Supporting information

PPV-Based Conjugated Polymer Nanoparticles as a Versatile Bioimaging Probe: A Closer Look at the Inherent Optical Properties and Nanoparticle−Cell Interactions

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EXPERIMENTAL SECTION

Characterization Methods

The molecular weight distribution of MDMO-PPV and CPM-PPV-*co*-MDMO-PPV were measured via a Tosoh EcoSEC (Tessenderlo, Belgium) system HLC-8320GPC, comprising an autosampler, PSS guard column SDV of 50 × 7.5 mm, followed by 3 PSS SDV analytical linear XL columns of 300 × 7.5 mm (5µm) at 40 °C (column molecular weight range is $1 \times 10^2 - 1 \times 10^6$ g·mol^{-1}) and a differential refractive index detector, Tosoh EcoSEC RI, using THF as eluent with a flow rate of 1 mL·min^{-1}. The used flow marker was toluene and calibration was performed using linear narrow polystyrene standards of $470 - 7.5 \times 10^6$ g·mol^{-1} obtained from PSS Laboratories (Valkenburg, The Netherlands) (MHKS for precursor ($\alpha = 0.67605$ and $k = 0.000142$ ml·g^{-1}) as well as conjugated MDMO-PPV ($\alpha = 0.809$ and $k = 0.00002$ ml·g^{-1}) were applied for molecular weight analysis). To determine the copolymer composition of CPM-PPV-*co*-MDMO-PPV, ^{1}H NMR spectra were recorded in $CDCl_3$ on a Varian Inova 300 spectrometer (Chemnitz, Germany) (300 MHz and 75MHz respectively, 5 mm probe). ATR−IR spectra were collected with a Bruker (Brussels, Belgium) Tensor 27 FT−IR spectrophotometer.

The study of both size and morphology of the conjugated nanoparticles was performed with TEM on a Tecnai G2 spirit twin, FEI (Zaventem, Belgium), at an accelerating voltage of 120 keV. TEM images were processed with ImageJ software. The respective samples were drop casted and dried on copper grids. In parallel, the hydrodynamic diameter and polydispersity as well as zeta-potential were determined with dynamic light scattering using ZetaPALS equipment, Brookhaven Instrument Cooperation (Waddinxveen, The Netherlands). The solid content of all samples was determined by thermogravimetric analysis.

The optical fingerprint of both nanoparticles and molecularly dissolved polymers as well as the absorption integrity over a time period of 12 months was measured in 1 cm quartz cuvettes using the Cary 5000 UV−vis−NIR spectrophotometer, Agilent Technologies (Diegem, Belgium), and the Jobin Yvon Fluorolog3 Tau fluorescence spectrophotometer, Horiba (Lier, Belgium). The emission spectra were corrected for the wavelength dependence of the throughput and sensitivity of the detection channel. The absorption extinction coefficient of the materials was calculated using the Beer-Lambert law by varying the concentration of nanoparticles in water or polymer in $CHCl_3$. The resulting extinction coefficients could be used to determine the absorption cross-section. The polymers and nanoparticles' quantum yield (QY) was measured using rhodamine 6G (in H_2O, QY = 0.95) as a fluorescence reference. Five dilutions were prepared for all samples (MDMO-PPV molecularly dissolved in $CHCl_3$, MDMO-PPV NPs in H_2O, CPM-PPV-*co*-MDMO-PPV molecularly dissolved in $CHCl_3$ and CPM-PPV-*co*-MDMO-PPV NPs in H_2O), as well as for the standard. The most concentrated one had an absorbance of 0.1 at 488 nm excitation for the NPs and 500 nm for the molecularly dissolved polymers, which were optically matched to the standard. Emission spectra were collected for all samples at their respective excitation wavelengths after which the absorption versus the integral of emission spectra for each dilution and sample were plotted and trend lines fitted. The resulting slope values (m) as well as the refractive indexes (η) of the liquids were used to determine the quantum yield of the sample according to the following formula.

$$QY_{SAMPLE} = QY_{STANDARD} \times \frac{m_{SAMPLE}}{m_{STANDARD}} \times \frac{\eta_{SAMPLE^2}}{\eta_{STANDARD^2}}$$

The nanoparticle and reference dye lifetime was measured through fluorescence lifetime imaging microscopy (FLIM) using a commercial Zeiss LSM 510 META NLO scan head, Zeiss, mounted on an inverted laser scanning microscope Axiovert 200M equipped with a LD C-Apochromat 40×/1.1 W Korr UV−vis−IR water immersion objective. Excitation was performed with a femtosecond pulsed titanium-sapphire MaiTai DeepSee laser, Spectra-Physics (Utrecht, The Netherlands), with an output wavelength of 970 nm (~5 mW average radiant power at the sample position). The emission light was detected using non-descanned detection: the fluorescence was directed using a dichroic mirror NFT 490 and the 480−520 nm band pass filter towards a photomultiplier tube (PMT), Hamamatsu 7422 (Mont-Saint-Guibert, Belgium). This PMT was connected to an SPC830 card, Becker and Hickl (Berlin, Germany), synchronized by the scan pulses from the confocal laser scanning microscope. The instrument response function was recorded using potassium dihydrogen phosphate micro-crystals, background subtracted and used in the analysis of the FLIM data. The data were obtained and exponentially fitted using SPCImage 2.9 data analysis software, Beckr and Hickl. An average lifetime of five independent measurements was taken. The same equipment was employed together with the Zeiss excitation fingerprinting macro window software for determining the two-photon excitation spectrum of both MDMO-PPV and CPM-PPV-*co*-MDMO-PPV NPs air-dried on microscope slides. Images were processed using AIM 4.2 software. The photobleaching kinetics were determined for both nanoparticle samples as well as reference dyes, with a fixed absorbance of 0.1 at their excitation λ_{max} (**Table S*1***). The fluorescence intensity signal was collected at their respective emission λ_{max} on regular time intervals (10 s) for a time period of 3600 s with a constant excitation power at their excitation λ_{max} using the conventional fluorescence spectrophotometer set-up. The effect of oxidative stress on conjugated nanoparticles was evaluated by incubating the sample with 0.1 wt % of H_2O_2 and measuring the change in absorbance.

Table S1. Excitation and Emission Maxima of Nanoparticle Samples and Reference Dyes in H_2O.

sample	$\lambda_{excitation\ (nm)}$	$\lambda_{emission\ (nm)}$
MDMO-PPV NPs	495	590
CPM-PPV-*co*-MDMO-PPV NPs	500	592
rhodamine B	554	573
rhodamine 6G	527	550
FITC	491	516

Cell Culture

HeLa cells were cultured using culture medium (Table S2) in a T25 flask, stored at 37 °C in a 5% CO_2 incubator and spliced after reaching 80% confluence.

Table S2. Cell Lines and Respective Culture Solutions.

cell line	medium	supplements
HeLa	DMEM	1% P/S
		10% FCS

Cytotoxicity Assays

MTT assay. HeLa cells were seeded at a density of 10000 cells per well, together with 100 µL of culture solution, in a 96-well flat-bottom plate. After 24 h of incubation at 37 °C in a 5% CO_2 incubator, the cells were washed with 1×PBS. A total of six wells per condition were taken and 100 µL of the reagents representing different required conditions were added to the respective wells: blank, 100% cell death (1:9.1 dilution in IMDM culture solution of a 340 mM solution of SDS in ultrapure water), 100 µg/mL of NPs in IMDM culture solution

(IMDM medium, 10% FCS, 1% P/S), 50 μg/mL, 10 μg/mL and 5 μg/mL. After the incubation period of 24 h the cells were washed three times with 1×PBS. The MTT solution (10 μL MTT + 100 μL IMDM culture solution) was added to the wells and incubated for 4 h at 37 °C, 5% CO_2, after which 100 μL of SDS solution (1 g SDS in 10 mL of 0.01 M HCl) was added to each well and the plate was put back in the incubator for another 18 h. The solution was transferred to a dark plate and the absorbance was detected at λ_{ex} = 570 nm with a FLUOstar OPTIMA plate reader, BMG LABTECH. The experiment was performed in triplicate.

Confocal Laser Scanning Microscopy Imaging

HeLa cells were seeded at 15000 cells per well in a μ-Slide 8 well plate, Ibidi, and left to incubate for 24 h in a 5% CO_2 incubator at 37 °C. The cells were washed with 1×PBS and incubated with 200 μL of 50 μg/mL of NPs in IMDM culture medium for another 24 h. As negative control IMDM culture medium was added. The cells were rinsed three times with 1×PBS at 37 °C. Next, 200 μL of fixation/extraction/permeabilization buffer (4 v/v % PFA supplemented with 0.3 v/v % triton x-100 and 5.0 w/v % sucrose) was added for 2 h at RT on a shaker at 50 rpm. The cells were washed three times with washing buffer (1×PBS containing 0.1% triton x-100) after which 200 μL of blocking buffer (1×PBS containing 0.1% triton x-100 and 2% Bovine Serum Albumine) was added and the cells were put on a shaker for 1 h at 50 rpm on RT. Next the cells were incubated with 200 μL of primary antibody (mouse monoclonal anti-α-tubulin, 1:1000 in blocking buffer) for 1 h at RT on 50 rpm. The cells were rinsed three times using washing buffer for 5 min at 50 rpm. Next, the cells were incubated for 1 h with 200 μL of secondary Ab (donkey antimouse Alexa Fluor 488, 1:250 in blocking buffer) at 50 rpm, RT. The cells were washed with washing buffer for 5 min at 50 rpm and incubated with 200 μL of DAPI (1:25 in blocking buffer) for 1 h at RT. The cells were rinsed three times with washing buffer for 5 min at 50 rpm and covered with 2 drops of

mounting medium to prevent photobleaching of the dyes. The cells were visualized at RT with the Zeiss LSM510 META NLO mounted on an inverted laser scanning microscope (Zeiss Axiovert 200 M) and a 40×/1.1 water immersion objective. Excitation of the nanoparticles and tubulin was done at 543 nm and 488 nm respectively (3 µW maximum radiant power at the sample) with an Argon-ion laser. Excitation of DAPI was done at 730 nm (~5 mW average radiant power at the sample position) with the femtosecond pulsed titanium-sapphire MaiTai DeepSee laser. Emission was detected using band-pass filters 565−615 (NPs), 390−465 nm (DAPI) and 500−550 nm (tubulin). The resulting 1024×1024 images with a pixel size of 0.06 µm were recorded using a pixel dwell time of 375 µs. A fixed pinhole size of 240 µm (tubulin), 600 µm (NPs) and 1000 µm (DAPI) was used. Images were processed using AIM 4.2 and ImageJ software.

RESULTS

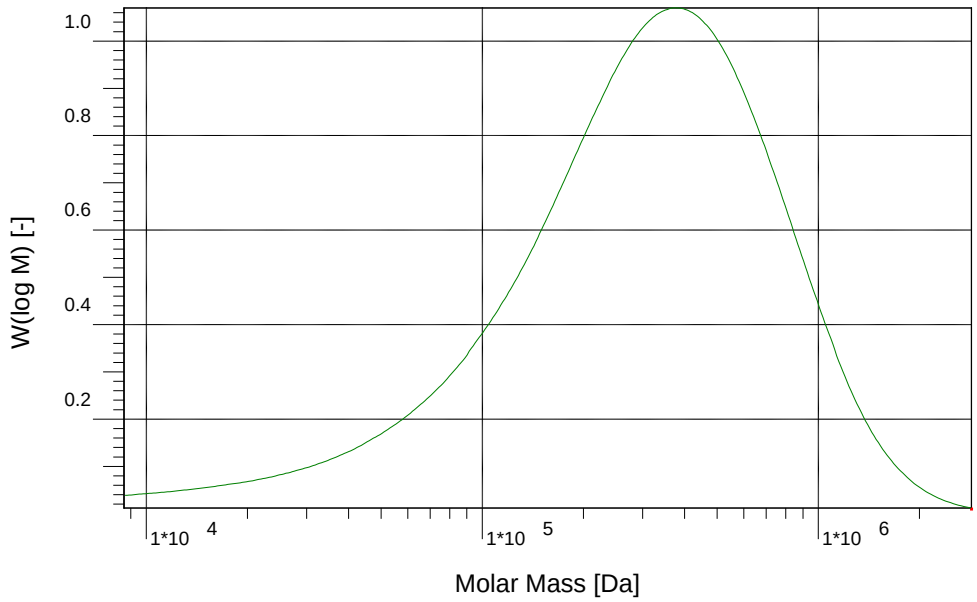

Figure S1. Molecular weight distribution of synthesized MDMO-PPV.

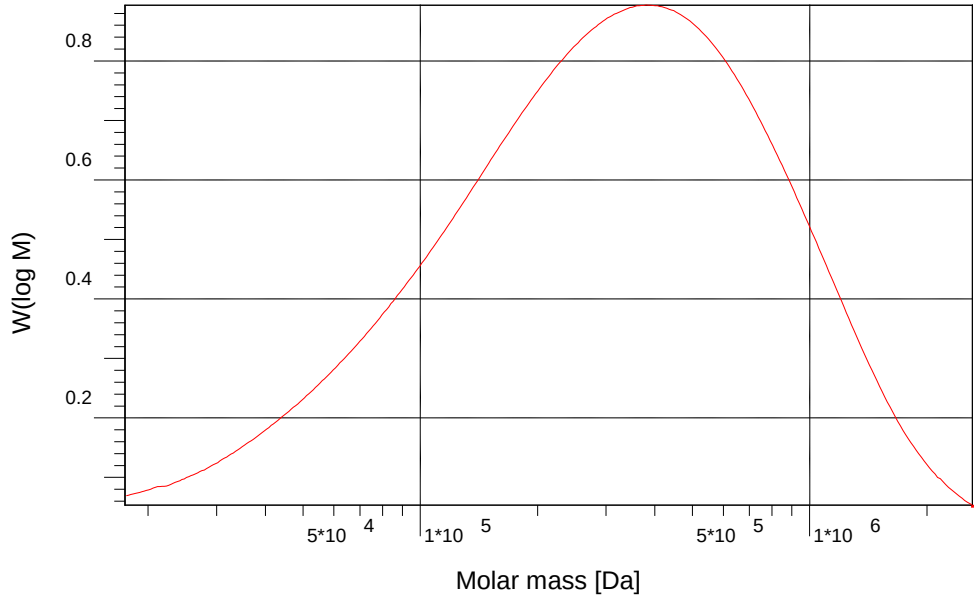

Figure S2. Molecular weight distribution of synthesized CPM-MDMO-PPV.

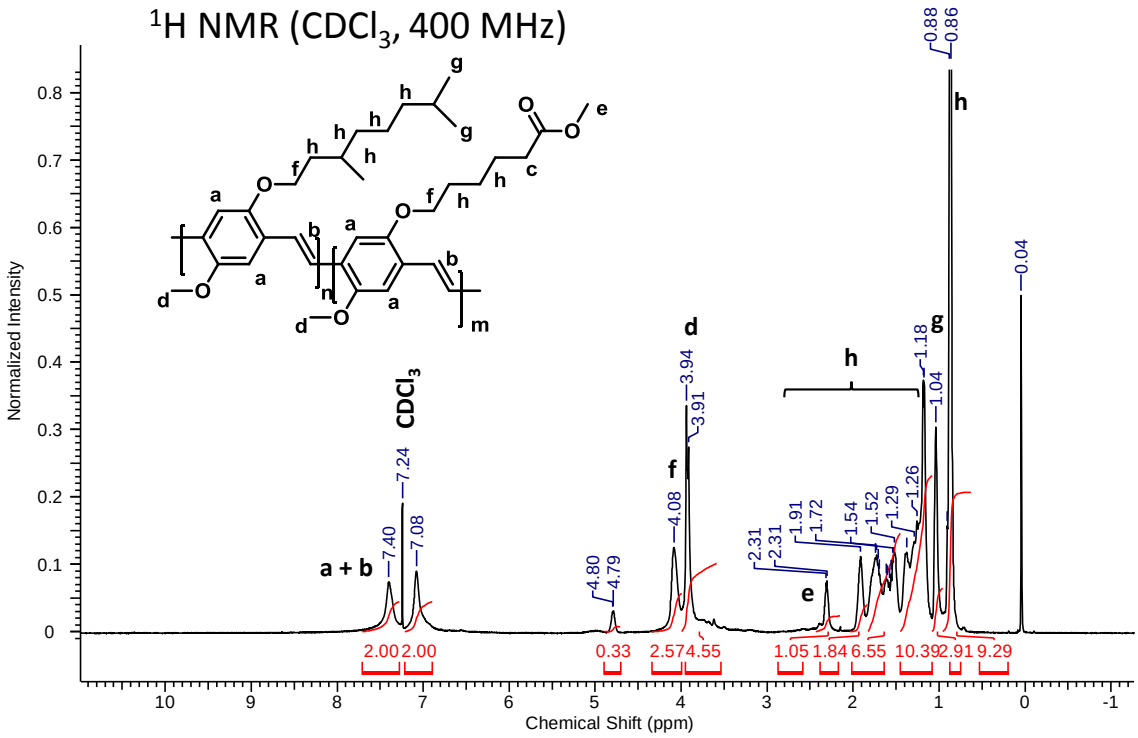

Figure S3. ^{1}H-NMR spectrum of CPM-MDMO-PPV.

^{1}H NMR ($CDCl_3$): δ = 1.91−0.86 (m, 30H); 2.31 (t, 2H); 3.94 (s, 5H); 4.09 (s, 3H); 7.08 (m, 2H); 7.40 (m, 2H).

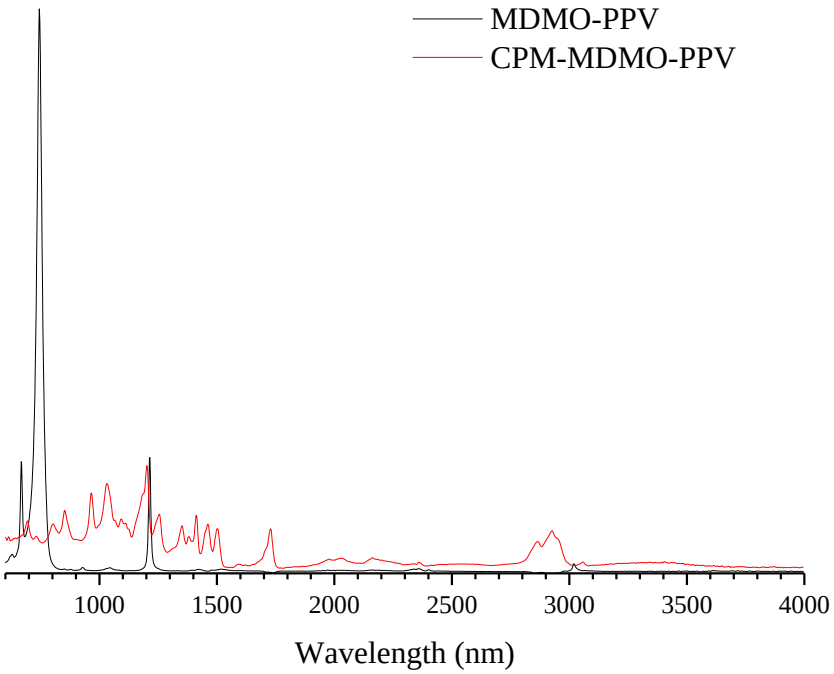

Figure S4. Infrared spectra of MDMO-PPV (black) and CPM-MDMO-PPV (red).

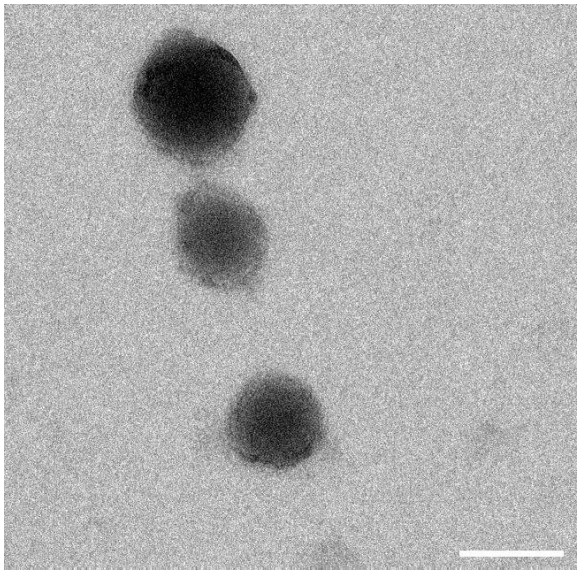

Figure S5. TEM image of MDMO-PPV NPs. Scale bar = 50 nm.

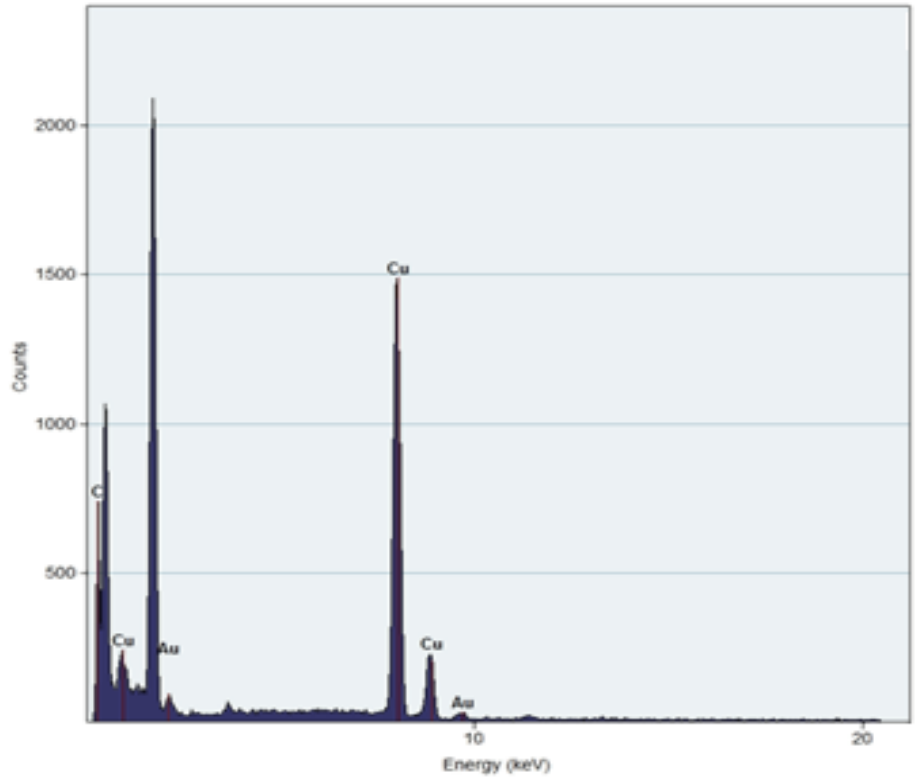

Figure S6. EDX spectrum of CPM-MDMO-PPV NPs functionalized with gold Ab.

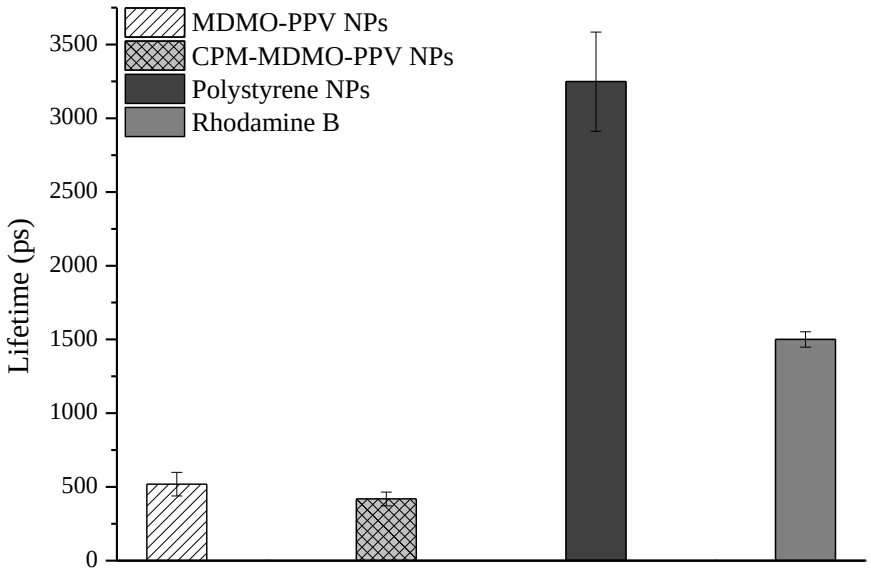

Figure S7. Lifetime measurement of MDMO-PPV and CPM-MDMO-PPV NPs in comparison to reference samples polystyrene NPs and rhodamine B.

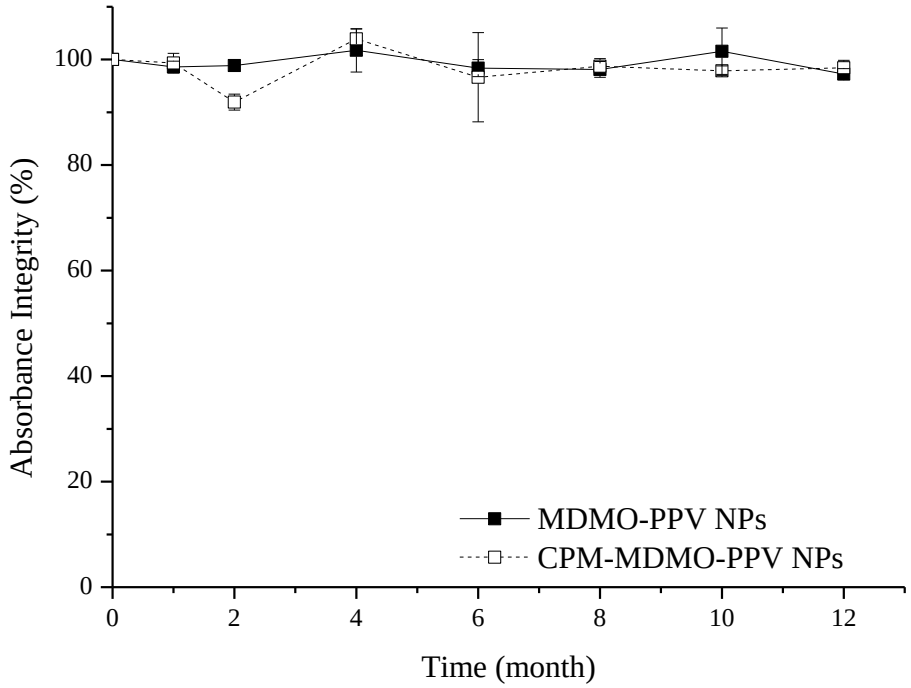

Figure S8. Absorbance integrity measurement of MDMO-PPV and CPM-MDMO-PPV NPs over a time period of 12 months as compared to a reference sample measured after synthesis/washing.

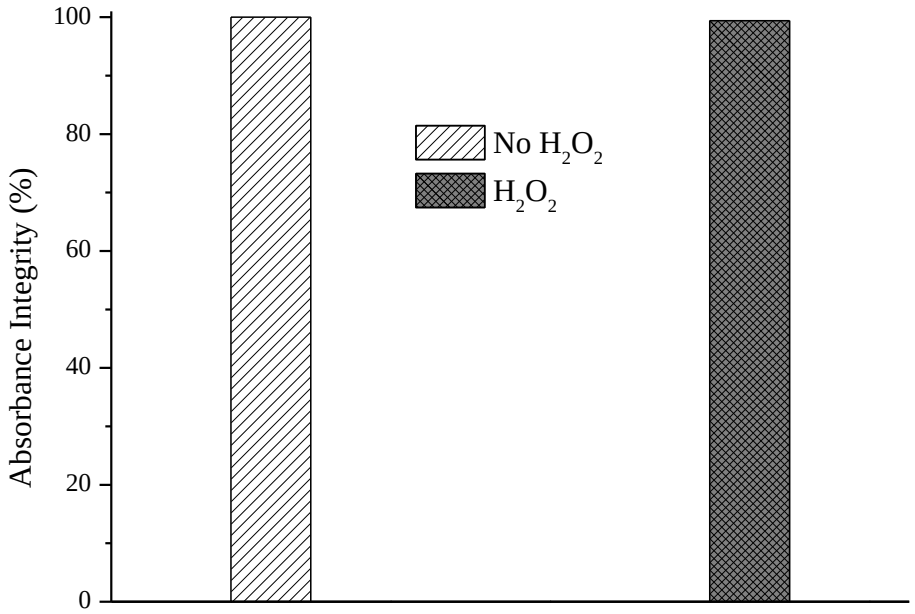

Figure S9. Absorbance integrity measurement of MDMO-PPV NPs after 24 h of exposure to a 0.1 wt % H_2O_2 solution as compared to a reference sample.

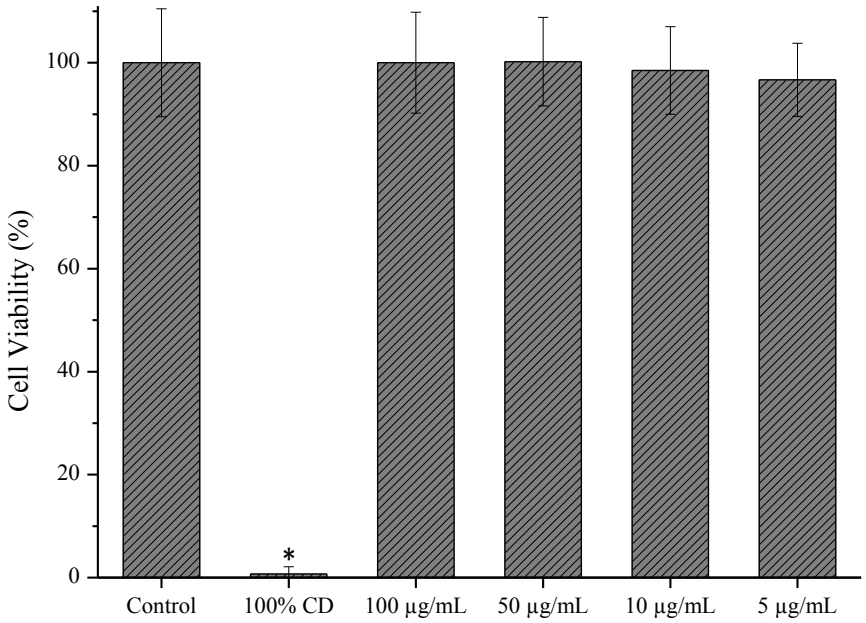

Figure S10. Dose-dependent cytotoxicity of MDMO-PPV NPs after 24h of exposure determined by the MTT assay in HeLa cells. Data are expressed as percent of control mean ± SD (N = 3).

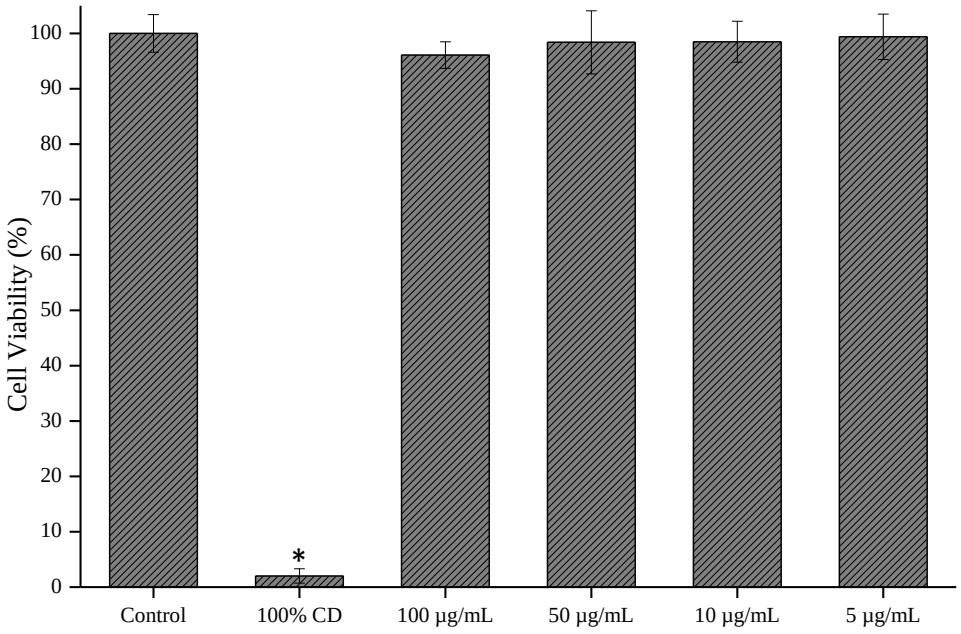

Figure S11. Dose-dependent cytotoxicity of MDMO-PPV NPs after 24h of exposure determined by the Alamar blue assay in HeLa cells. Data are expressed as percent of control mean ± SD (N = 3).

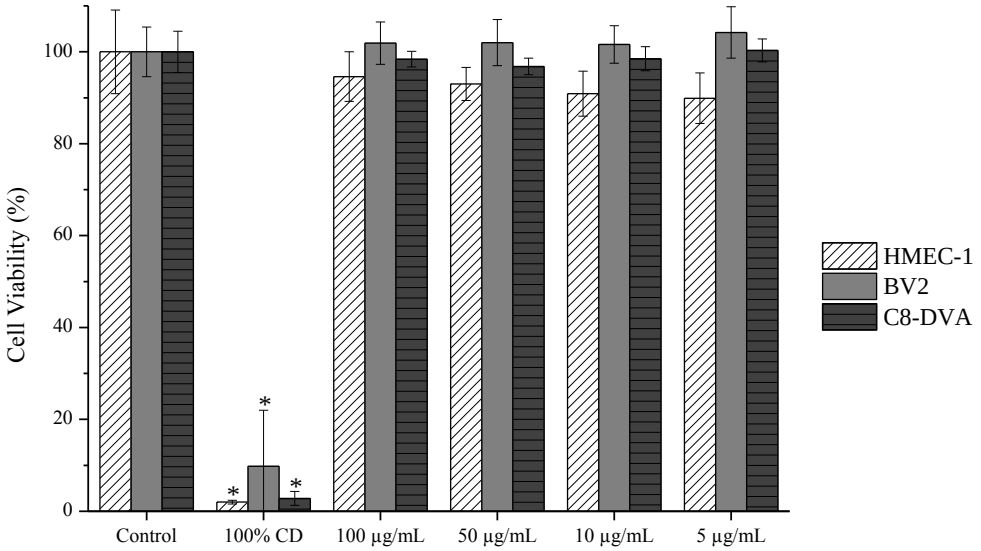

Figure S12. Dose-dependent cytotoxicity of CPM-MDMO-PPV NPs after 24h of exposure determined by the Alamar blue assay in HMEC-1, BV-2 and C8-D1A cells. Data are expressed as percent of control mean ± SD (N = 3).

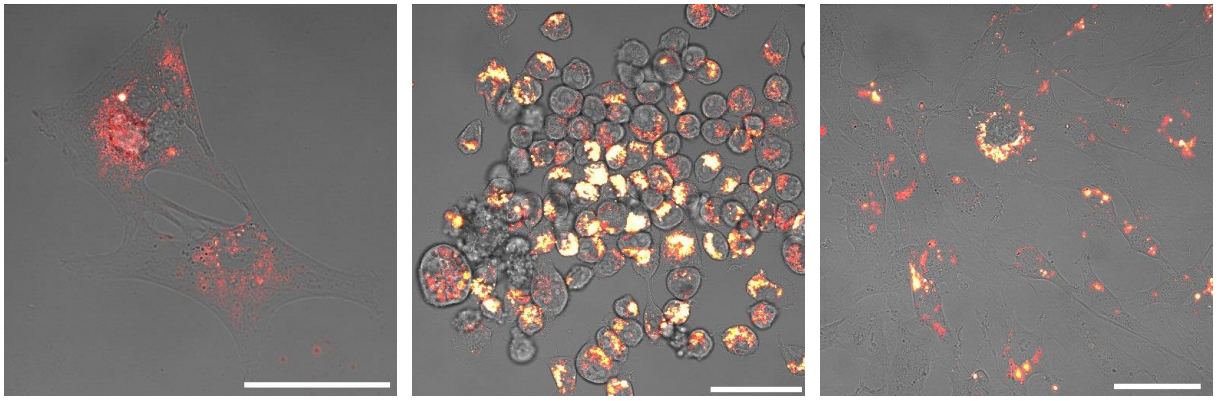

Figure S13. Confocal microscope image of C8-D1A (*left*), BV-2 cells (*middle*) and HMEC-1 cells (*right*) treated with CPM-MDMO-PPV-based NPS (*red*) for a time period of 20h. Scale bars = 50 µm.

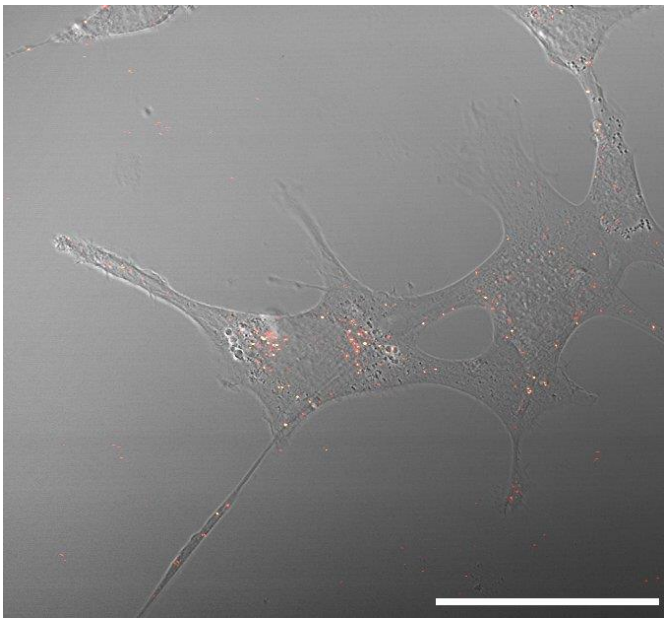

Figure S14 Confocal microscopy image of MDMO-PPV NPs (*red*) taken up by C8-D1A cells after 24 h of incubation. Scale bar = 50 μm.

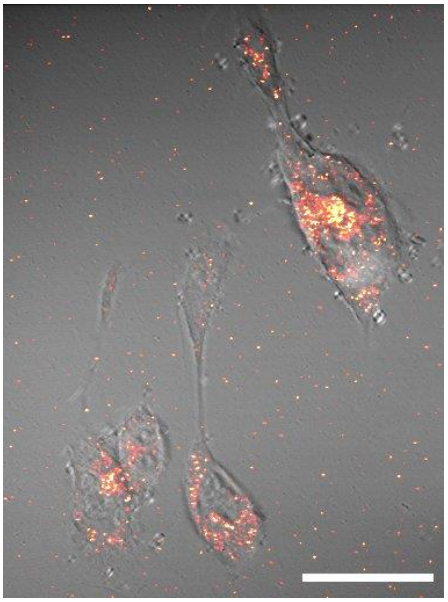

Figure S15. Confocal microscopy image of MDMO-PPV NPs (*red*) taken up by BV2 cells after 24 h of incubation. Scale bar = 25 μm.

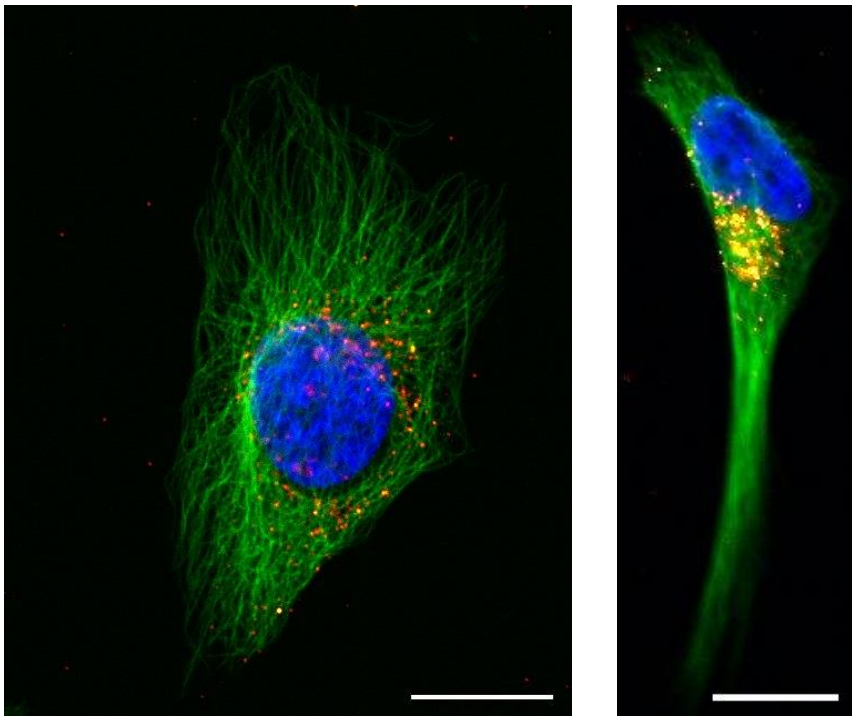

Figure S16. Confocal microscopy images of MDMO-PPV NPs (*red*) taken up by HeLa cells after 24 h of incubation. Additional staining of cell nucleus (*blue*) and tubulin (*green*). Scale bars = 15 µm.