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Pdots, a new type of nanoparticle, bind to *m*THPC via their lipid modified surface and exhibit very high FRET efficiency between the core and the sensitizer

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Pdots are a new type of nanoparticle which exhibit strong potential for future applications in biophysics and cell biology. They are composed of organic chromophoric polymers, whose surfaces can be modified with different amphiphilic polymers, such as PEGylated lipids to make them very stable as colloids in water. We demonstrate in this manuscript that the lipid nano-coating around the Pdot can bind very efficiently to amphiphilic molecules, such as photosensitizers *e.g.* *meso*-tetrahydroxyphenylchlorin (*m*THPC). As a result the sensitizer is brought into very close contact with the cores of the Pdots, and resonance energy transfer from the core to the sensitizer is very efficient; in some cases it is close to 1. We show the spectroscopic properties of two types of Pdots; their sizes, which are in the 13–47 nm range, depend on the kind of polymer and the length of the PEGylated lipid chains that wrap it. We measured the efficiency of FRET by investigating the decrease in donor intensity or its lifetime upon binding with *m*THPC. We also show the relative yields of singlet oxygen that are obtained via two pathways: by exciting the Pdots which transfer the energy to the attached sensitizer, or by exciting the sensitizer directly. This methodology could be used to enhance the use of a photosensitizer by employing both pathways in parallel.

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Introduction

Nanoparticles have attracted great interest due to their promising potential for applications in biological sciences and other biomedical research. Among the important nanostructures that have found use in biology and medicine one can list quantum dots (QDs),^{1,2} nanoparticles of gold, silicon, silica, metal oxides and several forms of carbon and the recently advanced semiconducting polymer dots (Pdots). Nanoparticles are used as drug-delivery vehicles, biosensors, imaging tools and markers, where their special chemical and optical properties are employed for analytical and diagnostic purposes.³

Quantum dots (QDs), especially, have the most wide-spread uses in scientific applications. They have narrow and size-dependent absorption and emission peaks with a considerable Stokes shift. Their absorption band is so wide that multiple excitation sources are not required for different QD types and sizes.^{4,5} They have thus

been extensively used as highly sensitive chemical and biological sensors and in live cell imaging.^{6–12} Semiconducting polymer dots (Pdots), which we describe here, on the other hand, are nanoparticles that are obtained by mixing high molecular weight chromophoric polymers with various amphiphilic molecules such as phospholipids.^{13,14} They share some properties with QDs, such as exhibiting a very strong absorption spectrum and strong brightness, narrow emission bands and symmetric emissions with improved brightness and photostability.^{15–21} They do not have size-dependent absorption and emission spectra.

Photodynamic therapy (PDT)^{22–25} is a relatively new selective method in cancer treatment. PDT is a promising approach for cancer treatment, where a photosensitizer dye, upon exposure to light of the required wavelength, generates the excited level of molecular oxygen, singlet oxygen (¹O₂), as well as other radical species. ¹O₂ is highly cytotoxic, causing oxidative destruction of the cells in which it has been generated and damaging the cell membrane and internal organelles. Illumination also elicits the dye's fluorescence, which is used to mark the borders of a tumor. Due to the preferential uptake of porphyrins by malignant tissue, the local illumination and the short life of the active species that are generated, no harmful effects are observed away from the tumor. PDT has been used for skin, lung, breast, bladder and other tumors, and has also been applied to eliminate viruses and bacteria.

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The attachment of photosensitizers to nanoparticles and their use as a combined system to carry out PDT have been proposed. The mode of attachment has spanned from covalent binding to a “loose” electrostatic binding. However, both methods were not pursued further and expanded.^{26,27} In this study, we propose a new approach to facilitate nanoparticle–sensitizer interaction by employing Pdots. The additional advantage of Pdots is that they are coated by an amphiphilic shell to make them water-miscible, and this coating can also be used as a nano-environment into which the amphiphilic or hydrophilic photosensitizer can intercalate non-covalently, but with a high binding constant. If the polymers of the Pdots, the donor, the sensitizer, and the acceptor, are chosen such that the donor's emission spectrum and the sensitizer's absorption spectrum exhibit good overlap, a fluorescence radiation energy transition (FRET)^{28,29} can occur. We therefore anticipate that since the absorption spectrum of a Pdot's material is broad, a large fraction of visible light will be absorbed by the Pdots, and will be channelled into the sensitizer *via* a FRET mechanism. The crucial point here is that one can gain from the Pdots' absorption of light by absorbing wavelengths shorter than those absorbed by the sensitizer, because the Pdots' band is so broad so that their overall optical cross-section is high. If the FRET efficiency is high, the overall funnelled energy to the sensitizer might compensate for the FRET efficiency. These considerations have to be taken into account when using the Pdot–sensitizer composite structure for a more efficient mechanism of biological sensitization.

Our aim in this paper is to demonstrate the spectroscopic properties of two new types of Pdots, with broad absorption in the visible range, up to the near infrared. We demonstrate their spectroscopic properties and also their efficient FRET interaction with the well-established photosensitizer *meso*-tetrahydroxyphenylchlorin (*m*THPC).

Experimental

Chemicals

Polymers poly[2-methoxy-5(2-ethylhexyloxy)-1,4-phenylene-vinylene] (ADS100RE, MEH-PPV; $M_w \geq 100\,000$), and poly[2-methoxy-5(2-ethylhexyloxy)-1,4-(1-cyanovinylphenylene)-*co*-{2,5-bis(*N,N'*-diphenylamino)-1,4-phenylene}] (ADS100RE, CN-PPV-DPD; M_w 15 000–50 000) were obtained from American Dye Source (Quebec, Canada). Their molecular structures can as be seen in Fig. 1.

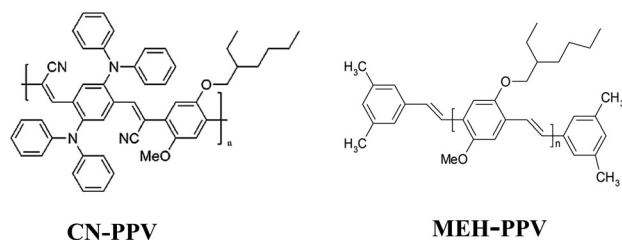


Fig. 1 Chemical structures of polymers: MEH-PPV and CN-PPV.

The following phospholipids were used for the amphiphilic shell: 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethyleneglycol)-350] (PEG₃₅₀-PE), which contains 7 PEG units and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy (polyethyleneglycol)-2000] (PEG₂₀₀₀-PE), containing 45 PEG units. They were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Additionally, we used natural egg-yolk lecithin, from Avanti Polar Lipids, in which the composition of fatty acids is as follows: 33% palmitic (C16:0), 31% oleic (C18:1), 13% stearic (C18:0), and 15% linoleic (C18:2). The remaining 8% is a mixture of several other fatty acids. *meso*-Tetrahydroxyphenylchlorin (*m*THPC) was prepared by one of us (MS), as described by Bonnett *et al.*³⁰

9,10-Dimethylantracene (DMA) was purchased from Sigma-Aldrich (St Louis, MO). Tetrahydrofuran was purchased from Bio-Lab (Jerusalem, Israel) and dichloromethane was purchased from Carlo Erba Reagents (Rodano, Italy); both were of analytical grade.

Preparation of Pdots

The Pdots were synthesized by two main routes: the miniemulsion and the reprecipitation method. The miniemulsion method uses ultrasonication to form stable miniemulsions containing small droplets of the polymer solution. While the reprecipitation method takes advantage of the conformational change brought about by the introduction of the hydrophobic polymer into an aqueous solvent.

Particle synthesis using the miniemulsion method: 2 mg of the conjugated polymer MEH-PPV was placed in 15 mL of dichloromethane (DCM) and was stirred for 48 hours to maximize its solubilization. The solution was filtered using a 0.2 μm membrane filter. Next, 4 or 7 mg of PEG₃₅₀-PE or PEG₂₀₀₀-PE, and 5.22 or 3.45 mg of lecithin were added, respectively, to maintain a constant PEGylated PE-to-lecithin ratio. The solution was mixed for 10 minutes and was then added to 20 mL of once-distilled water, under constant mixing. After sonication of the solution for 30 seconds using a Ti-probe sonicator (MSE Soniprep 150, Crawley, UK) it became homogeneous. The solution was paper-filtered immediately and centrifuged for 30 min at 2500 rpm. At this stage, the solution separated into two phases, an upper aqueous phase and a lower organic phase, which was discarded. To determine the Pdot concentration in the aqueous suspension, we measured the amount of substance that was lost throughout the preparation procedure at each step. The yields of the prepared Pdots were 76% for the PEG-PE₃₅₀ coating and 54% for the PEG₂₀₀₀-PE coating. The concentrations of the Pdots' dye material in the final aqueous stock suspensions were 1.52 and 1.09 mg mL^{-1} , respectively. The aqueous suspensions remained stable for several weeks.

Particle synthesis using the reprecipitation method was applied to prepare coated nanoparticles composed of the polymer MEH-PPV utilizing tetrahydrofuran (THF). The quantities of the materials were identical to those used in the previous preparation method. The preparation process until the sonication stage was identical to the preparation with DCM. Sonication of the solution was carried out for 30 seconds in an ultra-sonication bath (Elmasonic s 30, 37 kHz, Singen, Germany). After sonication the solution was flushed with nitrogen to remove the THF and

was centrifuged for 30 min at 2500 rpm. The yields of preparation were 42% for the PEG-PE₃₅₀ coating and 47% for the PEG₂₀₀₀-PE coating. The concentrations of the Pdots' dye material in the final aqueous suspensions were 1.15 and 1.06 mg mL⁻¹, respectively.

Nanoparticles were also prepared with the polymer CN-PPV with THF. The procedure was identical to the preparation with the MEH-PPV polymer. The yields of preparation were 94% for the PEG₃₅₀-PE coating and 93% for the PEG₂₀₀₀-PE coating. The concentrations of the Pdots' dye material in the final aqueous suspensions were 1.89 and 1.86 mg mL⁻¹, respectively.

Characterization of the Pdots: size and surface

Dynamic light scattering (DLS). Analyses were performed using a Cordouan Technology VASCO particle size analyzer (Nano Instruments Ltd, Pessac, France), with a laser wavelength of 658 nm. All data were obtained in a multi-acquisition mode, on 100 measurement runs with a time step of 50 s. In order to obtain good statistical information on the size dispersion of the samples, NanoQTM software of VASCO was operated in the multi-acquisition mode with each correlogram acquisition processed by the Padé–Laplace inversion algorithm.³¹

Atomic Force Microscopy (AFM). All scanning probe microscopy scans were performed using a MultiMode AFM with Nanoscope V electronics (Bruker AXS SAS, Santa Barbara, CA). The root-mean-square roughness (R_q) was calculated from $2 \times 2 \mu\text{m}^2$ micrographs. All samples were scanned with a tapping mode, using a FESP silicon probe (force constant 1–5 N m⁻¹, Bruker). The fast scan direction was perpendicular to the cantilever's long axis, and the images were captured in the retrace direction with a scan rate of 1 Hz at pixel resolution of 512 samples/line. Before analysis of the images, first order “flatten” and “plane fit” functions were applied to each image. The roughness was determined using Nanoscope analysis software.

High Resolution Scanning Electron Microscopy (HR-SEM). The surface morphology of the Pdots was assessed by HR-SEM (Magellan 400L, FEI) using an accelerating voltage of 5 kV with surface iridium coatings of approximately 30 nm thickness.

Spectroscopic measurements

Absorption spectra were registered on a Shimadzu (Kyoto, Japan) UV-2501PC UV-visible spectrophotometer. Fluorescence excitation and emission spectra, and fluorescence time-drive traces were measured using a Perkin-Elmer LS-50B digital fluorimeter (Norwalk, CT).

Singlet oxygen quantum yield

We prepared aqueous suspensions of Pdots at concentrations of 10 $\mu\text{g mL}^{-1}$ and 2 μM 9,10-dimethylantracene (DMA). DMA was used as a target for singlet oxygen, since it reacts selectively and efficiently with it to form the non-fluorescent 9,10-endoperoxide (DMAO₂). A diode-pumped solid state laser (Ningbo Lasever Inc., Ningbo, China) beam at 473 nm was chosen as its radiation wavelength was in an absorption band of the Pdots. In a similar way we choose a diode-pump solid state laser beam at 650 nm which is suitable for the absorption

of *m*THPC. A power meter (Ophir Nova, Jerusalem, Israel) was used to determine the power of the laser. A fluorescence time-drive experiment was run while irradiating the sample using the laser, and the decreasing intensity of DMA, due to its photo-oxidation, was monitored. The sample was stirred with a magnet during the time-drive run to ensure a proper homogeneous spread of light, DMA and the products of its decay throughout the cuvette.

Graphic and curve-fitting analyses

The rate of photon absorption by the sensitizer, k_{pho} , is given by eqn (1):

$$k_{\text{pho}} = \frac{0.98 \cdot P(1 - 10^{(-\text{abs} \cdot L)})}{E \cdot V} \quad (1)$$

where P is the power of the laser in mW, abs is the optical density of the sample at the irradiated wavelength, L is the path length traversed by the laser beam through the sample, E is the Einstein units of light energy per second per watt of light at the irradiating wavelength and V is the volume of the sample in the cuvette in mL. The factor 0.98 corrects for the light reflected at the air/liquid interface.

The fluorescence intensity decay of DMA was fitted to an exponential equation using Origin (Microcal Software, Northampton, MA) as described in eqn (2):

$$I_{\text{DMA}} = Ae^{-k_{\text{DMA}} \cdot t} \quad (2)$$

The quantum yields (QY) of the production of singlet oxygen are proportional to the rate of the photodestruction of DMA, k_{DMA} , normalized by light absorption, k_{pho} of eqn (1), as described in eqn (3):

$$\text{QY}_{\text{Pdot}/m\text{THPC}} \propto \left(\frac{k_{\text{DMA}}}{k_{\text{pho}}} \right)_{\text{Pdot}/m\text{THPC}} \quad (3)$$

Fluorescence quantum yields

To determine the fluorescence quantum efficiency of the Pdots, a comparison was performed with rhodamine 6G as a standard, whose quantum efficiency in ethanol is known to be 0.94.³²

Quantum efficiencies of the Pdots were estimated using eqn (4):

$$Q_{\text{PD}} = \frac{\text{OD}_{\text{ref}}}{\text{OD}_{\text{PD}}} \cdot \frac{I_{\text{PD}}}{I_{\text{ref}}} \cdot \frac{n_{\text{PD}}^2}{n_{\text{ref}}^2} \cdot Q_{\text{ref}} \quad (4)$$

where Q_{PD} and Q_{ref} are the quantum efficiencies of the fluorescence of the Pdots and rhodamine 6G respectively, I_{PD} and I_{ref} are their integrated fluorescence intensities, respectively, OD_{PD} and OD_{ref} are their absorption coefficients, and n_{PD} and n_{ref} are the refractive indices of the materials in water and ethanol, respectively.

Binding constants, K_b

The binding constant (K_b)³³ of *m*THPC to the Pdots was measured by monitoring the fluorescence intensity of *m*THPC, in aqueous solution, upon addition of increasing amounts of Pdots. The titration curve was used to monitor K_b . Following each added

batch, an overnight incubation period was found to be sufficient to achieve equilibrated binding.

FRET efficiency

We calculated the FRET efficiency from the various Pdots to *m*THPC bound to them, by two methods: measuring the change in fluorescence intensity of the donor, under stable conditions, or by measuring the donor's fluorescence lifetime, under similar conditions. Both are shown in eqn (5).

$$E = 1 - \frac{I_{DA}}{I_D} = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6} = 1 - \frac{\tau_{DA}}{\tau_D} \quad (5)$$

I_{DA} and I_D are the emission intensities of the donor molecules in the presence and absence of the acceptor molecule (or the donor's fluorescence lifetime with and without the acceptor, τ_{DA} and τ_D), respectively. r is the distance between the donor and acceptor pair and R_0 is the Förster distance, which is the distance between the donor and acceptor at which the energy transfer is (on average) 50% efficient. R_0 depends on J – representing the extent of overlap between the donor's fluorescence spectrum and the acceptor's absorption spectrum (eqn (7) below), κ^2 – the orientation factor, n – the refractive index of the medium, and on Q_0 – the fluorescence quantum yield of the donor in the absence of the acceptor, as described in eqn (6).³⁴

$$R_0^6 = 8.79 \times 10^{-5} \cdot \kappa^2 \cdot n^{-4} \cdot Q_0 \cdot J \quad (6)$$

$$J = \int f_D(\lambda) \cdot \epsilon_A(\lambda) \cdot \lambda^4 d\lambda \quad (7)$$

$f_D(\lambda)$ is the wavelength-dependent donor's normalized emission spectrum and $\epsilon_A(\lambda)$ is the extinction coefficient spectrum of the acceptor, in units $M^{-1} cm^{-1}$, and λ is the wavelength in nanometers. R_0 is expressed in this equation in Ångstroms.

Time-resolved fluorescence measurements

A home-built system at Bar Ilan University employs Time-Correlated Single Photon Counting. The excitation source has a femtosecond mode-locked Ti:sapphire laser (Chameleon Ultra II, Coherent, Santa Clara, CA). The laser pulse width was 140 fs before doubling. A pulse selector (A.P.E, Berlin) was used to reduce the basic 80 MHz pulse rate to 4 MHz.

The output frequency was multiplied by a flexible second- and third-harmonics generator (A.P.E., Berlin). The second harmonic was used for excitation at 480 nm. The emission was collected using a polarizer at a magic angle relative to the excitation polarizer at 590 nm. Measurements were taken at 25 °C. The emission wavelength was selected by a double 1/8 m subtractive monochromator, with an emission slit width of 32 nm (DIGIKROM CM112), and directed to the surface of a PMT (Hamamatsu, R9880U-210) biased at –1100 V. A single-photon counting board (SPC 630, Becker & Hickel GmbH) fed *via* a pre-amplifier (HFAC-26DB 0.1UA) and triggered by a photodiode (PHD-400N) was used. Life Time Analysis was done by the Marquardt nonlinear least-squares method.³⁵

Results

In this paper, we explored the spectroscopic, energy-transfer and structural properties of new nanoparticles, Pdots, as a tool in biophysical studies. Pdots have strong light absorption and we will show they also have a high efficiency for transferring energy to non-covalently attached molecules, such as sensitizers to generate singlet oxygen. This approach aims to use Pdots to increase the effectiveness of lipophilic molecules, such as sensitizers, that attach to the Pdots. The enhancement arises from the fact that the Pdots absorb light in a very broad spectral range and might transfer energy by FRET to the attached molecule, which absorbs light at a higher wavelength. Naturally, each excitation of the sensitizer *via* FRET will be somewhat less efficient than the direct excitation. However, the polymer can utilize a much broader wavelength range, exhibiting a high integrated absorption cross-section. Thus, the FRET route may add a lot to the photosensitization process because of the wide range of light wavelengths that could be used, even non-coherent white light.

Spectroscopic properties of the MEH-PPV Pdots

We coated the Pdots that were composed of MEH-PPV with 60% lecithin and 40% PEG-PE, to make them water-dispersible. This coating stabilizes the particles in water and forms the amphiphilic nano-environment into which the amphiphilic sensitizer intercalates.

We show a comparison of the behaviour of Pdots that were produced by using the reprecipitation method (with THF solution) and the miniemulsion method (with DCM solution). In addition, we examined the influence of the two types PEGylated lipid coatings, which differ in the PEG-PE chain length (350 and 2000) for each of the different preparation methods. The absorption and fluorescence spectra of the Pdots were prepared in the two different solvents, and with the two coatings are presented in Fig. 2. The absorption peak of the Pdots is at 500 nm while the central fluorescence peak is at 590 nm.

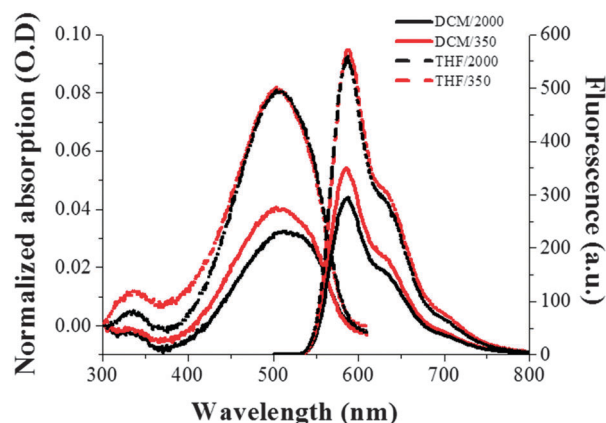


Fig. 2 The absorption and fluorescence spectra of Pdots prepared with two types of solvents and two lipid coating lengths: full line – preparation with DCM solution; dotted line – preparation with THF solution. Black line – 2000 long coating; red line – 350 long coating.

The figure demonstrates that the spectra of the Pdots with the different coatings are identical, thus, the type of coating does not change the spectroscopic qualities of the polymer. In addition, the figure illustrates that the spectra of Pdots obtained by both types of preparation (THF and DCM) are also identical. Therefore, the type of preparation does not affect the spectral qualities of the polymer either. In contrast, there is a difference in the fluorescence intensity of the Pdots of the two preparation methods: the Pdots prepared with the THF solution have higher fluorescence intensity than those prepared from the DCM solution. We assume that since THF is more polar than DCM, the contact area between the water phase and organic solvent during the preparation of the Pdots is different. These differences may affect the folding of the polymer within the globule. In addition, it is possible that preparing the Pdots in a sonication bath compared to much more powerful probe sonication influences the polymer in a different way because of the stronger heat effect and cavitation with probe sonication.

Additionally, we examined the fluorescence quantum yields of the Pdots, in comparison with rhodamine G6, whose fluorescence quantum yield in ethanol is 0.95. The results were the following: the calculated fluorescence quantum yields of Pdots prepared in DCM were 0.045 and 0.04 with lipid PEG-PE₃₅₀ and PEG-PE₂₀₀₀ coatings, respectively. In contrast, when the Pdots were prepared in the THF solution, the fluorescence quantum yields obtained were 0.164 and 0.225 for the same lipids, respectively. Thus, the type of preparation affects the quantum yield of Pdots. This result supports the hypothesis that the polymer MEH-PPV is better dissolved in THF solution than in DCM, which affects the production of the Pdots, and there is a possibility that the difference in the preparation methods of the Pdots influences the polymer in a different way. In contrast, the different lengths of the coating do not show a significant difference in results.

The size of the particles

In order to characterize the size of the Pdots we used AFM, which presents nanometric sizes according to the different type of preparation and coating. We discovered that when the Pdots were prepared from the THF solution, their sizes were ~10 and ~20 nm with coatings of PEG₃₅₀-PE chain lengths and PEG₂₀₀₀-PE chain lengths, respectively. In contrast, preparing the Pdots with DCM gave sizes of ~20 and ~40 nm for the same coatings, respectively. When we measured the surfaces of the Pdots by HR-SEM, we obtained sizes similar to those measured by AFM and the ratio between the Pdots with the different coating lengths was preserved. When we measured the diameter of the particles by means of DLS, we obtained an identical ratio between the Pdots with the different coating lengths, but the mean sizes were larger than those measured by AFM and HR-SEM. We attribute this to the inclusion of the hydrodynamic layer around the particles that moves with them and not just the diameter of the core of the particles. The mean results obtained by the various measurement techniques are shown in Table 1.

Fig. 3 presents a qualitative comparison of the Pdots with different coating lengths prepared from the THF solution.

Table 1 Mean size distributions of the Pdots from different preparation methods and coating lengths, as measured by AFM, HR-SEM and DLS

	Reprecipitation method		Miniemulsion method	
	PEG ₃₅₀ -PE in nm	PEG ₂₀₀₀ -PE in nm	PEG ₃₅₀ -PE in nm	PEG ₂₀₀₀ -PE in nm
AFM	25	13	41	17
HR-SEM	26	14	42	18
DLS	31	23	48	27

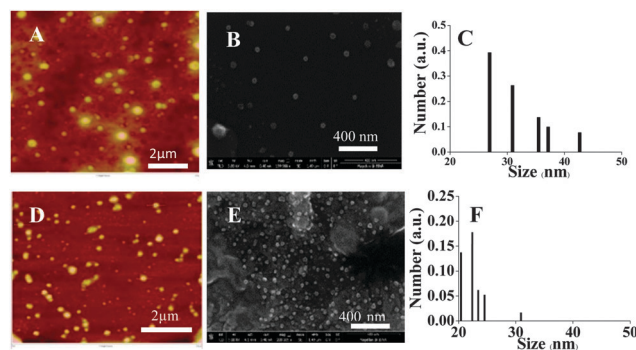


Fig. 3 The sizes of Pdots prepared from THF solution. (A) and (D) are AFM images of the Pdots with PEG₂₀₀₀-PE and PEG₃₅₀-PE coatings, respectively. (B) and (E) are HR-SEM images of Pdots with PEG₂₀₀₀-PE and PEG₃₅₀-PE, respectively. (C) and (F) show the results of the mean sizes of Pdots with PEG₂₀₀₀-PE and PEG₃₅₀-PE lipid coatings, respectively, as measured by DLS.

Illustrations (A–C) represent Pdots coated by PEG₂₀₀₀-PE and illustrations [D–F] represent Pdots with a PEG₃₅₀-PE coating.

Energy transfer between Pdots and *m*THPC – FRET efficiency

In this section we show the efficiency of energy transfer between the Pdots and the sensitizer which is attached to them. We linked the Pdots with a known photosensitizer, *m*THPC, which is commonly used in biological photosensitization and is known for its high efficiency for the production of singlet oxygen.^{26,36} The central absorption peak of this photosensitizer is at 430 nm and an additional peak is at 650 nm, while the fluorescence peak is at 653 nm. Therefore, the fluorescence peak of the Pdots overlaps nicely with the absorption band of *m*THPC and they are suitable to be used as a donor-acceptor pair and the efficiency of the energy transfer between them can be calculated.

To get the most efficient energy transfer, we first measured the *m*THPC binding constant, K_b , to Pdots, by titrating the *m*THPC with the Pdots and tracing the fluorescence intensity. These results are shown in Table 2. The intuitive way to

Table 2 The *m*THPC binding constant, K_b , to the Pdots made by various methods

K_b of <i>m</i> THPC to Pdots composed of:	Method	
	Reprecipitation method ($\mu\text{g mL}^{-1}$) ⁻¹	Miniemulsion method ($\mu\text{g mL}^{-1}$) ⁻¹
PEG ₃₅₀ -PE	0.585	0.0876
PEG ₂₀₀₀ -PE	0.469	0.07

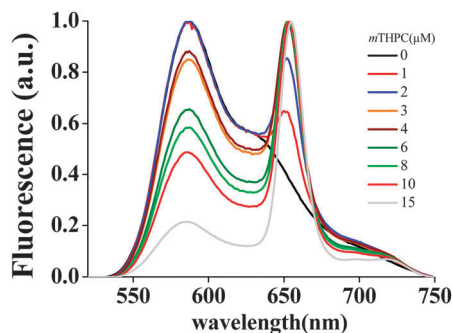


Fig. 4 Fluorescence of $10 \mu\text{g mL}^{-1}$ MEH-PPV-PEG₃₅₀-PE Pdots with 0–15 μM *m*THPC (λ_{exc} = 485 nm).

understand K_b is that at a Pdot concentration of $1/K_b$, half of the *m*THPC is bound.

Table 2 shows the binding constant of *m*THPC to Pdots at extremely low concentrations of Pdot. The low binding constants are expected considering the high hydrophobicity of *m*THPC.

In order to see the effect of energy transfer between the Pdots and *m*THPC, we excited the Pdots at 485 nm, a wavelength at which *m*THPC has very low absorption and the absorption is mostly due to the Pdots. We have also found this wavelength to show the highest ratio of Pdot-to-*m*THPC absorption. The samples contained Pdots at fixed concentrations of $10 \mu\text{g mL}^{-1}$ when the reprecipitation method was used and $60 \mu\text{g mL}^{-1}$ when the miniemulsion method was used, accordingly with the K_b ratio. We then added to the samples an increasing concentration of *m*THPC (0–15 $\mu\text{g mL}^{-1}$). Fig. 4 shows, as a comparative example, the energy transfer process between Pdots, prepared by the miniemulsion method with PEG₃₅₀-PE coating, and *m*THPC. The intercalation process of *m*THPC to the coating of the Pdots is slow and reaches equilibrium after up to a day, which is the time it took to achieve stable fluorescence intensity of *m*THPC, therefore we had to wait until the Pdot-*m*THPC system had equilibrated. Fig. 4 demonstrates that the fluorescence intensity of the Pdots decreases as the concentration of *m*THPC in the solution increases and demonstrates FRET-driven emission.

In order to assess, quantitatively, the results of the FRET process between the Pdots and *m*THPC, we calculated the efficiency of the energy transfer between them. This efficiency is based on calculation of the relative area of the decreasing fluorescence curve as a result of adding *m*THPC to the solution. Table 3 presents the results of the energy transfer, calculated using eqn (5). The table demonstrates that the type of Pdot preparation does not significantly affect the efficiency of the energy transfer, when Pdots with identical coatings are compared. However, when the same types of Pdot with different coating lengths are compared, a clear trend is observed: a longer coating induces more efficient FRET, until they coincide. We assume that due to the length of the PEGylated lipid coating, a more “dense” nanosized bundle of lipid and PEG chains is formed on the surface of the nanoparticle. This coating, which is much longer for the PEG₂₀₀₀-PE than for PEG₃₅₀-PE, affects the efficiency of FRET.

Table 3 The mean efficiency of the energy transfer between Pdots with lipid coatings of PEG₂₀₀₀-PE and PEG₃₅₀-PE ($10 \mu\text{g mL}^{-1}$ of reprecipitation method Pdots and $60 \mu\text{g mL}^{-1}$ of miniemulsion method Pdots) and an increasing concentration of *m*THPC (0–15 $\mu\text{g mL}^{-1}$)

<i>m</i> THPC (μM)	Miniemulsion method		Reprecipitation method	
	PEG ₂₀₀₀ -PE	PEG ₃₅₀ -PE	PEG ₂₀₀₀ -PE	PEG ₃₅₀ -PE
0	0	0	0	0
1	0.27	0.25	0.27	0.25
2	0.31	0.30	0.34	0.32
3	0.39	0.32	0.42	0.34
4	0.49	0.37	0.56	0.41
6	0.58	0.39	0.62	0.53
8	0.61	0.48	0.71	0.58
10	0.67	0.59	0.72	0.68
15	0.81	0.79	0.85	0.80

In addition to calculating the FRET, we calculated the Förster radius: the distance at which the FRET efficiency is 50%. The results when the Pdots were prepared with THF were 39.9 Å and 42.8 Å for the PEG₃₅₀-PE coating and the PEG₂₀₀-PE coating, respectively. In contrast, when the Pdots were prepared with DCM, the Förster radius was 33.0 Å and 32.3 Å for the two coatings, respectively. Overall, both preparation methods and both PEG-lipid coatings generate Pdots that, when binding *m*THPC, exhibit very efficient FRET. The Förster radius increases when using Pdots prepared with THF, indicating a more efficient FRET. This result is derived from the difference in the fluorescence quantum yields between the various solvents. In contrast, the length of the coating has no significant effect.

In this case we could not employ measurements of the lifetime of the Pdots as a tool to evaluate the FRET efficiency, using eqn (5), because the decay rate of the Pdots was very fast and the instrument does not measure lifetimes less than 1 ns with good accuracy.

Singlet oxygen quantum yield

The production of singlet oxygen is the final aim and indication of success of the overall process of the transduction steps of energy, by direct excitation of the sensitizer or *via* FRET from the Pdot donors, and can constitute a measure for the FRET process. Measurement of the singlet oxygen quantum yield was done by means of singlet oxygen quencher 9,10-dimethylantracene (DMA). DMA is known for its very efficient chemical reaction with singlet oxygen so it serves as a quantitative singlet oxygen trap. DMA is fluorescent, and when it reacts with singlet oxygen, it forms a non-fluorescent peroxide.³⁷ We followed the fluorescence intensity of DMA by exciting it at 360 nm, the probing wavelength, and the fluorescence intensity was measured at 430 nm, the major fluorescence peak of DMA. The decay of the DMA was examined in a solution with Pdots alone. This solution was illuminated with a laser diode at a wavelength of 473 nm. In addition, we examined two solutions that contained the pair *m*THPC-Pdots, with one solution illuminated with a laser diode at a wavelength of 473 nm in order to measure the singlet oxygen quantum yield due to the energy transfer between the Pdots and the *m*THPC. The second solution was illuminated with a laser diode at a wavelength of 650 nm, a wavelength that matches

Table 4 The relative singlet oxygen production by the pair *m*THPC–Pdts over Pdts alone when excited at a wavelength of 473 nm (first line) compared to the production of singlet oxygen by the pair *m*THPC–Pdts, when excited at a wavelength of 473 nm, over *m*THPC–Pdts excited at a wavelength of 650 nm (second line), with the various coating lengths

	Miniemulsion method		Reprecipitation method	
	PEG ₃₅₀ -PE	PEG ₂₀₀₀ -PE	PEG ₃₅₀ -PE	PEG ₂₀₀₀ -PE
$QY_{\text{Pdts}+m\text{THPC}}/QY_{\text{Pdts}}$ (EX: 473 nm)	3.26 ± 0.13	6.41 ± 0.27	3.61 ± 0.13	6.27 ± 0.23
$QY_{\text{Pdts}+m\text{THPC}}$ (EX: 473 nm)/ $QY_{\text{Pdts}+m\text{THPC}}$ (EX: 650 nm)	0.33 ± 0.01	0.59 ± 0.026	0.61 ± 0.04	0.79 ± 0.05

the absorption peak of the *m*THPC, in order to measure the singlet oxygen quantum yield by direct excitation of *m*THPC.

The relative yield of singlet oxygen production and the error in the different types of preparation and lengths of coating were calculated using eqn (1)–(3), as shown in Table 4.

Table 4 demonstrates that when the *m*THPC–Pdts composite structures were excited at 473 nm, where the Pdts absorb, there was a stronger generation of singlet oxygen compared to Pdts alone (first line in the table). This means, surprisingly, that the Pdts themselves generate singlet oxygen, though the generation of singlet oxygen is more productive when FRET occurs. In contrast, when the production of singlet oxygen is caused by the *m*THPC–Pdts composite structure, which is excited at a wavelength that matches the excitation of the Pdts, 473 nm, and the energy is transduced by FRET, it is less efficient than *m*THPC in the composite structure, which is excited directly (second line). The much higher concentration of Pdts than *m*THPC caused almost complete binding and therefore the yield of $^1\text{O}_2$ in Table 4 is independent of the extent of binding. The two values of relative $^1\text{O}_2$ production change between 0.61 and 0.79, which reflects the FRET efficiencies, as all of the numbers are normalized.

In addition, Pdts that were coated with longer PEG–lipids had a stronger photosensitizing effect than with the short PEG–lipid chains. This difference occurs in both types of preparation. We attribute this difference to the fact that the longer coating chains form a denser and thicker nanosized bundle around the inner core of the polymeric chromophore. This form creates a larger volume into which singlet oxygen can diffuse before escaping into the outside aqueous phase, and thus there is a better chance of meeting the target DMA molecule and reacting with it. We have observed this behaviour abundantly with photosensitizers in liposomal membranes.³⁸

These results show a difference between the two types of preparation of Pdts. The solvent in which the polymer is dissolved before creating the Pdts affects its folding in the Pdts core and also the fluorescent quantum efficiency of the polymer, as discussed above. This effect, as shown, influences the other parameters, such as singlet oxygen production, FRET efficiency, *etc.*

As we have mentioned above, the advantage of using the pair *m*THPC–Pdts in the dyad is the bigger light collection efficiency of the organic polymer in the Pdts, due to its broad and strong absorption spectrum. As a result, even if the FRET efficiency to the *m*THPC is slightly less than 1, the overall use of the wide light bandwidth of the Pdts, in parallel to direct light absorption by the *m*THPC brings about a more efficient

generation of singlet oxygen by the dyad than by the *m*THPC alone. At the concentrations that we used, namely $10 \mu\text{g mL}^{-1}$ MEH-PPV and $3 \mu\text{M}$ *m*THPC, at which the FRET efficiency was $\sim 39\%$ (Table 3), the Pdts constituted 68% of the integrated absorption cross section in the whole region between 300 and 700 nm and *m*THPC contributed only 32%. This result proves the advantage in employing FRET in order to collect light energy using Pdts.

Spectroscopic properties of the CN-PPV Pdts

We produced Pdts from the polymer CN-PPV, shown in Fig. 1. This polymer emits in the deeper red wavelength range, a region that is currently missing in this class of chromophores. The deeper red emission also enables better overlap with longer-wavelength absorbing photosensitizers. This polymer was dissolved only in the THF solution, due to the results obtained with the polymer MEH-PPV, which were better with the reprecipitation method (THF solution) than the miniemulsion method (DCM solution). The CN-PPV Pdts were coated with 60% lecithin and 40% PEG-PE for water stabilization. At this stage, we examined the influence of the same two types of coatings, which differ in the length of the PEG-PE chain.

The absorption, excitation and fluorescence spectra of the Pdts with the different coatings are presented in Fig. 5. The absorption peaks of the Pdts are at 390 nm while the central fluorescence peak is at 624 nm. This was observed more clearly when the fluorescence excitation spectrum was taken to eliminate the scattering. The figure demonstrates that the spectra of the Pdts with the two types of coatings are identical, thus, the type of coating does not change the spectroscopic qualities of the polymer.

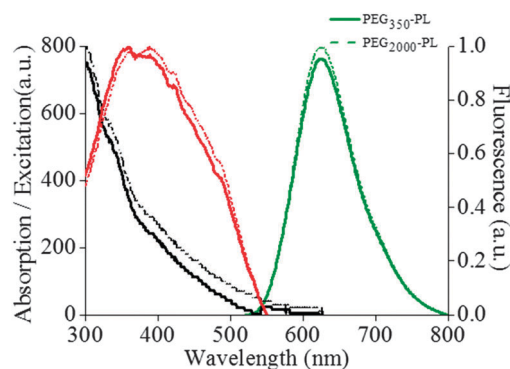


Fig. 5 Black line – absorption, red line – excitation and green line – fluorescence spectra of CN-PPV-Pdts prepared with two lipid coating lengths: dotted line – PEG₂₀₀₀-PE; solid line – PEG₃₅₀-PE.

We measured the fluorescence quantum yield of these Pdts by comparison with rhodamine G6. The results were that the quantum yields were not influenced by the length of the Pdts' coating chain and were 0.27 and 0.23 for the PEG₃₅₀-PE and PEG₂₀₀₀-PE coating, respectively. Thus, the length of the coating lipid does not result in a significant difference, just as we observed with the MEH-PPV Pdts.

The size of the particles

To characterize the size of the Pdts, we used AFM, which presented a small difference between the two types of Pdts coated with lipid chains of lengths 350 and 2000; the results were ~15 nm and ~20 nm, respectively. The mean was taken by counting 100 particles. In order to characterize the morphology of the Pdts, we used HR-SEM and obtained practically identical sizes to those obtained by AFM. As with the MEH-PPV Pdts, the sizes measured by means of the DLS were larger, as a consequence of inclusion of the hydrodynamic layer. Table 5 presents the mean size distribution of the Pdts measured using various instruments.

Fig. 6 presents a qualitative comparison of the Pdts with different coating lengths. The comparison was made by means of AFM, HR-SEM and DLS instruments. Illustrations [A–C] represent Pdts coated with PEG₃₅₀-PE and illustrations [D–F] represent Pdts coated with PEG₂₀₀₀-PE.

Energy transfer between Pdts and *m*THPC – FRET efficiency

As with the polymer MEH-PPV, we linked the Pdts composed of CN-PPV to the *m*THPC. To get the most efficient energy transfer, we first measured the *m*THPC binding constants, K_b , to Pdts. The K_b of *m*THPC to Pdts composed of PEG₂₀₀₀-PE

was 0.661 ($\mu\text{g mL}^{-1}$)⁻¹ and for PEG₃₅₀-PE was 0.399 ($\mu\text{g mL}^{-1}$)⁻¹. Since the polymer CN-PPV has measurable intrinsic fluorescence, we could measure its efficiency as a donor to *m*THPC. Fig. 7 demonstrates the lifetime of the Pdts alone and the Pdts in the presence of *m*THPC. The DO line represents the experiment with the donor-only and the DA curve represents the experiment with the donor-acceptor. Analysis of the fluorescence time-resolved FRET experiments is based on curve fitting methods. We obtained the average fluorescence lifetime of Pdts with increasing concentrations of *m*THPC. The average lifetime of the samples was determined from multiexponential analysis of the emission decay as described in eqn (8):

$$\langle\tau\rangle = \sum_i a_i \tau_i \quad (8)$$

where τ_i and a_i are the lifetime and the relative amplitude of the *i*th decay component respectively. The quality of the fitted curves to the experimental data is judged by the minimization of the χ^2 values. The strong decay rate of the DA curve shown in Fig. 7 is due to the FRET effect.

Another measurement that points to the FRET efficiency is the decreasing fluorescence intensity of the Pdts. In order to quantitatively evaluate the FRET process between the Pdts and *m*THPC, we excited the Pdts at a wavelength of 485 nm. The sample contained Pdts, which served as a donor, at a fixed concentration of 10 $\mu\text{g mL}^{-1}$; to this we added increasing concentrations of *m*THPC, which served as the acceptor (0–15 $\mu\text{g mL}^{-1}$). In this case as well, the linking of *m*THPC to the coating of the Pdts needed long equilibration (up to a day). Table 6 presents the mean results of the energy transfer efficiency between the Pdts and *m*THPC: by measuring the donor's decreasing lifetime or by its decreasing fluorescence intensity (see eqn (5)). The table demonstrates that the type of coating affects the efficiency of the transfer and that better efficiency of the energy transfer occurs with a coating of 2000 in length. This supports the postulation that we raised earlier: the coating with longer lipids “helps” bring the *m*THPC molecule closer to the polymer. It can also be seen that because of a better overlap between the donor's emission, which occurs at longer wavelengths, and the acceptor's absorption spectra, FRET efficiency in this case reaches almost unity.

We calculated the Förster radii (using eqn (6)) and found them to be 46.7 Å and 44.4 Å for Pdts with the PEG₃₅₀-PE and PEG₂₀₀₀-PE coatings, respectively.

Singlet oxygen quantum yield

We measured the production of singlet oxygen, the important parameter when Pdts are considered for photosensitization by a FRET mechanism. The process of singlet oxygen generation by illumination the Pdts followed by FRET to *m*THPC is demonstrated in Fig. 8.

At this stage too, the decay of the singlet oxygen target, DMA, was examined under several conditions: a sample containing Pdts alone illuminated by a laser diode at a wavelength of 473 nm; a sample containing the pair *m*THPC–Pdts in which singlet oxygen was generated *via* FRET after illuminating the Pdts solely; and one in which singlet oxygen was produced by

Table 5 Mean size distribution of the Pdts with different lengths of the lipid coating as determined by different techniques

	PEG ₂₀₀₀ -PE in nm	PEG ₃₅₀ -PE in nm
AFM	20	15
HR-SEM	20	16
DLS	27	22

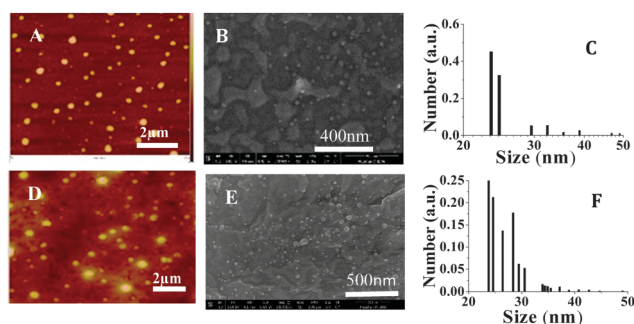
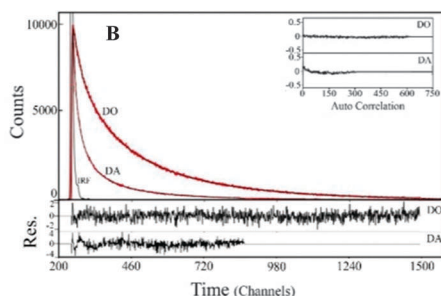


Fig. 6 The sizes of the Pdts. (A) and (D) are AFM images of the Pdts with PEG₃₅₀-PE and PEG₂₀₀₀-PE coatings, respectively. (B) and (E) are HR-SEM images of Pdts with PEG₃₅₀-PE and PEG₂₀₀₀-PE coatings, respectively. (C) and (F) show the results of the mean size of Pdts with PEG₃₅₀-PE and PEG₂₀₀₀-PE lipid coatings, respectively, as measured by DLS.

A CN-PPV (PEG ₃₅₀ -PE)					
<i>m</i> THPC (μM)	τ ₁ (a ₁)	τ ₂ (a ₂)	τ ₃ (a ₃)	< τ > (ns)	χ ²
0	14.92 (0.23)	4.70 (0.22)	0.73 (0.22)	6.87	1.1
1	12.93 (0.18)	3.51 (0.24)	0.56 (0.29)	4.73	1.11
2	12.71 (0.16)	3.59 (0.24)	0.57 (0.32)	4.27	1.25
3	12.29 (0.15)	3.16 (0.24)	0.51 (0.34)	3.79	1.16
4	11.46 (0.12)	2.07 (0.23)	0.41 (0.44)	2.75	1.31
5	9.83 (0.08)	2.09 (0.23)	0.33 (0.56)	1.7	1.29
6	9.31 (0.09)	1.87 (0.24)	0.29 (0.61)	1.52	1.37
7	8.99 (0.07)	1.68 (0.21)	0.25 (0.70)	1.18	1.42



C CN-PPV (PEG ₂₀₀₀ -PE)					
<i>m</i> THPC (μM)	τ ₁ (a ₁)	τ ₂ (a ₂)	τ ₃ (a ₃)	< τ > (ns)	χ ²
0	14.3 (0.21)	3.99 (0.21)	0.58 (0.31)	5.49	1.09
2	12.65 (0.17)	3.58 (0.21)	0.50 (0.36)	4.18	1.05
3	11.8 (0.14)	3.07 (0.20)	0.44 (0.43)	3.21	1.12
5	9.88 (0.09)	2.65 (0.21)	0.43 (0.43)	2.27	1.23
6	9.38 (0.10)	2.56 (0.23)	0.39 (0.51)	2.1	1.28

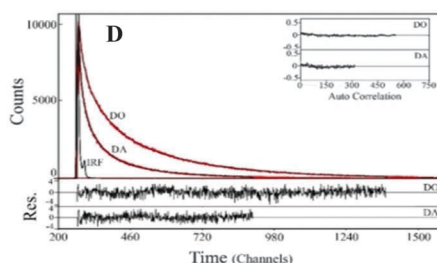


Fig. 7 Fluorescence decay parameters for CN-PPV Pdts with increasing concentrations of *m*THPC. The excitation was measured at 480 nm, where the Pdts's intrinsic chromophore absorbs almost exclusively and the emission was measured at 600 nm. (τ) is the average life-time of the Pdts. (A) and (C) demonstrate the mean lifetime of the donor, with PEG₃₅₀-PE and PEG₂₀₀₀-PE coatings, respectively and the result of χ² tests. (B) and (D) demonstrate the fluorescence decay curves of the Pdts. The residuals (bottom) and the corresponding autocorrelation functions (upper right) served as indicators for the quality of the fit of the experimental data to a multiexponential function.

direct illumination of *m*THPC. The relative yield of singlet oxygen production and the error in the different types of

Table 6 The efficiency of energy transfer between the Pdts with different coating lengths at a fixed concentration of Pdts (10 μg mL⁻¹) with increasing concentrations of *m*THPC (0–15 μg mL⁻¹) determined either by measuring the donor's fluorescence intensity or by the fluorescence lifetime. The data are averages of results obtained from 3 independent experiments

<i>m</i> THPC (μM)	PEG ₂₀₀₀ -PE		PEG ₃₅₀ -PE	
	By donor's fluorescence intensity	By donor's fluorescence lifetime	By donor's fluorescence intensity	By donor's fluorescence lifetime
0	0	0	0	0
1	0.19	0.31	0.12	0.2
2	0.3	0.37	0.19	0.23
3	0.38	0.44	0.26	0.41
4	0.45	0.59	0.42	0.5
6	0.67	0.77	0.66	0.78
8	0.82	0.9	0.76	—
10	0.91	—	0.9	—
15	0.98	—	0.97	—

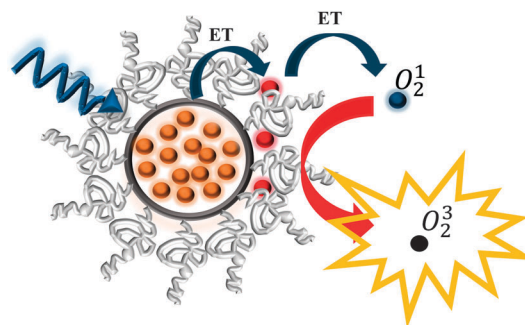


Fig. 8 Pdts nanoparticle enhance the singlet oxygen production by FRET to the photosensitizer.

Table 7 The relative singlet oxygen production by the pair *m*THPC–Pdts over Pdts alone when excited at a wavelength of 473 nm (first line) as compared to the production of singlet oxygen by the pair *m*THPC–Pdts, when excited at a wavelength of 473 nm, over the pair *m*THPC–Pdts excited at a wavelength of 650 nm (second line) for different coating lengths

	PEG ₃₅₀ -PE in nm	PEG ₂₀₀₀ -PE in nm
QY _{Pdts+mTHPC} /QY _{Pdts} (EX: 473 nm)	2.38 ± 0.08	3.07 ± 0.1
QY _{Pdts+mTHPC} (EX: 473 nm)/QY _{Pdts+mTHPC} (EX: 650 nm)	0.92 ± 0.05	0.99 ± 0.05

preparation method and length of coating were calculated by eqn (1)–(3), as shown in Table 7.

The first line of Table 7 demonstrates that the production of singlet oxygen by illumination of *m*THPC is 2–3 times more efficient than that achieved by illuminating CN-PPV Pdts with PEG₃₅₀-PE and PEG₂₀₀₀-PE coatings, and relying on FRET to transduce the energy to *m*THPC, respectively. Still, these CN-PPV Pdts turn out to have some efficiency in producing singlet oxygen.

However, more importantly, when we compare the efficiency of singlet oxygen generation by illuminating the Pdts followed by FRET to *m*THPC, relative to direct illumination of *m*THPC, the two yields are practically identical (see the second line of

the table, in which absorbance and laser powers are all normalized). This proves that the efficiency of the resonance energy transfer step is extremely high at almost one and both pathways, by FRET or direct excitation of the sensitizer, are equally efficient.

However, it should be taken into consideration that the indirect way leading to the production of singlet oxygen *via* FRET from the Pdots might practically be more important than by direct excitation of the photosensitizer. The reason for this is that the cross-section for absorption of light by the photosensitizer is small because it is limited to a small region of spectral light; the sensitizer absorbs in a small range of long wavelengths. However, the chromophore that constitutes the polymeric semiconductor has a very broad absorption spectrum, spreading over more than 150 nm. Therefore, the integrated cross-section for its absorption of light within that range is much higher. This is the reason for the high brightness of the Pdots and their absorbance, as well as the highly efficient FRET to the sensitizer bound to them.

Conclusions

Pdots are known as nanoparticles composed of partially-conductive polymers. By mixing them with PEGylated phospholipids they can become water miscible and form stable colloids. After their preparation, the Pdots are stable for months and present very good absorption and fluorescence. In this article, we present two types of Pdots, prepared from different polymers: MEH-PPV and CN-PPV. We coated them with a mixture of lipids which endows them with this miscibility, but it also generates a lipid layer into which amphiphilic molecules can intercalate. In this paper we demonstrate that the photosensitizer *m*THPC binds very efficiently to this lipid coating of the Pdots. The good binding and overlap between the emission spectrum of the Pdots and the absorption spectrum of the *m*THPC increase the efficient energy transition from the Pdots to the photosensitizer. We demonstrated that as a result of the energy transfer, *m*THPC serves as a sensitizer which is excited directly, and both indirectly, by FRET from the Pdots.

We also showed that there are detailed differences in the Pdot properties according to the modes of preparation. These express themselves in the fluorescence intensity, in the binding constant and in the size of the Pdots. Using the reprecipitation method yielded better results in these respects. This indicates that the initial solvent in which the polymer is prepared does influence the formation of the Pdots and their characteristics, and may even be trapped in the organic polymer's core. The ability to find the solvent in which the polymer will "feel" best is key to efficiently creating Pdots.

A comparison between the Pdots prepared from the different polymers revealed differences in size and generation of singlet oxygen. The generation of singlet oxygen by the Pdots prepared from CN-PPV was greater than the generation of singlet oxygen by the Pdots prepared from MEH-PPV. Additionally, singlet oxygen generated by the transfer of energy between the Pdots

and the photosensitizer was practically identical to the singlet oxygen created by the photosensitizer itself. This confirms the usage of the composite structure (*m*THPC-Pdots) efficiency with the wide spectral absorption of the Pdots and the maximal efficiency of the generation of singlet oxygen.

We also showed the effect of the length of the lipid chains that were used to wrap the organic core. The best results were obtained with the PEG₂₀₀₀-PE coating. The difference in size can be explained by the spatial arrangement of the coating. The PEGylated phospholipid which composes the coating arranges itself in the shape of a corm, so the longer the chain length of the coating, the larger the corm and the larger the Pdots. In contrast, when the coating is smaller, the corm created is smaller so the volume obtained is smaller. The differences in the efficiency of energy transfer and generation of singlet oxygen can be explained by the fact that the entanglement created by the length of the coating "helps" the photosensitizer molecule, *m*THPC, to get closer to the core of the polymer. This process causes a more efficient FRET and a greater generation of singlet oxygen.

This article demonstrates that it would be worthwhile to examine the use of Pdots in the field of biology. Future studies could examine whether the Pdots can improve the photodynamic treatment by serving as antennas for a wide range of wavelengths and not only in the red absorption range that exists today.

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