A conjugated polyelectrolyte-based fluorescence sensor for pyrophosphate†

Xiaoyong Zhao, Yan Liu and Kirk S. Schanze*

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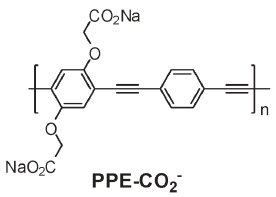
A new fluorescence turn-on sensor consisting of PPE-CO_2^-/Cu^{2+} shows high selectivity for pyrophosphate over other anions and is used to develop a real-time assay for alkaline phosphatase.

Because pyrophosphate is involved in many important biological processes such as cellular signal transduction and protein synthesis,[1] fluorogenic sensors for detecting pyrophosphate (PPi) in aqueous solution have been the focus of considerable research. "Fluorophore–spacer–receptor" sensors based on signal transduction mechanisms such as photoinduced electron transfer (PET)[2] or monomer–excimer formation[3] have been reported. "Turn-on" sensors[4] that rely on indicator displacement have also been designed. However, in these systems small molecule dyes are used as the indicators, therefore the sensitivity to PPi is limited due to comparatively inefficient quenching of the indicators by the receptors.

Herein, we report a novel fluorescence competition assay for PPi that consists of an anionic conjugated polyelectrolyte (CPE), PPE-CO_2^- (shown in Scheme 1) and cupric ion. This simple sensor system is selective for PPi in the presence of 12 other anions in 0.01 M HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) buffer at pH 7.5. In addition, due to the signal amplification imparted by the CPE, the sensor responds even at very low PPi concentration. Although CPEs have been used for detecting a variety of analytes,[5] this is the first application of a fluorescent CPE for sensing PPi.

As reported in an earlier communication,[6] PPE-CO_2^- was synthesized by a "precursor route"[7] involving palladium-mediated Sonogashira polymerization to afford a C_{12} diester protected polymer followed by a base-promoted hydrolysis of the ester groups. Gel permeation chromatography analysis of the ester precursor polymer affords M_n = 127 K and PDI = 2.3 (X_n = 185). In the HEPES buffer solution (pH 7.5), the absorption and fluorescence spectra of PPE-CO_2^- are essentially the same as those of the polymer in pure water,[8] indicating the polymer is aggregated in the HEPES buffer solution.



PPE-CO_2^-

Scheme 1

Previously, we[6] and others[9] have studied the interaction of CPEs bearing carboxylate side groups with metal ions. In the course of this investigation we compared the quenching of PPE-CO_2^- fluorescence by 9 divalent metal ions in HEPES buffer solution. Interestingly, we find that Cu^{2+} is a much more efficient quencher compared with the other metal ions, including Ca^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+} and Pb^{2+} (see supporting information†). The Stern–Volmer constant (K_{SV}) for Cu^{2+} quenching is *ca.* 10^6 M^{-1}, which is comparable to the quenching efficiency of PPE-CO_2^- by methyl viologen.[6] The strong and selective quenching by Cu^{2+} likely arises because the metal coordinates with the carboxylate groups of PPE-CO_2^-, and it efficiently quenches the singlet exciton *via* charge and/or energy-transfer mechanisms.[10]

The efficient quenching of PPE-CO_2^- by Cu^{2+} suggested that this system might be useful as a "turn-on" sensor for anions that coordinate with Cu^{2+}. Given that binding between PPi and metal ions is the basis of many sensor designs, we anticipated that addition of PPi to a PPE-CO_2^-/Cu^{2+} solution would disrupt the polymer–metal complex, leading to recovery of the polymer's fluorescence. Using a HEPES buffer solution containing 5 μM PPE-CO_2^- (polymer repeat unit concentration) and 10 μM Cu^{2+} (1 : 2 PPE-CO_2^-/Cu^{2+} molar ratio), fluorescence titration experiments were carried out with a variety of mono- and divalent anions, including PPi. At this PPE-CO_2^-/Cu^{2+} ratio, the intensity of the polymer's fluorescence was quenched by more than 98% at λ_{max} = 530 nm compared to the intensity of a solution containing only polymer. The PPE-CO_2^-/Cu^{2+}/buffer stock solutions were prepared one night before the anion titration experiments, and there was no observable precipitate present in the solutions. Fig. 1a

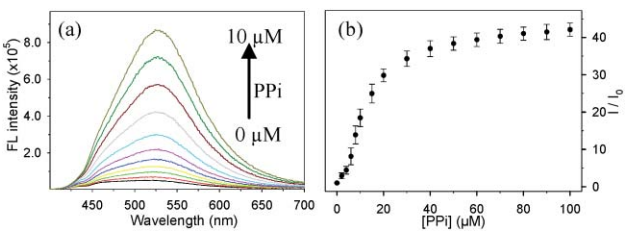


Fig. 1 (a) Fluorescence spectra of a solution of PPE-CO_2^-/Cu^{2+} (5 μM/10 μM) titrated with PPi in 0.01 M HEPES buffer at pH 7.5, 25 °C. (b) Intensity enhancement (I/I_0) at 530 nm titrated with PPi (0–100 μM). Excitation at 380 nm.

Department of Chemistry, University of Florida, FL 32611, USA. E-mail: kschanze@chem.ufl.edu; Fax: +1-352-392-2395; Tel: +1-352-392-9133

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shows the fluorescence response of a PPE-CO_2^-/Cu^{2+} solution concomitant with the addition of PPi at pH 7.5. Titration of PPi into the PPE-CO_2^-/Cu^{2+} (5 μM/10 μM) solution results in a continuous recovery of the polymer's fluorescence intensity, and at 10 μM of added PPi (1 equivalent relative to [Cu^{2+}]) a 17-fold enhancement of fluorescence intensity is observed. Titrations over the low concentration range (0–1 μM) indicate that the analytical detection limit for PPi is 80 nM (see supporting information†). As shown in Fig. 1b, the full titration curve of adding 0–100 μM PPi displays a sigmoidal progression, indicating that multiple equilibria are involved in the process.[11] These equilibria likely involve dissociation of the PPE-CO_2^-/Cu^{2+} complex and formation of the Cu^{2+}/PPi complex. The most significant fluorescence increase is seen with addition of PPi up to 20 μM (30-fold enhancement). Above that concentration the increase occurs more gradually, and at [PPi] = 50 μM (5 equivalents added) approximately 85% of the polymer's initial fluorescence intensity is recovered (38-fold enhancement). (Nearly 100% of the fluorescence intensity is recovered at 100 μM [PPi].) During the entire PPi titration, there is no shift of the emission maximum, and UV–Vis absorption exhibits only a small blue shift (< 5 nm). This finding suggests that PPi acts mainly by sequestering Cu^{2+}, and that no significant changes in the polymer aggregation state occur during the anion titration.

The selectivity of the PPE-CO_2^-/Cu^{2+} sensor for PPi was evaluated by carrying out the same fluorescence titration with other anions including monovalent anions (F^-, Cl^-, Br^-, I^-, HSO_4^-, NO_3^-, HCO_3^-, $H_2PO_4^-$, $CH_3CO_2^-$) and divalent anions (SO_4^{2-}, CO_3^{2-}, HPO_4^{2-}). Addition of 50 μM of any of these anions induced only a small change of the PPE-CO_2^- fluorescence intensity (typical change was less than 2%). Fig. 2 compares the response of PPE-CO_2^-/Cu^{2+} to addition of 50 μM of 13 different anions. This presentation shows that the sensor is highly selective to PPi with an intensity enhancement of 38-fold, compared with all of the other anions tested, including phosphate (Pi: $H_2PO_4^-$ and HPO_4^{2-}). The inset shows a photograph of the fluorescence from the PPE-CO_2^-/Cu^{2+}/anion solutions acquired under UV-illumination. The photograph clearly shows that the fluorescence of the polymer is very strong in the presence of PPi, whereas it is very weak in the presence of the other anions. Since selectivity to PPi in the presence of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) is important for some biosensor applications, the response of the PPE-CO_2^-/Cu^{2+} sensor to these two organic anions was also checked. Not surprisingly, addition of ATP elicits a very similar fluorescence response compared to PPi. However, the fluorescence response to ADP is markedly lower than that of PPi; at saturation, addition of ADP leads to only 25% recovery of the initial fluorescence intensity whereas as noted above addition of PPi leads to > 95% fluorescence recovery.

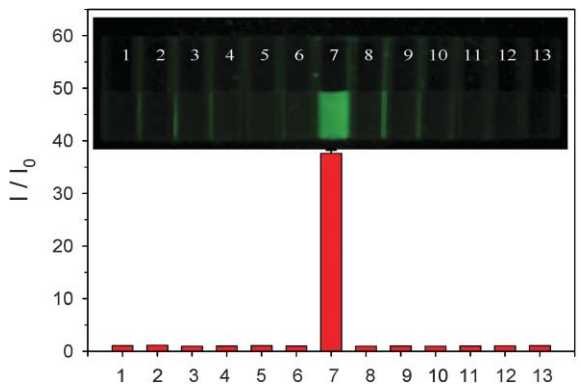

Fig. 2 Fluorescence response of PPE-CO_2^-/Cu^{2+} (5 μM/10 μM) to various anions at 50 μM concentration in 0.01 M HEPES buffer at pH 7.5, 25 °C (1, F^-; 2, Cl^-; 3, Br^-; 4, I^-; 5, HPO_4^{2-}; 6, $H_2PO_4^-$; 7, $P_2O_7^{4-}$; 8, $CH_3CO_2^-$; 9, HSO_4^-; 10, NO_3^-; 11, HCO_3^-; 12, SO_4^{2-}; 13, CO_3^{2-}). Inset shows a photograph of the sensor/anion solutions illuminated with a UV-lamp.

Taken together, the studies with the various anions suggest that the sensor response to PPi (and to ATP) arises by chelation of the diphosphate moiety to Cu^{2+}, which effectively sequesters the metal ion, disrupting its ability to bind to the carboxylate groups and quench the fluorescence of PPE-CO_2^-.[12] The hypothesis that the diphosphate unit acts by binding to and sequestering Cu^{2+} is further supported by the observation that addition of 10 μM (1 equivalent) of the strong chelator ethylenediaminetetraacetic acid (EDTA) to the PPE-CO_2^-/Cu^{2+}/HEPES solution leads to complete recovery of the polymer's fluorescence intensity.

In order to demonstrate the potential of the CPE-based PPi sensor in a bioanalytical application, a real-time turn-off assay was designed to monitor the activity of alkaline phosphatase (ALP). Mammalian ALP is widely distributed in human and animal tissues and it plays an important role in certain pathological conditions.[13] Since ALP catalyzes the hydrolysis of PPi to Pi at physiological pH, we used the PPE-CO_2^-/Cu^{2+} fluorescent sensor to detect the enzyme's activity. In a typical ALP assay, a solution containing 3 μM of PPE-CO_2^-, 6 μM of Cu^{2+} and 12 μM of PPi in 0.01 M HEPES (pH = 7.5) was prepared and incubated for 10 minutes at 37 °C. Following the addition of an aliquot of ALP (bovine intestinal mucosa), the fluorescence intensity of PPE-CO_2^- was monitored at 525 nm as a function of time (λ_{exc} = 390 nm). As shown in Fig. 3, after addition of ALP the polymer's fluorescence intensity steadily decreases due to enzyme-catalyzed hydrolysis of PPi. As expected, the fluorescence intensity decrease accelerates as the ALP concentration increases. This initial study clearly demonstrates that PPE-CO_2^-/Cu^{2+} provides an effective, real-time fluorescence assay for ALP activity. Moreover, it is evident that this system should be applicable to other enzyme assays involving PPi and Pi.

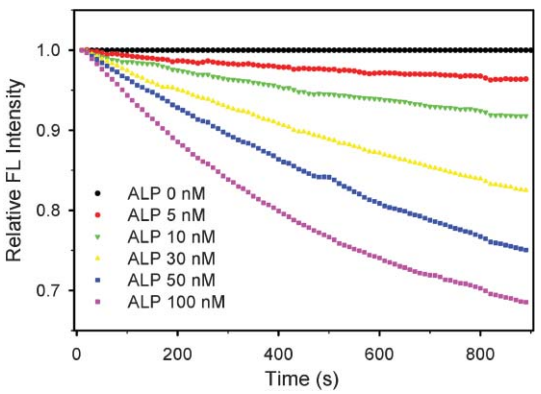

Fig. 3 ALP assay using PPE-CO_2^-/Cu^{2+} system ([PPE-CO_2^-] = 3 μM, [Cu^{2+}] = 6 μM, [PPi] = 12 μM in 0.01 M HEPES buffer, pH 7.5, 37 °C). Emission was monitored at 525 nm (λ_{exc} = 390 nm).

In summary, we report a new fluorescence turn-on sensor composed of a conjugated polyelectrolyte and Cu^{2+}. This system can detect PPi at nanomolar concentration with high selectivity over many inorganic anions, including Pi. On the basis of this finding a real-time turn-off assay for alkaline phosphatase was developed and tested under physiological conditions.

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