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Nano-Conjugate Fluorescence Probe for the Discrimination of Phosphate and Pyrophosphate

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Dedicated to the memory of Manfred Kämper (1933–2007)

Abstract: We describe a pyrophosphate (PPi) probe that is based on a fluorescent dicarboxylate-substituted poly(*para*-phenyleneethynylene) (PPE) and 10 nm cobalt–iron spinel nanoparticles (NPs) in aqueous media. The spinel NPs efficiently quench the fluorescence of the PPE at a concentration of 20–30 pmol. Addition of phosphate anions to the PPE–NP construct displaces the quenched PPE to give rise to a fluorescent response; we found that PPi and phosphate (Pi) have significantly different binding affinities for the self-assembled materials. We can discern >40 nM PPi in the presence of 0.1 mM Pi at pH 7, which suggests that these assemblies may be useful in bio-analytical applications. This displacement assay was used to effectively determine the ability of pyrophosphatase to hydrolyze PPi to Pi.

Keywords: cobalt · fluorescence · iron · nanostructures · phosphates · pyrophosphate

Introduction

Human bodily processes rely upon the combination of a host of different small molecule inorganic compounds at specific concentrations. Therefore, simple and reliable chemical probes for inorganic anions are generally desirable. Severe medical conditions, such as arthritis and Mönckeberg's arteriosclerosis (MA), occur when the concentration of pyrophosphate (PPi) is abnormal.

MA[1–6] is a form of vascular calcification caused by the unwanted deposit of hydroxyapatite in blood vessels.[7] Vascular calcification observed in MA is *not* due to the excess concentration of calcium and/or phosphate (Pi) ions, but the relative lack of PPi.[8] On the other hand, an excess of PPi can deposit calcium–PPi crystallites, leading to a form of arthritis. Therefore, the facile detection of PPi levels in the presence of Pi is of interest. The classic analytical determination of Pi is based on colorimetric molybdate and related assays.[9,10] However, PPi can not be quantitatively detected by these methods. Instead, enzymatic assays are deployed in clinical settings[11,12] to obtain PPi concentrations in blood serum. These assays are sensitive and reliable, but elaborate involving radioactively labeled reagents. A simple and rapid fluorescence-based assay for PPi would be attractive for its expeditious chemical quantification.

The determination of phosphate-type anions including PPi has been pursued with small and medium-sized fluorescent dyes.[13–22] The concepts that have been developed mostly use ammonium, guanidinium, thiourea, calixpyrrole and similarly tailored N-H species, which are able to hydrogen-bond phosphate and related anions. These receptors have been heavily investigated, but seem to work best in polar protic or polar aprotic media and not in water.[13–15,21] To achieve sensing of phosphate in serum, Anslyn removed the native protein content of serum by ultracentrifugation to ensure an unperturbed assaying process with a supramolecular probe.[15a]

In further developments Anzenbacher demonstrated the sensing of Pi and PPi in water using polyurethane-immobi-

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lized sensor dyes.[13c,d] However, his reported affinities for phosphate and pyrophosphate are similar and millimolar aqueous concentrations of either anion are needed to elicit a fluorescence response.

Blood serum concentrations of Pi are millimolar, whereas PPi concentrations are only micromolar and ATP is present in serum in nanomolar quantities. Therefore, it is of conceptual interest to obtain reporter systems that discern Pi and PPi. PPi sensors in water exploit either the complexation of multiply amine- or pyridine-appended fluorophores with a zinc salt or the reaction of carboxylate poly(*para*-phenyleneethynylene) **1** with a copper salt into composite probes. These probes bind PPi quite nicely, particularly if the fluorophores are coordinated to two zinc ions: PPi is chelated by the zinc ions held in place by suitable binding appendages.[16,19] While the competition between PPi and Pi binding has not been carefully examined in these systems, it seems that Pi/PPi ratios of up to 100 can be tolerated and micromolar amounts of PPi are detected in 0.01 M HEPES buffer. However, the zinc-based sensors might be less efficient when sensing Pi/PPi under more realistic conditions. The concentration of Mg^{2+} and Ca^{2+} is significant in serum and could interfere with the process, since both bind tightly to any phosphate-type anions and may also de-complex the probes. The masking of serum–Ca^{2+} or serum–Mg^{2+} with either cyclen or fluoride anions may not be able to solve this problem, since one could expect that the metallofluorophores might also suffer from decomplexation or metal exchange.

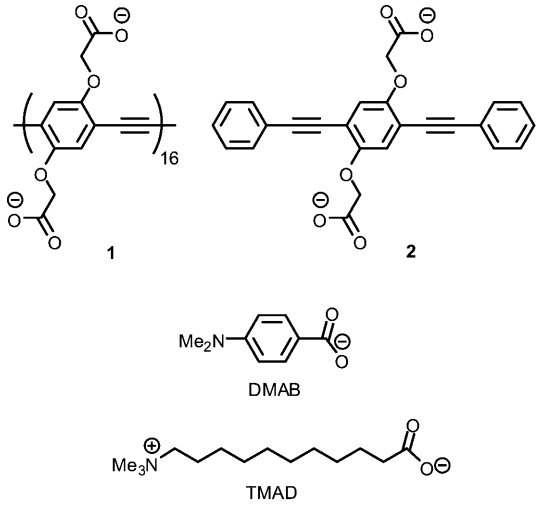
Alternative, simple, variable, modular, sensitive, robust, and easily modifiable methods for successful serum Pi/PPi analyses are desirable. A promising approach to determining Pi/PPi concentrations employs a self-assembled poly(*para*-phenyleneethynylene)–nanoparticle (PPE–NP) construct that is specific and sensitive for phosphate and phosphate-related anions in aqueous media and appears insensitive to most other anions. The concept expands our earlier notion that the addition of a suitable cofactor to a conjugated polymer is valuable to either detect or discern transition metals, proteins, or bacteria.[23,24] Similarly, using conjugated polymers with suitable cofactors in the form of matching–mismatching single-stranded DNA was successfully implemented by Bazan[25] and Leclerc[26] and also exploited by Schanze[27] to detect and quantify DNA.

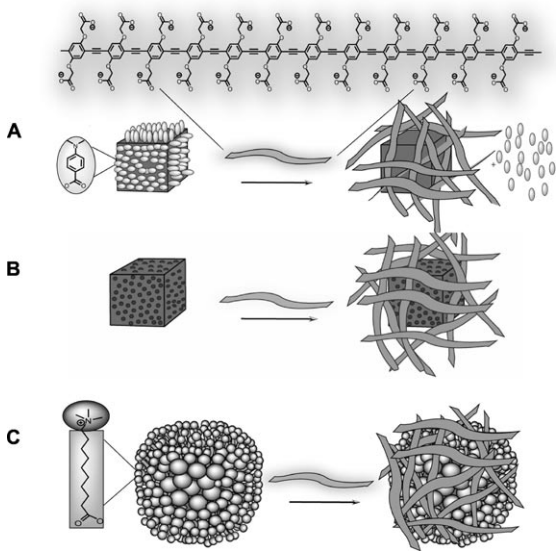
Herein we describe the self-assembly of cube-shaped magnetic cobalt spinel ferrite NPs ($CoFe_2O_4$)[28] with the conjugated polyelectrolyte **1**[29,30] and the use of this simple non-fluorescent material as a turn-on displacement probe for phosphate-type anions.[31] The electrostatically bound PPE is efficiently displaced from the NP surface forming a fluorescent solution upon addition of phosphate anions. The PPE–NP constructs detect PPi in the presence of a large excess of Pi.

These initial results from our experiments only serve as a proof of concept, since we have neither optimized the structure, charge density, or the size of the used PPE nor have we manipulated the surface properties of the spinel ferrite NPs to achieve more sensitive detection of PPi in aqueous media.

Results and Discussion

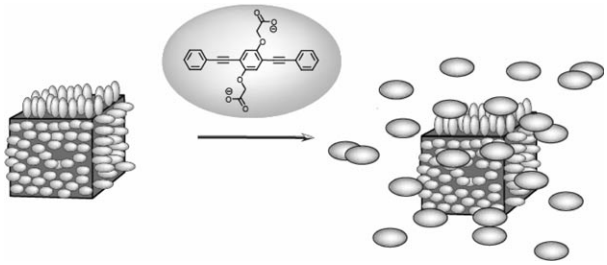
Upon mixing **1** (5×10^{-6} M) with 10 nm diameter cobalt ferrite spinel NPs $(CoFe_2O_4)_x$ in piperazine-1,4-bis-(2-ethanesulfonic acid) (PIPES)-buffered solutions (50 mM, pH 7.2, 0.1 M $KClO_4$), the fluorescence of **1** (degree of polymerization, $P_n=16$) was quenched to 10% of its original intensity in the presence of 20 pM of the NP. A probable quenching mechanism is Förster resonance energy transfer (FRET) from the excited state of **1** to the NP, but other mechanisms such as electron transfer cannot be excluded either. As PPEs have very short emissive lifetimes (0.3–0.5 ns), dynamic quenching processes are generally thought not to play any significant role. The observed quenching in PPEs is purely static.[24c,32] The NPs have a strong absorption at 463 nm, which overlaps with the emission maximum of **1**. Quenching is not unexpected, since gold NPs extinguish the fluorescence of conjugated polymers with high efficiency in assemblies produced from electrostatic interactions.[33–35] The multivalent display of the carboxylate in **1** stabilizes the PPE–NP constructs (Scheme 1) as one NP quenches the fluorescence of approximately 10^4 PPE chains. To support the claim of multivalency between **1** and the NPs we investigated the interaction of the NPs with a monomer **2**. Even at NP concentrations above 1 nM the fluorescence of **2** was not appreciably quenched and the constructs formed from **2** and the NPs were not further investigated. The other possible reason this compound is not quenched might be poor spectral overlap with the absorption of the NP; however, the absorption spectrum of the NPs ranges over the whole spectrum from 250 to 700 nm. The emission maximum of the trimer is around 400 nm, and therefore there is full spectral overlap.





Scheme 1. Schematic representation for the PPE–NP construct and fluorescence quenching with conjugated polymers. A) Displacement of the DMAB by **1**. B) Coordination of **1** to an unprotected NP (red dots are small inorganic counter-anions that are not replaced efficiently). C) Addition of **1** to TMAD functionalized NPs. In this case the TMAD is not released but **1** forms a complex with the cationic ammonium head group.

To understand the interactions between **1** and the NPs, we examined the fluorescence quenching of PPE **1** not only by the dimethylaminobenzoic acid (DMAB)-functionalized NPs but also with propionate-modified, unmodified, and 11-(trimethylammonium)undecanoate (TMAD)-modified NPs (Schemes 1 and 2). In Figure 1 the Stern–Volmer plots of the quenching of **1** by DMAB- and TMAD-functionalized NPs are shown. The DMAB-functionalized NPs give the most efficient quenching, while the TMAD-functionalized



Scheme 2. Inefficiency of small fluorophores in replacing DMAB from the NPs.

NPs were comparatively inefficient in quenching the fluorescence of the PPE **1**. NP concentrations at which the fluorescence drops to 10% of its original value ($[Q]_{90}$) of **1** (5×10^{-6} M) is 20 pM for the DMAB-functionalized NPs, while a concentration of approximately 2 μM of the TMAD-functionalized NPs is necessary to reach the same $[Q]_{90}$.

The concentration of **1** was 5×10^{-6} M based on the molecular weight of the repeating unit. All experiments were performed in a PIPES buffered solution. Scheme 1 illustrates the interpretation of our results. In the case of the DMAB-functionalized NPs, the aniline nitrogen atom of DMAB is uncharged as its pK_a values are 2.6 and 5.0 in water. When coordinated to the NPs, the apparent pK_a of the DMAB may change and better estimated as the pK_a of the hypothetical zwitterion, which was calculated/estimated to be 4.3 according to Wepster et al.[36] As a consequence, even coordinated to the NP, we do not assume that the coordinated DMAB is protonated at the amine at pH 7.2, the conditions under which the experiments were performed. At this pH DMAB exists as a carboxylate, while TMAD exists as an uncharged zwitterion. We interpret the large differences in the $[Q]_{90}$ values between DMAB- and TMAD-functional-

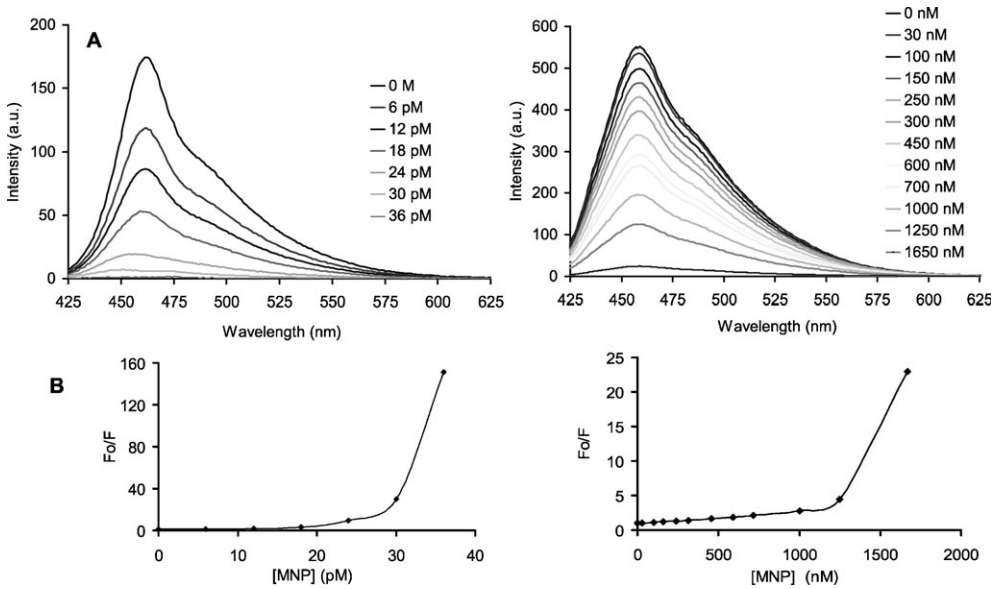


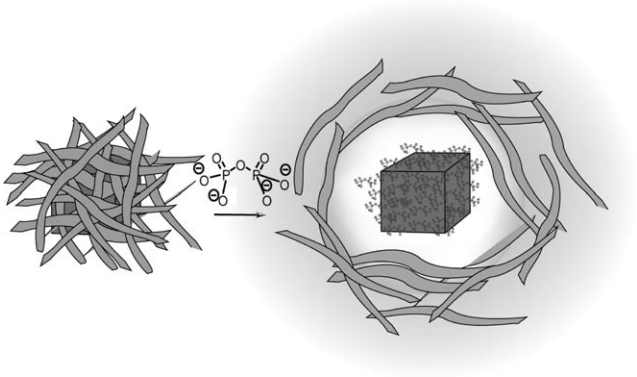
Figure 1. A) Fluorescence quenching and B) Stern–Volmer plot of PPE **1** (5×10^{-6} M) by 10 nm NPs $(CoFe_2O_4)_x$ stabilized by DMAB (left) or TMAD (right). The Stern–Volmer curves show a fluorescence intensity ratio, F_0/F and the different absolute initial intensities as show here have no bearing upon the Stern–Volmer plots as they are—by definition—internally calibrated.

ized NPs such that the DMAB ligand is displaced, and **1** is coordinated to a "naked" NP through displacement of DMAB. In the TMAD case the interaction of the NP to **1** is attenuated by the interspersed, positively charged TMAD, which is not removed by the addition of **1**; instead a weak complex forms between the TMAD-complexed NP and **1** (Scheme 1).

For native, unfunctionalized NPs the $[Q]_{90}$ is 2 nM. The surface of the "unfunctionalized" nanoparticles are "studded" with inorganic counterions (chloride, hydroxide) and residual surfactant molecules from the particle synthesis.[28] Negatively charged surfaces result,[28b] which very likely attenuate the interaction of the NP with the PPE **1**.

To get a further insight into these processes, we investigated the DMAB- and TMAD-functionalized NPs by IR spectroscopy before and after the addition of the PPE **1**. Photoacoustic IR experiments (Figure 2) demonstrate that the addition of the PPE **1** to the DMAB-functionalized NPs leads to displacement of a significant fraction of the bound DMAB from the NPs, while according to the same surface IR measurements, the NPs coated with TMAD only exhibit minimal changes. The PPE **1** therefore does not displace the TMAD from the NPs, but is electrostatically bound to the outer sphere of the still fully TMAD-coated NPs.

Fluorescence quenching of **1** by the low concentrations of the DMAB-coated NPs suggested that the PPE–NP constructs could be used as turn-on nanosensors for negatively charged analytes by displacement of the PPE from the NPs. Scheme 3 shows the proposed working mechanism of such



Scheme 3. Working principle of the nanoparticle based displacement assay. On the left hand side is the quenched PPE–NP construct, and on the right hand side is the now PPi-decorated NP and the displaced and now fluorescent PPE.

an assay. The PPE–NP constructs, formed by the combination of the DMAB–NPs and **1** are nonfluorescent. If **1** is displaced from the NP selectively by an analyte through strong binding to the NP, fluorescence recovery should be observed, and the PPE–NP constructs would act as a displacement assay. As the functionalization of both the spinel NPs and the conjugated polymer are facile, constructs with a variety of binding characteristics and strengths will be available by simple combination of aqueous solutions of NP and conjugated polymers. Such PPE–NP constructs might have potential use in bio-analytical applications. By combining differently substituted PPEs with a variety of NPs one could imagine an almost limitless range of properties (Scheme 1). Figure 3 shows the exposure of DMAB-functionalized NP to various anions (2 mM) in PIPES buffered solutions (50 mM, pH 7.2). Only Pi anions disassemble **1** from the NPs, probably due to their high charge density and affinity

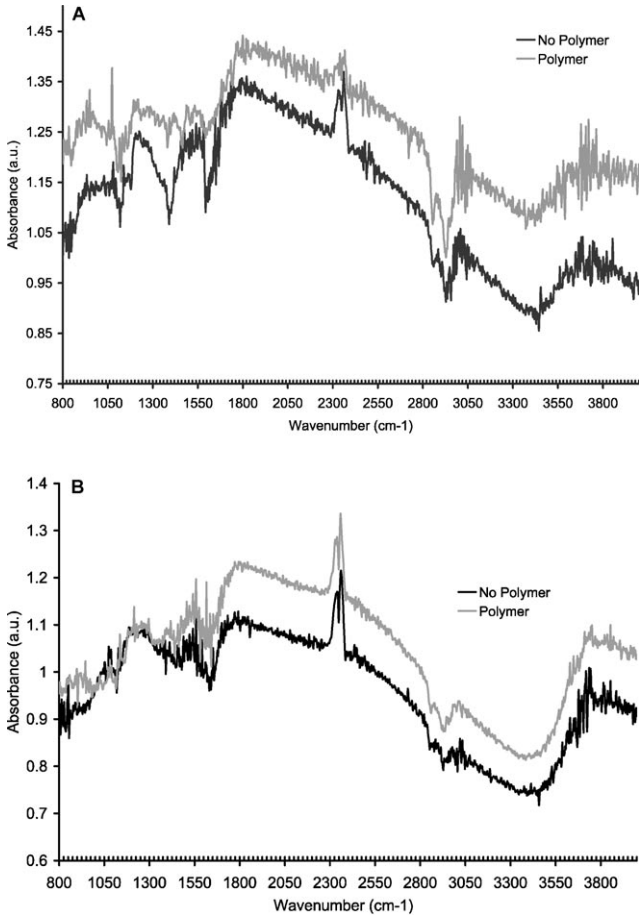


Figure 2. Photoacoustic IR spectra of cube-shaped functionalized spinel NPs before and after addition of polymer **1**. A) DMAB-functionalized NPs before and after addition of PPE **1**. B) TMAD-functionalized NPs before and after addition of PPE **1**. In A) the IR spectra change significantly upon addition of **1**, while in B), the addition of **1** does not induce much change. The large band at 2300 cm^{-1} is due to atmospheric carbon dioxide.

Figure 3. Photograph of fluorescence recovery of the PPE–NP constructs in PIPES buffer (50 mM) upon the addition of different anions. Photograph was taken under black light (Ex = 365 nm) and photographed using a Canon EOS digital camera equipped with an EFS 18–55 mm lens. Concentration of the DMAB-functionalized NPs was 30 pmol; concentration of **1** was 5×10^{-6} M a) Control; b) NaF; c) NaCl; d) NaBr; e) NaI; f) Na_2HPO_4; g) Na_2SO_4; h) CH_3COONa; i) $NaNO_3$; j) $NaNO_2$. The concentration of each anion is 2 mM.

towards the exposed cobalt and iron sites on the NPs, giving significant fluorescence recovery from the PPE–P constructs. The addition of biologically relevant cations (K^+, Na^+, Zn^{2+}, Mg^{2+}, Ca^{2+}) in mM concentrations did not disassemble the PPE–NP constructs. We have added these cations as their perchlorates and there was no fluorescence gain. From our experience with nanoparticles, only at concentrations >200 mM of NaCl does disruption of the PPE–NP constructs occur under "turn-on" of the fluorescence. Because the constructs were uniquely responsive to Pi, we suspected that other, more highly charged phosphate species such as adenosine triphosphate (ATP) and PPi ions would also disassemble the PPE–NP constructs (Figure 4). A remarkable

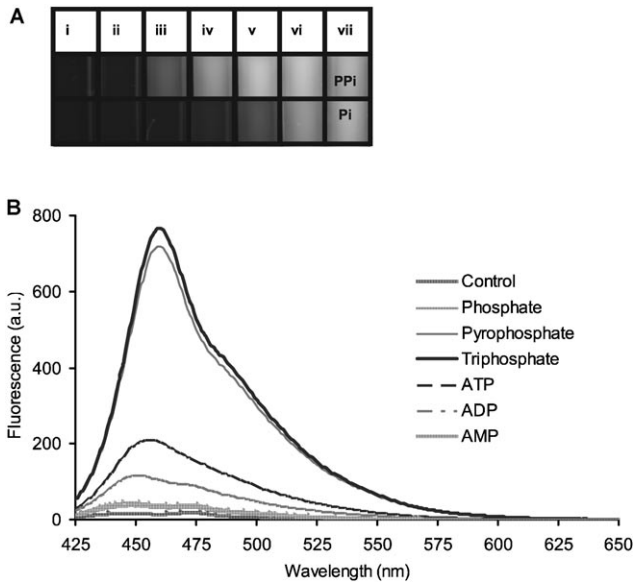


Figure 4. A) Photograph of the fluorescence recovery of the PPE–NP constructs in PIPES buffer (pH 7.2, 50 mM) upon the addition of $P_2O_7^{4-}$ (PPi) and PO_4^{3-} (Pi) ions i) Control, ii) 2×10^{-7} M, iii) 2×10^{-6} M, iv) 2×10^{-5} M, v) 2×10^{-4} M, vi) 2×10^{-3} M, vii) 2×10^{-2} M. Photograph was taken under black light (Ex = 365 nm) with a Canon EOS digital camera equipped with an EFS 18–55 mm lens. B) Fluorescence spectra of the solutions of the PPE–NP construct in PIPES buffered solutions (pH 7.2, 50 mM) upon the addition of phosphate type anions. Concentrations of the phosphate and related anions are 4 μM. Concentration of the NPs was 30 pmol; concentration of **1** was 5×10^{-6} M.

fluorescence increase was found upon the addition of PPi or ATP to the PPE–NP constructs. Figure 4a shows two sets of photographs. The top set displays the PPE–NP construct in the presence of increasing amounts of PPi, while the lower panel displays the same constructs in the presence of increasing amounts of Pi. For PPi, turn-on of the fluorescence is visible at a PPi concentration of 2 μmol, while for Pi very weak fluorescence is observed at a concentration of 200 μmol. Triphosphate, while also reactive, is of less interest in serum as its concentration is considerably less than that of pyrophosphate or diphosphate. Similarly, the concentration of ATP in the blood stream is lower by a factor of 1000 or so than that of pyrophosphate and therefore the sensing of triphosphate seems to be of lesser interest as well as beyond the sensitivity of our assay.

Biological fluids contain metal cations for which ATP and PPi have a high binding affinity (Mg^{2+}, Ca^{2+}). In the presence of Mg^{2+} and Ca^{2+} ions (2 mM), the fluorescence recovery of the PPE–NP construct by ATP and PPi was diminished, but still sufficiently high to detect either anion at micromolar concentrations (Figure S3 in the Supporting Information). For enhancing the sensitivity, Mg^{2+} and Ca^{2+} ions could be partially masked by the addition of fluoride anions in the forms of KF or NaF. Neither K^+ nor Na^+ ions interfere with the detection of PPi. The fluorescence recovery for added PPi is much greater than that for ATP.

In potential bio-diagnostic applications for the determination of PPi, a possible approach is to first determine the cumulative phosphate response by the PPE–NP constructs. Then, the PPi is hydrolyzed to Pi by addition of pyrophosphatase; this step is followed by re-determination of the fluorescence by using the solution in which the PPi had been hydrolyzed into Pi. The difference in fluorescence recovery would be attributed to the presence of PPi. As a proof of concept we checked the suitability of the PPE–NP constructs to monitor the hydrolysis of PPi to Pi by pyrophosphatase (Figure 5), in the presence of Mg^{2+} ions.[37] Our test consisted of monitoring the fluorescence modulation from the PPE–NP constructs upon responding to the decrease in the concentration of PPi in the enzymatic assay.[38]

The time-dependent decrease of PPi was measured by addition of small aliquots (20 μL) of the pyrophosphatase/PPi solution to the PPE–NP construct (2 mL). The kinetics were recorded by using solutions of PPi in tris- buffer (50 mM, pH 8.0), adding 0.4–2.0 "units" of pyrophosphatase (Figure 5). The hydrolysis was monitored upon the change of the fluorescence in the PPi–PPE–NP solution.[39]

The saturation time is a period in which full hydrolysis of PPi to Pi was achieved. The test showed the saturation time varied correlating to the amount of the enzyme units, which indicated that relative quantification of the active enzyme was easily achieved by measuring the change in the fluorescence due to the presence of the PPE–NP constructs. Figure 5 shows the experimental design and the results. In the current setup, the final concentration of Pi is 40 μM, while the beginning concentration of PPi is 20 μM. Under these specific concentration conditions, the fluorescence recovery from Pi is quite low, and we reach a fluorescence intensity plateau. To provide a true assay, one would have to invest a significant amount of effort to understand the underlaying rate equations, equilibrium constants, and so forth.

A critical question for MA (Figure 6) is the determination of small amounts of PPi in the presence of a large excess of Pi as found in blood serum. Aqueous solutions containing 0.1 mM Pi were spiked with increasing amounts of PPi at physiological pH. At a concentration of 0.1 mM, Pi does partially disrupt the PPE–NP constructs leading to a modest increase in absolute fluorescence intensity. If solutions that contain 0.1 mM Pi and 400 nM of PPi are investigated, a sufficient amount of additional PPE is released to give rise to a

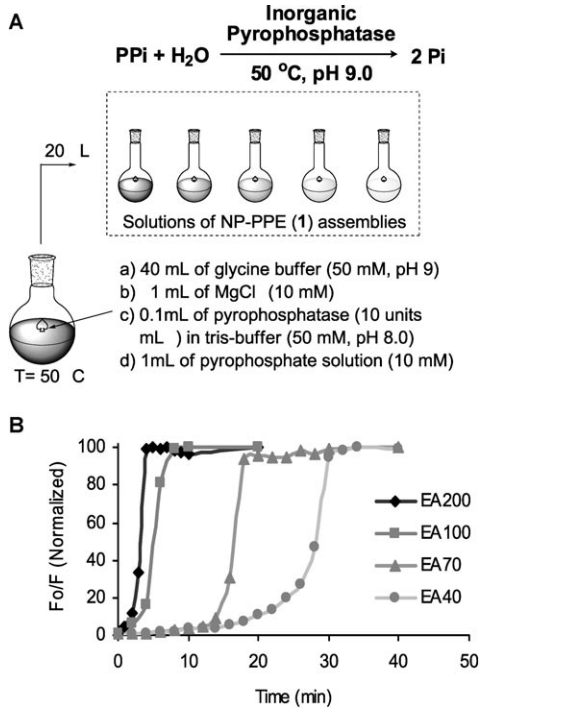


Figure 5. A) Kinetics of the enzymatic assay of pyrophosphatase monitored by the PPE–NP construct. B) Concentration of the DMAB-functionalized NPs was 30 pmol; concentration of **1** was 5×10^{-6} M. F_o is the fluorescence intensity from the solution of the constructs, from which 20 μL of the assay solution at $t_o=0$ was transferred to the PPE–NP construct solution, and F is the fluorescence intensity from the solution of the constructs from which 20 μL of the assay solution at time t was transferred to the construct solution. EA200-EA40 indicates units of pyrophosphatase used in the enzymatic assays. EA200 = 2 units, EA100 = 1 unit, EA70 = 0.7 unit, and EA40 = 0.4 unit. If only enzyme is added, there is no change in fluorescence intensity.

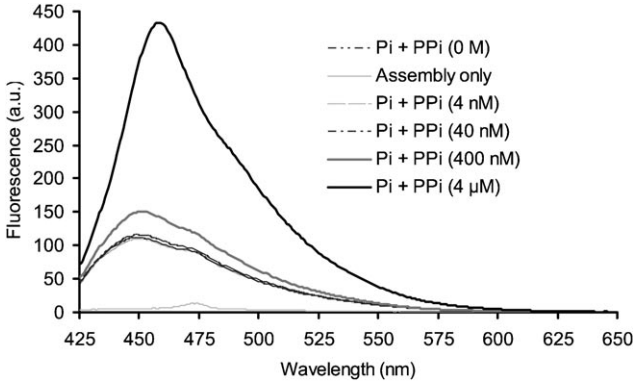


Figure 6. Fluorescence spectra of the solutions of the PPE–NP constructs upon the addition of PPi ions in the presence of 0.1 mM of HPO_4^{2-}/$H_2PO_4^-$ ions at pH 7.2. Concentration of the NPs was 30 pmol; concentration of **1** was 5×10^{-6} M.

strong signal. The addition of Pi increases emission about 50 times, while 4 μM PPi only adds an additional fourfold increase. The reason for this behavior is that the PPE is relatively short and polydisperse. As a consequence, Pi is able to displace some of the shorter PPE chains and lead to some restoration of the fluorescence. One can see that the λ_{max} of the emission is somewhat blue shifted when adding 0.1 mmol Pi, suggesting that the shorter chains are liberated first.

As the ratio of Pi/PPi is >250 in blood serum, any proposed sensing scheme must show a selectivity of PPi/Pi significantly above 500. The herein-presented PPE–NP constructs can detect PPi in a solution that is above 40 nM in PPi and 0.1 mM in Pi, demonstrating that their selectivity against PPi/Pi is in the range of >500, that is, PPi can be detected in the presence of a >500-fold excess of Pi by using a simple self-assembled PPE–NP construct.

Conclusion

DMAB-functionalized NPs $(CoFe_2O_4)_x$ combine with **1** to furnish nonfluorescent PPE–NP constructs that act as simple, self-assembled, convenient fluorescence turn-on probes for PPi at high nanomolar concentrations. An imminent application of this scheme is the successful monitoring of phosphatase activity in vitro. The PPi assay is robust as it can be performed in the presence of 0.1 mM Pi in aqueous solution at pH 7.2 and shows excellent selectivity for PPi. The combination of conjugated polymers with different surface-coated NPs should provide PPE–NP constructs of consanguine structure with varying analytical capabilities for the detection of Pi and related anions. Using a small ensemble of PPE–NP constructs as "chemical tongues"[40] might help to discriminate biologically important species in serum, saliva, and other biological fluids that have been freed from native protein by ultracentrifugation. The trivial ease of the PPE–NP construct assembly combined with the broad array of NPs and conjugated polymers available should make these constructs powerful, yet simple, versatile biodiagnostic tools, in which the recognition element (nanoparticle) is separated from the transmission element (conjugated polymer) and therefore independently addressable. As a next step we will examine PPE–NP constructs as nanomaterial-based[41–45] PPi and Pi sensors in clinical settings, and while the direct sensing of PPi is not possible, a suitable strategy would be to use a difference method (vide supra) to determine the concentration of PPi. The concentration ranges and the differences in sensitivity of our PPE–NP constructs towards different phosphate-type anions are similar to the concentrations of these anions that are found in biological media. Therefore this approach should be feasible.

Experimental Section

Detailed experimental procedures, and emission spectra for the employed constructs are to be found in the Supporting Information.

Acknowledgement

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[1] C. M. Shanahan, N. R. B. Cary, J. R. Salisbury, D. Proudfoot, P. L. Weissberg, M. E. Edmonds, *Circulation* **1999**, *100*, 2168.
[2] D. Proudfoot, C. M. Shanahan, P. L. Weissberg, *J. Pathology* **1998**, *185*, 1.
[3] A. I. Al-Absi, B. M. Wall, C. R. Cooke, *Am. J. Kidney Dis.* **2004**, *44*, e73.
[4] C. E. B. Couri, G. A. da Silva, J. A. B. Martinez, F. D. A. Pereira, F. J. A. de Paula, *Cardiovascular Disorders* **2005**, *5*, 34.
[5] K. A. Lomashvili, W. Khawandi, W. C. O'Neill, *J. Am. Soc. Nephrol.* **2005**, *16*, 2495.
[6] E. Chantelau, *VASA* **2001**, *30(S58)*, 15.
[7] H. Fleisch, R. G. Russell, F. Straumann, *Nature* **1966**, *212*, 901.
[8] *Biomaterials Science* (Eds.: B. Ratner, A. S. Hoffman, F. J. Schoen, J. E. Lemons), Academic Press, London, **1996**.
[9] H. Eibl, W. E. Lands, *Anal. Biochem.* **1969**, *30*, 51.
[10] S. L. Leiboff, *J. Biol. Chem.* **1928**, *79*, 611.
[11] G. Lust, J. E. Seegmiller, *Clin. Chim. Acta* **1976**, *66*, 241.
[12] C. P. Cheung, R. Suhadolnik, *J. Anal. Biochem.* **1977**, *83*, 61.
[13] a) D. Aldakov, P. Anzenbacher, *Chem. Commun.* **2003**, 1394; b) P. Anzenbacher, M. A. Palacios, J. Kursikova, M. Marquez, *Org. Lett.* **2005**, *7*, 5027; c) M. A. Palacios, R. Nsishiyabu, M. Marquez, P. Anzenbacher, *J. Am. Chem. Soc.* **2007**, *129*, 7538; d) G. Zyryanov, M. A. Palacios, P. Anzenbacher, *Angew. Chem.* **2007**, *119*, 7995; *Angew. Chem. Int. Ed.* **2007**, *46*, 7849.
[14] a) P. Plitt, D. E. Gross, V. M. Lynch, J. L. Sessler, *Chem. Eur. J.* **2007**, *13*, 1374; b) H. Miyaji, W. Sato, J. L. Sessler, *Angew. Chem.* **2000**, *112*, 1847; *Angew. Chem. Int. Ed.* **2000**, *39*, 1777.
[15] a) S. L. Tobey, E. V. Anslyn, *Org. Lett.* **2003**, *5*, 2029; b) S. L. Tobey, B. D. Jones, E. V. Anslyn, *J. Am. Chem. Soc.* **2003**, *125*, 4026; c) T. Gunnlaugsson, A. P. Davis, J. E. O'Brien, M. Glynn, *Org. Lett.* **2002**, *4*, 2449; d) E. Quinlan, S. E. Matthews, T. Gunnlaugsson, *J. Org. Chem.* **2007**, *72*, 7497.
[16] a) D. H. Lee, S. Y. Kim, J.-I. Hong, *Angew. Chem.* **2004**, *116*, 4881; *Angew. Chem. Int. Ed.* **2004**, *43*, 4777; b) D. H. Lee, J. H. Im, S. U. Son, Y. K. Chung, J.-I. Hong, *J. Am. Chem. Soc.* **2003**, *125*, 7752; c) H. N. Lee, Z. Xu, S. K. Kim, K. M. K. Swamy, Y. Kim, S.-J. Kim, J. Yoon, *J. Am. Chem. Soc.* **2007**, *129*, 3828; d) Y. J. Jang, E. J. Jun, Y. J. Lee, Y. S. Kim, J. S. Kim, J. Yoon, *J. Org. Chem.* **2005**, *70*, 9603; e) K. M. K. Swamy, S. K. Kwon, H. N. Lee, S. Kumar, J. S. Kim, J. Yoon, *Tetrahedron Lett.* **2007**, *48*, 8683; f) N. J. Singh, E. J. Jun, K. Chellappan, D. Thangadurai, R. P. Chandran, I. C. Hwang, J. Yoon, K. S. Kim, *Org. Lett.* **2007**, *9*, 485; g) X. Y. Zhao, Y. Liu, K. S. Schanze, *Chem. Commun.* **2007**, 2914.
[17] a) R. G. Hanshaw, S. M. Hilkert, H. Jiang, B. D. Smith, *Tetrahedron Lett.* **2004**, *45*, 8721; b) S. Patterson, B. D. Smith, R. E. Taylor, *Tetrahedron Lett.* **1997**, *38*, 6323.
[18] a) L. Fabbrizzi, N. Marcotte, F. Stomeo, A. Taglietti, *Angew. Chem.* **2002**, *114*, 3965; *Angew. Chem. Int. Ed.* **2002**, *41*, 3811; b) S. Mizukami, T. Nagano, Y. Urano, A. Odani, K. Kikuchi, *J. Am. Chem. Soc.* **2002**, *124*, 3920.
[19] D. H. Vance, A. W. Czarnik, *J. Am. Chem. Soc.* **1994**, *116*, 9397.
[20] P. D. Beer, P. A. Gale, *Angew. Chem.* **2001**, *113*, 502; *Angew. Chem. Int. Ed.* **2001**, *40*, 486.
[21] For reviews on designed phosphate type anion sensors see: a) T. Gunnlaugsson, M. Glynn, G. M. Tocci, P. E. Kruger, F. M. Pfeffer, *Coord. Chem. Rev.* **2006**, *250*, 3094; b) E. J. O'Neil, B. D. Smith, *Coord. Chem. Rev.* **2006**, *250*, 3068; c) E. A. Katayev, Y. A. Ustynyuk, J. L. Sessler, *Coord. Chem. Rev.* **2006**, *250*, 3004.
[22] a) X. Zhang, L. Guo, F. Y. Wu, Y. B. Jiang, *Org. Lett.* **2003**, *5*, 2667; b) Y. Kubo, M. Kato, Y. Misawa, S. Tokita, *Tetrahedron Lett.* **2004**, *45*, 3769.
[23] a) I.-B. Kim, U. H. F. Bunz, *J. Am. Chem. Soc.* **2006**, *128*, 2818; b) C.-C. You, O. R. Miranda, B. Gider, P. S. Ghosh, I.-B. Kim, B. Erogan, S. A. Krovi, U. H. F. Bunz, V. M. Rotello, *Nature Nanotech.* **2007**, *2*, 318; c) R. L. Phillips, O. R. Miranda, C.-C. You, V. M. Rotello, U. H. F. Bunz, *Angew. Chem.* **2008**, *120*, 2628; *Angew. Chem. Int. Ed.* **2008**, *47*, 2590.
[24] For general reviews see: a) S. W. Thomas, G. D. Joly, T. M. Swager, *Chem. Rev.* **2007**, *107*, 1339; b) D. T. McQuade, A. E. Pullen, T. M. Swager, *Chem. Rev.* **2000**, *100*, 2537; c) U. H. F. Bunz, *Chem. Rev.* **2000**, *100*, 1605.
[25] B. Liu, G. C. Bazan, *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 589.
[26] H.-A. Ho, M. Leclerc, *J. Am. Chem. Soc.* **2004**, *126*, 1384.
[27] M. R. Pinto, K. S. Schanze, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 7505.
[28] a) Q. Song, Z. J. Zhang, *J. Am. Chem. Soc.* **2004**, *126*, 6164; b) E. L.-H. Heintz, Ph.D. Thesis, Georgia Institute of Technology, Atlanta, **2004**.
[29] I.-B. Kim, A. Dunkhorst, U. H. F. Bunz, *Langmuir* **2005**, *21*, 7985.
[30] I.-B. Kim, A. Dunkhorst, J. Gilbert, U. H. F. Bunz, *Macromolecules* **2005**, *38*, 4560.
[31] S. Wiskur, H. Ait-Haddou, J. J. Lavigne, E. V. Anslyn, *Acc. Chem. Res.* **2001**, *34*, 963.
[32] a) J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 2nd ed., Springer, New York, **2004**; b) Q. Zhou, T. M. Swager, *J. Am. Chem. Soc.* **1995**, *117*, 12593.
[33] C. Fan, S. Wang, J. W. Hong, G. C. Bazan, K. W. Plaxco, A. J. Heeger, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 6297.
[34] Since the quenching of the fluorescence of **1** by the NP could be due to internal filter effects as a consequence of the absorption of the NP, we measured the emission spectra of the solutions of the PPE–NP assemblies after precipitation using a magnet. Under a magnetic field, free NPs and PPE **1** bound NPs were removed. The supernatant was effectively nonfluorescent after addition of NPs [40 pM], indicating the formation of the PPE–NP assemblies. The PPE–NP assemblies are easily re-dispersed into the solution by shaking in the absence of a magnetic field. The model compound **2** was not quenched by the NPs. The electrostatic interaction between **2** and NPs is insufficient to form a stable complex in the presence of buffer.
[35] T. Yavuz Cafer, J. T. Mayo, W. W. Yu, A. Prakash, J. C. Falkner S. Yean, L. Cong, H. J. Shipley A. Kan, M. Tomson, D. Natelson, V. L. Colvin, *Science* **2006**, *314*, 964.
[36] B. Van de Graaf, A. J. Hoefnagel, B. M. Wepster, *J. Org. Chem.* **1981**, *46*, 653.
[37] a) J. K. Heinonen, R. J. Lahti, *Anal. Biochem.* **1981**, *113*, 313; b) the Sigma quality control test procedure for the enzymatic assay of inorganic pyrophosphatase was employed.
[38] A small aliquot of the assay was transferred to the solution of the assemblies at set time intervals. The fluorescence of the solution is measured after 20 min of incubation time. The initial concentration of sodium PPi in the assay was 1.6 mM [10 mM]. One "unit" of pyrophosphatase will liberate 1.0 μmol of inorganic Pi per minute at pH 9.0 at 50 °C. The fluorescence recovery from the solution gradually decreased with increasing incubation time. Beyond a specific incubation time of the assay (3 min for EA200; 5 min for EA100; 18 min for EA70, 30 min EA 40), the fluorescence intensity of the solution remained constant, indicating that all of the PPi had been hydrolyzed by the enzyme. No further fluorescence recovery was observed.
[39] Where a small aliquot of the assay solution was transferred to the test solution of the assemblies. Note that the addition of the enzyme in the solution of the assemblies does not change any fluorescence. The fluorescence intensity was measured until the intensity did not change further. Once the enzyme hydrolyzes all of pyrophosphate ions in the assay (or at very low concentration of PPi ions), the fluorescence of the solution of the assemblies remains unchanged. The normalized F_o/F was plotted as a function of incubation time in each experiment.

[40] J. J. Lavigne, E. V. Anslyn, *Angew. Chem.* **2001**, *113*, 3212; *Angew. Chem. Int. Ed.* **2001**, *40*, 3118.
[41] N. L. Rosi, C. A. Mirkin, *Chem. Rev.* **2005**, *105*, 1547.
[42] P. Alivisatos, *Nat. Biotechnol.* **2004**, *22*, 47.
[43] W. C. W. Chan, S. Nile, *Science* **1998**, *281*, 2016.
[44] C.-Y. Zhang, H.-C. Yeh, M. T. Kuroki, T.-H. Wang, *Nat. Mater.* **2005**, *4*, 826.
[45] I. L. Medintz, A. R. Clapp, H. Mattoussi, E. R. Goldman, B. Fisher, J. M. Mauro, *Nat. Mater.* **2003**, *2*, 630.

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