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A highly sensitive and selective antioxidant probe based on a bi-modally functionalized conjugated polyelectrolyte†

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A new water-soluble anionic conjugated polyelectrolyte with a nitroxide radical covalently linked to the sulfonated poly(phenylene ethynylene) backbone (PPE-SO₃) is reported. This radical-functionalized PPE-SO₃ (RF-PPE-SO₃) demonstrates fluorescence and electron spin resonance (ESR) bimodal signaling function and shows sensitive and selective response to antioxidants.

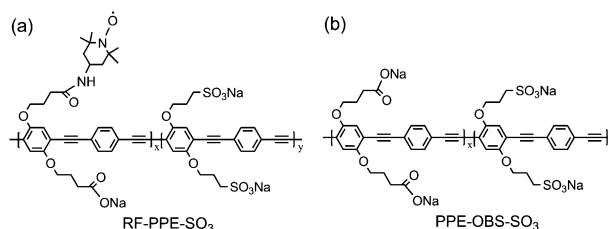
Conjugated polyelectrolytes (CPEs) are characterized by a delocalized electronic structure with ionic solubilising side groups. CPEs bear numerous merits in terms of chemo- and biosensors, such as water solubility, large extinction constants, high emission quantum yields, structurally controllable optical properties, and ultrafast exciton transport, an intrinsic trait that results in analyte-derived fluorescence signal amplification, named as amplified quenching or superquenching.^{1,2} Therefore, in recent years, CPEs-based chemo- and biosensors have attracted increasing attention and have been applied in sensing a wide range of chemical species including metal ions,^{3–7} anions,^{8,9} small biomolecules,¹⁰ proteins,¹¹ DNA,^{12,13} etc.

Conventional fluorescent probes based on CPEs recognise analytes usually through electrostatic interaction.^{3,4,14} As a result, these probes usually bear limited selectivity. In order to eliminate electrostatic interaction and enhance selectivity, functionalized CPEs have been designed and synthesized for detecting specific targets, such as hydrogen peroxide and glucose,¹⁵ proteases,^{16,17} bacteria,¹⁸ metal ion and glutathione reductase activity.¹⁹ However, little attention has been devoted to CPEs functionalized with a free radical. The 2,2,6,6-tetramethylpiperidine-*N*-oxide free radical (TEMPO) functionalized polythiophene thin film has recently been reported by Kunz and Wolf²⁰ and its charging and discharging capabilities for applications in organic-based batteries have been investigated.

Herein we report a newly designed and synthesized water-soluble nitroxide radical-functionalized sulfonate-substituted poly(phenylene ethynylene) (RF-PPE-SO₃, Scheme 1a) as an electron spin resonance (ESR) and fluorescence bi-modal probe.

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Scheme 1 Chemical structures of RF-PPE-SO₃ (a) and PPE-OBS-SO₃ (b).

The 4-amino-2,2,6,6-tetramethylpiperidine-*N*-oxide free radical (4A-TEMPO) was chosen as the functional group because of its dual-functional property. As a paramagnetic group, the nitroxide moiety quenches the excited state of the fluorophore efficiently, keeping the probe's prefluorescent property,^{21–23} and the stable nitroxides are highly active scavengers of radicals.^{24,25} Nitroxide free radical scavenging or redox chemistry returns the probe to its natural fluorescent state and so the tethered nitroxide-fluorophore molecule is described as a prefluorescent nitroxide probe.²⁶ To date, more and more types of prefluorescent nitroxide probes have been reported, such as nitroxide-quinoline,²⁷ nitroxide-quantum dots,^{22,28} nitroxide-phthalocyaninosilicon²⁹ and nitroxide-lanthanide.³⁰ It is expected that the labelled nitroxide radical group in the RF-PPE-SO₃ can act as an effective quencher of the excited singlet state of the conjugated polyelectrolyte and thus makes the RF-PPE-SO₃ an off-on fluorescence switch when the radical moiety is reduced or traps a free radical to transform into a diamagnetic hydroxylamine. Due to transformation of the paramagnetic group to a diamagnetic one, an on-off ESR response can also be expected. PPE-OBS-SO₃ (Scheme 1b) as a nonradical-functionalized control was also synthesized. The mechanism of the signaling is discussed and the practicability of this newly synthesized bi-modal probe is demonstrated by its sensitive and selective response to antioxidants.

Fig. 1 illustrates the absorption and emission spectra of the as-prepared PPE-OBS-SO₃ and RF-PPE-SO₃ both in the absence and presence of ascorbic acid (AA). The absorbance of PPE-OBS-SO₃ and RF-PPE-SO₃ is dominated by a broad and intense band with $\lambda \approx 440$ nm, which is typical of PPE-type conjugated polymers.^{14,31} Strong emission of the PPE-OBS-SO₃ was observed around 520 nm with excitation maximum around 438 nm and an absolute quantum yield (A-QY) of 0.16 in aqueous solution was measured. As expected, the RF-PPE-SO₃ was

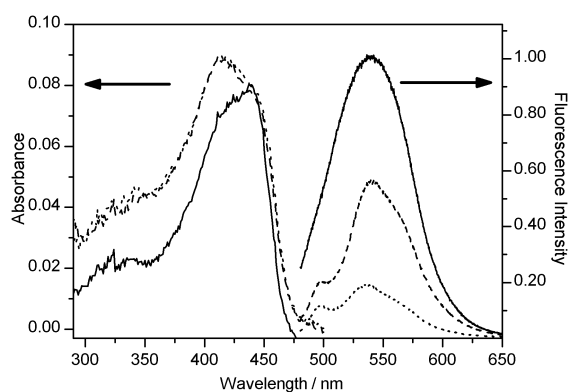


Fig. 1 The absorption (left) and emission (right) spectra ($\lambda_{\text{ex}} = 438$ nm, slit width: 3 nm/3 nm) of 10 μM of PPE-OBS-SO₃ (solid line) and RF-PPE-SO₃ in the absence (dotted line) and presence (dashed line) of 20 μM of AA in water.

hypofluorescent with an A-QY of 0.01. After the side-chain nitroxide radical being reduced by AA to a diamagnetic hydroxylamine, named as HA-PPE-SO₃, a recovery of fluorescence in HA-PPE-SO₃, relative to RF-PPE-SO₃, was observed (A-QY = 0.07), without obvious change of emission wavelength and profile, and the absorption spectra did not show obvious change either. The off-on fluorescence intensity was proportional to the concentration of AA, and accompanying with the fluorescence intensity increase was an ESR signal decrease of the RF-PPE-SO₃ (Fig. 2). Bimodal response of RF-PPE-SO₃ to carbon-centered radicals, photochemically produced by azobisisobutyronitrile (AIBN, Scheme S2, ESI[†]), was also observed (Fig. S1, ESI[†]). Trapping methyl radicals produced photochemically changed the side-chain nitroxide radical to a stable diamagnetic *O*-methylhydroxylamine, and continuous fluorescence increase was observed as the photo-reaction was taking place, until a maximum was reached. The radical trapping was confirmed by a synchronous decrease of the ESR signal originated from the side-chain nitroxide radical in the RF-PPE-SO₃. In a control experiment using PPE-OBS-SO₃ instead of RF-PPE-SO₃ in reaction with AA, neither off-on fluorescence increase nor on-off ESR signal decrease was observed (data not shown).

Intermolecular collisional quenching of PPE-SO₃ with TEMPO had previously been reported.^{32,33} According to the

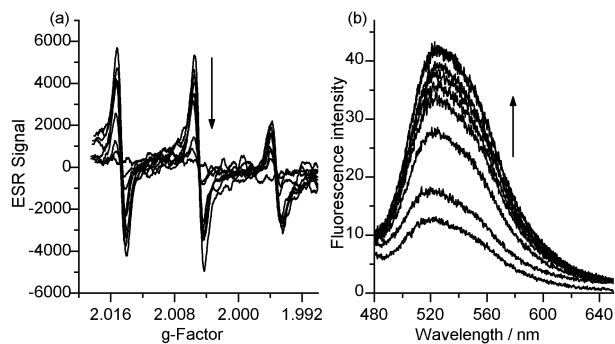


Fig. 2 ESR (a) and fluorescence (b) spectra of RF-PPE-SO₃ (1 mM) in the presence of various concentrations of AA (0–2.9 mM) ($\lambda_{\text{ex}} = 438$ nm, slit width: 1.5 nm/1.5 nm).

early observation, obvious quenching of PPE-SO₃ emission was achieved at a considerable high concentration of TEMPO (mM magnitude, about 3–4 order of that of PPE-SO₃). This low quenching efficiency might have been attributed to an intermolecular mechanism. With a ratio of 1 : 330 of [TEMPO] to [RF-PPE-SO₃] (synthesis of RF-PPE-SO₃, ESI[†]), the side-chain labelled TEMPO in RF-PPE-SO₃ demonstrated a much higher quenching efficiency. This high quenching efficiency should be attributed to the enhanced intramolecular quenching effect in the polymer. Static fluorescence quenching of the sulfonate-substituted polyfluorene derivative (PFP-SO₃) by 4-(trimethylammonium)-2,2,6,6-tetramethylpiperidine-1-oxyl iodide based on electrostatic interaction has also been reported.³³ There has been no paper concerning an intramolecular quenching mechanism reported, to the best of our knowledge. In this work, we have investigated the quenching mechanism of the side-chain labelled nitroxide radical on the fluorescence of RF-PPE-SO₃. Since no obvious changes in absorption spectrum and emission spectrum of RF-PPE-SO₃ were observed in the presence of AA, we first excluded the possibility that the fluorescence quenching/recovery is due to conformational changes that might have resulted from radical labelling. Energy migration fluorescence quenching has long been observed.³⁴ The precise mechanism of energy migration depends upon the given system. Resonance energy transfer (RET) and photoinduced electron transfer (PET) dominating quenching have been observed.^{35,36} In both mechanisms, RET and PET, the fluorescence lifetime should be shortened owing to an additional deactivation pathway. The results show (Fig. 3) that the fluorescence lifetime of RF-PPE-SO₃ ($\tau = 0.2$ ns) is far shorter than that of PPE-OBS-SO₃ ($\tau = 5.8$ ns). Because of lacking a favourable spectral overlap between the emission spectrum of the conjugated polyelectrolyte backbone and the absorption spectrum of the side-chain nitroxide radical group, the RET mechanism could not dominate the quenching. We assume that the lifetime shortening has resulted from the quenching of the excited singlet state of RF-PPE-SO₃ by the side-chain labelled paramagnetic nitroxide radical, 4A-TEMPO, an acceptor of electron, *via* an intramolecular photoinduced electron-transfer from the excited-state of the conjugated polyelectrolyte backbone to the ground-state of a side-chain nitroxide radical when the exciton migrates to the 4A-TEMPO labelled monomer. Reducing 4A-TEMPO to diamagnetic

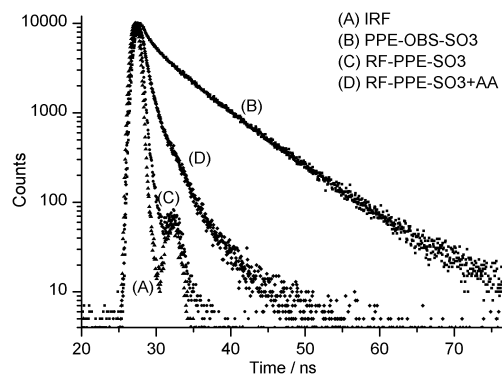


Fig. 3 Fluorescence decays ($\lambda_{\text{ex}} = 457$ nm, $\lambda_{\text{em}} = 518$ nm) of PPE-OBS-SO₃ (B), RF-PPE-SO₃ in the absence (C) and presence (D) of AA; the instrument response function (IRF) (A) is also presented.

hydroxylamine, a poor acceptor of electron, eliminated the electron-transfer pathway and, thus, the fluorescence lifetime of RF-PPE-SO₃ became longer ($\tau = 1.1$ ns) in the presence of AA.

AA is a diprotic acid ($pK_{a1} = 4.10$ and $pK_{a2} = 11.97$) and its oxidation potential changes as a function of pH. The fluorescence intensities of RF-PPE-SO₃ in the presence and absence of AA as pH ranged from 2 to 11 were measured, respectively. The fluorescence recovery efficiency F/F_0 (defined as the ratio of the fluorescence intensity of RF-PPE-SO₃ in the presence (F) and absence (F_0) of AA) reached maximum at about pH 8, and the profile of the F/F_0 -pH plot is similar to that of pH-dependent potential of AA (Fig. S2, ESI[†]) in a range of pH 2 to 9. The decrease in fluorescence recovery efficiency in more alkaline media might have resulted from electrostatic repulsion between the polyanion and negatively charged AA.

AA was used as a model molecule for further examining the practicability of RF-PPE-SO₃ in sensing antioxidants. Results show that the plot of fluorescence recovery efficiency (F/F_0) versus the concentration of AA (Fig. S3, ESI[†]) is linear in the range of 20–120 nM ($r = 0.9992$, $n = 7$) with a detection limit of 5 nM based on a signal-to-noise (S/N) of 3, indicating that RF-PPE-SO₃ can serve as a quantitative and sensitive probe for AA. The relative standard deviation for 11 replicate detections of 60 nM AA was 3.89%, which indicated acceptable precision. Other antioxidants were also studied, such as glutathione (GSH) and L-cysteine (CySH), and RF-PPE-SO₃ also shows off–on fluorescence responses to these antioxidants (Fig. S4, ESI[†]).

The fluorescence response behaviours of RF-PPE-SO₃ on other various amino acids were also examined. As shown in Fig. S5 (ESI[†]), fluorescence off–on response was not observed to Gly, Ala, Arg, Asp, Cystine (Cyt), Leu, Phe, Ser, Thy and Val, even at a much higher amino acid to probe concentration ratio, under the same conditions. Due to weak reducibility of Tyr, a fluorescence recovery was observed in Tyr. Fluorescence off–on response to hydroquinone (HQ), which has a hydroxide group in the benzene ring as Tyr, was also observed. These results demonstrate that the fluorescence recovery efficiency is related to the reducibility of antioxidants and RF-PPE-SO₃ could be used as a sensitive generic probe of antioxidants. Selective detection of AA can be achieved by eliminating interferences resulted from thio-based molecules, such as CySH and GSH, by a thiol-blocking reagent, *N*-ethylmaleimide (NEM, Fig. S6, ESI[†]).

In summary, we report the design and synthesis of a fluorescence and ESR bimodally functionalized conjugated polyelectrolyte-based probe for antioxidants. The as-prepared RF-PPE-SO₃ is hypofluorescent and high ESR, due to the paramagnetism of the labelled nitroxide radical. On recognising an antioxidant or a radical target analyte, the nitroxide radical is reduced to a diamagnetic group, and gives off–on fluorescence and on–off ESR bimodal signaling. The enhanced intramolecular quenching of the nitroxide radical to the excited singlet state of RF-PPE-SO₃ makes highly sensitive signaling possible.

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