

Supplementary information for

Microwave-driven synthesis of bisphosphonate nanoparticles allows *in vivo* visualisation of atherosclerotic plaque.

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1. Experimental.

Materials

Iron (III) acetylacetonate $\text{Fe}(\text{acac})_3$, 1,2- Dodecanediol, Oleylamine, benzyl alcohol, potassium permanganate, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysulfosuccinimide sodium salt (NHS) and Neridronate sodium trihydrate were purchased from Sigma Aldrich. Oleic acid was purchased from Fisher Scientific UK.

Microwave-assisted synthesis of oleic acid coated iron oxide nanoparticles (1)

In the microwave flask was added 0.5 g of $\text{Fe}(\text{acac})_3$, 1.4 ml of oleic acid, 0.6 ml of oleylamine, 0.3 g of 1,2-dodecanediol and 10 ml of Benzyl alcohol. The mixture was stirred at 60°C for 2 min in the microwave system at power max. After that, the temperature was increased from 60°C to 180°C and stirred during 20 min. Then, nanoparticles were collected using a magnet and washed three times with EtOH. Finally, nanoparticles were resuspended in 10 ml of chloroform.

Microwave-assisted synthesis of azelaic acid coated iron oxide nanoparticles (2)

0.28 mmol of KMnO_4 and 0.81 mmol of BTACl were dissolved in a mixture of $\text{H}_2\text{O}:\text{CHCl}_3$ (3:2 ml). The resultant solution was added to a 5 ml aliquot of OA-IONP in the microwave adapted flask. The mixture was heating at 105°C and 300W for 9 minutes. Then, 10 ml of phosphate buffer pH=2.9 were put into the flask and the microwave protocol repeated. After cooling step, nanoparticles were recovered using a magnet and washed twice with 5 ml of 10% NaHSO_3 . Nanoparticles were washed three more times with 1% NaOH and finally redispersed in phosphate buffer pH=7.0 yielding a stable aqueous dispersion.

Synthesis of Neridronate iron oxide nanoparticles (3)

To a 2 ml aliquot of NP-azelaic acid (3 mg ml⁻¹ in Fe) suspended in PBS 1X was added 12 mg of EDC (0.06 mmol) and 15 mg of Sulfo-NHS (0.07 mmol). The mixture was shaken in a vortex at r.t for 35 min. Then, nanoparticles were destabilized using a magnet and washed two times to avoid the presence of phosphates from PBS buffer and re-dispersed in HEPES 1mM pH= 7.7 buffer. 5 mg (0.02 mmol) of sodium neridronate were immediately put into the solution and the mixture was shaken in a vortex for 2 h and the resulting nanoparticles were separated with a magnet and washed with water. This last washing process was repeated four times to yield a stable water colloid.

Characterization of the nanoparticles

-Dynamic light scattering and Zeta potential

Hydrodynamic size and evolution of the Zeta potential against pH for the hydrophilic suspensions were evaluated in a Zetasizer NANO-ZS device (Malvern Instruments) provided with a He–Ne laser of 633 nm wavelength. Hydrodynamic size was measured at pH 7. Zeta potential was measured in a special Zeta potential cuvette at pH 7.

-Magnetic resonance characterization

Relaxometric properties were investigated by measuring T_1 and T_2 protons relaxation times at different dilutions. The relaxation time measurements were carried out in a Minispec MQ60 (Bruker) at 37°C and a magnetic field of 1.5 T. From the graph of the Fe-concentration dependent relaxation times, the relaxivities r_1 and r_2 were determined for nanoparticles **2** and **3**.

-FTIR characterization

Spectra were recorded in a Spectrum 400, Perkin Elmer, equipped with a simple universal IR sampling, directly from the solid nanoparticles.

-Nanoparticles uptake, Flow Cytometry Assay and Perls' Staining.

Approximately 1×10^6 control and treated cells were collected after 24 hours of incubation (10, 50 and 100 µg/ml neridronate-functionalised nanoparticles).

Cell uptake

Cells were trypsinised and measured in PBS. A total of 7,000 events were recorded for each sample using the BD FACSCanto™ II system. Forward scatter (FSC) diode detector, and photomultiplier tube (PMT) SSC detector was used in the assay. Samples

are analysed with BD FACSDiva™ Software and an average of the medians and standard deviations was calculated using FlowJo Software.

Different cell populations in a flow cytometer can be distinguished by the forward-scattered light (FSC) and side-scattered light (SSC) related to cell size and cell internal complexity respectively. SSC is increased in cells treated for 24 hours with neridronate-functionalised nanoparticles allowed us to define nanoparticle-cell interaction.

Additionally, cell culture was stained with Perls' Staining to detect SPIO-labeled cells. Slides were stained with 1% aqueous potassium ferrocyanide in 2% solution of HCl for 15 min and counterstained with nuclear fast red.

Cell lines proliferation and Cytotoxicity assays.

C57BL/6 mouse adult fibroblasts (MAFs) were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 5% fetal bovine serum (FBS), 1% penicillin-streptomycin and 1 mM sodium pyruvate in a humidified atmosphere of 5% CO₂ at 37°C. Cytotoxicity and nanoparticle uptake were assessed in MAFs exposed to NPs at different concentrations and times of incubation. Control cells were treated with vehicle (PBS 1x).

After culturing for 24h, 48h and 72 h, the cells were trypsinized and cell proliferation was assessed using the Neubauer counting-chamber under the optical microscope. Cell viability is determined by adding DNA binding dyes. DAPI was then added to a final concentration of 0.001% (w/v), and cells were analyzed by flow-cytometry using the BD FACSCanto™ II system. All experiments were performed in triplicate.

2. Figures.

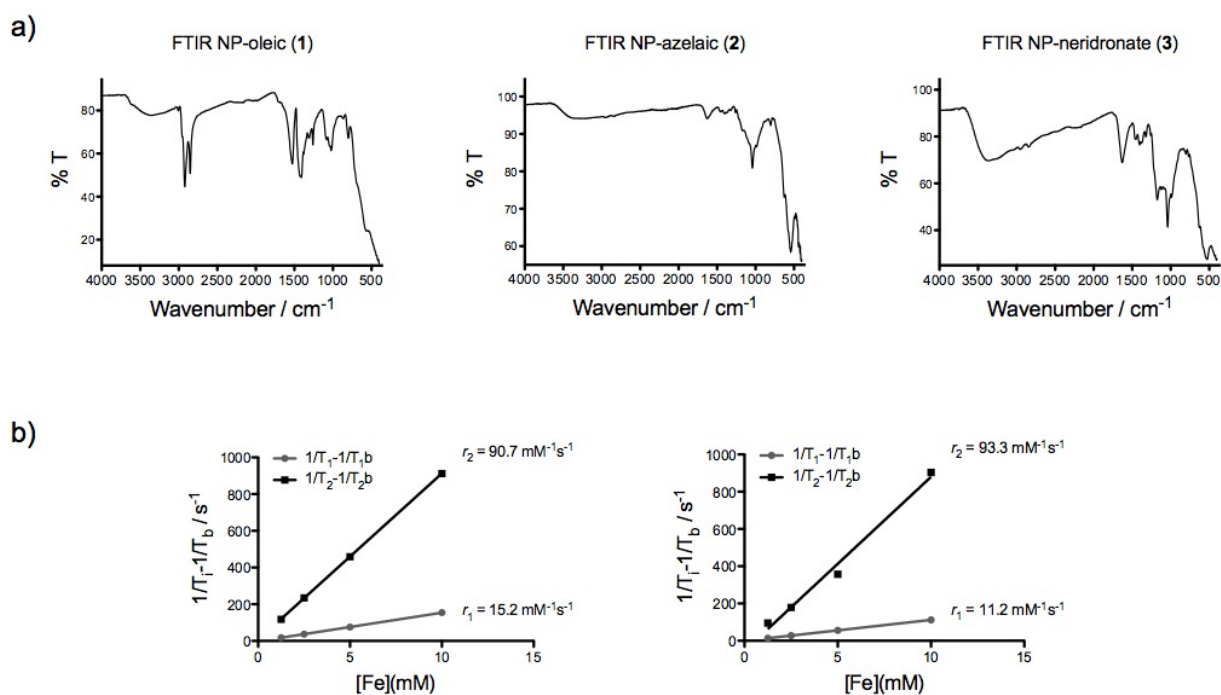


Figure S1. a) Infrared spectra for the three nanoparticles synthesised in this work; b) relaxivity study for nanoparticles 2 (left) and 3 (right).

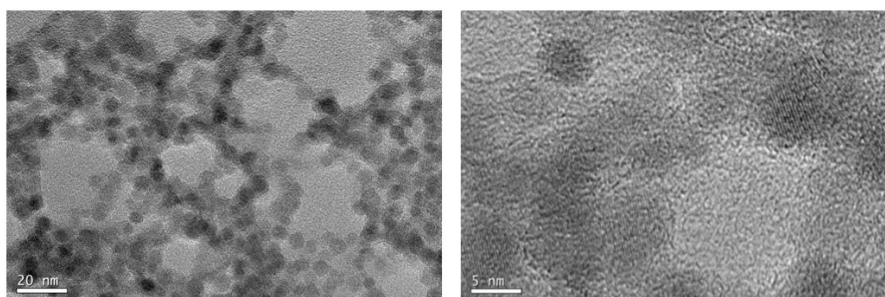


Figure S2. TEM images, at two magnifications, for nanoparticles 2.

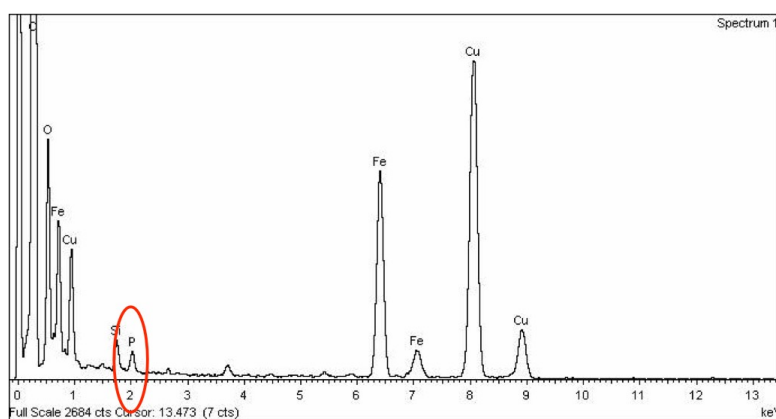


Figure S3. EDX spectrum for nanoparticles 3.

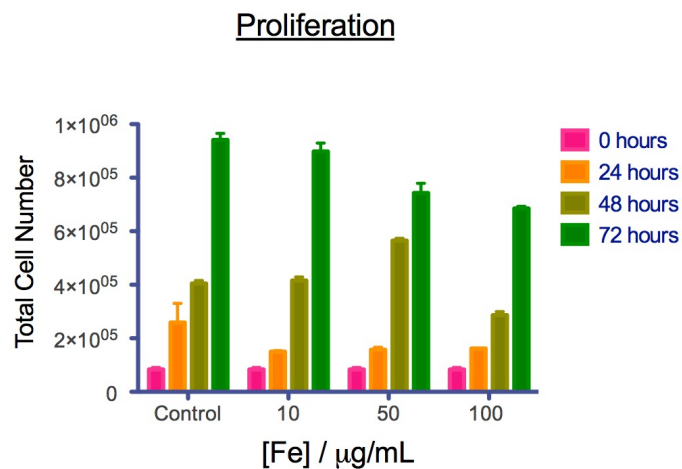


Figure S4. Cell proliferation experiment for three concentrations of nanoparticles **3** and three incubation times.

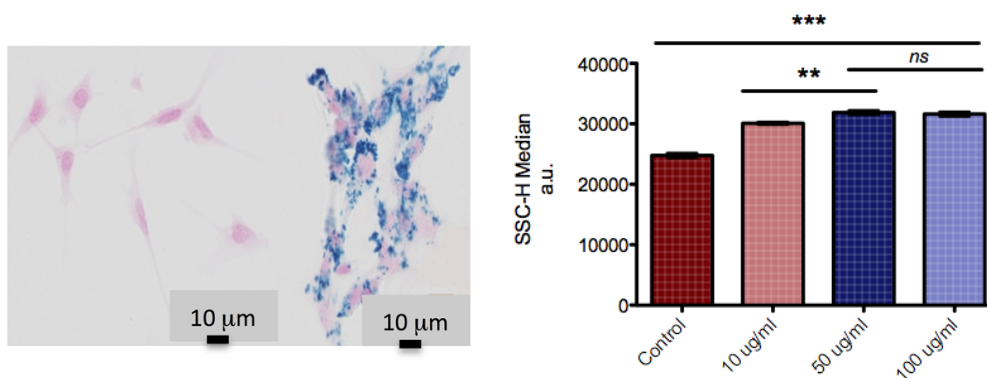


Figure S5. a) Perls' staining of Control cells (left) and labeled neridronate-functionalised nanoparticles cells (right, positive cells in blue) after 24 hours of incubation ($C=50 \mu\text{g/ml}$). Bar= $10 \mu\text{m}$; b) Data are shown for Side scatter (SSC-H) with different concentrations of neridronate-functionalised nanoparticles (10, 50 and $100 \mu\text{g/ml}$) for 24 hours.

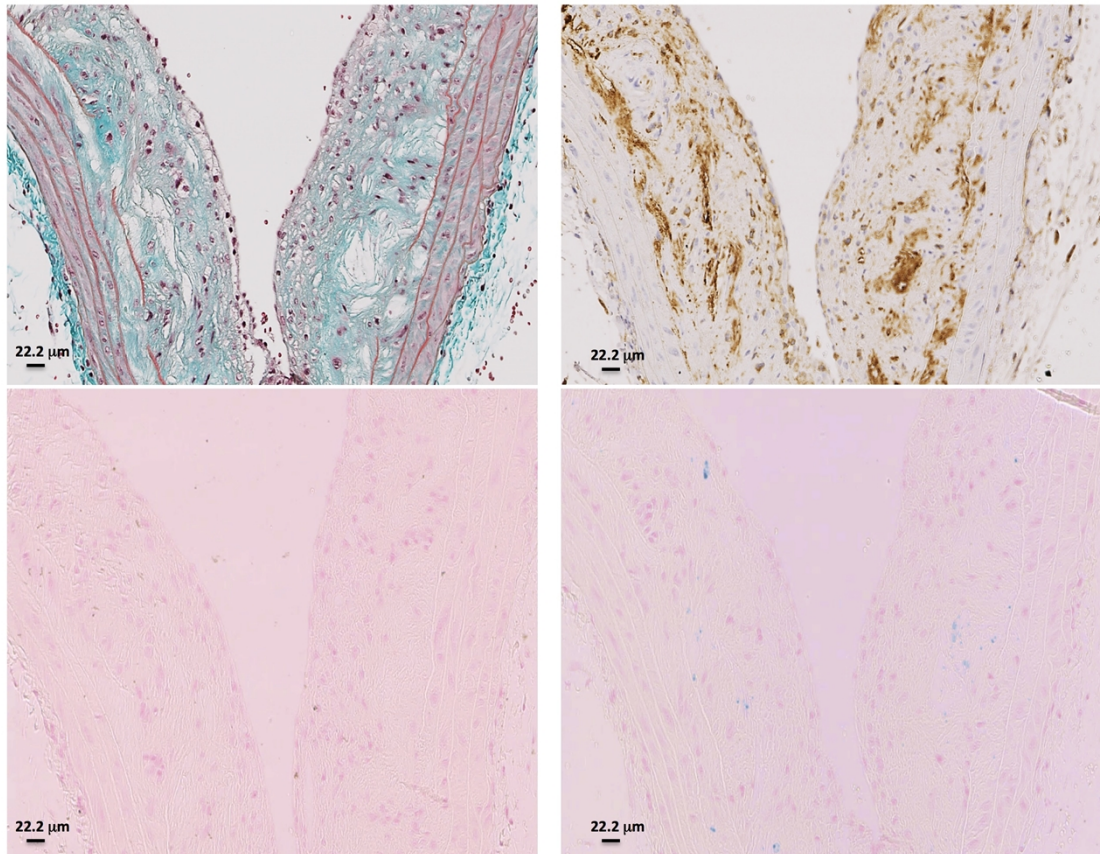


Figure S6. Histology of aorta plaque sections stained, from left to right, for fibrosis, macrophages, calcium vesicles and iron deposits.

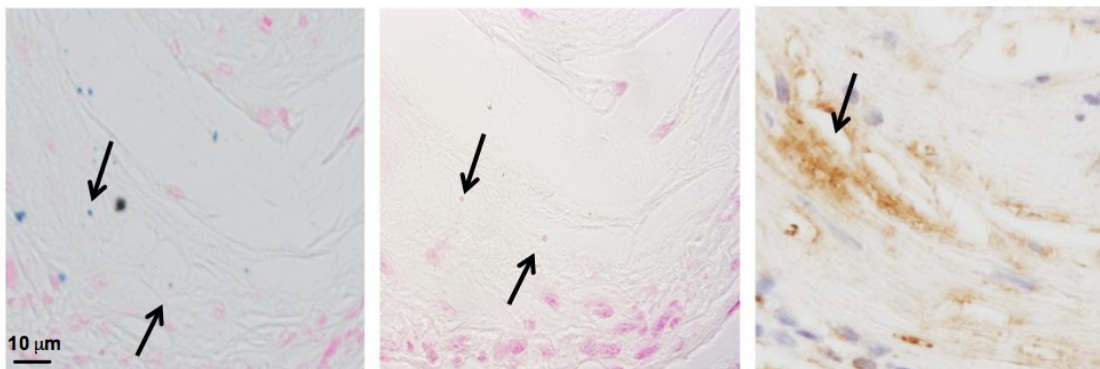


Figure S7. Histology of carotids plaque sections stained, from left to right, for iron, calcium vesicles and macrophage.