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Radical Scavenging Mediating Reversible Fluorescence Quenching of an Anionic Conjugated Polymer: Highly Sensitive Probe for Antioxidants

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The anionic conjugated polymer poly{1,4-phenylene[9,9-bis(4-phenoxybutylsulfonate)]fluorene-2,7-diyl} (PFP-SO_3^-) can form a complex with cationic quencher 4-(trimethylammonium)-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (CAT1) through electrostatic interactions. The fluorescence of PFP-SO_3^- is efficiently quenched by CAT1 with a Stern−Volmer constant (K_{sv}) of 2.3×10^7 M^{-1}. The dynamic quenching rate constant of 8.85×10^{16} M^{-1} s^{-1} shows that the static quenching, as supported by PFP-SO_3^-/CAT1 charge pairing, is the dominant quenching mechanism. Either by hydrogen abstraction, or by reduction, the transformation of the paramagnetic nitroxide radical into diamagnetic hydroxylamine inhibits the quenching, and therefore, the fluorescence of anionic conjugated polymer is recovered. The fluorescence recovery can be used to probe the processes of hydrogen transfer reaction from antioxidants to radicals and the reduction of radical by antioxidants. These assays benefit from the sensitivity of optical signals from conjugated polymers. The PFP-SO_3^-/CAT1 assembly can also be used as a platform to sense ascorbic acid in water with high selectivity and sensitivity.

Introduction

In recent years free radical reactions have received much attention due to their involvment in polymer and food degradation and oxidative damage of DNA, protein, and lipids in a variety of disorders, diseases, and cancers.[1−3] Antioxidants function as radical scavengers to play a vital role in the inhibition of these danger radical reactions.[4] As a consequence, it is very important to study their radical scavenging processes and antioxidant capabilities. The mechanism of the radical scavenging by antioxidants is based on the trapping of radicals by hydrogen transfer[5,6] or redox reactions.[3,7] These reactions can be monitored via the ESR signal changes of the radicals[8] or via fluorescent signal changes of fluorophores convalently linked to radical quenchers.[5,7,9] However, the ESR analysis requires large sample quantities and lacks sensitivity. Fluorescent methods based on the dual fluorophore−nitroxide probes require the connection of fluorophores and nitroxide radicals by appropriate chemical reactions. It is still needed to develop new sensitive, simple, and reliable assay methods to probe the radical scavenging by antioxidants.

In comparison to small molecule counterparts, the electronic structure of the conjugated polymer coordinates the action of a large number of absorbing units. As first discovered by Swager and colleagues,[10] the excitation energy along the whole backbone of the conjugated polymer transferring to the quencher results in the amplified fluorescence quenching. Therefore, conjugated polymers can be used as the optical platform in highly sensitive chemical and biological sensors. Recently, we and others have utilized this optical property of conjugated polymers to detect DNA, RNA, protein, and metal ions.[11−18] These assays benefit from the sensitivity of optical signals from conjugated polymers and the simplicity of fluorescence measurement techniques. Herein, we demonstrate a simple and highly sensitive

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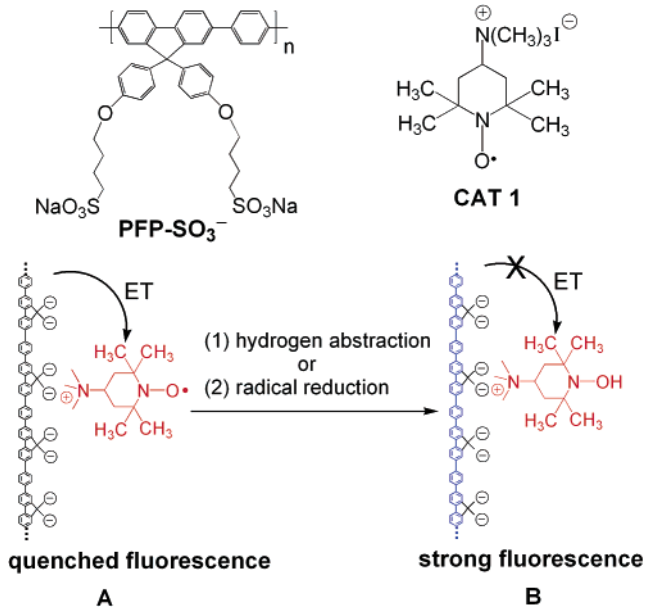
fluorescent probe on the basis of reversible fluorescence quenching of an anionic conjugated polymer mediated by radical scavenging. It can be used to probe the processes of hydrogen transfer reactions from antioxidants to radicals and the reduction reaction of radicals by antioxidants. The same probe can also be used to detect antioxidant capabilities of a variety of antioxidants. Due to the excellent antioxidant capability of ascorbic acid, a fluorescent sensing for ascorbic acid is realized with high sensitivity and selectivity.

Experimental Section

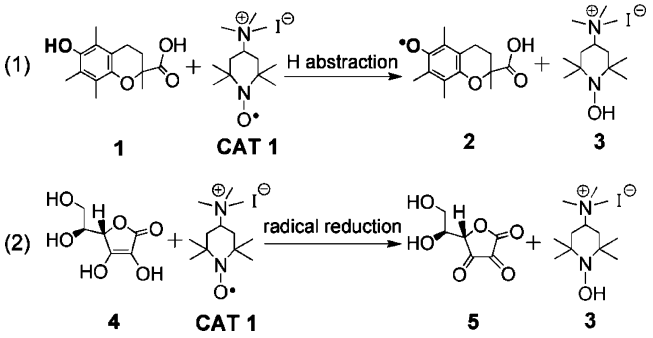
Materials and Instruments. The CAT1 was obtained from Molecular Probes. The (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) was purchased from Aldrich. The gluotathione (reduced) was obtained from Amresco. The ascorbic acid was purchased from 3rd Chemical Reagent Factory of Tianjin, China. The synthesis of PFP-SO_3^- is available in the literature.[19]

The UV−vis absorption spectra were recorded on a Jasco V-550 spectrometer. Fluorescence measurements were done in 3 mL polystyrene cuvettes and on a F-4500 (Hitachi) fluorometer equipped with a Xenon lamp excitation source. The excitation wavelength was 376 nm. All experiments were done in phosphate buffer solution (5 mM, pH 7.4). Water was purified using a Millipore filtration system.

Scheme 1. Schematic Representation of Radical Scavenging Assays and Chemical Structures of the Anionic Conjugated Polymer (PFP-SO_3^-) and Cationic Nitrioxide Radical Quencher (CAT1)



Scheme 2. Radical Scavenging Reactions of CAT1 by Trolox and Ascorbic Acid



Fluorescence Quenching Experiment. The quenching experiment was performed by successive additions of CAT1 to the solution of PFP-SO_3^- ([PFP-SO_3^-] = 1.0×10^{-6} M) in phosphate buffer at room temperature, and the fluorescence spectra were measured.

Fluorescence Recovery Experiment. The trolox ([trolox] = 1.0×10^{-4} M) or ascorbic acid ([ascorbic acid] = 2.0×10^{-4} M) was added into the solution of CAT1 ([CAT1] = 5.0×10^{-5} M) in phosphate buffer. After the sample was incubated for 35 min for trolox or 10 min for ascorbic acid, the PFP-SO_3^- was added and the fluorescence spectra were measured.

Fluorescence Recovery as a Function of Concentrations of Antioxidants. A series of mixed solutions of CAT1 ([CAT1] = 5.0×10^{-5} M) and antioxidants with different concentrations ((0−3.0) $\times 10^{-4}$ M) in phosphate buffer were prepared at room temperature. After the samples were incubated for 35 min for trolox or 10 min for other antioxidants, PFP-SO_3^- was added and the fluorescence spectra were measured. The plot of fluorescence intensity at 422 nm for PFP-SO_3^- as function of trolox concentrations was then obtained.

Fluorescence Recovery as a Function of Reaction Times. A series of mixed solutions of CAT1 ([CAT1] = 5.0×10^{-5} M) and trolox ([trolox] = 1.0×10^{-4} M) or ascorbic acid ([ascorbic acid] = 2.0×10^{-4} M) in phosphate buffer were prepared at room temperature. After the samples were incubated for certain time, the

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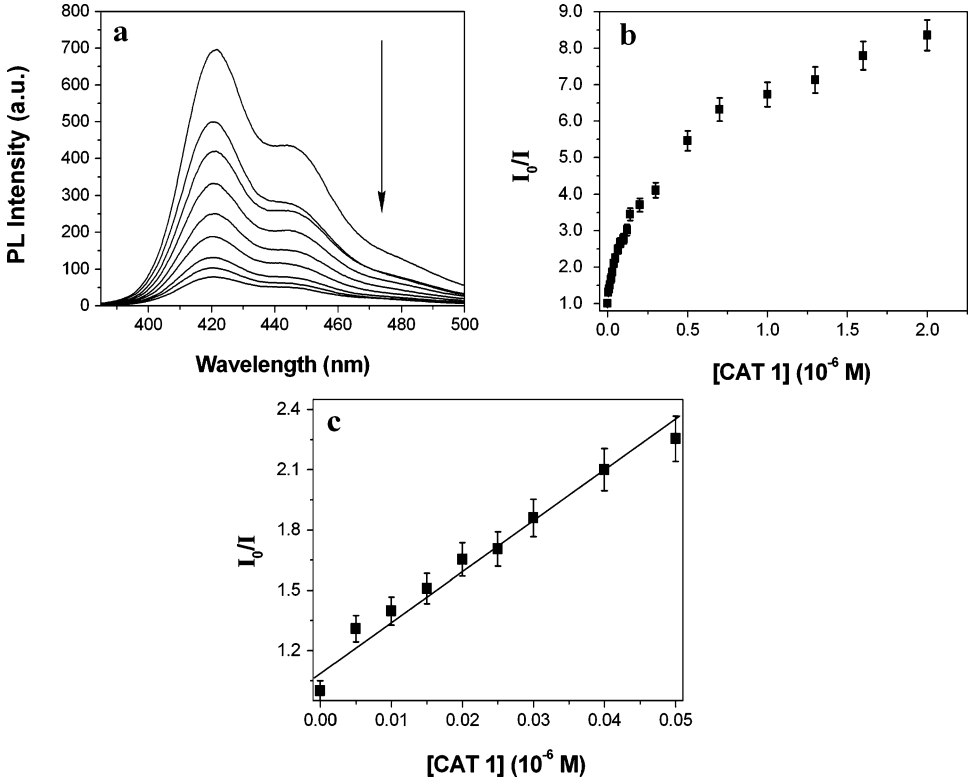

Figure 1. (a) Fluorescence emission spectra of PFP-SO_3^- in phosphate buffer solution (5 mM, pH = 7.4) with successive additions of CAT1 ([PFP-SO_3^-] = 1.0×10^{-6} M in RUs, [CAT1] = 0 to 2.0×10^{-6} M). (b) K_{sv} plot of PFP-SO_3^- in the presence of CAT. (c) K_{sv} plot in low CAT1 concentration (0−50 nM) profiles. The excitation wavelength is 376 nm. The error bars represent the standard deviation of four measurements conducted at each CAT1 concentration.

PFP-SO_3^- was added and the fluorescence spectra were measured. The plot of fluorescence intensity at 422 nm for PFP-SO_3^- as function of the time of hydrogen transfer or radical reduction process was then obtained.

Results and Discussion

Design of the Fluorescent Probe for Radical Scavenging. Our new fluorescent probe for radical scavenging is illustrated in Scheme 1. The anionic poly{1,4-phenylene-[9,9-bis(4-phenoxybutylsulfonate)]fluorene-2,7-diyl} (PFP-SO_3^-)[19] can form the complex with cationic radical quencher CAT1 through electrostatic interactions. PFP-SO_3^- is one of the polyfluorene derivatives that exhibit higher thermal stability and photoluminescence quantum efficiency. These polyfluorene derivatives have higher energy band gaps (2.8 eV) with higher oxidation potentials (∼1.5 V) and redox potentials (∼−2.2 V).[20] Taking into account the oxidation potentials (∼0.63 V) and redox potentials (∼−0.62 V) of nitroxide radicals,[21] the fluorescence of PFP-SO_3^- can be efficiently quenched by CAT1 via a charge-transfer process[22] (Scheme 1A). Either by hydrogen abstraction or by reduction, the transformation of the paramagnetic nitroxide radical into diamagnetic hydroxylamine inhibits the quenching, and therefore, the fluorescence of PFP-SO_3^- is recovered (Scheme 1B).

The (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) (**1**)[5] and ascorbic acid (**4**)[7] are chosen as proofs of the concept for the hydrogen abstraction and reduction reactions, respectively (Scheme 2). CAT1 can abstract a hydrogen atom from trolox (**1**) to produce hydroxylamine **3**. Ascorbic acid can reduce CAT1 to hydroxylamine **3**, and itself is oxidated to the dehydroascorbic acid **5**.

Study on Amplified Fluorescence Quenching. Although CAT1 has been used as the quencher to small fluorophores,[22] its quenching ability to conjugated polymers has never been reported. To investigate the quenching ability of nitroxide radical CAT1, the fluorescence quenching of PFP-SO_3^- ([PFP-SO_3^-] = 1.0×10^{-6} M in repeat units (RUs)) was thus examined by CAT1 in phosphate buffer solution (5 mM, pH 7.4) (Figure 1a). The quenching efficiency is related to the Stern−Volmer constant, K_{sv}, and is determined by monitoring measurable changes in the fluorescence via the Stern−Volmer equation:[23]

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

Here I_0 is the fluorescence emission intensity in the absence of the quencher, I is the fluorescence emission intensity in the presence of the quencher, and [Q] is the quencher concentration.

The quenching of PFP-SO_3^- by CAT1 shows downward curvature in the Stern−Volmer plot (I_0/I vs [CAT1]) at higher CAT1 concentration (Figure 1b), which indicates that not all PFP-SO_3^- molecules are accessible by electrostatic

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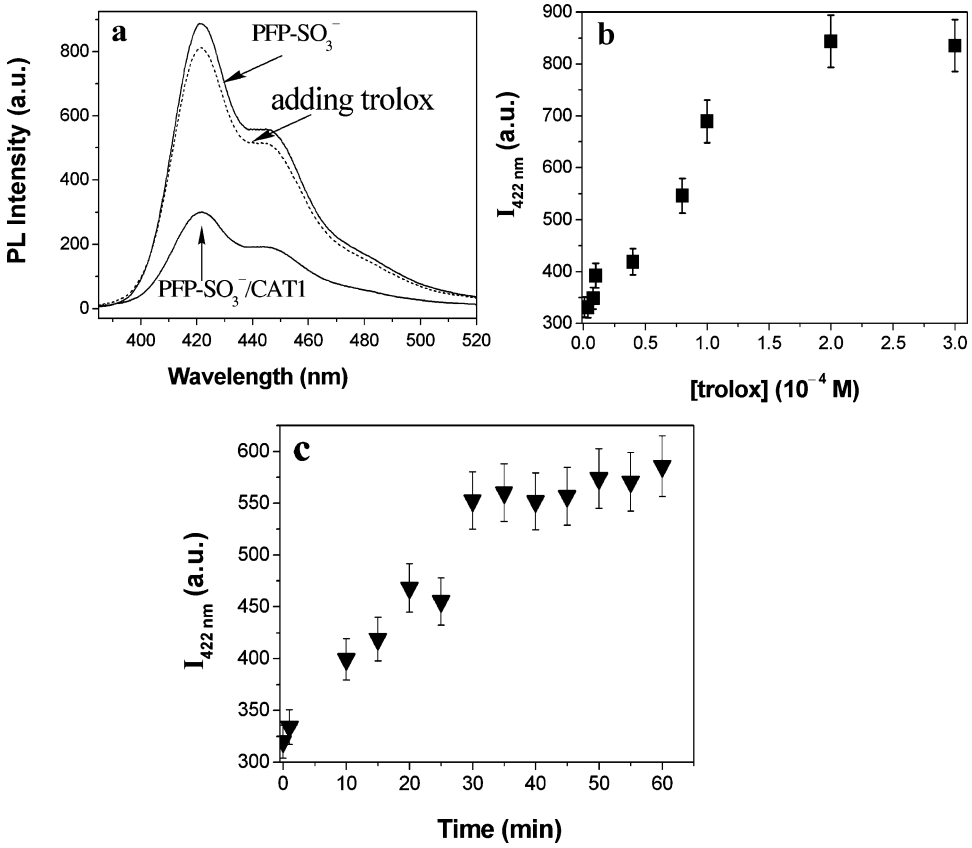

Figure 2. (a) Emission spectra of PFP-SO_3^- and PFP-SO_3^-/CAT1 in the absence and presence of the trolox. (b) Fluorescence intensity at 422 nm of PFP-SO_3^-/CAT1 as function of trolox concentrations ([trolox] = (0−3.0) × 10^{-4} M). (c) Fluorescence intensity at 422 nm of PFP-SO_3^-/CAT1 in the presence of trolox as function of the incubating time. The excitation wavelength is 376 nm, and measurements are performed in phosphate buffer (5 mM, pH 7.4) ([PFP-SO_3^-] = 1.0 × 10^{-6} M in RUs, [CAT1] = 5.0 × 10^{-5} M, [trolox] = 1.0 × 10^{-4} M). The error bars represent the standard deviation of four measurements conducted at each trolox concentration or time.

interactions to this charged quencher in this case. At low concentrations of CAT1, a linear Stern−Volmer plot is obtained with the K_{sv} value of 2.3 × 10^7 M^{-1} (Figure 1c). The dynamic quenching rate constant (k_q) can be calculated from eq 2[23] using the K_{sv} value and fluorescence lifetime (τ) of the PFP-SO_3^-.

$$k_q = \frac{K_{sv}}{\tau} \tag{2}$$

The fluorescence lifetime for PFP-SO_3^- is approximately 0.26 ns,[19] which provides a quenching rate constant of 8.85 × 10^{16} M^{-1} s^{-1}. This value is several orders of magnitude above those possible for diffusion-controlled quenching.[23,24] Static quenching, as supported by PFP-SO_3^-/CAT1 charge pairing, is therefore the dominant quenching mechanism. The quenching efficiency is about 80% at 10^{-7} M CAT1, which shows that the remarkably low concentrations of CAT1 are effective in quenching the fluorescence of PFP-SO_3^-. This provides us the chance to probe radical scavenging and detect antioxidants with high sensitivity.

Assay for the Hydrogen Transfer Process. As shown in Figure 2a, upon addition of the CAT1 ([CAT1] = 5.0 × 10^{-5} M), the fluorescence of PFP-SO_3^- ([PFP-SO_3^-] = 1.0 × 10^{-6} M in RUs) is efficiently quenched. The trolox is added and allowed to equilibrate for 35 min, which reverses the quenching due to the scavenging of nitroxide radical via hydrogen transfer from the trolox to CAT1. The fluorescence recovery exhibits dependence upon the concentration of the trolox (Figure 2b). It should be noted that the amount of the trolox necessary to produce obvious fluorescence recovery is remarkably lower in the range 10−100 μM. Figure 3a shows that the hydrogen transfer process from trolox to CAT1 is very slow. The quenching recovery increases in the time range from 0 to 30 min and reaches a plateau after 35 min.

Assay for the Radical Reduction Process. Figure 3a shows that, for additions of ascorbic acid ([ascorbic acid] = 2.0 × 10^{-4} M) to the solution of PFP-SO_3^-/CAT1 ([PFP-SO_3^-] = 1.0 × 10^{-6} M in RUs, [CAT1] = 5.0 × 10^{-5} M), the fluorescence of PFP-SO_3^- is significantly recovered. It was found that the recovery proceeded to a completion in about 0.5−3.5 min, which is much faster than the hydrogen transfer process from trolox to CAT1 (Figure 3b). We noted that the fluorescence of PFP-SO_3^- was recovered by 200% instead of the expected 100%. It is known that the oxygen dissolved in solvents is a quencher,[25] and so we bubbled the solution of PFP-SO_3^- in phosphate buffer with nitrogen for 2 h and the intensity of PFP-SO_3^- increased by about 100% (Figure 3a). It was found that the same fluorescence recovery was obtained if we only bubbled the phosphate buffer with nitrogen for 2 h and then added PFP-SO_3^- to the buffer

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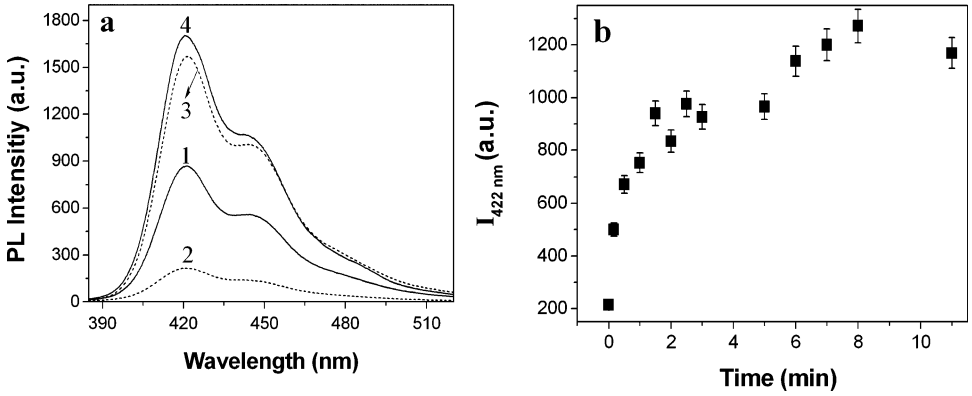

Figure 3. (a) Emission spectra of (1) PFP-SO_3^- under O_2, (2) PFP-SO_3^-/CAT1, (3) PFP-SO_3^-/CAT1 in the presence of ascorbic acid, and (4) PFP-SO_3^- under N_2. (b) Fluorescence intensity at 422 nm of PFP-SO_3^-/CAT1 in the presence of ascorbic acid as function of the incubating time. The excitation wavelength is 376 nm, and measurements are performed in phosphate buffer (5 mM, pH 7.4) ([PFP-SO_3^-] = 1.0×10^{-6} M in RUs, [CAT1] = 5.0×10^{-5} M, [ascorbic acid] = 2.0×10^{-4} M). The error bars represent the standard deviation of four measurements conducted at each time.

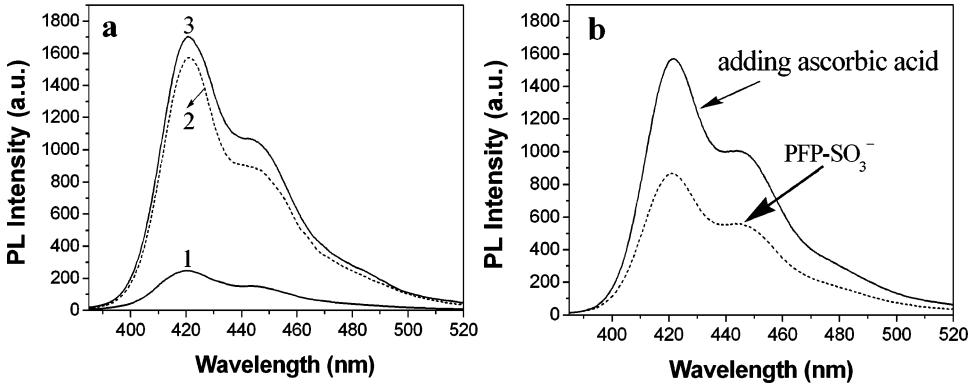

Figure 4. (a) Emission spectra of (1) PFP-SO_3^-/CAT1 under N_2, (2) PFP-SO_3^-/CAT1 in the presence of ascorbic acid under N_2, and (3) PFP-SO_3^- under N_2. (b) Emission spectra of PFP-SO_3^- in the absence and presence of ascorbic acid under O_2. The excitation wavelength is 376 nm, and measurements are performed in phosphate buffer (5 mM, pH 7.4) ([PFP-SO_3^-] = 1.0×10^{-6} M in RUs, [CAT1] = 5.0×10^{-5} M, [ascorbic acid] = 2.0×10^{-4} M).

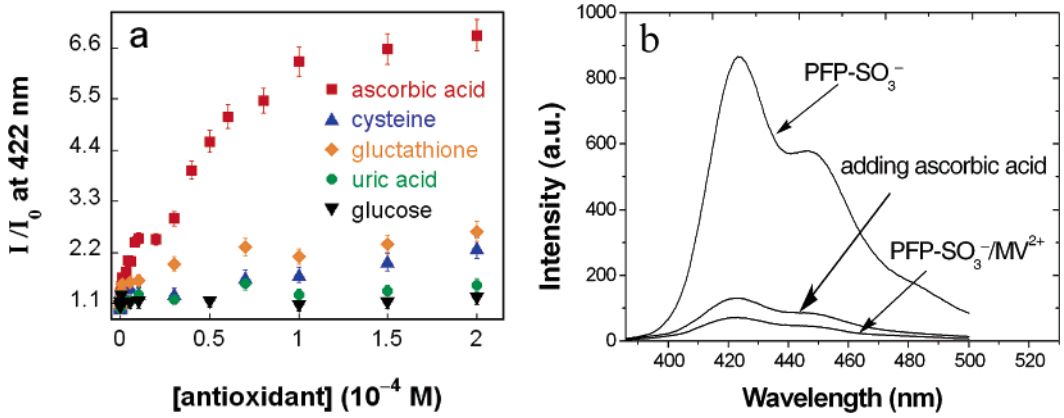

Figure 5. (a) Fluorescence intensity at 422 nm for PFP-SO_3^-/CAT1 as function of concentrations of antioxidants under O_2 ([antioxidants] = (0−2.0) × 10^{-4} M, [PFP-SO_3^-] = 1.0×10^{-6} M in RUs, [CAT1] = 5.0×10^{-5} M). The error bars represent the standard deviation of four measurements conducted at each antioxidants concentration. (b) Emission spectra of PFP-SO_3^-, PFP-SO_3^-/MV^{2+}, and PFP-SO_3^-/MV^{2+}/ascorbic acid under O_2 ([PFP-SO_3^-] = 1.0×10^{-6} M in RUs, [MV^{2+}] = 4.37×10^{-8} M, [ascorbic acid] = 2.0×10^{-4} M). The excitation wavelength is 376 nm, and measurements are performed in phosphate buffer (5 mM, pH 7.4).

solution. The effect of degassing on the fluorescence recovery due to a change in the state of aggregation of the PFP-SO_3^- induced by the degassing process is ruled out. That is, the oxygen dissolved in the buffer solution quenches half of the fluorescence of PFP-SO_3^-. A similar result was also reported in the literature.[26] Therefore, upon addition of ascorbic acid to the solution of PFP-SO_3^-/CAT1, the nitroxide radical and oxygen are all scavenged. To prove this dual scavenging mechanism, two additional experiments were employed with controlled single scavenging process for nitroxide radical or for oxygen only: (a) The addition of ascorbic acid ([ascorbic acid] = 2.0×10^{-4} M) to the solution of PFP-SO_3^-/CAT1 ([PFP-SO_3^-] = 1.0×10^{-6} M in RUs, [CAT1] = 5.0×10^{-5} M) in the absence of oxygen only results in 100% fluorescence recovery, where only nitroxide radical was scavenged (Figure 4a). (b) The addition of ascorbic acid to

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the solution of PFP-SO_3^- without CAT1 in the presence of oxygen results in about 100% fluorescence increase, where only the scavenging for oxygen was performed (Figure 4b).

Assay for Antioxidant Capability and the Sensor for Ascorbic Acid. To investigate the antioxidant capabilities of different antioxidants, the fluorescence recovery for PFP-SO_3^-/CAT1 under oxygen was examined by water-soluble antioxidants, such as ascorbic acid, cysteine, gluctathione, uric acid, and glucose. Figure 5a exhibits that fluorescence recovery for PFP-SO_3^-/CAT1 ([PFP-SO_3^-] = 1.0×10^{-6} M in RUs, [CAT1] = 5.0×10^{-5} M) depends on the concentration of antioxidants. For ascorbic acid the fluores--cence recovery is remarkably higher than those for other antioxidants, which shows the antioxidant capability of ascorbic acid is strongest in these water-soluble antioxidants. Ascorbic acid can be determined in the concentration range from 50 nM to 200 μM (Figure 5a). The control experiments were also done with a nonspecific quencher, *N*,*N*′-dimethyl-4,4′-bipyridinium (MV^{2+})[27] for ascorbic acid. As shown in Figure 5b, upon addition of the MV^{2+}, the fluorescence of PFP-SO_3^- is efficiently quenched. The ascorbic acid was added and allowed to equilibrate for 35 min, and almost no recovery was observed for the fluorescence of PFP-SO_3^-. These results confirm that the radical reduction of CAT1 by ascorbic acid specifically controls the fluorescence recovery of PFP-SO_3^-. Therefore, PFP-SO_3^-/CAT1 can be used as the platform in a highly sensitive ascorbic acid sensor with minor interference from other antioxidants.

Conclusion

In summary, the amplified fluorescence quenching of anionic PFP-SO_3^- by cationic CAT1 was employed with a Stern−Volmer constant of 2.3×10^7 M^{-1}. The dynamic rate constant of 8.85×10^{16} M^{-1} s^{-1} shows that the static quenching is the dominant quenching mechanism. Either by hydrogen abstraction or by reduction, the transformation of the paramagnetic nitroxide radical into diamagnetic hydroxylamine inhibits the quenching, and therefore, the fluorescence of anionic conjugated polymer is recovered. The fluorescence recovery can be used to probe the processes of hydrogen transfer reaction from antioxidants to radicals and the reduction reaction of radicals by antioxidants. These assays benefit from the sensitivity of optical signals from conjugated polymers. A highly sensitive ascorbic acid probe with minor interference from other antioxidants is also obtained.

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