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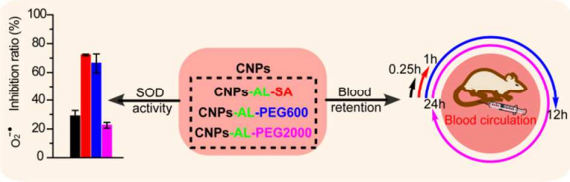
Table of contents entry



Using alendronate as anchor, functional molecules could be easily grafted onto ceria nanoparticle leading to enhanced bioproperties of the nanoparticles.

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Alendronate as robust anchor for ceria nanoparticle surface coating: facile binding and improved biological properties†

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The bisphosphonate group of alendronate was found to possess high binding affinity to ceria nanoparticles (CNPs). Using alendronate as anchor, functional molecules could be easily grafted onto CNPs leading to enhanced enzyme mimetic activity, reduced cytotoxicity, improved stability, prolonged blood circulation, and decreased liver and especially spleen accumulation of the nanoparticles.

Artificial enzymes such as metal complexes, cyclodextrins, porphyrins, polymers and dendrimers were developed to mimic natural enzymes flawlessly have many advantages including resistant to denaturation, easy to synthesize and low-cost.[1] Recently, metallic nanomaterials have shown their potentials in mimicking various enzymatic activities. For example, gold nanoparticls have exhibited RNase and oxidase activity,[2, 3] whereas V_2O_5 nanowires[4] and iron oxide nanoparticles[5] have shown peroxidase activity. Apart from above mentioned nanomaterials, an intensive research on CNPs have been pursued for its enzyme mimetic activity based on the coexistence of Ce^{3+} and Ce^{4+} at the nanoparticle surface.[6-8] Previous reports have shown the superoxide dismutase (SOD),[9] catalase[10] and oxidase[11] mimetic activities of CNPs, which have led to various biomedical applications such as oxidative stress protection,[12] treatment of dermal wounds,[13] ischemic stroke protection,[14] tissue regeneration,[15, 16] anti-radiation[17, 18] and anti-inflammatory.[19] Despite of these interesting biomedical applications, most of the CNPs used in previous studies were either naked[15, 16, 20-23] or weakly protected by surfactants.[17, 20, 24, 25]

It is well known that nanoparticles without sufficient surface protection inevitably encounter many obstacles *in vivo*,[8, 26, 27] especially aggregation and clearance by mononuclear phagocyte system (MPS) which finally lead to decrease of nanoparticle activity and shorten the nanoparticle blood circulation retention time. Previous reports have studied the coating of CNPs with hydrophilic polymers such as dextran,[28] chitosan[29] and poly (acrylic acid)[11, 30] to improve the nanoparticle stability. However, other polymers and functional molecules that inherently have no binding affinity with CNPs could not be steadily adsorbed at the nanoparticle surface. Especially, the polyethylene glycol (PEG) that could improve many of the biological properties of nanoparticles[31, 32] could not be directly grafted onto CNPs. Therefore, it is necessary to find a robust anchor to bridge up the nanoparticle and the PEG chain. Previous studies have used carboxyl,[33] phosphonate[34] and propanediol[35] as anchor to graft PEG onto CNPs. Though successful PEG coating have been claimed in these studies, none of them have shown the stability of the PEGylated CNPs under physiological condition. This could be ascribed to the weak binding affinity of the anchors with the nanoparticles. So far, there is only one study that has achieved stable PEGylated CNPs under physiological condition.[14] In this study, phospholipid was used as anchor to bind PEG onto CNPs through hydrophobic interaction between phospholipid tail conjugated to PEG and oleylamine adsorbed on CNPs. Although more than ten days of nanoparticle stability was reported by this method, the PEG chains were not truly anchored at the nanoparticle surface. Most importantly, this method is only applicable for CNPs originally protected by long alkyl chain, while the retained alkyl chain at the nanoparticle surface could induce uncertain side effects when applied *in vivo*. The limitations of the above mentioned methods inspired us to explore a robust anchor for CNPs surface coating meanwhile maintain or improve the biological properties of the nanoparticles.

An ideal anchor should have high binding affinity to CNPs and chemically inert for any catalytic reaction caused by CNPs since these nanoparticles possess several kinds of enzyme mimetic activities. Based on these principles, we investigated the feasibility of using alendronate as anchor for CNPs surface coating taking advantage of the characteristics of bisphosphonate and amine tail of the molecule. The P-C-P structure of bisphosphonate is highly stable and resistant to chemical and enzymatic hydrolysis. Most importantly, the bisphosphonate tail of alendronate could easily bind to mineral surface through the formation of multidentate coordination bond.[36] Besides, the amine tail at another side of alendronate could be effectively linked to carboxylated PEG by EDC/NHS chemistry. Based on these deductions, the alendronate anchor was linked to succinic acid (SA) and PEG diacid by EDC/NHS chemistry and the resulting ligand was used to replace the original coating layer at CNPs surface.

The CNPs were prepared by microemulsion or thermal decomposition method,[18, 37] and the CNPs surface coating was developed by probing the interaction between alendronate anchor and nanoparticles. In a two phase reaction system, CNPs prepared by microemulsion method dispersed in toluene were mixed with

alendronate solution containing Na_2CO_3 and the mixture was allowed to react at 80 ºC for 12 h. After reaction, the yellow colored CNPs transferred into water and the resulting alendronate anchored CNPs (CNPs-AL) were well dispersed in water. However, the nanoparticles precipitated from water without alendronate protection at the same reaction condition (Fig. 1a). There are two possible binding sites in alendronate including biphosphonate and primary amine end group. It is, therefore, necessary to know the binding group of alendronate ligand to CNPs for designing effective ligands for CNPs surface coating. If biphosphonate end binds to the CNP, then primary amine end of alendronate would easily be accessible and can be confirmed by reacting it with carboxylated fluorescent molecule. To test this hypothesis, CNP-AL nanoparticles were dialyzed in water for 24 h and the purified nanoparticles were reacted with Rhodamine B by EDC/NHS chemistry. After purification again by dialysis in phosphate buffer (5 mM pH 7.4) for 24 h, the color of the nanoparticles changed from yellow to pink and strong fluorescent signal was detected in the solution (Fig. S1), indicating the bonding of amine group to Rhodamine B and biphosphonate end to the CNPs. Based on this result, alendronate was conjugated to SA and PEG diacid with molecular weight of 600 and 2000 by EDC/NHS chemistry (see supporting information for details). The resulting ligands were used for CNPs surface modification through an in situ ligand exchange method, i.e. react the nanoparticles with unpurified anchor conjugated SA and PEG ligands.[38] As expected, alendronate conjugated SA and PEG easily transferred CNPs from toluene into water and the resulting CNPs-AL-SA, CNPs-AL-PEG600 and CNPs-AL-PEG2000 were well dispersed in water (Fig. 1a). The hydrodynamic diameter of the CNPs-AL-SA, CNPs-AL-PEG600 and CNPs-AL-PEG2000 were 10.4 ± 0.4, 15.2 ± 0.4 and 21.3 ± 0.5 nm (Fig. 1b) respectively. The TEM images revealed that after surface coating, the agglomerated CNPs were disassembled from each other suggesting improved dispersibility of the nanoparticles in water (Fig. 1c and Fig. S2). To demonstrate the universality of our method, the CNPs prepared by thermal decomposition method protected by oleylamine were also coated with PEG using the same procedure. Similar as the CNPs prepared by microemulson method, the alendronate conjugated PEG could easily stabilize the nanoparticles in water (Fig. 1d).

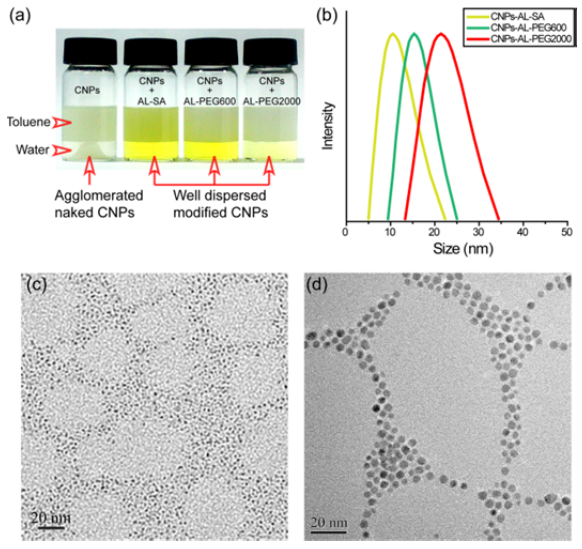

Fig. 1 (a) CNPs surface coating using alendronate as anchor. (b) The hydrodynamic size distribution of surface coated CNPs. The TEM images of CNPs-AL-PEG600 synthesized by microemulsion (c) and high temperature decomposition method (d) dispersed in water.

To confirm the existence of functional groups including alendronate, SA and PEG on CNPs, the nanoparticles were analyzed by nuclear magnetic resonance (NMR), thermo gravimetric analysis (TGA) and X-ray photoelectron spectroscopy (XPS). Though some of the active hydrogen atoms were sheltered by the nanoparticle in the NMR spectrum, the intensive characteristic chemical shift at 2.25 ($-CH_2-COO-$) for SA and 3.51 ($-CH_2-O-$) for PEG clearly proved the successful grafting of SA and PEG onto the nanoparticle surface (Fig. 2a). In TGA, obvious weight loss were observed between 320 °C to 480 °C that is within PEG decomposition range was detected for CNPs-AL-PEG600 and CNPs-AL-PEG2000 (Fig. 2b). The PEG ligand weight percentage for CNPs-AL-PEG600 and CNPs-AL-PEG2000 were 51.7% and 67.3% respectively. Accordingly, the calculated ligand/nanoparticle ratio for CNPs-AL-PEG600 and CNPs-AL-PEG2000 were about 71 and 54 respectively. According to XPS spectrum (Fig. 2c), CNPs did not show any phosphorus signal whereas phosphorus (2p) signals were detected in all of the surface coated CNPs suggesting successful binding of alendronate to the nanoparticle after ligand exchange reaction.

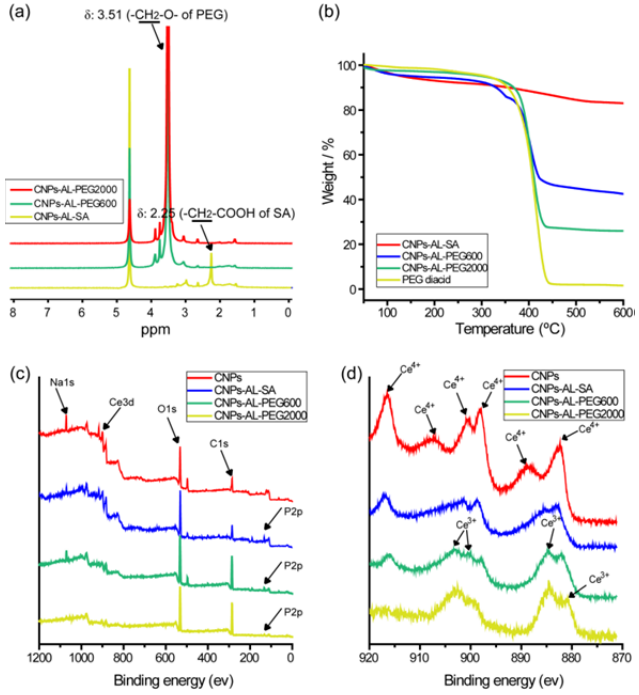

Fig. 2 (a) The NMR spectrums of surface coated CNPs. (b) the TGA graphs of surface coated CNPs. (c) The full XPS spectrums of CNPs before and after surface coating. (d) Selected XPS segments related to valence state of cerium ions with corresponding binding energy peaks for Ce^{3+} (880.20, 885.00, 899.50 and 903.50 ev) and Ce^{4+} (882.10, 888.10, 898.00, 900.90, 906.40 and 916.35 ev).

The enzyme mimetic activity of CNPs caused by the coexistence of Ce^{3+} and Ce^{4+} in the nanoparticle lattice is one of the main reasons for most of their biological effects. Therefore, it is necessary to verify if the surface coating procedures and anchoring group/ligands have any derogatory effects on the activity of the nanoparticles before universality of anchoring group/ligands can be claimed. The SOD activity of CNPs before and after surface coating was evaluated by inhibiting superoxide free radical generated by riboflavin under light illumination (Fig. 3a). The results indicated that the CNPs-AL-SA and CNPs-AL-PEG600 showed higher SOD activity than the naked CNPs. However, the SOD activity CNPs-AL-PEG2000 was lower than the naked CNPs (Fig. 3b). The increase in SOD activity

of CNPs-AL-SA and CNPs-AL-PEG600 could be ascribed to the increase of Ce^{3+} concentration in the nanoparticles[39] as the Ce^{3+} percentage in CNPs, CNPs-AL-SA and CNPs-AL-PEG600 were 37.3%, 41.6% and 49.3% respectively (Fig. 2d and Fig. S3). In addition, the improved dispersibility of the nanoparticle which led to better CNP surface accessibility of superoxide free radicals could be another important contributing factor for higher activity. Nevertheless, the CNPs-AL-PEG2000 that had higher Ce^{3+} concentration (50.9%) and similar dispersibility showed lower SOD activity. Particularly, the SOD activity of CNPs-AL-PEG2000 was even lower than the naked CNPs. This phenomena could be caused by the longer PEG chain (linear chain length of PEG600 and PEG2000 is about 4.9 nm and 16.3 nm respectively)[32] that obstructed further the superoxide free radical from reaching the nanoparticle surface. The CNPs-AL-SA showed highest activity by combining moderate Ce^{3+} concentration in the nanoparticles and very small anchored ligand length comparing to PEG. Based on these results, we concluded that the SOD activity of CNPs could be controlled by varying the thickness of coating layer at the nanoparticle surface.

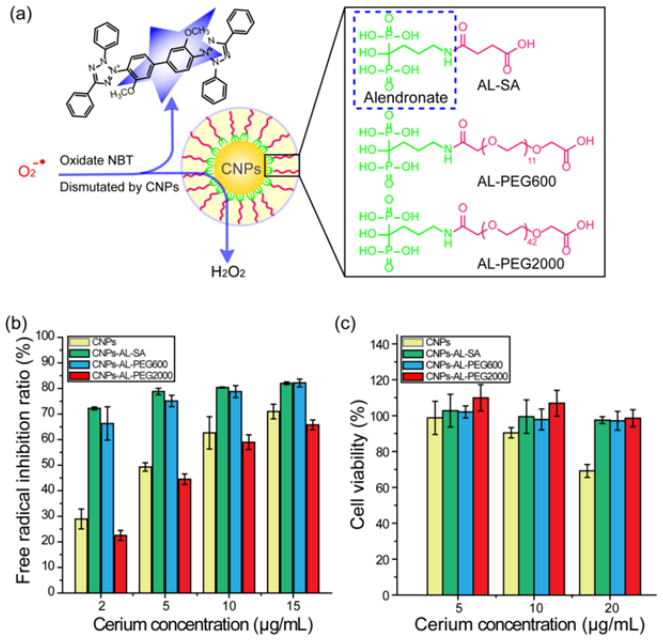

Fig. 3 (a) Schematic illustration of the dismutation of superoxide free radical by surface coated CNPs, the free superoxide free radical reacted with nitrotetrazolium blue chloride (NBT) to generate blue product which could be detected at 560 nm. (b) The superoxide free radical inhibition ratio of CNPs before and after surface coating. (c) The cell viability of CNPs before and after surface coating.

The surface coating strategy is regularly used to improve the biological properties of nanoparticle. Therefore, it is necessary to know if the surface coating truly changed the biological properties of CNPs. To demonstrate the stability of the surface coated CNPs in physiological condition, the nanoparticles were dispersed in phosphate buffered saline (PBS) containing 10% fetal bovine serum (FBS) and their size change was recorded by dynamic light scattering (DLS). The naked CNPs quickly precipitate out and the CNPs-AL-SA precipitate out in two days from the solutions suggesting the nanoparticles were unstable in physiological condition. However, the PEGylated CNPs exhibited excellent stability as no obvious size change was observed for CNPs-AL-PEG600 and CNPs-AL-PEG2000 in one month (Fig. S4 and Table S1). Cytotoxicity is one of the critical criteria for evaluation the safety of nanomaterials. Therefore, the CNPs before and after surface coating were submitted for cell viability assay at cerium concentration of 5, 10 and 20 μg/mL. As a result, full retention of cell viability was observed for CNPs-AL-SA, CNPs-AL-PEG600 and CNPs-AL-PEG2000, while the naked CNPs exhibited obvious inhibitory effects on HL7702 liver cells at cerium concentration of 10 and 20 μg/mL (Fig. 3c). These results clearly demonstrated the improved cell compatibility of the CNPs after surface coating.

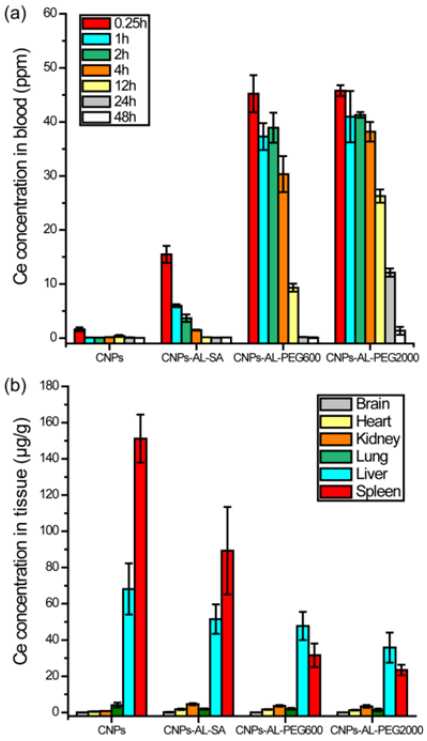

Fig. 4 (a) The *in vivo* blood circulation retention behavior of CNPs within 48 h after tail intravenous injection. (b) The organ distribution of CNPs before and after surface coating.

To evaluate the blood circulation retention time, all four types of nanoparticles were intravenously injected into mice with Cerium dose of 10 mg/kg body weight and the blood was collected from angular vein at 0.25, 1, 2, 4, 12, 24 and 48 h after injection. Concurring with expectation, the naked, AL-SA and PEGylated CNPs showed incremental blood circulation retention behavior as the blood Cerium concentration (BCC) for naked CNPs, CNPs-AL-SA, CNPs-AL-PEG600 and CNPs-AL-PEG2000 were 1.6 ± 0.4, 15.5 ± 1.6, 45.2 ± 3.4 and 45.8 ± 1.0 ppm respectively 15 min after injection (Fig. 4a and Table S2). Notably, the mice administered with CNPs-AL-PEG2000 retained high level of BCC (12.1 ± 0.8 ppm) even 24 h after injection. These results indicated that the PEGylated CNPs possess excellent "stealth" characteristic. To clarify if the blood circulation retention differences have any effects on the distribution of the nanoparticles in organs, the mice were sacrificed one week after injection and the nanoparticle in the important organs including brain, heart, kidney, lung, liver and spleen were analyzed. In general, the liver and spleen entrapped much more nanoparticles compare with the other organs. Whereas, the concentration (Fig. 4b and Table S3) and total amount (Fig. S5 and Table S4) of nanoparticle in liver and spleen for mice administered with naked CNPs and CNPs-AL-SA were much higher than CNPs-AL-PEG600 and CNPs-AL-PEG2000, especially in the

spleen. These results suggested that the PEGylation not only prolonged the blood circulation retention time of the CNPs but also changed the finial distribution of the nanoparticles in organs.

In conclusion, alendronate was found to possess high binding affinity to CNPs. Using alendronate as anchor, CNPs can easily be coated with SA, PEG and can be extended to other functional molecules. The surface coating has profound effect on modulating enzyme mimetic activity, stability, cytotoxicity, blood circulation and organ accumulation behaviors of the nanoparticles. The CNPs coated with SA and PEG600 showed enhanced SOD activity due to the increased Ce^{3+} in the nanoparticle and improved dispersibility of the nanoparticle, while the CNPs coated with PEG2000 showed decreased SOD activity owning to the obstruction of the substrates to the nanoparticle surface by the longer PEG chain. The CNPs coated with PEG were highly stable in physiological condition as almost no size increase was observed for these nanoparticles in one month. The surface coated CNPs retained full cell viability while the naked CNPs showed obvious cytotoxicity under experimental condition. Besides, the CNPs coated with PEG showed prolonged blood circulation retention time suggesting improved "stealth" property of the nanoparticles, which in turn led to the decrease of accumulation of nanoparticles in liver and especially spleen. This work not only provided a general method for CNPs surface coating but also clarified the influences of the surface coating procedures on the biological properties of the resulting nanoparticles. Based on these results, the surface coated CNPs could be rationally designed to further explore *in vivo* applications.

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