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Supporting Information

Highly stable monodisperse PEGylated iron oxide nanoparticle aqueous suspensions: a nontoxic tracer for homogeneous magnetic bioassays

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1 Experimental procedure

1.1 Materials

$FeCl_3 \cdot 6H_2O$ (98%), 1-octadecene (ODE, 90%), tetracosane (TC, 90%), hexane (95%), ethanol (99.8%), chloroform (99.9%), dopamine hydrochloride, sodium nitrite (97%), sulfuric acid (98%), celite (503), N-methylmorpholine, N, N'-Disuccinimidyl carbonate, ethanolamine, deuterium oxide (D_2O, 99.9 atom %D), N, N-Dimethylformamide (DMF) and Phosphate Buffered Saline (PBS, 100 mM, NaCl 0.138 M and KCl 0.0027 M, pH=7.4) solution were purchased from Sigma-Aldrich. Oleic acid (99%) and sodium oleate (97%) were purchased from TCI. $CDCl_3$ was purchased from Carl Roth GmbH. Hydroxyl-terminated poly(ethylene glycol) NHS ester (HO-PEG-NHS, M_w 3500 Da) was purchased from Jenkem Technology. Herceptin antibody was supplied by Max-Planck-Institute for Experimental Medicine, Göttingen, Germany. All the chemicals were utilized "as received" and without further purification.

1.2 Synthesis of oleic acid coated NPs

In a typical synthesis procedure, 2 mmol iron-oleate (synthesis recipe can be found in [1]), 6 mmol oleic acid and 10 mL solvent (1:1 w/w, ODE:TC)) were poured into a glass flask attached to a Schlenk line. Afterwards, the mixture was degassed and dried at $100°C$ for 10 min through a frequent evacuation and filling with argon. This step was carried out to eliminate any remaining traces of volatile impurities and water from the mixture. The resulting reddish transparent liquid was heated up to $357°C$ at a heating rate of $3°C$/min and refluxed for 60 min under flow of argon. The obtained black suspension was cooled to $60°C$, washed by adding a 3:1 acetone/hexane mixture and the particles were separated by centrifugation. Dissolving and precipitating of the particles via the solvent (hexane)-antisolvent (acetone) chemistry was repeated three times to remove the unreacted compounds. The oleic acid capped nanoparticles (NP-OA) were readily dispersed in chloroform or toluene.

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1.3 Synthesis of hydrophilic compounds

1.3.1 Nitrodopamine

Nitrodopamine was synthesized using the recipe reported by Malisova et al. [2]. Typically, dopamine hydrochloride (1.94 g, 10 mmol) and sodium nitrite (1.54 g, 22 mmol) were dissolved in 25 mL distilled water and cooled down to $0°$C in a salt-ice bath. Subsequently, 10 mL sulfuric acid (17.4 M) was added dropwise to the mixture and eventually a mustard colored precipitate was formed. The resultant mustard colored slurry was stirred at room temperature overnight, and then the precipitate was filtered and rinsed thoroughly with distilled water until no trace of acid was detected. Afterwards, it was rinsed again several times with methanol. The product was dried at $40°$C in high vacuum for two days and then stored in a vacuum desiccator (1,483 g, yield=63%). ^{1}H NMR (400 MHz, D_2O, δ): 7.73 (s, 1H, arom. H-d), 6.90 (s, 1H, arom. H-c), 3.32 (t, 2H, CH_2), 3.23 (t, 2H, CH_2). The NMR spectrum is shown in Fig. S1.

1.3.2 HO-PEG-nitrodopamine

Nitrodopamine (30 mg, 0.128 mmol) and N-methylmorpholine (20 mL) were loaded in a mixture of 10 mL ethanol and 2 mL chloroform. To this mixture, HO-PEG-NHS (500 mg, 0.14 mmol) was added and stirred at RT overnight. The crude product was filtered over celite (503), the filter material was washed with chloroform, and then the resultant clear green-yellowish solution was evaporated to dryness. The final product was dried at high vacuum and kept in a vacuum desiccator [2]. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.60 (s, 1H, arom. H-g), 6.93 (s, 1H, arom. H-f), 3.95 ppm (s, 2H, $CH_2 - c$), 3.2-3.8 (m, CH_2CH_2O, PEG-a,b; $2 \times$ CH_2-d,e). The recorded NMR spectrum is presented in Fig. S1.

1.4 Phase transfer: PEGylation of NP-OA nanoparticles

The NP-OA suspensions in chloroform were transferred into water using a robust and efficient ligand exchange reaction. Typically, 1 mL of the particle suspension containing 8.9 μmol (0.5 mg) iron was mixed with 3.9 μmol (14.2 mg) of HO-PEG-nitrodopamine, incubated at RT (Thermomixer comfort, Vaudaux-Eppendorf) for 20 h while being stirred at 800 rpm. Afterwards, 10 μL triethylamine was added to block the remaining active groups and then the mixture was incubated for another 4 h. After evaporating the solvent and drying the particles

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under vacuum at RT, the particles were dispersed in distilled water. Next, excessive unbound ligands were removed by ultra-centrifuging at 10000 g for 20 minutes. This process was repeated four times. The obtained pellets of the particles were dispersed in distilled water, filtered through a 200 nm syringe filter to eliminate big clusters, and stored at 4°C. To study the particle stability in physiologically relevant fluids, the PEGylated nanoparticles were transfered into PBS (100 mM, NaCl 0.138 M and KCl 0.0027 M, pH=7.4) using dialysis membranes with a cut-off of 10 kDa (Slide-A-Lyzer dialysis cassette, thermo scientific). The PEGylated particles are designated as NP-PEG.

1.5 Functionalization of NP-PEG particles with Herceptin

In order to activate the PEGylated NPs for a subsequent coupling with Herceptin antibodies, the hydroxyl terminated PEG moieties should be activated. Here, N, N'-Disuccinimidyl carbonate, a small homobifunctional NHS ester crosslinking reagent, was used to convert the hydroxyl terminal groups to NHS-carbonate ones using the protocol established by Hermanson [3]. In a typical experiment, firstly, the NP-PEG particles were transfered into DMF by centrifugation at 12000 rpm for 10 min. This process was repeated four times to completely exchange the solvent. After having the particles suspended in DMF ($\approx$ 0.5 mg_{NPs} mL^{-1}), 6 mg N, N'-Disuccinimidyl carbonate was added to the suspension and the mixture was incubated for 2 h at RT while being mixed at 800 rpm. Subsequently, the particles were washed three times with DMF to eliminate by-products. At last the particles were quickly washed with ice-cold water to eliminate any remaining traces of DMF prior to being redispersed in 1 mL phosphate buffer saline (coupling buffer, pH=7.4) containing 0.5 mg Herceptin and incubated for 2 h at RT while being gently shaken. Afterwards, 30 μL ethanolamine was added into the mixture to convert the unreacted NHS sites to the original hydroxyl groups. Next, an additional half an hour incubation was applied. The Herceptin functionalized particles were washed three times with the coupling buffer and finally dispersed in 1 mL distilled water and stored at 4°C.

1.6 Isolation and infection of bone marrow-derived macrophages

Macrophages were derived from bone marrow extruded from the femur and tibia of female C57BL/6 wild type mice. Bone marrow was flushed out of the bones by using ice-cold com-

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plete cell culture medium consisting of Dulbecco's Modified Eagle Medium (DMEM) medium (10 mM 2-(4-(2-Hydroxyethyl)- 1-piperazinyl)-ethansulfonic acid (HEPES), 2 mM L-glutamine, 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin and macrophage colony stimulating factor (PAN Biotech, Germany) in a concentration of 50 ng mL^{-1}) and cultured for 7 days at 37°C, 5% CO_2. At day 7, differentiated macrophages were harvested, counted, adjusted to 106 cell mL^{-1} and added to a 48-well tissue culture plate (250 mL $well^{-1}$) for infection. Macrophages were treated with the NP-PEG suspenions in DMEM medium at iron concentration varying from 1:10 (NP-PEG:DMEM) to 1:1000. The cells treated with 1μg mL^{-1} LPS (Sigma Aldrich, St. Louis, USA) served as a positive control and the non-treated ones as a negative control. Supernatants were collected after 24 h and used for ELISA and LDH determination.

2 Characterization

2.1 Structural and compositional properties

To study the crystallography of the synthesized particles, high resolution transmission electron microscopy (HRTEM) and selected area electron diffraction (SAED) analyses were performed utilizing a double spherical aberration corrected field emission microscope (JEOL, 200 keV). The samples were prepared by drying a drop of diluted particle suspensions on carbon coated TEM copper grids. To determine the amounts of different oxide phases, Mössbauer absorption spectroscopy was carried out on a ^{57}CoRh spectrometer equipped with a temperature controller, allowing us to sweep the operating temperature from 15 to 295 K. The dried particles were used for Mössbauer measurements.

2.2 Static and dynamic magnetic properties

Static maggnetization measurements were carried out on both NP-OA and NO-PEG suspensions and also on immobile NP-OA at 295 K using a Quantum Design Magnetic Property Measurement System (SQUID-MPMS). The immobile sample was prepared by solidification of 87 μL of the particle suspension with 87 mg of gypsum in polycarbonate capsules. Temperature dependent magnetization measurements were carried out on the immobile sample in an external DC magnetic field of 1 mT. All the presented magnetization data are corrected with

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respect to the diamagnetic effect of gypsum and solvents. Furthermore, the particle dynamic magnetic properties were studied by using an ac susceptometer (ACS) at a magnetic field amplitude of 95 μT and frequencies varying from 1 kHz to 1 MHz. ACS measurements were performed on the particle suspensions with an iron concentration of 10 mM.

The particle response to a rotating magnetic field (RMF) was measured utilizing our own built fluxgate-based setup [4]. The measurements were carried out on 150 μL particle suspensions with a rotating magnetic field of 1 mT and a frequency up to 5 kHz. The sample iron concentrations are given in the corresponding section in the main text.

2.3 Surface properties

Infrared (IR) spectroscopy (Bruker Tensor 27) was employed to study the particle surface properties prior and after the PEGylation. The ligand exchange success was evaluated by the IR technique too. To estimate the amounts of the bound OA and PEG molecules to the particle surface and their dissociation behavior, thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) (Netzsch, Thermisch Analyse, TG 209, Cell) were performed from $25°$C to $600°$C at a heating rate of $10°$C min^{-1} under flow of nitrogen (10 mL min^{-1}) and oxygen (20 mL min^{-1}).

2.4 Hydrodynamic size and stability assessment

The particle hydrodynamic size distribution prior and after the PEGylation and after Herceptin labeling was measured by photon-cross correlation spectroscopy (PCCS, Nanophox from Sympatec GmbH) at $90°$ backscattered mode. Temperature dependent PCCS measurements were carried out by heating up the particle aqueous suspensions from $25°$C to $40°$C in $5°$C steps with 10 min delay time prior to the respective PCCS experiment. In addition to PCCS, the phase lag obtained in RMF measurements were analyzed to gain more information about the particle hydrodynamic features.

2.5 ^{1}H NMR analysis

^{1}H NMR spectra were acquired with a Bruker Avance 400 MHz spectrometer at RT ($\approx$ 5 mg sample in D_2O or $CDCl_3$). Chemical shifts are given in ppm with respect to the residual solvent

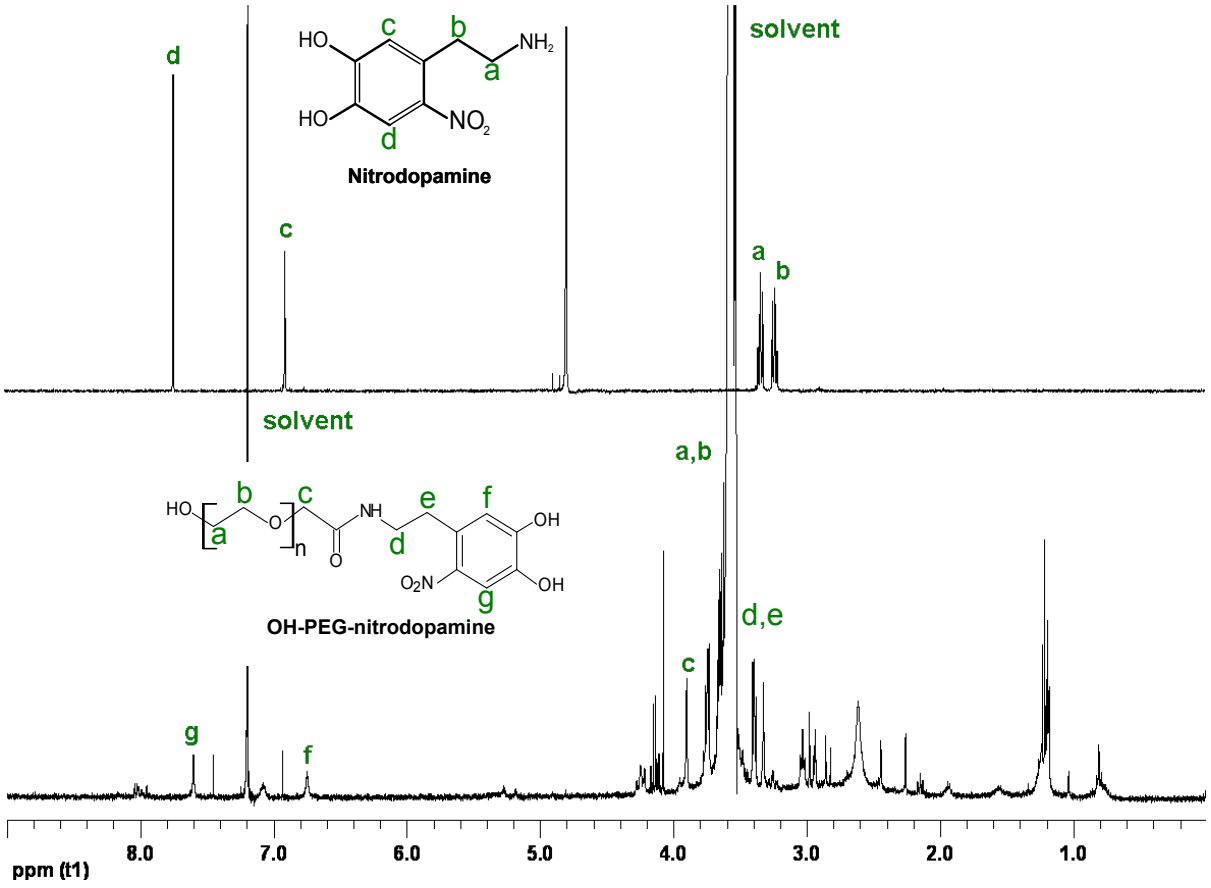

Fig. S 1 ^{1}H NMR spectra of nitrodopamine (400 MHz, D_2O, δ) and HO-PEG-nitrodopamine (400 MHz, $CDCl_3$, δ). The spectra were calibrated with respect to the solvent signal.

signals.

Fig. S1 presents the NMR spectra obtained for nitrodopamine and HO-PEG-nitrodopamine. In the nitrodopamine spectrum, the peaks corresponding to the aromatic ring protons can be seen at 7.63 (d) and 6.90 ppm (c). The peaks appearing at 3.32 (a) and 3.23 ppm (b) can be assigned to the methylene groups. The results match well with the previous findings by Malisova et al. [2]. The NMR spectrum of HO-PEG-nitrodopamine is more complex. The peaks related to the nitrodopamine ring protons (g and f) can be identified at 7.60 and 6.93 ppm. The peak at 3.90 ppm (c) can be attributed to the protons of the methylene groups (O-CH_2-(C=O)NH-)[2]. The PEG chain methylene groups (a and b) overlap with those corresponding to nitrodopamine and yet can be observed at 4.06 to 3.24 ppm. On the whole, the occurrence of an amide bond between nitrodopamine and PEG moieties can be concluded.

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2.6 Cytokine determination

The levels of interleukin (IL)-6 and interleukin (IL)-10 were determined by specific ELISA using matched antibody pairs and recombinant cytokines as the standards. Ninety six-well micro titer plates were coated with the corresponding purified anti-murine capture monoclonal anti-IL-6 or -IL-10 antibody (Pharmingen, San Jose, California, USA) at 2 μg mL^{-1} in sodium bicarbonate buffer overnight at 4°C. The wells were washed and then blocked with 3% bovine serum albumin-PBS before the cell culture supernatant samples and the appropriate standard were added to each well. Biotinylated rat monoclonal anti-IL-6 or -IL-10 antibody (Pharmingen) at 2 μg mL^{-1} was added as the second antibody. Detection was performed with streptavidin-peroxidase and the plates were developed using $3, 3', 5, 5'$-Tetramethylbenzidin (TMB).

2.7 LDH assay

The LDH level was measured in the supernatant of S. pyogenes-infected cells using the CytoTox 96 (Promega, USA) according to the manufacturer's instructions. Briefly, 50 μL supernatant of the treated macrophages was transferred into a 96-well enzymatic assay plate. Subsequently, 50 μL reconstituted substrate mix was added to each well and then the plate was incubated for 30 min at RT. Absorbance was measured at 490 nm. The cytotoxicity percentage was calculated as

[(sample LDH release - spontaneous LDH release)/(maximum LDH release - spontaneous LDH release)]$\times$ 100.

The spontaneous release is the amount of LDH released from the cytoplasm of uninfected macrophages whereas the maximum release is the amount released by total lysis of uninfected cells after treatment with 0.1% Triton X-100.

2.8 Particle uptake by macrophages: transmission electron microscopy and wide range parallel electron energy-loss spectroscopy (WR-PEELS) investigations

The particle uptake by macrophages was explored by a TEM microscope equipped with a Libra 120 plus energy-filter TEM (Zeiss, Germany) with a column-integrated Omega energy-

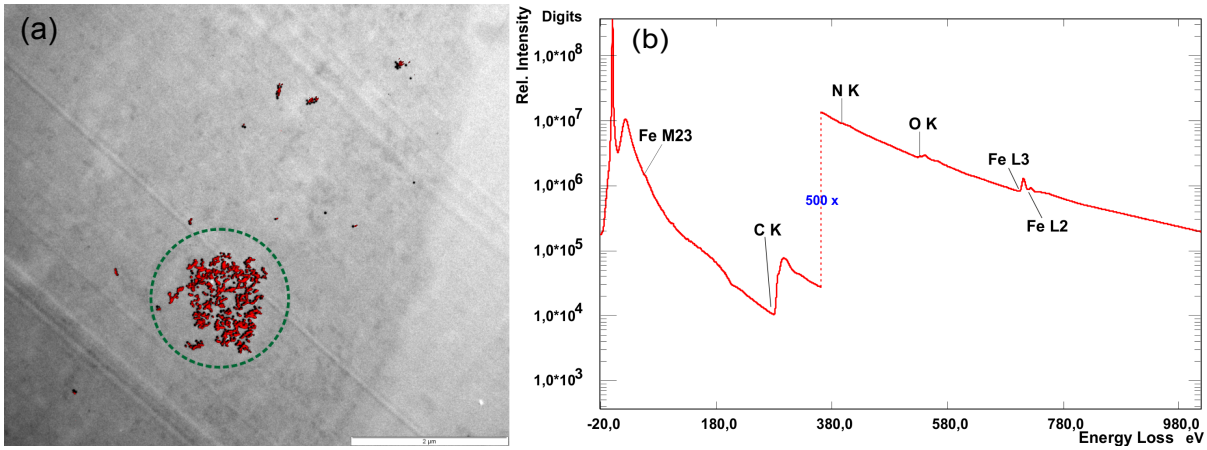

Fig. S 2 (a) ESI Fe-elemental map shown in red, superposed on the ultrastructural grey image of the motif, and (b) WR-PEELS spectrum taken from NP-PEG particles engulfed by macrophages.

filter. The samples were prepared according to the procedure established by Roming et al. [5] Possible deviations from the procedure caused by OsO_4 was excluded. The WR-PEELS was set from -20 eV to 1018 eV with an energy resolution of 1.3 eV (FWHM). Electron spectroscopic imaging (ESI) was utilized to obtain Fe-elemental maps. The ESI analysis was carried out based on the three-window method with the energy-slit set to 10 eV and the width to 711 eV, 690 eV (W1) and 660 eV (W2). The recorded images and analytical data were processed with iTEM-software (OSIS, Münster, Germany) and CorelDraw-Suite.

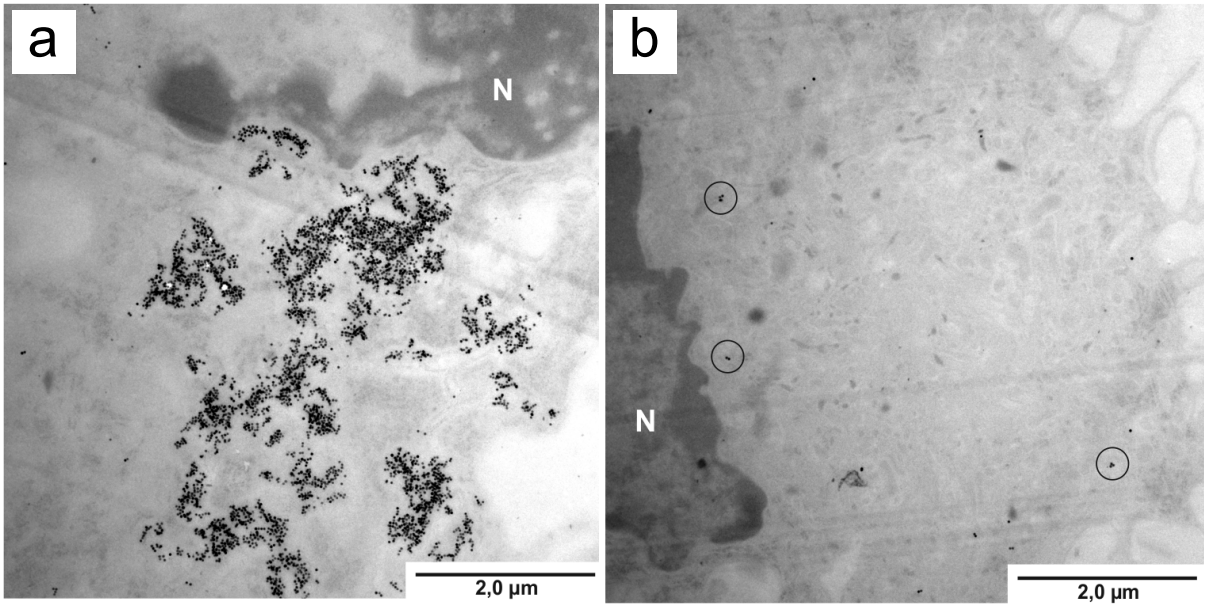

Fig. S 3 Enlarged TEM images of macrophages treated with NP-PEG suspensions in DMEM at iron concentrations of (a) 30 μg_{Fe} mL^{-1} and (b) 0.3 μg_{Fe} mL^{-1}.

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