Electronic Supplementary Material (ESI) for Journal of Materials Chemistry B.

SUPPLEMENTARY INFORMATION

Highly Cohesive Dual Nanoassemblies for Complementary Multiscale Bioimaging

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1. Nanoassembly colloidal stability in various media

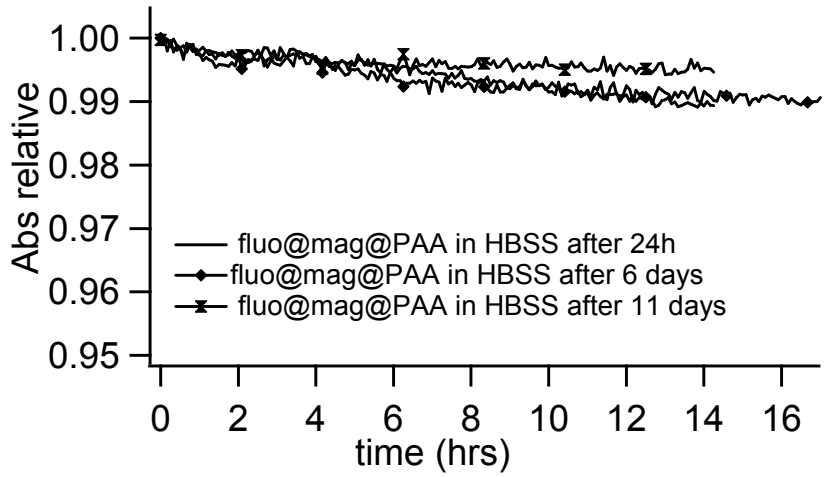

Figure S1. Relative absorbance at 450 nm of *fluo@mag@PAA* nanoparticles dispersed in HBSS buffer, recorded over a period of 14 h at 1 day, 6 days and 11 days after the solution was prepared. The presence of large agglomerates would lead to fast decantation and loss of absorbance, which is not observed here as absorbance changes by 1 % only.

2. Calculations of the number of superparamagnetic nanoparticles γ-Fe_2O_3 per nanoassembly *fluo@mag@PAA*

Number of dyes per FON

For all calculations, we have assumed that all dyes aggregated into spherical FON (average mean diameter D_H = 120 nm) after precipitation. The dye molecular volume was cZalculated to be 1.73 nm^3 from *ab initio* DFT calculations.

By knowing the concentration of the fluorophore stock solution (0.1 wt. %) and the exact volume (50 µL) injected in a solution of iron oxide nanoparticles (2.5 mL), the number of dyes per FON was thus calculated to be 5.24×10^5. From the dye number per FON and the total amount of dyes in the solution, the number of FON was valued to be 7.25×10^{10}.

Number of superparamagnetic nanoparticles

The average mean diameter of γ-Fe_2O_3 nanoparticles was measured to be 7 ± 0.1 nm.

Literature data such as the number of iron(III) ions per unit cell of crystalline maghemite and the volume of a single unit cell allowed us to calculate the number of iron(III) per nanoparticle, that was equal to 4970. ICP-OES titrations provided a total amount of 1.96×10^{18} iron(III) ions in the solution, hence we could value the total number γ-Fe_2O_3 nanoparticles to be 3.94×10^{14} in the solution.

Number of superparamagnetic nanoparticles par nanoassembly

We assumed identical magnetic coverage for all FON. From the previous calculations, the number of maghemite nanoparticle per FON could be inferred to be 5400 ± 230.

3. Two-photon excited fluorescence spectral detection and imaging

2.1. TPEF spectral detection

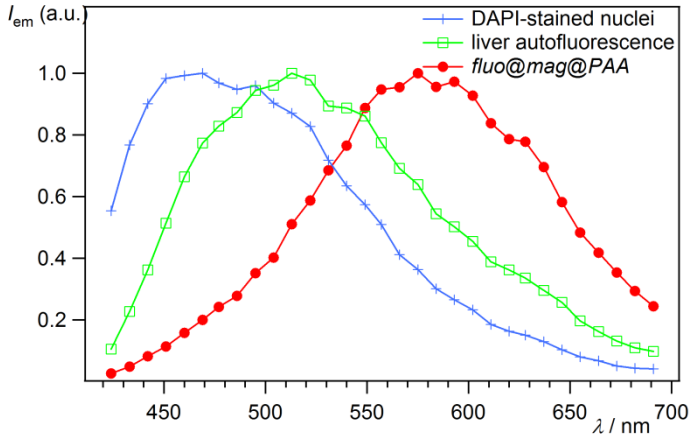

Figure S2. Spectral detection upon two-photon excitation at 830 nm of the fluorescence signal emitted by a 10 µm-thick section of mouse liver, stained with DAPI after fixation on glass coverslips.

2.2. TPEF imaging

KIDNEY

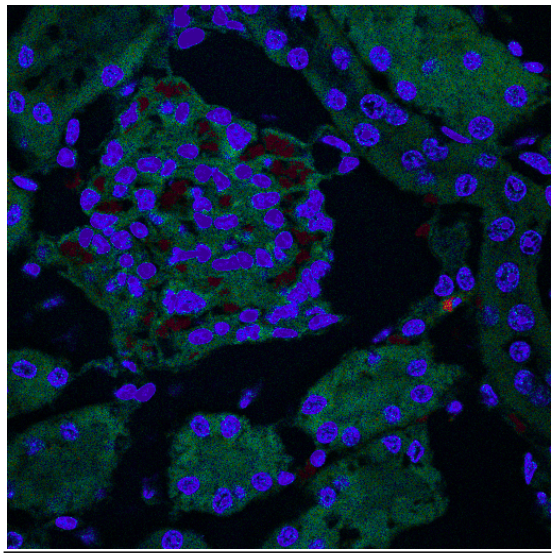

Figure S3. Two-photon excited fluorescence LSM image of a 10 μm-thick section of mouse kidney in channel mode (λ_{exc} = 830 nm). Only one red spot corresponding to the *fluo@mag@PAA* nanoassemblies is distinguishable in accord with the clearance process efficiently ensured by liver macrophages. The DAPI-stained nuclei appear blue while the rest of the tissue mostly emits in the green spectral range.

LUNG

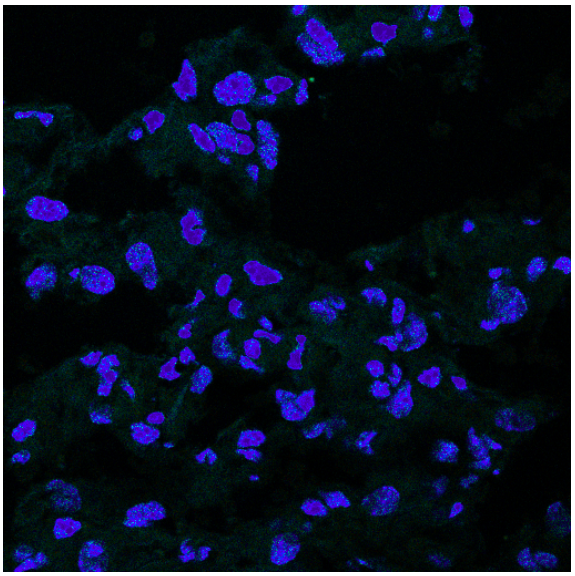

Figure S4. Two-photon excited fluorescence LSM image of a 10 μm-thick section of mouse lung in channel mode (λ_{exc} = 830 nm). No red spot corresponding to the *fluo@mag@PAA* nanoassemblies could be detected in accord with the clearance process efficiently ensured by liver macrophages.

HEART

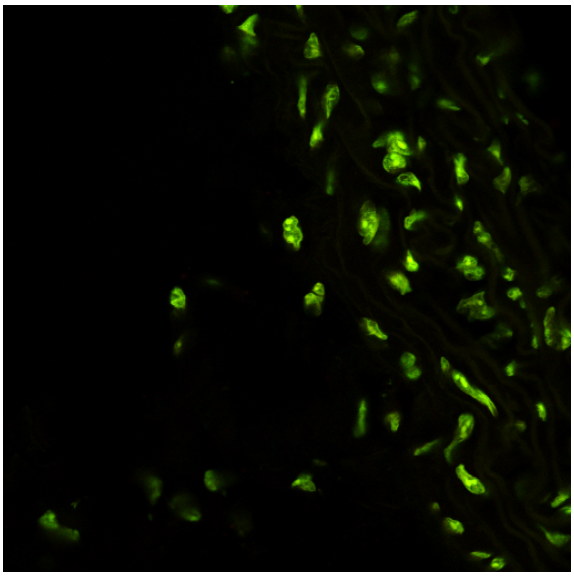

Figure S5. Two-photon excited fluorescence LSM image of a 10 µm-thick section of mouse heart in channel mode (λ_{exc} = 830 nm). No red spot corresponding to the *fluo@mag@PAA* nanoassemblies could be detected in accord with the clearance process efficiently ensured by liver macrophages.

LIVER

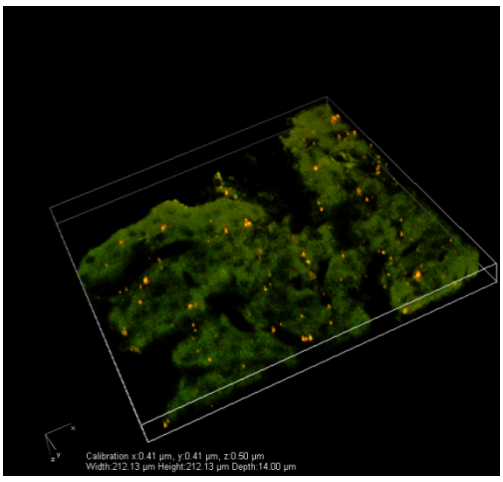

Figure S6. One-photon excited fluorescence LSM image of a 10 µm-thick section of mouse liver (λ_{exc} = 488 nm and λ_{em} > 510 nm). The *fluo@mag@PAA* nanoassemblies appear as orange dots imbedded in the green-autofluorescent liver. Spectral detection evidences an emission signal centered around 600 nm according to the emission properties of the *fluo@mag@PAA* nanoassemblies.

2.3. Fluorescence lifetime imaging (FLIM)

Fluorescence lifetime measurements were performed by means of the time-correlated photon counting module PicoHarp300 (PicoQuant) equipped with a picosecond pulsed laser diode LDH485 (λ_{exc} = 485 nm, fwhm <100 fs) which was directed to a Nikon A1R Si inverted microscope with a high

transmission oil-immersion microscope objective (Nikon, Plan Apo, 60×, NA = 1.4). Fluorescence time decay was recorded with a single-photon avalanche diode (SPAD) and analyzed by using SymPhoTime software (PicoQuant v 5.1.3.1).

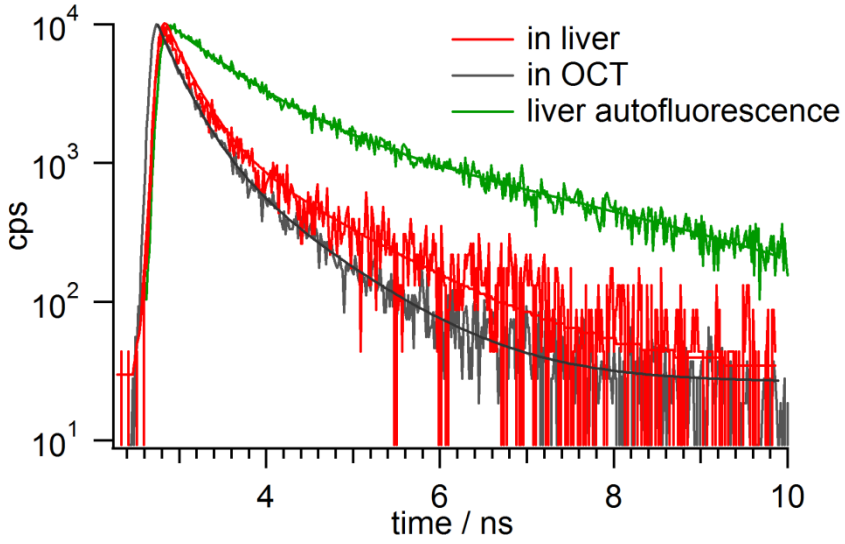

Figure S7. Fluorescence lifetime imaging of mouse liver section (λ_{exc} = 488 nm and λ_{em} > 510 nm) The fluorescence decays of *fluo@mag@PAA* nanoassemblies in liver (red) and dispersed in OCT (grey), to mimic the OCT-impregnated liver, were found very comparable. Upon multiexponential fitting, we obtained two main lifetime components at τ_1 = 1.28 ns and τ_2 = 0.28 ns in liver against τ_1 = 0.9 ns and τ_2 = 0.27 ns in OCT. By contrast, the autofluorescence decay of the liver devoid of *fluo@mag@PAA* nanoassemblies strongly differs and provided two main lifetime constants at τ_1 = 3.95 ns and τ_2 = 0.76 ns We can thus confidently ascribe the emissive orange dots to *fluo@mag@PAA* nanoassemblies and discard any significant contribution of potentially existing lipofuscin.

3. Tissue sectioning for optical and electron microscopy imaging

3.1. Organ extraction

The mice were sacrificed 24 h after the injection. Five organs (liver, spleen, kidney, lung and heart) were removed for *ex vivo* analyses in order to assess the biodistribution of the *fluo@mag@PAA* nanoassemblies. They were immersed in a bath of zinc-containing fixatives (Pharmingen, France) at 4°C for 48 h, and 4 wt. % paraformaldehyde - 20 wt. % sucrose solution at 4°C for 24 h. They were eventually frozen in OCT formulation (Optimum Cutting Temperature formulation made of water-soluble glycols and resins - Tissue-Tek) by using vapors of liquid nitrogen. Zinc-containing fixatives avoid fluorophore destruction during the fixing process. The frozen organs were stored at -80°C for 30 days before use.

3.2. Tissue sectioning for optical imaging

All organs were cut as 10 µm-thick sections at -20°C using a cryotome cryostat (Leica Biocut 2030). The sections were immediately deposited on superfrost glass slides, let dry for 2 h before being treated with Prolong (containing DAPI as a cell nucleus staining agent) and protected with an upper coverslit to allow for microscopy imaging. The sections were left at 4°C for at least 72 h before observation.

3.3. Tissue sectioning for TEM imaging

Preparation of sodium cacodylate buffer 0.2 mol.L^{-1}, pH = 7.4. Trihydrate sodium cacodylate (21.4 g), dihydrate calcium chloride (25 mg), and hexahydrate magnesium chloride (50 mg) were dissolved in distilled water (450 mL). The pH mixture was adjusted upon adding a 0.5 mol.L^{-1} HCl solution under magnetic stirring. Distilled water was eventually added to adjust the total volume to 500 mL.

Tissue fixation. The OCT-embedded tissues were primarily fixed with glutaraldehyde (2.5 wt. % / vol) for 2 h to ensure progressive thaw and structure preservation. They were subsequently washed with sodium cacodylate buffer for 10 min and postfixed with 2 % tetraoxide osmium for 1 h. They were finally rinsed twice in two distinct baths of fresh sodium cacodylate buffer for 5 min each.

Dehydration. Tissue dehydration was performed as follows: 50 % ethanol for 10 min, 70 % ethanol for 10 min, 90 % ethanol for 15 min, 100 % ethanol – 2 changes for 15 min each, propylene oxide – 3 changes for 15 min each.

Resin embedding. Resin infiltration was performed using a succession of a 1:2 mix of epoxy resin:propylene oxide for 30 min, a 1:1 mix of epoxy resin:propylene oxide for 30 min, and a 2:1 mix of epoxy resin:propylene oxide for 30 min. All samples were placed in fresh resin at ambient temperature overnight or at 4°C for 1-2 days. Polymerization was performed in an oven at 60°C for 48 h.

Sample (block) cutting. All blocks were cut as 70 nm-thin sections using a cryo-ultramicrotome (model Reichert Ultracut S) mounted with a diamond knife (Diatome). The sections were placed on the dark side of a holey carbon-coated copper grid (Delta Microscopies).

Post-staining. The grids were floated in a saturated solution of uranyl acetate in 50 % ethanol for 25 min at room temperature and rinsed briefly twice with Millipore water. They were floated on a drop of lead citrate for 10 min, in a water-free atmosphere achieved by the presence of sodium hydroxide pellets placed next to the lead citrate container. They were washed twice with 0.1 mol.L^{-1} sodium hydroxide and finally twice with Millipore water.

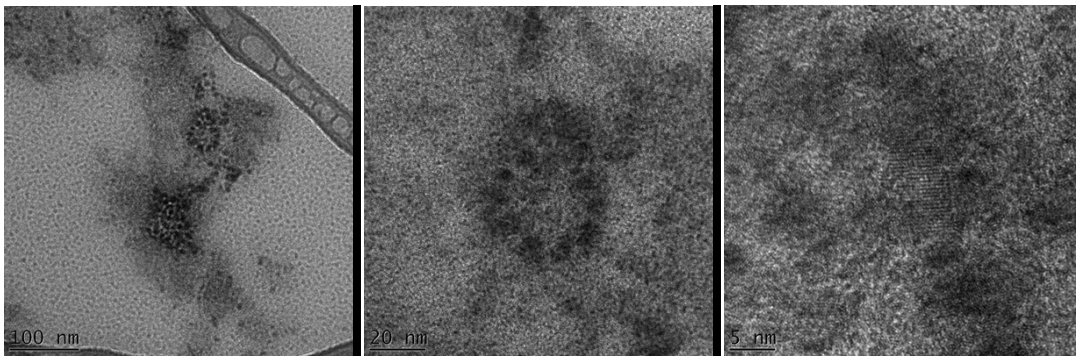

Figure S8. TEM image of a 70 nm-thin slice of mouse liver deposited on a holey carbon-coated grid. Increasing magnification from left to right: general view (left); zoom-in showing a circular arrangement of nanoparticles (middle); crystallographic planes, characteristic of γ-Fe_2O_3 crystalline nanoparticles, can be observed (right). EDX measurements aiming at evidencing the presence of iron from the maghemite nanoparticles failed due to the large hemoglobin content present in liver.