Supplemental Information

PEGylated PRINT Nanoparticles: The Impact of PEG Density on Protein Binding, Macrophage Association, Biodistribution, and Pharmacokinetics.

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Particle Characterization

Table S1 depicts electrophoretic mobilities measured at pH 5.5 of non-PEGylated, PEG mushroom and PEG brush nanoparticles.

Table 1 Calculations

Equation S1 was used explicitly to calculate the area occupied per PEG chain (A) for PLGA nanoparticles, where M_{PEG} is the molecular weight of the PEG graft, f is mass fraction of PEG in the particle, ρ is the density of the nanoparticle, N_A is Avogadro's number, and d is the diameter of the particle.[1] Using this value for A, D was then calculated using Equation 3 in the main text. Equation S2 was used only to calculate D for liposomal particles.[2] For this equation A_{lipid} is the area occupied per lipid and m is the mole fraction of PEG incorporated in the nanoparticle.[3] For all other nanoparticles listed in Table 1, Equations 1-3 were used.

PEG Density Calculations

To determine surface PEG density, we calculated the total number of PEG molecules and nanoparticles in each sample. The number of PEG molecules was calculated from the standard curve. The number of NPs was calculated using Equation S3, where the mass of NPs per well (m) was determined by TGA, the density of the nanoparticle (ρ_{NP}) is 1.1 g/cm^3 and the volume (V) determined by measurements from SEM images. Equation S4 was used to calculate PEG density (S), where the surface area (SA_{NP}) of a hydrated 80 nm x 320 nm nanoparticle was measured by fluid AFM. The area occupied by each PEG chain (A) is defined by Equation S5. Assuming that PEG occupies a circular footprint on the particle surface, the distance between PEG grafts (D) can be calculated using Equation S6.

PK Analysis

PK Analysis of the blood concentration data was conducted with PKSolver. The integration constants from the biexponential fit are in Table S2 and were used to calculate elimination half-life, clearance, and AUC for each particle type.

Biodistribution

Organ fluorescence was analyzed at each time point and expressed as percent recovered fluorescence. As in Figure 8 the trend holds (over time), as PEG density increases blood and splenic accumulation increases, whereas liver accumulation decreases (Figures S1- S5). We have displayed the data in two ways, as percent recovered fluorescence per organ or per gram of tissue. The trends stay the same; however, since the liver is a large organ, when the data is displayed per gram of tissue, the liver accumulation appears lower than the splenic accumulation.

Materials

Poly(ethylene glycol) diacrylate (M_n 700) ($PEG_{700}DA$), 2-aminoethyl methacrylate hydrochloride (AEM), diphenyl (2,4,6-trimethylbenzoyl)-phosphine oxide (TPO), bovine serum albumin (BSA) protein standards (2 mg/mL), trypsin, ethylenediametetraacetic acid (EDTA), and sucrose were purchased from Sigma-Aldrich. Thermo Scientific Dylight 488, 650 and 680 maleimide, Thermo Scientific HyClone fetal bovine serum (FBS), PTFE syringe filters (13 mm membrane, 0.22 μm pore size), dimethylformamide (DMF), triethylamine (TEA), pyridine, sterile water, borate buffer (pH 8.6), Dulbecco's phosphate buffered saline (DPBS) (pH 7.4), 1X phosphate buffered saline (PBS) (pH 7.4), acetic anhydride, and methanol were obtained from Fisher Scientific. Fluorescein-PEG(5k)-succinimidyl carboxy methyl ester (fluorescein-PEG_{5k}-SCM) and methoxy-PEG(5k)-succinimidyl carboxy methyl ester ($mPEG_{5k}$-SCM) were

purchased from Creative PEGWorks. Conventional filters (2 μm) were purchased from Agilent and polyvinyl alcohol (Mw 2000) (PVOH) was purchased from Acros Organics. PRINT molds (80 nm x 80 nm x 320 nm) were obtained from Liquidia Technologies. Tetraethylene glycol monoacrylate (HP_4A) was synthesized in-house as previously described.[4] Murine alveolar macrophage (MH-S) cells were purchased from American Type Culture Collection.

Methods

PRINT Nanoparticle Fabrication

The PRINT particle fabrication technique has been described previously in detail. [5] The pre-particle solution was prepared by dissolving 3.5 wt% of the various reactive monomers in methanol. The reactive monomers included: a cure-site monomer (an oligomeric PEG with a nominal molar mass of 700 g/mol terminally functionalized on both end groups with an acryloxy functionality); a hydrophilic monomer used to make up the majority of the particle composition (HP4A); an amine containing monomer (AEM) which served to provide the amine functionality used to conjugate PEG onto the surface of the PRINT particles; and in some cases a polymerizable fluorescent tag. In all cases a photoinitiator, TPO, was also added. Two different pre-particle solutions were used throughout the following studies. For quantifying PEG density and ITC studies the pre-particle solution was comprised of 68 wt% HP_4A, 20 wt% AEM, 10 wt% $PEG_{700}DA$, and 1 wt% TPO. For the remainder *in vitro* and *in vivo* studies, the pre-particle solution was comprised of 67.5 wt% HP_4A, 20 wt% AEM, 10 wt% $PEG_{700}DA$, 1 wt% TPO and 1.5 wt% Dylight maleimide (either 680, 650 or 488). Using a # 3 Mayer rod (R.D. Specialties), a thin film of the pre-particles solution was drawn onto a roll of freshly corona treated PET, using a custom-made roll-to-roll lab line (Liquidia Technologies) running at 12 ft/min. The solvent

was evaporated from this delivery sheet by exposing the film to a hot air dam derived from heat guns. The delivery sheet was laminated (80 PSI, 12 ft/min) to the patterned side of the mold, followed by delamination at the nip. Particles were cured by passing the filled mold through a UV-LED (Phoseon, 395 nm, 3 SCFM N_2, 12 ft/min). A PVOH harvesting sheet was hot laminated to the filled mold (140 ºC, 80 PSI, 12 ft/min). Upon cooling to room temperature, particles were removed from the mold by splitting the PVOH harvesting sheet from the mold. Particles were then harvested by dissolving the PVOH in a bead of water (1 mL of water per 5 ft of harvesting sheet). The particle suspension was passed through a 2 μm filter (Agilent) to remove any large particulates. To remove the excess PVOH, particles were centrifuged (Eppendorf Centrifuge 5417R) at ca. 21,000 g for 15 min, the supernatant was removed and the particles were re-suspended in sterile water. This purification process was repeated 4 times.

Nanoparticle Characterization

Stock particle concentrations were determined by thermogravimetric analysis (TGA) using a TA Instruments Q5000 TGA. TGA analysis was conducted by pipetting 20 μL of the stock nanoparticle solution into a tared aluminum sample pan. Samples suspended in water were heated at 30 °C/min to 130 °C, followed by a 10 minute isotherm at 130 °C. Samples suspended in DMF were heated at 30 °C/min to 170 °C, followed by a 10 minute isotherm at 170 °C. All samples were then cooled at 30 °C/min to 30 °C, followed by a 2 minute isotherm at 30 °C. TGA was also performed on a 20 μL aliquot of supernatant from a centrifuged sample of the stock nanoparticle solution to account for the mass of any stabilizer remaining in each sample. The concentration of stabilizer was subtracted from the concentration of stock particle solution to determine the actual particle concentration. Particles were visualized by scanning electron

microscopy (SEM) using a Hitachi S-4700 SEM. Prior to imaging, SEM samples were coated with 1.5 nm of gold-palladium alloy using a Cressington 108 auto sputter coater. Particle size and zeta potential were measured by dynamic light scattering (DLS) on a Zetasizer Nano ZS (Malvern Instruments, Ltd.).

Swelling of the particles in an aqueous environment was analyzed using fluid atomic force microscopy (AFM) with an Asylum Research MFP-3D atomic force microscope at room temperature. Height, phase, and amplitude images were acquired in water, in tapping mode, with a silicon nitride cantilever (Budget Sensors, k = 0.06 N/m) at a scan rate of 1 Hz. Samples for imaging were prepared by pipetting particle suspension onto a clean glass slide. The solution was allowed to evaporate in an effort to settle the nanoparticles onto the glass slide. A droplet of water was then placed upon the dried nanoparticles to re-hydrate them. A droplet of water was also placed upon the AFM tip. The two droplets were merged and images collected. The hydrated dimensions of the particles were then determined from the AFM images.

PEGylation Quantification

After purification, the particles were reconstituted in DMF following the centrifugation technique outlined above and the concentration of particles in DMF was determined by TGA. The particles fabricated contain free primary amine groups which were used as functional handles to react with a fluorescein-PEG_{5k}-SCM. The particles (1 mg NPs in 1 mL DMF) were reacted with TEA (100 µL) for 10 min at room temperature on a shaker plate (Eppendorf, 1400 rpm). The fluorescein-PEG_{5k}-SCM was dissolved in DMF and added to the reaction mixture (14 mg and 2 mg of fluorescein-PEG_{5k}-SCM for high and low PEG density, respectively). The reaction mixture was shaken overnight and then quenched with borate buffer (100 µL). The

nanoparticle solution was then washed 5 times with DMF via centrifugation. The PEGylated particles were characterized by SEM, DLS, and TGA. After conjugating fluorescently-tagged PEG_{5k} to the nanoparticle surface, the amount of PEG bound to the particle was assessed via fluorescence measurements. The fluorescein-PEG-NP solution was diluted 1:10 in borate buffer and pipetted (200 μL) into a Corning 96-well clear bottom plate in triplicate. The supernatant from the same nanoparticle solution was added to the 96-well plate using the same method. A serial dilution of unconjugated fluorescent-PEG_{5k} was utilized to create a standard curve. Fluorescence measurements (λ_{ex} = 494 nm; λ_{em} = 521 nm) of the 96-well plate containing the (1) PEGylated nanoparticle (2) supernatant and (3) standard curve were taken using a SpectraMax M5 plate-reader. The fluorescence in the supernatant was subtracted from the fluorescence observed from the nanoparticle suspension and the final fluorescence measurement was correlated to fluorescein-PEG_{5k} concentration through the standard curve.

PEGylation and Acetylation for in vitro and in vivo studies

For *in vitro* and *in vivo* studies, particles were PEGylated using the same procedure outlined above. However, instead of a fluorescein-$PEGK_{5K}$-SCM, a methoxy-PEG_{5K}-SCM was used. Following PEGylation, particles were acetylated with acetic anhydride to quench any unreacted amines and to yield a negative zeta potential. For acetylation, nanoparticles (1 mg NP in 1 mL DMF) were reacted with an excess (10 μL) of pyridine and acetic anhydride (7 μL). The reaction was carried out in a sonicator bath (Branson Ultrasonic Cleaner 1.4 A, 160 W) for 15 min, after which a second addition of acetic anhydride (7 μL) was added and the suspension was sonicated for another 15 min. Following acetylation, the particles were washed by centrifugation one time in DMF, followed by a borate buffer wash to neutralize any acetic acid side product,

and then 4 washes with sterile water. Post-acetylation, particles were analyzed by TGA, DLS and SEM.

Protein Binding Using Isothermal Titration Calorimetry (ITC)

The ITC experiments were performed at 37 ºC, using a VP-ITC microcalorimeter (GE MicroCal Inc., USA). Experiments were performed by injecting 20 μM solution of BSA in 1X PBS into a 2 mL sample cell containing nanoparticles at a concentration of 2 mg/mL in 1X PBS with a stirring speed of 300. A total of 44 injections were performed with a spacing of 240 s and a reference power of 10 μcal/s. Titration volumes of BSA were as follows: a first injection of 2 μL, followed by twenty eight injections of 5 μL, and fourteen injections of 10 μL. Binding isotherms were plotted and analyzed using Origin Software (MicroCal Inc., USA), where the ITC measurements were fit to a one-site binding model.

Macrophage Association Assay

Murine alveolar macrophage cells (MH-S) were used to investigate the uptake of nanoparticles as a function of surface PEG density. MH-S cells were plated at a density of 40,000 cells per well in a 24-well plate and were incubated at 37°C for 24 hours. Following 24 h, the dye-labeled (Dylight 650 or 680) nanoparticle samples (20 μg in 1 mL water) were incubated with the cells for 0.5, 2.5, 6, 24, and 48 h. At the set time points, cells were washed three times with 500 μL 1X PBS and detached by the addition of 1X trypsin/EDTA (300 μL) to each well. Following a 5 minute incubation (37°C), 1X DBPS/10% FBS (500 μL) was added to each well and was mixed vigorously. This final solution was then transferred to a polypropylene tube and analyzed using

a Dako CyAn flow cytometer with excitation and emission filters set to match that of the fluorescent dye incorporated into the particles. For each sample, 10,000 cells were measured.

In Vivo Studies

All experiments involving the mice were carried out in accordance with an animal use protocol approved by the University of North Carolina Animal Care and Use Committee. Female BALB/c mice (18-25 g, Jackson Laboratory) were dosed via tail vein injections of 12.5 mg of NPs per kg of mouse weight. The volume of injection ranged from 75 μL to 104 μL of (3 mg/mL) nanoparticle suspension in an isotonic sucrose solution (9.25 wt%).

Intravital microscopy (IVM) was used to assess the circulation profile of the three different particle types. Experiments were performed using an IV 100 laser scanning microscope (Olympus). The mouse was anesthetized with isofluorane and a tail vein catheter was applied. Hair was removed from the ear of the mouse with Nair, and the mouse was placed on a 37 ºC heated stage in the prone position and kept under anesthesia. The hairless ear was immobilized to an aluminum block with double-sided tape, and vasculature was visualized with a 488-nm laser. Mice were then dosed with Dylight 650-labeled NPs with varying PEG surface coverage. Fluorescence was measured using a 633-nm laser, and imaging scans were captured every 5 s for 2 hrs. For circulation analysis, the image files from each scan were exported to ImageJ. Following literature procedures, the images were stacked in groups of four, and fluorescent signal in each stack was analyzed in the region of interest.[6, 7] Background corrections were obtained using the initial fluorescence in the region of interest before injection.

In an effort to determine circulation half-life, blood draw and biodistribution studies were also completed. Injections and tissue/blood collection were performed with assistance of the

Animal Studies Core (UNC-CH). Mice were dosed with NPs or sucrose (control). For each particle type and control, we examined four mice per time point (5 min, 15 min, 30 min, 1 h, 3 h, and 24 h). At the various time points post-injection, mice were given a dose of ketamine/dexmedetomidine blend to deeply anesthetize them prior to cardiac puncture for blood collection. Blood was collected and stored in heparinized eppendorf tubes (Milian, USA). Mice were sacrificed and organs (liver, spleen, kidney, and lung) were harvested, weighed, and transferred to 6- or 12-well plates for fluorescence analysis with an IVIS Lumina imager (Caliper Life Sciences); excitation and emission filters were set to 675 nm and 720 nm, respectively. Heparinized blood was pipetted in 100 μL aliquots into black 96-well plates and imaged on the IVIS Lumina. In order to determine particle concentration in the blood, we performed serial dilutions (in triplicate) of particles in freshly harvested mouse blood and plotted a standard curve.

Pharmacokinetic analysis of the blood draw data was performed using PKSolver.[8] Data was fit to either a one- or two-compartment model, and the Akaike information criterion (AIC) was used to compare goodness of fit for each nanoparticle type.[9]

Equation S1

$$A = \frac{6M_{PEG}}{dN_A f \rho}$$

Equation S2

$$D = \sqrt{\frac{A_{lipid}}{m}}$$

Equation S3

$$\#NPS = \frac{m}{\rho_{NP}\, V_{NP}}$$

Equation S4

$$S = \frac{\#\ \text{PEGs}}{SA_{NP}}$$

Equation S5

$$A = \frac{1}{S}$$

Equation S6

$$D = 2\sqrt{\frac{A}{\pi}}$$

Table S1: Electrophoretic mobilities of nanoparticles measured by dynamic light scattering.

Sample	Mobility (cm^2/Vs)		
non-PEGylated	-2.02E-04	±	4.20E-06
PEG mushroom	-7.82E-05	±	1.41E-06
Peg brush	-1.11E-04	±	4.89E-06

Table S2: Constants of integration from two-compartmental model fit of blood concentration data with coefficient of determination (R^2).

	A	α	B	β	R^2
non-PEGylated	0.042	5.98	0.004	0.777	0.9996
PEG mushroom	0.080	4.82	0.075	0.045	0.9977
PEG brush	0.068	1.45	0.100	0.036	0.9990

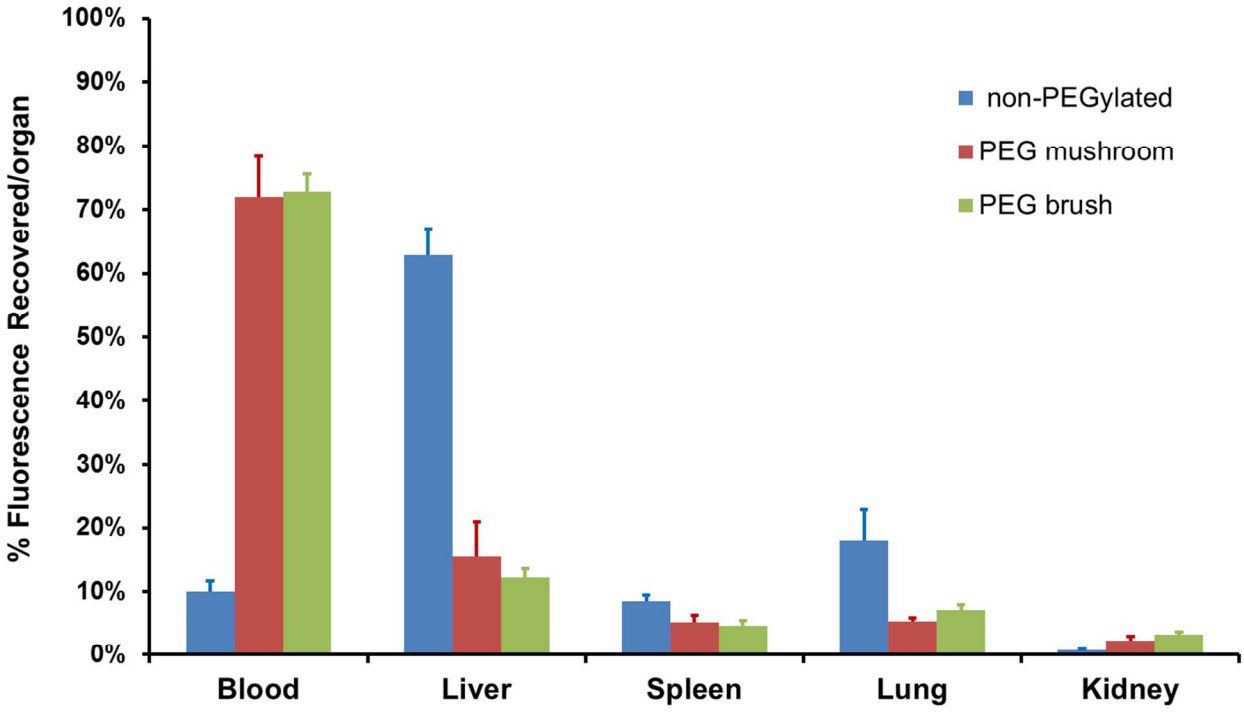

Figure S1. Biodistribution of nanoparticles at 0.083 h post-injection, expresses as percent recovered fluorescence per organ. Error bars represent standard deviation from n=4.

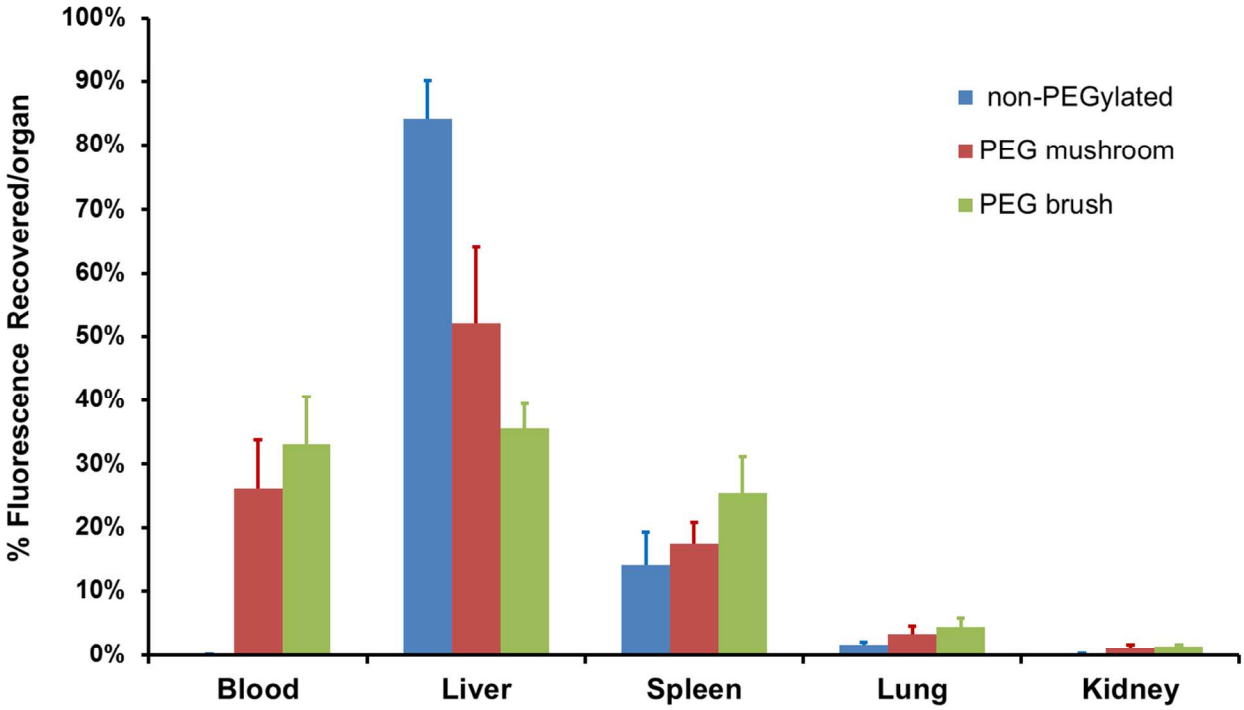

Figure S2. Biodistribution of nanoparticles at 3 h post-injection, expresses as percent recovered fluorescence per organ. Error bars represent standard deviation from n=4.

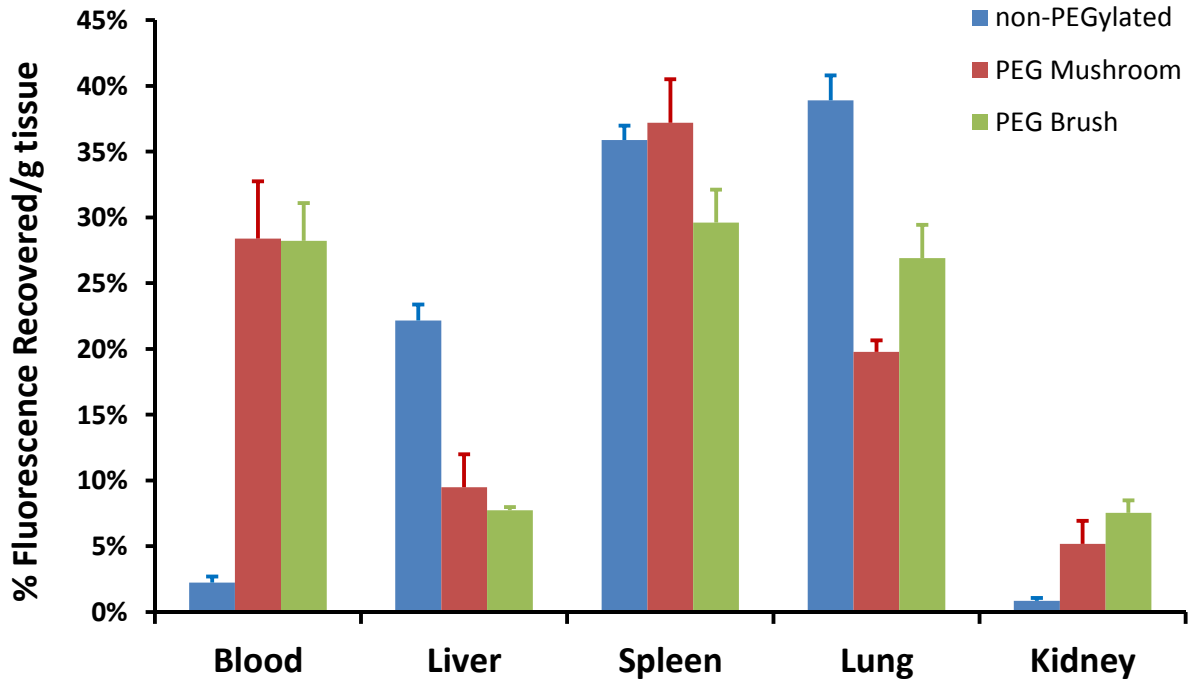

Figure S3. Biodistribution of nanoparticles at 0.083 h post-injection, expresses as percent recovered fluorescence per gram of tissue. Error bars represent standard deviation from n=4.

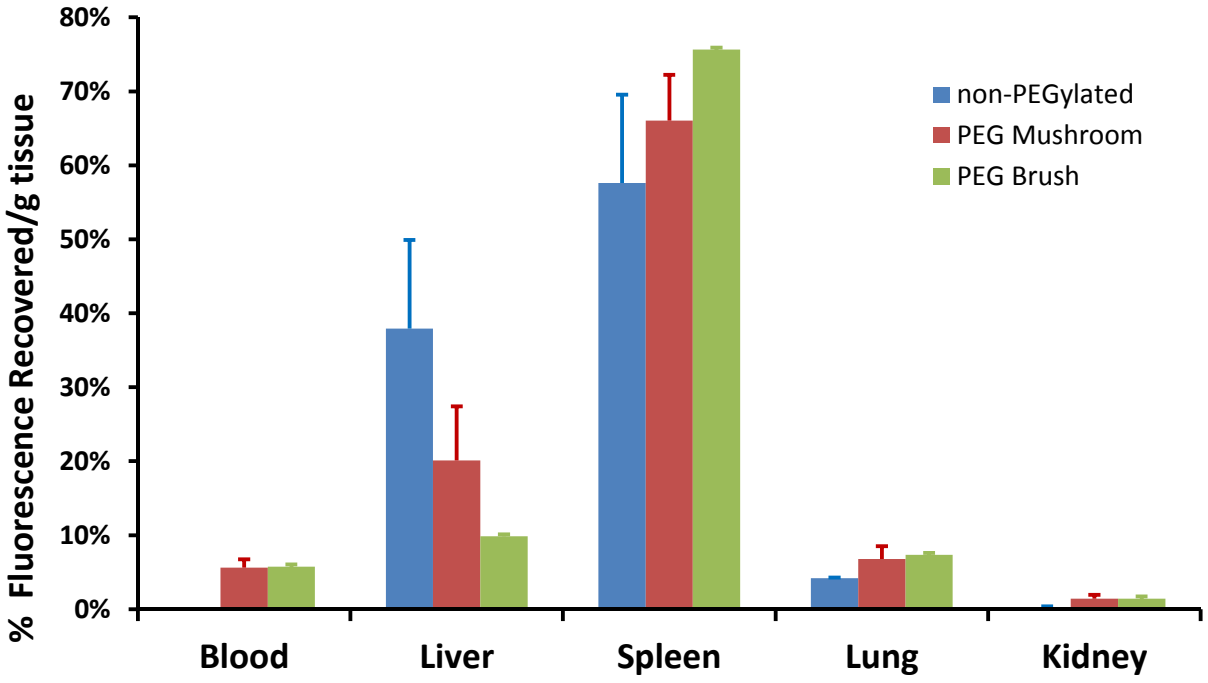

Figure S4. Biodistribution of nanoparticles at 3 h post-injection, expresses as percent recovered fluorescence per gram of tissue. Error bars represent standard deviation from n=4.

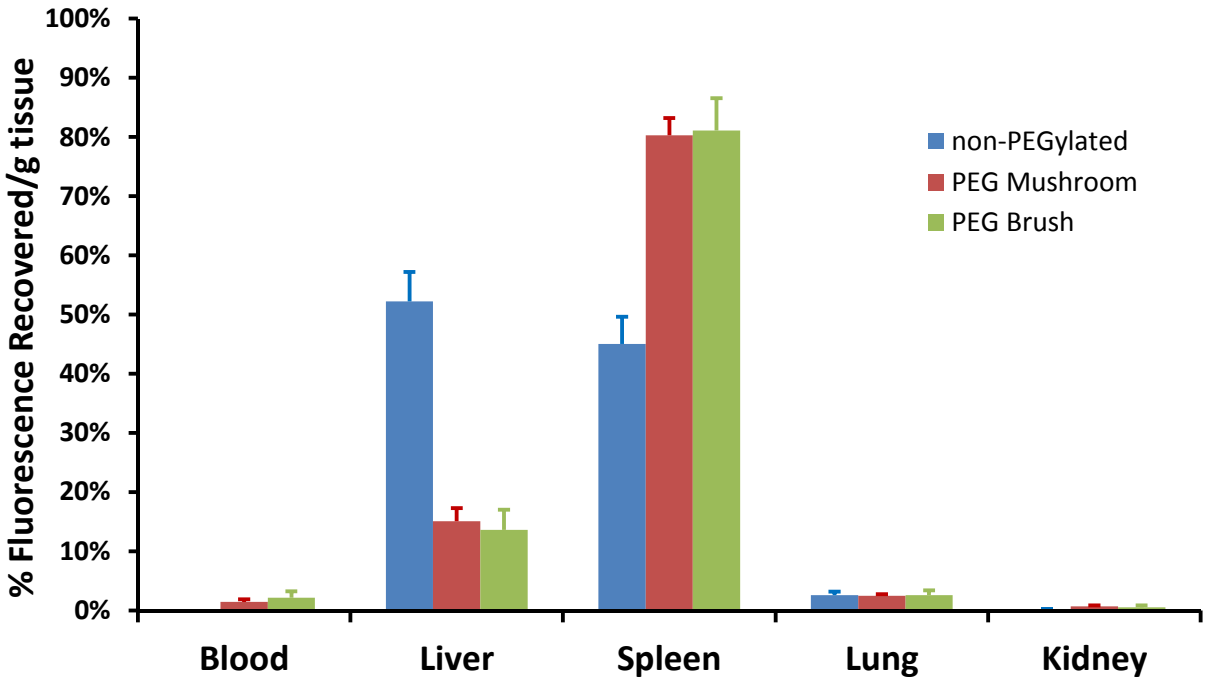

Figure S5. Biodistribution of nanoparticles at 24 h post-injection, expresses as percent recovered fluorescence per gram of tissue. Error bars represent standard deviation from n=4.

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