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Continuous and Sensitive Acid Phosphatase Assay Based on a Conjugated Polyelectrolyte

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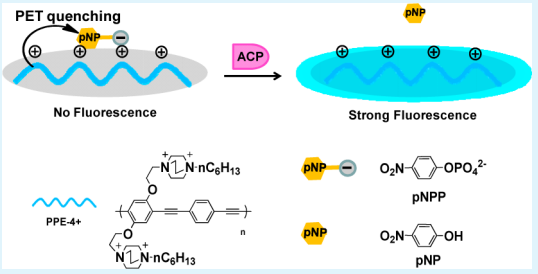
Supporting Information

ABSTRACT: We report a novel continuous and sensitive fluorescence turn-on assay for ACPs, which consists of a cationic conjugated polyelectrolyte (PPE4+) and a commonly used phosphatase substrate p-nitrophenyl phosphate (pNPP). The kinetics of the ACP catalyzed hydrolysis of the substrate pNPP was monitored by the fluorescence change of PPE4+ and corresponding kinetic parameters were derived to be consistent with the literature reports. The applications of PPE4+/pNPP-based ACP assay in high-throughput screening of ACP inhibitors and detection of prostatic acid phosphotase (PAP) in vitro were demonstrated.



KEYWORDS: *conjugated polymer, polyphenylene ethynylene, p-nitrophenyl phosphate, acid phosphatase, prostatic acid phosphotase, fluorescence assay*

1. INTRODUCTION

Acid phosphatases (ACPs), which non-specifically hydrolyze phospho esters in acidic environment, are widely distributed in mammalian body fluids and tissues. An abnormal level of serum ACP can be indications of several diseases, including prostate cancer, Gaucher disease, Paget's disease, thrombophlebitis, hyperparathyroidism, kidney disease, multiple myeloma and et al.[1] ACPs have thus been regarded as important biomarkers, for example, prostatic acid phosphatase (PAP) was established to be the first cancer biomarker for prostate cancer in early 1940's and has been used in prostate cancer diagnosis and post-surgical evaluation.[2] Some ACPs, such as phosphatidic acid phosphatase type 2C[3] and tartrate-resistant acid phosphatase (TRAP),[4] have attracted much attention as an emerging class of drug targets.[5,6] Therefore, development of convenient and sensitive ACP assays can find applications in both disease diagnosis and drug discovery.

Different phosphatase assays have been developed, among which either colorimetric substrate *p*-nitrophenyl phosphate (pNPP)[7] or fluorometric substrates such as 4-methyl-7-hydroxycoumarinyl phosphate (MUP),[8] 3,6-fluorescein diphosphate (FDP),[9] and 6,8-difluoro-4-methylum-belliferyl phosphate (DiFMUP)[10] are used. Some of the substrates, for example, pNPP and MUP, favor only continuous assays of phosphatases at alkaline pH, because their corresponding products can show only strong light absorption or fluorescence emission, respectively, at basic condition. However, most ACPs require a lower pH for enzymatic activity.[1] Such assays can not be applied in continuous real-time measurements since reactions have to be stopped by increasing the pH value before measurements. Although as an improvement, FDP and DiFMUP have been used in continuous ACP assays over a pH range from 5 to 9, the synthesis of these fluoregenic substrates is rather complicated.[9,10]

During the past two decades, conjugated polyelectrolytes (CPEs) have become a unique material to build novel chemo or biosensors for small molecules, metal ions, enzymes, proteins, DNAs, carbohydrates, and so on, mainly because of their amplified fluorescence quenching property.[11−18] Because enzyme detection and activity study is of high importance for the pathological screening and therapeutic development, there have been many reports on CPE fluorescence quenching-based assays of different disease-related enzymes, including thrombin, papain, enterokinase, protein kinase, caspase-3, 7 and 8, and *β*-secretase, et al.[13,19−23] Herein, we report a novel continuous and sensitive fluorescence turn-on assay for ACPs, which consists of a cationic CPE and a commonly used phosphatase substrate pNPP.

2. RESULTS AND DISCUSSION

The *p*-nitrophenyl moiety was reported[21] as an efficient quencher of polyphenylene ethynylene (PPE) when they were at close proximity through photo-induced electron transfer (PET) mechanism. Such property of PPE/pNP has

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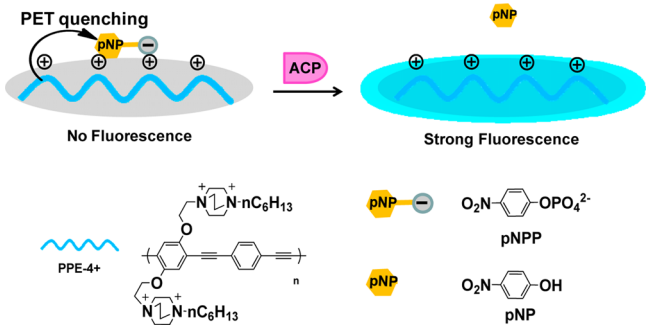
been applied in biosensing for peptidase, thrombin, and caspases.[19,21] As shown in Scheme 1, the substrate pNPP and

Scheme 1. Illustration of PPE4+/pNPP-Based ACP Assay



the product p-nitrophenol (pNP) are anionic and neutral, respectively. The negatively charged pNPP is supposed to strongly associate with the cationic PPE4+, which leads to an efficient fluorescence quenching. However, the quenching effect of the neutral pNP on the polymer is negligible because of much weaker interaction and fluorescence turn-on effect is thus expected to take place.

To test the feasibility of this new assay method, we compared the effects of pNPP and pNP on the fluorescence of PPE4+ in Hepes buffer under mild acidic condition. As shown in Figure 1a, additions of the substrate pNPP (0–20 μM) to 10 μM

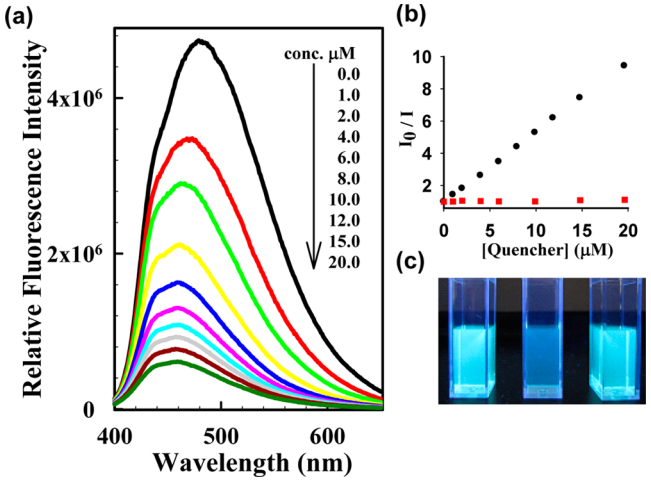


Figure 1. (a) Fluorescence quenching spectra of 10 μM PPE4+ by pNPP (0 to 20 μM) in 10 mM Hepes buffer (pH 5.2). (b) SV plots of PPE4+ quenching by pNPP (black circle) and pNP (red square), respectively. (c) Pictures taken by digital camera with UV light (365 nm) on samples: 10 μM PPE4+ alone (left), 10 μM PPE4+ with 20 μM pNPP (middle), and pNP (right), respectively.

PPE4+ resulted in a significant fluorescence intensity decrease. Specifically speaking, at the concentration of 20 μM, pNPP quenched almost 90% of the fluorescence of PPE4+. The Stern–Volmer (SV) plot (I_0/I vs the quencher's concentration, Figure 1b) is quite linear and the derived SV constant (K_{sv}) is about 4.3×10^5 M^{-1}. As a distinctive comparison, no obvious quenching took place when using the product pNP instead (Figure 1b,c).

ACP catalyzed conversion of pNPP to pNP was then monitored by PPE4+/pNPP assay in Hepes buffer for proof-of-concept. The substrate pNPP has been used in commercially available colorimetric ACP assays at about 200 μM in order to give the observable absorbance change. Similarly, about 100 μM of DiFMUP substrate is usually used in fluorometric ACP assays.[10] However, much less susbstrate was needed (only 10–20 μM pNPP) in our method to achieve obvious fluorescence intensity change mainly due to the amplified quenching property of PPE4+. As shown in Figure 2, adding 20 μM

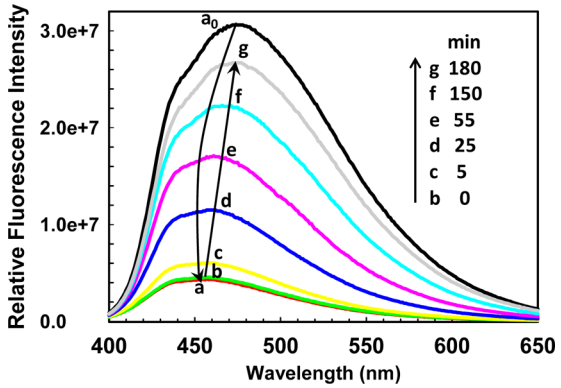


Figure 2. Fluorescence spectroscopic changes of PPE4+ with addition of pNPP and ACP. The excitation wavelength is 380 nm. Plot a_0, the spectrum of 10 μM PPE4+ in 10 mM Hepes buffer (pH 5.2) at 25 °C; plot a, after titration of 20 μM pNPP; plots b–g: spectra after addition of 20 nM ACP and collected as a function of time (0, 5, 25, 55, 120, and 180 min, respectively).

pNPP to PPE4+ resulted in a significant fluorescence quenching (a_0 → a). After adding a non--specific ACP from potato, there's no obvious emission increase (a → b), indicating that the enzyme itself didn't affect the polymer's fluorescence. As the enzymatic reaction proceeded, the emission of PPE4+ gradually increased and then reached a plateau, at which the fluorescence of the pre-quenched polymer was recovered up to 85% of the original intensity (b → g).

To demonstrate the feasibility of using PPE4+/pNPP assay for a continuous real-time ACP activity study, we investigated the ACP-catalyzed hydrolysis of pNPP as a function of time upon incubation with different ACP concentrations (0–20 nM). Figure 3a illustrates the increase of PPE4+ fluorescence intensity *vs* the incubation time at each enzyme concentration. It was observed that the fluorescence recovery of PPE4+ depended on the enzyme's concentration. Lower enzyme concentration led to less fluorescence recovery and slower initial reaction rate (Figure 3b,c, detailed calculation is described in the Supporting Information). The limit of detection (LOD) of ACP in our assay is determined to be about 0.17 nM, which is corresponding to 0.05-0.17 mU/mL, comparable to the literature reported methods.[24]

Kinetic parameters of ACP were further determined with assays at different initial pNPP concentrations (Figure 4). The Lineweaver–Burk plot was obtained using double reciprocal data of initial rate V vs substrate concentration $[S]_0$ (detailed calculations can be found in the Supporting Information). Among the parameters, the Michaelis constant K_m relates to the substrate's affinity to the enzyme, whereas the the ratio of k_{cat}/K_m is usually a measure of enzyme efficiency. The K_m value was determined to be 258.4 μM and k_{cat}/K_m was 6.1×10^4 M^{-1} S^{-1}, consistent with the literature reported data,[25,26] indicating that the polymer didn't affect the binding between the substrate

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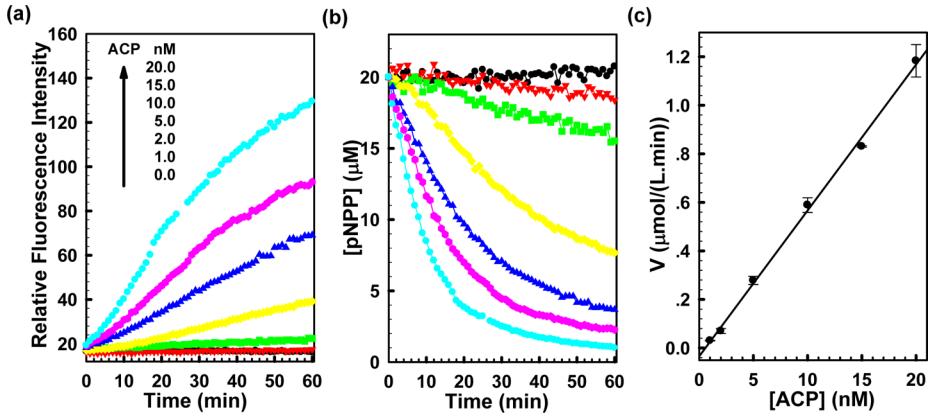

Figure 3. Fluorescence turn-on assays for ACP. (a) Real-time fluorescence turn-on effect of ACP assays. (b) Derived pNPP concentrations as the function of time. (c) Initial rate vs. enzyme concentration for ACP assays. Assays were carried out with 10 μM PPE4+ and 20 μM pNPP in 10 mM Hepes buffer (pH 5.2) at 25 °C with different concentrations of ACP (0, 1.0, 2.0, 5.0, 10.0, 15.0, and 20.0 nM).

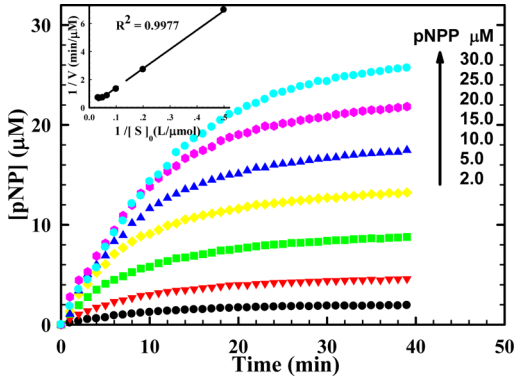

Figure 4. Derived concentrations of the product pNP as the function of time for ACP assays at different initial pNPP concentrations (2, 5, 10, 15, 20, 25, and 30 μM). (Inset: The Lineweaver–Burk plot). Assays were carried out using 20 nM ACP and 10 μM PPE4+ in 10 mM Hepes buffer (pH 5.2) at 25 °C. All experiments were conducted in triplicate.

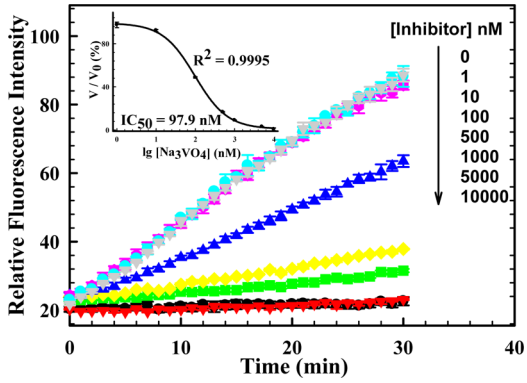

Figure 5. Fluorescence inhibition assay for ACP. (Inset: Plot of V/V_0 vs log[Na_3VO_4], in which V is the initial rate of ACP-catalyzed hydrolysis reaction in the presence of different amounts of inhibitor and V_0 was the rate in the absence of inhibitor.) Assays composed of 10 μM PPE4+, 20 μM pNPP and 20 nM ACP in buffer were pre-incubated with varying concentrations of the inhibitor Na_3VO_4 (0, 1, 10, 100, 500, 1000, 5000, and 10000 nM) for 15 min before fluorescence measurements. All experiments were conducted in triplicate.

pNPP with ACP. These results further confirmed that the PPE4+/pNPP based ACP assay can be applied in real time ACP activity measurement.

High-throughput screening (HTS) for effective ACP inhibitors and/or activators in vitro would greatly contribute to rapid drug development. Application of PPE4+/pNPP-based ACP assay as an in vitro screening tool of inhibitors was demonstrated using a known broad-spectrum phosphatase inhibitor Na_3VO_4. As shown in Figure 5, the initial hydrolysis reaction rates decreased with increased inhibitor concentrations. The IC_{50} value of Na_3VO_4 was obtained by sigmoidal fit of the data using SigmaPlot 10.0 and it was 97.9 nM, which is in a good agreement with the literature report.[27]

PAP levels in serum for most normal persons are in the range of 0 to 5.0 ng/mL.[28,29] Due to lower sensitivity of PAP test in predicting prostate cancer pathological stage or margin status, it has been replaced by prostate-specific antigen (PSA) test after its introduction in the 1980s. However, recent studies have re-evaluated the potential contribution of PAP test and found that PAP was a significant prognostic factor for patients with intermediate- and high-risk prostate cancer.[30] Since pNPP is a non-specific substrate for a wide range of phosphatases, quantitative analysis of PAP was able to be carried out by PPE4+/pNPP assays. As shown in Figure 6, they hydrolysis

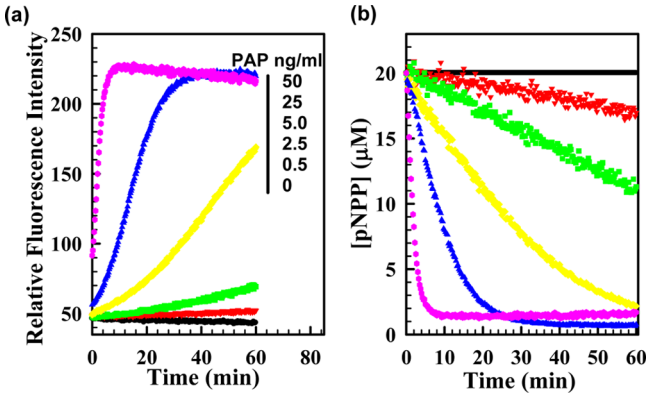

Figure 6. (a) Fluorescence turn-on effect of PAP assays. (b) Derived pNPP concentrations as a function of time for PAP assays. Assays were carried out with 10 μM PPE4+ and 20 μM pNPP in 10 mM Hepes buffer (pH 5.2) at 25 °C with different concentrations of PAP (0, 0.5, 2.5, 5.0, 25, and 50 ng/mL).

rate of pNPP showed PAP concentration dependency. It's worth to pointing out that the enzymatic reaction can be

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detected by our method when the PAP concentration was as low as 0.5 ng/mL.

3. CONCLUSION

In summary, we report a novel continuous and sensitive ACP assay composed of PPE4+ and a commonly used substrate pNPP. Applications of such assay in the ACP activity measurement and in vitro HTS for ACP inhibitors were demonstrated. This assay method can also be generalized to detect other ACPs, such as PAP, for further application in disease diagnosis and evaluation.

4. EXPERIMENTAL SECTION

Steady-State Fluorescence Quenching. Steady-state fluorescence quenching experiments of PPE4+ by pNPP and pNP were carried out by micro-titration in a fluorescence cuvette. In a typical titration quenching experiment, 2.0 mL solution of 10 μM PPE4+ in 10 mM Hepes buffer (pH 5.2) was placed in a 1 cm quartz fluorescence cell. The fluorescence of the polymer before and after addition of 1-10 μL aliquots of a concentrated quencher solution was recorded. The fluorescence quenching data were analyzed using the Stern−Volmer (SV) equation as in eq 1

$$\frac{I_0}{I} = 1 + K_{SV}[Q] \quad (1)$$

where I_0 is the initial fluorescence intensity of PPE4+ solution, I is the fluorescence intensity at any quencher concentration [Q], and K_{SV} is the SV constant.

Real-Time ACP Assay. The enzyme assays were carried out in 10 mM Hepes buffer (pH 5.2) at 25 °C. A typical procedure was carried out as follows: the solution of 10 μM PPE4+ was pre-quenched by pNPP in a Corning 96-well plate and the initial fluorescence intensity I_q at 500 nm was recorded. Either ACP or prostate acid phosphatase (PAP) was then added into the assay solution and the fluorescence intensity (It) as a function of time was recorded. All experiments were conducted in triplicate. Calculation details were included in the Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and calculation details. This material is available free of charge via the Internet at http://pubs.acs.org/.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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