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A New Conjugated Polymers-Based Combination Probe for ATP Detection Using Multisite-Binding and FRET Strategy

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A new conjugated polymers -based ratiometric combination probe was constructed for adenosine triphosphate detection by taking advantage of multisite-binding and fluorescence resonance energy transfer strategy. The method is rapid and highly selective, which can clearly discriminate ATP from persistent interferents such as ADP, AMP, other nucleoside polyphosphates and nucleobases.

The nucleoside polyphosphates (NPPs) that construct the basic genetic framework are essential and ubiquitous in almost all the cellular events.[1] Particularly, ATP can transport chemical energy for metabolism, and is usually referred to as "the primary energy currency'' of intracellular energy transfer.[2] Fluctuation in the ATP level has been found to cause many clinical diseases, for instance, Parkinson's disease and various malignant tumors.[2b, 3] In addition, ATP concentration as a key indicator has been widely used to monitor cell viability, food quality, and environment.[4] Therefore, to accurately detect and quantify ATP is an important goal for both biochemical and clinical applications.

The classical ATP detection methods including HPLC, NMR spectroscopy and capillary electrophoresis have been used for decades of years. However, compared with preferable fluorescence assay, these traditional methods usually are short of high sensitivity and selectivity, spatiotemporal resolution and satisfactory compatibility for visible and real-time imaging in biological system.[5] A commercialized ''luciferin-luciferase bioluminescence assay'' [6] for ATP sensing is sensitive but must use unstable and costly luciferin and luciferase, which limits its widespread use.[4] Recently, several small molecule or aptamer-based fluorescent ATP probes have been developed.[7] Small-molecule probes are mainly based on the electrostatic interaction between negatively charged phosphates of ATP and positively charged recognition groups, however, selectivity of these probes is not satisfying due to the interference from NPPs or negatively charged biomolecules.[8] Compared to small-molecule probes, aptamer-based probes are generally more selective and can be more readily and economically synthesized on a large scale. [9] However, the non-specific binding between aptamer and fluorophore may cause higher background. [10] Besides, most of the aptamer-based methods cannot distinguish adenosine from ATP. [11] Recently, a few probes with more than one recognition site had been developed to sense ATP in living cells. However, they worked in a high ATP concentration at a range of 0.1-10 mM.[3, 12]

In recent years, owing to their strong light-harvesting and signal-amplification properties, conjugated polymers (CPs) have attracted much attention in cell imaging, in vivo tumor targeting, selective recognition of biomolecules, drug carriers, and medical diagnostics/therapeutics.[13] Recently, some CPs-based ATP sensors, especially polythiophenes (PT) based sensors, have been reported based on electrostatic interactions between cationic probes and anionic phosphates of ATP.[7g, 13c, 14] Nevertheless, the electrostatic attraction is nonspecific. In addition, some of these probes utilized a fluorescence "turn-off" strategy or monitored ATP according to maximal absorption wavelength red-shift, which could suffer from the background interference or be less sensitive.

Herein, we designed and synthesized two conjugated polymers including phenylboronic acid (PBA)-modified PPE-PBA and quaternary ammonium-modified PFP-NMe_3^+, which were used as a new combination probe to sense ATP. The new neutral PPE-PBA can recognize ribose of ATP by covalent bond. Whereas, PFP-NMe_3^+ can interact with the negatively charged phosphates of ATP by electrostatic interaction. Owing to the dramatical overlap between emission spectrum of PFP-NMe_3^+ and absorption spectrum of PPE-PBA, the efficient FRET will occur when ATP is present and brings two CPs close by multi recognition sites. The new CPs-based combination probe based on multisite binding and FRET strategy could sensitively and selectively distinguish ATP from interferents, such as adenosine, ADP, AMP, NNPs and saccharides, etc..

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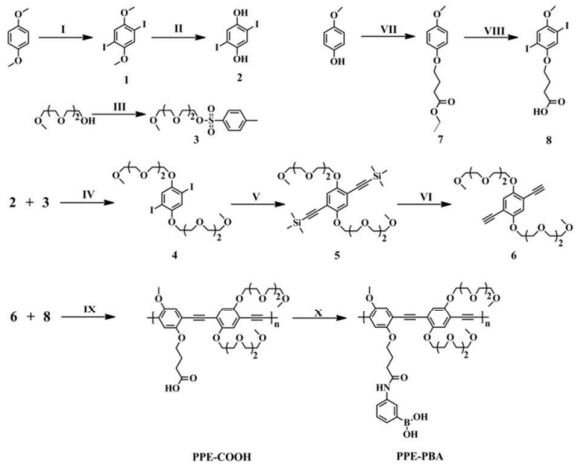
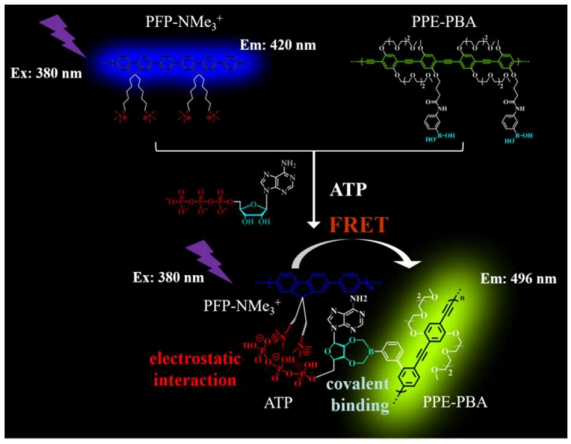


Scheme 1. Synthesis of PPE-PBA: (I) I_2, H_5IO_6, MeOH, 70 °C, 4 h; (II) BBr_3, CH_2Cl_2, -78 °C to r.t., 14 h; (III) TsCl, pyridine, CH_2Cl_2, 0 °C to r.t., overnight; (IV) K_2CO_3, acetone, DMF, 58 °C, overnight; (V) TSMA, $Pd(PPh_3)_2Cl_2$/CuI, triethylamine, THF, r.t., 4 h; (VI) K_2CO_3, MeOH/THF, r.t. 5 h; (VII) ethyl 4-bromobutyrate, K_2CO_3, DMF, 100 °C, 1 h; (VIII) I_2, KIO_3, con. H_2SO_4, AcOH, 120 °C, 3 h; (IX) $Pd(PPh_3)_4$/CuI, morpholine; (X) 3-aminophenylboronic acid, EDC/HOBt, triethylamine, DMF, 0 °C to r.t., overnight.

$PFP\text{-}NMe_3^+$ is commonly used as energy donor owing to its ideal fluorescence quantum yield in biosensors, which is prepared according to the previously reported procedure.[14a] The PPE-PBA is selected as the energy acceptor owing to its absorbance spectrum possibly overlaping the emission spectrum of $PFP\text{-}NMe_3^+$. The synthetic route for the PPE-PBA and its monomers and precursor are outlined in Scheme 1. [14c-e, 15] The PEG groups are introduced to the side chains of polymers to further improve the water-solubility and reduce nonspecific interactions and undesirable aggregation.[13c] To build a binding site between neutral poly (phenylene ethynylene) and ATP, the carboxyl groups are doped into side chains at a 50% molar ratio to afford the precursor polymer PPE-COOH firstly. Then, the 3-aminophenylboric acid, which acts as the ATP binding site, can covalently linked to the PPE-COOH by coupling amino groups with carboxyl groups to create the target PPE-PBA. According to the integral ratio from the ^1H- NMR spectrum of PPE-PBA, one can estimate around 25 % of carboxyl groups are modified with PBA groups.

The working principle of the probe is schematically represented in Scheme 2. When ATP is added into the probe solution, PPE-PBA can recognize the ribose of ATP by a covalent binding manner, whereas $PFP\text{-}NMe_3^+$ can keep close to the phosphate group of ATP by electrostatic interactions. Finally, a $PFP\text{-}NMe_3^+$/ATP/ PPE-PBA complex is achieved, which shortens the distance of CPs and enhances FRET efficiency.

The absorption and fluorescence spectra of $PFP\text{-}NMe_3^+$ and PPE-PBA in water are shown in **Fig. 1a**. The fluorescence emission spectrum of $PFP\text{-}NMe_3^+$ matches well with the absorption spectrum of PPE-PBA under excitation at 380 nm, making the FRET possibly occurs. [16] Note that upon binding with ATP, the fluorescence intensity (420 nm) of $PFP\text{-}NMe_3^+$ remarkably decreases and the fluorescence intensity (496 nm) of PPE-PBA increases (**Fig. 1b**), which demonstrates the



Scheme 2. Mechanism of the CPs-based combination probe for sensing ATP.

efficient FRET from $PFP\text{-}NMe_3^+$ to PPE-PBA occurs in virtue of ATP-bridged linking. Corresponding fluorescent photographs of polymers in the absence and presence of ATP were shown in **Fig. S1**, which is consistent with the fluorescence emission. To prove the FRET process, the emission lifetimes (τ) and the quantum yields (Φ_F) for the donor and acceptor were measured and summarized in Table S1. The Φ_F of $PFP\text{-}NMe_3^+$ decreased dramatically meanwhile that of PPE-PBA increased from 2.26% to 5.40% in the presence of ATP. As the energy donor, $PFP\text{-}NMe_3^+$ showed a shorter τ after mixing with PPE-PBA and ATP.[17] These data indicated that FRET occurs between $PFP\text{-}NMe_3^+$ and PPE-PBA. Our probe system applies a multisite-binding strategy, which allows a more efficient and selective sensor for the detection of ATP.

To further prove the recognition of ATP by the combination probe, the size distribution measurement was conducted by dynamic light scattering (DLS). Because conjugated polymers are amphiphilic macromolecules, they inevitably form very loose aggregations in aqueous solution. As shown in **Fig. 1c**, $PFP\text{-}NMe_3^+$, PPE-PBA and combination probe have average hydrodynamic diameters of 170.9, 105.8 and 142.5 nm, respectively. Upon adding ATP in the combination probe solution, the $PFP\text{-}NMe_3^+$/ATP/PPE-PBA complex shows an average hydrodynamic diameter of 887.0 nm, which means the combination probe recognizes the ATP and form bigger aggregation by virtue of ATP-bridged linking. These results indicate that ATP induces the formation of $PFP\text{-}NMe_3^+$/ATP PPE-PBA complex and activates FRET between the two CPs.

Considering that the CPs molar ratio is crucial for the fluorescence intensity ratio as well as detection sensitivity, we investigated the fluorescence ratio of PPE-PBA to $PFP\text{-}NMe_3^+$ (R ($I_{496\ nm}/I_{420\ nm}$)) under different CPs molar ratios ($PFP\text{-}NMe_3^+$/ PPE-PBA). Herein, the CPs molar ratio of 2:1, 3:1, 1:1 and 1:2 are studied. As shown in **Fig. S2a**, when the CPs ratio is 2:1, the best fluorescence enhancement is obtained, which exhibits lower background signal and most sensitivity compared with others. Therefore, 2:1 as the optimized CPs molar ratio is used for the following studies.

It is noted that the pH value of the buffer strongly influences the formation of covalent bonding between PBA and 1,2 -

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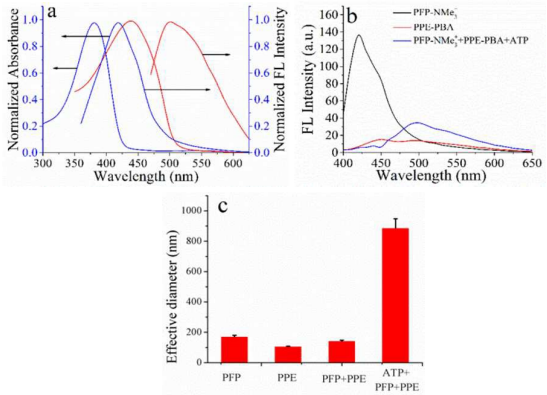

Fig. 1 (a) The normalized absorption and fluorescence spectra of PFP-NMe_3^+ and PPE-PBA in water. (b) Emission spectra of PFP-NMe_3^+, PPE-PBA and PFP-NMe_3^+/PPE-PBA in the presence of ATP in 10 mM HEPES buffer. [PFP-NMe_3^+] = 2 μM, [PPE-PBA] = 1 μM, [ATP] = 180 μM. λ_{ex} = 380 nm. (c) DLS results for PFP-NMe_3^+, PPE-PBA, PFP-NMe_3^+/PPE-PBA and ATP/PFP-NMe_3^+/PPE-PBA.

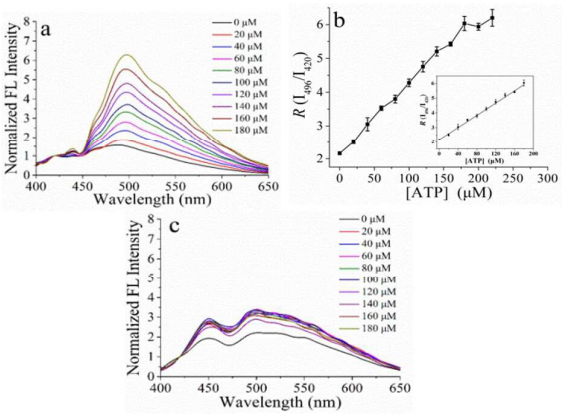

Fig. 2 (a) Normalized fluorescence spectra and (b) fluorescence intensity ratios (I_{496}/I_{420}) of combination probe with increasing concentrations of ATP. [PFP-NMe_3^+] = 2 μM, [PPE-PBA] = 1 μM. (c) Normalized fluorescence spectra of PFP-NMe_3^+ and PPE-COOH with increasing concentrations of ATP. [PFP-NMe_3^+] = 2 μM, [PPE-COOH] = 1 μM. λ_{ex} = 380 nm.

diol.[8] Thus, some pH values from 6.0 to 8.5 were tested. As shown in **Fig. S2b**, when pH of the buffer is 7.8, the fluorescence intensity ratio reaches to the maximun after the formation of PFP-NMe_3^+/ATP/PPE-PBA complex, which indicates that the weak basic environment is favorable for the formation of the complex. Thus, pH 7.8 is chosen for our detection system.

To demonstrate the feasibility of the CPs-based combination probe to sense ATP, the fluorescence ratio of combination probe was measured in HEPES buffer after adding an aliquot of concentration of ATP. It was reported that in weak base solution, about 50% of the boron centers often form anionic boronate species with hydroxide anions. The neutral form and a hydroxyboronate anion exist in equilibrium.[18] When blending PFP-NMe_3^+ and PPE-PBA in buffer, a weak electrostatic interaction between hydroxyboronate anion and quaternary ammonium cation slightly shortens their distance, which brings a weak FRET. As shown in **Fig. 2a**, with increasing the concentration of ATP from 0 to 180 μM, the ratio R gradually

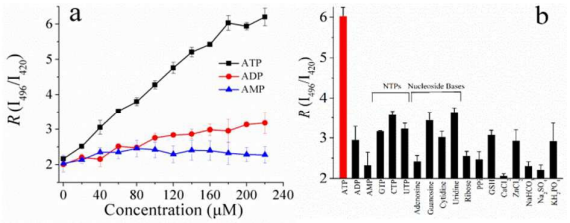

Fig. 3 (a) Dependence of the fluorescence ratio R of the combination probe on various concentrations of ATP, ADP and AMP. (b) The selectivity of combination probe toward ATP and other related biomolecules. [PFP-NMe_3^+] = 2 μM, [PPE-PBA] = 1 μM, [analytes] = 180 mM. λ_{ex} = 380 nm.

increases from 2.16 to 6.03. That means the combination probe recognizes the ATP and approach each other by ATP-bridged linking, leading to the FRET from PFP-NMe_3^+ to PPE-PBA occurring. The ratio R reaches a plateau when the concentration of ATP is 180 μM. **Fig. 2b** (inset) shows the corresponding linear equation is Y=0.021X+2.169 at a range of 0 to 180 μM (R^2 = 0.997), and the limit of detection for ATP is calculated to be 2.5 μM (S/N = 3),[19] which is about three orders of magnitude lower than that with a multisite-binding and rhodamine B-based fluorescent probe. [12] This result indicates that our new CPs-based combination probe is far more sensitive than small molecule probe with the similar multisite-binding, which is in virtue of the high light-harvesting and fluorescence quantum yield properties of CPs.

In addition, the control experiment was carried out in PFP-NMe_3^+/PPE-COOH (without PBA) solution before and after addition of ATP (**Fig. 2c**). In the absence of ATP, the ratio of the mixture is about 2 due to the weak electrostatic and hydrophobic interactions between the two CPs in HEPES buffer. After addition of ATP, no obvious ratio enhancement is observed even if the concentration of ATP reaches 180 μM. These data show that the covalent linking between PBA and ribose plays a key role for ATP detection.

It is well known that ADP and AMP can produce persistent interference in ATP detection. The response of the probe for ADP and AMP was thus examined by similar fluorescence titration experiments, and the results are presented in **Fig. 3a**. In the presence of AMP, the ratio hardly increases in the whole range of 0 ~ 180 μM, whereas the ratio increases only about 0.95 at presence of ADP. Note that the ratio dramatically enhances with increasing of phosphate groups number on the analyte molecules (ATP >> ADP > AMP), which owes to the higher negative charge density of the analyte making for stronger electrostatic interactions. Because of ATP having three phosphate groups, the strongest attraction between ATP and PFP-NMe_3^+ produces the most significant FRET efficiency. Obviously, the new probe is highly selective for ATP comparing with ADP and AMP. In addition, we investigated the selectivity of the sensor for ATP against its analogs, including CTP, GTP and UTP. **Fig. 3b** exhibits that there is a negligible change of ratio R in the presence of other analogs. The unequal hydrophobic nature of the nucleobase (A > G > C, U) may cause differential interactions between nucleoside triphosphates (ATP, GTP, CTP and UTP) and the backbones of CPs, which

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makes for the best selectivity for ATP.[20] Besides, the selective recognition of the probe to ATP may also be improved by a more effective π-π stacking interaction between the backbones of CP and adenine due to matched spatial orientations.[3] We also investigated the response of the new probe for nucleoside bases, such as adenosine, guanosine, cytidine and uridine. The ratio only increases a little no matter adding A, G, C or U to the mixture. In addition, PPi, ribose, various anions and bio-related metal ions were also chosen to examine the selectivity of the new probe. No obvious enhancement of ratio R is observed no matter whichever is present. Furthermore, we studied the interference from saccharides [21], such as glucose, fructose and mannose **(Fig. S3).** These saccharides do not make obvious interference on ATP detection. These results demonstrate that the novel approach exhibits excellent selectivity for ATP detection. Importantly, both the electrostatic interactions and covalent binding play a significant and synergetic role in specifically sensing ATP. The two are independent, which illustrates the high selectivity of the new probe.

Finally, we compared the performance of our new method with other methods reported recently in literatures. As shown in **Table S2**, although most of the reported ways showed lower LOD than that of our approach, they presented limited selectivity because they cannot distinguish NPPs and/or nucleotide bases. Taking advantage of the multisite-binding strategy, our CPs-based combination probe greatly improves the selectivity of ATP sensor. Although the multi-site sensors reported by Chang' group and Li' group presented a good selectivity, they are less sensitive.[3, 8] Therefore, our new method demonstrates an obvious advantage for ATP detection with high selectivity and good sensitivity.

In summary, we have designed a novel CPs-based combination probe for ATP detection based on multisite-binding and FRET mechanism. The cationic conjugated polymer PFP-NMe_3^+ functions as the energy donor while a PBA and PEG modified conjugated polymer PPE-PBA acts as the energy acceptor. When ATP was introduced, strong electrostatic attraction and covalent binding between the CPs and ATP functioned cooperatively to narrow the distance between two CPs, which brings the strong FRET from donor to acceptor. The significant enhancement of fluorescence ratio can be used to quantify the concentration of ATP. Our new method demonstrated a sensitive and highly selective detection for ATP by taking advantage of high light-harvesting and strong fluorescence properties of CPs and CPs-based FRET. This study should provide a novel strategy for designing composite CPs-based ratiometric probes to detect chemical and biological analytes via multisite interactions.

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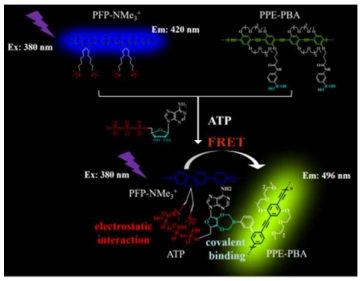

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