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Cellular uptake and activity of heparin functionalised cerium oxide nanoparticles in monocytes

S.R. Simon Ting [a,b,1], John M. Whitelock [a], Romana Tomic [a], Cindy Gunawan [b], Wey Yang Teoh [c], Rose Amal [b], Megan S. Lord [a,*]

[a] Graduate School of Biomedical Engineering, The University of New South Wales, Sydney, NSW 2052, Australia
[b] ARC Centre of Excellence for Functional Nanomaterials, School of Chemical Engineering, The University of New South Wales, Sydney, NSW 2052, Australia
[c] Clean Energy and Nanotechnology (CLEAN) Laboratory, School of Energy and Environment, City University of Hong Kong, Kowloon, Hong Kong Special Administrative Region

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ABSTRACT

Cerium oxide nanoparticles (nanoceria) are effective in scavenging intracellular reactive oxygen species (ROS). In this study nanoceria synthesized by flame spray pyrolysis (d_{XRD} = 12 nm) were functionalised with heparin via an organosilane linker, 3-aminopropyltriethoxysilane. Nanoceria were functionalised with approximately 130 heparin molecules per nanoparticle as determined by thermo gravimetric analysis. Heparin functionalised nanoceria were more effectively internalised by the human monocyte cell line, U937, and U937 cells that had been activated with phorbol 12 myristate 13-acetate (PMA) than bare nanoceria. The heparin functionalised nanoceria were also more effective in scavenging ROS than nanoceria in both activated and unactivated U937 cells. Heparin coupled nanoceria were found to be biologically active due to their ability to bind fibroblast growth factor 2 and signal through FGF receptor 1. Additionally, the heparin-coupled nanoceria, once internalised by the cells, were found to be degraded by 48 h. Together these data demonstrated that heparin enhanced the biological properties of nanoceria in terms of cellular uptake and ROS scavenging, while the nanoceria themselves were more effective at delivering heparin intracellularly than exposing cells to heparin in solution.

1. Introduction

The therapeutic potential of nanoparticles is vast and includes applications such as the delivery of conventional drugs to situations where the materials themselves are considered to be the therapeutic, which represents a new class of drugs. Advances in material synthesis have enabled the fabrication of nanostructured biomaterials with tuneable properties, while the incorporation of biological molecules such as proteins or glycosaminoglycans on the surface or within nanoparticles has the potential to enhance biological activities [1]. Cerium oxide nanoparticles (nanoceria) have been shown to be non-cytotoxic and react catalytically with reactive oxygen species (ROS) through facile cyclic oxidation states that switch between cerium (III) and cerium (IV) providing intracellular anti-oxidant properties [2–5]. Therefore, nanoceria can be considered as a new class of therapeutics due to the fact that it is the material itself that has the therapeutic effects once internalised by cells.

Surface modification of nanoparticles has been explored to improve cell-selectivity and stability [6,7]. The functionalisation of nanoparticles with naturally occurring polysaccharides has been explored for active targeting strategies as they have specific recognition for certain cell types and tissues. Glycosaminoglycans are key components of cell surfaces and the extracellular matrix and are negatively charged polysaccharides composed of repeating disaccharide units that can be sulphated at various positions along the polysaccharide chain [8,9]. Heparin is the most sulphated glycosaminoglycan composed of repeating units of uronic acids with an average of 2.7 sulfo groups per disaccharide [10]. With the exception of hyaluronan, glycosaminoglycans decorate proteins called proteoglycans which have diverse biological functions. Heparin decorates the protein core of the proteoglycan, serglycin, produced by mast cells [11] and is most widely known for its role in anticoagulation, however it also takes part in tumour metastasis, angiogenesis and inflammation. Heparan sulphate, a lesser sulphated form of heparin, has important roles in sequestering growth

* Corresponding author. Tel.: +61 2 9385 3910; fax: +61 2 9663 2108.
E-mail address: m.lord@unsw.edu.au (M.S. Lord).
[1] Present address: Centre for Health Technologies, University of Technology, Sydney, Ultimo, NSW 2007, Australia.

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factors in the extracellular matrix, protecting them from proteolytic degradation and presenting them to cell surface growth factor receptors [8,9,12,13]. The incorporation of heparin into nanoparticle systems has been explored through the encapsulation of heparin within the nanoparticle or on its surface for applications including the delivery of anticoagulant activity or growth factors or to reduce cytotoxicity. Additionally, heparin may achieve active targeting as cells have specific cell surface receptors that recognise heparin [14]. Heparin encapsulated poly(methylmethacrylate) nanoparticles have been explored to stimulate growth factor production [15,16] while heparin loaded poly(lactic-co-glycolic acid) nanoparticles have been reported to prolong the anti-factor Xa activity compared with intravenous administration of heparin [17]. Heparin coated iron oxide nanoparticles have been explored as a negative contrast agent for magnetic resonance imaging [18] while heparin functionalised superparamagnetic nanoparticles can delay phagocytosis and prevent blood clotting *in vitro* [19].

Few studies have investigated functionalising nanoceria to improve biological responses. Nanoceria have been functionalised with poly(ethylene glycol) (PEG) [20], which has been reported not to affect cell viability or ROS scavenging [21], as well as dextran, which exhibited pH-dependent ROS scavenging [22]. Additionally, nanoceria have been reported to be conjugated with PEG and an antibody for selective delivery to Aβ aggregates [23]. Changes in the surface chemistry of nanoceria have also been reported to provide tuneable responses in terms of protein adsorption and cellular uptake [24]. The present study sought to functionalise nanoceria with heparin to investigate the activity of these particles in a biological environment as both heparin and nanoceria exhibit antioxidant properties protecting cells from free-radical induced damage [2,25,26].

Therefore, the aims of this study were to functionalise nanoceria with heparin and to investigate the activity of these particles in terms of growth factor binding and signalling, cellular uptake and ROS scavenging in human monocyte cell line, U937.

2. Materials and methods

Chemicals were purchased from Sigma–Aldrich (Castle Hill, Australia) unless stated otherwise.

2.1. Synthesis of nanoceria

Cerium oxide nanoparticles (nanoceria) were synthesized using flame spray pyrolysis as described previously [2] with liquid precursor and sheath gas flow rates of 5 L/min each. The nanoceria were found to have a Brunauer-Emmett-Teller (BET) equivalent particle diameter of 7 nm and a diameter of 12 nm by X-ray diffraction (XRD) analysis.

2.2. Heparin functionalisation of nanoceria

Nanoceria were dispersed in anhydrous dimethyl formamide (DMF) at a concentration of 10 mg/mL using an ultrasonic bath for 15 min under ambient conditions in a round bottom flask sealed with a rubber septum to prevent moisture from entering the flask. Subsequently, 3-aminopropyltriethoxysilane (APTES) (0.25 mL, 1.07×10^{-3} mol) was added drop wise to the nanoparticle suspension and, the flask was resealed with rubber septum and stirred overnight at 45 °C with a magnetic stirrer. The APTES modified particles were purified by washing with DMF four times using a centrifuge at 20 °C. Acetone was used for the final wash to facilitate the removal of DMF. The particles were first dried in a fume hood for 16 h and later transferred to a vacuum oven for further drying at 40 °C for 16 h (Fig. 1A).

Porcine sub-intestinal mucosal heparin (180 IU/mg, molecular mass 18000 Da, 1.06 g, 5.89×10^{-5} mol) was activated by first dissolving into a 1:1 dimethyl sulfoxide (DMSO)/water mixture (10 mL). EDC (2.57×10^{-3} g, 1.34×10^{-5} mol) and NHS (1.35×10^{-2} g, 1.17×10^{-4} mol) were then added to the above mixture and stirred at 50 °C for 6 h. The heparin-NHS was added to the suspension containing APTES-nanoceria (200 mg) in 1:1 DMSO/water mixture and stirred for 3 days at ambient temperature. The heparin-APTES-nanoceria was purified by washing with 1:1 DMSO/water mixture four times. Acetone was used for the final wash to facilitate the removal of DMSO and water. The particles were first air dried in a fume hood for 16 h and later transferred to a vacuum oven for further drying at 40 °C for 16 h (Fig. 1B).

2.3. Characterisation of heparin functionalised nanoceria

A Perkin Elmer Spotlight 400 Attenuated total reflectance-fourier transform infra-red spectroscopy (ATR-FTIR) was used to measure changes in the surface chemical structure of the nanoceria following functionalisation with APTES and heparin. Spectra were recorded between 650 and 4000 cm^{-1}. ^{13}C cross-polarization with the magic angle spinning (CP/MAS) nuclear magnetic resonance (NMR) analysis was performed using a Bruker Avance III 300 solid state NMR operating at 100 MHz. A Thermo gravimetric analyzer (TGA) 2950HR V5.4A operating in an air atmosphere with a heating rate of 5 °C min^{-1} between 20 and 1000 °C was also carried out to determine the percentage of organic grafted onto nanoceria. By applying the formula, $N = X N_A \rho V / M_w$, where N is the number of molecules on each nanoparticle, X is the percentage weight loss, N_A is Avogardo's number, ρ is the density of the nanoparticle and V is the volume of one nanoparticle and M_w is the molecular weight of the molecule.

2.4. Growth factor binding and signalling activity of heparin functionalised nanoceria

BaF32 cells are from an IL-3-dependent and heparan sulphate proteoglycan deficient myeloid B cell line that has been stably transfected with fibroblast growth factor receptor (FGFR) 1c [8,27]. BaF32 cells represent a model system developed to identify heparin structures that interact with FGFs and their receptors. The readout of this assay is cell proliferation which indicated the formation of ternary complexes on the cell surface between heparin/heparan sulphate, FGF2 and FGFR1c [28]. BaF32 cells were maintained in RPMI 1640 medium containing 10% (v/v) foetal bovine serum (FBS), 10% (v/v) WEHI-3BD conditioned medium, 100 U/mL penicillin and 100 μg/mL streptomycin. WEHI-3BD cells were maintained in RPMI 1640 medium supplemented with 2 g/L sodium bicarbonate, 10% (v/v) FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, and the conditioned medium was collected three times per week and stored at −20 °C until it was required. For the mitogenic assays, the BaF32 cells were transferred into IL-3 depleted medium for 24 h prior to experimentation and seeded into 96-well plates at a density of 2×10^4 cells/well in the presence of medium only, 30 nM heparin, nanoceria or heparin functionalised nanoceria (12 μg/mL, equivalent to 30 nM heparin) either in the presence or absence of 0.03 nM FGF2. Cells exposed to heparin and FGF2 were used as a positive control for the assay, as this combination is known to induce cell proliferation, while cells exposed to each of the treatments in the absence of FGF2 were used as a negative controls. Background absorbance readings were also obtained for each of the treatments in the absence of cells. Cells were incubated for 96 h in 5% CO2 at 37 °C, and the number of cells present was assessed using the MTS assay. The MTS reagent (Promega, Madison, USA) was added to the cell cultures 6 h prior to measurement of the absorbance at 490 nm.

2.5. Culture of U937 cells

The human leukaemic monocyte lymphoma cell line, U937, cell line was maintained in a monocytic cell suspension in RPMI-1640 culture medium containing 10% (v/v) foetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified incubator (5% CO_2/95% air atmosphere at 37 °C). Treatment of U937 cells with phorbol 12-myristate 13-acetate (PMA) mimics the activation/differentiation of monocyte *in vivo* [29]. Cells were activated for 72 h with the addition of 100 nM PMA to the culture medium, and will be referred to herein as activated U937 cells.

2.6. Cell proliferation analysis

U937 and activated U937 cells were seeded in 6-well tissue culture polystyrene plates at a density of 2×10^5 cells/well in 3 mL medium. Cells were incubated for 4 h prior to the addition of nanoceria at a concentration of 200 μg/mL or without nanoceria. Cell viability was analysed at 24, 48 and 72 h after the addition of nanoceria using an automated cell viability analyser (ViCell, Beckman Coulter, Sydney, Australia) that is based on trypan blue exclusion dye analysis.

2.7. Reactive oxygen species analysis

The level of cellular ROS was measured using the intracellular peroxide-dependent oxidation of 2′, 7′ − dichlorodihydrofluorescein diaceteate (DCFH-DA) to form a fluorescent compound, 2′ 7′-dichlorofluorescein (DCF). U937 and activated U937 cells were seeded in 6-well tissue culture polystyrene plates at a density of 2×10^5 cells/well in 3 mL medium and incubated for 4 h prior to the addition of 50 or 200 μg/mL nanoceria or heparin-functionalised nanoceria. The presence of ROS was determined at 24, 48 and 72 h after the addition of nanoparticles. Cells exposed to medium only were analysed at each of the time points to determine the normal level of ROS in the cells. Cells exposed to 4% (w/v) ethanol at each of the time points were used as a control for dead cells and cellular debris for the flow cytometry analysis. Activated U937 cells were removed from culture dishes by trypsinisation and resuspended in Dulbecco's phosphate buffered saline, pH 7.4 (DPBS) while U937 cells did not require trypsinisation to be removed from the culture dishes. Cells were