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Controlled PEGylation of Monodisperse Fe_3O_4 Nanoparticles for Reduced Non-Specific Uptake by Macrophage Cells**

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Fe_3O_4 nanoparticles are emerging as promising candidates for drug delivery and biomedical imaging applications due to their ultra-fine sizes, biocompatibility and superparamagnetic properties.[1,2] However, two common problems are confronted when applying these particles for *in vivo* applications:[1,3,4] their destabilization due to the absorption of plasma proteins and non-specific uptake by reticular-endothelial system (RES), like macrophage cells. Due to their large surface area, when exposed in a physiology environment, magnetic nanoparticles tend to interact with plasma proteins, causing size increase that often results in serious agglomeration. These particles are also considered as an intruder by the innate immunity system and can be readily recognized and engulfed by the macrophage cells. In both cases, the particles will be removed from the blood circulation system and lose their function quickly, leading to dramatic reduction in efficiency in nanoparticle based diagnostics and therapeutics. To inhibit the plasma coating and escape from the RES for longer circulation times, the nanoparticles are usually coated with a layer of hydrophilic and biocompatible polymer such as dextran,[5] dendrimers,[6] polyethylene glycol (PEG),[7] or polyethylene oxide (PEO).[8]

This surface decoration step is even more important when facing Fe_3O_4 nanoparticles generated from organic solution synthesis. Nanoparticles synthesized using these methods[9] are monodisperse and single crystalline with higher magnetic moment than those prepared from co-precipitation of a Fe(II) and Fe(III) salt mixture in basic aqueous solution.[10,11] But these monodisperse particles are hydrophobic, and surface modification is necessary to make them hydrophilic and stable against agglomeration and non-specific uptake in a biological system. In previous search for controlled surface modification of monodisperse Fe_3O_4 nanoparticles, several different functionalization methods have been applied to covert hydrophobic nanoparticles into hydrophilic ones.[7,12,13] A recent report shows that monodisperse Fe_3O_4 nanoparticles can be conjugated to a breast cancer-targeting antibody, Herceptin, via 2,3-dimecaptosuccinic acid and sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1 carboxylate (sulfo-SMCC) and utilized as magnetic probes for breast cancer cell imaging applications.[14] However, few of these studies succeed in showing stability of the particles in physiology environment in terms of evasion from agglomeration and macrophage uptake – an understanding that is essential for target-specific cell recognition and cell entry.

Here we report a systematic study of functionalization of monodisperse 9 nm Fe_3O_4 nanoparticles with PEG. PEG is an amphiphilic polymer and is commonly regarded as a non-specific interaction reducing reagent.[4] It has been widely used for the conjugation with proteins to extend their circulation time.[15] In this study we successfully anchored PEG on the monodisperse Fe_3O_4 nanoparticles through a covalent bond, and the resulted particles demonstrate negligible aggregation in cell culture condition and much reduced non-specific uptake by macrophage cells. Coupled with the desired biomolecules, these stable nanoparticles should have great potentials for biomedical applications.

The coating chemistry is outlined in Scheme 1. Dopamine (DPA) was first linked with one of the carboxylic groups in the PEG diacid via the EDC/NHS chemistry. The DPA moiety was proved to have high affinity to the Fe_3O_4 surface.[16] Through DPA, the PEG was covalently anchored on the surface of the particles by replacing the oleate and oleylamine. The stability of these particles was monitored by their size change in phosphate buffered saline (PBS) plus 10% fetal bovine serum (FBS) in a normal cell culture condition. The non-specific uptake of the particles was analyzed by measuring the Fe concentration within RAW 264.7 macrophage cells after incubation of the nanoparticles with these cells.

The 9 nm monodisperse Fe_3O_4 nanoparticles were synthesized according to a published procedure[16] that was further modified so that a one-step synthesis of Fe_3O_4 nanoparticles could yield the desired sizes. Figure 1a shows a transmission electron microscopy (TEM) image of the Fe_3O_4 nanoparticles synthesized using this modified procedure. The nanoparticles are monodisperse with a standard deviation of less than 7%. The as-synthesized nanoparticles are coated with a layer of oleate and oleylamine.[16] To convert the nanoparticles from hydrophobic to hydrophilic, we first linked dopamine (DPA) with one COOH group in PEG diacid (HOOC-PEG-COOH) via EDC/NHS chemistry to give HOOC-PEG-DPA, which was then used to replace oleate/oleylamine around the particles in $CHCl_3$/DMF solution. In order to study the effect of

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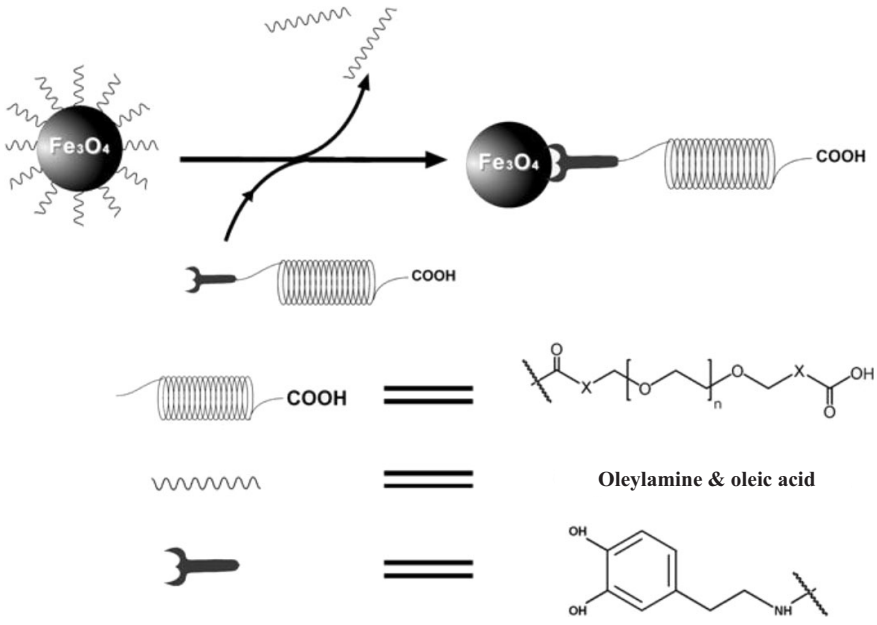
ADVANCED MATERIALS



Scheme 1. Surface modification of Fe_3O_4 nanoparticles via DPA-PEG-COOH. X=$CH_2NHCOCH_2CH_2$ for PEG_{3000}, PEG_{6000}, PEG_{20000}. X is not present in PEG_{600} – the bonds on both sides of the X are directly linked.

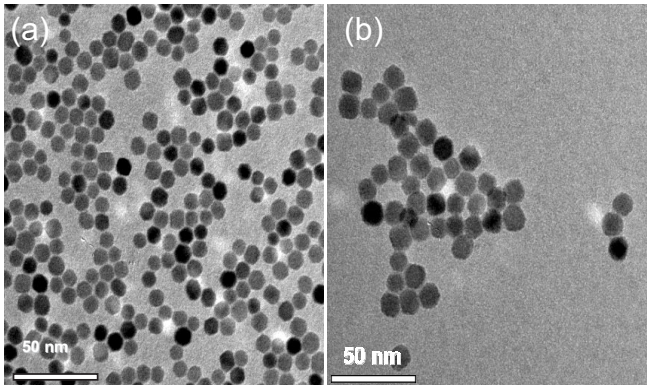


Figure 1. TEM images of the Fe_3O_4 nanoparticles before (a) and after (b) ligand exchange with DPA-PEG_{6000}.

PEG length on reducing non-specific uptake, a series of PEG diacid molecules with molecular weight (*M*) being 600, 3000, 6000 and 20000 were coupled to dopamine and further on the surface of the nanoparticles as shown in Scheme 1. After ligand exchange, the nanoparticles were able to disperse in water, forming a clear solution. TEM images of the PEG coated nanoparticles were obtained by evaporating water from the dispersion on amorphous carbon coated copper grid. Figure 1b shows TEM image of Fe_3O_4 nanoparticles coated by PEG_{6000}. Comparing Figure 1a and b, one can see that there is no obvious change in core size after surface modification with DPA-PEG. The PEG coating thickness around the nanoparticles was characterized with dynamic light scattering (DLS) that measures the hydrodynamic diameter of the nanoparticles in their dispersion state. The measurement results in water are given in Figure 2. It can be seen that before modification, the nanoparticles in hexane have an overall size around 11 nm. This is close to the simple addition of the dimensions from the core (9 nm) and the shell (2 nm – the length of the oleate and oleylamine molecules). After modification, the sizes of the particles increase to around 40, 50, 70 and 90 nm for PEG_{600}, PEG_{3000}, PEG_{6000}, PEG_{20000} coated nanoparticles, respectively, indicating the PEG molecules are incorporated in the surface coating. Zeta potentials of PEG-particles in water (Supporting Information, Table S1) also show that all the particles are negatively charged.

The surface coating of the PEG-Fe_3O_4 nanoparticles was further characterized by thermal gravitational analysis (TGA) and infrared (IR) spectroscopy. In the TGA analysis, the as-synthesized nanoparticles show two peaks at around 230 and 410 °C (Supporting Information, Fig. S1), accounting for the mass loss due to the evaporation of oleic acid or oleyl amine on the nanoparticle surface. But after ligand exchange, these two peaks disappear and are replaced by a strong desorption at about 360 °C. Comparing with the TGA of the free DPA-PEG ligand (Supporting Information, Fig. S2), one can see that this mass loss is caused by the evaporation of the DPA-PEG ligand anchored on the surface of the particles. The successful ligand exchange was also proven by IR analysis (Supporting Information, Fig. S3). The as-synthesized nano-

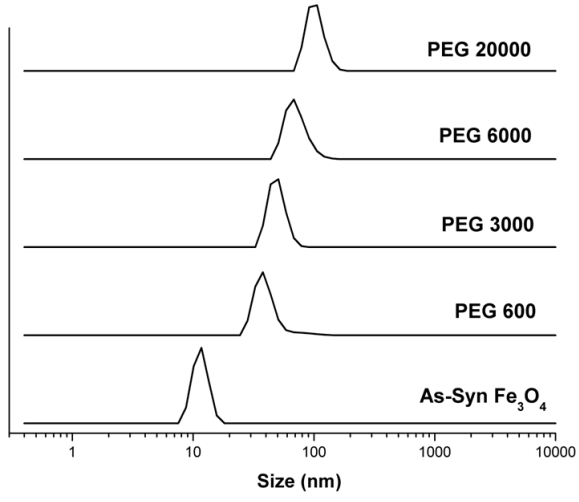


Figure 2. Hydrodynamic sizes of the Fe_3O_4 nanoparticles coated with different surfactants. The sizes were measured from the aqueous solution of the nanoparticles by DLS.

particles show two main absorption peaks in IR spectrum: one is at ~3500 cm^{-1}, which is attributed to the COOH and NH_2 group on oleic acid and oleylamine; and the other one is around 2800 cm^{-1}, arising from the stretching vibration of C–H. After modification, however, all four sets of nanoparticles exhibit a new peak at ~1100 cm^{-1}, which is due to the characteristic stretching vibration of C–O–C group from PEG.

Stability of the PEG coated Fe_3O_4 nanoparticles was tested in PBS plus 10% FBS at the incubation temperature 37 °C. DLS was used to track the size change of the nanoparticles during the incubation. Figure 3 shows the hydrodynamic diameters of the nanoparticles coated with various PEGs in PBS plus 10% FBS. It can be seen that the nanoparticles shows a ~10–20 nm size increase compared with those in

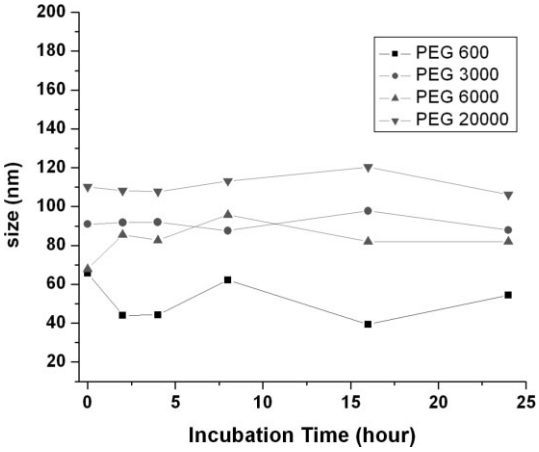


Figure 3. Hydrodynamic size changes of the DPA-PEG coated Fe_3O_4 nanoparticles incubated in PBS plus 10% FBS at 37 °C for 24 h.

water (Fig. 2). This is attributed to the interaction of nanoparticles with the FBS in the incubation medium. However, with the existence of the dense PEG coating, this interaction did not cause further agglomeration and all four sets of the nanoparticles show excellent stability without obvious size increase during an incubation period of 24 hours. The TEM images of the nanoparticles after incubation are similar to those before the modification (data not shown), indicating that DPA-PEG indeed offers a robust coating around the Fe_3O_4 nanoparticles, making them sustain from the cell culture condition.

To study the uptake of the PEG-coated Fe_3O_4 nanoparticles by the innate immune system, we incubated these particles with the RAW 264.7 cells, which are one kind of mouse macrophage cell line, at three different concentrations: 0.1 mg Fe mL^{-1}, 0.01 mg Fe mL^{-1} and 0.001 mg Fe mL^{-1}, and measured Fe concentrations within the cells after the incubation by inductively coupled plasma – atomic emission spectrometry (ICP-AES) analysis. For comparison, dextran-coated Fe_3O_4 nanoparticles with the same Fe concentration were also incubated with the RAW 264.7 cells and the Fe concentration within the cells was measured. The RAW 264.7 cells grown without nanoparticles were used as control. The results from the 0.01 mg Fe mL^{-1} of PEG-coated samples are shown in Figure 4. It can be seen that the dextran-coated nanoparticles give the highest uptake, followed by PEG_{600}-coated nanoparticles, of which the uptake is about 30%–50% of that from the

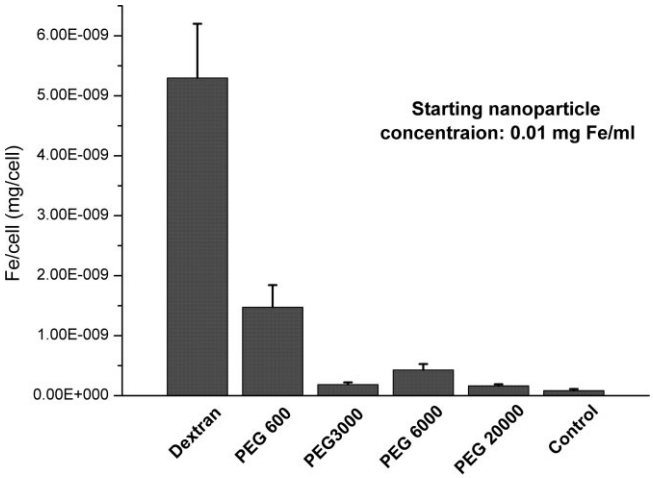


Figure 4. Macrophage uptake assay of the DPA-PEG coated Fe_3O_4 nanoparticles with initial Fe concentration at 0.01 mg Fe/mL.

dextran-coated ones. For PEG_{3000}-, PEG_{6000}-, and PEG_{20000}-coated nanoparticles, their uptakes are comparative with the background, indicating negligible uptake of these nanoparticles by the macrophage cells. At concentration of 0.1 mg Fe mL^{-1}, however, the uptakes of all kinds of particles increase about 20 times, indicating that the uptake is concentration dependent, which is consistent with previous observations.[17,18] PEG with molecular weight higher than 3000 give dense coating over the surface of the nanoparticles, and thus the length of PEG chain becomes insignificant factor in terms of non-specific uptake. The comparatively higher uptake of PEG_{600}-coated nanoparticles is likely caused by their less covered particle surface.

This report demonstrated that a DPA-PEG based ligand was readily synthesized and was efficient in stabilizing monodisperse 9 nm Fe_3O_4 nanoparticles made from the high temperature organic phase synthesis. The hydrophilic nanoparticles keep the morphology of the Fe_3O_4 core and are stable in water or in a physiological environment with non-detectable agglomeration. More importantly, these particles show much less uptake by macrophage cells, meaning that these particles can escape from the innate immune system. The multiple carboxylic groups on the particle surface allow conjugation onto the particles with a variety of macromolecules through the formation of amide bond at mild conditions. This makes these particles ideal candidates for the enhancement of targeting efficiency in drug delivery and for higher signal-to-noise ratio in MRI. Work on using these particles for bio-imaging, bio-detection and drug delivery is underway.

ADVANCED MATERIALS

Experimental

Iron (III) acetylacetonate, $Fe(acac)_3$, α,ω-bis{2-[(3-carboxy-1-oxopropyl)amino]ethyl} polyethylene glycol (M = 3000, 6000 and 20000), polyethylene glycol diacid (M = 600), dopamine hydrochloride, sodium carbonate and organic solvents used in the syntheses were purchased from Sigma Aldrich. *N*-hydroxysuccinimide (NHS) and *N*,*N*′-dicyclohexylcarbodiimide (DCC) were from Pierce Biotechnology. All the buffers and media were from Invitrogen Corp. Water was purified by Millipore Milli-DI Water Purification System. All the dialysis bags were purchased from Spectrum Laboratories, Inc.

Synthesis of Fe_3O_4 Nanoparticles. The synthesis of Fe_3O_4 nanoparticles followed the procedure published previously [16]. 2 mmol of $Fe(acac)_3$ was dissolved in a mixture of 10 ml benzyl ether and 10 mL oleylamine. The solution was dehydrated at 110 °C for 1 h, and was quickly heated to 300 °C and kept at this temperature for 2 h. 50 mL of ethanol was added into the solution after it was cooled to room temperature. The precipitate was collected by centrifugation at 8000 rpm and was washed with ethanol 3 times. Finally, the product (150 mg) was redispersed in hexane, and ~0.25 mL oleic acid was added to stabilize the particles.

Surface Modification of Fe_3O_4 Nanoparticles. PEG diacid 20 mg (this amount is for PEG diacid 3000; for other PEG diacids, same moles were used), NHS (2 mg), DCC (3 mg) and dopamine hydrochloride (1.27 mg) were dissolved in a mixture solvent containing $CHCl_3$ (2 mL), DMF (1 mL), and anhydrous Na_2CO_3 (10 mg). The solution was stirred at room temperature for 2 h before Fe_3O_4 nanoparticles (5 mg) were added, and the resulting solution was stirred overnight at room temperature under N_2 protection. The modified Fe_3O_4 nanoparticles were precipitated by adding hexane, collected by a permanent magnet and dried under N_2. The particles were then dispersed in water or PBS. The extra surfactants and other salts were removed by dialysis using a dialysis bag (MWCO = 10000) for 24 h in 1× PBS or water. Any precipitation was removed by a 200 nm syringe filter. The final concentration of the particles was determined by ICP-AES analysis.

Dextran coated nanoparticles were synthesized by co-precipitation of $FeCl_2$ and $FeCl_3$ in aqueous solution with the presence of ammonium hydroxide according to an earlier publication [11].

Nanoparticle Characterization. TEM measurements were taken on a Philips EM 420 (120 kV). The hydrodynamic diameters of the nanoparticles were measured by Malvern Zeta Sizer Nano S-90 dynamic light scattering (DLS) instrument. TGA analysis was done by TA Instruments Q500 Thermogravimetric Analyzer. IR studies were run on ATI Mattson Infinity Series FTIR Spectrophotometer.

Cell Uptake Experiment. Raw 264.7 cell lines were cultured in RPMI 1640 media (with Glutamine and Phenol Red) with 10% FBS and 1% antibiotics in T25 culture flasks. Before the test, the growth medium was removed. The cells were washed twice with PBS before Fe_3O_4 nanoparticles coated with different PEG's in growth media, each with different concentrations (0.1 mg Fe mL^{-1}, 0.01 mg Fe mL^{-1}, 0.001 Fe mL^{-1}) were added. Cells grown without any particles were used as control. The cells were then incubated for 4 h at 37 °C, 5% CO_2, washed with PBS twice and redispersed in RPMI. The cell concentrations were determined by hemacytometry and the Fe concentrations were determined by ICP-AES.

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