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Complete separation of magnetic nanoparticles via chemical cleavage of dextran by ethylenediamine for intracellular uptake

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Experimental details

Synthesis of bare SPIONs

Double distilled H_2O (ddH_2) was deoxygenated with N_2 gas for 20 min prior to all experiments. 33.79 g $FeCl_3 \cdot 6H_2O$ and 12.43 g $FeCl_2 \cdot 4H_2O$ were dissolved in 250 mL ddH_2O to make iron stock solution ($[Fe^{3+}]$ = 0.5 M, $[Fe^{2+}]$ = 0.25 M). 1.6 mL iron stock solution (Fe content 1.2 mmole) was topped up with ddH_2O to a volume of 38 mL at 4 °C. 2 mL ice cooled NH_4OH was poured into the iron solution under vigorous magnetic stirring, during which black precipitates appear immediately. The reaction mixture was heated to 70 °C and kept for 30 min to complete the reaction. The black precipitates were collected with a rare earth magnet and washed three times with ddH_2O to remove the unreacted ions and salt. Finally, as-prepared SPIONs was re-dispersed in ddH_2O and stored at 4 °C for further experiments.

Particle formation within dextran matrix

Dextran coated SPIONs (D-SPIONs) were prepared *in-situ* coprecipitation, in which dextran absorption takes place simultaneously on SPIONs. Desired amount Dextran T-10 (variable from 0.1 g to 3 g) was dissolved in 1.6 mL iron stock solution (Fe content 1.2 mmole) and top up with ddH_2O to 38 mL at 4 °C. The mixture was purged by N_2 for 30 min in ice bath to eliminate O_2 prior to reaction. 2 mL ice cooled ammonia was poured into the mixture at 4 °C under vigorous stirring and kept for 10 min. The clear brownish yellow solution turned to black immediately indicating the formation of SPIONs. The mixture was heat up to 70 °C and kept for 30 min. During the reaction, N_2 was flow through the flask to prevent oxidation. Failure of N_2 flow can cause oxidation of the final product resulted in a reddish brown non-magnetic/paramagnetic precipitates. After the mixture was cooled to room temperature (R. T.), the black precipitate was collected by a rare-earth magnet and the supernatant was removed by decantation. The black precipitate was washed with dd H_2O for 3 times and re-dispersed in ddH_2O with sonication for 2 min to form black colloid. With increasing amount of dextran T-10, very stable black colloidal suspension was formed, with no precipitate can be collected with rare-earth magnet or laboratory centrifuge (up to 14,000 r.p.m.). Black colloid was dialysis against 5 L ddH_2O to remove excessive salts and unbound dextran.

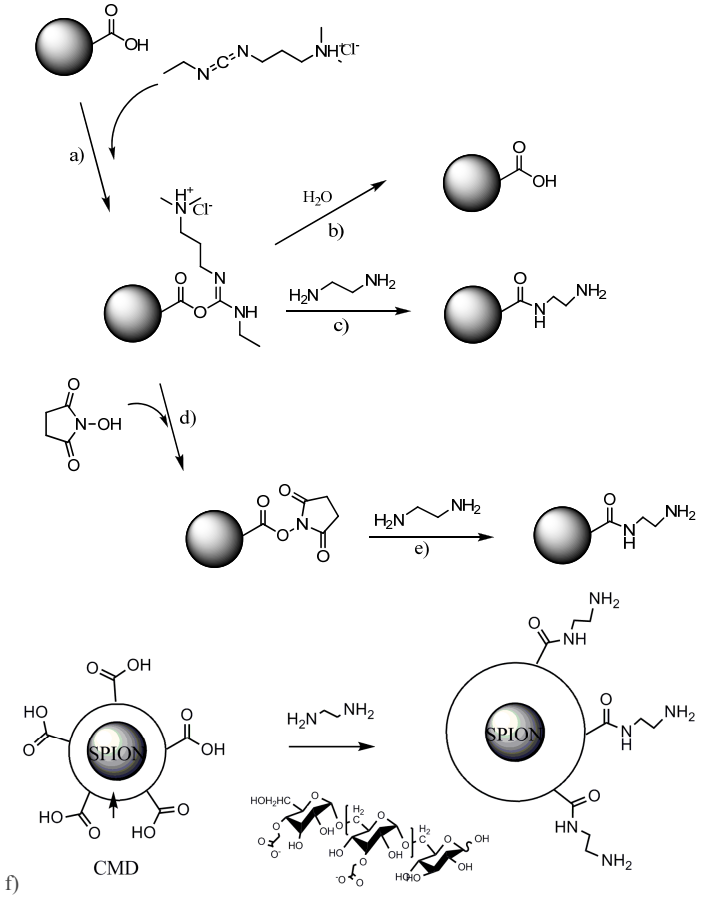
Dextran shell modification: crosslinking, carboxymethylation and diamine modification

Crosslinked dextran coated SPIONs (CLD-SPIONs) were prepared as following procedures: in a fume hood, desired amount of epichlorohydrin (ECH) was added to 20 mL suspension, containing variable amount of D-SPIONs (iron content from 0. 3 – 1.2 mmole) dispersed in 2.5 M NaOH solution. The mixture was incubated for 24 hrs at R. T., with continuous shaking to promote the interaction between aqueous and organic (ECH) phase. After crosslinking reaction is completed, black colloidal suspension was dialyzed against 5 L ddH_2O to remove ECH and excessive salts. The iron content of the CLD-SPIONs dispersion was determined by UV-Vis spectrometer measurement which is described next section.

For carboxymethylation, CLD-SPIONs colloid (Fe content 0.2 - 1 mmole) was re-dispersed in 10 mL 0.1 M NaOH solution, which was under continuous stirring for 1 hr at R. T. while purging with N_2. 0.443g monochloroacetic acid (MCA) was slowly added to the mixture, and the mixture was heat up to 60 °C in a water batch and kept for 90 min while N_2 was kept flowing to prevent oxidization. After the reaction was completed, the as-prepared CMD-SPIONs was dialyzed against 5 L ddH_2O to remove the excessive salts and kept at 4 °C for further experiment and characterization.

Ethylene diamine hydrochloride (EDA) was employed to amine activate the carboxymethyl functionalities and dual crosslinked SPIONs through 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) linker chemistry. 10 mg EDC and 6 mg *N*-hydroxysulfosuccinimide (NHS) was added to 2 mL CMD-SPIONs suspensions and stirred for 15 min at R. T., followed by addition of 3.5 mL EDA (in 10 times excessive amount) into the reaction mixture and incubation for various time at R. T. (up to 7 days). Reaction was stopped by dialysis against 2 L ddH_2O to remove unreacted EDA. The as-prepared AMD-SPIONs were stored at 4 °C for further experiment and characterizations.

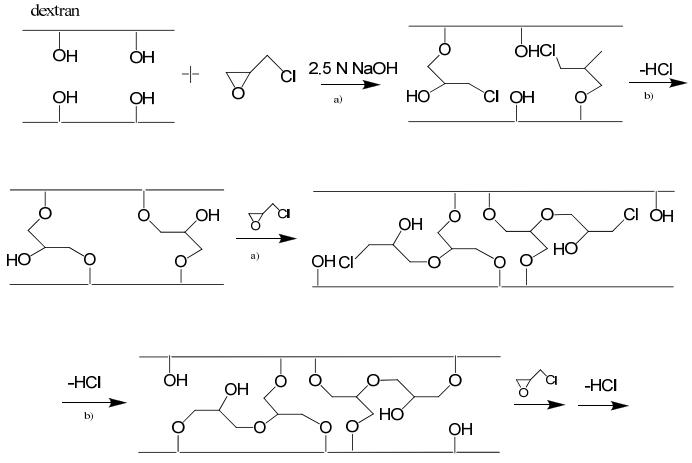
1 mg fluorescein isothiocynate (FITC) was dissolved in 1 mL dimethyl sulfoxide (DMSO) to make FITC stock solution (1 mg/mL), stored in dark at 4 °C. 5 μL FITC stock solution was diluted 10 times with ddH_2O prior to use in dark, 50 μL FITC working solution was slowly added to AMD-SPIONs dispersed in 2 mL 0.1 N phosphate buffer (pH = 9.0) and incubated for 4 hrs in dark at R. T. by occasionally shaking. FITC conjugated SPIONs were dialyzed against 2 L ddH_2O in dark at 4 °C to remove excessive FITC.



S. Scheme. 1. Schematical illustration of diamine functionalization of carboxyl functionalities of CMD-SPIONs: a) EDC reacts with carboxyl group on CMD-SPIONs forming an amine-reactive *O*-acylisourea intermediate, which may undergo b) regeneration of carboxyl group or c) reaction with amine molecules (EDA) forming a stable amide bond. d) NHS stabilizes the amine reactive intermediate by converting it into an amine reactive NHS-ester intermediate, hence increasing the efficiency of the EDC mediated reaction. e) amine group at one end of EDA react with NHS-ester functionalized SPIONs intermediate yielding a stable amide bond and leaving the other amine group intact. f) simplified reaction scheme.

Mechanisms of particles separations by"dual-crosslinking" and amine activation by diamine

The particles are not separated after crosslinking of dextran shells by epichlorohydrin(ECH), in which the reaction only takes place inter- or intra-molecularly on the dextran coated SPION surfaces as a chain reaction as illustrated below, hence form a cage-like structure on the SPION surfaces. Firstly, the opened epoxy group of ECH is reacted with hydroxyl groups on glucan units of dextran to form free chlorohydrin fragments on the side chains of dextran molecules. Afterward, dehydrochlorination reaction takes place between hydroxyl group on dextran and free chlorohydrin fragment, to form a crosslinked structure bearing a new hydroxyl group at the terminal. The chance of attachment of unabsorbed dextran in the solution is very low (negligible).



In the case of "dual-crosslinking" and amine activation by diamine reaction, it is possible that crosslinking process takes place on the surface of carboxymethylated dextran crosslinked SPIONs (CMD-SPIONs) to link the free CMD in the solution or only one terminal amine groups participate the reaction resulting in the other one available for further use.

Transmission electron microscopy (TEM)

The morphologies and sizes of nanoparticles were examined on two transmission electron microscope (TEM) model JEOL 2100F (200 kV) and 1230 (80 kV, Japan) depending on different purpose: the physical sizes of iron oxide cores were measured on JEOL 2100F (200 kV); and both core and shell structures were observed under JEOL 1230 (80 kV). TEM samples were prepared by placing a few drops of nanoparticles suspension onto a standard carbon-coated grid and then dried at ambient conditions. The TEM images were processed by Image-J software to determine the particle size and size distribution.

Zeta potential measurement

The surface charge of nanoparticles is determined by photon correlation spectroscopy (PCS), carried out with Zeta-Sizer HA300 (Malvern, UK). For zeta-potential measurement, all samples were diluted with ddH_2O into ~10 μg/mL iron concentration, and the pH was adjusted with 0.1 N NaOH or 0.1 N HCl to the range of pH3 to pH12, respectively. All the samples were injected through standard syringes.

Cell culture techniques

MG-63 cell line, a human osteosarcoma cell line was used in the *in vitro* cell tests. MG-63 cell line was cultured in Dulbeccos Modified Eagles Medium (DEME) supplemented with 10 % fetal bovine serum (FBS), 1 % 200 mM L-glutamine and 1 % penicillin/streptomycin at 37 °C in a humidified atmosphere with 5 % CO_2. Cells were grown in 25 cm^2 cell culture flask with 15 mL complete DEME medium that was changed every two or three days until 90 % confluence was achieved.

Acute cytotoxicity determined by MTT assay

The cytotoxicity of samples were assessed by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay. MTT assay is a cell proliferation assay, based on the ability of a mitochondrial dehydrogenase enzyme in viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystal. The number of surviving cells is directly proportional to the level of the formazan product created, which can then be quantified by reading absorbance at 570 nm with a multiwell scanning spectrophotometer (ELISA reader). Briefly, Cells were incubated with SPIONs containing media at desired iron concentration for 24 hrs. 10 μL MTT solution was added to the medium and the mixtures were incubated for 4 hrs at 37 °C, followed by addition of 100 μL DMSO to dissolve the purple crystals with gentle pipetting. The absorbance was measured with an ELISA reader at 570 nm. All control cells were cultured with complete medium only.

Cellular internalization analysis by confocal microscopy

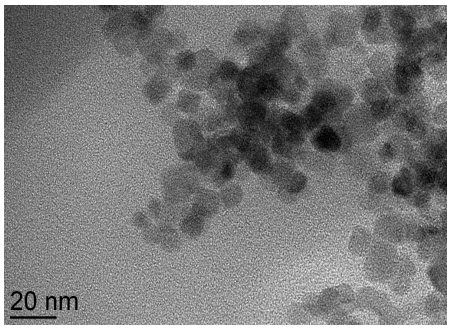
Cell membrane was stained by PKH26 red fluorescent cell linker kit according to the standard protocol given by the supplier. Briefly, MG 63 cells were trypsinized, collected and counted on a cytoheamatometer as described above. 2×10^7 cells were washed once with medium without serum in a polypropylene tube, and collected by centrifuge at 400 rpm for 5 mins. The cell pellet was re-suspended in 1 mL Dilutent C and quickly mixed with 1 mL freshly prepared 4×10^{-6} mole PKH26 dye in Diluent C in a polypropylene tube and incubated for 5 min at 25 °C . 2 mL FBS was added to the mixture to stop the reaction and followed by addition of 4 mL complete medium. Cells were centrifuged for 400 rpm for 10 mins at 25 °C and the loose pellet was washed with complete medium for further three times. Finally, 10 mL complete medium was added to the washed cell pellet and cells were counted again on a cytpheamatometer. Cells were plated at 5,000 cells/well in a 24-well flat-bottom plate in 2 mL complete cell culture medium each well for 24 hrs at 37 °C, 5 % CO_2.

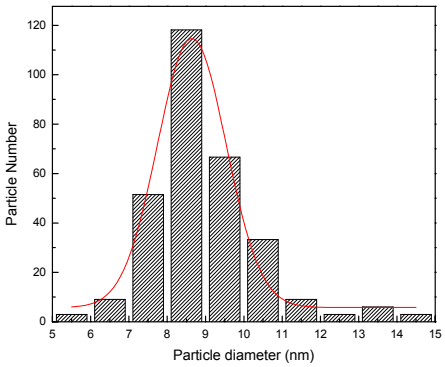
Fluorescent dye tagged particles was added to MG 63 cell cultures and incubated in dark at 37 °C, 5 % CO_2 for 15 hrs and 24 hrs time intervals. The excessive and unbound particles were washed away by PBS for three times, and followed by fixation of cells by 4 % glutaraldehyde in PBS at 25 °C for 20 mins. The fixed cells were examined under a laser scanning confocal microscope. Cell images were collected under the 40 X oil immersion objective lenses, and no post-acquisition enhancing processing was performed.

Particle size evolution

For more definite proof of the particle size and monodispersibility of AMD-SPIONs, the histogram of particle distribution of each samples were shown.

a)





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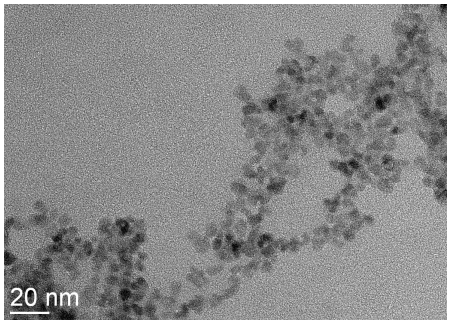

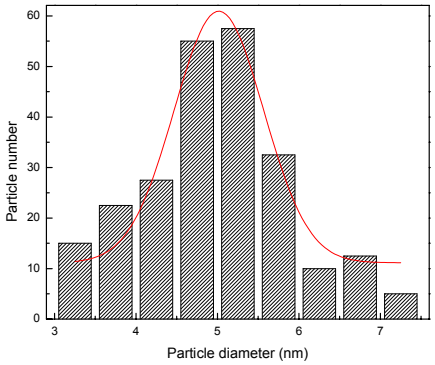

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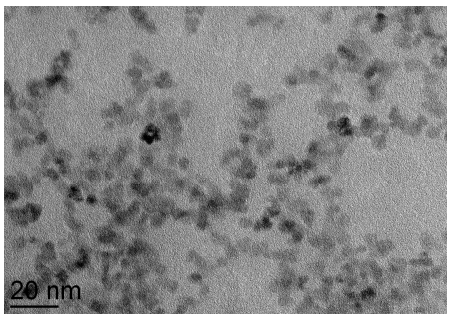

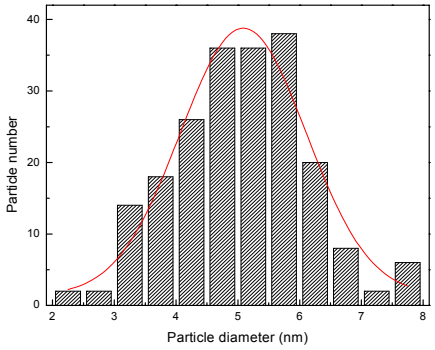

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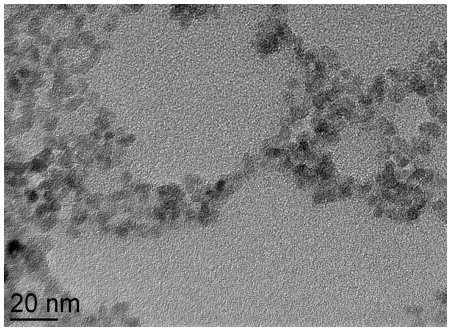

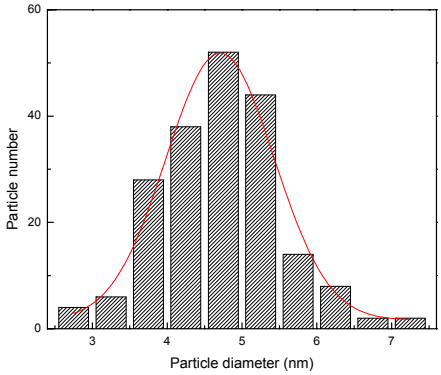

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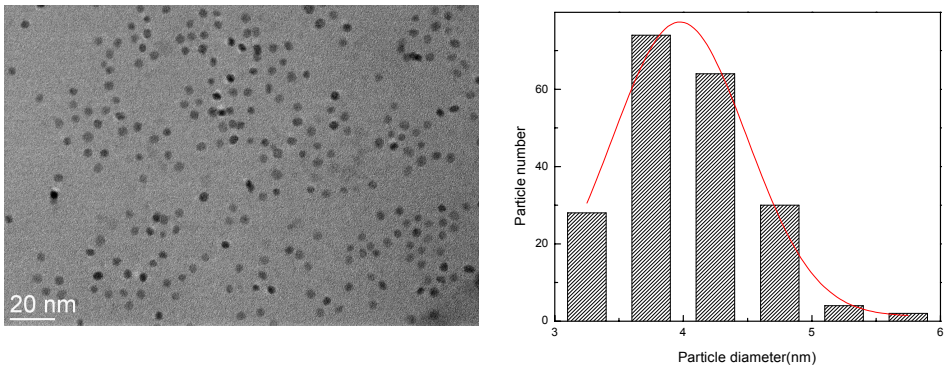
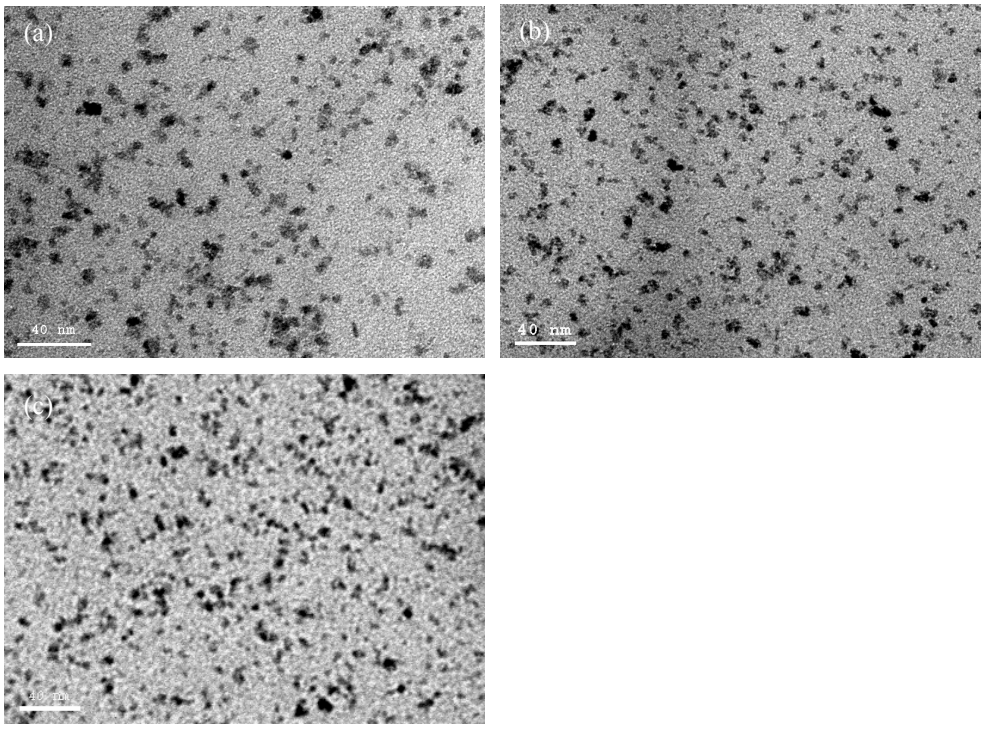


S. Figure. 1. TEM images (left) and sample particle size distribution histograms (right) of a) B-SPIONs, b) D-SPIONs, c) CLD-SPIONs , CMD-SPIONs and e) AMD-SPIONs

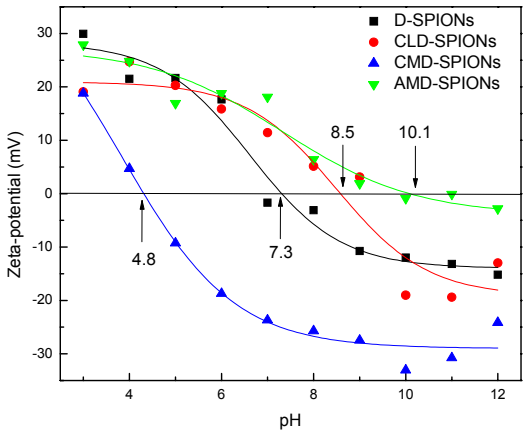
To investigate the proposed mechanism, AMD-SPIONs which were incubated with EDA for varied period were examined under a TEM with 80 kV, which enable the polymer visible. Hence it is reasonable that the TEM images are not as clear as TEM images taken under high voltage. The TEM images of AMD-SPIONs activated with EDA clearly show the tendency to increasing monodispersibility of core SPIONs from Day 1 to Day 7. With 1 day and 4 day incubation, particles still shown some degree of agglomeration, indicating EDA reaction is not completed yet to separate all SPIONs. Especially in Day 1, particles are still clumped together as small aggregates. However after 7 days incubation, SPION cores become perfectly dispersed with minimum agglomeration, although some of amine activated dextran coating is still adhered.



S. Figure. 2. TEM images of AMD-SPIONs with EDA activation for (a) 1 day, (b) 4 days and (c) 7 days. All the TEM images were taken by a JEOL 1230 TEM (beam current 68 μA and accelerating voltage 120 kV). With such relative low voltage, one should be able to visualize the polymer coating as light grey due to lower density of electron, compared to iron oxide cores shown as black colour under TEM. After 7 days incubation, SPION cores become perfectly dispersed with minimum agglomeration, although some of amine activated dextran coating is still adhered. With 1 day and 4 day incubation, particles still shown some degree of agglomeration, indicating EDA reaction is not completed to separate all SPIONs.

Surface charge analysis

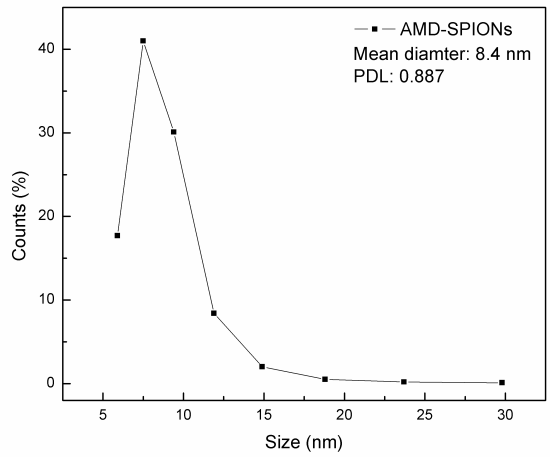
Zeta-potential of D-SPIONs, CLD-SPIONs, CMD-SPIONs and AMD-SPIONs as a function of pH have been shown to confirm the presence of carboxylic groups and amine groups on the surface of CMD-SPIONs and AMD-SPIONs respectively. The D-SPION curve shows a pH-dependency of zeta-potential and an isoelectric point (IEP) pH_{IEP} = 7.3. In D-SPIONs, dextran molecules are physically adsorbed onto surface of iron oxide, which may dissociate resulting in exposure of iron oxide to the medium. At aqueous system, exposed iron oxide surface is hydrated and covered by Fe-OH groups. At low pH, Fe-OH group react with H^+ and yield Fe negatively charged Fe-O^-. The IEP of CLD-SPIONs is determined pH_{IEP} = 8.5. In CLD-SPIONs suspension, physical absorbed dextran is crosslinked by ECH, which makes a tighter association between iron oxide particles and dextran molecules and less exposure of iron oxide surface to the environment. The zeta-potential of CLD-SPIONs has smaller values in the whole range of pH compared with the D-SPIONs, indicating that dextran molecules prevent aggregation through steric repulsion. The successful activation of carboxymethyl groups on coating changes the interfacial properties of nanoparticles. IEP value of CMD-SPIONs shifts to pH_{IEP} = 4.3, is attributed to the presence of carboxylic groups of CMD. The acid-base equilibrium favors carboxylate anion side at higher pH range, above pH 8, the zeta-potential of CMD-SPIONs is stabilized around -30 mV, suggesting the all carboxyl groups exsit in the carboxylate anion form, even increase the environmental pH, the surface charge is not changed. The IEP left shift is a qualitative indicator for successful carboxymethylation, the quantitative degree of substitution (DS) of CMD is not determined in this experiment. After diamine conjugation, the IEP dramatically shifted to pH_{IEP} = 10.1, suggesting most of the carboxyl groups are converted into amine groups. In the range of pH 3 to pH 10, the primary amine groups are in the protonated form, and as pH increases protonation reduces. Above pH 10, the zeta-potential of AMD-SPIONs are stabilized around 0 mV, indicating that all the amine groups exist in the form of $–NH_2$. Hence zeta-potential measurement confirm the both carboxyl and amine activation of SPIONs, open up the possibility of conjugating imaging dyes or targeting ligands or imaging agents for further applications or studies.



S. Figure. 3. Zeta-potential of D-SPIONs, CLD-SPIONs and CMD-SPIONs and AMD-SPIONs dispersed in water against pH from pH3 to pH12.

Dynamic light scattering (DLS)

DLS technique was used to determine the hydrodynamic size of the final AMD-SPIONs. As shown below the mean diameter of AMD-SPIONs was determined about 8.4 nm with PDL = 0.887. As the hydrodynamic size of a particle includes the coating layer (in this case amine activated dextran) that interact with water, the hydrodynamic size is always bigger that the physical " core" size determined by TEM (4.4 nm).



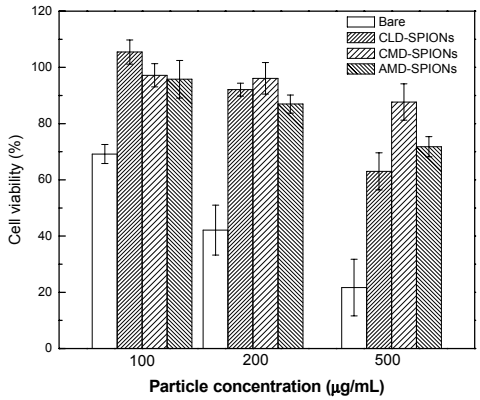
S. Figure. 4. The hydrodynamic size determined by dynamic light scattering technique (DLS), with Malvern zeta-sizer HA 3000 (Malvern, UK).

Cytotoxicity study of SPIONs

At low iron concentration (100 μg/mL), less than 70 % cells survived after 24 hrs B-SPIONs treatment, at highest iron concentration (500 μg/mL) around 40 % cell viability was observed, hence B-SPIONs is not suitable for direct bio-related applications, which is in agreement with other studies showing B-SPIONs have toxic effect over a range of cell types. It is reasonable to coat SPIONs with biocompatible molecules, such as dextran and dextran depravities to significantly improve the cell viability and attachment to the supporting surfaces.

The histogram of cell survival rate of MG 63 cells under CLD-SPIONs, CMD-SPIONs and AMD-SPIONs treatment for 24 hrs were shown. The coating materials of all three samples were considered to be similar, expect for surface charges. According to the zeta-potential curves of all samples described in the previous chapter, the surface charge of CLD-SPIONs (neutral), CMD-SPIONs(negative surface charges) and AMD-SPIONs (positive surface charge) at physiological pH 7.4 were determined to be +7 mV, -27 mV and +12 mV, respectively. None of coated SPIONs had shown appreciably toxicity over osteoblast cells at relatively low concentration (100 μg/mL and 200 μg/mL) in this work. CLD-SPIONs showed slightly promotional effect over MG 63 cells at low concentration of 100 μg/mL. at highest concentration of CLD-SPIONs (500 μg/mL), 60 % cell viability was observed.

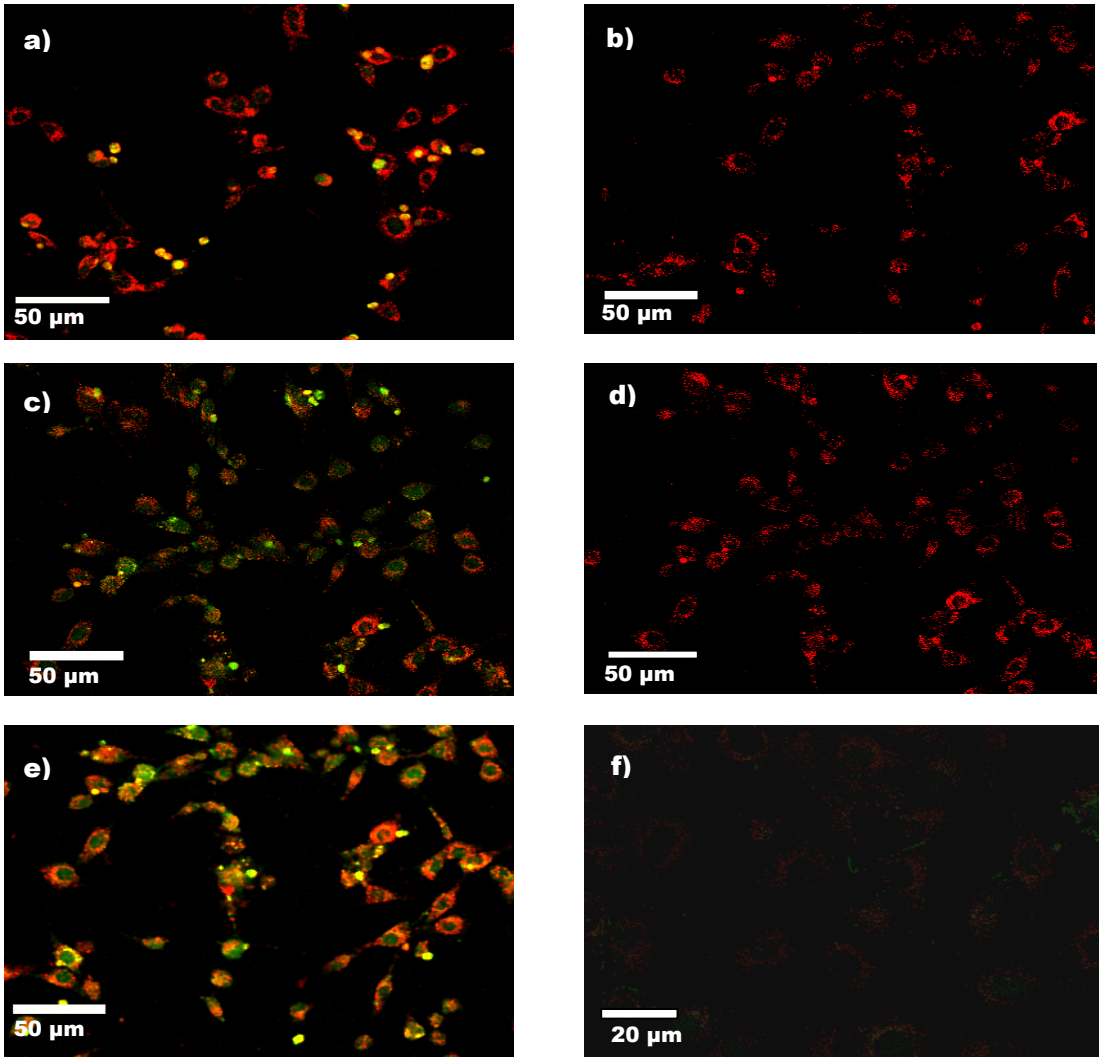
At iron concentration of 100 μg/mL, CMD-SPIONs and AMD-SPIONs treated cells had shown similar cell survival rate of 96 % and 95 %. The cytotoxicity of CMD-SPIONs kept unchanged with increasing particle concentration, at highest concentration of 500 μg/mL, cell survival rate exceeded 85 %. The cytotoxicity of AMD-SPIONs increased dramatically, at 100 μg/mL Fe content, cell survival rate reached 97 % and at 200 μg/mL Fe concentrations, cell viability still achieved 87 %, at highest concentration of 500 μg/mL, cell viability was reduced to 72 %. In conclusion, MTT assay shows that both CLD-SPIONs and CMD-SPIONs do not exert acute adverse effect on cells even at high dosage, suggesting that both of them are applicable as nanocarriers for *in vivo* applications.



S. Figure. 5. The histogram of cell viability after 24 hrs treatment with bare SPIONs, CLD-SPIONs, CMD-SPION and AMD-SPIONs in MG-63 cell at iron concentration of 100 μg/mL, 200 μg/mL and 500 μg/mL. Cell viability (%) after 24 hrs incubation with bare SPIONs, CLD-SPIONs, CMD-SPIONs and AMD-SPIONs was determined by MTT assay.

The fluorescent overlay images of MG-63 cells after incubation with FITC tagged AMD-SPIONs for different time intervals on the left column and control cells images with out SPIONs co-incubation on the right. We had uses PKH 26 as membrane specific dyes to stain cell membranes, for observation of cell morphologies. AMD-SPIONs treated cells up to 24 hrs shows no noticeable morphological abnormality in comparison with negative controls, indicative of very low or no adverse effect of such particles with cells, in consistent with the results of MTT cytotoxicity assays.

The overlay confocal microscopy images revealed that AMD-SPIONs formed bright small clusters around the cells after 8 hrs incubation with MG-63 cells, possibly at physiological condition; individual particles start to form small clusters and uptake by MG-63 cells. Considering the fact that there are not specific targeting agents decorated on the surface of SPIONs, it is reasonable to postulate that the cellular interaction between negatively charged plasmas membrane and positively charged AMD-SPIONs was initiated by electrostatic interaction. At 8 hrs interval, it clearly shows that the green fluorescence clustered in adjacent to cells. At time interval of 15 hrs incubation, a more evenly distributed green fluorescence was observed inside cells, indicating after internalization, the agglomeration of particles break down again possibly due to the environmental pH changes. It has been reported that dextran decorated particles are uptake by cells via non-specific endocytosis in which they go through endosome/lysosomes pass ways. It is a known fact that the pH inside endosome and lysosomes are acidic (pH 5.5), that attributed to the fine re-dispersion of particles. After 24 hrs incubation, stronger green fluorescence was observed, indicating more particles were being endocytosed.



S. Figure. 6. Confocal images of MG-63 cells stained with red membrane dye PKH26 incubated with 20 µg/mL FITC conjugated AMD-SPIONs (green coloured) for a) 8 hrs; c) 15 hrs and e) 24 hrs; with control cells without incubation with nanoparticles b) 8 hrs and d) 15 hrs. f) is a enlarged image of MG-63 cells treated with FTIC conjugated AMD-SPIONs for 24 hrs at 37 °C, 5 % CO_2.