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Quencher–fluorophore ensemble for detection of pyrophosphate in water

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Abstract—We present a new example of on–off switching using a fluorescent PPi sensor system based on the quencher–fluorophore ensemble, which shows a moderate selectivity for PPi ions among other anions in an aqueous solvent.

In recent years, considerable efforts have gone into developing selective and sensitive chemosensors that can detect biologically important anions.[1] In particular, anions such as pyrophosphate ($P_2O_7^{4-}$, PPi) and adenosine triphosphate (ATP), which play vital roles in several bioenergetic and metabolic processes, are significant targets that must be conventionally monitored.[2] Therefore, several groups have considered the detection and discrimination of these anions as important research subjects.[3]

Among various chemosensors, fluorescent chemosensors offer a number of advantages such as high sensitivity, low cost, easy detection, and versatility.[1,4] The molecular ensemble, which is composed of a signaling unit (indicator) bound to a binding site (receptor) by noncovalent interactions, has attracted considerable attention.[1e,g,3a,c,e,f] In particular, one important advantage of the ensemble approach is that 'on–off' switching is achieved by the simple mixing of the indicator and receptor.[1e,g,3a,c,e,f]

In this Letter, we present a new example of on–off switching using a fluorescent PPi sensor system based on the quencher–fluorophore ensemble, which shows a moderate selectivity for PPi ions among other anions in an aqueous solvent.

The quencher–fluorophore ensemble examined in this study is composed of two parts. One is a quencher–receptor conjugate (Q–R) that comprises a quencher and a target binding site. The other is a fluorophore–substrate conjugate (F–S) that shows weak coordination to the binding site of a quencher–receptor conjugate and displays fluorescence quenching. However, when the target substrate (PPi) is added to the mixture of Q–R and F–S (F·Pi), the weakly bound F·Pi is expelled and the original fluorescence of F·Pi is regenerated.

Generally, in the molecular ensemble approach, the indicator is directly coordinated to the metal and/or ionic moiety, to achieve on–off signaling.[1g,3a,c,e,f] Therefore, in many cases, several requirements must be satisfied; only a limited number of metal ions are used for the binding site because some metal ions such as Cu^{II} can quench the fluorescence, and the indicator must fit well to the binding site.[3c,5] However, this quencher–fluorophore ensemble approach can use various metal ions and indicators without the former restriction, because the fluorescence on–off mechanism simply depends on the distance between the fluorophore and the quencher.

Moreover, indicators with different affinity constants can be easily synthesized because diverse substrates can be introduced into the fluorophore–substrate conjugate. Therefore, the best conditions for the discrimination of a target substrate are easily produced by simple synthesis. For example, if the affinity trend of a certain receptor (R) for substrates (A, B) is A > B > C > D, a selective fluorescent ensemble for A is easily developed by the introduction of B into the fluorophore–substrate conjugate part. The ensemble is disturbed only by A, because only A can expel the fluorophore-B conjugate from the receptor. Therefore, only A induces the change in fluorescence emission (Scheme 1).

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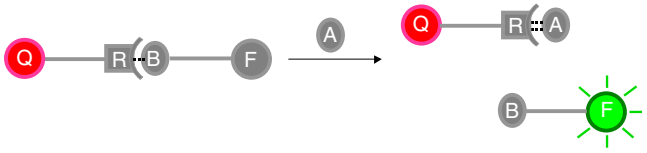


Scheme 1. Schematic representation of quencher (Q)-fluorophore (F) ensemble approach. (R: receptor, A and B: substrates, affinity trend of R: A > B).

The quencher (Q–R)-fluorophore (F·Pi) ensemble for the detection of PPi is outlined in Scheme 2.

As the receptor part of Q–R, a Zn^{2+} complexation with a bis(2-pyridylmethyl)amine (DPA) moiety is employed. Phosphate is selected as the substrate part of F·Pi because its affinity with the receptor part is weaker than that of PPi.

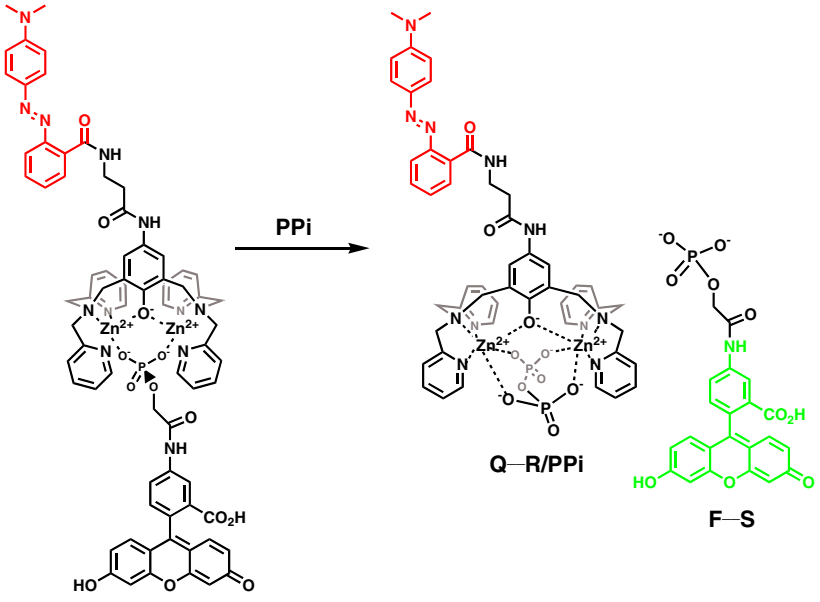
Methyl red of Q–R and fluorescein of F·Pi, which are well-known dyes used in molecular beacons, are selected as a quencher–fluorophore pair.[4e]

Synthesis of the quencher–receptor conjugate (Q–R) is described in Scheme 3. Compound **2** and 4-aminophenol were introduced to glycine by a consecutive EDC coupling reaction. The resulting phenol **5** was reacted with DPA and *p*-formaldehyde to give **1**, which was finally treated with $Zn(NO_3)_2$ to give Q–R as the product.[6]

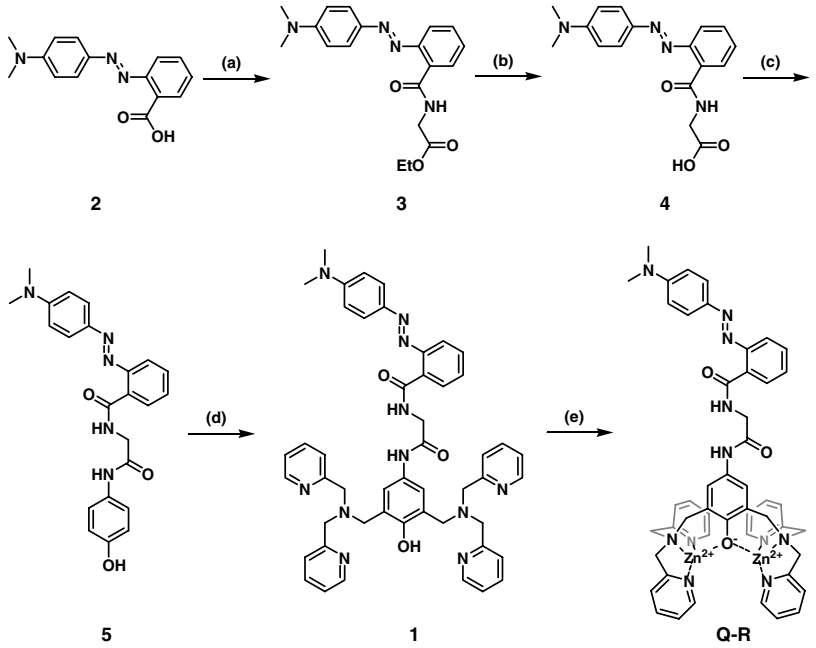
Synthesis of the fluorophore–substrate conjugate (F·Pi) is described in Scheme 4. Attachment of pivaloyl-protected 5-aminofluorescein onto **6** was accomplished by an EDC coupling reaction. Consecutive removal of the benzyl and pivaloyl protecting groups provided F·Pi.[7]

First, the effect of Q–R on the fluorescence emission spectrum of F·Pi (1 μM) was investigated in an aqueous solution of 10 mM HEPES buffer (pH 7.4) at 25 °C. When Q–R was added to the aqueous solution of F·Pi, the fluorescence emission of the fluorescein of F·Pi decreased in a dose-dependent manner. An increase in the Q–R concentration resulted in complete quenching of the emission. The curve fitting of the titration profiles was consistent with a 1:1 adduct and the apparent association constant (K_a) was estimated to be 5.6×10^5 M^{-1} for Q–R/F·Pi (Fig. 1).[8]

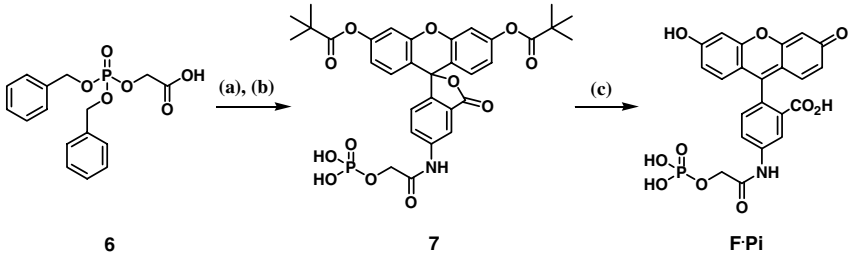
The effect of anions (sodium salts) on the fluorescence emission spectrum of the Q–R/F·Pi ensemble was investigated in an aqueous solution of 10 mM HEPES buffer (pH 7.4) at 25 °C (Fig. 2). When PPi was added to the aqueous solution of the ensemble, the fluorescence emission was dramatically enhanced. An increase in the PPi concentration of up to 3 equiv relative to the Q–R concentration resulted in a 45-fold enhancement of fluorescence. The lowest limit of the binding constant between Q–R and PPi was estimated to be 10^8 M^{-1}.[9a] However, the addition of 3 equiv of ATP resulted in only an 18-fold enhancement of fluorescence. In the case of ADP, the Q–R/F·Pi ensemble showed a relatively small emission change (an 11-fold increase) upon addition of 3 equiv of ADP. The binding constant for ATP was estimated to be less than $\sim 10^7$ M^{-1}.[9a] When a 60-fold excess of Pi and AMP was added, Q–R/F·Pi showed only a subtle emission change (a 5-fold increase). Further, the addition of $CH_3CO_2^-$ and F^- did not lead to an emission enhancement, even after the addition of 100 equiv of each anion (Fig. 2).



Scheme 2. Chemical structures of a quencher–receptor conjugate (Q–R) and a fluorophore–substrate conjugate (F–S = F·Pi) used in an ensemble approach.



Scheme 3. Synthesis of the quencher–receptor conjugate (Q–R). Reagents and conditions: (a) EDCI, glycine ethylester·HCl, CH_2Cl_2; (b) aq KOH, EtOH/THF; (c) EDCI, 4-aminophenol, CH_2Cl_2; (d) bis(2-pyridylmethyl)amine, *p*-formaldehyde, EtOH/H_2O; (e) $Zn(NO_3)_2{\cdot}6H_2O$, H_2O, MeOH.



Scheme 4. Synthesis of the fluorophore–substrate conjugate (F·Pi). Reagents and conditions: (a) EDCI, pivaloyl-protected 5-aminofluorescein, distilled CH_2Cl_2; (b) Pd/C, H_2, MeOH/EtOAc; (c) aq NaOH, MeOH.

The selective fluorescence enhancement for PPi can be explained on the basis of Scheme 2. Before the addition of a target anion (PPi) into the Q–R/F·Pi ensemble, the fluorophore and the quencher are held in close proximity to each other through the phosphate of F·Pi binding to the Zn^{2+}-DPA moiety of Q–R; therefore, there is no fluorescence. If a target (PPi) is added to a solution containing a molecular ensemble, F·Pi is displaced from Q–R by PPi, which is the competing target analyte. PPi is the most strongly binding with Q–R among other nucleotides (ADP, ATP) having the same PPi units, because the total anionic charge density of 4 O–P oxygens involved in the complexation of PPi with Q–R is relatively bigger than that of 4 O–P oxygens of ATP.[9a] Consequently, F·Pi exhibits the original fluorescence because fluorescein is separated from methyl red. The selectivity trend of the dinuclear Zn^{2+}-DPA moiety (receptor part of Q–R) is $PPi > ATP > ADP > AMP \sim Pi > CH_3CO_2^- \sim F^-$.[9]

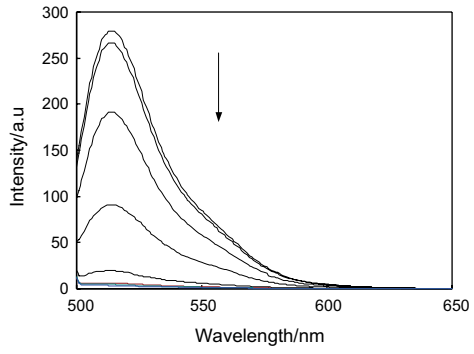


Figure 1. Fluorescence change of F·Pi (1 μM) upon the addition of Q–R (6.1 μM) in an aqueous solution of 10 mM HEPES buffer (pH 7.4) at 25 °C: Q–R (μL) = 0, 10, 30, 50, 70, 100, 130, 180, 250.

In summary, we have developed a Q–R/F·Pi ensemble, which shows a moderate selectivity for, and a high affinity with, PPi in an aqueous solution. The quencher–fluorophore approach described in this study can be utilized for the detection of various analytes through the competitive binding interaction between the target analyte and fluorophore-attached substrate for the quencher–receptor conjugate.

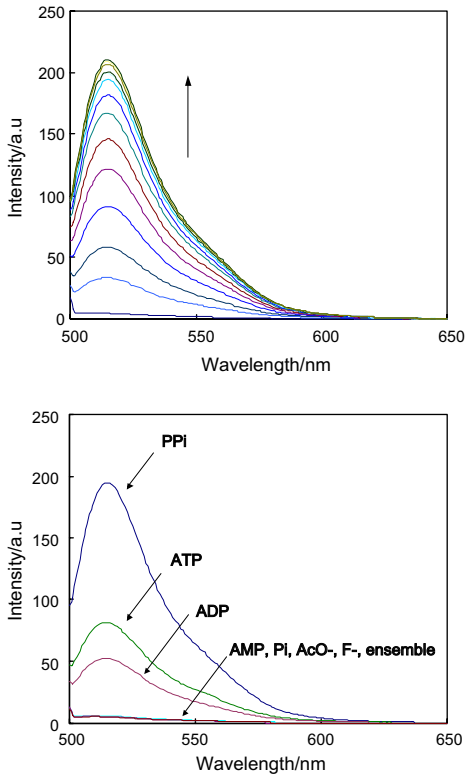


Figure 2. (top) Changes in fluorescence emission for Q–R/F·Pi ensemble ([Q–R] = 6.1 μM, [F·Pi] = 1 μM) after the addition of PPi (0.21 mM) in an aqueous solution of 10 mM HEPES buffer (pH 7.4) at 25 °C: PPi (μL) = 0, 10, 20, 40, 60, 90, 140, 210, 310, 510, 1010, 2010, 4010, 7810. (bottom) Fluorescence emission spectra of Q–R/F·Pi ensemble in 10 mM HEPES buffer (pH 7.4) at 25 °C in the presence of various anions (20 μM).

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Supplementary data

Synthesis and spectral data of new compounds. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2007.05.006.

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6. Spectral data of **1**: ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.90 (s, 6H, $(CH_3)_2N$–), 3.67 (s, 4H, –PhOHCH_2N–), 3.75 (s, 8H, –NCH_2Py), 4.23 (d, $J = 3$ Hz, 2H, –CONHCH_2–), 6.66 (d, $J = 9$ Hz, 2H, $(CH_3)_2$NPhH$_2$–), 7.23 (t, $J = 12$ Hz, 4H, –NCH_2Py), 7.47–7.60 (m, 8H, –NCH_2Py, CONHPhHOH–, N_2PhH$_2$–), 7.68 (t, $J = 12$ Hz, 4H, –NCH_2Py), 7.76 (d, $J = 9$ Hz, 1H, –N_2PhH–), 7.95 (d, $J = 9$ Hz, 2H, $(CH_3)_2$NPhH$_2$–), 8.00 (d, $J = 9$ Hz, 1H, –N_2PhH–), 8.48 (d, $J = 6$ Hz, 4H, –NCH_2Py). ^{13}C NMR (75 MHz, DMSO-d_6): δ 44.25, 54.33, 58.89, 59.28, 111.92, 116.28, 122.73, 122.81, 123.16, 123.24, 123.90, 124.34, 126.53, 129.69, 130.54, 137.17, 137.33, 143.03, 149.17, 150.26, 153.29, 158.67, 158.93, 166.60, 167.23. HRMS (FAB): *m/e* Calcd for $C_{49}H_{49}N_{11}O_3$ $[M+H]^+$ 840.4098; found, 840.4110. Mass data of Q–R: HRMS (FAB): *m/e* Calcd for $C_{49}H_{48}N_{11}O_3{\cdot}2Zn_2NO_3^+$ $[M]^+$ 1090.2281; found, 1090.2310.
7. Spectral data of **7**: ^{1}H NMR (300 MHz, CD_3OD): δ 1.35 (s, 18H, $CO_2C(CH_3)_3$), 4.49 (s, 2H, OCH_2CONH), 6.71 (d, $J = 6$ Hz, 2H), 6.87 (s, 2H), 7.02 (s, 1H), 7.10 (d, $J = 6$ Hz, 2H), 7.65 (s, 1H), 8.25 (s, 1H). HRMS (FAB): *m/e* Calcd for $C_{32}H_{32}NO_{12}P$ $[M+H]^+$ 654.5872; found, 654.1740. ^{1}H NMR data of F·Pi: ^{1}H NMR (300 MHz, DMSO-d_6): δ 4.43 (d, $J = 9$ Hz, 2H), 6.44 (d, $J = 9$ Hz, 2H), 6.76 (m, 4H), 7.21 (d, $J = 9$ Hz, 1H), 8.04 (d, $J = 9$ Hz, 1H), 8.44 (s, 1H).
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