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# Aspartic acid functionalized water-soluble perylene diimide as "Off–On" fluorescent sensor for selective detection $Cu^{2+}$ and ATP

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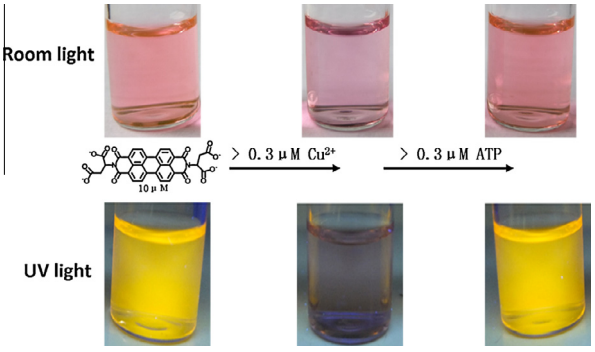
## HIGHLIGHTS

- Water soluble PDI was used in fluorescence sensor $Cu^{2+}$ and ATP in pure aqueous solution.
- The 0.3 μM fluorescence sensitivity is one of the highest.
- Concentration- and pH-dependence spectra were investigated.

## GRAPHICAL ABSTRACT

Water soluable aspartic acid functionalized perylene diimide (PDI) was used as turn-off fluorescence sensor for Cu(II) ion. The PDI–copper complex is very sensitive turn-on fluorescence sensor for ATP molecule.

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## ABSTRACT

Aspartic functionalized water-soluble perylene diimide, *N,N′*-di(2-succinic acid)-perylene-3,4,9,10-tetracarboxylic diimide (PASP) has two absorbance maximums at 527 and 498 nm ($\varepsilon \approx 1.7 \times 10^4$ L cm$^{-1}$ mol$^{-1}$) and two emission peaks at 547 and 587 nm respectively. Emission intensities decrease with the increase of PASP concentrations in 20–100 μM ranges. Spectral titrations demonstrate that each PASP can coordinate to two $Cu^{2+}$ ions in the absence of HEPES buffer. Its stability constant is estimated to be about $1.0 \times 10^{12}$ L$^2$ mol$^{-2}$ at pH 7.20 and its coordinate stoichiometry increased to 7.5 in the same pH in the presence of HEPES buffer. The emission of PASP will be completely quenched upon formation of $Cu^{2+}$ complex. The lowest "turn-off" fluorescence detection limit was calculated to be 0.3 μM $Cu^{2+}$. PASP–Cu solution was used as a "turn-on" fluorescence biosensor to detect ATP. The sensitivity towards ATP is 0.3 μM in 50 mM HEPES buffer at pH 7.20, which is one of the most sensitive fluorescence sensors.



## Introduction

In recent years, the development of optical chemosensors for selective detection of biologically and environmentally important ionic species or bio-molecular compounds have gained attention in the research community [1–8]. Copper is very interesting in this regard because it is indispensable in its interactions with certain proteins to produce numerous metalloenzymes such as superoxide dismutase, cytochrome c oxidase and tyrosinase that are critical for life. However, copper is toxic when its concentration exceeds cellular needs and has been linked to neurodegenerative diseases such as Alzheimer's disease and Wilson's disease [9–11]. Owing to the simplicity and high sensitivity of their responses, fluorescent chemosensors were widely investigated for the detection and measurement of transition-metal ions such as copper. Detection of Cu (II) by variety of synthesized colorimetric or fluorescent probes, such as amide/amine, calixarene, nanoparticle, coumarins,

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porphyrin, rhodamine, spiropyrane, polypeptide and G-quadruplex, have been reported [7,8,12,13]. It is well known that paramagnetic Cu(II) will significantly quench fluorescent and thus Cu(II) may acts as turn-off fluorescent chemosensor. Even though great advancements in the field of $Cu^{2+}$ ion chemosensors have been achieved, there is still a demand to develop new indicators with improved sensitivity, especially fluorescent sensors with high efficiency in the visible spectrum with a specific selectivity toward $Cu^{2+}$. The perylene diimides (PDIs) have strong absorption with high molar absorption coefficients and long fluorescence lifetime and near uniform fluorescence quantum yields [14] and are widely used as fluorescent sensors [15–18].

ATP has a key role as the major energy currency in many biological processes [19–21]. Its concentrations are tightly regulated under normal conditions. Aberrant ATP levels have been associated with particular diseases such as angiocardiopathy due to the excessive production of ATP by creatine kinase [22]. Accurate detection and quantification of ATP is an important goal for both biochemical and clinical applications. The minimum response concentration of a series of fluorescence resonance energy transfer (FRET)-based indicators was 7.4 μM–3.3 mM [23]. Numerous metal complexes, especially $Zn^{2+}$ complexes, were also used as sensors for ATP [15,24–31]. The lowest detectable ATP concentration is about 0.5 μM for $Zn^{2+}$–anthracene complex [32] and 5.88 μM for $Zn^{2+}$-dipicolylamine-functionalized polydiacetylene monomer [33]. Fluorescent silent Cu(II) complexes as turn-on fluorescence sensors to respond strong coordinating ligands, such as $CN^-$ [34,35], thiols [36,37], $S^{2-}$ [38] and nitric oxide [39], have been reported.

In this paper, we report a water-soluble perylene diimide PASP that acts as a fluorescent "turn-off" sensor to sensitively and selectively recognize $Cu^{2+}$ in aqueous solutions. In addition, the fluorescence silent PASP–Cu complex solution acts as an in situ "turn-on"fluorescent biosensor for ATP molecules.

## Experiment

### *Apparatus*

UV–Vis absorption spectra were performed with a Puxi TU-1900 spectrometer with a 1.0 cm quartz cell equipped with a temperature-controlled water bath (All of the UV–Vis experiments were performed at 25 °C). Luminescent spectrum was carried out on a Shimadzu RF-5301 spectrophotometer (For all fluorescence measurements, excitation and emission slit widths were 3 nm and 3 nm, respectively. The emission spectra were excited at 500 nm and with the low sensitivity).

### *Materials*

PASP ($C_{32}H_{18}O_{12}N_2{\cdot}5H_2O$) was synthesized by an improved method according to literatures [40,41]. $^1H$ NMR spectra and Elemental analysis was used to characterize the purity of PASP. All other inorganic reagents are A.R. grade from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The biological chemicals used were bought from Shanghai Sangon Co., Ltd. (Shanghai, China). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was supplied by Aladdin Co., Ltd. (Shanghai, China). All of the chemical regents were used as obtained without further purification. All of the solutions in the experiments were prepared with sub-boiled water distilled in all-quartz apparatus.

### *Sample preparation*

0.01 M and 0.001 M PASP stock solution were prepared in aqueous solution with some solid NaOH. The pH of the stock solution was about 11. The stock solutions (0.01 M) of the metal nitrate (or chlorate) and biological molecules (or ions) of ATP, ADP, AMP, $HPO_4^{2-}$, $HCO_3^-$, $Cl^-$ were prepared in aqueous solution. All test samples were prepared by dilute required amount of stock solution (<130 μL) with 3.0 mL of 50 mM HEPES buffer solution. PASP–Cu (II) (10 μM PASP, 100 μM Cu (II) aqueous solution was used for in situ biosensor of ATP. The complex solution was stored 12 h in dark before measurement.

### *Measurement procedures*

Fluorescence of PASP at different concentrations was complemented by adding different amount of PASP stock solution into 3.0 mL HEPES buffer solution (pH 7.20). pH values were adjusted with 6.0 or 2.0 M HCl, and/or NaOH solution. Metal sensor test solutions were prepared by placing 3.0 mL 10 μM PASP in 50 mM HEPES buffer (pH 7.20) solution into a cuvette, adding an appropriate aliquot of metal ion stock solution. Biosensor tests were performed the same way as metal sensor tests except PASP–Cu complex solutions were used instead of PASP solution.

## Results and discussion

### *Fluorescence spectra of PASP at different concentrations*

PASP exhibits good solubility in alkali aqueous due to the electrostatic repulsion between its multiple negative charges.

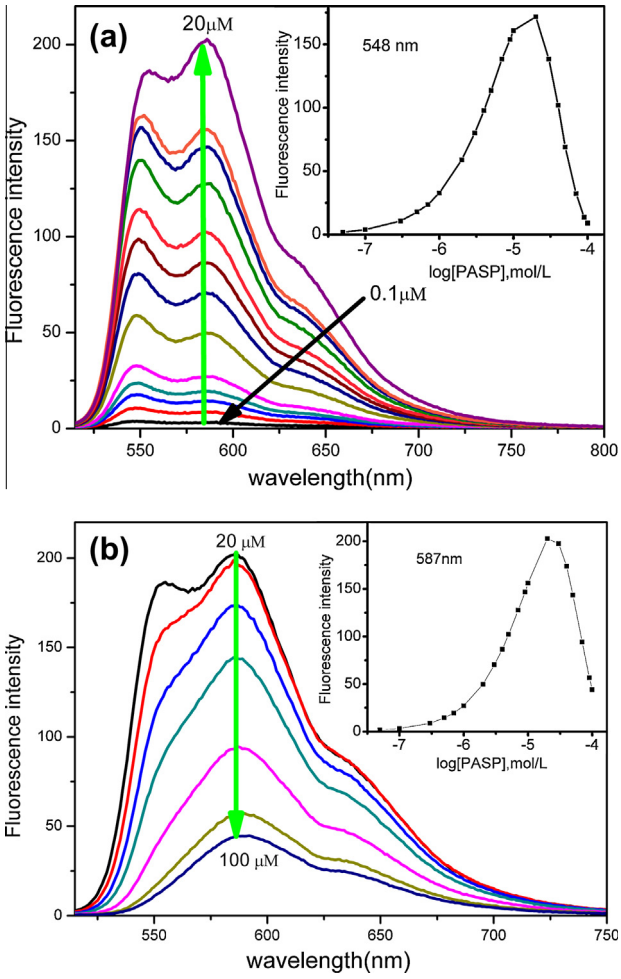



**Fig. 1.** Fluorescence spectra of PASP at low (<20 μM) and high (>20 μM) concentrations in HEPES buffer (50 mM, pH 7.20): (a) 0.1–20 μM and (b) 20–100 μM.

However, the large conjugate aromatic ring has a strong tendency towards aggregation via π–π and/or hydrophobic interaction in aqueous solution in different concentration. Monomers exhibit normal Frank–Condon progression with the ratio of 527 and 498 nm absorption $A^{0-0}/A^{0-1} \sim 1.6$, aggregated molecules have inversed intensity distribution with $A^{0-0}/A^{0-1} \leqslant 0.7$ [42].

Fluorescence spectra can also be used to monitor the solution behavior of PASP in HEPES buffer (50 mM, pH 7.20). There are two emission peaks at around 548 and 587 nm (Fig. 1). The 548 nm emissions are attributed to the monomer, while the 587 nm emissions can be assigned to aggregated form of PASP. At a low concentration (<20 μM), the 548 nm emission (monomer) is obviously stronger than the 587 nm emission (aggregation). At a higher concentration (>20 μM), the emission of the aggregated form at 587 nm is stronger than the monomer emission at 548 nm. When the concentration is lower than 20 μM, PASP exists mainly in monomer form. However, the aggregation in pH 7.20 HEPES buffer is insignificant. Based on these spectra results and considering the fact that the emission at 548 nm has same overlap with the 527 nm absorbance, one can conclude that the 548 nm emission were reabsorbed by ground-state PASP molecules in high concentrations (>20 μM). That is the reason why 548 nm emissions gradually disappeared at very high concentrations. However, the 587 nm emission has much less overlap with the 527 nm absorbance as the 587 nm emission had significantly less decreases than that of 548 nm emission. Reabsorption is predominate in concentration >20 μM.

### *Fluorescence spectra of PASP at different pH*

The present form as well as the spectra of PASP depends on pH [40]. Fig. 2 is the fluorescence spectra of 10 μM PASP in HEPES buffer (50 mM) at a different pH but pH did not change emission position. However, fluorescence intensities are highly dependent on pH. It was worthy to mention that the fluorescence of PASP was completely quenched when pH reached 6.43 or lower due to insolubility (Fig. 2). The emission intensity is constant in pH 7.0–7.6, but decreases in pH 7.6–8.5. The emission intensity is essentially constant in pH 8.5–11.5. At pH 11.5 or higher, the emission intensity starts to decrease. The succinic acid has $pK_{a1}$ and $pK_{a2}$ of 4.21 and 5.64 respectively. Minimum pH to remove both protons from succinic acid is ~pH 7.64, which agrees well with the emission plateau of pH in the 7.5–12 region.

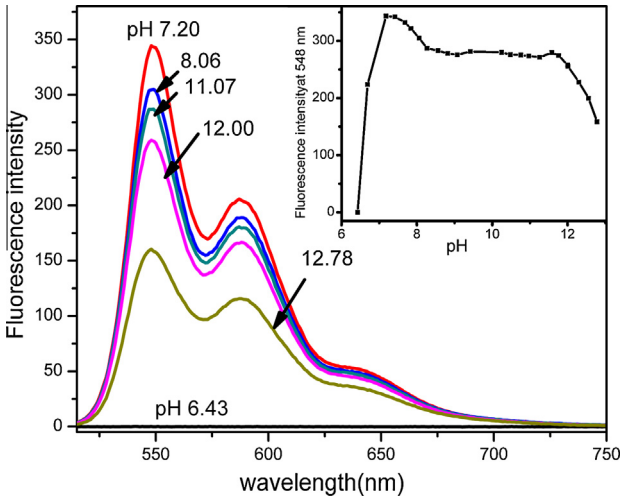



**Fig. 2.** Fluorescence spectra of PASP (10 μM) in HEPES buffer (50 mM) at different pH. Inset: Dependence of the emission intensity of PASP at 548 nm at different pH.

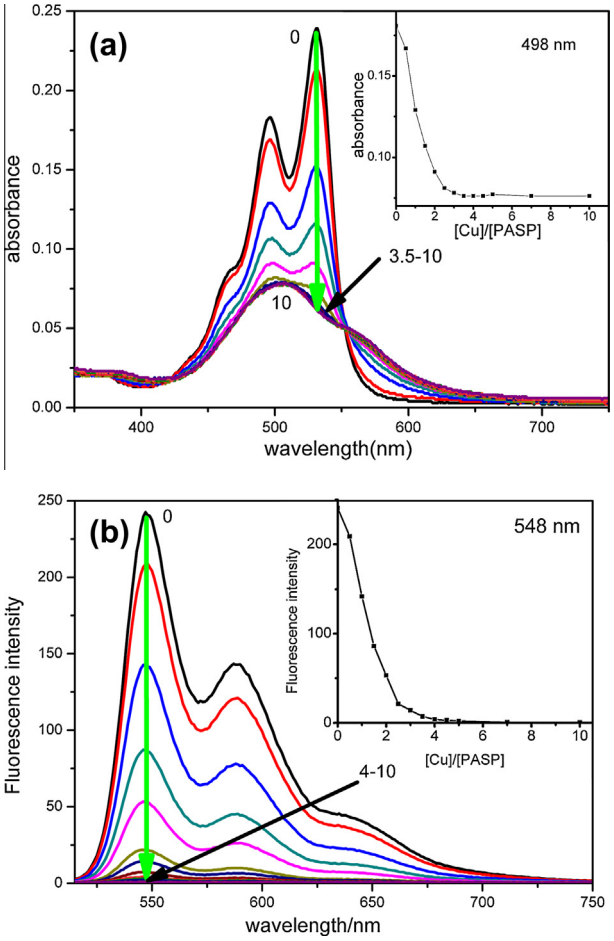
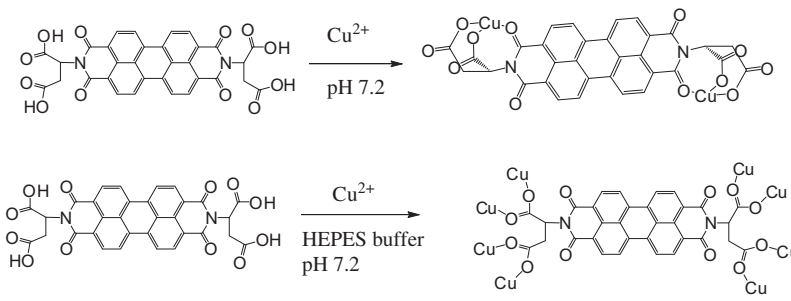



**Fig. 3.** (a) Absorption and (b) fluorescence spectra of PASP (10 μM) in presence of different $Cu^{2+}$ concentration (the ratio [Cu]/[PASP] was from 0 to 10 in aqueous solution at pH ~ 7.20. The insets show the relationship between the intensity and the [Cu]/[ PASP] at 498 nm absorption and 548 nm emission.

### *"Turn-off" fluorescent sensor of PASP to $Cu^{2+}$*

It is known that multiple L-aspartic acid pendants in one fluorophore facilitate binding with metal cation [41,43]. Fig. 3 compares the absorption and emission spectra of PASP (10 μM) in the absence and presence of $Cu^{2+}$ ions at pH 7.20 without buffer. It can be seen from Fig. 3a that by increasing $Cu^{2+}$ concentrations, the absorption bands at 498 nm and 527 nm of PASP decrease gradually and a new broad shoulder at 506 nm is developed. These spectra indicate that $Cu^{2+}$ can interact with PASP in aqueous solution. Fluorescence spectra are shown in Fig. 3b. By adding $Cu^{2+}$ to PASP aqueous solution, the fluorescence of PASP was quenched gradually. The fluorescence quenching was most likely caused by the photo-induced electron transfer (PET) [16]. $Cu^{2+}$ may also intensify the π–π stacking among perylene moieties and facilitates the formation of non-emitting PASP aggregation with the low-energy excitonic transition that decreases PASP emission [44–47]. The intensities of 527 nm absorbance and 548 nm emissions depend on the $Cu^{2+}$ concentrations. The turning point appeared at PASP: $Cu^{2+}$ = 1:2 for both absorbance and emission in the absence of HEPES buffer (Fig. 3, inset). The binding stoichiometry between PASP and Cu is 1:2, which is similar to literature data [15,16]. From the experimental absorbance/emission at 1:2 ratio, PASP–copper (II) complex dissociates 11–14% and the observed formation constant of $Cu_2$PASP is estimated $1.0 \times 10^{12}$ $L^2$ $mol^{-2}$. This number is obviously larger than that of copper succinate mononuclear




**Scheme 1.** Proposed binding modes of PASP with $Cu^{2+}$ in the absence and presence of HEPES buffer in pH 7.20.

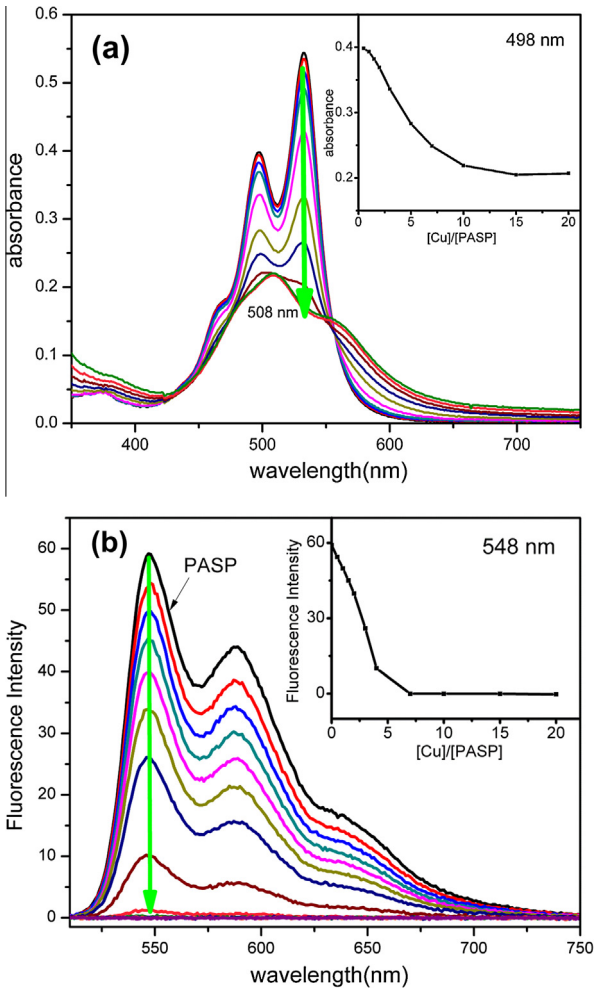



**Fig. 4.** (a) UV–Vis absorption spectra and (b) fluorescence spectra of PASP (10 μM) in the presence of $Cu^{2+}$ (0–20 equiv.) in HEPES buffer solution (50 mM, pH 7.20). The insets show the relationship between the intensity and the [Cu]/[PASP] at 498 nm absorption and 548 nm emission.

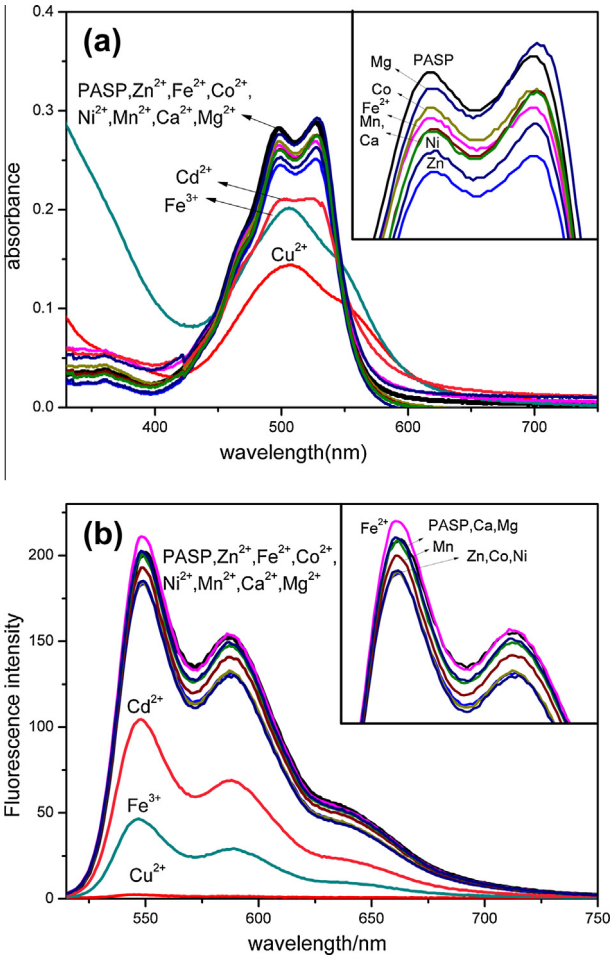



**Fig. 5.** Absorption (a) and fluorescence (b) spectra of PASP (10 μM) in the presence of 100 μM metal ions ($Cu^{2+}$, $Zn^{2+}$, $Fe^{3+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Mn^{2+}$, $Cd^{2+}$, $Ca^{2+}$, $Mg^{2+}$) in HEPES buffer (50 mM, pH 7.20).

complex ($10^{4.5}$) [48], and it is possible that the amide oxygen atom has some interaction with $Cu^{2+}$ (Scheme 1).

Fig. 4 shows absorption and emission spectra of PASP–copper (II) systems in HEPES buffer (50 mM, pH 7.20). Although the shape of the spectra is almost identical to those without HEPES buffer, the coordination stoichiometry increased to 7–8 (Metal:Ligand) in the presence of HEPES buffer rather than 2 in the absence of HEPES. This indicates that HEPES buffer had interaction with Cu (II). It is possible that each carboxylate oxygen atom binds one Cu (II); the proposed binding mode of PASP with $Cu^{2+}$ in the presence of HEPES buffer was shown in Scheme 1.

From Figs. 3 and 4, we see the completely quenching of the 548 nm emissions requires different amount of Cu (II) ion in the absence and presence of HEPES buffer in neutral solution (pH 7.20). 10 equivalents copper (II) is enough to completely quench the 548 nm emission in the presence of HEPES buffer. Fig. 5 is the UV–Vis and emission spectra of 10 μM PASP in the presence of 100 μM metal ions in HEPES buffer (50 mM, pH 7.20). From Fig. 5, both the shape and intensity change significantly in the presence of $Cu^{2+}$, $Fe^{3+}$ and $Cd^{2+}$. In the presence of $Cu^{2+}$ or $Fe^{3+}$ ions, the absorbance at 498 and 527 nm decreased and the absorption maximum shifted to a longer wavelength. The 498 nm absorbance in free PASP shifted to 506 nm in $Cu^{2+}$ or $Fe^{3+}$ complexes. Fluores-

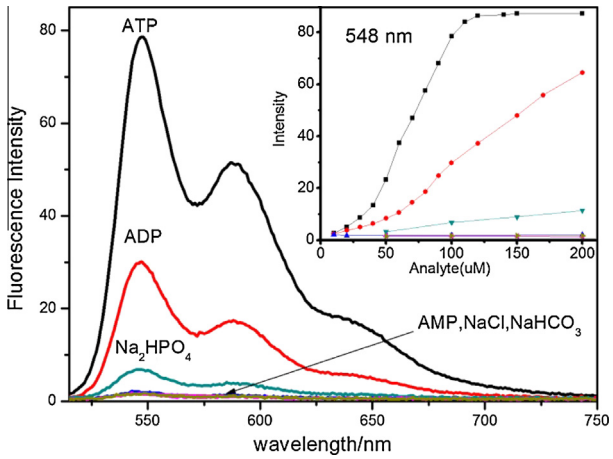



**Fig. 6.** Fluorescence spectra of PASP (10 μM) and $Cu(NO_3)_2$ (100 μM) in the presence of biological molecules and ions (100 μM) in HEPES buffer (50 mM, pH 7.20).

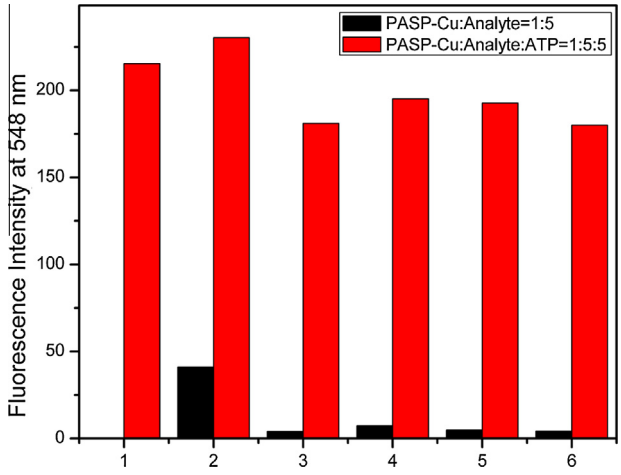



**Fig. 7.** Fluorescence response of PASP (10 μM)- $Cu(NO_3)_2$ (100 μM) at 548 nm upon addition of various anions in HEPES buffer (50 mM, pH 7.2). Black bars: upon addition of 5 equiv. of anions; Red bars: the integrated fluorescence response upon subsequent addition of 5 equiv. of ATP. (1) ATP; (2) ADP; (3) AMP; (4) $HPO_4^{2-}$; (5) $HCO_3^-$; (6) $Cl^-$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cence spectra shows 10 equivalent Cu (II) can completely quench the 548 nm emission. 10 equivalent $Fe^{3+}$ and $Cd^{2+}$ quench 77% and 50% of ligand emission. From UV–Vis and fluorescence spectra, the interaction of $Zn^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Mn^{2+}$,$Ca^{2+}$, $Mg^{2+}$ with PASP is negligible in 10 μM PASP and 100 μM metal ions in HEPES buffer (50 mM, pH 7.20).

Buffered solution of 10 μM PASP and 100 μM $Zn^{2+}$, $Fe^{2+}$, $Co^{2+}$, $Ni^{2+}$, $Mn^{2+}$, $Ca^{2+}$ or $Mg^{2+}$ binary complex have fluorescence emission at 548 nm. When the binary complexes were further added 100 μM $Cu^{2+}$, the fluorescence was also completely quenched. An equivalent competitive metal ion does not affect the absorbance and emission of $Cu^{2+}$ complex. The selectivity of PASP toward $Cu^{2+}$ suggests that PASP is a good potential fluorescence probe to the Cu (II) determination. The lowest limit detection was calculated to be 0.3 μM according to 3 sigma rules. However, excess $Fe^{3+}$ may have caused some interference.

### *PASP–Cu as a "turn-on" fluorescent biosensor of ATP*

Colorimetric and fluorescent sensing of biologically important anions have attracted considerable interest in recent years [15,17,41]. ATP has a strong affinity towards $Cu^{2+}$ ions with respect to multiple phosphates [49,50]; one can expect that the introduction of ATP into fluorescent quenched PASP–Cu solution would competitively bind with $Cu^{2+}$ ions, disrupting the PASP–Cu complex. As a result, free PASP molecules are released, leading to the recovery of PASP fluorescence, thus turning on a detectable fluorescent signal. To investigate the sensor ability of the PASP–Cu (II) system, 10 μM PASP and 100 μM $Cu(NO_3)_2$ mixtures were selected as the biosensor for a variety of biological molecules and ions. Fig. 6 shows the fluorescence spectra in the presence of 100 μM biological molecules or ions in HEPES buffer (50 mM, pH 7.20). ATP displayed a remarkable enhancement of fluorescence intensity at 548 nm and 587 nm. ADP can also enhance the fluorescence intensity but less significant than that of ATP. $Na_2HPO_4$, $NaH_2PO_4$, AMP, $NaHCO_3$ and NaCl essentially have no effect on the emissions at 548 nm (fluorescence silent). The observed selectivity of PASP–Cu (II) toward ATP suggests that PASP–Cu (II) has a potential application in sensing ATP in aqueous solution. As shown in Fig. 6, when the ATP concentration increased, the maximum fluorescence intensity at 548 nm and 587 nm gradually increased. As an equimolar amount of ATP relative to $Cu^{2+}$ was added to the solution, the fluorescence intensity recovered 100% of the initial intensity of free PASP. Extra ATP will not change in emission intensity further. These results indicate that the competitive binding of ATP to

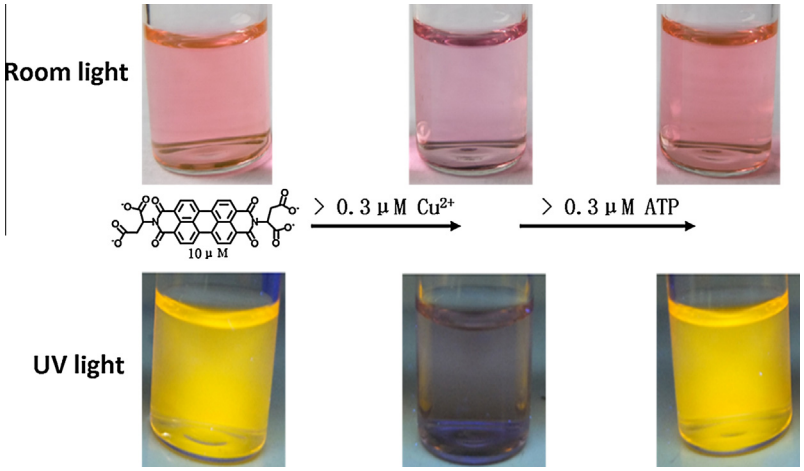



**Fig. 8.** Photographs of PASP (10 μM) as a "Turn-On" sensor to $Cu^{2+}$ (>0.3 μM) and a "Turn-Off" sensor to ATP (>0.3 μM) in the room light and UV light.

$Cu^{2+}$ results in the dissociation of the PASP–Cu (II) complex, which is responsible for the reappearance of the fluorescence due to the equilibrium moving to the free PASP. Notably, there exists a good liner relationship between emission intensity and ATP concentration around 30–100 μM ($R^2$ = 0.993), indicating that this approach is applicable for quantitative detection of ATP. The intercept is 25 μM, which agrees well with Fig. 4, [PASP]/[Cu] = 1/7.5 in HEPES buffer. The limit of detection was calculated to be 0.3 μM according to 3 sigma rules [51], which is one of the top sensitivity fluorescent Cu complex sensors [41]. Fig. 7 showed the detection of PASP–Cu to ATP in the existence of other biological molecules or ions ($ADP, AMP, H_2PO_4^-, HPO_4^{2-}, HCO_3^-$ and $Cl^-$) were still sensitive enough.

Fig. 8 is a schematic photograph illustrating color change from PASP to PASP–Cu and PASP–Cu–ATP consecutively in the room light and UV light. PASP acts as a "turn-off" fluorescence sensor that detects Cu (II) selectively while PASP–Cu (II) complex can be a "turn-on" fluorescence sensor detects ATP sensitively.

## Conclusion

The pH- and concentration-dependent absorbance and emission spectra of water-soluble PASP were studied. PASP can selectively coordinate to $Cu^{2+}$ in the presence of other competing metal ions. Each PASP can coordinate to two $Cu^{2+}$ ions in the pure aqueous solution. Fluorescence was completely quenched upon formation of Cu (II) complex, thus PASP can act as "turn-off" sensor to detect Cu (II) selectively. The lowest detection limit was 0.3 μM in HEPES buffer solution. PASP–Cu complex was used as "turn-on" sensor to detect ATP selectively. The sensitivity towards ATP is 0.3 μM in HEPES buffer, which is one of the top sensitivity fluorescence sensors.

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