LETTER

# Two-Photon Excited Fluorescence of a Conjugated Polyelectrolyte and Its Application in Cell Imaging

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**ABSTRACT** The two-photon excited fluorescence of a conjugated polyelectrolyte (CPE), $PPESO_3$, was studied in methanol and in water. The photophysical and amplified quenching properties of the CPE observed under two-photon excitation were comparable to the results obtained under one-photon excited conditions. Two-photon fluorescence microscopy performed with $PPESO_3$-coated silica nanoparticles in HeLa cells provided images with significantly improved resolution compared to one-photon microscopy, demonstrating the utility of the CPE as a fluorescent probe in two-photon fluorescence cell imaging.

**KEYWORDS:** two-photon absorption • conjugated polyelectrolytes • near-infrared • cell imaging

## INTRODUCTION

Conjugated polyelectrolytes (CPEs) combine the properties of conjugated polymers with water solubility (1). While the conjugated backbone allows efficient exciton delocalization, which forms the basis for amplified quenching (2, 3), the ionic side chains afford the advantage of processability from aqueous solution. The fluorescence of CPEs is generally quenched very efficiently by oppositely charged species such as metal−organic ions and biomolecules (1, 4). CPEs have been explored as biosensors because of their selectivity in binding specific molecules, and they have found applications in optoelectronic devices (1, 5−11). Recent developments in the field of CPEs have shown that this class of compounds could also serve as efficient light-activated biocidal agents (12, 13).

Conjugated polymers feature nonlinear optical properties, and their two-photon excitation-induced fluorescence has been documented by a few groups (14−31). Tailoring the properties of CPEs for two-photon absorption (2PA) is important because it could enhance their application in biological systems. The influence of the solvent and the concentration dependence of 2PA cross sections of conjugated polymers in organic solvents has been reported by Wang et al. (26). A recent study from Narayanan et al. describes the application of a fluorescent conjugated polymer as an efficient chemosensor for trinitrotoluene (TNT) under two-photon excited conditions (27). Their work combines the phenomenon of amplified quenching of the polymer fluorescence with two-photon excitation to develop a highly sensitive sensor for TNT in the vapor phase. In the present communication, we report two-photon excited fluorescence from a water-soluble, poly(phenyleneethynylene)-based CPE, amplified quenching, and its application to two-photon fluorescence microscopy (2PFM) imaging in HeLa cells. The photophysical properties of the CPE, $PPESO_3$, have been studied by our group and others (32−34).

## MATERIALS AND METHODS

**Materials.** Silica microspheres of average diameter 300 ± 10 nm were purchased from Bangs Laboratories. Poly(dimethyldiallylammonium) chloride (PDDA) was purchased from Aldrich, and the water-soluble, anionic conjugated polyelectrolyte (CPE), $PPESO_3$, was synthesized following a previously reported procedure (32). HeLa cells were purchased from America Type Culture Collections (Manssas, VA).

**Methods.** ***Polymer-Coated Silica Particles.*** The polymer-coated silica nanospheres were prepared using the layer-by-layer method described by Kim et al. (35). PDDA and $PPESO_3$ were used as the polycation and the fluorescent polyanion, respectively (1 mM in water). PDDA was allowed to adsorb on the negatively charged surface of the nanospheres through electrostatic interactions. Subsequent exposure of the PDDA-coated silica nanospheres to oppositely charged $PPESO_3$ results in silica/PDDA/$PPESO_3$ spheres. Additional bilayers were deposited by successive treatments with PDDA and $PPESO_3$. Between layer coatings, the remaining unadsorbed polyelectrolytes were separated by centrifugation (3650*g*) and washing with deionized water (Milli-Q, 18 MΩ).

**Differential Interference Contrast (DIC) Image and One-Photon Fluorescence Microscopy (1PFM).** Conventional DIC and conventional 1PFM images were obtained using an inverted microscope (Olympus IX70) equipped with a QImaging cooled CCD (model Retiga EXi) and tungsten or mercury excitation lamps (100 W). One-photon confocal fluorescence images of the fixed cells were obtained using a custom-made filter cube (Ex, 377/50; DM, 409; Em, 525/40). Both images were collected with a 60× oil immersion microscope objective (UPLANSAPO 60×, NA = 1.35, Olympus).

**2PA and Imaging.** 2PA of $PPESO_3$ in methanol and aqueous solutions were determined over a broad spectral region by the two-photon unconverted fluorescence method relative to

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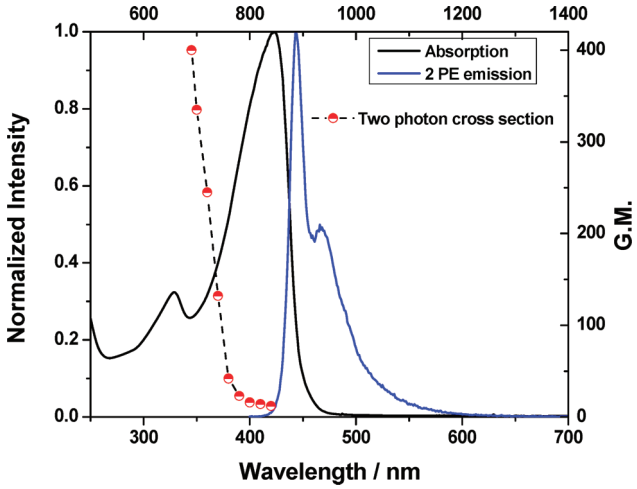
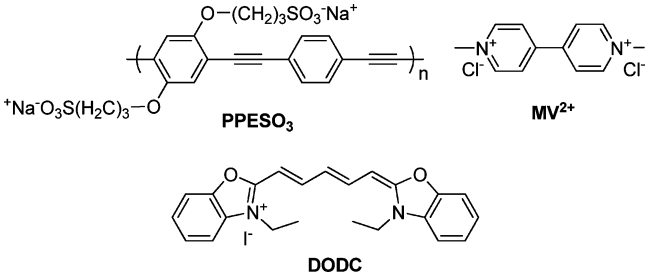



FIGURE 1. Linear and two-photon photophysical properties of $PPESO_3$ ($c = 28\ \mu M$; $\varepsilon = 57\ 000\ M^{-1}\ cm^{-1}$) polymer in methanol. Normalized one-photon absorption spectrum (black line), normalized two-photon excited emission spectrum (blue line), and 2PA spectrum (black line and circle symbol). Note that the units on the right-hand side ($y$ axis) are G.M. per polymer repeat unit.

## Scheme 1. Chemical Structures of CPE and Quenchers




rhodamine B in methanol (Figures 1 and S-2 in the Supporting Information). A PTI QuantaMaster spectrofluorimeter coupled with a Ti:sapphire femtosecond laser (Coherent MIRA 900 with pulse duration, ~200 fs, tuning range 700−1000 nm, and 76 MHz repetition rate) was used.

2PFM images were collected on a modified Olympus Fluoview FV300 microscope system coupled to a Coherent MIRA Ti:sapphire laser, which was used for 2PA measurements. A short-pass emission filter (FF01-694/SP-25, Semrock) was placed in the microscope scan head to avoid background irradiance from the excitation source. Consecutive layers, separated by 0.2 $\mu$m, were recorded to create a 3D reconstruction from overlaid 2PFM images. The two-photon-induced fluorescence was collected with a 60× oil immersion microscope objective (UPLANSAPO 60×, NA = 1.35, Olympus).

## RESULTS AND DISCUSSION

$PPESO_3$ (Scheme 1) is an anionic polymer that is soluble in both water and alcohol and exhibits blue or green fluorescence. In methanol, the polymer shows a strong fluorescence and a well-defined 0−0 band with $\lambda_{max} = 442$ nm, whereas a broad, red-shifted band with diminished quantum yield is observed in water. The spectral data suggest that in water the polymer is strongly aggregated, whereas in methanol, a "good solvent", it exists as individual chains with minimal aggregation.

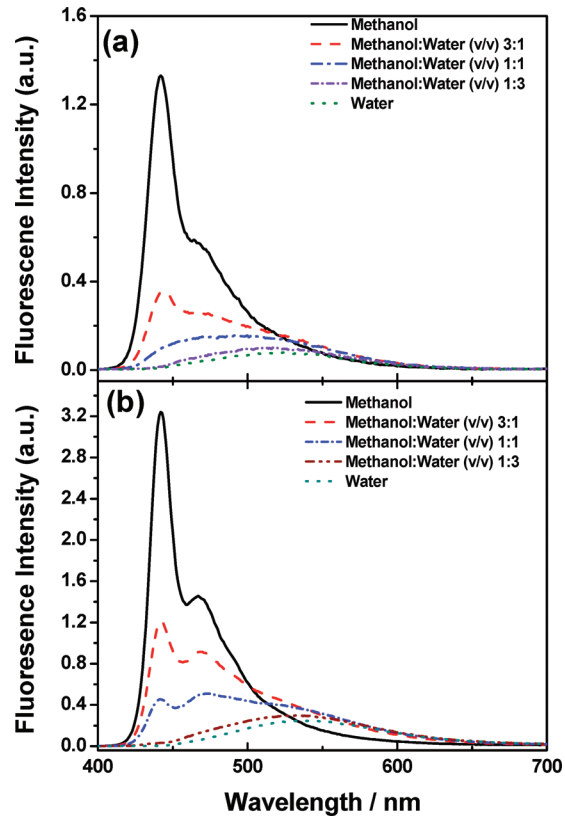



FIGURE 2. Fluorescence spectra of 10 $\mu$M $PPESO_3$ in varying proportions of methanol and water at $\lambda_{ex}$ = (a) 740 nm and (b) 380 nm.

2PA of $PPESO_3$ in methanol was determined by monitoring the upconverted fluorescence, and the spectrum is shown along with the 2PA excited fluorescence spectrum in Figure 1. First, note that the 2PA excited fluorescence spectrum ($\lambda_{ex} = 740$ nm) exhibits $\lambda_{max} = 440$ nm, and it is essentially identical with that observed under 1PA excitation, indicating that the emitting state produced by 2PA is the same as that produced by 1PA. The 2PA cross section was determined relative to rhodamine B in methanol; the onset of 2PA is at ~800 nm, and the cross section increases monotonically and reaches ~400 G.M. at 690 nm (36). Comparing the 2PA and 1PA spectra (plotted for comparison in Figure 1) reveals that the onset of 2PA occurs to the blue of the longest wavelength $\pi-\pi^*$ transition in the 1PA spectrum (when compared as $2\lambda$ for the 2PA spectrum). This is consistent with the notion that the lowest-energy transition ($1A_g \rightarrow 1B_u$) is one-photon-allowed but two-photon-forbidden; enhanced 2PA occurs on the blue side of the 1PA-allowed transition, and it is likely due to absorption into the second singlet state ($1A_g \rightarrow mA_g$). Similar observations have been made in the 2PA spectra of other centrosymmetric conjugated polymers (30, 37).

In order to determine if the aggregated and molecularly dissolved states of $PPESO_3$ exhibit similar fluorescence under one- and two-photon excitation, we examined the solvent dependence of the fluorescence spectra in methanol/water mixtures, and the results are shown in Figure 2. For both 2PA and 1PA excitation (parts a and b of Figure

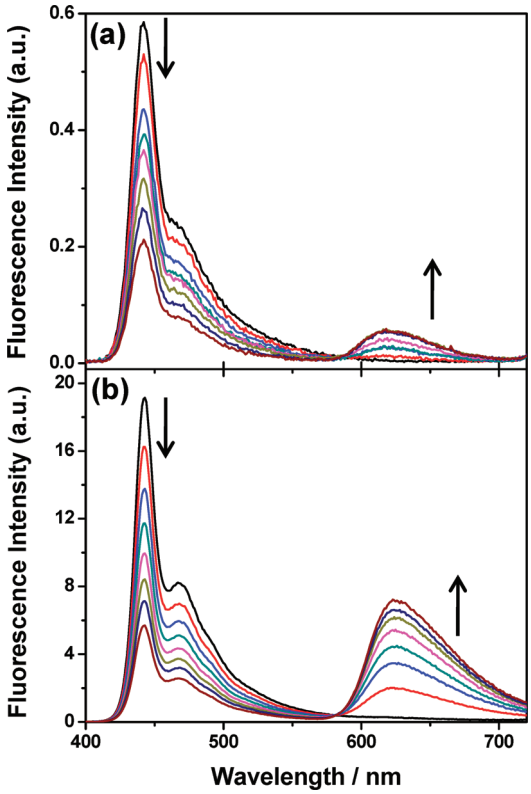



**FIGURE 3. Fluorescence quenching of $PPESO_3$ (10 $\mu$M in methanol) by DODC (0.1−10 $\mu$M) at $\lambda_{ex}$ = (a) 740 nm and (b) 380 nm.**

2, respectively), it is seen that, as the volume fraction of water increases in the solvent, the fluorescence intensity decreases and the spectra become broader and undergo a significant red shift. The response of fluorescence to the solvent is very similar for 2PA and 1PA excitation, indicating that the photophysics of the fluorescent state, including exciton transport and trapping by aggregate states, remains the same when the polymer is excited by 2PA.

Much of the interest in CPEs is motivated by their use as fluorescent sensors that respond to analytes present at very low concentration. The sensitive fluorescence response is a result of the "amplified quenching effect" (2), whereby oppositely charged quencher ions are able to quench CPE fluorescence with Stern−Volmer (SV) quenching constants ($K_{SV}$) in excess of $10^7$ $M^{-1}$. Amplified quenching is attributed to the electrostatic interaction between an oppositely charged polymer and the quencher (ion pairing) as well as the delocalization and rapid transport of the singlet exciton over the $\pi$-conjugated CPE chain. In the present study, we sought to determine whether the amplified fluorescence quenching properties of $PPESO_3$ are also observed when the CPE is excited by 2PA.

Thus, parallel quenching studies were carried out under 1PA and 2PA excitation using $PPESO_3$ and the cationic electron-transfer quencher methylviologen ($MV^{2+}$) and the cationic cyanine dye energy-transfer quencher (DODC).

The SV fluorescence quenching of $PPESO_3$ ($c = 10$ $\mu$M) by $MV^{2+}$ under 1PA and 2PA excitation gave rise to similar results (see Figure S-3 in the Supporting Information). At low [$MV^{2+}$] (<2 $\mu$M), the SV plots are nearly linear, and the $K_{SV}$ values are $3.8 \times 10^6$ and $3.0 \times 10^6$ $M^{-1}$ under 2PA and 1PA excitation, respectively. In both cases, at higher [$MV^{2+}$], the SV plots show upward curvature, and the observed quenching efficiency was slightly larger under 2PA excitation. Overall, the results indicate that the amplified quenching effect is clearly active when $PPESO_3$ is excited by 2PA; this result shows that the CPE could be used in sensing schemes with infrared excitation.

We also examined the quenching of $PPESO_3$ by the energy acceptor DODC. The dye was selected because in previous work we have shown that the dye quenches by a fluorescence resonance energy-transfer (FRET) mechanism, and consequently the polymer can be used to sensitize the dye fluorescence (33). As shown in the Supporting Information (Figure S-3), the addition of DODC results in very efficient quenching of the $PPESO_3$ fluorescence under 2PA excitation, with $K_{SV} \sim 2.5 \times 10^6$ $M^{-1}$, which is slightly larger than that observed under 1PA excitation ($K_{SV} \sim 1.5 \times 10^6$ $M^{-1}$). Interestingly, we also compared the ability of $PPESO_3$ to sensitize the DODC fluorescence under 1PA and 2PA excitation, and the results are shown in Figure 3. Here it is seen that the addition of a low concentration of DODC results in quenching of the $PPESO_3$ fluorescence with a concomitant increase of fluorescence from DODC at longer wavelength ($\lambda \sim 640$ nm). While the intensity of the sensitized fluorescence is somewhat weaker under 2PA excitation, the control experiments show that near-infrared excitation (740 nm) of DODC in the absence of $PPESO_3$ results in virtually no upconverted fluorescence. This result indicates that $PPESO_3$ can act as a relatively efficient "two-photon" upconverting sensitizer. It is likely that this 2PA

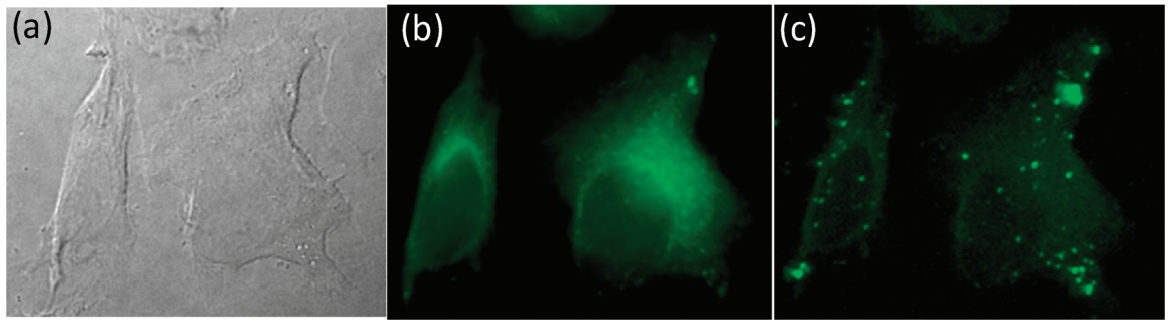



**FIGURE 4. Images of HeLa cells incubated with $PPESO_3$ silica nanoparticles (20 $\mu$g/mL, 2 h): (a) DIC, exposure time 20 ms; (b) 1PFM image, 130 ms (filter cube: Ex, 377/50; DM, 409; Em, 525/40); (c) one layer of the 2PFM image (Ex, 750 nm; power, 30 mW).**

FRET sensitizing scheme will apply to other cationic dyes that are able to effectively associate with the anionic CPE in solution. In addition, because a number of CPE sensor schemes are based on FRET to an ion-paired dye, the result suggests that these sensors may operate under 2PA excitation using near-infrared photons.

One important application of 2PA excited fluorescence lies in its utility in cellular imaging by 2PFM. Given that the fluorescence of $PPESO_3$ can be excited efficiently in the near-infrared by 2PA, we set out to demonstrate that the polymer could be used for intracellular imaging. Thus, $PPESO_3$-coated silica nanoparticles (300 nm) were fabricated by using the layer-by-layer technique, as described previously (35). HeLa cells were incubated with the $PPESO_3$-coated particles (20 $\mu$g/mL for 2 h), and the labeled cells were subsequently imaged by using DIC and 1PFM and 2PFM, respectively; see Figure 4) (38).

As seen in Figure 4c, 2PFM images of the labeled HeLa cells ($\lambda_{ex}$ = 750 nm) feature bright fluorescence from the polymer-coated silica particles present in the cell. A comparison of the images in parts b and c of Figure 4 makes it clear that the 2PFM images provide considerably improved contrast and spatial resolution compared to those obtained by 1PFM; indeed, it is possible to detect the emission from individual $PPESO_3$-coated nanoparticles in the 2PFM images (Figure 4c). Inspection of the image reveals single particles as well as aggregates, and the particles appear to be preferentially localized in endosomes in the cytosolic region of the cell. As shown in the Supporting Information, a 3D reconstruction of the cell image is possible (Figure S-5). These imaging experiments demonstrate that the water-soluble CPE $PPESO_3$ can be imaged under two-photon excitation, either as a coating on nanoparticles (Figure 4c) or as the free polymer (Figure S-4 in the Supporting Information). The significance of this result is that it opens up the possibility of using water-soluble conjugated polymers as two-photon excited imaging agents and the possibility of applying their superior sensing properties (which arise from the amplified quenching effect) to intracellular sensing and imaging.

In summary, we have demonstrated that the anionic CPE $PPESO_3$ exhibits moderately strong 2PA in the near-infrared region, with an onset at ca. 800 nm. The absorption is shifted blue relative to the lowest allowed 1PA transition, indicating that 2PA excites the polymer into the second singlet state, which is forbidden in the 1PA spectrum. The photophysical and amplified quenching properties of the polymer are essentially identical under 1PA and 2PA, indicating that the same fluorescent state is populated via 2PA. We demonstrated that the polymer can serve as a two-photon sensitizer for a long-wavelength-emitting cyanine dye, as well as its application in cellular imaging using 2PFM. These studies provide the blueprint for the application of poly(phenyleneethynylene)-based CPEs as sensors, fluorescent imaging agents, and singlet oxygen sensitization with near-infrared excitation.

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**Supporting Information Available:** Methods on cell line, cytotoxicity assay, preparation of cells for imaging studies, SV plots, and a 3D image of HeLa cells reconstructed from 2PFM. This material is available free of charge via the Internet at http://pubs.acs.org.

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