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. Once released to the near-neutral (pH 7.4) cytosol, endogenous H2O2 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 2NF (Figure 2A).

The synthesis of the Pt-isotripeptide 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 1 is performed in solution catalyzed by CuI, to a monomeric self-assembling peptide 2ff, which assembles into the nanofibers 2NF (Figure 2A). The nanofiber is composed of three functionalities (Figure 2A): (1) a pro-assembling isopeptide (ISA) caged by an immolative boronic acid group, (2) a transporter peptide, trans-activator of transcription (TAT), that interacts with cellular membranes, enabling cellular uptake and endosomal release. This peptide carries a SHA group that binds to the boronic acid via a pH responsive dynamic covalent bond. (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 its specific 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. Once released to the near-neutral (pH 7.4) cytosol, endogenous H2O2 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 2NF (Figure 2A).

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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. The increase to near-neutral pH within the cytosol empowers the oxidation of the boronic acid by endogenous H2O2, resulting in its immolation, thus converting 1 to iso-2 and initiating the rearrangement into 2. The kinetics of the H2O2-triggered formation of 2 in solution was investigated by LC−MS (Figures 2B−D and S28). In aqueous solution, incubation with H2O2 for 20 min resulted in the complete disappearance of 1 and the emergence of two new peaks with retention times (tR) 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 [2 + H]1+ and 991.3 for [2]2+ (Figure 2C and Figure S29). In comparison with 2 obtained via direct synthesis (tR = 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 H2O2 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 H2O2 (Figure S30). Similar conversion kinetics is observed when 1 was incubated with H2O2 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 dπ(Pt)→π*(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 d2 interactions between platinum(II) centers in neighboring molecules of 2, suggesting a proclivity for self-assembly in PB forming 2NF.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 (λmax = 792 nm) with a 1.5-fold higher...
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 H2O2-induced conversion and subsequent assembly of 2 into 2NF in PB. Treatment of 1·TAT with H2O2 (0.5 mM) led to a red shift in the λmax with a concomitant increase in emission intensity (Figure S33). The conversion is completed in 280 min with the final emission profile resembling 2NF, in agreement with the LC−MS study.

**Amyloid-Like Nanofibers 2NF Form with High Molecular Order.** The self-assembly of the complexes was visualized using transmission electron microscopy (TEM).
were observed for $2_{\text{NF}}$ 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 $2_{\text{NF}}$ showed diffraction arcs with a 3.3 Å lattice spacing, in agreement with the intermolecular distance of $\pi$-$\pi$ 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 $2_{\text{NF}}$ 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 $2_{\text{NF}}$ 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 $2_{\text{NF}}$ 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 group42 is also observed for $2_{\text{NF}}$, 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 $2_{\text{NF}}$ at the molecular level was further examined using NMR. At 298 K, $2_{\text{NF}}$ 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 $2_{\text{NF}}$ revealed NOE cross-peaks between the protons on the tpy group and the phenyl ring of the alkynyl ligand ($H^f/H^p$ and $H^l/H^p$) and between the non-neighboring protons on the tpy moiety ($H^f/H^l$, $H^p/H^l$, and $H^p/H^f$) (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 $2_{\text{NF}}$ 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→$2_{\text{NF}}$ 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, collinear 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 $2_{\text{NF}}$ can be detected through early changes in the cytoskeleton, whose formation is tightly coupled to glycolytic pathways.43,47 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 $2_{\text{NF}}$ has to be reached for actin restructuring. Actin ﬁlaments in cells are regulated by Rho-GTPases,49 which are accumulated on the lateral membranes in cultured mammalian epithelial cells.51 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.55 As the equilibrium of GTP and ATP is typically maintained by nucleoside-diphosphate kinases,56 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 $2_{\text{NF}}$ on the main mechanisms of cellular metabolism, AGlyc and OxPhos pathways, which are core upstream processes involving at least 10% of the human proteome.57 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 S5). Treatment of both cell lines with 1-TAT that transforms into $2_{\text{NF}}$ 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 $2_{\text{NF}}$ 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.45,46 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, $2_{\text{NF}}$ 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→$2_{\text{NF}}$ 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.45,46,47

Therefore, the AGlyc of A549 and MDA-MB-231 cells was measured using their extracellular acidification rate (ECA($R_{HCO_3}$)), 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 space58 (Figure 5F). The cells first underwent glucose starvation to account for the basal non-glycolytic
acidiﬁcation, followed by the addition of saturating amounts of glucose to trigger glycolysis. The formation of the nanoﬁbers at 25 μM shuts down glycolysis of A549 and MDA-MB-231 to 6 ± 1 and 13 ± 3%, respectively (Figure S1). The capability of cells rewiring their metabolism47 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 ± 1.6 mpH/min (A549) as well as +8.0 ± 1.5 mpH/min (MDA-MB-231) (Figure 5J). 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 5J). 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 → 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.46 Indeed, treated A549 and MDA-MB-231 cells showed concentration dependent reduction in HDAC activity at 4 h, with 25 μM 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 μM, 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 μM) concentration (Figure 5). 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 IC_{50} values of 1-TAT → 2NF on both cell lines (60.6 μM/A549; 58.5 μM/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 → 2NF showed similar cytotoxicity toward non-cancerous CHO and HEK cell lines (IC_{50} = 49.8 and 72.3 μM 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 2NF nano fiber. 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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