Supporting Information

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Different PEG-PLGA Matrices Influence In Vivo Optical/Photoacoustic Imaging Performance and Biodistribution of NIR-Emitting π-Conjugated Polymer Contrast Agents

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Materials and Methods

Reagents, chemicals and kits were obtained from Sigma-Aldrich when not stated otherwise.

Size, size distribution and particle surface
Sizing and electrophoretic mobility was performed with a Malvern ZetaSizer ZS Nano (Malvern Instruments Ltd, UK.). Nanoparticle size was measured using dynamic light scattering (DLS) (633 nm He-Ne laser and a backscatter detector with a measurement angle of 173°). Size analysis was performed at 37°C and at 70 µg/mL total solids concentration. The zeta potential was measured at 25°C, at a final polymer concentration of 20 µg/mL in 10 mM NaCl in standard electrophoresis cuvettes (DTS1070, Malvern Instruments GmbH) measured in a ZetaSizer ZS Nano (Malvern Instruments Ltd, UK).

Suspension properties
PH value was assessed potentiometrically, tonicity was measured with a Semi-Micro Osmometer K7400 (Knauer).

Nanoparticle stability
Size and zeta potential of the NPs produced were measured immediately after production and after a 24-hour incubation period at 37°C in water or in DMEM+10 % FBS (CCM) to assess colloidal stability. After incubation, the formulations were further diluted with double-distilled water (70 µg/mL total solids) and measured using DLS at 37°C and a dispersant viscosity of 0.7000 cP. Formulations incubated in CCM (0.525 mg/mL) were diluted with water (10.5 μg/mL) and measured by nanoparticle tracking analysis (NTA) with the 532 nm laser unit. The NTA measurements were performed for 60 s with manual shutter and gain adjustment. The camera level was set to 12 and the detection threshold to 14. Three batches of each formulation were evaluated. Each sample was measured three times. The mean values calculated by the NTA software and the standard deviation refer to the arithmetic values of all particles analyzed.

Product yield and conjugated polymer content analysis
The product yield was determined gravimetrically by freeze-drying certain volumes of the nanoparticle suspensions. Fluorophor content of the nanoparticle suspensions was measured by fluorescence spectroscopy (FluoroMax-4, HORIBA Scientific). The fluorophore mass fraction in the final product was calculated by dividing the quantified fluorophore mass (spectrometry) by the gravimetrically obtained mass of the filtered batches. The relative coefficient of variation (CV) was calculated to evaluate the uniformity of the fluorophore content. The CV is the quotient of the coefficient of variation and the square root of the number of samples. This value is usually expressed as a percentage and indicates sample uniformity when values are below 5 %.

Foreign and particulate matter
The European Pharmacopoeia test has been adapted to provide a simple method for visually assessing the quality of parenteral preparations for visible particles. Before the test was performed, all labels on the container were removed, the outside was washed and dried. The container was then carefully swivelled and rotated, ensuring that no air bubbles were introduced. The container was then inspected at the
observation station for about 5 s in front of the white panel, followed by observation in front of the black panel. Any visible particles present were recorded.

**Endotoxin contamination detection and quantification**

The pH of the nanoparticle suspension was adjusted to 7.0 (± 0.5) with diluted 1.0 N NaOH. According to the kit instructions (Pierce LAL Chromogenic Endotoxin Quantification Kit; Thermo Scientific), standard curve, quality control, inhibition/enhancement control and nanoparticle formulations were prepared and dispensed in a pre-warmed (37°C) sterile 96-well microplate. In each well 50 µL of LAL reagent was added and incubated for 10 minutes. Subsequently, 100 µL pre-warmed chromogenic substrate solution was added, shaked gently and incubated for 6 minutes at 37 °C. Finally, 50 µL of stop reagent (25 % acetic acid) was added and the plate gently shaked. The absorbance was measured at 405 nm on a plate reader. The background corrected standard curve was used to determine the endotoxin concentration of each sample. For the experimental animals used, the maximum acceptable endotoxin value was calculated taking into account the maximum dose per hour, body weight and the human pyrogenic threshold dose of endotoxin per kg body weight[1].

**Detection of bacterial contamination**

Luria broth agar was dissolved, autoclaved and poured into Petri dishes under sterile conditions. The pH of the study sample was 7.0 (± 0.5) after adjustment with diluted 1.0 N NaOH. 50 µL nanoparticle sample and controls (negative: sterile water; positive: E. Coli Nissle 1917; mutaflor suspension) were spreaded on the agar surface using bacterial spreaders. Dishes were incubated at 37 °C and the potential formation of bacterial colonies was monitored over 72 h.

**Residual solvents**

24 h after production a solid-phase microextraction (SPME) followed by gas chromatography mass spectrometry (GCMS-QP2010S from Shimadzu) method was used to determine the level residual THF within the formulation. This method uses a fibre coated with an extraction phase. The fiber was exposed to the gas phase over the nanosuspension equilibrated in a test tube in a 50 °C water bath for 3 minutes and then mounted on the GC/MS unit for analysis. The used column (5 % diphenyl - 95 % dimethylpolysiloxane) was an Optima 5 MS - 0.25 µm. The carrier gas used was hydrogen with a column flow of 1.16 mL/min, the sampling time was 1 minute. The injector temperature was 200 °C and the column temperature 40 °C. The program was programmed to initially hold this temperature constant for 5 minutes, then increase to 280 °C at a rate of 100 °C/minute and finally hold the temperature for 1 minute. The total time of the program was 8.4 minutes. The ion source temperature was 250 °C, the interface temperature was 300 °C, the solvent cut time was 1 minute and the mass range 35.00 - 100.00 m/z.

**Photoluminescence quantum yield measurement**

Photoluminescence quantum yield (PLQY) equals the number of photons emitted, divided by the number of photons absorbed. PLQY was measured with the FluoroMax 4 using the PTI K-Sphere in the sample compartment. A 10 mm quartz cuvette was used for the measurement. Internal filter effects due to reabsorption must be avoided, so the sample was diluted so that the absorption at the excitation wavelength was less than 0.1. In order not to oversaturate the photomultiplier detector (PMT), the signal strength was checked in the real time control (RTC).
Neutral-density filter (ND) were used on the excitation side to reduce the intensity on the Rayleigh scatter to stay within the linear range of the detector (1 million and 1.5 million CPS on the raw S1 signal). Slits on the excitation and emission monochromators were set to 3 nm in the sample measurement. Integration time was set on 1 s. The area balance factor (ABF) needed for the PLQY tool was assessed by measuring the spectrum in question once with and once again without the required filter combination. The ABF is the quotient of the integrated curves of the relevant spectra.

**In Vitro optical imaging of nanoparticles in a tissue-mimicking phantom**

The polyurethane mouse shaped XFM-2 Phantom includes additional scattering particles and dyes to simulate the optical properties of living tissue. This alternative method makes it possible to plan animal experiments more efficiently, reduce their extent or even avoid them altogether. The polyethylene rods containing a fluorescent probe embedded in the tip are inserted into a slot in the phantom to imitate an internal fluorescent light source. The slots are located at a ventral depth of 4.1 mm and an axial depth of 32.8 mm. SBR values were acquired from samples with fluorophore masses ranging from 0.125 – 1 µg from n=3 sample batches. Photobleaching was assessed by measuring the SBR of each sample using 10 measurements over a period of 10 days and calculating the % decrease in SBR from the first and last measurement values.

**In vitro and in vivo photoacoustic instrumentation**

The system was equipped with a linear array of ultrasound transducer at a central frequency 21 MHz (MX 250) and 2 fiber optic bundles on either side of the transducer for the illumination. The fiber bundle was coupled to a tunable Nd:YAG laser (680 to 970 nm) with a 20 Hz repetition rate. The laser pulse width is 6-10 nanoseconds, pulse peak energy varies between 50 - 60mJ depending on the wavelength but the system does pulse-pulse to energy correction.

**HeLa cell culture, implantation into mice**

HeLa cells (American Type Culture Collection (ATCC)) were cultured in a 75 cm² flask in Dulbecco’s Modified Eagle Medium (GIBCO) (DMEM, supplemented with 10% (v/v) heat inactivated fetal bovine calf serum (FBS) and 1% (v/v) penicillin streptomycin). The culture flasks were kept in an incubator at 37 °C, 5% CO2 and 95% relative humidity. Mice received HeLa tumor cells implantation by subcutaneous injection (150 µL) in the right flank. Subsequently tumor size was monitored daily. Injected cells were in log phase and fed on the previous day. Two million HeLa cells were suspended in 50 mL of 50% v/v mixture of Matrigel supplemented with DMEM +10% FBS, 1% pen/strep (100 U/mL penicillin and 100 µg/mL streptomycin). The tumors were grown (approximately 10–15 days) until a single aspect was 7–9 mm before used for optical imaging experiments.

**In vivo fluorescence imaging**

The IVIS Spectrum imaging system was used for real-time whole animal fluorescence imaging of the contrast agents at 9 time points over 5 days. Three regions of interest of equal areas were chosen for assessment: 1) the tumor to assess passive accumulation of the fluorophore in the tumor area, 2) the abdominal region to assess liver accumulation and 3) an area of skin at the neck to assess peripheral fluorophore concentrations.
Serum protein adsorption
Sample preparation for serum protein adsorption was carried out according to a modified method described by Gossmann et al. To ensure reproducibility of the SDS-PAGE analysis the ratio of total particle surface area to mouse serum concentration was kept at 17.5 mL/m² for all four nanoparticle formulations. The formulations were diluted with 0.22 µm filtered bidistilled water to a constant particle surface concentration (0.04 m² in 500 µL) and this dispersion was incubated with 700 µL mouse serum for 30 minutes at 37 °C with constant agitation of 1000 rpm in a Thermomixer® comfort (Eppendorf AG, Hamburg, Germany). The NP were separated from the supernatant by centrifugation at 20,000 x g for 1 h. The supernatant was discarded and the particle pellet was resuspended in filtered bidistilled water and washed by three centrifugation steps at 20,000 x g for 1 h.

SDS-PAGE analysis of adsorbed serum proteins
After protein corona formation and final washing step the NP pellet was resuspended under shaking (1000 rpm, 22 °C) in 40 µL laemmli sample buffer overnight to desorb the proteins from the NP surface. Subsequently, the samples were centrifuged again (60 min, 20,000 x g) in order to separate the desorbed proteins from the NP. The supernatant was transferred into a new tube and heated for 5 min at 95 °C to denature the proteins. The pellet was again resuspended in 40 µL sample buffer, the suspension was transferred into a new tube and heated for 5 min at 95 °C to denature the proteins which were still remaining on the nanoparticle surface after overnight desorption in reducing loading buffer. Following that, a 15% SDS-polyacrylamide gel was prepared and the samples as well as the protein standard (PageRuler, 10 to 200 kDa, Thermo Scientific and prestained PageRuler, 10 to 170 kDa, Thermo Scientific), negative control, positive control and diluted mouse serum were applied in the gel wells. As negative control, NP were used which were incubated in water instead of serum. All other steps were performed in the same way. As positive control CNPPV NP were used which have a significantly higher zeta potential than the other NP formulations and therefore are much more likely to bind serum proteins.

After run, the mini gel was fixed 2 h in 10% TCA solution and then stained with colloidal Coomassie Brilliant Blue solution overnight. Background was destained in bidistilled water. To improve the sensitivity of protein detection in the gel a silver staining was performed additionally.

J774.A1 nanoparticle uptake study
The macrophage cell line J774.A1 was cultured in a 75 cm² flask in cell culture medium containing, Dulbecco’s Modified Eagle Medium (GIBCO) (DMEM, supplemented with 10% (v/v) heat inactivated fetal bovine calf serum (FBS) and 1% (v/v) penicillin streptomycin) in an incubator at 37 °C, 5% CO2 and 95% relative humidity. The J774.A1 cells were removed from the culture flask by careful scraping. The cells were seeded into a 96 well plates at density of 3.0 × 10⁴ cells/well in CCM (without phenol red). After 48 hours, the cells were washed with 37°C preheated PBS and then incubated with 200 µL of the nanoparticle suspension (0.743 mg/mL) diluted in either DMEM or mouse serum for 30 minutes in the incubator, DMEM was used as a control.

After washing the cells were treated with Triton (1% v/v) aiming cell digestion or stained with Hoechst (0.01% v/v) for cell counting. Finally, the cells treated with Hoechst were washed and fixed with paraformaldehyde solution in PBS (4 % v/v).
The absorbance of Triton digested cells was measured at 669 nm with a Cytation 5 (BioTek, Winooski, USA) and corrected by subtracting the background (untreated cells). Finally, the absorbance was calculated using the calibration lines of the respective formulations. Cell count performed with the Gen5 software. The experiment was repeated with three cell passages (#15-18), each formulation was tested n=15 in total.

**Blood parameters**
Under deep terminal anaesthesia at day 5 after i.v injection of CPN suspension, blood was drawn via cardiac puncture (23G needle) to investigate the impact of acute exposure of living animals to the CPNs. Complete blood chemistry, liver and kidney function parameters were assessed (n=3 in each group). Cervical dislocation was performed immediately following blood withdrawal of about 1 mL.

**Ex vivo organ optical imaging**
Major organs and tumor tissues were imaged ex vivo. Necropsy was performed by a midline incision ventrally from caudal to rostral aspects of the mouse through both the skin and peritoneum. Organs (lung, heart, liver, spleen, kidneys, ovary and tumor tissue) were subsequently removed, rinsed in saline and imaged in the IVIS Spectrum in the optimal wavelength setting, 645/840 nm.

**Histology of organs**
The most common histopathological staining system to visualise different cellular structures is Hematoxylin and Eosin (H&E), which was also used in this study. Hematoxylin stains the nuclei blue since it binds to nucleic acids in the cell nucleus, while eosin stains the cytoplasm pink. Using the staining method, tissue samples can be examined for their morphology and provide information on the health status of the laboratory animals. Major organs as lung, heart, liver, spleen, kidneys, ovary and tumor tissue were paraffin-embedded for H&E staining. Paraffin-embedded tissue samples were cut into 4 μm slices on the microtome. After 10 minutes in histol and 5 minutes in isopropanol, the sections were dewaxed via a descending alcohol route beginning with 95% ethanol over 80%, 70%, 50% to 50%. Subsequently, the sections were stained for 30 seconds with hematoxylin and 5 minutes in acidified 0.1% eosin solution. Before covering, the samples were drained via an ascending alcohol series, poured over with mounting medium (Roti-Histokitt, Carl Roth, Karlsruhe, GER) and finally covered with a coverslip. The tissues were examined using the Cytation 5 (BioTek, Winooski, USA) images were examined with the Gen5 software.
Size distribution by DLS of nanoparticle formulations

Figure S1: Size distribution of nanoparticles containing PCPDTBT (PEG$_{2kDa}$-PLGA$_{4kDa}$ (A); PEG$_{5kDa}$-PLGA$_{55kDa}$ (B); PEG$_{2kDa}$-DPPE (C) incubated over 24 h at 37 °C in either water measured with DLS. Colors represent measurement time points, red: 0h; black: 12h; blue: 24h after incubation.

Absorbance spectra

Figure S2: Absorbance spectra of CPN formulations (PEG$_{2kDa}$-PLGA$_{4kDa}$ (orange); PEG$_{5kDa}$-PLGA$_{55kDa}$ (gray); PEG$_{2kDa}$-DPPE (dark red)) measured 24 h after incubation at different temperatures, 20 °C (A); 37 °C (B) or 85 °C (C).
Figure S3: Decrease of SBR of different formulations in the phantom mouse during fluorescence imaging in the IVIS spectrum, as a function of number of excitations and time. In a PE rod 10 µL (2.1 mg/mL) of the formulations were imaged immediately after production and subsequently after 24 h and 72 h at 745 nm/840 nm 10 times in immediate succession (A). The batches of formulations were kept in the dark at room temperature for 14 days, then the experiment was repeated (B).
Protein corona assessment

The gel electrophoresis was performed in triplicates.

SDS-PAGE number 1

Figure S4.1: Qualitative visualization of protein corona composition using SDS-PAGE. Analysis of nanoparticle supernatant after incubation with serum, washing and heating for 5 minutes at 95°C (A) and analysis of the resuspended nanoparticle pellet in reducing loading buffer after heating 5 minutes at 95°C (B).
Figure S4.2: Qualitative visualization of protein corona composition using SDS-PAGE. Analysis of nanoparticle supernatant after incubation with serum, washing and heating for 5 minutes at 95°C (A) and analysis of the resuspended nanoparticle pellet in reducing loading buffer after heating 5 minutes at 95°C (B).
Figure S4.3: Qualitative visualization of protein corona composition using SDS-PAGE. Analysis of nanoparticle supernatant after incubation with serum, washing and heating for 5 minutes at 95°C (A) and analysis of the resuspended nanoparticle pellet in reducing loading buffer after heating 5 minutes at 95°C (B).
Figure S5: Weight of dissected mice organs - lung, heart, liver, spleen and kidney harvested at day 5 after tail vein injection of CPN-suspension.
Figure S6: Microscopic histopathological hematoxylin and eosin (H&E) stained images of tissue sections of removed mouse organs - liver, spleen, kidney, heart, lung, ovary and tumor tissue harvested at day 5 after tail vain injection of CPN-suspension. No cellular or tissue damage was observed after 5 days.
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