Communication

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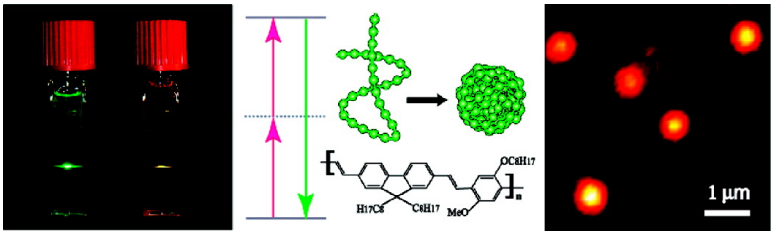

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Conjugated Polymer Dots for Multiphoton Fluorescence Imaging

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Multiphoton fluorescence microcopy has recently emerged as a powerful technique for three-dimensional imaging in biological systems.[1−4] The nonlinear dependence of excitation probability on light intensity results in a highly localized excitation and improved spatial resolution. The small effective excitation volume also reduces background signal due to autofluorescence and the fluorescence of dye molecules outside of the laser focal volume, while the ability to employ near-IR wavelengths for excitation can reduce photodamage to the sample as well as facilitate imaging of biological specimens due to the near-transparency of many tissues in this spectral range.[4] The widespread adoption of multiphoton fluorescence imaging and microscopy has been hindered by the bulky and expensive pulsed laser sources typically required for excitation due to the relatively low multiphoton excitation cross sections of available dyes.[5,6] Interest in the development of brighter probes has led to the design and synthesis of dyes with two-photon action cross sections larger than 1000 Göppert-Mayer units (GM).[7] Gold nanorods have also been demonstrated as contrast agent for in vitro and in vivo two-photon luminescence imaging, and the two-photon action cross sections were determined to be ∼2000 GM.[8] Colloidal CdSe quantum dots appear to be very promising probes for two-photon microscopy due to their excellent photostability and large two-photon action cross sections (from 2000 to 47 000 GM),[9] although single particle blinking and a significant fraction of "dark" nanoparticles are drawbacks for some applications.[10]

Conjugated polymers are known to possess high absorption coefficients and high fluorescence efficiency, which have led to a wide range of applications in optoelectronic thin film devices.[11,12] The extraordinary light-gathering power of conjugated polymers is evidenced by the first reported direct determination of the optical absorption cross section of a single molecule at room temperature, in which an optical cross section of the conjugated polymer MEH-PPV in the vicinity of 10^7 M^{-1} cm^{-1} was determined.[13] We have recently developed and characterized highly fluorescent nanoparticles consisting of one or more hydrophobic conjugated polymers.[14,15] These "CPdot" nanoparticles represent a new class of highly fluorescent probes with potential applications for biosensing and imaging. In this Communication, we determine the two-photon excited fluorescence properties of CPdots and explore their application for multiphoton fluorescence microscopy. Our measurements of the nonlinear optical properties of the CPdots indicate extraordinarily high two-photon action cross sections—in one case as high as 2.0×10^5 GM, the highest reported to date for a particle of comparable size. Detection of two-photon excited fluorescence of single nanoparticles was demonstrated using relatively low laser power, demonstrating the great potential of these CPdots for multiphoton fluorescence imaging applications.

The conjugated polymers employed in this study (Figure 1a) are the polyfluorene derivative poly(9,9-dihexylfluorenyl-2,7-diyl) (PDHF, average MW 55 000), the copolymer poly[{9,9-dioctyl-2,7-divinylenefluorenylene}-alt-*co*-{2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylene}] (PFPV, average MW 270 000), and the polyphenylenevinylene derivative poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene] (MEH-PPV, average MW 200 000). The polymers were purchased from ADS Dyes, Inc. and used without further purification. Stable, aqueous dispersions of CPdots were prepared using a reprecipitation method described previously.[14,15] The nanoparticle size can be controlled over the range of 5−50 nm by varying the concentration of the precursor solution. Unlike inorganic semiconductor nanoparticles, the particle size does not affect the shape of the absorption and fluorescence spectra of CPdots. Instead, an increase in particle size largely results in an increase in the optical cross section per particle.[14,15]

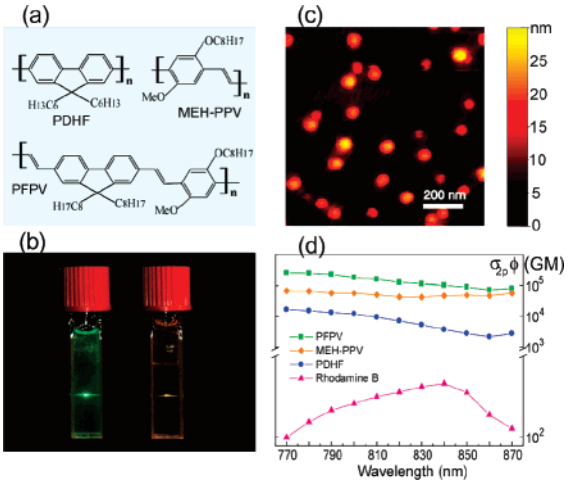

Figure 1. (a) Chemical structures of the conjugated polymers. (b) Photograph of the fluorescence from aqueous CPdot dispersions under two-photon excitation of an 800 nm mode-locked Ti:sapphire laser. (c) A typical AFM images of the PFPV dots on a silicon substrate. (d) Semilog plot of two-photon action cross sections ($\sigma_{2p}\phi$) versus the excitation wavelength for CPdots and rhodamine B (reference compound).

The CPdots exhibit one-photon absorption in the wavelength range of 350−500 nm. However, when the output of a mode-locked Ti:sapphire laser (100 mW, 800 nm, 100 fs) is focused into aqueous dispersions of PFPV and MEH-PPV dots (8 ppm, 3 nM), strong fluorescence in the vicinity of the focus is clearly visible (Figure 1b). Power-dependent excitation efficiencies provide further evidence for the two-photon excited fluorescence (data shown in Supporting Information). At a given laser intensity, I, two-photon fluorescence intensity is proportional to $\sigma_{2p}\phi I^2$, where σ_{2p} is the two-photon absorption cross section and ϕ is the fluorescence quantum yield. A convenient measure of the two-photon fluorescence brightness is the two-photon action cross section, $\sigma_{2p}^* = \sigma_{2p}\phi$.[4,9] A custom-built photon-counting spectrometer was used to determine the two-photon action cross sections as a function of excitation wavelength. The measurements were performed on CPdots of ∼20 nm diameter (Figure 1c), consisting of PDHF, PFPV, and MEH-PPV, respectively. The experimentally determined two-photon action cross sections (λ_{ex} = 770 nm) are 1.4×10^4, 5.5×10^4, and 2.0×10^5 GM for PDHF, MEH-PPV, and PFPV

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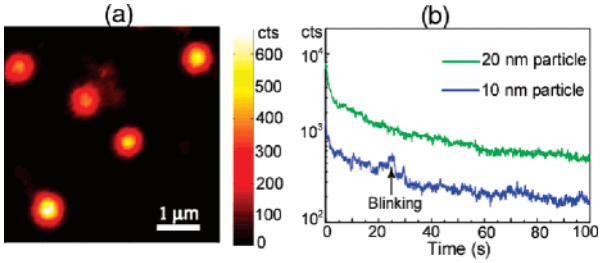

Figure 2. (a) A 5 μm × 5 μm fluorescence image of single PFPV dots immobilized on a glass coverslip obtained using two-photon excitation (800 nm). (b) Photobleaching kinetics of single PFPV dots under two-photon excitation. No obvious blinking was observed for ~20 nm PFPV dots, while it is often observed for smaller particles (<10 nm).

dots, respectively (Figure 1d). The two-photon action cross section for MEH-PPV dots is about 1 order of magnitude larger than that of the molecular solution,[16] consistent with the particle size results that indicate 10−20 molecules per particle. Significantly, the results show that, as two-photon fluorescent probes, the PFPV dots are 3−4 orders of magnitude brighter than conventional fluorescent dyes[5] and an order of magnitude brighter than quantum dots.[9] It is somewhat surprising that PFPV dots were determined to have the highest brightness of the three polymers, given that under one-photon excitation PDHF dots are brighter than both PFPV and MEH-PPV dots. However, the higher two-photon cross sections of PFPV and MEH-PPV are consistent with theoretical and experimental results indicating that π-conjugated systems with alternating donor-π-donor structures exhibit relatively large two-photon absorption cross sections.[7,17] The alkoxy side groups in PFPV and MEH-PPV act as electron donors, forming the donor-π-donor motif associated with relatively high two-photon cross sections, while PDHF does not possess alkoxy side groups.

To demonstrate the potential of the CPdots for multiphoton fluorescence imaging, single particles on a glass substrate were imaged using a custom-built confocal fluorescence microscope employing the attenuated output of a mode-locked Ti:sapphire laser (800 nm, 100 fs) for excitation. Figure 2a shows a 5 μm × 5 μm fluorescence image of the PFPV dots sparsely dispersed on a glass coverslip. Each bright spot in Figure 2a corresponds to a single PFPV dot. The high per-particle brightness is evident in the relatively low average laser power (260 μW, at the sample) employed to obtain high contrast images. Typically, pulsed laser light is required to generate sufficient two-photon fluorescence signal for single fluorophore detection. However, on the basis of these results, we estimate it should be possible to obtain two-photon fluorescence images of single CPdot nanoparticles using ~10 mW CW laser illumination provided by an inexpensive semiconductor diode laser. Indeed, focusing several tens of milliwatts of 800 nm CW laser light (*Ti:sapphire laser operating in CW mode*) onto a single layer of nanoparticles generated fluorescence that was readily visible to the unaided eye.

Single conjugated polymer molecules typically exhibit complex photophysics such as fluorescence intermittence (blinking) and photon antibunching.[18,19] Single particle fluorescence kinetics traces (Figure 2b) indicated no observable blinking for 20 nm PFPV dots, while it was often observed in smaller particles (<10 nm), consistent with a recent single particle results.[20] As fluorescent probes for imaging or single particle tracking, the relatively steady fluorescence of CPdots compares favorably to that of quantum dots, which typically exhibit pronounced blinking on time scales of milliseconds to hundreds of seconds.[10] Analyses of single particle kinetics traces indicate that approximately ~10^6 photons per particle (~10 nm diameter) were detected prior to photobleaching. This is lower than the photostability under one-photon excitation (~10^7 photons detected), consistent with prior observations that single fluorophores exhibit lower photostability under two-photon excitation than under one-photon excitation.[21] Silica encapsulation would likely improve photostability.[14]

In summary, we report on a new class of two-photon fluorescent nanoparticles, CPdots, which exhibit the largest two-photon action cross sections reported to date for particles of comparable size. Demonstration of single particle imaging using relatively low laser excitation levels demonstrates the potential utility of CPdots for multiphoton fluorescence microscopy applications and raises the possibility of employing small, inexpensive near-infrared diode lasers for two-photon excited fluorescence imaging.

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Supporting Information Available: Experimental details of CPdot preparation and characterization, measurements of two-photon action cross sections, single particle imaging, and complete author list of refs 7 and 17. This material is available free of charge via the Internet at http://pubs.acs.org.

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