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Protein adsorption and cellular uptake of cerium oxide nanoparticles as a function of zeta potential

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Abstract

The surface chemistry of biomaterials can have a significant impact on their performance in biological applications. Our recent work suggests that cerium oxide nanoparticles are potent antioxidants in cell culture models and we have evaluated several therapeutic applications of these nanoparticles in different biological systems. Knowledge of protein adsorption and cellular uptake will be very useful in improving the beneficial effects of cerium oxide nanoparticles in biology. In the present study, we determined the effect of zeta potential of cerium oxide nanoparticles on adsorption of bovine serum albumin (BSA) and cellular uptake in adenocarcinoma lung cells (A549). The zeta potential of the nanoparticles was varied by dispersing them in various acidic and basic pH solutions. UV–visible spectroscopy and inductively coupled plasma mass spectrometry (ICP-MS) were used for the protein adsorption and cellular uptake studies, respectively. Nanoceria samples having positive zeta potential were found to adsorb more BSA while the samples with negative zeta potential showed little or no protein adsorption. The cellular uptake studies showed preferential uptake for the negatively charged nanoparticles. These results demonstrate that electrostatic interactions can play an important factor in protein adsorption and cellular uptake of nanoparticles.

Keywords

Cerium oxide nanoparticles; Protein adsorption; Cellular uptake; Zeta potential; Electrostatic interactions

1. Introduction

It is useful to improve the knowledge of the mechanisms of biological interaction of materials with cells and tissues, and consequently to be able to improve the performance of biomaterials. Recent advances in nanotechnology have resulted in the generation of various nanostructured biomaterials which have promising and highly controllable properties for medical purposes, ranging from diagnosing diseases to providing novel therapies [1–4]. Due to large surface area/volume ratio nanoparticles tend to agglomerate and adsorb proteins. When bound to proteins, they may be quickly cleared by macrophages before they can reach target cells. One possible approach to increase the circulation time of nanoparticles in blood stream is to modify the

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particles surface to disperse them and minimize or eliminate protein adsorption [5]. Synthetic adsorbents have been examined under different conditions such as solution pH and protein concentration for adsorption of proteins and in many cases, the mechanism of protein adsorption has been attributed to electrostatic interaction [6,7]. The electrostatic interaction of the nanoparticles can be controlled by variation in their surface charges, which can be determined by measuring the zeta potential of these particles. In metal oxides, zeta potential can be altered by changing the pH, conductivity, or concentration of a formulation component [8].

Recently, we have found cerium oxide nanoparticles to be very useful in biomedical applications. Treatment of rat neuron cells with nanoceria increased the cultured lifespan and reduced the cell injury induced by hydrogen peroxide or UV light *in vitro* [9]. Other potential biological uses of nanoceria have emerged from studies showing protection of primary cells from the detrimental effects of radiation therapy [10], prevention of retinal degeneration induced by intracellular peroxides [11] and neuroprotection to spinal cord neurons [12].

In the present study, we analyzed effects of zeta potential variation of nanoceria particles on protein adsorption and cellular uptake using an *in vitro* cell culture lung cell model (A549). A better understanding of protein adsorption will improve the ability to control conformation, orientation, and stability which will allow us to make tailored surfaces for bioactivity. Serum albumin is the most abundant protein found in human blood. The knowledge of adsorption of albumin to ceria surfaces is very important because once in the body, blood proteins will adsorb to the particles and cells will then react with the adsorbed proteins on the particles which will ultimately affect cellular uptake and can alter biochemical activity [13]. To determine binding of ceria to albumin, we used bovine serum albumin (BSA) as a model.

Our goal in this study is to determine how surface charge modification (measured by zeta potential) of cerium oxide nanoparticles (nanoceria) affects protein adsorption and cellular uptake of these particles. Considering the beneficial effects of the cerium oxide nanoparticles in biological applications, a study related to their protein adsorption and cellular uptake is very important.

2. Experimental

2.1. Cerium oxide nanoparticles synthesis

Cerium oxide nanoparticles were synthesized using two different wet-chemical synthesis processes namely, microemulsion and hydrothermal synthesis technique. The detailed process for microemulsion synthesis of nanoceria is given elsewhere [14]. The nanoparticles powder was obtained by washing the precipitate of nanoceria several times with acetone and water to remove the surfactant used in the synthesis process. The hydrothermal synthesis process of nanoceria is given below.

Cerium nitrate was dissolved in NANOpure water (resistance: 18.2 MΩ) to obtain 0.1 M solution. Equal volume of 0.5 N ammonium hydroxide solution was then added with stirring at about 300 rpm. The solution was then heated in an oven at 110 °C to evaporate all the water in the solution and the cerium oxide powder obtained was then heated at 300 °C for an hour and then furnace cooled. The powder was then transferred into a Teflon-lined stainless-steel autoclave containing 2.0 N sodium hydroxide solution up to 80% of its total volume and heated at 120 °C for 24 h under autogenous pressure. The system was then allowed to cool down to room temperature. Finally, the resulting solution was titrated with HCl to bring the pH of the solution to 7.0 and then the excess liquid was evaporated off resulting in hydrothermal ceria nanoparticles.

The obtained nanoceria particles were characterized using high-resolution transmission electron microscopy (HRTEM). The HRTEM images were obtained with Philips (Tecnai series) transmission electron microscope operated at 300 keV.

2.2. pH buffer treatment and zeta potential measurements

The zeta potential of the synthesized nanoceria was varied by mixing 5 mg of the nanoceria with 5 ml of buffer solution and stirring the solution using magnetic stirrer for 30 min. Following, nanoceria particles were centrifuged and re-dispersed in 5 ml of NANOpure water and stirred once again for 30 min. Water was used as the dispersant for the particles to measure their zeta potential. The zeta potential of the particles was measured by the Zetasizer (Nano-ZS) from Malvern Instruments and its software, Dispersion Technology Software (DTS).

The pH buffers used were 1, 1.9, 3, 5, 7, 8, 11, and 13. All of these buffers were hand made to ensure that each one had the same ions in solution. Each pH buffer had 25 ml of 0.2 M KCl solution and 75 ml of NANOpure water. HCl or NaOH solutions were added as necessary to adjust the pH. Acidic pH buffer treatment of ceria particles in theory will create a positive zeta potential because of the increase in the H^+ ion concentration, and basic buffer treatment will cause a negative zeta potential because of the increase in OH^- ion concentration [15].

2.3. Protein adsorption

To determine protein adsorption, 2.5 ml of nanoceria solution, 2 ml of NANOpure water and 0.5 ml of 2 mg/ml BSA were added together and stirred vigorously with a magnetic stirrer for 2 h. The nanoceria particles were centrifuged and the concentration of BSA was determined in the supernatant using UV–visible spectroscopy (Cary 1E UV-Visible Spectrophotometer, Varian Analytical Instruments) by determining the absorbance maximum at 280 nm wavelength. A calibration curve was prepared using known concentrations of BSA and is shown in Fig. 1 [7,16–18]. The protein adsorbed on the nanoceria particles was calculated using following equation:

$$q = \frac{(C_i - C_f)V}{m}, \quad (1)$$

where C_i and C_f are the initial BSA concentration and the BSA concentration in the supernatant after adsorption studies, respectively; V is the total volume of the solution (5 ml); and m is the weight of the nanoceria particles added into the solution.

2.4. Cellular uptake

Six samples of cerium oxide nanoparticles were used for cellular uptake, which are described in Table 1. Adenocarcinoma lung cells (A549) were cultured in Dulbecco's Modified Eagle Media (DMEM) containing 10% fetal bovine serum. Cells were treated with 1.72 $\mu\text{g}/\text{ml}$ cerium oxide nanoparticle concentrations for each particle preparation. The cells were incubated at 37 $^{\circ}\text{C}$ in a humidified incubator containing 5% CO_2 atmosphere for 2 days and then collected and washed with PBS to remove excess media. Since nanoceria particles are not detectable by fluorescence microscopy, inductively coupled plasma mass spectrometry (ICP-MS) measurements were conducted on the nanoceria-treated cells to determine the amount of nanoceria taken up by the cells.

3. Results and discussions

3.1. HRTEM analysis of nanoceria

Fig. 2 shows the bright field HRTEM images of both microemulsion and hydrothermal nanoceria samples. While the microemulsion nanoceria particles are only 3–5 nm in size, the hydrothermal nanoceria particles are 8–10 nm in size. The hydrothermal process creates

different size nanoparticles because of the heat treatment and increased pressures involved, whereas the microemulsion method is at room temperature. Lattice fringes shown in both the figures indicate the crystalline nature of the particles. Due to smaller size, the microemulsion nanocereria tend to agglomerate more compared to the hydrothermal nanocereria.

Ceria fluorite structure has three low-index planes, namely very stable $\{1\ 1\ 1\}$ plane, the less stable $\{1\ 1\ 0\}$ plane and the higher energy $\{0\ 0\ 1\}$ plane [19]. The $\{1\ 1\ 0\}$ and $\{0\ 0\ 1\}$ planes are less stable compared to the $\{1\ 1\ 1\}$ planes. Therefore $\{1\ 1\ 1\}$ planes are inherently less reactive [20–22]. Yang et al. [23] found that adsorption of CO on $\{1\ 1\ 1\}$ is weak, whereas there are both weak and strong adsorptions on the $\{1\ 1\ 0\}$ plane. Therefore, if planes with higher surface energy could be generated and stabilized, they could provide more sites for chemical adsorption. Fig. 2 show dominantly $\{1\ 1\ 1\}$ planes for the synthesized nanocereria samples, which are observed when the nanoparticles are oriented along the $[1\ 1\ 0]$ direction. This can be useful in achieving better protein adsorption.

3.2. Zeta potential studies

Fig. 3 gives the zeta potential of both hydrothermal and microemulsion nanocereria as a function of pH of the buffer solution used for treatment. It shows that the zeta potential of the as prepared hydrothermal nanocereria is positive while that of microemulsion-based nanocereria is negative. This difference in the zeta potential is due to the chemicals involved in the synthesis process. In case of microemulsion process, use of NH_4OH for nanoparticles synthesis results in -16.26 mV zeta potential. On the other hand, the ceria nanoparticles were treated with HCl solution in the last step during hydrothermal synthesis process leading to $+36.60$ mV zeta potential for the as synthesized nanoparticles.

The isoelectric point (IEP) is the pH of a dispersion medium of a colloidal suspension at which the colloidal particles carry no net charge. The colloidal system is the least stable at the IEP as there are no inter-particle repulsive forces due to absence of particle surface charges. Practically, all metal oxides become charged by the adsorption of hydrogen ions (H^+) or hydroxide ions (OH^-), while remaining neutral at a specific pH. In case of the microemulsion nanocereria the IEP is approximately 4.5 and is about 9.5 for hydrothermal nanocereria. The difference in the IEP is due to different zeta potentials of the as synthesized nanoparticles.

The correlation of zeta potential to pH is important to know so that one can predict how the varying pH inside the human body will affect the surface charge of the nanocereria particles. Furthermore, because proteins are abundant in the human body it is essential to know how pH, zeta potential, particle size, and synthesis procedure affects protein adsorption onto ceria nanoparticles. Because of the positive zeta potential, the particles can be expected to enhance the removal of negatively charged substances from the liquid solution.

3.3. Protein adsorption studies

The surface chemistry of biomaterials has great effects on the protein adsorption process [24]. Protein adsorption on various materials has been widely studied and it has been found that, factors such as electrostatic interaction, hydrophobic interaction and specific chemical interactions between protein and the adsorbent play important roles. Selective adsorption of proteins on various synthetic adsorbents has been examined under different conditions (such as solution pH and protein concentration) and for many proteins the mechanisms of selective adsorption has, in several reports, been attributed to the electrostatic interaction [6,18,25].

The comparison of BSA adsorption and zeta potential as obtained in Fig. 4 clearly indicates that positive zeta potential for nanocereria particles favors the protein adsorption. Both the microemulsion as well as hydrothermal nanocereria samples having positive zeta potential

displayed good protein adsorption. The negatively charged samples did not significantly adsorb protein. The higher protein adsorption for the hydrothermal nanoceria can be attributed to the positive zeta potential obtained for the as synthesized nanoparticles due to the use of HCl treatment.

The surface charge property of a material influences the protein adsorption process under physiological conditions. By varying the surface charges one can vary the electrostatic interaction between the protein and the adsorbent for selective adsorption of a particular protein [26]. Factors such as pH and solution electrolyte concentration have a considerable impact on the strengths and types of electrostatic charges on the adsorbent and thus can lead to different protein and surface interactions under different conditions. Burns and Zydney [27] investigated the effect of solution pH on the transport of globular proteins with different surface charge characteristics and showed that adsorbent charge does have a significant effect on the protein adsorption. They found that there is a strong attractive interaction between α -chymotrypsinogen which has a strong positive charge and the negatively charged Biomax membrane.

The BSA protein charge is dependant on pH and ionic strength of the solution. At the IEP of BSA (pH 4.78), its structure is more hydrophobic and compact and adsorption occurs mainly due to hydrophobic interactions. Above the IEP of BSA, it has negative charges [26,28] and the electrostatic forces are dominant over hydrophobic interactions. The BSA adsorption studies in the present study were carried out in water (pH 7.0) which gives negative charges on BSA. The attractive forces between the positively charged nanoceria particles and the negatively charged BSA protein lead to protein adsorption while the repulsive forces between the negatively charged nanoparticles and BSA lead to no protein adsorption.

A protein adsorption of 112.65 mg/g of nanoceria was obtained for the hydrothermal nanoceria with zeta potential of 40.20 while microemulsion ceria samples with zeta potential of 32.20 and 43.64 adsorbed 59.32 and 67.40 mg of protein per gm of nanoceria, respectively (Fig. 4). The hydrothermal nanoceria with zeta potential of 40.20 mV adsorbed much more protein than microemulsion nanoceria with a higher zeta potential of 43.64 mV. This may be due to intrinsic positive zeta potential of the hydrothermal nanoceria. Also, the hydrothermal nanoceria dispersion in water was more stable with low agglomeration. This can lead to greater effective specific surface area for the hydrothermal nanoceria compared to the microemulsion nanoceria to give more protein adsorption.

In addition, the nanoceria samples showed increased protein adsorption with increasing zeta potential which further confirms that the electrostatic forces is the primary interaction for BSA adsorption in the present study. The microemulsion nanoceria sample with -17.72 mV charge adsorbed small amount of the protein. This behavior shows that protein adsorption is not only due to electrostatic interactions, but also due to van der Waals, hydrophobic, hydrophilic, structural and steric interactions between the protein and the adsorbent as well as between the protein molecules. Robertson and Zydney [29] reported that proteins could be adsorbed on surfaces with the same charge. Nonetheless, majority of the samples showed electrostatic interaction as the main driving force for the protein adsorption.

3.4. Cellular uptake of nanoceria

Targeted entry into cells is an important area of research in drug delivery and therapy [30–32]. Site specific delivery of drugs and therapeutics can significantly reduce drug toxicity and increase therapeutic effects. Nanoparticles could be used as effective delivery vehicles for intracellular targeting and have been extensively studied for delivering drugs, genes and vaccines [33,34]. Understanding the interactions of nanoparticles with cells is crucial for improving their behavior *in vivo* and *in vitro*. A better understanding of uptake into cells and

the potential for degradation in intracellular compartments is a key gap in nanotechnology that needs to be filled. In order to increase the intracellular uptake of nanoparticles transfection agents have been used [35]. However these agents are toxic molecules and not approved for clinical use [36]. Also coating the particles by appropriate bio-adhesive materials such as polyvinyl alcohol (PVA), poly(ethylene glycol) (PEG), poly (D, L-lactic-co-glycolic acid) can greatly improve their cellular uptake [37–40].

The uptake of nanoparticles by cells can be viewed as a two-step process: first a binding step on the cell membrane and second the internalization step [41]. The attachment of the particles to the cell membrane as the first step seems to be most affected by the surface charge of the particles [36]. Therefore we studied the uptake of cerium oxide nanoparticles as a function of the amount of surface charge on these particles without using any transfection agent.

A549 cells were treated with different concentrations of cerium oxide nanoparticles dispersions. The results of the cellular uptake showed that the A549 cells treated with concentrations lower than 1.72 $\mu\text{g/ml}$ of ceria nanoparticles had little or no cellular uptake (see Fig. 5). Fig. 6 shows the nanoceria uptake by A549 cells treated with the 1.72 $\mu\text{g/ml}$ concentration of microemulsion and hydrothermal nanoceria dispersions having different zeta potentials (see Table 1 for the samples details). Microemulsion ceria treated with pH 13 solution proved to have the highest cellular uptake at a concentration of 1.56 $\mu\text{g/l}$. The zeta potential of this sample was -43.10 mV. The hydrothermal nanoceria with negative zeta potential also showed higher cellular uptake compared to the one with positive zeta potential. Similar results of higher cellular uptake for anionic nanoparticles of iron oxide by HeLa cells were shown by Wilhelm et al. [41]. Based on these results, surface charge of the nanoparticles seems to be a key determining factor for cellular uptake.

Nanoparticles show a high affinity for cellular membrane mainly due to electrostatic interactions [41]. After the adsorption of the nanoparticles on the cellular membrane, the uptake occurs via several possible mechanisms like, pinocytosis, non-specific or receptor-mediated endocytosis or phagocytosis [36]. It is however known that cell membranes possess large negatively charged domains, which should repel negatively charged nanoparticles. The zeta potential studies on the A549 cells indicated mean zeta potential of -10.2 mV with a deviation of ± 19.7 mV. This indicates that there are few cationic sites for adsorption of the negatively charged particles. Wilhelm et al. [41] suggested that the negatively charged particles bind at the cationic sites in the form of clusters because of their repulsive interactions with the large negatively charged domains of the cell surface. In addition, the nanoparticles, already bound on the cell surface present a reduced charge density that may favor adsorption of other free particles. Thus, the high cellular uptake of negatively charged nanoparticles is related first to the non specific process of nanoparticles adsorption on the cell membrane and second to the formation of nanoparticles clusters. Studies by Limbach et al. on human lung fibroblast cells also indicated that cells rapidly absorb negatively charged ceria nanoparticles [42].

In the present study, the smaller size of the microemulsion ceria (3–5 nm) as compared to the hydrothermal nanoceria (8–10 nm), may explain its efficient uptake. Although the mechanism for better cellular uptake of negatively charged nanoparticles needs further study, the adsorption of the negatively charged particles at the positively charged sites via electrostatic interaction can lead to localized neutralization and a subsequent bending of the membrane favoring in turn endocytosis for cellular uptake [41]. In conclusion, simple surface charge modification of the cerium oxide nanoparticles can be useful to alter their cellular uptake. Thus, it is possible to localize the nanoparticles to specific intracellular targets (lysosomes, cytoplasm, mitochondria etc.) by modifying their surface charge [34,43].

4. Conclusions

Cerium oxide nanoparticles were synthesized by microemulsion and hydrothermal process. While the microemulsion process led to negative zeta potential, the hydrothermal process gave positive zeta potential to the as-synthesized nanoparticles. Thus, the zeta potential of the resulting nanoceria particles is dependent on the synthesis process. The IEP was found to be approximately 4.5 and 9.5 for the microemulsion and hydrothermal nanoceria, respectively. Electrostatic interactions may be the main driving force for the protein adsorption and cellular uptake of the nanoceria. The positive zeta potential for the nanoceria samples was favorable for albumin adsorption while negative zeta potential was found to be favorable for the nanoparticles uptake in the A549 cells. The negative charges carried by the BSA affect the protein adsorption. The cellular uptake of the cerium oxide nanoparticles is also dependent on the surface charge. The results of this study can allow future research to better tailor nanoparticle preparations by simple surface charge modifications.

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Fig. 1. Standard curve for BSA concentration measurements. UV–visible spectroscopy measurements were carried out for known concentrations of BSA at the absorbance maximum of 280 nm. The absorption coefficient, ϵ was determined to be $0.7224 \text{ mg ml}^{-1} \text{ cm}^{-1}$.

Fig. 2. Bright field HRTEM images of (a) microemulsion nanocereria (particle size: 3–5 nm) and (b) hydrothermal nanocereria (particle size: 8–10 nm). Arrows indicate the dominant 111 planes of the nanoparticles on the TEM grid which are observed when the nanoparticles are oriented along the $[1\ 1\ 0]$ direction. Such orientation can be useful in achieving better protein adsorption.

Fig. 3.

Variation in the zeta potential of the nanoceria samples as a function of the pH of the buffer solution. Inherent zeta potential was negative for microemulsion nanoceria and positive for hydrothermal nanoceria. The isoelectric points (IEP) of 4.5 and 9.5 were observed for the microemulsion and hydrothermal nanoceria, respectively.

Fig. 4. Amount of BSA adsorbed on the (a) microemulsion and (b) hydrothermal nanoceria samples as a function of its zeta potential. * Indicates no significant protein adsorption was observed for the sample. Positively charged nanoparticles favored BSA adsorption and showed increased protein adsorption with increasing zeta potential.

Fig. 5. Cellular uptake of the nanoceria samples in A549 cells for different concentrations of nanoceria treatment. Inset gives the expanded plot of the results at smaller concentrations of nanoceria. * Indicates no significant cellular uptake was observed for the sample (details of the samples are given in Table 1). Cells treated with concentrations lower than 1.72 µg/ml of ceria nanoparticles had little or no cellular uptake.

Fig. 6. Cellular uptake of the (a) microemulsion and (b) hydrothermal nanoceria samples in A549 cells versus their zeta potential. Samples with negative zeta potential showed higher cellular uptake compared to the one with positive zeta potential.

Table 1

Description of the nanoceria samples used for cellular uptake studies

Sample	Description	Zeta potential (mV)
CeO ₂ -1	As-synthesized microemulsion ceria in water	-16.26
CeO ₂ -2	Microemulsion ceria treated with pH 13.0 buffer	-43.10
CeO ₂ -3	Microemulsion ceria treated with pH 1.0 buffer	45.01
CeO ₂ -4	As-synthesized hydrothermal ceria in water	36.60
CeO ₂ -5	Hydrothermal ceria treated with pH 13.0 buffer	-42.46
CeO ₂ -6	Hydrothermal ceria treated with pH 1.0 buffer	41.59