Supporting Information for:

Bisphosphonate-anchored PEGylation and Radiolabeling of Superparamagnetic Iron Oxide: Long-Circulating Nanoparticles for *In Vivo* Multimodal (T1 MRI-SPECT) Imaging

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1) Synthesis of PEGylated USPIOs obtained using a co-precipitation method (PEG(5)-BP-USPIO$_{copr}$)

Iron oxide nanoparticles (USPIO$_{copr}$) were synthesised using a well established co-precipitation method (Kang, Y. *et al.*, *Chem. Mater.*,1996, 8, 2209). To PEGylate USPIO$_{copr}$, an aliquot of the ferrofluid at pH 7 containing approximately 1 mg of iron oxide was mixed with 10 mg of PEG(5)-BP or PEG(5)-COOH. The two dispersions were sonicated for 15 min and then transferred to a 30 kDa size-exclusion filter and subsequently washed several times with water to remove unbound PEG polymers. After 3-4 washing steps, the sample containing PEG(5)-COOH precipitated as a result of the weak binding. On the other hand, the sample containing PEG(5)-BP remained in dispersion without signs of aggregation for more than 4 washings and was fully recovered from the filter. IR studies confirmed the presence of the polymer (Figure S1) after the purification process. DLS studies over 7 days confirmed enhanced stability in saline compared to non-PEGylated ferrofluids (Figure S1). In addition, zeta-potential measurements at neutral pH confirmed a neutral surface for PEG(5)-BP-USPIO$_{copr}$ (-0.1 mV) compared to USPIO$_{copr}$ (43.5 mV).

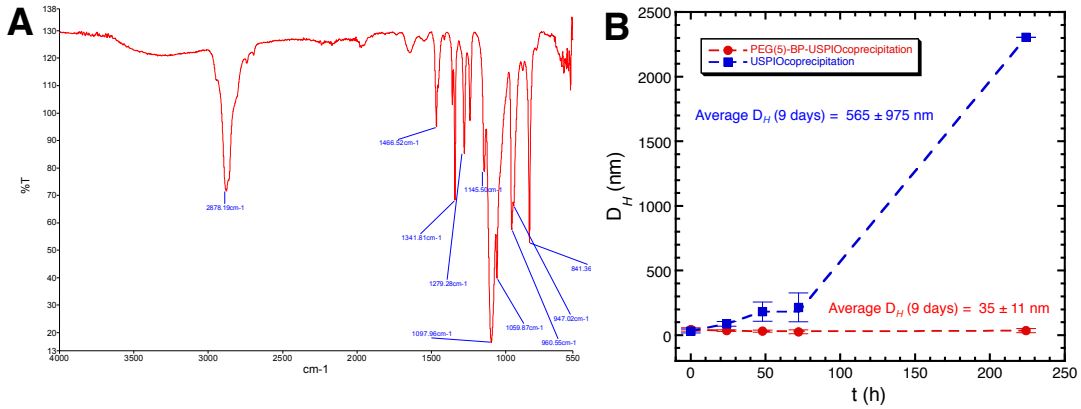


Figure S1. (A) IR spectrum of PEG(5)-BP-USPIO$_{copr}$ showing the characteristic PEG vibrations (B) DLS stability study in saline with PEG(5)-BP-USPIO$_{copr}$ (red) and 'naked' USPIO$_{copr}$ (blue) over 9 days. PEG(5)-BP-USPIO$_{copr}$ (red) remained stable whereas the non-coated USPIO$_{copr}$ precipitated over time.

2) Attempted synthesis of PEG(5)-COOH-USPIO:

Oleylamine-coated USPIOs (1 mg) and PEG(5)-COOH (10 mg) were added to 2 mL of DCM in an open glass vial and the mixture sonicated until the solvent evaporated (~15 min). To the remaining residue were added 4 mL of water resulting in a clear brown solution. This mixture was washed with 4 mL of hexanes to remove oleylamine. This process was repeated 2 more times followed by removal of hexanes by evaporation. The final mixture was filtered through a 0.2 μm hydrophilic PTFE filter, followed by several cycles of washing/concentrating using a Vivaspin 2 centrifugal filter (30 kDa molecular weight cut-off) using water to remove excess PEG(5)-COOH. The solution became cloudy after 4 cycles of washing-redispersion in water, confirming the loss of PEG(5)-COOH leading to the formation of iron oxide aggregates that precipitated in water.

3) IR studies of PEG(5)-BP

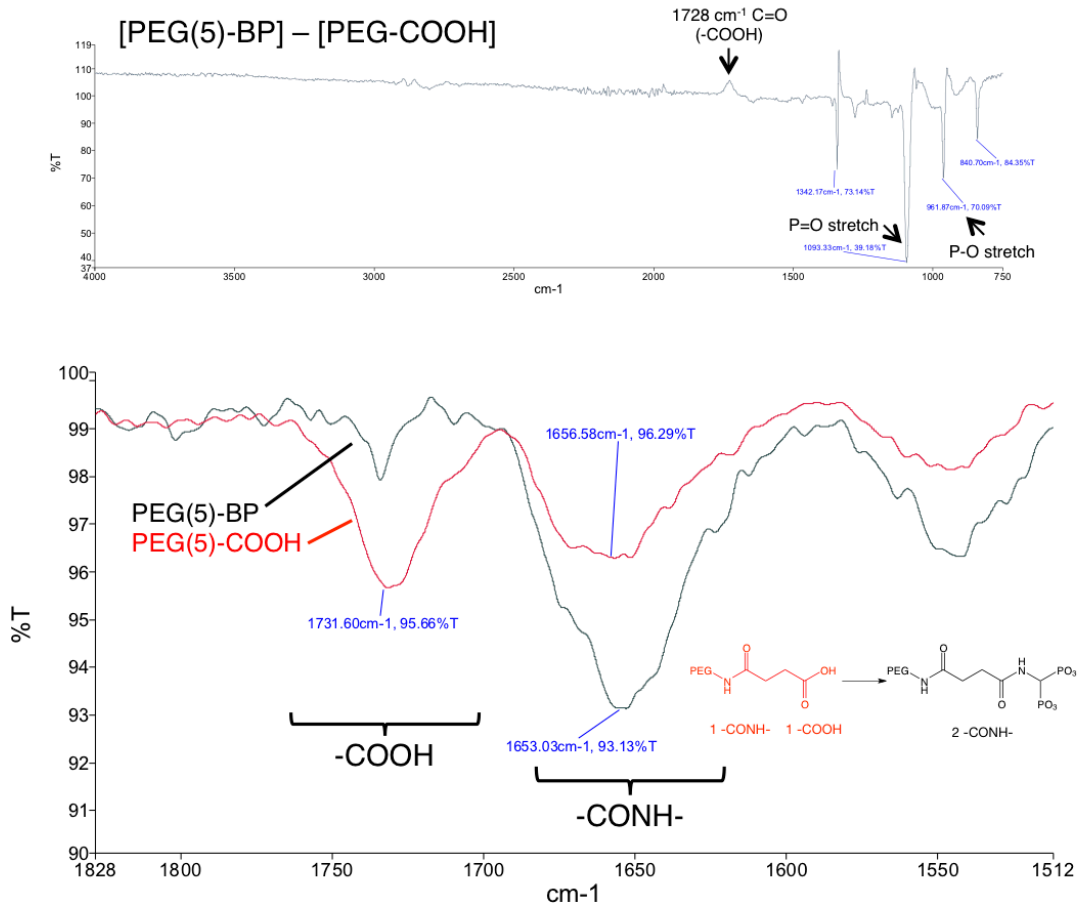


Figure S2. IR studies with PEG(5)-BP. Top: Difference IR spectrum (starting material – product → PEG(5)-COOH – PEG(5)-BP) showing the presence of BP-related peaks, as well as a positive peaks as a result of the disappearance of the COOH vibration and 1730 cm^{-1}. Bottom: C=O stretching area of the of the IR spectra of PEG(5)-COOH and PEG(5)-BP, showing the expected disappearance of the COOH peak upon formation of the new amide bond and expected increase of the CONH band.

4) PEG(5)-BP-USPIO PEG density calculations:

(*NOTE*: These calculations assume USPIOs are spheres of a fixed diameter (5.5 nm) and composed of Fe_3O_4)

- **Using the amount of non-bound PEG(5)-BP after purification:**

- Calculation of number of Fe atoms/USPIO and mass of each USPIO

The mass of each 5.5 nm USPIO is 4.5 x 10^{-19} g, by using the density of Fe_3O_4 (5.17 g/cm^3).

Thus, as the molecular weight of Fe_3O_4 is 232 g/mol, the number of moles is 1.939 x 10^{-21} moles Fe_3O_4, and the number of Fe_3O_4 units per USPIO is 1.939 x 10^{-21} x N_A = 1168. Hence, the number of Fe atoms/USPIO is 1168 x 3 = 3504 Fe atoms/USPIO

- Calculation of number of PEG molecules bound to USPIOs

Using the mass of the non-bound PEG(5)-BP, obtained from the washings during purification of PEG allowed us to calculate that 2.4 mg of PEG(5)-BP was bound to the USPIOs.

Hence, using the average MW of PEG(5)-BP value of 5307 g/mol, we can calculate that this corresponds to approximately 2.72 x 10^{17} molecules.

- Calculation of number of USPIO nanoparticles

Method 1. The Fe concentration of the PEG(5)-BP-USPIO dispersion was 26.62 mM (ICP-MS) in 300 μL, which corresponds to 7.98 x 10^{-6} moles and 4.81 x 10^{18} Fe atoms. Hence, 4.81 x 10^{18} Fe atoms/3504 atoms/NP = 1.37 x 10^{15} NPs

Method 2. The mass of USPIOs used for the synthesis was 1.6 x 10^{-3} g (after removing the mass of oleylamine, see TGA data below), hence dividing by 4.5 x 10^{-19} g/NP calculated earlier gives us 3.6 x 10^{15} NPs.

- Calculation of number of PEGs/USPIO NPs

Using method 1. 2.72 x 10^{17} PEGs/1.37 x 10^{15} NPs = **198 PEGs/NP**
Using method 2. 2.72 x 10^{17} PEGs/3.6 x 10^{15} NPs= **76 PEGs/NP**

- Calculation of theoretical maximum density of BPs/USPIO NPs

The surface area of a 5.5 nm sphere is 95 nm^2. The footprint of a BP is approximately 0.85 nm^2 (calculated from a computational model using Chem3D, Cambridge Software). Using these values, the theoretical maximum number of BPs that can bind to a 5.5 nm sphere is **112**.

- **Derived from TGA measurements:**

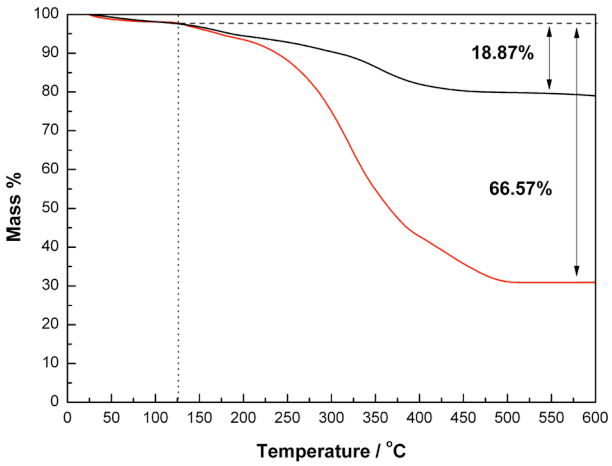


Figure S3. TGA study with oleylamine-SPIOs (black line) and PEG(5)-BP-USPIOs (red line). The heating rate was 10 °C/min under a N_2 flow. The vertical line indicates the temperature at which we consider most of the absorbed water has evaporated (125 °C).

Assuming that the weight loss before 125 °C is due to absorbed water (Figure S3), we can calculate that 67% of the total weight of PEG(5)-BP-USPIO is due to PEG(5)-BP, which corresponds to 2.68 mg PEG(5)-BP (note that this value is close to the 2.4 mg of non-bound PEG(5)-BP we recovered from the reaction). This corresponds to (2.68×10^{-3} g/5307 g/mol) x $N_A = 304.2 \times 10^{15}$ molecules of PEG(5)-BP.
On the other hand, we know from the TGA study (Figure S1) that out of the 2 mg of oleylamine-USPIOs, 19% corresponds to oleylamine. Hence, 1.6 mg is the weight of the Fe_3O_4 NPs in oleylamine-USPIOs, and consequently in PEG(5)-BP-USPIOs (all USPIOs were capped with PEG(5)-BP and transferred into the aqueous phase), which corresponds to 3.55×10^{15} USPIOs using the value of 4.5×10^{-19} g/USPIO, calculated in the previous section. By dividing 304.2×10^{15} molecules of PEG(5)-BP by 3.55×10^{15} USPIOs we obtain that each USPIO is bound to **86 PEG(5)-BP molecules**, which corresponds to **0.9 PEG/nm^2**, using the surface area of a 5.5 nm diameter sphere.

5) XPS spectrum

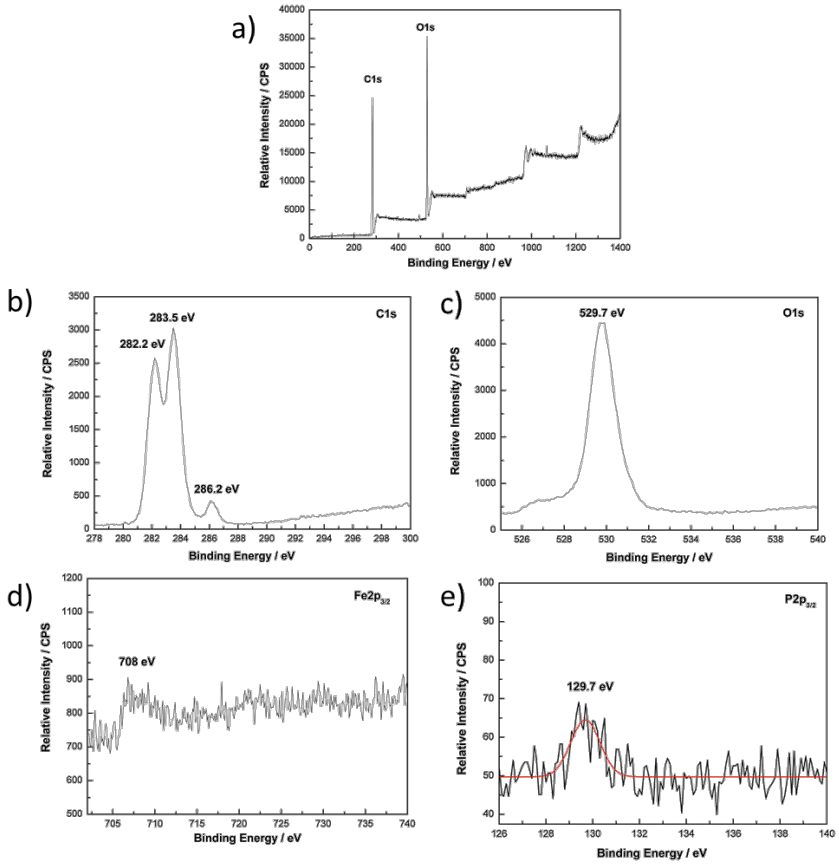
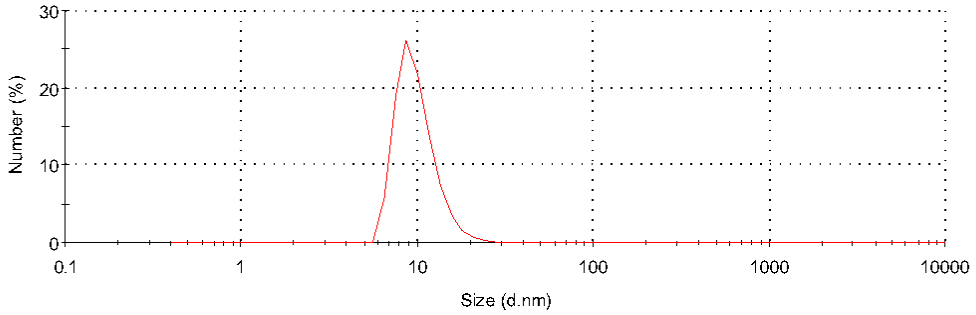


Figure S4: XPS spectrum of PEG(5)-BP-USPIO. The full spectrum (a) shows a dominant signal from PEG chain and carbon dioxide absorbed on the surface. Carbon was found at 282.2 eV, 283.5 eV and 286.2 eV, other three elements C(b), O (c), Fe(d) and P(e) were found at 529.7 eV, 708 eV and 129.7 eV, respectively. Weak signal of iron and phosphorus was due to the coverage of PEG chain and the low abundance of P.

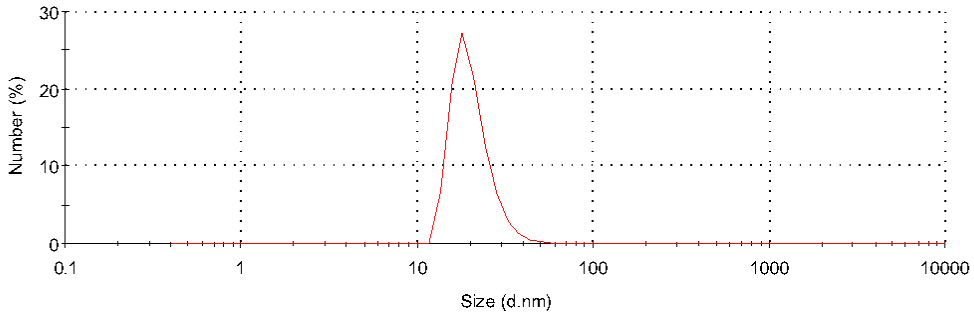
1) Wagener K., Batich C., Kirsch B., Wanigatunga S. J. Polym. Sci. Part A 27, 2625 (1989)
2) Beamson G., Briggs D. High Resolution XPS of Organic Polymers: the Scienta ESCA300 Database (1992)

6) DLS and zeta potential studies

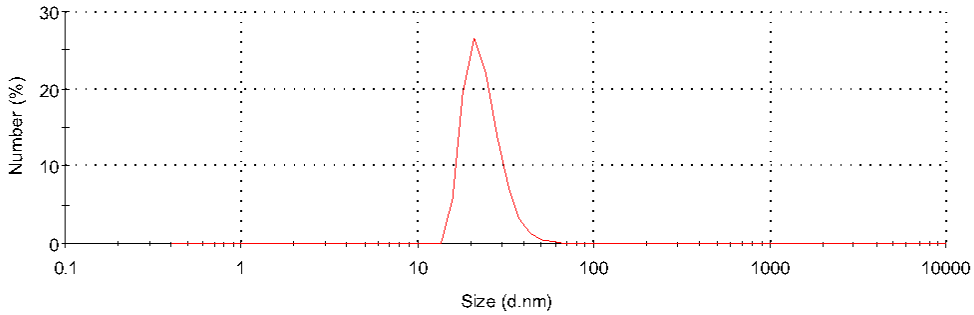
1. DLS of Oleylamine-USPIOs (in dichloromethane)



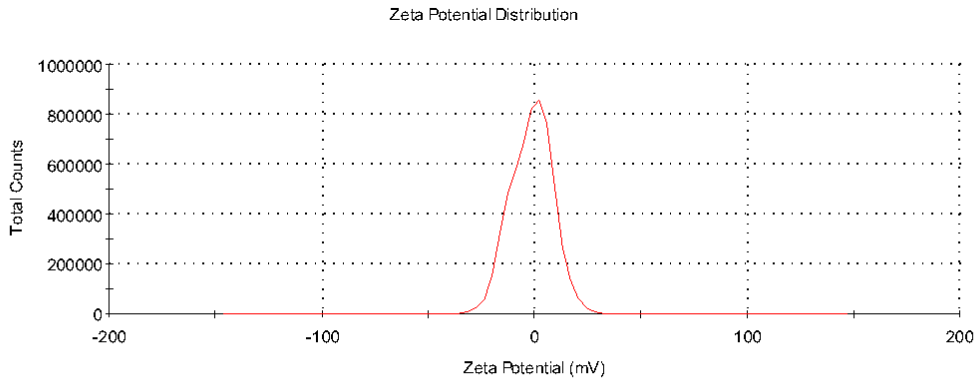
2. DLS of PEG(5)-BP-USPIOs (in saline)



3. DLS of PEG(5)-BP-USPIOs (in saline) after 7 months



4. Zeta-potential of PEG(5)-BP-USPIOs at pH 7.4 (in PBS)



5. Temperature stability study over 4 h at 50°C in saline (please note a different batch of PEG(5)-BP-USPIOs with slightly larger D_H was used for this study)

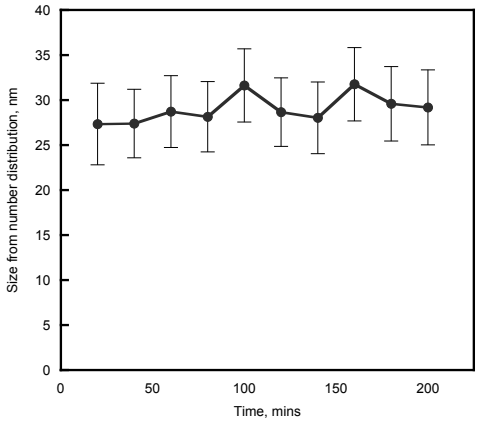


Figure S5. DLS stability study at 50°C in saline

7) Magnetic properties of PEG(5)-BP-USPIOs

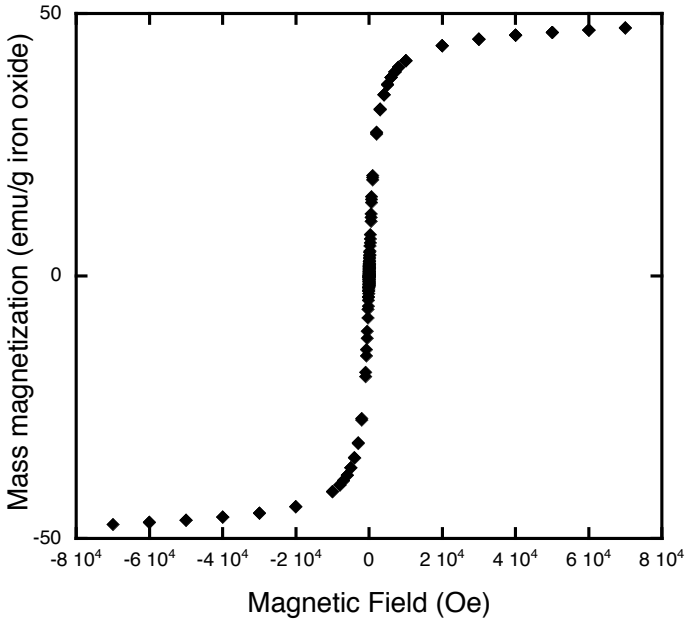


Figure S6. Magnetization at several magnetic fields at 300 K for PEG(5)-BP-USPIOs showing superparamagnetic behaviour and a M_s of 51 emu/g iron oxide. The weight of iron oxide was calculated by subtracting the mass of PEG as calculated by TGA.

8) Accumulation of USPIOs in liver after injection of PEG(5)-BP-USPIO

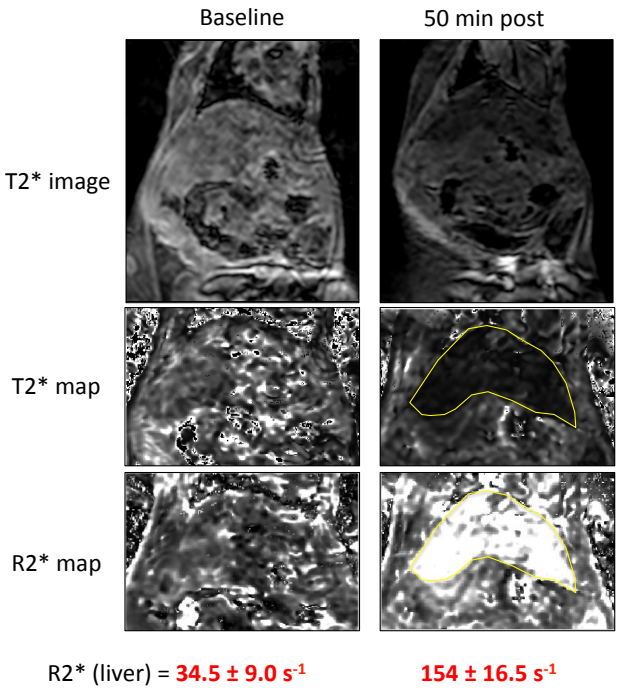


Figure S7. T2* mapping of PEG(5)-BP-SPIOs before (left) and 50 min after (right) showing accumulation of particles in liver (highlighted area)

9) SPECT-CT of radiolabeled Feraspin XS

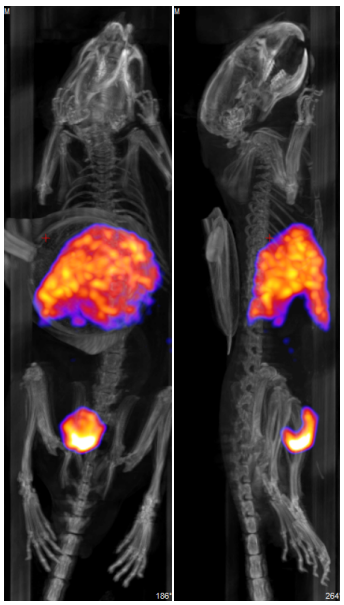


Figure S8. SPECT-CT images of a mouse injected with 3 MBq of radiolabeled Feraspin XS™ 50 min after injection showing accumulation of particles in liver and the bladder. The USPIOs were radiolabeled using Tc-99m-DPA-ale (30 min incubation at 37°C followed by purification by size-exclusion filtration (10 kDa MWCO)

10) SPECT-CT of radiolabeled PEG(5)-BP-USPIO - Kidneys uptake

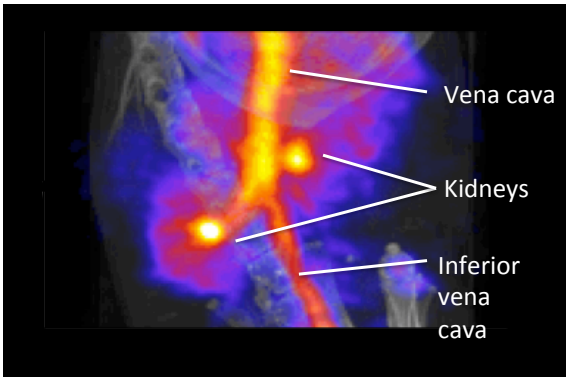


Figure S9. SPECT-CT images of a mouse injected with 20 MBq of radiolabeled PEG(5)-BP-USPIO 40 min after injection. Close up of the abdominal area showing high signal in the kidneys. For a full-body image see Figure 6 in the main text.

11) *In vivo* experiment with radiolabeled PEG(5)-BP-USPIO - Size exclusion chromatogram of urine at final time point (3.3 h)

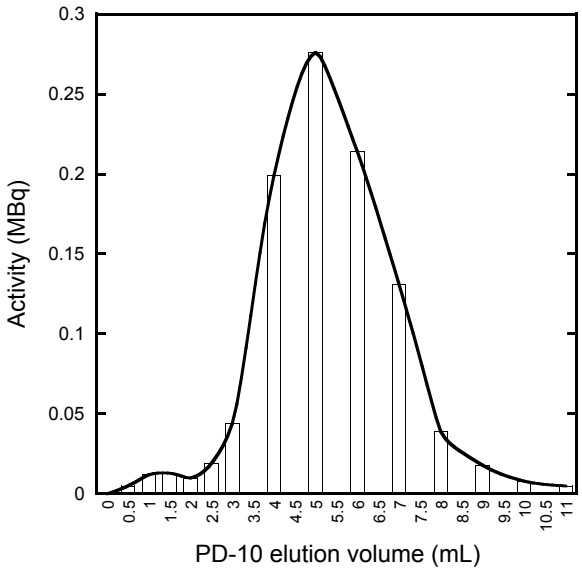


Figure S10. Size exclusion (PD10) chromatogram of a urine sample after 3.3 h injection of PEG(5)-BP-USPIO (Please note that 0.5 mL fractions were collected until a volume of 3 mL, whereas 1 mL fractions were collected after this volume). Most of the activity elutes as Tc-99m-DPA-ale (peak at 5 mL) whereas a small radioactive peak at a peak at 1-1.5 mL suggests some PEG(5)-BP-USPIOs elute intact.