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Nucleopeptide Assemblies Selectively Sequester ATP in Cells

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Abstract

Selectively interacting with ATP by synthetic molecules under physiological condition promises many useful applications but remains a challenge. Here we report that assemblies of nucleopeptides selectively sequester ATP in complex conditions (e.g., serum and cytosol). We develop assemblies of nucleopeptides that selectively sequester ATP over ADP. Counteracting enzymes interconvert ATP and ADP to modulate the nanostructures formed by the nucleopeptides and the nucleotides. The nucleopeptides, sequestering ATP effectively in cells, slow down efflux pumps in multidrug resistance cancer cells, thus boosting the efficacy of an anticancer drug. Examining additional 11 nucleopeptides (including D- and L-enantiomers) yields five more nucleopeptides that differentiate ATP and ADP via either precipitation or gelation. As the first example of using assemblies of nucleopeptides for interacting with ATP and disrupting intracellular ATP dynamics, this work illustrates the use of supramolecular assemblies to interact with small and essential biological molecules for controlling cell behaviors.

Graphical Abstract

Keywords

self-assembly; nucleopeptide; enzyme switch; targeting metabolism; hydrogel

Adenosine triphosphate (ATP), one of the most important biological anions, plays crucial roles in many cellular processes, including cellular respiration,^[1] energy transduction,^[2] enzyme catalysis,^[3] and signaling.^[4] Therefore, selective binding or sequestration of these polyphosphate species under biological conditions would help elucidate their roles in

relevant physiological events and provide a powerful way to control cellular processes. Much efforts have focused on developing specific receptors, such as synthetic host-guest receptors,^[5] RNA-aptamers,^[6] bis-Zn based artificial receptors,^[7] recombinant proteins and synthetic peptides,^[8] for recognizing ATP. Among these strategies, protein engineering or peptide fragment mimetic is a direct approach for ATP recognition or sequestration.^[8a, 8b] Although some of them exhibit high affinity toward ATP in water^[8c, 9] or phosphate-free buffers (e.g., HEPES^[7a, 10]), these molecules are largely ineffective for recognizing ATP in complex physiological conditions (i.e., PBS, human serum, and cell milieu). Therefore, the development of molecules for selectively sequestering ATP in complex medium is still limited, and their applications in cells remain unexplored. We have found that a nonapeptide formed a hydrogel instantly upon the addition of ATP.^[11] This rather unexpected observation indicates that it is feasible to use assemblies of small molecules to interact with ATP. To increase the specificity towards ATP, we decided to explore the use of supramolecular assemblies of nucleopeptides for selectively sequestering ATP in complex physiological conditions.

Nucleopeptides, as the covalent conjugates of nucleobases and peptides,^[12] exhibit excellent biocompatibility, good biostability, facile functionality, readily self-assemble in water, and promise useful biological and biomedical applications, including as antibiotics,^[13] hydrogelators,^[14] molecular recognition,^[15] and promoters for cell uptake of nucleic acids,^[16] and protease resistant peptide derivatives to accelerate the degradation of certain peptide nanofibrils.^[17] Most importantly, bearing nucleobase(s), nucleopeptides would exhibit appreciable affinity to adenosine on ATP even under physiological condition due to the well-known Watson-Crick base pairs in the context of cells and in developing supramolecular materials.^[18]

To explore the possibility of using nucleopeptides for selectively sequestering ATP, we introduced thymine to cap the N-terminal of a nonapeptide that instantly formed a hydrogel upon the addition of ATP.^[11] Our study shows that the assemblies (rather than monomers) of a nucleopeptide (**NP1**) (Figure 1A) selectively sequester ATP over ADP in complex physiological conditions, evidenced by phase transition. Hexokinase (HK)^[19] and creatine phosphokinase (CPK)^[20] are able to control the cycle of ATP/ADP, thus modulating phase transition (from precipitate to solution and vice versa) and reversibly changing the morphologies of assemblies of **NP1** and the nucleotides in PBS (Figure 1B). Most importantly, **NP1** exhibits selectivity towards ATP under physiological conditions, including in serum and in cells. Being incubated with multiple drug resistance cancer cells, **NP1** slows down the efflux of an anticancer drug (e.g., doxorubicin (Dox)), results in long cellular retention of Dox, thus boosting the anticancer activity of Dox against Dox-resistance cancer cells (Figure 1C). Examining 11 analogs of **NP1** identifies five more nucleopeptides that differentiate ATP and ADP via either precipitation or gelation. As the first use of assemblies of small molecules for selectively sequestering ATP, this work opens up a new approach for rationally design supramolecular assemblies for sequestering (or recognizing) small biological molecules in complex physiological conditions to mimic the functions of proteins and to control cell behaviors.

We designed nucleopeptide **NP1** (Figure 1A) according to the following rationale: (i) a D-nonapeptide, ffkfkklkl, consists of ff for increasing self-assembly ability, kkfkklkl for interacting with phosphate group^[11] on ATP, and D-amino acids (f = D-phenylalanine, k = D-lysine, and l = D-leucine) for proteolytic resistance; (ii) thymine, capping the N-terminal of nonapeptide, ensures affinity to adenosine on ATP. After obtaining **NP1** by solid phase peptide synthesis (SPPS),^[21] we first examined the ability of **NP1** for differentiating ATP and ADP in PBS buffer. **NP1** forms a clear solution, which becomes precipitates after the addition of ATP (Figure 2). But **NP1** remains as a transparent solution in the presence of ADP. As revealed by transmission electron microscopy (TEM), **NP1** forms short nanofibers with length of 40 ± 5 nm and width of 4 ± 2 nm, which, in the presence of ATP, turn into uniform nanofibers with several hundred nanometers in length and 7 ± 2 nm in width, which likely further aggregates to precipitate. ADP interacting with **NP1** only results in short nanofibers with diameter of 5 ± 2 nm, which remains soluble. Moreover, **NP1** differentiates ATP and ADP in human serum (i.e., ATP interact with **NP1** to form precipitates in serum, but **NP1** remains soluble in serum upon the addition of ADP (Figure S1)), suggesting that the designed nucleopeptides should function under complex physiological conditions.

As revealed by the circular dichroism (CD) spectra and the analysis of the CD by DichroWeb,^[22] **NP1**, at 0.4 wt%, presents predominantly in α -helical conformation (45%) with 28% of β -sheet and 19% of unordered structures (Figure 2D, Figure S2). Adding ATP slightly increases the α -helix conformation content of **NP1** to 50%, and adding ADP slightly decreases the α -helix of **NP1** to 40%. The red shift of two negative peaks of 200 nm and 223 nm in the presence of ATP (or ADP) agrees with that ATP or ADP enhances the assemblies of **NP1**. At 0.05 wt%, **NP1** (Figure 2E) exhibits dominantly unordered structures (55%). The presence of ATP or ADP slightly reduces the portion of unordered structures, but significantly increases the percentage of α -helix from 0% to 9.5% or 15.0%, respectively. Static light scattering (SLS) of **NP1** hardly changes until the concentration of **NP1** is above 0.2 wt% (Figure S3), suggesting that **NP1** starts assemble to form detectable nanofibers at the concentrations higher than 0.2 wt%. These results indicate that (i) the assemblies of **NP1** interact stronger with ATP or ADP than the monomer of the **NP1** does; (ii) ATP or ADP affects the secondary structure of **NP1**, thus resulting in different assemblies (Figure 1). Most importantly, these results confirm that **NP1** interacts with ATP and ADP differently in PBS buffer.

To further examine the ability of **NP1** to differentiate ATP and ADP in the presence of other biological molecules (e.g., metabolites and enzymes), we employed a pair of counteracting enzymes to interconvert ATP and ADP for controlling the self-assembly of **NP1**. The pair of enzymes are hexokinase (HK), which phosphorylates hexoses and generates ADP by transferring the phosphate group from ATP to glucose, and creatine phosphokinase (CPK), which catalyzes the generation of ATP from ADP in the presence of phosphocreatine (Pcr). With glucose, **NP1** plus ATP remains as a precipitate, though TEM reveals more bundles of nanofibers with the fibril diameters of 8 ± 2 nm (Figure 3A). Adding HK hydrolyzes ATP to ADP, which turns the precipitates into a clear solution and the long nanofibers into short (20–100 nm) and thin (4 ± 2 nm) fibers (Figure 3B). With Pcr, **NP1** plus ADP remains as a clear solution, containing short nanofibers of 3 ± 2 nm in width (Figure 3C). The addition of

CPK to the solution turns ADP into ATP and the solution into precipitates, which consist of long nanofibers with the diameters of 11 ± 2 nm (Figure 3D). These results indicate that **NP1** is able to sequester ATP when ATP forms via cellular metabolism.

Expression of ATP-dependent efflux pumps in cancer cells plays a crucial role in multiple drug resistance (MDR).^[23] Moreover, the concentration of ATP is usually several millimolar in human cells.^[24] ³¹P NMR spectra indicated that **NP1** can slow down the hydrolysis rate of ATP by ALP (Figure S5). Therefore, we tested the ability of **NP1** for sequestering ATP in MDR cancer cells. After confirming that **NP1** selectively sequester ATP in the buffer containing cellular major components (e.g., various proteins and glycans) (Figure S6), we treated MES-SA/dx5 cells with **NP1** in the presence of Dox. Five hours after changing to the medium from with to without Dox, most of Dox remains inside the MES-SA/dx5 cells treated with **NP1**. But there is little Dox in the MES-SA/dx5 cells without **NP1** treatment (Figures 4A and S7). These results suggest that the assemblies of **NP1** likely sequester ATP inside cells, thus preventing the efflux of Dox by the efflux pump driven by ATP. Encouraged by these results, we further evaluated anti-proliferation efficiency of Dox in combination of **NP1** against MES-SA/dx5 cells. Exhibiting little cytotoxicity by itself, **NP1**, significantly increases the cytotoxicity of Dox against MES-SA/dx5 cells in a dose-dependent manner (Figures 4B, S8 and S9). Specifically, the addition of **NP1** at the concentration of 500 μ M (or 200 μ M) increases the cell death caused by Dox from 46% to 92% (or 67%) (Figures 4B and S8). Moreover, **NP1** exhibits dose-dependent effect in enhancing the anticancer efficiency of Dox, agreeing with that the assemblies of **NP1** sequester ATP in vitro (Figure S3).

We also used TEM to image live cells incubated without or with **NP1**. The cells incubated with **NP1** exhibits nanofibrous structures having diameters of 7 ± 2 nm, which are similar with the TEM image of the cell free experiment (Figures 5A and S10). Then, we measured the cellular ATP levels in Dox untreated or treated conditions in the presence of **NP1**. The cells maintain ATP levels after the treatment of **NP1** within 5 h, while the control cells increase the ATP levels (Figure 5B). Because **NP1** exhibit little cytotoxicity, this result suggests that **NP1** affects the metabolic processes of ATP. To further demonstrate the interaction between **NP1** and ATP inside cells, we synthesized NBD labelled **NP1** (**NP1-NBD**) as a structural analog of **NP1**, which retained ability for sequestering ATP (Figure S11). CLSM images (Figures 5C and S12) indicate that almost all ATP overlapped with **NP1-NBD**, and the fluorescence of ATP hardly changes with time (Figure S13). These results together further confirm the designed nucleopeptide selectively sequesters ATP to boost anticancer drug activity in MDR cells.

To correlate the structures of nucleopeptides with the ability of sequestering ATP, we synthesized 11 analogues of **NP1** and tested their phase transition in the presence of ATP or ADP (Scheme S2 and S3, Table S2). **NP2**, as an L-enantiomer of **NP1**, which becomes precipitates after the addition of ATP, but remains as a transparent solution in the presence of ADP (Figure S14). TEM reveals that the amorphous nanostructures formed by **NP2**, transform into spherical like structures after addition of ATP with width of 29 ± 2 nm, which further interact with each other to form 3D network (Figure S14) and precipitate from the

solution. On the contrary, ADP interacts with **NP2** to results in nanofibrous structure with width of 10 ± 2 nm. Different morphologies of the nanostructures resulted from the solutions of **NP1** and **NP2** likely originate from their chiralities, which minimally affect their ability to differentiate ATP and ADP, evidenced by macroscopic phase transition during ATP/ADP cycle (Figure S17). Systematically shortening the lengths of **NP1** or **NP2** results in four more nucleopeptides (**NP3** to **NP6**, Schemes S2 and S3) that differentiate ATP and ADP via either precipitation or gelation (Figures S19 to S21). These results indicate that the efficacy of the assemblies of nucleopeptides for sequestering ATP depends more on the length than on the chirality and more on self-assembling ability than on the number of charges of the nucleopeptides.

In conclusion, illustrating the use of assemblies of nucleopeptides for selectively sequestering ATP in complex conditions, this work provides a novel approach to modulate the functions of ATP in cells, agreeing with the notion that locally increasing the concentration of small molecules^[25] would be a powerful strategy for modulating biological processes. In essence, the use of the interconversion of ATP to ADP to control the dynamics and filaments of **NP1** mimics the actin filament formation, in which ATP activates G-actin polymerization and hydrolysis of ATP to ADP destabilizes the actin filament.^[26] Thus, the reversible morphology transition of nucleopeptide during ATP/ADP cycle, which controlled by counteracting enzymes, may act as a starting point for mimicking self-assembly/disassembly process of actin. These findings not only provide an alternative strategy for potentially targeting metabolism of cancer cells by assemblies of small molecules,^[6c] but also extend the supramolecular assemblies, which are bountiful from the development of gelators,^[27] as an effective entities for recognizing cellular bioactive molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. (A) Structure of a nucleopeptide (**NP1**) to sequester ATP selectively. (B) Illustration of assemblies of **NP1** interaction with ATP or ADP and the reversible phase transition of the assemblies controlled by a pair of counteracting enzymes. (C) Plausible mechanism of assemblies of **NP1** in a multidrug resistance cell to sequester ATP, thus slowing down drug efflux and boosting drug efficacy.

Figure 2.

TEM images of **NP1** (0.4 wt%) in PBS buffer (pH7.4) (A) without or (B, C) with 1 equiv. of (B) ATP and (C) ADP. Insets: the corresponding optical images. Scale bar is 50 nm. CD spectra of **NP1** at (D) 0.4 wt% and (E) 0.05 wt% with or without ATP or ADP (equal molar with **NP1**).

Figure 3.

NP1 assemblies with ATP in the presence of glucose (A) without or (B) with addition of HK. **NP1** assemblies with ADP in the presence of Pcr (C) without or (D) with the addition of CPK. Insets are the corresponding optical images. The concentration of **NP1** is 0.4 wt%, equal molar ATP or ADP is added. All experiments are performed in 100 mM PBS buffer, pH7.4 for 24 h. Scale bar in (A) to (D) is 100 nm.

Figure 4.

(A) CLSM images show the inhibition of Dox efflux by **NP1** in MES-SA/dx5 cells at 0 h (changing to fresh medium without Dox after washing 3 times) and 5 h (further incubation after changing medium). Scale bar is 20 μ m. (B) MES-SA/dx5 cell viability that treated with Dox or the mixture of **NP1** and Dox (5 μ M) for 48 h (n=3, ***: $p < 0.001$).

Figure 5.

(A) TEM images of cell components collected from live cells without (control) or with the incubation of **NP1** for 5h. Scale bar is 100 nm. (B) ATP concentrations in the live cells incubated with **NP1** (500 μ M), **NP1** plus Dox (10 μ M), Dox (10 μ M), and culture medium ($n = 3$, the asterisks indicate the difference between 1 h with 3 or 5 h. ns: non-significant, *: $p < 0.05$, **: $p < 0.01$). (C) CLSM images of MES-SA/dx5 cells incubated with Alexa-ATP and **NP1-NBD** (50 μ M). Scale bar is 5 μ m.