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Discrimination of adenine nucleotides and pyrophosphate in water by a zinc complex of an anthracene-based cyclophane†

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Combining metal-anion coordination and π-π stacking interactions, zinc complex of a novel anthracene-based cyclophane was designed to recognise adenine nucleoside polyphosphates. This complex was found to show selective fluorescence enhancement for ATP, ADP, AMP and PPi in neutral aqueous solution. Among them, ADP induced the largest fluorescence change to the complex, while ATP showed the strongest binding affinity to the complex. This property was used to sense ATP in the presence of excess amounts of other phosphates such as ADP, AMP, PPi and Pi.

Introduction

Phosphate anion species are abundant in biological systems where they play many important roles, and the development of receptors and sensors for them is of great importance due to their biological significance.[1] Among them, adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP) and adenosine 5'-monophosphate (AMP) are particularly important nucleotides since they play vital roles in living systems.[2] For example, ATP is not only well-known as a universal energy currency in all biological systems for metabolism, but also as an extracellular signalling mediator in many biological processes.[3] ADP and AMP are also important for their roles in bioenergetics, metabolism, and transfer of genetic information.[4] In particular, ADP is the common product of the most fundamental biological reactions catalysed by ATPases and kinases.[5] Besides, inorganic pyrophosphate (PPi) is also important because it involved in many enzyme-catalysed biosynthesis and metabolic processes, and most of them produce PPi as a hydrolysis product of nucleoside polyphosphates such as ATP.[6] Given the biological importance of these phosphate anion species, continuous efforts have been made to develop chemosensors for these phosphates.[1,5,7-10]

Cyclophanes possess a defined cavity size to bind guest molecules, and this unique property makes them very promising for biomolecular recognition.[11,12] Recent studies showed that anthracene-based cyclophanes exhibited good recognition properties for nucleotides.[13] One feature of this type system is capable of offering additional π-π stacking interactions between nucleic base and anthracene group to obtain selectivity. However, the reported systems often use relatively weak electrostatic interactions as main force to bind nucleotides, thus low binding affinities (10^3-10^4 M^{-1}) were generally observed.[13] In contrast, metal complex of cyclophanes can bind anions more tightly via metal-anion coordination chemistry, and this property has been used by us[14] and other groups[15] to create effective sensors for anions in water. Among the efforts for the development of chemosensors for phosphate species, metal complexes especially dinuclear zinc complexes as the binding motif are particularly useful, and they have become an indispensable part of phosphate sensor designs.[7,8] For example, many dinuclear zinc complexes have been developed as effective chemosensors for phosphate anion species in water.[16] More recently, an anthracene-based macrocylic dinuclear zinc complex has been reported to show high selectivity for nucleoside polyphosphates, especially for ATP/ADP.[17] Based on this, dinuclear zinc complex of anthracene-based cyclophanes could be reasoned as a good platform for fluorescent sensing of nucleotides.

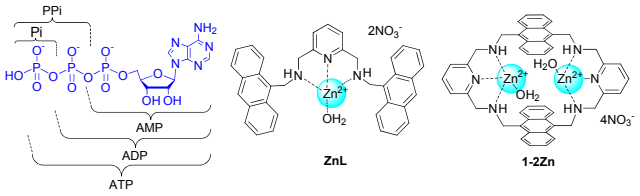


Fig. 1 Nucleotides (ATP, ADP and AMP), pyrophosphate (PPi), orthophosphate (Pi), receptor **ZnL** and **1-2Zn**.

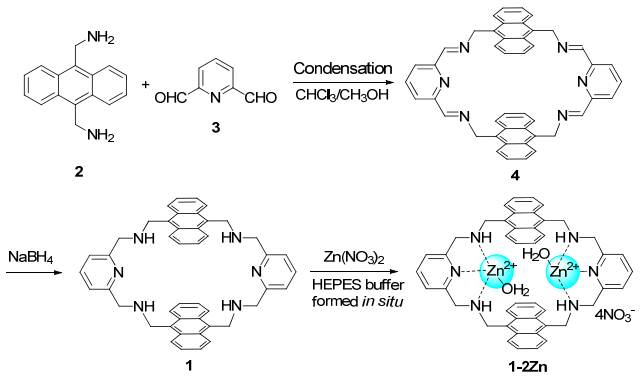
We recently reported a simple, readily available mononuclear zinc complex (**ZnL**, Fig. 1), which appends two anthracene groups and was unexpectedly found to show high selectivity for ADP with a unique increased fluorescence response in neutral aqueous solution.[18] We proposed that the selectivity of **ZnL** for ADP over ATP and AMP is an outcome of the combination of the metal anion coordination with the π – π stacking interactions between the adenine group in ADP with one of the anthracene group in **ZnL**, in which the well matched distance between the molecular size of ADP and **ZnL** plays an important role.[18] This interesting property and our continuous interest in phosphate recognition and sensing[19] prompted us to extend the structure of **ZnL** to a new anthracene-based dinuclear zinc macrocyclic complex **1-2Zn** (Fig. 1). We expected this new complex to show good sensing properties for nucleoside polyphosphates. Indeed, we found that **1-2Zn** showed selective responses for ATP, ADP, AMP and PPi, which enables this receptor to discriminate these phosphate species from orthophosphate (Pi) and many other

anions in water. Notably, **1-2Zn** showed the most intensified fluorescence response for ADP, but showed the highest binding affinity (> 10^6 M^{-1}) for ATP among the test analytes in neutral aqueous solution (see below). It is also worth noting that **1-2Zn** possesses the ability to discriminate AMP from Pi, which is important, because most of the developed phosphate chemosensors showed similar optical changes for these two phosphate species so that they could not distinguish between them.[8] Based on our previous work on **ZnL**,[18] we proposed the most possible reason for this selective sensing property of **1-2Zn** is that this complex may have such characteristics of combining the metal anion coordination interactions and the π – π stacking interactions between the adenine group in ATP, ADP and AMP with the anthracene group in **1-2Zn**.

Results and discussion

1. Design and synthesis

Based on our recently reported results,[18] we designed the dinuclear zinc complex structure of **1-2Zn**. Because of the characteristics of a dinuclear metal centre, we expected that this complex not only can provide tight binding affinities for phosphates species, but also can increase the water solubility of the receptor compared to that of **ZnL**. In addition, the anthracene groups can act as the fluorescent signalling unit and offer π – π stacking interactions units for the adenine group of ATP, ADP and AMP.



Scheme 1 Synthesis of **1-2Zn**.

The preparation of **1-2Zn** is outlined in Scheme 1. A symmetrical macrocyclic cyclophane compound **1**, the ligand of **1-2Zn**, can be readily prepared in good yield by [2+2] condensation of 9,10-bis(aminomethyl)anthracene **2** and 2, 6-pyridinedicarboxaldhyde **3** followed by reduction of the resulting Schiff base intermediate **4** using $NaBH_4$ (Scheme 1). Both **2**[20] and **3**[21] are known compounds, and they are prepared according to the previously published procedures. Structural identification of the Schiff base **4** and the cyclophane **1** were confirmed by NMR, IR and HR-MS spectroscopy. Detailed synthetic procedures and structure characterizations are given in the experimental section and in the ESI†. The macrocyclic dinuclear zinc complex **1-2Zn** was obtained *in situ* by mixing receptor **1** with two equiv of $Zn(NO_3)_2$ in water, hence the isolation and purification procedure was avoided (see below).

2. The fluorescent property of 1 for metal ions

The fluorescent property of cyclophane **1** was first investigated. The polyamine structure of **1** allows it can be dissolved in wholly water to micromolar concentrations with addition of moderate amount of hydrochloric acid (to make a 20 μM stock solution of **1** in water, ~4 equiv of HCl was added). Due to the adjacent amine as the quencher via a photoinduced electron-transfer (PET) process, **1** (10 μM) displays a week fluorescence around 420 nm in an aqueous HEPES buffer (10 mM, pH = 7.2) solution. Since receptor **1** contain two binding sites for metal ions, the effect of various metal ions (20 μM) on the fluorescence of **1** (10 μM) was tested. As shown in Fig. S1 (ESI†), only the addition of Zn^{2+} slightly intensified the fluorescent intensity of the **1** solution. In contrast, other metal ions such as Co^{2+}, Cu^{2+}, Hg^{2+}, Ag^{+}, Ni^{2+}, Cd^{2+} quenched the fluorescence, while addition of Li^{+}, Na^{+}, Mg^{2+} etc. showed no effect. Detailed titration of $Zn(NO_3)_2$ to receptor **1** (10 μM) showed that the fluorescence of **1** was gradually intensified upon the addition of Zn^{2+} (Fig. S2a, ESI†), which indicates Zn^{2+} coordination to the receptor with cancellation of the PET process.[16a] However, the fluorescence intensification of **1** (10 μM) does not saturate with 2 equiv of Zn^{2+}, but more than 250 equiv of Zn^{2+} are required for saturation under this condition (Fig. S2a, ESI†), indicating that the binding of Zn^{2+} to receptor **1** is not very tight. This is probably because the electrostatic repulsion between the positively charged first Zn^{2+} coordination site and the incoming second Zn^{2+} cation makes the binding of the second Zn^{2+} more difficult.[16a] To verify a dinuclear zinc complex is formed between **1** and Zn^{2+}, Job's plot was investigated, and indeed the results showed a 1:2 binding mode between **1** and Zn^{2+} (Fig. S2b, ESI†).

3. Facilitation of Zn^{2+} coordination by ATP, ADP and PPi

Interestingly, under the same conditions, when Zn^{2+} was added to the receptor **1** (10 μM) in the presence of 2 equiv of ATP,[22] the fluorescence enhancement was much more sharply saturated and the fluorescence saturation started almost at the addition of 2 equiv of Zn^{2+} (Fig. S2c, ESI†). This indicates that coordination of Zn^{2+} to the receptor **1** is greatly facilitated by ATP. Besides, this result also suggests that the fluorescence enhancement induced by ATP is mainly attributable to the phosphate-assisted coordination of the second Zn^{2+}, if compared to the fluorescence enhancement of Zn^{2+} titration shown in Fig. S2a (ESI†). This is because the second Zn^{2+} binding site of receptor **1** is partially free in the absence of ATP due to a not strong enough binding between receptor **1** and Zn^{2+}, so that PET quenching is more obvious to lessen the anthracene fluorescence. However, when ATP is present, coordination of the second Zn^{2+} to receptor **1** is facilitated, which suppressed PET quenching and recovered the fluorescence intensity[16a], thus the fluorescence enhancement was much more sharply saturated during Zn^{2+} titration.

The same facilitation effect was also observed in the presence of a relatively more excess amount of ADP and PPi (Fig. S2d and 2e, ESI†). In contrast, almost no facilitation effect was observed even in the presence of 50-fold excess of inorganic phosphate (Pi). These results suggest that coordination of Zn^{2+} to the receptor **1** can be selectively induced by certain phosphate derivatives (such as ATP and ADP), in other words, Zn^{2+} complex of receptor **1** could be used to detect certain phosphates selectively under aqueous neutral conditions.

4. The sensing property of 1-2Zn

Inspired by this selective facilitation effect, we reasoned that although a large excess of Zn^{2+} is required for saturation of the two binding sites of receptor **1**, a system (**1-2Zn)** that simply prepared by mixing of receptor **1** and 2 equiv of Zn^{2+} *in situ* could be used as a fluorescence probe to sense biologically relevant phosphate anion species selectively. Hence, **1-2Zn** was prepared in this way without any additional isolation and purification procedures. Fluorescent titrations of **1-2Zn** (10 μM) upon addition of different anions in aqueous HEPES buffer (10 mM, pH 7.2) at 25 ºC were then tested. Indeed, **1-2Zn** was found to show interesting sensing properties. As shown in Fig. S3 (ESI†), the fluorescence of **1-2Zn** was found gradually intensified until saturation upon the addition of ATP, ADP, AMP and PPi. In contrast, almost no fluorescence changes were observed upon the addition of other anions (all as sodium salts) such as PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$, $PhOPO_2^{2-}$, F^-, Cl^-, Br^-, I^-, NO_3^-, SO_4^{2-}, HCO_3^-, $CH_3CO_2^-$, citrate, N_3^-, $S_2O_7^{2-}$, ClO_4^- (Fig. S3, ESI†). These results indicate that **1-2Zn** is selective for ATP, ADP, AMP and PPi among these anions. In addition, the fluorescence of the resulting solutions for ATP and ADP in Fig. S3 are stable (within 1 hour was tested), indicating ATP or ADP hydrolysis mediated by **1-2Zn** was not observed in a short time scale. The typical fluorescent titration of **1-2Zn** (10 μM) upon addition of ATP (0–100 μM) is shown in Fig. 2a. This titration data obey a typical 1:1 binding saturation curve (inserted in Fig. 2a, $R^2 = 0.99161$), and Job's plot also indicates the formation a 1:1 host-guest complex for ATP (Fig. 2b).

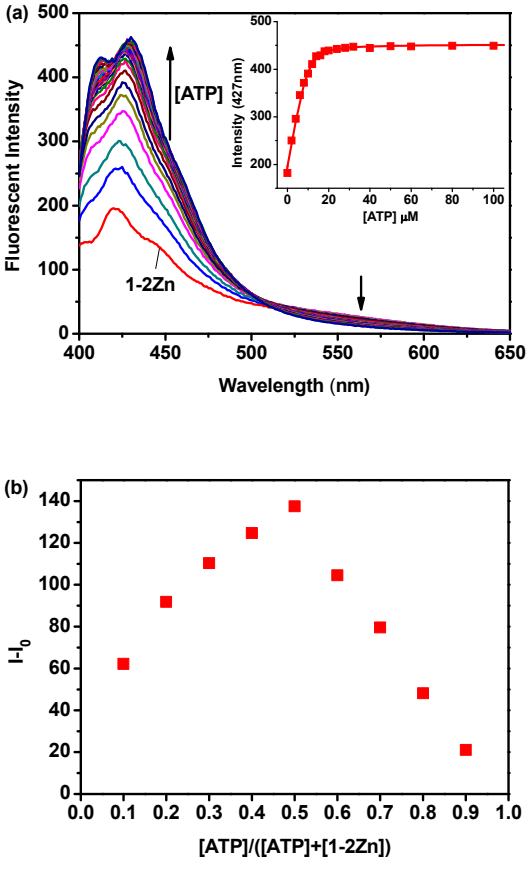


Fig. 2 (a) Fluorescent titrations of **1-2Zn** (10 μM) upon addition of ATP (0 – 100 μM). Inset: Fluorescence intensity changes of **1-2Zn** at 427 nm as a function of ATP concentration, the red line is generated by curve fitting using a 1:1 binding mode.[23] (b) Job's plot examined between **1-2Zn** and ATP, [**1-2Zn**] + [ATP] = 10 μM. λ_{em} = 427 nm. All experiments were measured in aqueous solution of 10 mM HEPES buffer (pH 7.2) at 25 °C (λ_{ex} = 380 nm).

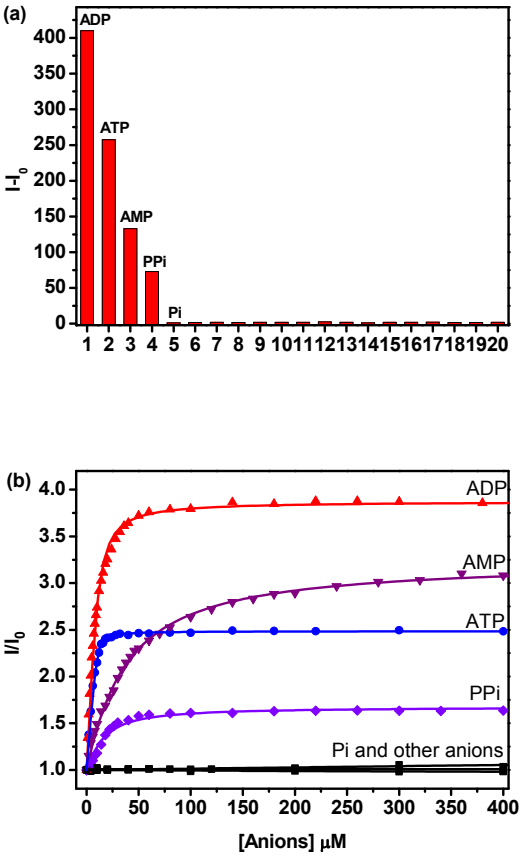


Fig. 3 (a) The fluorescence intensity change ($I-I_0$) of **1-2Zn** (10 μM) at 427 nm upon addition of 2 equiv of various anion species. Anions from 1-20 are: 1. ADP, 2. ATP, 3. AMP, 4. PPi, 5. PO_4^{3-} (Pi), 6. HPO_4^{2-}, 7. $H_2PO_4^-$, 8. $PhOPO_3^{2-}$, 9. F^-, 10. Cl^-, 11. Br^-, 12. I^-, 13. NO_3^-, 14. SO_4^{2-}, 15. HCO_3^-, 16. $CH_3CO_2^-$, 17. Citrate, 18. N_3^-, 19. $S_2O_7^{2-}$, 20. ClO_4^-. (b) The relative fluorescence intensity (I/I_0) changes of **1-2Zn** (10 μM) at 427 nm upon titration of 0–40 equiv of various anions. All experiments were measured in aqueous solution of 10 mM HEPES buffer (pH 7.2) at 25 °C (λ_{ex} = 380 nm). Solid lines in (b) are generated by curve fitting of the data using a 1:1 binding mode.[23]

Table 1 Apparent association constant (K_a) of **1-2Zn** for several phosphate anion species in an aqueous HEPES buffer (10 mM, pH 7.2) at 25 °C [a].

Anion	K_a (M^{-1})
ATP	$(2.0 \pm 0.3) \times 10^6$
ADP	$(3.9 \pm 0.3) \times 10^5$
AMP	$(3.1 \pm 0.1) \times 10^4$
PPi	$(9.1 \pm 1.1) \times 10^4$
other anions	ND[b]

[a] All anions were added as sodium salts. K_a was determined by fitting the fluorescent titration curve as shown in Fig. 3b. [b] Not determined due to too small changes of fluorescence intensity.

The selectivity of **1-2Zn** can be well illustrated in Fig. 3, in which Fig. 3a shows the fluorescence intensity change ($I-I_0$) of **1-2Zn** (10 μM) at 427 nm upon addition of 2 equiv of various anion species and Fig. 3b shows the relative fluorescence intensity (I/I_0) changes (λ_{em} = 427 nm) of **1-2Zn** (10 μM) upon addition of 0-40 equiv of various anions. We can see that **1-2Zn** showed selective fluorescence enhancement for ATP, ADP, AMP and PPi. Among them, addition of ADP caused the most fluorescence intensity changes to **1-2Zn** under the same conditions. However, analysis of the titration data by nonlinear curve fitting[23] showed that **1-2Zn** has the strongest binding affinity for ATP. The apparent association constants (K_a) value for the complexation of **1-2Zn** with ATP is determined to be $(2.0 \pm 0.3) \times 10^6$ M^{-1}, while that with ADP, PPi and AMP is $(3.9 \pm 0.3) \times 10^5$ M^{-1}, $(9.1 \pm 1.1) \times 10^4$ M^{-1} and $(3.1 \pm 0.1) \times 10^4$ M^{-1}, respectively (Table 1). The K_a value for the complexation of **1-2Zn** with other anions cannot be accurately determined due to too small fluorescence changes. Therefore, **1-2Zn** is more selective for ATP from the point view of binding affinity.

The big differences in binding affinities of **1-2Zn** to different phosphate anions may allow us to sense ATP in the presence of excess ADP, AMP and inorganic phosphate. This is practically important because ATP is coexisting with these phosphates in biological systems. To test this feasibility, the fluorescence changes of **1-2Zn** upon addition of ATP in the presence of excess amount of these phosphate species were investigated. Fig. 4 shows the fluorescence changes of the **1-2Zn** (10 μM) solution upon addition of ATP in the presence of 100 equiv of Na_3PO_4 (Pi) under aqueous neutral conditions. We can see that detection of ATP using **1-2Zn** in the presence of large excess of Pi is still effective. Besides, addition of ATP to **1-2Zn** (10 μM) in the presence of excess amounts of PPi (10 equiv), AMP (20 equiv) and ADP (5 equiv) also resulted in the displacement of these phosphates by ATP, and accompanied by obvious fluorescent signal changes (Fig S4, ESI†). These results clearly indicate the feasibility of using **1-2Zn** to sense ATP in the presence of ADP, AMP, PPi and Pi. Although it maybe difficult to infer the concentration of ATP if the concentration of other nucleotides such as ADP is unknown or is changing, the above results indicate that **1-2Zn** can be used as a potential fluorescent sensor for ATP.

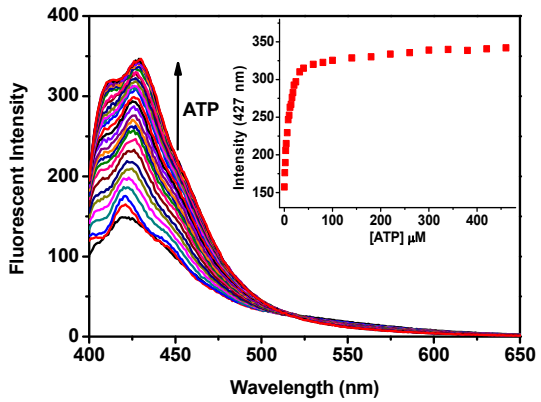


Fig. 4 Fluorescent spectra changes of **1-2Zn** (10 μM) upon addition of ATP (0–450 μM) in the presence of Na_3PO_4 (1000 μM) in aqueous solution of 10 mM HEPES buffer (pH 7.2) at 25 °C (λ_{ex} = 380 nm). Pot of fluorescent intensity changes at 427 nm against ATP concentrations is inserted.

The tighter binding of **1-2Zn** with ATP over ADP and AMP can be explained by the fact that ATP has more negative charges (as ATP^{4-}) in neutral solution to interact with the metal ions centre of **1-2Zn**. Interestingly, our system showed that the binding affinity of **1-2Zn** for ATP is higher than that of PPi. This is quite different from that of many dinuclear zinc complexes reported recently,[7,19c] which often showed higher binding affinities for PPi over ATP. This may indicate that except the similar metal coordination interactions between the phosphate anions and **1-2Zn**, additional π-π stacking interactions between the adenine group of ATP and the anthracene group of **1-2Zn** may exist during their complexation.[11a] The big difference of the sensing behavior of **1-2Zn** between AMP and Pi also supports the existing of π-π stacking interactions, otherwise, **1-2Zn** is most likely to show similar response towards them. As for the larger fluorescence enhancement of **1-2Zn** for ADP over ATP, this is probably similar to our previous work,[18] the well matched distance between the molecular size of ADP and the complex led to stronger π-π stacking interactions between the adenine group in ADP over ATP with the anthracene group in **1-2Zn**, thus resulting a larger effect on the anthracene fluorophore. Although efforts failed to achieve the crystal structure of the complex of **1-2Zn** with ATP, and the poor solubility of **1-2Zn** in water prevents us from further NMR studies, the selective sensing responses of **1-2Zn** for ATP, ADP, AMP are most likely attributable to the existing of π-π stacking interactions based on the above analysis, however, this does not exclude other possible reasons such as caused by steric, hydrophobic surface or geometry effect, etc. Nevertheless, the property of combining metal-anion coordination and π-π stacking interactions should be very useful to design effective nucleotide sensors.

Experimentals

General

All reagents were purchased from commercial suppliers and used without further purification. All solvents were purified prior to use. N-(2-hydroxyethyl)piperazine-N'-(2-ethane-sulfonic acid) (HEPES) was used to prepare buffer solution and all solutions were prepared with distilled water that had been passed through a Millipore-Q ultrapurification system. Melting points were determined using an X-4 apparatus and are not corrected. IR spectra were recorded on a Perkin-Elmer Spectrum BX FT-IR spectrophotometer as KBr pellets and were reported in cm^{-1}. NMR spectra were measured on Varian Mercury 600 instruments, operating at 600 MHz for ^1H NMR and 150 MHz for ^{13}C NMR. Coupling constants (J values) are reported in hertz. Electrospray mass spectra (ESI-MS) were acquired on Agilent 1100 Series LC/MS ion trap mass spectrometers and 6530 Accurate-Mass QTOF spectrometer coupled to an Agilent HPLC 1200 series (Agilent Technologies). Fluorescent spectra were recorded immediately after mixing the receptor and the analytes without any delay on an Agilent Cary Eclipse fluorescence spectrophotometer with a temperature controller. Standard quartz cuvettes with a 10 mm lightpath were used for all fluorescent spectra measurements.

Synthesis of 4

To a stirring solution of 118 mg (0.5 mmol) of 9,10-bis(aminomethyl)anthracene in 40 ml of CH_3OH at room

temperature was added drop by drop over 30 min a solution of 2,6-pyridinedicarboxaldehyde (67.5 mg, 0.5 mmol) in a mixture of $CHCl_3$ and CH_3OH (20 ml of each). The resulting mixture was then stirred at room temperature for 24 hours. The yellow solid was collected on a filter and washed with CH_3OH for many times. Further drying in a vacuum afforded **2** as a yellow solid (150 mg, yield 80%), mp > 300 ºC; ^{1}H NMR (600 MHz, $CDCl_3$): 5.94 (8H, s, $4CH_2$), 7.39 (8H, d, J = 7.2 Hz, ArH), 7.49 (4 H, d, J = 7.8 Hz, PyH), 7.82 (2 H, t, J = 7.8 Hz, PyH), 8.01 (8 H, d, J = 7.2 Hz, ArH), 8.21 (4 H, d, J = 8.4 Hz, CH=N). IR (KBr, cm^{-1}): 3065, 2886, 1645 (s), 1585, 1446, 1345, 1306, 1275, 1180, 1034, 993, 962, 792, 725 (s). HRMS (MALDI-TOF): m/z calcd for $C_{46}H_{34}N_6Na^+$ (M + Na^+) 693.2737, found 693.2735 (100%) and calcd for $C_{46}H_{34}N_6K^+$ (M + K^+) 709.2477, found 709.2465. Due to poor solubility, ^{13}C NMR spectrum of **2** was not collected.

Synthesis of 1

To a stirring solution of **2** (120 mg, 0.179 mmol) in a mixture of CH_3OH (60 ml) and $CHCl_3$ (10 ml) was slowly added 0.15 g of $NaBH_4$ in portions over two hours. The mixture was then refluxed over night. The solvent was evaporated and H_2O (60 ml) was added. The formed solid was collected on a filter and washed with H_2O, dried under vacuum to afford **1** as a yellow solid (110 mg, yield 91%), mp > 300 ºC; ^{1}H NMR (600 MHz, $CDCl_3$): 4.16 (8H, s, $4CH_2$), 4.63 (8H, s, $4CH_2$), 7.01 (8H, d, J = 7.2 Hz, ArH), 7.32 (4H, d, J = 7.8 Hz, PyH), 7.71 (2H, t, J = 7.8 Hz, PyH), 8.07 (8H, d, J = 6.6 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): 45.77, 55.44, 121.37, 124.34, 125.40, 129.84, 131.35, 136.91, 159.49. IR (KBr, cm^{-1}): 3323, 3063, 2883, 1590, 1575, 1448, 1180, 1154, 1109, 765. MS (ESI): 679.6 (M + H^+); HRMS (ESI) m/z calcd for $C_{46}H_{43}N_6^+$ (M + H^+) 679.3544, found 679.3551.

Conclusions

In summary, we have developed a new metal complex of an anthracene-based cyclophane system (**1-2Zn**), which was found to be a selective fluorescent sensor for ATP, ADP, AMP and PPi in neutral aqueous solution. This system is readily available, and shows strong binding affinity for ATP and the most intensified fluorescence enhancement for ADP. This property can be attributed to the combination of metal-anion coordination with π-π stacking interactions between the adenine and anthracene group and this strategy should be useful for designing effective sensors for nucleotides.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Facilitation of Zn^{2+} coordination to **1** by ATP, ADP and PPi, and additional fluorescence studies. See DOI: 10.1039/b000000x/

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