

Restored Endothelial Dependent Vasodilation in Aortic Vessels after Uptake of Ceria Coated Silica Nanoparticles, *ex vivo*

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Abstract

Ceria nanoparticles (CeNPs) have attracted considerable interest in the treatment of a number of conditions associated with increased production of reactive oxygen species (ROS), due to their unique antioxidant properties. We have previously demonstrated the attenuation in vasodilation after uptake of silica nanoparticles (SiNPs). Hence, we investigated whether ceria coating of SiNPs would improve the magnitude of vasodilation. Ceria coated SiNPs (CeSiNPs) were fabricated and fully characterised and their direct influence on vasodilator responses of aortic vessels examined, *ex vivo*. We demonstrate that while SiNPs significantly attenuate endothelial-dependent (acetylcholine-ACh) vasodilation, their surface modification with CeNPs leads to significant improvement in dilator responses (n=5, p<0.001, at most ACh concentrations). These findings have implications in the fabrication of biocompatible nanoparticles for medical intervention. Furthermore, CeSiNPs may represent novel therapeutic tools for the protection and treatment of conditions where attenuated dilator responses are observed.

Keywords: Nanoparticles; Silica; Ceria; Free-radical; Vasodilation

Introduction

Ceria (Cerium oxide; CeO₂) nanoparticles have attracted considerable interest in the treatment of conditions associated with increased production of reactive oxygen species (ROS), due to their unique antioxidant properties. Ceria nanoparticles (CeNPs) have been shown to protect against ischaemic stroke [1], and protect cells against radiation damage [2]. The redox property of CeNPs is due to the large number of surface oxygen vacancies giving it a radical scavenging property which has been described to be more efficient than the biological antioxidant, superoxide dismutase (SOD) [3]. Silica nanoparticles (SiNPs) have potential biomedical applications, but may themselves generate ROS upon cellular uptake [4,5]. Indeed, we have demonstrated that exposure of aortic vessels to SiNPs attenuated vasodilation, which improved after co-incubation in SOD, suggesting a role for ROS in quenching nitric oxide (NO) [6]. In the present study, we fabricated and characterised ceria coated SiNPs (CeSiNPs) and compared their influence on vasodilator responses of aortic vessels with SiNPs *ex vivo*. We suggest that ceria coating of SiNPs will restore vasodilator responses, hence increase their biocompatibility for use in therapeutic intervention.

Experimental

Materials and solutions

All reagents and agonists were purchased from Sigma-Aldrich (UK). Millipore water was used for all experiments. Physiological Salt Solution (PSS) was prepared using the following chemical composition [mM]: 119 NaCl, 4.7 KCl, 1.2 MgSO₄·7 H₂O, 25 NaHCO₃, 1.17 KH₂PO₄, 0.03 K₂EDTA·2H₂O, 5.5 glucose and 1.6 CaCl₂·2H₂O; pH 7.4. Potassium Physiological Salt Solution (KPSS-60 mM) was prepared using the following chemical composition [mM]: 78.2 NaCl, 60 KCl, 1.2 MgSO₄·7 H₂O, 25 NaHCO₃, 1.17 KH₂PO₄, 0.03 K₂EDTA·2H₂O, 5.5 glucose and 1.6 CaCl₂·2H₂O; pH 7.4 (as previously described was prepared as previously described [6]).

Synthesis and characterisation of nanoparticles

SiNPs were synthesised via a sol-gel precipitation method as

previously described [6], the ceria precursor and ceria nanoparticulate shell was grown on the SiNPs surface as previously described by Oh et al. [7]. For ceria nanoparticles, ceria precursor (3 mL) was added to water (30 mL) while stirring, then stirred vigorously over 30 min. The pH of the solution was adjusted to 9.1 and stirred for 3 hours 35 min at 60°C. The product was collected by centrifugation (6000 rpm/30 minutes) and then washed with water several times. The NP size was determined by photon correlation spectroscopy (DLS, Malvern Zetasizer nano ZS instrument, UK). Briefly, the suspended NPs were placed in a polystyrene cuvette to give 1 mL 0.02% solution. DLS of NPs was measured in distilled water and also in PSS solution. The hydrodynamic size was measured by an infra-red light passing through the sample and any resulting scattered light was detected. Zeta potential (zetasizer nano ZS Malvern Instrument, UK) of the NPs was measured by placing the suspension containing 0.02% solution in the disposable zeta capillary cell. The zeta potential was obtained in the automatic mode by detecting the electrophoretic mobility produced by laser Doppler velocimetry. The potentials were determined for NPs in both distilled water and PSS. NPs were prepared for transmission electron microscopy (TEM) as follows: a 100 µL drop of NP solution suspended in water was placed onto a holey carbon coated TEM copper grid (Agar Scientific Ltd., UK). The copper grid was dried in an oven at 50°C, for 2 hours. The NP sizes were further confirmed using TEM (Philips TechnaiTM12 Biotwin TEM) analysis.

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Vascular functional studies

Thoracic aortic arteries of male Wistar rats (150-250 g) were excised after humane killing by stunning and cervical dislocation following institutional approval and in accordance with European Commission Directive 86/609/EEC guidelines (n=11 animals; one vessel per animal). Approximately 3-4 mm aortic rings were mounted in an organ-bath system (gassed PSS in 95% O₂; 5% CO₂; 35°C), as previously described and tension recorded using Lab chart 6 (Power lab, AD Instruments, UK) [6]. Vessels were pre-constricted with high K⁺ (60 mM, KCl). Responses to endothelium-dependent dilator agonist were examined by adding cumulative doses of acetylcholine (ACh; 0.01-100 μM), before and after incubation with NPs for 30 min. The final concentration of SiNPs, and CeSiNPs placed in the organ-bath experiments were 1.96×10¹² NPs mL⁻¹. Vessels were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.3. and processed for TEM. Briefly, the vessels were allowed to stand in the solution for 2 hours at 22°C. The samples were post-fixed with reduced osmium (OsO₄ 1% + K₄Fe(CN)₆ 1.5%) for 1 hour, then dehydrated in a series of alcohols, infiltrated with TAAB LV resin and polymerized for 24 hours at 60°C. Ultrathin 70 nm sections were cut with Leica 'Ultracut S' ultra microtome and placed on copper grids. The grids were observed in Tecnai 12 Biotwin TEM at 80 kV.

Statistical analysis

Data are expressed as mean ± standard error of mean (SEM) with 'n' representing the number of vessels. Dilator responses are expressed as percent relaxation. Concentration response curves were assessed using statistical package for the social sciences (SPSS; version 19). The difference between groups at a given concentration was tested by one way analysis of variance (ANOVA) with Bonferroni corrections. Statistical significance is taken as P<0.05.

Results

Characterisation of nanoparticles

The SiNPs were mono dispersed with an average diameter of 47± 8 nm as demonstrated by TEM (Figure 1A). The addition of ceria around the SiNPs increased the diameter to 50± 4 nm with the individual CeNPs size of 3.8± 1.2 nm (Figures 1B-1D). The energy dispersive spectroscopy (EDS) of SiNPs confirms the presence of silica (Figure 1E and 1F). The CeSiNPs contain both silica and ceria (Figure 1G). The hydrodynamic diameters measured with dynamic light scattering (Figure 2) are of similar size ranges to the particle size observed by electron microscopy. The size of the SiNPs in water was 51.57 nm which increased to 83.88 nm in PSS. Upon ceria coating of the SiNPs, the size increased to 112.5 nm in water and 111.0 nm in PSS. The size of the ceria nanoparticles

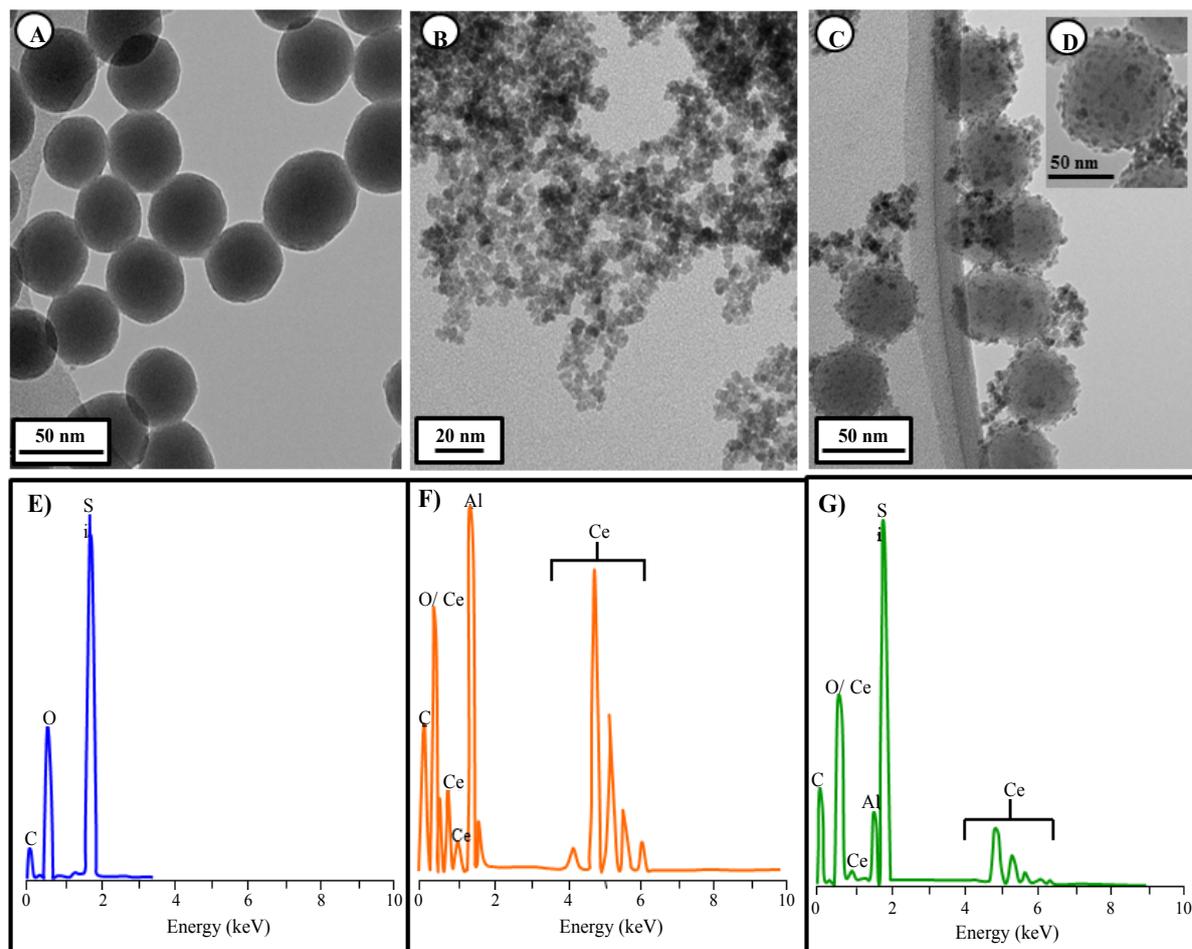
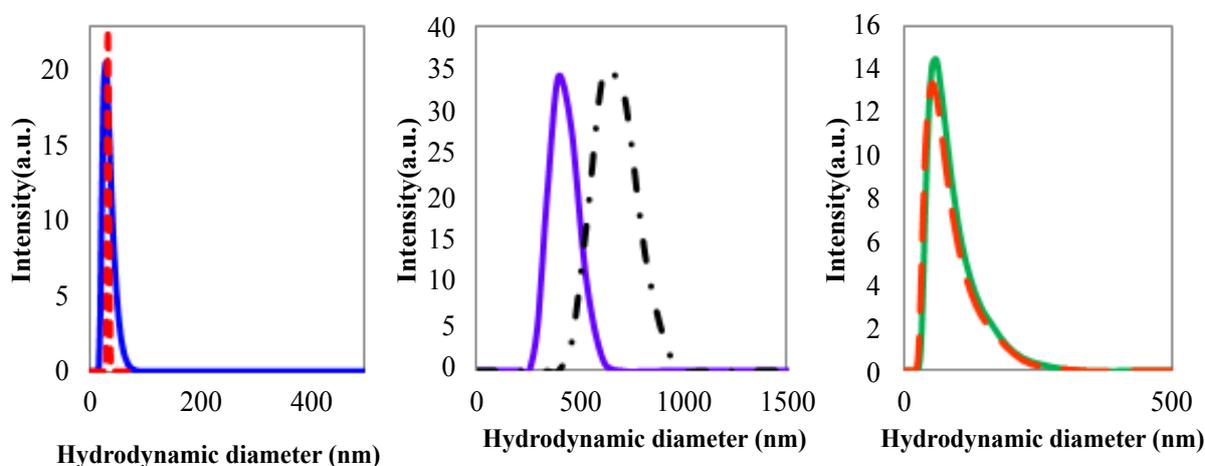


Figure 1: TEM image of A) SiNPs (47 ± 8 nm); B) CeNPs; C) CeSiNPs (50 ± 4 nm), D) enlarged image of CeSiNP; EDS analysis for E) SiNPs; F) CeNPs; G) CeSiNPs.



Summary

Sample	SiNPs in water	SiNPs in PSS	CeNPs in water	CeNPs in PSS	CeSiNPs in water	CeSiNPs in PSS
Count rate	153.4 kcps	211.7 kcps	197.4 kcps	187.9 kcps	309.1 kcps	464.4 kcps
Cumulants results						
Z-Average size	48.32 d,nm	79.65 d,nm	1004 d,nm	1858 d,nm	93.35 d,nm	91.67 d,nm
PDI	0.047	0.032	0.877	0.939	0.158	0.159
PDI width	10.48 d,nm	14.29 d,nm	939.8 d,nm	1801 d,nm	37.08 d,nm	36.52 d,nm
Distribution result	Mean/ Area	Mean/ Area				
Peak 1	51.57 d,nm / 100.0%	83.88 d,nm / 100.0%	408.7 d/nm / 100.0%	642.2 d,nm / 100.0%	112.5 d,nm / 100.0%	111 d,nm / 100.0%
Keys						

Figure 2: Dynamic light scattering profile of the synthesized nanoparticles, in water and PSS.

alone in water was 408.7 nm which increased slightly to 642.2 nm in PSS. The poly-dispersion index of the SiNPs indicates that there was a small degree of size variation, which is consistent with previous reports for fabricating small size particles using the standard Stöber method. The DLS of bare SiNPs were in agreement with the actual particle diameter. The CeSiNPs remained stable after being placed in PSS as the hydrodynamic diameter remained similar. The concentration of NPs held in the solution for DLS analysis was 0.02%, while for the organ-bath experiment the concentration was far smaller. The zeta potential values for the SiNPs was -37 mV in water, but increased to -15.5 mV in PSS. Similarly, the zeta potential for the CeSiNPs was -32.2 mV in water, which increased to -12.9 mV after suspension in PSS.

Influence of nanoparticles on endothelium-dependent vasodilator responses

All vessels demonstrated constriction to high potassium solution (KPSS, n=11). Incubation in SiNPs (3.03 ± 0.22 g tension and 2.58 ± 0.24 g tension, before and after incubation respectively), or CeSiNPs ($3.03 \pm$

0.27 g tension and 2.96 ± 0.32 g tension, before and after incubation respectively) had no influence on the magnitude of the constrictor response. All pre-constricted vessels dilated to ACh in a dose dependent manner. Incubation in amorphous SiNPs or CeNPs alone (1.96×10^{12} NPs mL⁻¹) caused a significant attenuation in dilation as compared to PSS alone (Figure 3). Incubation in CeSiNPs (50 ± 4 nm) at 1.96×10^{12} NPs mL⁻¹ led to a significant improvement in dilator responses (Figure 3). TEM transverse sections demonstrated uptake of both SiNPs and CeSiNPs into the cytoplasm of endothelial cells [ECs]. CeSiNPs were identified within 80% of ECs (Figure 4).

Discussion

In the present study, we demonstrate that coating SiNPs with ceria significantly improves endothelial-dependent vasodilation, in viable aortic vessels, *ex vivo*. Incubation in SiNPs or CeNPs alone attenuated dilation. The NPs were rapidly internalised by ECs, as previously demonstrated *in vitro* [8]. There was no evidence of translocation through the elastic lamina or smooth muscle cell layers.

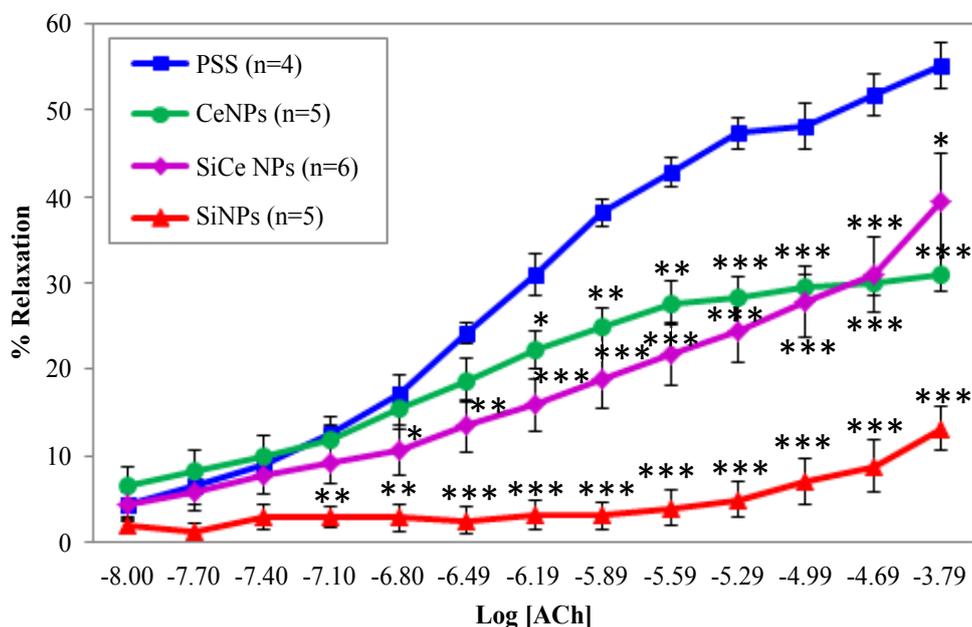


Figure 3: The influence of SiNPs, CeSiNPs and CeNPs on endothelium-dependent vasodilator responses. Control is PSS alone. 'n' is number of vessels. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, error bars=SEM.

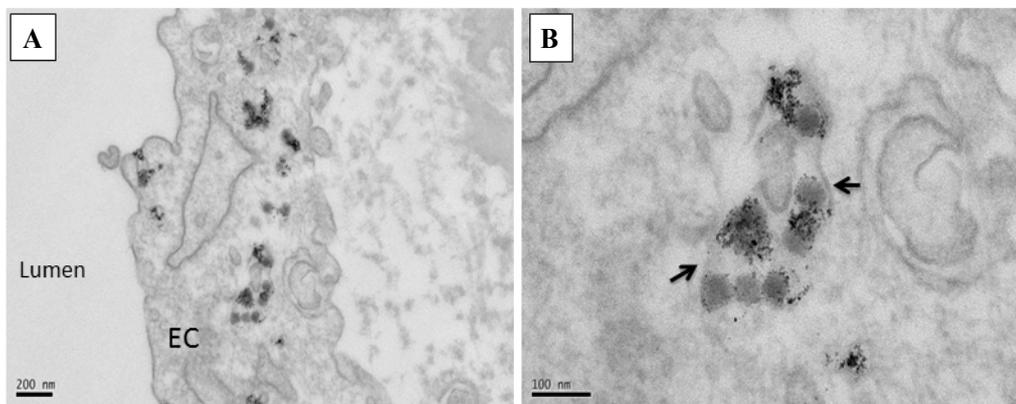


Figure 4: Representative TEM images showing uptake of CeSiNPs within endosomal structures (arrows) at, A) 9300 mag, and B) 30000 mag, by endothelial cells (ECs) lining aortic vessels.

The zeta potentials confirm that SiNPs and CeSiNPs were stable in both water and physiological salt solution (PSS). When the suspensions were placed in water the zeta potentials were greater than -30 mV. However, when suspended in PSS the potential increased to the unstable region of ~-14 mV as expected due to the high level of ions contained within the physiological solution. The net charge of the nanoparticle influences the ions which surround the interfacial region, and thus leads to the formation of a layer of concentrated counter ions surrounding the surface. This is known as the electrical double layer. The ions which are closely attached to the nanoparticle surface are referred to as the Stern layer. In the outer region the ions are less firmly attached and this is referred to as the diffused region. There is a natural boundary which exists within the diffused layer, and as the nanoparticle moves so do the ions that are within this boundary. However, ions that are beyond this boundary do not move with the nanoparticle. This

boundary is called the surface of hydrodynamic shear or the slipping plane and the potential which exists at this boundary is referred to as the zeta potential. The presence of ions within the media results in the formation of a less structured hydration layer surrounding the nanoparticles and thus lowers zeta potential [6]. Accordingly, the zeta potential of our fabricated nanoparticles was reduced when the nanoparticles were suspended in the high salt solution, PSS.

The attenuated dilation due to SiNP uptake, observed in the present study, may be related to the NP's generation and stimulation of intracellular ROS. These can act as scavengers of NO, the major vasodilator in aortic vessels. SiNPs have a high concentration of surface OH groups and this may play an important role in ROS generation, which has previously been demonstrated in a number of cell types *in vitro*, including human umbilical vein endothelial cells (HUVECs)

[4], lung sub-mucosal cells [9] and human keratinocyte cells [10]. Additionally, we have previously demonstrated that attenuated dilator function due to SiNP (71 ± 6 nm) uptake by aortic vessels *in vitro*, could be improved by co-incubation of the SiNPs with SOD, supporting a role for SiNPs in the generation of intracellular ROS [6]. The resultant oxidative stress has been shown to be a major cause of nanotoxicity, leading to endothelial dysfunction, DNA damage and apoptosis [10]. For example, Duan et al. [4], were able to demonstrate that SiNP incubation with HUVECs led to a dose and time dependent cytotoxic influence on these cells (both necrosis and apoptosis) through DNA damage and cycle arrest, as well as inhibition of SOD and glutathione peroxidase enzymes. The attenuated dilation after uptake of CeNPs alone may be due to their aggregated state (hence increase in size) since uptake of very small size mono-dispersed CeNPs has documented antioxidant properties in isolated cells [2]. Within the vasculature, CeNPs alone may bind NO, decreasing NO bioavailability, as has been recently shown after CeO₂ NP inhalation studies [11]. The fact that the ceria metal can exist in variable oxidation states (3+ and 4+), means that the cerium oxide nanoparticle can create 'defects' or oxygen vacancies within its lattice structure. This allows it to act as an oxygen buffer, in response to changes in physical parameters. Hence, when ROS are generated on the surface of SiNPs, the valency of the cerium dioxide nanoparticles can change spontaneously from 3+ to 4+ enabling it to bind ROS (OH- moieties) [12].

We demonstrate that while SiNPs of ~50 nm attenuate endothelial-dependent relaxation, ceria coating of these SiNPs leads to significant improvement in dilator responses. This study will inform the future fabrication of biocompatible SiNPs for use in imaging diagnostics. Furthermore, CeSiNPs may represent novel therapeutic tools for the protection and treatment of conditions where reduced dilator responses are observed.

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