Macroscopic and Fluorescent Discrimination of Adenosine Triphosphate via Selective Metallo-hydrogel Formation: A Visual, Practical, and Reliable Rehearsal toward Cellular Imaging

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S Supporting Information

ABSTRACT: With use of simple terpyridine zinc nitrate complexes, intriguing visual recognition of adenosine triphosphate (ATP) via selective coordination assembly leading to two-component metallo-hydrogel formation has been realized. With intensive fluorescent study and density functional theory calculations, it may be inferred, besides the selective metal–ligand interaction between Zn center and phosphate groups, the intramolecular π -stacking between the planar nucleobases of ATP and the metal-hybrid aromatic ring of pincer complex strongly affected the geometry of the coordinated adducts and possible molecular self-assembly process, which constitute a completely new sensing strategy in comparison with the conventional approaches. Furthermore, in light of extreme sensitivity of pincer zinc complexes toward ATP at micromolar scale (1.85 μ M) and remarkable fluorescent enhancement (ca. 44-fold) upon ATP addition, the feasibility of the low cytotoxicity pincer zinc complexes in monitoring ATP in HeLa cells has been fulfilled with confocal fluorescence microscopy.

KEYWORDS: adenosine triphosphate, cellular imaging, fluorescence enhancement, metallo-hydrogel, visual recognition

1. INTRODUCTION

As an intriguing challenging task, the development of novel molecular sensing systems for substances of biological significance, especially under aqueous conditions, has recently received considerable attention.¹ The difficulty of sensing is not only the competitive interaction of water molecules with the artificial sensor but also how to selectively amplify a weak signal of discrimination.² For example, as one of the most important nucleotides, adenosine triphosphate (ATP) plays key roles in intracellular energy transfer and also functions as an extracellular signaling mediator in various metabolic processes.^{3,4} The deficiency of ATP is consequently correlative with a plethora of pathogenesis.^{5–7} However, the selective

probing of ATP in the presence of other bioactive substances in physiological media is still challenging. Numerous artificial fluorescent receptors bearing functional groups readily forming hydrogen bonding have been consequently developed.^{8–12} In some cases, positively charged substitutions were introduced to strengthen the hydrogen-bonding effects to anions. The relatively low sensitivity and poor selectivity of sensing are usually observed due to the interference of the sugar moiety in ATP along with the competitive interaction of water, which

Received: May 16, 2016

Accepted: July 15, 2016

Published: July 15, 2016

Figure 1. (a) Synthesis of pincer zinc complexes **2a** and **2b** and (b) the resulting crystal structure of **2a**·MeOH obtained after direct evaporation.

limited their feasibility.¹³ When the coordination affinity of the phosphate anion to the various metal centers is taken into account, the metal–ligand coordination is considered as a second strategy to probe ATP.^{14–19} Several fluorescent mono- and bimetallic complexes were synthesized, and applied in the molecular recognition of ATP along with other bioactive phosphates without selectivity.^{1,10,12,20} Despite these achievements, only a few examples of visual discrimination of ATP along with its homologues via colorimetric methods have been reported,^{21–25} and the specific protocol for ATP detection from a mixture containing adenosine diphosphate (ADP), adenosine monophosphate (AMP), and other phosphates is still regarded as an extremely challenging task due to their similar structures, especially under aqueous conditions.

Functional molecular gels represent one kind of smart soft matter,^{26,27} which recently also exhibit potential as a valuable platform for molecular recognition.²⁸ Hamachi and co-workers first applied molecular organo-hydrogels as efficient supports to immobilize an artificial sensor for phosphate derivatives analysis in 2005.²⁹ Liu and co-workers subsequently reported the visual discrimination of ATP in preference to ADP and AMP via cationic organogels collapse in alcoholic aqueous media, although the limit of detection was relatively high (6.25×10^{-3} M).³⁰ However, to the best of our knowledge, there are no reports of the molecular discrimination of ATP from other phosphates using selective metallo-hydrogels formation in physiological media as a sensor system. The incorporation of transition metals can endow the characteristics associated with the metals into the gel systems, and provide a good opportunity to subtly control the molecular assembly process and

microscopic response to external stimuli.^{31–37} Our group recently demonstrated unprecedented visual discriminations of enantiomers, positional isomers, and mercapto analogues via selective phase transformation of molecular metallogels based on rational designed pincer complexes.^{38–42} Following our research interests in exploring the feasibility of metal pincer complexes in catalysis, sensing, and molecular switches,^{38–44} we sought to explore the possibility of selectively visual discrimination of ATP under the aqueous environment by using simple terpyridine-Zn(II) complexes, study the mechanism, and investigate their possible applicability toward cellular imaging.

2. RESULTS AND DISCUSSION

Initially, pincer zinc complexes **2a** and **2b** containing weakly bound nitrate anions (Figure 1a) were designed for ATP recognition. Complex **2a** was readily obtained almost in a quantitative yield by reaction of 2-furyl terpyridine **1a** with $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ in methanol at room temperature for 24 h. Transparent rectangular single crystals suitable for X-ray analysis were obtained by slow evaporation of the resulting methanol solution. As illustrated in Figure 1b, one nitrate anion was not coordinated to the zinc center of complex **2a**; the oxygen atom of methanol solvent was coordinated instead. The ^1H NMR (CD_3OD) spectrum study showed two sets of signals and suggested a dynamic equilibrium between the complexes **2a** and **2a**·MeOH in the solution state. The ratio of two complexes was slightly influenced by the variation of test temperature (Figures S38–S40 of the Supporting Information). Subsequently, the investigation on the gelation ability of

Figure 2. Scanning electron microscopic morphologies of (a) the precipitates of **2a**/H₂O (2 wt %), (b) [**2a** + 0.25 equiv of AMP]/H₂O (2 wt %), (c) [**2a** + 0.25 equiv of ADP]/H₂O (2 wt %), and (d) gel [**2a** + 0.25 equiv of ATP]/H₂O (2 wt %). TEM images of (e) gel [**2a** + 0.25 equiv of ATP]/H₂O (2 wt %) and (f) gel [**2a** + 0.5 equiv of K₃PO₄]/H₂O (1 wt %).

complex **2a** and **2b** in a number of organic solvents as well as pure water was therefore carried out. No gel was formed after regular heating and cooling procedures for the gel preparation;⁴² precipitates or clear solutions were obtained instead (Table S1). Nucleotides like ATP contain phosphate, sugar, and planar nucleobase, three major fragments, which not only may bind to the metal center due to the strong affinity coefficient of phosphate group to zinc center¹⁵ but also may form hydrogen bonding and π - π interactions to facilitate molecular self-assembly leading to the gel formation. Therefore, ATP and its analogues were involved in the gelation tests. Only solution or precipitates were obtained in the most selected organic solutions (such as MeOH, dimethylsulfoxide, and dimethylformamide). An opaque white two-component metallo-hydrogel was finally obtained by cooling a hot aqueous mixture containing 2 wt % complex **2a** and 0.25 equiv of ATP. However, the addition of ADP and AMP under the identical operation did not result in metallo-hydrogel formation, and only the white precipitates were observed (Table S2). Although the samples containing **2b** and 0.25 equiv of AMP, ADP, or ATP (2 wt %) were not completely dissolved after heating under the identical gelation operation, partial gel formation was observed with the soluble part of the sample containing ATP. The observation of these differences allows the first selective visual recognition of ATP out of its bioactive homologous via selective gel formation.

To confirm this macroscopic distinguishable phenomenon, the microscopic morphology study on the samples of gelation tests with pincer complex **2a** were performed with scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The precipitates of **2a**/H₂O (2 wt %) revealed irregular bulky crystalline rods (Figure 2a). Similar but dense crystalline rods were observed with the precipitate of [**2a** + 0.25 equiv of AMP]/H₂O (2 wt %, Figure 2b). When ADP was involved, the images containing microcrystalline blades along with cubic crystals and fibers were found (Figure 2c). In contrast, amorphous sheets were observed in the SEM image of the gel [**2a** + 0.25 equiv of ATP]/H₂O (2 wt %, Figure 2d), which consisted of very condensed cross-linked nanofibers, further proved by using TEM study (Figure 2e). Therefore, the selective molecular recognition of ATP from its homologues appeared due to the long and dense nanofibers formed by the

coordination adduct containing complex **2a** and ATP via self-assembly, which were able to immobilize water molecules within the network to form stable metallo-hydrogels.

With these exciting results in hand, a set of control experiments was carried out. A number of phosphates, including sodium pyrophosphate (PPi), potassium phosphate (K₃PO₄), dipotassium phosphate (K₂HPO₄), potassium phosphate monobasic (KH₂PO₄), etc., were selected to probe the role of the phosphate group during the gelation process (Table S3). Upon the addition of 1 equiv of K₃PO₄, complex **2a** or **2b** was difficult to dissolve in water even under severe heating. Decreasing the amount of K₃PO₄ to 0.5 equiv resulted in the formation of a translucent white gel [**2a** + 0.5 equiv of K₃PO₄]/H₂O (1 wt %) after cooling the heated mixture. To our delight, even with 0.1 equiv of K₃PO₄, a robust gel was still obtained. When the concentration of **2a** was increased to 2 wt %, the sample could not be completely dissolved by heating; however, the dissolved part also formed a gel after cooling and resting operation. TEM revealed very dense nanofibers which was much broader than that observed in the gel [**2a** + 0.25 equiv of ATP]/H₂O (2 wt %, Figure 2e,f). To exclude ion effects, a series of potassium and sodium salts containing other anions were used in the control experiments. The gel formation only occurred in the samples containing K₂HPO₄ or KH₂PO₄; other salts all resulted in precipitates or solutions under the identical gelation operation (Table S3). This outcome indicated the key role of phosphates during the gel formation, which was also consistent with previous studies. Therefore, this system can act as a simple, straightforward, and practical visual discrimination of phosphates from other anions.

Subsequently, a study of thermodynamic property of the phosphates containing metallo-hydrogels was carried out. The gel-to-sol phase-transition temperatures (T_g) of the selected gel samples were determined by the "test-tube-inversion method".⁴⁵ In comparison with our previous reported metallo-gels,^{38–41} all the gel samples were relatively robust and survived the heating over 100 °C. For instance, the T_g value of gel [**2a** + 0.5 equiv of K₃PO₄]/H₂O (1 wt %) was 115 °C. The plot of T_g was steadily increased along with the addition of K₃PO₄ from 0.1 to 0.5 equiv. When the phosphate amount was further increased to 0.6 equiv, the T_g was decreased to 103 °C (Figure S11). Similar results were also observed with the gel

Figure 3. (a) Visual discrimination of ATP out of its homologues via selective gel formation; (b) calculated geometries of three adducts formed by complexes **2a** with AMP, ADP, and ATP, respectively.

[**2a** + ATP]/H₂O (2 wt %), which indicated the amount of ATP exhibited a strong impact on the thermo-stability of metallogel. The addition of 0.25 equiv of ATP resulted in the highest T_g value (Figure S10).

A series of rheological studies were performed to quantify the gels' mechanical and thixotropic characteristics. In the case of gel [**2a** + 0.25 equiv of ATP] (2 wt %), the elastic storage modulus (G') was much greater than the loss modulus (G'') when the shear strain was <4.0% (a value of yielding point). Beyond this level, the gel gave a strain overshoot:⁴⁶ when the G' value was going to further decrease, the value G'' would increase first and then decrease during the range of 6.3%–20.0%. At large strain amplitudes (>13%), the gel showed a catastrophic disruption accompanied by a reversal of the viscoelastic signal ($G'' > G'$) (Figure S12). Subsequently, the shear strain of 1.0% was applied to characterize viscoelastic behavior of the gel samples with increasing angular frequency. As shown in Figure S13, the dynamic frequency sweep rheometry data revealed a kind of classic shear viscosity curve. The slightly increasing values of G' and almost no change in G'' along with the steadily increasing frequency suggested that the energy storage process occurred without obvious energy dissipation and the metallo-hydrogel possessed very good elasticity. The complex viscosity (η^*) was decreased along with the increasing frequency, indicating the shearing thinning phenomena occurred and thixotropic character of the gel. Similar results were obtained for the gel [**2a** + 0.5 equiv of K₃PO₄] (1 wt %); however, the strength of relative parameter was much lower (Figures S14 and S15).

The X-ray crystal structure analysis of complex **2a**·MeOH revealed planar pincer complex **2a** efficiently packed via two types of intermolecular π -stacking (Figure S17). Two molecules interacted to form a unit via weak π – π interaction between the furan ring and the center pyridine ring of the second molecule (3.629 Å). The units were further assembled into linear aggregates via the second π – π interaction between the furan ring and the side pyridines of the second units (3.627 Å). In combination with the results obtained by X-ray diffraction (XRD) of the xerogels [**2a** + 0.25 equiv of ATP] and [**2a** + 0.5 equiv of K₃PO₄], the signals at 2θ around 23° with corresponding distance at 3.7–3.9 Å, we may suggest that the

intermolecular π -stacking and metal–metal interactions of the adducts formed by the pincer complex and phosphates may be responsible for the molecular self-assembly leading to the gel formation (Figures S18 and S19). However, the difference between the thermal and mechanical characteristics of the gels containing ATP and K₃PO₄ indicates additional interactions may exist during the self-assembly process of complex **2a** with ATP.

Density functional theory (DFT) calculation was therefore applied to further explore the possible interactions of three coordination adducts formed by complex **2a** with AMP, ADP, and ATP, respectively, with the basis set of 6-31+G(d).⁴⁷ The results indicated that intramolecular π -stacking played an important role in the geometry of three coordination adducts. Because of the intramolecular π -stacking between planar nucleobase moiety and the metal-hybrid aromatic ring of complex **2a**, three adducts all seem to adapt in the folded geometries (Figure 3b). However, the calculated energies at 298 K for three folded coordination adducts formed by AMP, ADP, and ATP were –7.6, 0.1, and 2.2 kcal/mol, respectively. Mulliken charge analysis indicated that the charges on the terminal oxygen atoms of AMP were the most negative, while those of ATP were the least negative in the folded forms. In consideration that the coordination between zinc and oxygen was ionic, more negative charge should be on the oxygen atom, which would result in a stronger coordination bond leading to the adduct formation. It would be expected that the coordination adducts formed by complex **2a** with AMP or ADP intended to adopt folded forms, which were unfavorable for the formation of metallogel via molecular self-assembly. In the case of the coordination adduct formed by complex **2a** and ATP, the intramolecular π -stacking between the two aromatic rings was relatively weaker; to form stable adduct, liner geometry may be preferred, which may allow the possibility of intermolecular self-assembly leading to the gel formation.

At last, UV–vis and fluorescent studies were carried out, which are considered as powerful tools to probe the mechanism for molecular recognition.^{48,49} No apparent changes were observed in the UV–vis spectra of **2a** (3.69×10^{-5} M) after 0.25 equiv of ATP, ADP, AMP, or 0.5 equiv of selected potassium salts addition (Figures S21 and S22). At 298 K,

Figure 4. (a) Emission spectra of **2b** (3.69×10^{-5} M, $\lambda_{\text{ex}} = 448$ nm) in fresh pure deionized water with pH at 7 with 0.25 equiv of AMP, ADP, ATP, or 0.5 equiv of different anions and (b) **2b** (3.69×10^{-5} M) in fresh pure deionized water with pH at 7 with various concentrations (μM) of ATP ($\lambda_{\text{ex}} = 448$ nm).

Figure 5. Confocal fluorescence microscopy images of (a) HeLa cells incubated with $5 \mu\text{M}$ complex **2b** for 15 min (control) and then further incubated with $50 \mu\text{M}$ ATP (b) for 1 h and (c) for 6 h (red channel: $\lambda_{\text{ex}} = 488$ nm; fluorescence collected window: 550–750 nm). (d) Overlay image of frames (c) with bright field image (scale bar = $10 \mu\text{m}$); (e) overlay image of complex **2b** existed HeLa cells (control) incubated for 6 h with bright field image; and (f) overlay image of complex **2b** existed HeLa cells (control) incubated for 6 h and then adding $50 \mu\text{M}$ ATP incubated for another 6 h, with bright field image (scale bar = $10 \mu\text{m}$); (g) time-dependent fluorescence intensity of complex **2b** existed HeLa cells in the presence of $50 \mu\text{M}$ ATP (A, for 0–6 h) and complex **2b** existed HeLa cells (B, for 0–6 h) and then adding $50 \mu\text{M}$ ATP (C, for 6–12 h); and (h) HeLa cells in the colocalization experiment (scale bar = $10 \mu\text{m}$).

pincer complex **2b** (3.69×10^{-5} M) exhibited yellow color and three major bands at 284, 323, and 405 nm were observed. According to the previous reports,⁵⁰ the lowest energy absorption bands of complex **2b** at 405 nm (black line) was assigned to the intraligand charge transfer (ILCT) (Figure S23). Although only slightly red shifts (ca. 2 nm) were observed with three samples after ATP (purple line), ADP (green line), or AMP (red line) addition, the intensity of all three was weaker than that of pure complex **2a**, especially in the

case of ATP. However, no obvious change in the spectra was found with other anions (Figure S24).

In light of gel [**2a** + 0.25 equiv of ATP] being weak fluorescent, fluorescent study was further investigated. Upon excitation at 284 nm, planar compound **2a** exhibited a strong fluorescent emission at 450 nm.⁵⁰ In contrast to the selected anions, which showed no observable alternation (Figure S21), the addition of 0.25 equiv of ATP resulted in a slight but noticeable quenching of the emission at 450 nm (11%, Figure S28). Because of the photoinduced electron transfer (PET)

effect of dimethylamino-phenyl group, when the aqueous solution of complex **2b** was excited at 448 nm, only very weak fluorescent emission was observed at 617 nm, which may be considered as a better sensor system to study the influence after nucleotides addition. To our delight, a dramatic fluorescence enhancement (ca. 44-fold) at 617 nm was found in the mixture of complex **2b** and 0.25 equiv of ATP, whereas much less enhancement (ca. 14-fold) was observed after ADP addition and almost no change was found with the sample containing AMP and other anions (Figure 4a). The linear form geometry (Figure 3a) of the coordination adduct formed by complex **2b** and ATP may be suitable for further molecular self-assembly resulted in fluorescence enhancement due to aggregation-induced emission (AIE). Subsequently, other biological species including selected amino acids and peptide were also involved; no apparent influence on the fluorescence was observed (Figure S30), which may highlight its potential application toward cell imaging. The detection limit of ATP in water with pincer complex **2b** was found at micromolar scale (ca. 1.85 μM , Figure 4b), which was much lower than the average concentration of ATP in mammalian cells.⁵¹ Additionally, a good linearity varied the concentration of ATP from 0 to 18.45 μM and relative fluorescent intensity of complex **2b** at 617 nm was observed (Figure S32).

3. BIOLOGICAL APPLICATION

To test the cytotoxicity of complex **2b**, a standard 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay was performed (Figure S36). The IC_{50} of complex **2b** was 120 μM , which indicated its low cytotoxicity under the test conditions. With use of confocal fluorescence microscopy, the possibility of complex **2b** to detect ATP in HeLa cells was carried out. HeLa cells were incubated in the presence of 5 μM complex **2b** for 15 min at 37 $^{\circ}\text{C}$ and then washed with Dulbecco's modified eagle medium (DMEM) three times to remove the excess complex **2b**. The cells were further incubated in fresh cell culture medium (DMEM + 20% FBS) for the visual detection of ATP existing in the HeLa cells. As a control, the cells only showed very faint fluorescence without external addition of ATP (Figure 5a). Then 50 μM ATP was added into the biosystem (A), and after being incubated for 1 h, the cells displayed a significant fluorescent enhancement in the intensity (Figure 5b). To study the relationship of fluorescence enhancement of [**2b** + ATP] along with the incubated duration, the time-dependent fluorescence intensity was monitored for an additional 5 h. As shown in Figure 5g-A, the fluorescence intensity gradually increased and arrived at a plateau after 4 h, which featured a 16-fold fluorescent enhancement (Figure 5c,d and 5g-I). In the case of no external ATP addition system (B), only a moderate enhancement (8-fold) was observed after 6 h of incubation (Figure 5e and 5g-B vs 5g-A), which demonstrated that complex **2b** can efficiently detect the original ATP in the HeLa cells. After 50 μM ATP was added to the system (C), further enhancement (ca. 2.3-fold) in fluorescence intensity was also observed after incubation for an additional 6 h (Figure 5f and 5g-C). The aforementioned results suggested that complex **2b** was photostable and showed strong fluorescent enhancement within biological media, which also confirmed the applicability of complex **2b** for the ATP detection in the living cells. In addition, the colocalization experiment was also implemented with the HeLa cells containing Hoechst 33342, which revealed that the targeting position of complex **2b** toward ATP was

mainly in cytoplasm (Figure 5h). Furthermore, according to the colocalization experiment of HeLa cells in the presence of Cy7 (mitochondria tracker) and complex **2b**, the fluorescence was actually distributed in mitochondria of the cell (Figure S36). This result could be further confirmed by flow cytometric analysis of isolated mitochondria (Figure S37), indicating a new and potentially powerful protocol for real-time imaging mitochondrial ATP changes in cells.

4. CONCLUSIONS

A selective macroscopic and fluorescent recognition of ATP from its homologues was successfully developed by using simple terpyridine zinc complexes via selective metallo-hydrogel formation. After detailed thermodynamic characterization, rheology, microscopy, and XRD studies of the resulting metallo-hydrogels, the plausible mechanism of this intriguing selective recognition was intensively investigated. With use of fluorescent study and DFT calculations, unlike the conventional molecular sensing strategies, along with the coordination ability of phosphate group to the zinc center, the intramolecular π -stacking between the planar nucleobases of ATP and the metal-hybrid aromatic ring of pincer complex strongly affected the geometry of the coordinated adducts and possible molecular self-assembly process, which may lead to the selective metallo-hydrogel formation and remarkable fluorescent enhancement (ca. 44-fold), therefore, providing a new approach for selective ATP discrimination. In consideration that cytotoxicity of complex **2b** and the detectable concentration of ATP in the water, both are relatively low; the potential application of this simple zinc pincer complex in monitoring ATP in HeLa cells has been successfully realized, which further supported that the visual discrimination of ATP via selective metallo-gel formation could be considered as a visible, practical, and reliable in vitro rehearsal toward cellular imaging.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.6b05804.

Synthetic detail, ^1H NMR, ^{13}C NMR, and ESI-MS spectra, single-crystal study (CCDC 1060488), sol–gel transition temperature, viscoelasticity and XRD study, UV/vis and fluorescent spectra, SEM and TEM morphologies, and cellular imaging (PDF)

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Author Contributions

T.T. and W.F. conceived and designed the experiments; W.F. performed the synthesis and experiments; C.L., Y.L., and X.B. contributed to the data confirmation and analysis; Z.L. performed the DFT calculations; F.Y. and L.C. contributed to the cell image test; all authors discussed the results; T.T. and W.F. wrote the paper; T.T. supervised the work.

Funding

The National Natural Science Foundation of China (No. 91127041, 21172045, and 21572036).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Financial support from the National Natural Science Foundation of China (No. 91127041, 21172045, 21405172, and 21572036), the Shanghai International Cooperation Program (14230710600), the Doctoral Fund of Ministry of Education of China (20130071110032), Ministry of Science and Technology of China (2016YFA0202902), the Shanghai Leading Academic Discipline Project (B108), and Department of the People's Republic of Chemistry Fudan University is gratefully acknowledged.

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