Fluorescent ratiometric sensing of pyrophosphate *via* induced aggregation of a conjugated polyelectrolyte†

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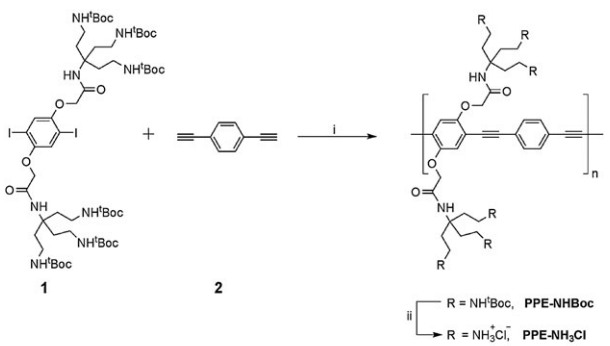
Direct detection of pyrophosphate (PPi) in aqueous solution is demonstrated using a cationic poly(phenylene-ethynylene) with polyamine side chains. Pyrophosphate-induced polymer aggregation causes a significant spectroscopic change, which in turn allows quantification of dissolved PPi using ratiometric signals.

Conjugated polyelectrolytes (CPEs) are a class of water-soluble polymers characterized by a π-conjugated backbone with ionic solubilizing side groups.[1,2] Chen *et al*. reported the first example of using PPV-SO_3^- as an ultrasensitive probe for the biotin–avidin binding event.[3] Ever since, this family of materials has been intensively studied for chemical and biological sensor applications owing to their superior signal amplification compared to small molecule dyes.[4] Regardless of the nature of various analytes, *e.g*. DNA, proteins and ions, the signal transduction has been mainly based on three different mechanisms: photoinduced electron transfer quenching, fluorescence resonance energy transfer, and analyte-induced perturbation of polymer chain conformation.[5] Many sensory systems based on these mechanisms rely on fluorescence intensity change at a single wavelength or require labeling of the probe and/or the analyte with special dyes. In contrast, signal transduction *via* analyte-induced polymer aggregation has received less attention.[6] This mechanism takes advantage of the multivalent interaction between the polymer and the analyte to induce absorption and/or fluorescence modulation. Thus it does not require any extra labeling steps and allows the analytes to be extended to molecular species without optical or quenching properties.

Pyrophosphate is an anion that plays a significant role in many biological processes, such as cellular ATP hydrolysis, DNA and RNA polymerizations and many enzymatic reactions.[7,8] It is also known that abnormal PPi levels can cause vascular calcification leading to severe medical conditions.[9] In an earlier contribution, we demonstrated the first example of a PPi sensor based on an anionic CPE with carboxylate side chains.[10] The sensor design combines the efficient intrachain/interchain exciton migration of CPEs with association–dissociation of the fluorescence-quenching cupric ion, affording PPi detection in the nanomolar range. The system shows high sensitivity and selectivity to PPi in buffered aqueous solution; however, like many intensity-based sensors, the sensory response is subject to interferences such as variation of polymer concentration, solution conditions and instrument settings. Herein, we report a ratiometric sensor design for pyrophosphate (PPi) which utilizes two distinct fluorescence signals corresponding to the "free chain state" and "aggregate state", respectively. The current sensor shows high selectivity to PPi over phosphate (Pi) in aqueous solutions. Since discrimination between PPi and Pi is of crucial importance to assays for detecting many enzymes,[11] we believe the current system using a ratiometric response will provide a facile platform for CPE-based enzyme assays with built-in corrections.

In a series of studies, we have shown that poly(phenylene-ethynylene)s (PPEs) with linear ionic groups (SO_3^-, CO_2^-, and NR_3^+) undergo spontaneous aggregation in aqueous solution as signaled by a broad emission band with low quantum yields (~5%).[12–14] In order to retain the optical properties of these materials in aqueous solution, we designed and synthesized a diiodo-monomer (**1**) with branched polyamine side chains, as shown in Scheme 1. This monomer was prepared through multi-step synthesis (see ESI†) and then co-polymerized with 1,4-diethynylbenzene (**2**) under standard Sonogashira coupling conditions.[13] The polymerization is a typical step-growth condensation yielding an organic-soluble polymer (**PPE-NHBoc**) with a number-averaged molecular weight of 24 kD and a polydispersity of 4.3. The neutral polymer was then hydrolyzed using 4 M HCl in dioxane affording the water-soluble polymer, **PPE-NH_3Cl** with a quantitative yield. The complete removal of the *tert*-butyloxycarbonyl (^{t}Butyl) groups was confirmed by the absence of the singlet at δ 1.40 ppm assigned to the protecting group (^{t}Boc) in the ^{1}H NMR spectrum and the disappearance of carbamate (NHCOO^{t}Butyl) absorption at 1692 cm^{-1} in the infrared spectrum (Fig. S1 and S2, ESI†).

The absorption and emission spectra of **PPE-NH_3Cl** exhibit a muted solvent dependence in comparison to its analogue



Scheme 1 Synthesis of cationic poly(phenylene-ethynylene) with branched polyamine side groups.

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polymers with linear ionic groups. Specifically, many CPEs with linear side chains exhibit optical properties that are strongly dependent on the solvent.[2] Typically, addition of water (“poor solvent”) to methanol (“good solvent”) results in the emergence of a new absorption band at longer wavelength and the replacement of a strong emission band at shorter wavelength with a red-shifted, weak and broad band. In clear contrast, **PPE-NH$_3$Cl** shows a negligible change of the absorption band with a maximum at 405 nm in water (pH $\sim$ 6.0) the same as that in methanol solution, as shown in Fig. 1. For both solutions, a strong and sharp fluorescence assigned to the 0–0 band with λ_{max} = 432 nm is observed for the polymer. The polymer retains approximately 50% of its quantum efficiency in water (Φ = 0.23) compared to that in methanol (Φ = 0.45) and maintains a similar vibrational structure. The optical properties were found to be strongly dependent on the solution pH (Fig. S3, ESI†). For pH $\leq$ 6.5, the polymer shows similar spectra as in deionized water, and varying pH in this range resulted in minimal spectral changes. An abrupt and significant change in both absorption and emission spectra is observed between pH 7.0 and 8.0. Further increasing of pH only leads to slight spectral changes. The potentiometric titration curve (Fig. S4, ESI†) of the polymer indicates a three-step deprotonation process around pH 4, 8 and 10, which corresponds well with the pK_a values of the polyamine ligand.[15] Combined with the spectral changes of the polymer solution with pH, it is clear that deprotonation of the side chains leads to a significant reduction of the electronic repulsion and induces aggregation of polymer chains through hydrophobic and π–π stacking interactions.[16]

To investigate the analyte-induced aggregation for PPi sensing, we next examined the response of this polymer to PPi in 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffered solution at pH = 6.5. Fig. 2 shows the absorption and fluorescence spectra of **PPE-NH$_3$Cl** upon the addition of PPi. As the PPi concentration increases, the absorbance at λ_{max} = 400 nm decreases and an absorption band at λ_{max} = 430 nm emerges to become the dominating peak. Consistent with our earlier results and others,[12,17] this red-shift corresponds to aggregation-induced planarization of phenylene-ethynylene backbone. In addition, a well-defined isosbestic point at λ = 410 nm is observed suggesting a conversion between the “free chain state” and the “aggregate state” of the polymer chains. The fluorescence spectra also reveal the clear transition between the two states. The strong blue emission

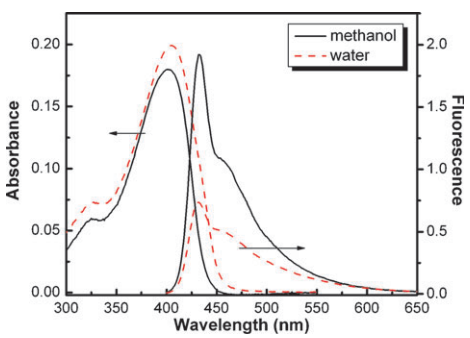

Fig. 1 Absorption and emission spectra of **PPE-NH$_3$Cl** in methanol and water ([**PPE-NH$_3$Cl**] = 4 μM).

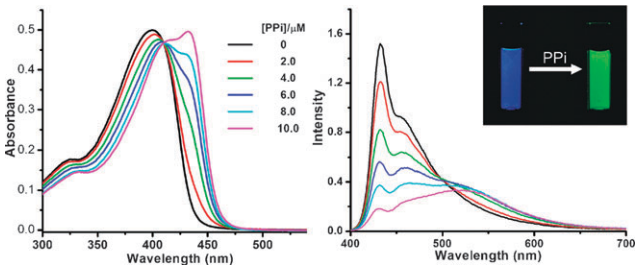

Fig. 2 Absorption (left) and fluorescence (right) spectra of **PPE-NH$_3$Cl** in buffered solutions (pH = 6.5) with increasing PPi concentration ([**PPE-NH$_3$Cl**] = 10 μM). Inset: fluorescence images of the solutions before and after the addition of PPi (1 equiv.).

with resolved vibrational structure (433–455 nm) decreases in intensity with increasing PPi concentration accompanied by enhancement of the green emission band at λ_{max} = 520 nm. This large red-shift ($\sim$90 nm) of the emission spectra is due to the efficient intermolecular exciton coupling among polymer chains with close proximity, leading to a lower energy “aggregate state”. The blue-to-green transition is visually readily observable under UV illumination (Fig. 2, inset).

Discrimination between PPi and Pi is an important issue for PPi sensing as the two anions coexist under many circumstances.[8] To demonstrate the feasibility of our current system to selectively detect PPi, we tested the response of **PPE-NH$_3$Cl** to Pi under the same conditions. As expected, neither absorption nor emission spectra show distinct changes to Pi even at higher concentrations (Fig. S5, ESI†). We ascribed the different response to the ability of PPi to “crosslink” the polymer chains carrying polyamine ligands, thus inducing polymer aggregation, as illustrated in Fig. 3. Although Pi can also neutralize the polymer charges, clearly, it is unable to induce the interpolymer interactions. We have also examined the sensing selectivity of the current CPE sensor to other inorganic anions including halide ions, carbonate, sulfate and biologically important anions, such as adenosine monophosphate (AMP), adenosine diphosphate (ADP) and adenosine triphosphate (ATP). It is found that the current sensor is highly selective to PPi compared to a number of tested inorganic ions. However, the polymer’s fluorescence is affected somewhat by ADP and ATP (Fig. S6, ESI†), therefore interference by these species must be considered in developing assays based on the **PPE-NH$_3$Cl**/PPi sensor.

Fig. 4 shows the ratiometric response of both the absorption (A_{430}/A_{400}) and fluorescence (I_{510}/I_{455}) signals to the PPi

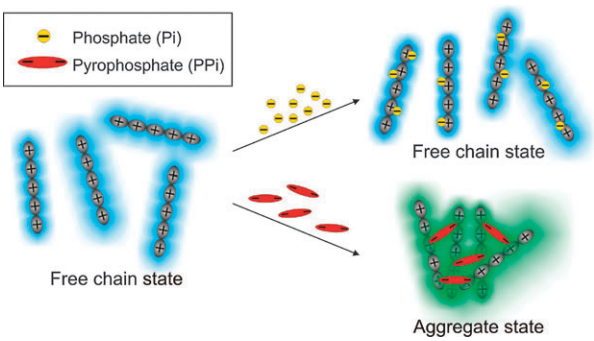

Fig. 3 Schematic illustration of PPi-induced polymer aggregation.

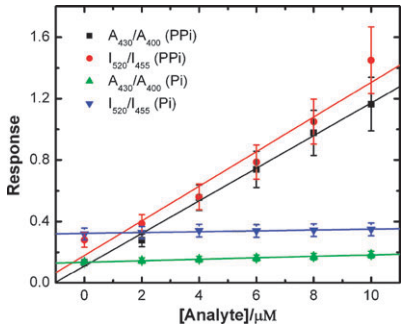

Fig. 4 The ratiometric signals based on the absorption and fluorescence spectra of **PPE-NH$_3$Cl** in buffered solutions with the addition of PPi or Pi.

concentration. A linear dependence of both signals was observed in the PPi concentration range (0–10 μM). Importantly, the use of a ratiometric signal allows quantification of PPi concentration without the need to utilize a Stern–Volmer based calibration curve which is generated *ex situ* where varying experimental conditions and artifacts such as sample bleaching can affect the signal calibration.[11a] Also, the dual response from both absorption and emission is of additional benefit for accurate analyte detection. In contrast to the effects seen with PPi, addition of Pi to the polymer solution leads to negligible changes. This high selectivity for PPi over Pi enables the current system to be used in biological assays in which these two anions are involved. We estimate that the detection limit of the current sensor to PPi is 340 nM (Fig. S7, ESI†). Although the detection limit is slightly higher compared to the sensor based on Stern–Volmer quenching,[10] the ratiometric signaling enables a simple and continuous method with *in situ* calibration for measuring PPi concentration.

In summary, we have synthesized a cationic poly(phenyleneethynylene) with branched polyamine side chains and investigated its application as a solution-based PPi sensor. This polymer features a strong blue emission band with well-resolved vibronic structure in water in contrast to many PPEs with linear solubilizing groups. In buffered aqueous solution, the spectroscopic properties of the polymer are sensitive to the concentration of PPi with high selectivity over Pi and other inorganic anions due to the analyte-induced aggregation mechanism. The blue-to-green fluorescence change is readily visible by the unaided eye. In addition, ratiometric signals obtained from absorption and/or fluorescence spectra can be easily and directly calibrated to provide the PPi concentration. We are currently developing biological assays for enzymes with PPi as substrates using this system and we believe similar design principles can be applied to other non-quenching anionic species of interest.

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