Covalent Surface Modification of Cerium Oxide Nanoparticles Promising for Nanomedicine Application

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Abstract
Cerium Oxide Nanoparticles (CNPs) have been emerged as a potential nanomedicine for treatment of several diseases including cancer. CNPs have the natural tendency to aggregate or agglomerate at its bare state and led to sedimentation at biological environment. Since the biological environment is essentially aqueous, nanoparticle surface modification using suitable biocompatible hydrophilic chemical moiety is highly desirable for its stable aqueous dispersion. In this report, we have used (6-{2-[2-(2-Methoxy-ethoxy)-ethoxy]-ethoxy}-hexyl)triethoxysilane to engineer the CNP’s surface for production of improved grade nanoparticles for potential nano-biological
application. The surface modified nanoparticles were produced by ammonia-induced ethylene glycol assisted precipitation method and were characterized using high resolution transmission electron microscopy, powdered x-ray diffraction analysis and dynamic light scattering techniques. The interaction of functional moiety with the surface of nanoparticle was studied using powerful cross polarization magic angle sample spinning solid state nuclear magnetic resonance spectroscopy. The prepared nanoparticles demonstrated improved aqueous dispersibility and more importantly a covalent interaction among the functional moiety and the surface of the nanoparticle. The surface modified nanoparticles should be promising for nanomedicine applications.

Key words

Cerium Oxide nanoparticle; covalent surface modification; aqueous dispersion; nanomedicine

1. Introduction

Introduction of nanotechnology in medicine has attracted an unprecedented number of nanomaterials for their therapeutic application [1]. For example, carrier as well as non-carrier based nanoparticles have been well investigated for their potential application in nanomedicine [2]. Cerium (Ce) oxide nanoparticles (CNPs) as a non-carrier metal oxide nanoparticle has recently demonstrated its potential interest in biology and nanomedicine [3-5]. Due to the magical auto-regeneration of Ce valence states (Ce$^{3+}$ and Ce$^{4+}$) in CNPs, these nanoparticles can act as both pro-oxidant and an antioxidant [4]. CNPs in (+3) oxidation state have shown to scavenge superoxide radical anion in living cells mimicking the activity of superoxide dismutase (SOD),
while at (+4) oxidation state, it facilitates the decomposition of H₂O₂ to O₂ and H₂O, mimicking the role of catalase [4]. CNPs have shown to scavenge the reactive oxygen species (ROS) and have shown its promise to cure several oxidative stress related disorders [5]. CNPs have also shown their inhibitory effect on cancer progression being toxic to tumor cells and nontoxic to stromal cells and were investigated as a potential nanomedicine for cancer treatment [6-8]. Optimal therapeutic performance of these nanoparticles needs a well and stable biological dispersion throughout the duration of treatment and remains a major obstacle in its application [5-9].

Due to large number of surface defects, CNPs have natural tendency to aggregate at their bare state, if not functionalized [9]. The aggregation results in quick sedimentation, deposition in reticuloendothelial system (RES) and toxicity [10]. Non-functionalized CNPs in high ionic medium such as phosphate buffer saline (PBS) and cell culture medium have shown to aggregate and form larger particles within a few hours [9-11]. The sedimentation goes faster with increasing of the nanoparticle concentration. In order to overcome these drawbacks, synthesis of biocompatible CNPs in pure water, citrate, cyclodextrin, polymers and surface modification using few functionalizing moieties such as polyethylene glycol and heparin have already been reported [7, 12-14]. Most of those protecting agents were found to be either readily washed off or reduce the catalytic activity of the nanoparticles upon exposure to physiological condition and ended up with short circulation time in in vivo environment [9, 15].

Therefore, it is important to search for new functionalizing moieties as well as in situ surface engineering strategies for production of improved grade CNPs for
nanomedicine application. Furthermore, the physicochemical characterization of surface modified CNPs is pre-requisite for the adequate assessment of their biological application. Uses of organosilanes have already been reported to engineer the surface of magnetic and silica nanoparticles [16, 17]. Surface engineering of the CNPs could be possible via silanization under defined and optimized reaction condition [18]. Possible functionalization of CNPs with hydrophilic biocompatible silane moieties could improve the aqueous dispersibility of the nanoparticles [18-19].

In this report, 6-{2-[2-(2-Methoxy-ethoxy)-ethoxy]-ethoxy}-hexyl)triethoxysilane (MEEETES) was used as a hydrophilic biocompatible silane for in situ surface modification of surface of CNPs produced via ammonia-induced ethylene glycol (EG) assisted precipitation method. The surface modification was carried out in a single and two step synthetic procedure and the modified nanoparticles were characterized using a variety of complementary techniques including powerful $^{13}$C cross polarization magic angle sample spinning (CP-MAS) solid state-nuclear magnetic resonance (Solid state-NMR) to get a better understanding on surface modified CNPs.

2. Experimental

2.1 Preparation and surface modification of Cerium oxide nanoparticles (CNPs)

CNPs were synthesized by ammonia (NH$_3$)-induced ethylene glycol (EG) assisted precipitation method [18]. Briefly, 7.8 mL (0.12 mol) of EG (99%, Sigma Aldrich) was added to 92.2 mL of de-ionized water (Millipore Water System) in a 250 mL of two neck round bottom distilling flask at 50 °C in a silicone bath reflux condenser system kept under constant magnetic stirring at 350 rpm. 5.16 g (0.012 mol) of Cerium (III) nitrate hexahydrate (Ce(NO$_3$)$_3$·6H$_2$O) (99.99%, Sigma Aldrich) was
added into the EG/H₂O solution at EG to Ce³⁺ molar ratio of 10:1. After complete dissolution of Ce(NO₃)₃·6H₂O, 5 mL of aqueous ammonia (NH₄OH) (29.44%, Fisher Scientific) was added to the solution and the pH was adjusted to 9.6. The solution was kept under constant stirring at 750 rpm and allowed to react until it became yellow (as a confirmation of CNP formation). The reaction volume was subjected to vacuum filtration using standard Whatman (Ø = 110 mm and grade 589/3) filter paper over a Buchner funnel. The filtered materials were subjected to alternate wash with ethanol and de-ionized water (6 times) and dried overnight in a fume hood. The dried materials were crushed and grinded in a ceramic mortar using a pestle and freeze-dried using a freeze-drier (Labconco). The freeze-dried nanoparticles were again crushed and grinded using the ceramic mortar and pestle to ensure the production of fine and powdered CNPs.

Surface modified CNPs were produced by functionalization of MEEETES to the surface of CNPs in in situ. For production of CNPs modified with MEEETES functionalities (MEEETES-CNPs), the above method of preparation of CNPs was first followed. After the confirmation of CNP formation, the surface modification was followed as the immediate step of reaction. Instead CNP recovery, 400 µL of MEEETES (20 mM) (SiKEMIA) was added to entire 100 mL of reaction volume under an inert atmosphere and was allowed to react for overnight. Once the color of the solution turned to milky yellow (as an indication of MEEETES-CNP formation), the reaction was stopped. MEEETES-CNPs were recovered via centrifugation (17000 rpm, 10 m, 4 °C) using a high speed refrigerated centrifuge (Avanti J-26XP, Beckman Coulter). The nanoparticles were further washed using mixture of
ethanol and H$_2$O using centrifugation at identical condition. The pellets in centrifuge tubes were dispersed in de-ionized water using sonication and were freeze-dried using freeze-drier. The freeze-dried nanoparticles were crushed and grinded using the ceramic mortar and pestle to ensure the production of fine and powdered MEEETES-CNPs.

2.2 Characterization

2.2.1 Transmission electron microscopy (TEM)

TEM and selected area electron diffraction (SAED) of nanoparticles were carried out using an FEI Titan 80–300kV (ST) with the field-emission gun operating at 300 kV. TEM in conjunction with energy-dispersive X-ray analysis (EDXA) were carried out to investigate the elemental information of nanoparticles.

2.2.2 X-ray diffraction (XRD)

Powdered XRD measurement of the nanoparticles were performed in a $\theta$-$\theta$ mode from 20 to 80 degrees (20) by a Bruker D8 advance X-ray diffractometer using Cu K$\alpha$ radiation ($\lambda = 0.154$ nm) operated at 40 kV and 40 mA in the Bragg–Brentano geometry with a linear position-sensitive detector (an opening of 2.9°).

2.2.3 Dynamic light scattering (DLS)

DLS and zeta potential measurement of nanoparticles were performed by Zetasizer Nano (Malvern) at 37 °C using a 1 cm path length quartz cuvette. The size distributions and sizes measured were measured and sizes were expressed as mean ± standard deviation (SD) (n=3).

2.2.4 CP-MAS $^{13}$C NMR
CP-MAS $^{13}$C NMR experiment on CNPs and MEEETES-CNPs was performed on a Bruker Avance III 400 MHz spectrometer equipped with a triple-resonance 4 mm Bruker MAS probe. $^{13}$C CP-MAS NMR spectra were recorded at a resonance frequency of 100.622 MHz under 14 kHz spinning rate and were normalized with CNPs. The temperature for all experiments was kept at 298 K. The cross-polarization CP contact time was set to 2 ms employing ramp.100 for variable amplitude CP. Bruker Topspin 3.0 software was used for data collection and spectral analysis.

3. Results and Discussion

The surface modification of CNP was carried out in two distinct steps of a single synthetic procedure [18], which involves the initial step of hydroxylation of CNP surface using EG and subsequent reaction of MEEETES with the exposed surface hydroxyl (-OH) groups. Figure 1 shows the TEM and high resolution-TEM (HR-TEM) images of the nanoparticles.

The microscopic images of the nanoparticles revealed the production of extremely small polyhedral shape nanoparticles of 5-10 nm size. No significant size difference was observed among the individual nanoparticles of CNPs and MEEETES-CNPs, indicated that the surface modification did not affect the individual nanoparticle size. The results demonstrated the functional ligand moieties were extremely small. TEM images demonstrated the significant differences in dispersion of CNPs at their functionalized and non-functionalized state. MEEETES-CNPs showed more homogeneous dispersion as well as less aggregation than CNPs. The information obtained from TEM was further validated using DLS measurement (Figure 2).
DLS cannot discriminate between inorganic and organic materials and measures overall nanoparticle size i.e. the hydrodynamic diameter of the nanoparticle at their best possible aggregated state [20]. The aggregate size or hydrodynamic diameter (d.nm) was found to have a single significant peak of average particle size (mean Z-Ave) of (200.6±0.5) nm for CNPs and (96.8±2.2) nm for MEEETES-CNPs respectively (Figure 2 B). The results indicated that the nanoparticle aggregation was significantly reduced after the surface functionalization process and have better aqueous dispersibility than CNPs.

Figure 3

Figure 3 showed the zeta potential distribution of CNPs and MEEETES-CNPs. The measured zeta potential values of CNPs and MEEETES-CNPs were (-32.0 mV) and (-42.3 mV) respectively. Zeta potential is related to the charge on the surface of nanoparticle and is an essential indicator of the stability of nanoparticles in a colloidal system [21]. In principle, if all the particles in suspension have a large negative or positive Zeta potential they will tend to repel each other and there is no tendency to flocculate or aggregate. However, if the particles have low Zeta potential values then there is no force to prevent the particles coming together and there is higher tendency to flocculate. The general dividing line between stable and unstable suspensions is generally taken at either +30mV or -30mV. Particles with Zeta potentials more positive than +30mV or move negative than -30mV are normally considered stable and stability increases with increasing positive or negative value. MEEETES-CNPs indicated greater stability than CNPs in colloidal state. Higher colloidal stability of nanoparticles is extremely important for safe use of nanoparticles for use in nanomedicine [21]. The improved water dispersibility and
higher colloidal stability of MEEETES-CNPs could be due to the surface hydrophilicity in combination with electrostatic and steric repulsion among the nanoparticles [19].

The SAED of both nanoparticles showed ring-like patterns (Figure 1: B and F). The images revealed the high crystallinity and ordered structure of the nanoparticles lattice planes. Both SAED and HR-TEM images demonstrated the lattice fringes can be clearly observed for both the nanoparticles. The powdered XRD pattern of CNPs and MEEETES-CNPs indicated the diffraction peak of the nanoparticles were indexed as CeO₂ phase (Figure 2A). The characteristic diffraction peaks marked respectively by their indices (111), (200), (220), (311), (222), (400), (331) and (420) should be well indexed to the FCC fluorite structure of available CNPs [22]. More importantly, the characteristic peaks did not change (other than intensity and minor variation in width) after the surface modification process. This indicated that the crystalline structure of the nanoparticles was unaltered after surface modification. Moreover, it was noticed that the peaks were broad as an indication of the formation of very fine particles in the nanoscale regime and is in strong agreement with the results obtained from TEM.

The EDXA analysis of CNPs and MEEETES-CNPs elementally demonstrated the presence of additional “Si” in MEEETES-CNPs (Figure 1: D and H) as a preliminary confirmation of the presence of MEEETES in surface modified CNPs. In order to get a better understanding over the presence and chemical interaction of functional moiety to the nanoparticle surface, $^{13}$C CP-MAS solid state NMR was used to study the chemical composition and molecular structure of surface modified CNPs.
Figure 4

Figure 4 shows the $^{13}$C CP-MAS spectrum of MEEETES-CNPs with annotated peaks and a representative chemical structure of MEEETES. NMR spectrum of MEEETES-CNPs shows seven peaks that can be assigned to the different carbon atoms of MEEETES moiety. It was expected that ethoxy groups of MEEETES would give two very strong almost identical NMR signals at 18.2 ppm (CH$_3$-CH$_2$-O-), and at 58.4 ppm (CH$_3$-CH$_2$-O-) [23, 24]. The absence of those signals in the spectrum indicated that all alkoxy silanes were bonded to the surface of the nanoparticle since the nanoparticles were washed thoroughly upon surface functionalization. All other signals were assigned according to published reports and are in agreement with structure proposed [23, 24]. It could be noticed though, that the signal at 14.08 ppm, corresponding to (-Si-CH$_2$-) group, was distinctly broader than other signals. This might be because not all MEEETES molecules have bonded with all three Si-O-Si bonds, but some only with two or even one, like R-Si(OH)(Si-O-)$_2$ or R-Si(OH)$_2$(Si-O-) (Figure 5). However this doesn’t contradict with the conclusion that all functional MEEETES moieties are strongly covalently bonded to the surface of nanoparticles.

Figure 5

4. Conclusion

For nanomedicine applications, nanoparticles need to be stable with respect to aggregation and well dispersible in aqueous media. The report provides a generic approach to engineer the surface of Cerium Oxide nanoparticle with small sized biocompatible silane moiety for stable nanoparticle dispersions in aqueous media. As
an important part of the conclusion, it is worth noted that the binding of these functional chemical moiety to the nanoparticle surface are covalent, which means that they cannot be easily desorbed from the nanoparticle surface and should demonstrate better stability and enhanced circulation time in biological environment. The prepared surface modified Cerium Oxide nanoparticles should be useful for nanomedicine and several other nanobiological application.

Conflict of interests

The author declares that there is no conflict of interest regarding the publication of this research article.

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References


**Figure Captions**

**Figure 1.** TEM (A and E), HR-TEM (C and G), SAED (B and F) and EDXA analysis (D and H) of CNPs (A, B, C and D) and MEEETES-CNPs (E, F, G and H), Red arrow: presence of additional element “Si” in EDXA of MEEETES-CNPs.

**Figure 2.** Figure 2. Powdered X-ray diffraction (A) and nanoparticle hydrodynamic size distribution (B) of CNPs and MEEETES-CNPs.

**Figure 3.** Zeta potential value distribution of CNPs (A) and MEEETES-CNPs (B) in aqueous medium.

**Figure 4.** The $^{13}$C CP-MAS spectrum of MEEETES-CNPs with annotated peaks and the chemical structure of MEEETES as a reference.

**Figure 5.** Schematic illustration for formation of MEEETES-CNPs and possible chemical structure of MEEETES-CNPs.
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