Synthesis of β-cyclodextrin conjugated superparamagnetic iron oxide nanoparticles for selective binding and detection of cholesterol crystals

Supporting Information

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Content

I. Materials and instrumentation

Carboxymethyl-β-CD sodium salt was purchased from Sigma. ConA-HRP and 3,3′,5,5′-tetramethylbenzidine (TMB) were the products of Invitrogen and ACROS, respectively. Other reagents including dextran (molecular weight distribution: 9000-11000), β-CD (≥ 97%), cholesterol (cell culture degree, ≥ 99%), iron (III) chloride hexahydrate ($FeCl_3$•$6H_2O$, 99%), iron (II) chloride tetrahydrate ($FeCl_2$•$4H_2O$, 99%) and epichlorohydrin (99%) were purchased from Sigma-Aldrich and used without further purification. Ammonium hydroxide (28-30 wt%) was purchased from CCI (Columbus Chemical Industries, INC.) and used as received. Water used is highly pure deionized with a resistance higher than 18 MΩ.

UV-vis measurements were carried out on a UV-4001 spectrometer (HITACHI HIGH-TECHNOLOGIES Co., Japan). DLS measurements were performed on a Zetasizer Nano Series aparatus (Malvern, UK). Images of cholesterol crystals were obtained from an Olympus CKX41 inverted light microscope and an Olympus FluoView FV1000 confocal laser scanning microscope. TGA was measured on a NETZSCH instrument (STA 449 F3 Jupiter, USA) under a nitrogen atmosphere. Magnetic moments of the NPs were measured on a SQUID magnetometer from Quantum Design, Inc. TEM images were collected on a JOEL 2200FS electron transmission microscope.

II. Experimental procedures

Preparation of needle-like cholesterol crystals

The needle-like cholesterol crystals used in this study were prepared according to the procedures reported in the literature.[1]

*Synthesis of β-CD-conjugated superparamagnetic iron oxide nanoparticles (β-CD-NP **2**)*

To a round bottom flask which was immersed in a water-ice bath, a freshly prepared stock solution of 4.5 g dextran and 0.3259 g iron (III) chloride hexahydrate (1.2 mmol) in 8 mL H_2O were added through a 0.22 μm filter (MILLEX®GS filter unit). The filter was further rinsed with 2 mL H_2O. A stock solution of 0.2391 g iron (II) chloride tetrahydrate (1.2 mmol) in 0.9 mL H_2O was prepared freshly and filtered through a 0.22 μm filter, from which 0.45 mL was injected into the flask dropwise under nitrogen and strong stirring. After stirring for half an hour, 0.45 mL ice-cold ammonium hydroxide was added dropwise and the mixture was then heated to ~ 80 °C. Reaction was allowed for 90 min. Upon completion, the reaction mixture was cooled to room temperature and the excess dextran and inorganic ions were removed by ultrafiltration to give the dextran-coated iron oxide nanoparticles. The total volume of this nanoparticle solution was concentrated to 20 mL

by ultrafiltration and filtered through a 0.22 μm filter, to which 20 mL 5 M NaOH and 4 mL epichlorohydrin were added and the mixture were stirred at room temperature for 24 hours to crosslink the dextran on the nanoparticles.[2] The nanoparticles were purified by dialysis against deionized water with over 10 exchanges of water during two days to give NP **1**. The volume of NP **1** was concentrated to 30 mL by ultrafiltration, to which 8 mL ammonium hydroxide (28-30 wt%) was added and the mixture was reacted at 37 °C for 36 h giving NH_2-NP **1**.

Commercially available carboxymethyl-β-cyclodextrin sodium salt (average three carboxymethyl groups per β-CD, Sigma-Aldrich) was treated with acid resin Amberlite® IR 120 to form carboxymethyl-β-CD. Carboxymethyl-β-CD (0.311 g) was dissolved in water (4 mL) and acetonitrile (2.5 mL), to which N-methyl morpholine (NMM, 100 μL) and 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT, 60 mg) were added successively. After stirring at room temperature for 1 hour, a solution of NH_2-NP **1** (55 mg in 15 mL H_2O) was added and the mixture was stirred at room temperature for 20 hours, followed by neutralization with Dowex H^+ resin. The resin was filtered out and the as-produced β-CD-SPION was purified by dialysis.

For conjugation with the fluorescent probe, 10 mg FITC dissolved in 5 mL DMSO was added to a solution of β-CD-SPION (50 mg in 25 mL H_2O) and this mixture was stirred at room temperature for ~ 2 days. The excess of FITC was removed by dialysis.

Any potentially remaining amine groups on β-CD-SPION and FITC-β-CD-SPION were capped with methoxy acetic acid by amidation reaction using CDMT and NMM as mentioned above producing NP **2** and FITC-NP **2**.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA plate (Nunc MaxiSorp) was coated with cholesterol crystals by evaporating an ethanol solution of cholesterol (1 mg/mL, 200 μL per well) and then blocked with 5 wt % BSA (300 μL/per well) at 37 °C for 2 h. After washing three times with PBS (presaturated by cholesterol), nanoparticle solutions with varying concentrations were added (200 μL per well) and the plate was incubated at 37 °C for 16 h. The plate was washed three times with PBS before ConA-HRP (0.1 μg/mL, prepared in 1 wt % BSA/PBS) was added (300 μL per well). The plate was incubated at 4 °C for 2 h and then washed three times with PBS. TMB (composition: 5 mg TMB + 2 mL DMSO + 18 mL citric buffer + 20 μL H_2O_2) was then added (200 μL per well) and the plate was stored in dark for 15 minutes for colour development. After the blue colour appeared, the reaction was quenched by addition of 0.5 M H_2SO_4 (100 μL per well). The solutions were transferred to a new

ELISA plate by taking 200 µL from each well and the absorbance at 450 nm was recorded on an iMark™ Microplate Reader.

Prussian blue staining of cholesterol crystals

To each glass bottle containing 5 mg cholesterol crystals, 1 mL of each nanoparticle solution (0.8 mg/mL) were added. The samples were incubated at 37 °C for 16 h. The cholesterol crystals were washed with water for at least three times. Freshly prepared 10 wt % $K_4Fe[CN]_6$ and 20 wt % HCl were mixed with a volume ratio of 1 : 1 and added to each bottle (1 mL per bottle). The samples were left for 15 min for colour development before further characterizations.

Rabbit tissue staining without exogenous cholesterol crystal deposition and TEM study

Atherosclerotic rabbit aorta tissues were placed in 1.5 mL Eppendorf tubes. One group of tissue received NP **1** (200 µl) while the other received NP **2** (200 µl) at the same concentration (0.8 mg/ml). The tissues were incubated at 37^{o}C for 12 h after which the nanoparticle solutions were removed and the tissues were washed with PBS (5 times).

For Prussian blue staining, after the final wash, fixing solution (paraformaldehyde/glutaraldehyde 2.5 % each in 0.1 M sodium cacodylate buffer pH 7.4) was added to each tissue (400 µl). The tissues were incubated for 12 h at 0^{o}C, followed by washing with PBS (3 times) and drying on a lyophilizer. The tissues were then dipped for 10 seconds in a 1:1 solution of 10% potassium ferrocyanide trihydrate ($K_4Fe(CN)_6.\ 3H_2O$) and 20% HCl solutions. The tissues were then washed with water for four times. A blue colour was observed on the areas bearing iron oxide nanoparticles.

For TEM study, fixing solution (paraformaldehyde/glutaraldehyde 2.5 % each in 0.1 M sodium cacodylate buffer pH 7.4, 400 µl) was added to each tissue after NP incubation and washing. The tissues were incubated in the fixing reagent for 4 h at room temperature. The fixing solution was removed, and the tissues were washed twice (5 min each) with 0.1 M sodium cacodylate buffer at room temperature. The tissues were incubated in 2% osmium tetraoxide solution for 2 h at room temperature followed by washing once (5 min) with 0.1 M sodium cacodylate buffer. The tissues were then incubated in an ascending series of hexylene glycol solutions (30%, 50%, 70%, 90% hexylene glycol in 0.1 M sodium cacodylate buffer, 15 min each at room temperature). Note that ethanol was avoided in this protocol and replaced by hexylene glycol to minimize the solubilization of the cholesterol deposits in the plaque. The tissues were incubated with 100% hexylene glycol with swirling (3 times, 10 min each at room temperature), which were then infiltrated and embedded in 100% Durcupan resin, and blocks were made on silicone molds. Sections were

collected on TEM copper grids. Images were obtained on a JOEL 100 CXII (Japan electron optics laboratories) (Fig. 3b, c). X-ray photoelectron spectroscopy (XPS) data was collected on a JOEL 2200FS TEM (Fig. S8).

Prussian blue staining of rabbit tissue with exogenous cholesterol crystal deposition

Two rabbit aorta tissues were mounted on charged glass slides. For each slide, one half of the tissue was treated with an ethanol solution of cholesterol (1 mg/mL; 2 × 0.5 μL). After ethanol was evaporated, the tissues were treated with NP **1** and **2**, respectively at the same iron content (0.78 mg/ml, 30 μL/tissue). The tissues were covered with glass cover slips and transferred to a box containing deionized water and then incubated at 37 °C for 24 h. The cover slips were removed and the tissues were washed with water four times. The tissues were transferred to a Coplin Chamber containing 10% $K_4Fe[CN]_6$ solution and incubated for 10 min, then transferred to a Coplin Chamber containing a mixture of 10 wt % $K_4Fe[CN]_6$/20 wt % HCl with a volume ratio of 1:1 for 20 min (Gomori's modified Prussian blue). The tissues were washed with water for four times. Nuclear fast red (50 μL) was added above each tissue and incubated for 5 min. The tissues were rinsed with distilled water for four times. Images were collected on an Olympus CKX41 inverted light microscope (Fig. S7).

MRI of the nanoparticle adsorbed cholesterol crystals

For MRI, the nanoparticle adsorbed cholesterol crystals were embedded in polyacrylamide gels. MRI measurements were carried out on a GE 3T Signa HDx MR scanner (GE Healthcare, Waukesha, WI) with a wrist coil. 3D FSPGR T2*-weighted images were acquired with the following parameters: flip angle 15°; echo times (TEs) 5.9 ms, 14.5 ms, 23.0 ms, 31.6 ms, 40.2 ms, 48.7 ms, 57.3 ms, and 65.9 ms; time of repetition (TR) 72.5 ms; receiver bandwidth (rBW) 15.6 kHz; field of view (FOV) 6 cm; slice thickness 1.5 mm; number of slices 16; acquisition matrix 256 × 256; number of excitation (NEX) 1; and scan time 1 min 39 s.

Determining the R_2 values for the NP **2***

Five different dilutions of NP **2** were prepared to a final volume of 5 ml in 15 ml-centrifuge tubes (Corning). The tubes were placed on a polystyrene tube holder. All MRI experiments were carried out on a GE 3T Signa® HDx MR scanner (GE Healthcare, Waukesha, WI). To evaluate the R_2* characteristics of the nanoparticles, the following parameters were used: head coil, 3D fast spoiled gradient recalled echo sequence, flip angle = 15°, 16 TEs = 2.1 ms, 4.6 ms, 7.0 ms, 9.4 ms, 11.8 ms, 14.3 ms, 16.7 ms, 19.1 ms, 21.5 ms, 24.0 ms, 26.4 ms, 28.8 ms, 31.2 ms, 33.7 ms, 36.1 ms, and 38.5 ms, TR = 41.9 ms, rBW = ± 62.5 kHz, FOV = 16 cm, slice thickness = 1.5 mm, number

of slices = 16, acquisition matrix = 256 × 256, NEX = 1, and scan time = 1 min 55 sec. [Fe] was plotted against (1/T2*), and R_2* is the slope of the generated straight line (Fig. S5).

III Figures and Tables

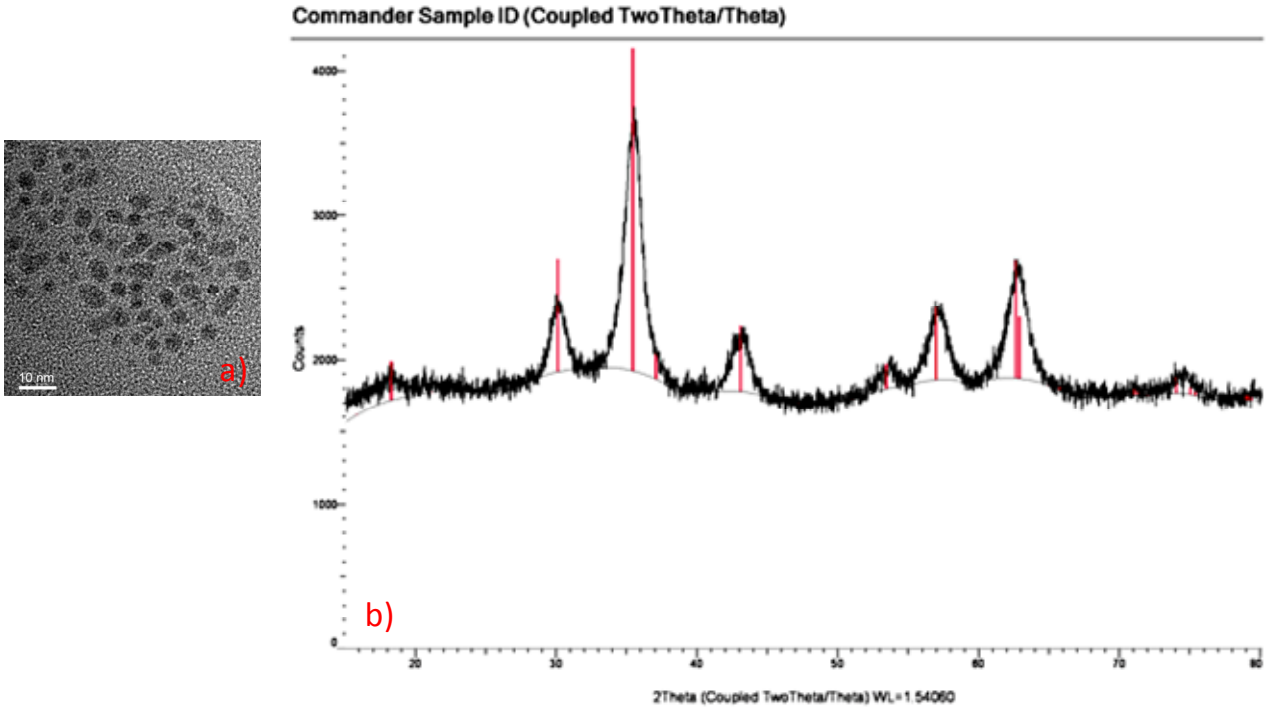

Fig. S1. a) TEM image of β-CD conjugated SPION (NP **2**) showing average core size of the NP was 6 nm. b) Power XRD spectrum of NP **2** confirming the core of NP **2** was Fe_3O_4 magnetite. The red lines are the magnetite peak patterns from the database.

Table S1. Zeta potential and hydrodynamic diameters of various nanoparticles.

	Zeta potential (in H_2O, mV)	Hydrodynamic diameter (nm)
NP **1**	-42.0	
NH_2 NP **1**	-12.0	42.0
NP **2**	-40.2	56.8

NH_2 NP **1** had less negative zeta potential than NP **1**, which was consistent with amine functionalization of the NP surface since the amino groups should be protonated in pH neutral media thus reducing the negative zeta potential values of NPs. The zeta potential of NP **2** became more negative following carboxymethyl β-CD reaction as the amino groups on NH_2 NP **1** were neutralized upon forming the non-charged amide bonds. After β-CD conjugation, the average hydrodynamic diameter of the NPs increased from 42.0 nm to 56.8 nm, consistent with the additional coating. The hydrodynamic diameter was larger than the diameter shown by TEM. This is because TEM measures the size of the NP core under vaccum, while the hydrodynamic diameter is a measure of the size of the core, the total coating as well as the hydrated layer in solution.

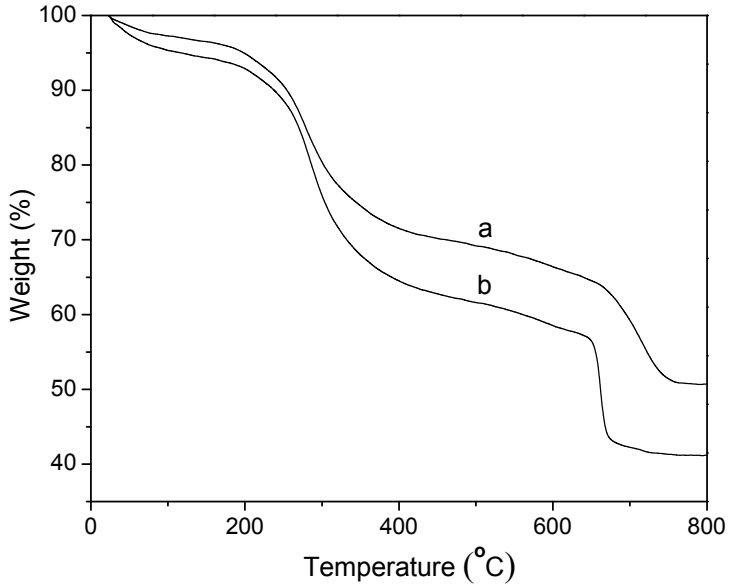

Fig. S2. TGA of iron oxide nanoparticles of NH_2-NP **1** (curve a) and NP **2** (curve b). NP **2** showed an extra 10% weight loss when the sample was heated to 800 °C due to β-CD conjugation. Based on TGA data before and after β-CD conjugation, it can be determined that 42% of the NP **2** weight came from Fe_3O_4 core, 39% came from the dextran coating and 19% was due to β-CD.

The number of β-CD molecules on each NP **2** particle was estimated as the following:

The lattice volume of magnetite is 592 $Å^3$ and Z (the number of Fe_3O_4 per lattice) = 8. The average core diameter of NP **2** is 6 nm by TEM. Assuming the NP is a sphere, the number of lattices in one particle is 191 and the number of Fe_3O_4 (MW 232) molecule in each particle is 1528. Therefore, since the weight ratio of core to β-CD is 42% : 19%, there are on average 125 β-CD molecules (MW 1308) per NP.

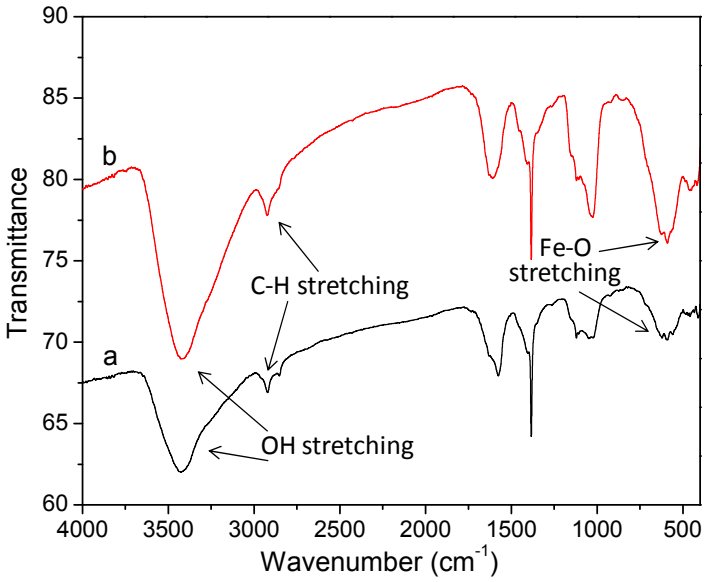

Fig. S3. FTIR of iron oxide nanoparticles before (curve a) and after (curve b) β-CD conjugation.

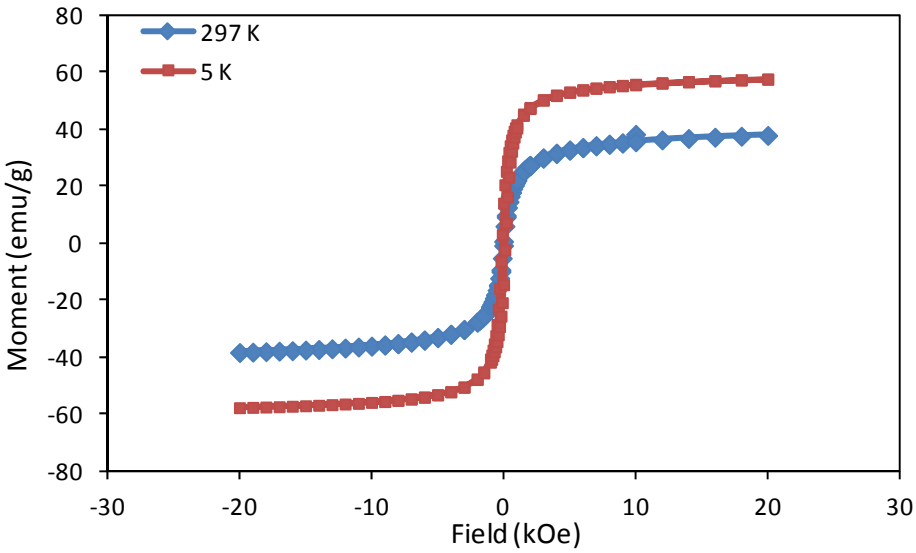

Fig. S4. Field-dependent magnetization of NP **2** at 297 K (blue line) and 5 K (red line) using SQUID. NP **2** exhibited superparamagnetic properties as evident from the absence of a hysteresis loop.

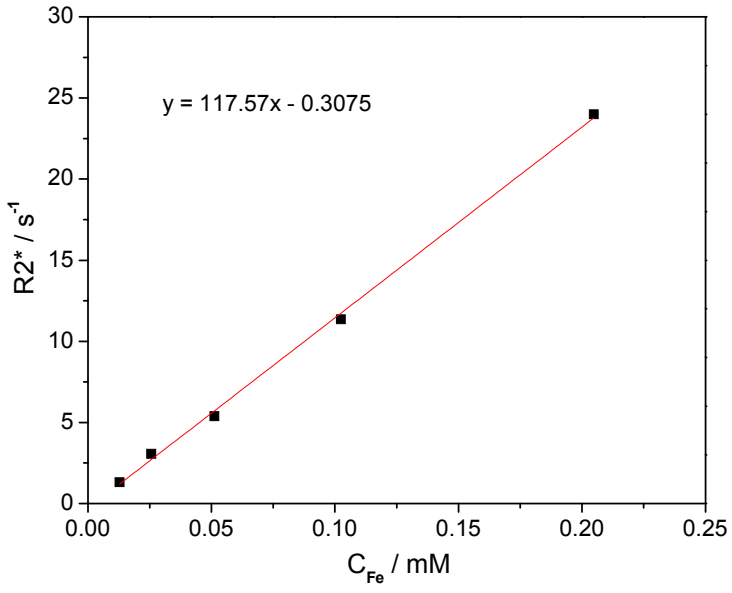

Fig. S5. Relaxivity measurements for β-CD conjugated NP **2**. (The relaxivity of NP **1** was similar to that of NP **2**.)

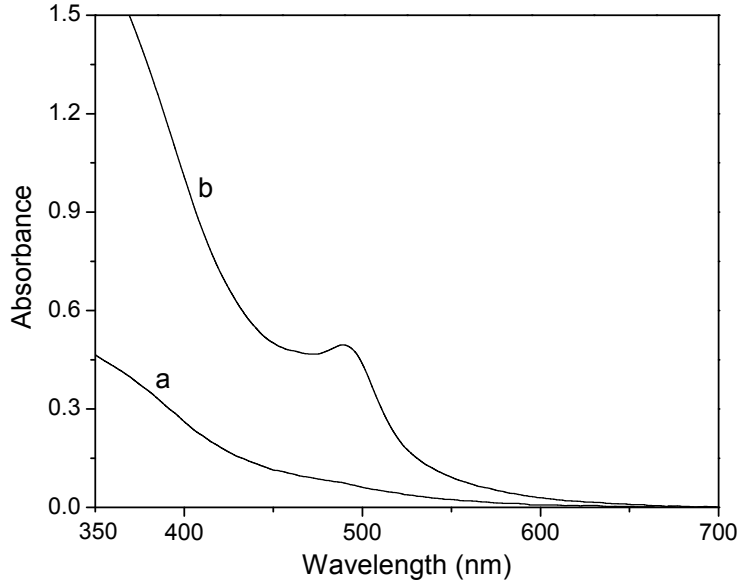

Fig. S6. UV-vis absorption curves of β-CD conjugated SPION (NP**2**, curve a) and FITC-labelled β-CD conjugated SPION (FITC-NP**2**, curve b). The nanoparticle concentration is 0.4 mg/mL in both cases. Based on the UV-vis absorbance, there are on average 16 FITC on each FITC-NP **2**.

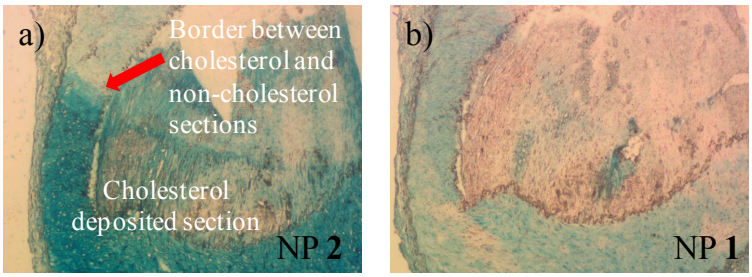

Fig. S7. Light microscopy images of rabbit aorta tissues incubated with an aqueous solution of a) NP **2** and b) NP **1** for 24 hours, treated with Prussion blue and counter stained with nuclear fast red. In both cases the NP concentrations were 0.78 mg/mL. Cholesterol crystals were deposited to the bottom sections of both tissues. The tissues were about 1 cm long.

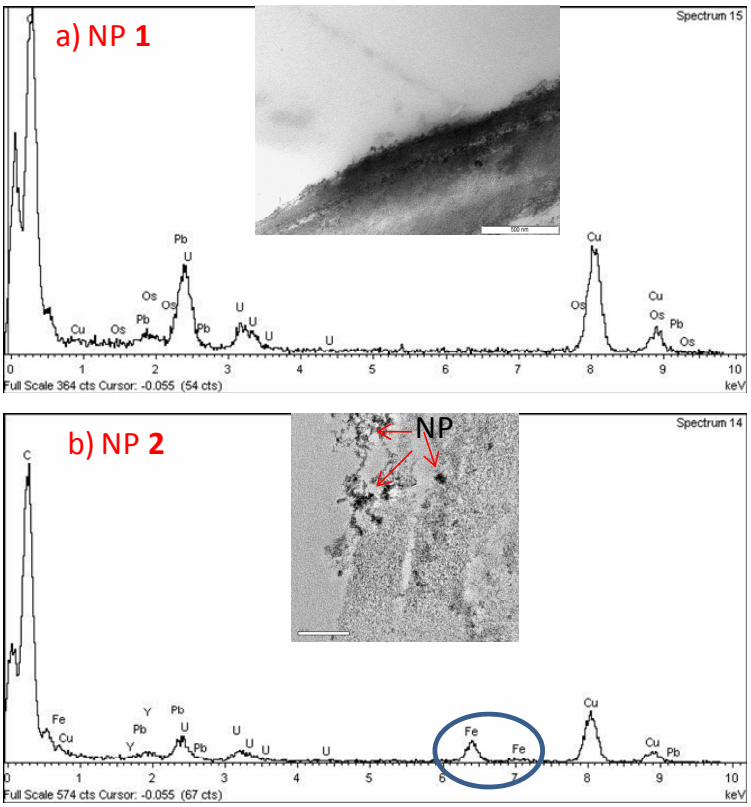

Fig. S8. X-ray photoelectron spectroscopy spectra of atherosclerotic tissues incubated with a) NP **1** and b) NP **2**. Iron was clearly detected in the lower spectrum supporting the observation of NP in the corresponding TEM images. Cu was from the copper grid, U was from the uranium acetate staining, Os was from the osmium tetraoxide used for tissue processing.

References

1. Elizabeth, A.; Joseph, C. and Ittyachen, M. A. *Bull. Mater. Sci.*, 2001, **24**, 431.

2. Kamat, M.; El-Boubbou, K.; Zhu, D. C.; Lansdell, T.; Lu, X.; Li, W. and Huang, X. *Bioconjugate Chem.*, 2010, **21**, 2128.