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Cerium oxide based nanozymes: Redox phenomenon at biointerfaces

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Cerium oxide nanoparticles (nanoceria) are known to exhibit enzymelike activity, such as biological catalase, oxidase, superoxide dismutase, and peroxidase enzymelike activities. Catalytically active nanoceria offer several advantages over natural enzymes, such as controlled synthesis at low cost, tunable catalytic activities, as well as high stability against stringent physiological conditions. Exploiting these properties, several biomedical applications, such as biosensing, immunoassay, drug delivery, radiation protection, and tissue engineering, have been maneuvered. This review article provides a comprehensive summary of reported biological enzymelike activities of nanoceria and the possible mechanism of catalysis. It is also discussed that what physicochemical properties (surface charge, size, surface chemistry, and enzymelike activity) of nanoceria are altered when exposed to biologically relevant buffers and even in the mammalian cell cytoplasm. Based on various reports provided in the literature, these important issues need to be addressed. It has also been deliberated that in mammalian cells variations in properties of nanoceria observed are due to the intrinsic catalytic activity or as a result of downstream effects that could have emerged after the particles have interacted with biologic/environmental components. Such studies could be helpful in providing a better understanding the nanoceria's multienzyme-like activities and potential applications in the biomedical fields with special reference to damaging effects of free radicals in biological systems. *© 2016 Author(s). All article content, except where otherwise noted, is licensed under a Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).* [http://dx.doi.org/10.1116/1.4966535]

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I. INTRODUCTION

The first element of the lanthanide group, Cerium, with 4f electrons has attracted attention from researchers from the areas of material's science, chemistry, biology, and engineering. Cerium oxide is a fluorite crystalline structure and has recently emerged as a possible material for several applications in biomedical sciences.[1,2] Nanoceria has been utilized in glass polishing, fuel additives, catalysts, solid-oxide fuel cells, solar cells, and potential pharmacological materials.[3–6] Cerium oxide has also been an attractive material for various industrial applications such as automobile exhaust gas treatments; oxidative couplings of methane and water–gas shift reactions.[7] Among biological applications, nanoceria has shown promising results in free radical mediated disease such as autoimmune diseases that involve oxidative stress and multiple sclerosis (MS).[8] MS is a complex disease characterized by patterns of disease advancement, which results in loss of myelin sheath coated neurons in the central nervous system. Neurons lacking the insulating myelin sheath cannot convey nerve impulses that allow communication between neurons and muscles. This result, MS patients suffer paralysis and suffer the loss of motor function. The reactive oxygen species (ROS) generate oxidative stress in the central nervous system and damage the myelin sheath and macromolecules.[9] Treatment of nanoceria to animal models of MS resulted in fewer motor nerve defects and fewer severe clinical symptoms than untreated animals.[1] Nanoceria has been used for providing protection to the retina of eyes since last ten years.[10,11] The small volume of the eye makes them unique organ for the study of remedial agents in which a few curative agents can be effective. It has been seen that nanoceria primarily accumulates in the retina of healthy rats where they retain for more than one year. Long term accumulation of nanoceria did not cause any negative effect on retinal function, which indicates that nanoceria in even small doses can exhibit the protective effects against the free-radical mediated destructive effects on the retina.[12] Experimentally, primary cell culture models of retinal neurons were protected from H_2O_2 induced cell death by pre-exposure with nanoceria.[13]

A. Role of free radicals and need of universal antioxidant in biology

It is well known that the environment was oxygen-deficient until 2.3 billion years ago, and the Earth had only small amount of molecular oxygen for biological organisms.[14] The emergence of oxygen leads to the generation of oxygen-based free radicals in the bio-organisms. It has already been established that biology produces a range of ROS, such as superoxide ($O^{2\bullet-}$) and H_2O_2, by chemical reactions between oxygen and adrenalin and dopamine, etc., auto-oxidizable biomolecules.[15] Similarly, nitrogen-based reactive nitrogen species (RNS) are also generated in the biological organisms due to biochemical reactions.[16] The production of free radicals in the human body is essential for redox signaling and cellular homeostasis.[17] A plethora of

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mechanism is known by which body monitors and keeps balance between the production and deactivation of free radicals. This helps the body to minimize the free radical's insults or repair the damages. Generally, it has been observed that the reaction of a free radical with a nonradical biomolecule such as protein, lipid, carbohydrate, and nucleotide results in the further formation of free radicals, which in turn react with a new biomolecule and thus produce free radical chain reaction.[18] The exposure of excess ROS results in lipid peroxidation, oxidative stress, oxidation of biomolecules, deoxyribonucleic acid (DNA) strand break, and protein-DNA strand exchange/cross-linking.[19,20] As a result of these, cells undergo abnormal cellular, metabolic, and signaling pathways. According to Harman's "Free Radical Theory of Aging," the free-radical damage leads to the degeneration of DNA, proteins, and other biomolecules, eventually leading to aging and ultimately death of mammals.[21] Further, the free radicals are also contributed to the basis of over hundreds of diseases, including neurodegenerative diseases, cardiovascular, sickle cell, and chronic fatigue syndrome.[22–25]

B. Need of inorganic antioxidants

Antioxidants are known to protect the tissues from damages imposed by free radicals or improve the different disease states of tissues. The human body has a vast network of the endogenous antioxidant system, which can be classified into enzymatic and nonenzymatic types.[26] The enzymatic antioxidants include superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase, peroxiredoxins, etc., whereas nonenzymatic antioxidants include glutathione, uric acid, coenzyme Q, melatonin, α-tocopherol, ascorbic acid, carotenes, etc.[27,28] There are some elements, which have no direct antioxidant function, but they are required for the activity of biological antioxidants; these elements are copper, selenium, zinc, iron, and manganese.[29] The natural enzymes in living systems are excellent biocatalysts, specific to substrates, and speed up the rate of biochemical reactions to several folds under a defined range of temperature. However, the catalytic activities of natural enzymes are frequently found to be sensitive to the heavy metals (such as sodium azide) based inhibitors. Further, the catalytic activity of natural enzymes is also controlled by its limited stability in harsh environmental condition, and the applicability is limited by the high cost of synthesis, isolation, and purification.[30] Therefore, the discovery and development of an alternative mimetic system are highly desired. In this context, the synthesis of inorganic nanomaterials (NMs) exhibiting biological enzymelike properties is currently a growing area of research with special interest to the biological applications.[30–33] These NMs offer a range of advantages over natural enzymes such as controlled synthesis at low cost, tunability in catalytic activities, and stability against stringent conditions. The high surface to the volume ratio of NMs offers better catalytic efficiency than natural enzymes.[33] So far, several nanoparticles (NPs), such as gold nanoparticles (AuNPs), V_2O_5, Pt-Pd-Fe_3O_4, nanoceria, carbon nanotubes, graphene oxide, and iron oxide NPs, have been shown to exhibit the natural enzymelike property.[32,34–39] Among them, nanoceria has been well studied as promising enzyme mimics under *in vitro*, *in vivo*, and animal models[6,40–42] (Table I).

II. BIOLOGICAL ENZYME-LIKE ACTIVITIES OF NANOCERIA

A. Nanoceria as SOD mimic

In the biological system, one electron reduction of the oxygen molecules generates superoxide, which is one of the key-free radicals.[52] In biological organisms, SOD enzyme is involved in the repair of cells and reduces the damage caused by superoxides. SOD enzyme catalyzes the disproportionation of superoxide to hydrogen peroxide and O_2. Similar disproportionation reaction with nanoceria was the first observation by Self and colleagues.[48,53] The first report on nanoceria showing enzymelike activity came in 2007 by Korsvik *et al*. Through x-ray photoelectron spectroscopy study, it was argued that inherent Ce^{+3}/Ce^{+4} valence switching ability of nanoceria makes its possibility as SOD mimics. Further, the cycling between Ce^{+3} and Ce^{+4} oxidation states and regeneration of Ce^{+3} atoms on the surface of nanoceria creates the possibility of its long-term use. They also investigated the two preparations of nanoceria having high and low proportion ratios of Ce^{+3}/Ce^{+4} and found that high proportion of Ce^{+3}/Ce^{+4} ratio exhibited high SOD mimicking activity.[48] It has been well documented that nanomaterials interact with the surrounding biomolecules and usually modify the surface, which results in ineffective catalytic activity.[49,54] The interaction of nanoceria with biologically relevant media was comprehensively studied by Singh and coworkers.[49] They showed that nanoceria upon reaction with phosphate buffer, a commonly used biological buffer in cell culture and animal models, show a concentration-dependent shift in the absorbance pattern (>50 μM) when observed under UV-Vis spectra.[54] The absorbance peak centered at ~250 nm (due to Ce^{+3} in nanoceria) was shifted to 275 nm (due to cerium phosphate) when 100 μM nanoceria were exposed to phosphate concentration of greater than 50 μM. Further, the SOD activity recorded after the incubation of nanoceria with phosphate, resulting in the loss of SOD activity of nanoceria, was observed when nanoceria was pre-exposed to phosphate of more than 50 μM. This observation clearly indicates that the intrinsic properties of nanomaterials could be affected by the components of biologically relevant buffers and components. It was interesting to note that pre-exposed nanoceria to phosphate resulted in increased catalase activity in a concentration-dependent manner. Similar to phosphate, other anions such as carbonate and sulphate anions were also investigated for any loss in SOD activity of nanoceria. Interestingly, no change in UV-Vis spectra pattern and concomitantly neither drop in the SOD activity nor the emergence of catalase activity were observed. The results obtained imply that phosphate is present in the cell

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TABLE I. Summary of enzymelike activities of nanoceria shown in cell culture and animal models.

Nanoceria synthesis and size	Type of activity	Experimental model	Dosage	Results/remarks	References
Dextran-coated nanoceria (5, 12, 14 and 100 nm)	Oxidaselike activity	A549	0.5–10 μM	Dextran coated nanoceria can catalyze the oxidation of organic dyes and small molecules at slightly acidic *p*H in the absence of hydrogen peroxide. Conjugation of nanoceria with targeting ligands makes it efficient detection tool in immunoassays.	43
Protein G-polyacrylic acid coated nanoceria	*p*H tunable oxidaselike activity	MCF-7 and A549	1–20 μM	Nanoceria can oxidize the amplifu at neutral *p*H to the stable fluorescent product resorufin by inhibiting the further oxidation to nonfluorescent product resazurin. This property can be used to develop the cell-based ELISA system.	36
Water based nanoceria (3–8 nm) with lower Ce^{3+}/Ce^{4+} ratio	Nitric oxide radical scavenging activity	N/A	50–1000 μM	Nanoceria with a low level of Ce^{+3} instigates $^{\bullet}NO$ scavenge by the formation of an electropositive nitrosyl ligand as a result of electron transfer from $^{\bullet}NO$ to a Ce^{+4}. Nanoceria incubated with phosphate also having the $^{\bullet}NO$ scavenging ability.	44
Polyacrylic acid coated nanoceria (5 nm)	Peroxidaselike activity	N/A	0.5–10 mmol/l	Nanoceria exhibits H_2O_2 concentration dependent peroxidase-like activity which provides a colorimetric approach for glucose sensing	45
Water based nanoceria (18–35 nm)	Peroxynitrite decay activity	N/A	10–100 μM	Nanoceria accelerates the decomposition of peroxynitrite ($ONOO^-$) that is independent of Ce^{+3}/Ce^{+4} ratio on nanoparticles surface. The decay of $ONOO^-$ by nanoceria was favored by the interaction of nanoceria with carbonate radical that is generated during $ONOO^-$ decomposition.	46
Water based nanoceria (3–7 nm)	Free radical scavenging activity	Murine insulinoma (beta TC-tet)	50–100 μM	Nanoceria protects the mammalian cells against the influence of oxidative stress and improves the cellular survival.	47
Water based nanoceria (3–5 nm) by varying Ce^{3+} and Ce^{4+} ratio	SOD mimetic activity	N/A	10 μM–10 nm	Nanoceria with higher Ce^{+3}/Ce^{+4} ratio attributed as SOD mimics.	48
Bare nanoceria with high Ce^{4+}/Ce^{3+} ratio (10 nm)	Catalase mimetic activity	N/A	100 μM	Catalase mimetic activity of Ce^{+4} is not altered by incubation with phosphate anions, change in *p*H, and composition of cell culture media.	49
Water based nanoceria (5–8 nm)	Catalase and SOD mimetic activity	24 months old male rats	1 mg/kg body weight	Nanoceria induces an increase in levels of sex hormones, sperm count quality, and activation of spermatogenesis via reduction of oxidative stress.	50
Bare nanoceria (3–5 nm)	Free radical scavenging activity	Human colon cells (CRL 1541)	1–100 nM	Nanoceria protects the gastrointestinal epithelium from radiation-induced damage by increasing the production of SOD and acting as a free radical scavenger.	51

cytoplasm in abundance and can alter the superoxide radical scavenging property of nanoceria, which can only scavenge the peroxides after exposure to phosphate. This observation suggests that nanoceria interact differently with different components of biologically relevant buffers and result in change in properties.[55] It has also been reported that nanomaterials incubated in protein complex, undergo formation of protein corona on the surface of particles, where some protein molecules are in dynamic motion and certain are strongly associated with nanoparticle surface.[56,57] Additionally, the research groups of Seal and Self studied the role of adsorbed protein [bovine serum albumin (BSA)] and polymer [polyethylene glycol (PEG)] over the SOD mimetic activity of nanoceria. Both these studies answered many of the unfamiliar questions and kept nanoceria in a special category of inorganic nanocatalyst. The observed results showed that the inherent SOD mimetic activity of nanoceria was not compromised even after the coating of BSA or PEG, which makes this nanoparticle significantly different than other particle types. Generally, nanoparticles

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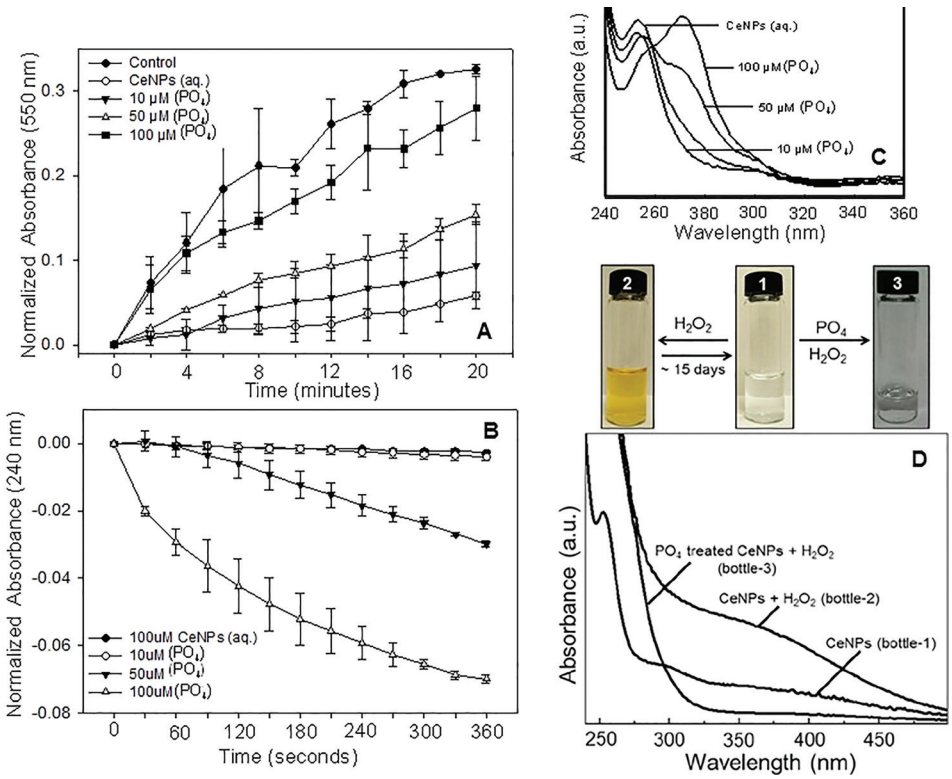


FIG. 1. (a) Dose-dependent shift in the surface oxidation state of nanoceria by treatment with phosphate anions (PO_4). Nanoceria (100 mM) were dispersed in a range of concentrations of phosphate (10, 50, and 100 mM) and incubated for 24 h before SOD mimetic (a) catalase mimetic (b) activity and UV-visible spectroscopy (c) measurements from the resulting suspensions. A kinetic measurement of catalaselike activity was analyzed by following H_2O_2 degradation at 240 nm. The formation of cerium phosphate arrests Ce in Ce^{+3} state, thereby blocking the oxidation by hydrogen peroxide. Nanoceria (1 mM) and phosphate treated nanoceria (1 mM) were treated with H_2O_2 (0.2 M) and incubated for 24 h followed by UV-Vis spectra measurements (d). Reprinted with permission from Singh *et al*., Biomaterials **32**, 6745 (2011). Copyright 2011 Elsevier.

after coating with biomolecules or polymers become passive and do not react with the surrounding species. On one hand, this is useful as nanoparticles reactivity with surrounding ions and species can be controlled; however, the intrinsic properties are diminished. Conversely, surface coating of nanoceria does not compromise with intrinsic properties and biocompatibility. Probably, due to these reasons, the investigation on the enzymelike activity of nanoceria was continuously explored and more biological mimicking actions are reported.

B. Nanoceria as catalase mimic

Catalase, found in all the living organisms, is known to degrade hydrogen peroxide, a harmful oxidizing agent. Nanoceria has also been discovered by Self and coworkers to exhibit redox state-dependent biological catalase mimetic activity in 2010.[58] The authors established the catalaselike activity of nanoceria (in high Ce^{+4}/Ce^{+3} ratio) by several methods such as Amplex Red assay; UV-Vis spectrophotometer based monitoring of H_2O_2 at 240 nm and monitoring of released oxygen by an oxygen electrode. Later, Celardo *et al*. proposed a possible mechanism which involves two half reactions, where one molecule of H_2O_2 reacts with nanoceria having high Ce^{+4} and reducing it to Ce^{+3} by releasing protons and oxygen.[59] Next, another H_2O_2 molecule could bind to the vacant oxygen site and oxidize Ce^{+3} back into Ce^{+4} and release water molecules. The two processes proposed by authors are cyclic and similar to the process found in catalase enzyme. Similar to SOD-like activity, catalaselike activity of nanoceria in the biologically relevant buffer was also studied by Singh and coworkers.[49,54] In this study, it was reported that catalase mimetic activity of nanoceria (high Ce^{+4}) is resistant to the exposure of different *p*H (from 2 to 10), the chemical composition of cell culture media and phosphate anions. The exposure of nanoceria to above conditions did not alter the UV-Vis spectra pattern and catalaselike activity (Fig. 1). This result was also supported by no significant change in the particle size and zeta potential after incubating them in the solutions of varying *p*H (up to *p*H 10). A similar trend was observed when nanoceria were exposed to phosphate (up to 100 μM concentration); however, phosphate concentration above 100 μM resulted in a drastic increase in size ($\sim$3000 nm) and zeta potential (-22 mV). This was suggested due to the excess amount of negatively charged phosphate anion in the suspension. It is reported that cerium (Ce^{+3}) has high affinity to phosphate anions, which has been shown that "Ce" ions can be used as the better capture agent for inorganic phosphate released during the phosphatase enzyme-mediated hydrolysis of phosphate-containing substrates.[60] Therefore, it is interesting to note that the association of phosphate with Ce ions from nanoceria is redox state-dependent and exhibits

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high affinity to Ce in the +3 oxidation state than Ce ions in the +4 oxidation state. Given the abundance of phosphate anions in the biological system, it is likely that the radical scavenging properties of internalized nanoceria would be influenced by cellular concentration of phosphate. Therefore, based on *in vitro* studies, it is suggested that free radical scavenging property of nanoceria is unaffected by *p*H and phosphate concentration. However, detailed *in vivo* studies are required to establish that these particles are universal inorganic antioxidant.

C. Hydroxyl radical scavenging nanoceria

Hydroxyl radicals are one of the extremely reactive and short-lived oxidants produced in biological organisms.[61] Unlike superoxide, which can be scavenged by SOD enzyme, there is no enzyme, which can eliminate the hydroxyl radicals from the organisms. The reported methods for scavenging of hydroxyl radicals include endogenous antioxidant such as SOD, glutathione peroxidase, catalase, melatonin, and vitamin E.[62,63] Parallel to the Self and coworkers, Hickman and coworkers in 2007 explored that subnanometer (2–5 nm) sized nanoceria can exhibit neuroprotective effects from hydrogen peroxide treated adult spinal cord model system by degrading the hydrogen peroxide and preventing from oxidative insult.[64] They proposed that the protective effect of nanoceria on the spinal cord model was due to the free-radical scavenging activity. These free radicals were generated due to hydrogen peroxide and probably the species were hydroxyl radicals. They found that when colorless nanoceria (Ce^{+3}) was treated with hydrogen peroxide, there was a clear change in color from colorless to dark orange color, which indicate the conversion of Ce^{+3} into Ce^{+4}. Interestingly, when this orange-colored solution was kept in the dark for ~3–4 weeks, the color returned to the original state, which was argued the regeneration of nanoceria from Ce^{+4} to Ce^{+3}. By the year 2011, nanoceria gained the popularity as one of the best inorganic enzyme mimic and further to it, the mechanistic aspect of its enzymelike activity, enticed many researchers' attention. Perez *et al*. in 2011 investigated and elaborated that regenerative ability of nanoceria as a *p*H dependent process.[36] His research group reported that under physiological and basic *p*H conditions, nanoceria could retain their regenerative ability; however, at acidic *p*H did not. The antioxidant properties of nanoceria cited above are inferred from its ability to scavenge hydroxyl radicals. In 2011, Xue *et al*. added important information toward the mechanism and showed direct evidence for hydroxyl radical scavenging activity of nanoceria using the methyl violet assay.[65] The authors reported that nanoceria could only partially scavenge the hydroxyl radical, and the activity was the size-dependent with possible involvement of Ce^{+3} at the surface of nanoceria.

D. Nanoceria as peroxidase mimetic

Peroxidases are known to contain a heme as a cofactor in their active sites, or redox-active cysteine or selenium containing cysteine residues (selenocysteine).[66] There are several types of peroxidases found in living organisms such as glutathione peroxidase, myeloperoxidase, haloperoxidase, lactoperoxidase, peroxiredoxin, etc. These classes of enzymes catalyze the conversion of hydrogen peroxide to water and oxygen. Recently, nanomaterials has been shown to exhibit peroxidaselike activity and have shown potential to replace natural peroxidase enzymes by offering many advantages such as high stability and low cost of synthesis.[67] Singh and coworkers have reported that peroxidaselike activity of AuNPs can be synergistically enhanced by adenosine triphosphate (ATP) molecules.[31] It has been shown that transition metal ions such as Fe is a common peroxidase mimic, where they generate hydroxyl radicals through a general mechanism similar to that in Fenton's chemistry.[68] The generated hydroxyl radicals react with substrates and oxidize them in colored product. Although in 2008, Heckert *et al*. reported that cerium ions could also exhibit Fenton-like reaction and produce hydroxyl radicals,[69] their applications were not realized until recently. Later in 2011, it was investigated by Perez and other research groups that nanoceria could exhibit peroxidase-like activity.[36] Based on this property of nanoceria, colorimetric method for glucose detection and other applications in the areas of environmental chemistry, biosensors, and nanomedicines have been developed (Fig. 2).[6,70] Peroxidases belong to oxidoreductase class of enzyme and are widely present in living organisms.[71] They catalyze the reduction of peroxide and concurrently oxidize various substrates. Exploiting this strategy several assays have been designed. Substrates such as colorless 3,3,5,5-tetramethyl benzidine (TMB) and 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid are oxidized in colored product in the presence of peroxidases, which have been shown to sense and detect biomolecules.[39,72]

E. Nanoceria as oxidase mimetic

Oxidase enzyme, belongs to the oxidoreductases class of enzymes, catalyzes the oxidation-reduction reaction which involves molecular oxygen as electron acceptor. The reaction involves donation of hydrogen atom and reduction of oxygen to water or hydrogen peroxide. The important oxidases found in living organisms are cytochrome C-oxidase, glucose oxidase (GOx), cytochrome-P450 oxidase, and xanthine oxidase.[73] Oxidaselike activity of nanoceria was first reported by Asati *et al*. and revealed that at acidic *p*H nanoceria quickly oxidize a series of organic substrates even in the absence of any oxidizing agent.[43] They synthesize dextran coated nanoceria and reported that the oxidaselike activity was dependent on particle size and thickness of polymer coating. Further, they designed an immunoassay based on folate conjugated nanoceria to target the folate expressing cancer cells by catalytic oxidation of colorimetric substrates. Generally, these substrates are used as a horseradish peroxidase substrate in many immunoassays and this peroxidase-catalyzed reaction H_2O_2 is required as the electron acceptor; however, nanoceria oxidize the substrates even in the

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absence of substrates. In order to understand the issue that nanoceria acts as an oxidase or an oxidant during organic reagent oxidation, Xu *et al*. have proposed that property of nanoceria could be controlled by *p*H of the suspension.[74] They further corroborate their hypothesis with the previous observation made by Perez *et al*. that nanoceria under physiological and alkaline *p*H recycles between Ce^{+3} and Ce^{+4} oxidation states and thus able to maintain the catalytic property. However, under acidic *p*H environment, nanoceria reacts with H^{+} ions and produces dissoluble cerium ions and acts as an oxidant. Contrary to this, investigations carried out by Singh and coworkers suggest that nanoceria, either in Ce^{+3} or Ce^{+4} oxidation states, do not compromise with the antioxidant property under acidic *p*H environment. In fact, the nanoparticle synthesis is both art and science; therefore, synthesis method of nanoceria also controls the properties. Other parameters such as temperature, impurities, lattice doping, surface modification, and particle size could influence the physicochemical properties of nanoceria.

F. Nanoceria is phosphatase mimic

Phosphatase is an enzyme which is known to remove phosphate groups from their substrates by hydrolyzing phosphoric acid monoesters into free phosphate ions.[75] Alkaline phosphatase is a common phosphatase found in living organisms. Phosphatase plays several important roles in biological processes such as cell proliferation, signal transduction, metabolism, and cell–cell communications. The above mentioned enzymelike activity of nanoceria has attracted more nanozyme activity exploration by researchers worldwide. Among them, the phosphataselike activity of nanoceria was first reported by Kuchma *et al*. explored the reactivity of nanoceria with phosphate ester bonds of *p*-nitrophenol phosphate (pNPP), ATP, *o*-phospho-L-tyrosine and DNA.[76] They calculated the activity of the bond cleavage for pNPP at *p*H 7 to be 0.860 ± 0.010 nmol *p*-nitrophenol/min/μg nanoceria. Interestingly, when nanoceria (Ce^{+3}) bind to plasmid DNA, no cleavage products were detected. However, nanoceria (Ce^{+4}) exposure exhibited the ability to break phosphorus-

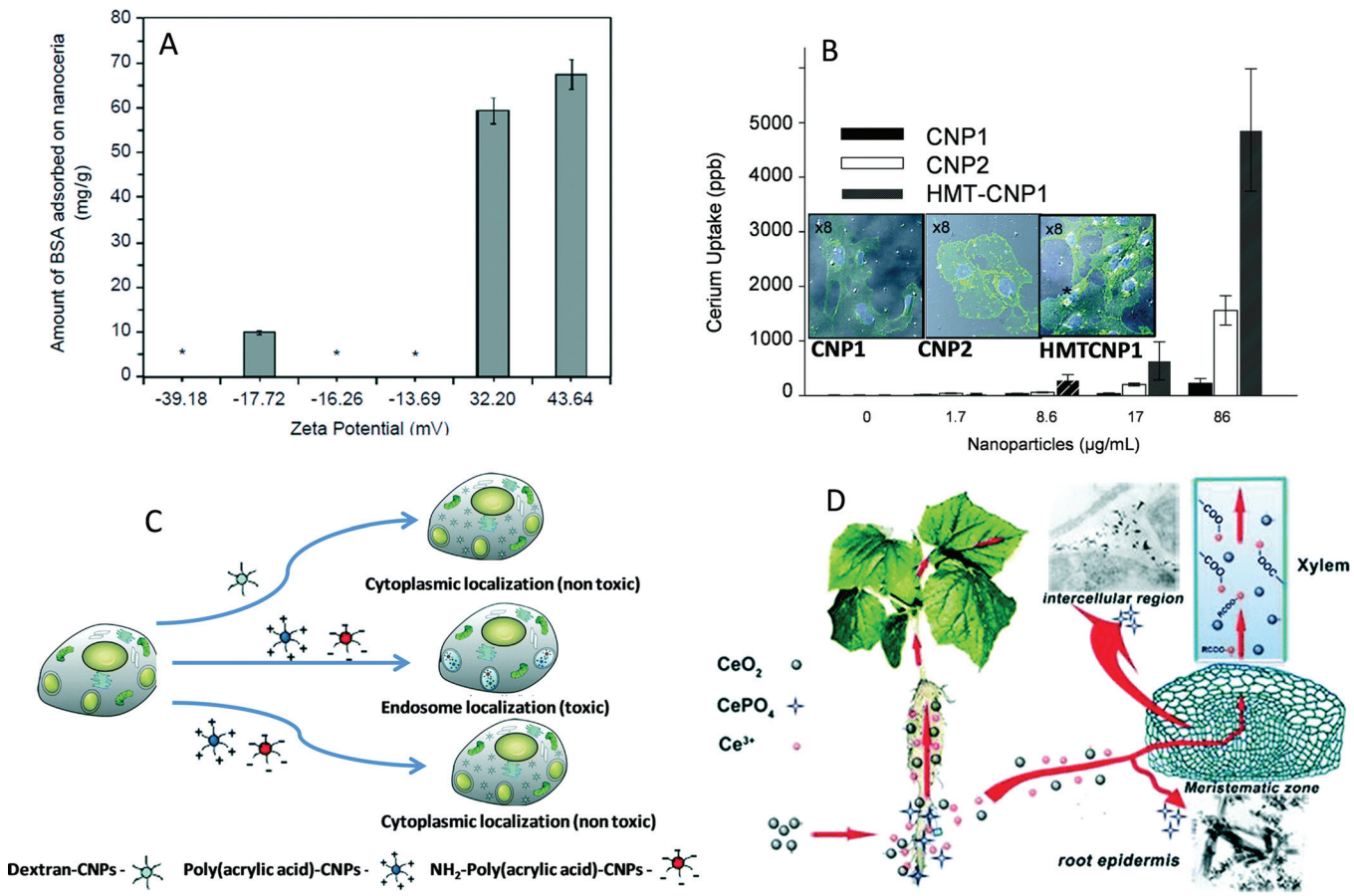


FIG. 2. (a) Amount of BSA adsorbed on the microemulsion nanoceria samples as a function of its zeta potential. * indicates that no significant protein adsorption was observed for the sample. Positively charged nanoparticles favored BSA adsorption and showed increased protein adsorption with increasing zeta potential. (b) Increased uptake of HMT-nanoceria1 in HUVEC cells as measured by ICP-MS. (c) Polymer-coated nanoceria's cell internalization, localization, and proposed toxicity mechanism. Neutral dextran coated nanoceria (zeta potential–0) internalized and localized mostly in the cytoplasm of cells and hence it was not cytotoxic. Aminated poly(acrylic acid) coated nanoceria (+ve surface charge) and poly(acrylic acid) coated nanoceria (−ve surface charge) can localize either into the cytoplasm or the lysosomes, depending on the type of cells. When the nanoceria localized to the lysosome, the low *p*H of this organelle activates the nanoparticle's oxidaselike activity, exhibiting toxicity. Aminated poly(acrylic acid) coated nanoceria (−) or poly(acrylic acid) coated nanoceria (+) that localized into the cytoplasm displayed no cytotoxicity (d) schematic of biotransformation of cerium oxide nanoparticles in cucumber plant. Reprinted with permission from Kumar *et al*., Environ. Sci.: Nano **1**, 516 (2014). Copyright 2014 Royal Society of Chemistry.

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oxygen bonds. Although this was an exciting discovery, any concrete application based on phosphatase activity of nanoceria has not been explored so far.

G. Nanoceria for scavenging nitrogen based radicals

Nanoceria is probably the sole nanoparticle which is reported to scavenge both the oxygen and nitrogen-based free radicals with almost same extent. RNS scavenging by nanoceria is a relatively newer area of research and so far only few reports are available. In 2014, Estevez *et al*. have reported the scavenging of RNS by nanoceria in brain tissues.[77] However, Dowding *et al*. were first to reported that nanoceria (with the lower level of Ce^{+3}) can scavenge the nitric oxide radicals ($NO^{\bullet}$) in 2013.[78,79] They performed the $NO^{\bullet}$ scavenging experiment by making ferrous form of hemoglobin (Hb) to interact with $NO^{\bullet}$. S-nitroso-*N*-acetyl penicillamine was used to generate $NO^{\bullet}$ and conversion of ferrous form of Hb to the ferric form of Hb by $NO^{\bullet}$. Dowding observed that nanoceria containing high Ce^{+3} oxidation state did not scavenge the $NO^{\bullet}$ suggesting that no interaction between $NO^{\bullet}$ and nanoceria. However, the exposure of nanoceria with high Ce^{+4} inhibited the ability of $NO^{\bullet}$ to oxidize Hb in a dose-dependent manner. When compared with a known scavenger (DEMPO) of $NO^{\bullet}$, similar pattern was observed. This result suggests that nanoceria exhibit redox state-dependent interaction with $NO^{\bullet}$. Additionally, the authors further confirmed the $NO^{\bullet}$ scavenging property of nanoceria by another method based on fluorescence emission of NONOate, another $NO^{\bullet}$ donor, in the presence or absence of nanoceria. The addition of nanoceria (high Ce^{+3}) showed no effect on the ability of dissolved $NO^{\bullet}$ to oxidize the copper fluorescein conjugate (Cu-FL), a specific detector of produced $NO^{\bullet}$. However, when nanoceria (high Ce^{+4}) was exposed, oxidation of Cu-FL was prevented, and the fluorescent signal was decreased in the dose-dependent manner to nanoceria.

Peroxynitrite ($ONOO^-$) is a nitrogen based strong oxidizing agent, generated generally from the reaction between superoxide and nitric oxide radicals. Generally, $NO^{\bullet}$ is nontoxic *in vivo*; however, recently, it has been reported that the cause of toxicity is attributed to the formation of the potent oxidant $ONOO^-$. It is interesting to report here that compared to superoxide and $NO^{\bullet}$, $ONOO^-$ is highly reactive and more potent to oxidize DNA, proteins, lipids, and other biomolecules. It was reported by Dowding *et al*. that nanoceria significantly accelerated the decay of $ONOO^-$ shown by three independent methods.[79] It was also shown that the ability of nanoceria to interact with $ONOO^-$ is independent of the oxidation state of Ce on the surface of nanoceria. In one of the methods, the protection against the nitration of proteins by nanoceria was estimated by using peroxynitrite-induced protein tyrosine nitration of BSA using a specific antibody for 3-nitrotyrosine (3-NT). High 3-NT immunereactivity was observed due to the production of nitrated tyrosine on BSA surface. However, when the same experiment was carried out in the presence of nanoceria (high Ce^{+4}) or glutathione, a significant decrease in tyrosine nitration was observed.

Based on the above discussion, it is clear that 2007 to 2014 was the period of investigation of new enzymelike activities and mechanism behind these actions exhibited by nanoceria. However, the currently above reported properties of nanoceria are being translated in many biological applications. Some of the applications are discussed in Sec. III.

III. BIOLOGICAL APPLICATIONS OF NANOCERIA

Nanoceria has recently emerged as a fascinating and lucrative material with tremendous application in biomedical sciences such as biosensing, bioassays, cancer diagnostics, drug delivery, environmental engineering, and scaffold for cell/tissue growth. The various applications of nanoceria have been broadly divided into the following two subsections biosensors and therapeutics.

A. Nanoceria as biosensors

1. H_2O_2 detection

Nanoceria has shown excellent reactivity toward H_2O_2, which has helped researchers to design biosensor based on colorimetric reaction without the use of enzymes. As it has been well established that nanoceria (Ce^{+3}) reacts strongly with H_2O_2 and oxidizes to nanoceria (Ce^{+4}) almost immediately, thus yielding yellow color.[54] It has also been shown that this reactivity increases with a decrease in nanoceria's crystallite size due to the increase in the surface area as well as the amount of Ce^{+3} at the surface.[80] Gaynor *et al*. have studied this phenomenon and showed using density functional theory that adsorption of peroxide and oxygen molecules on nanoceria creates new states in the electronic structure, leading to transitions absorbing in the visible region of the electromagnetic spectrum.[81] They used self-assembled monolayer (SAM) of poly(4-vinylpyridine) for immobilizing the nanoceria on a glass substrate. Through UV-Vis absorption measurement, they showed that this system could sense H_2O_2 as low as 10 μM. It was also shown that after the first use, immobilized nanoceria could be baked at 80 °C to regenerate the sensor for reuse. Nanoceria-based composites are also shown to exhibit H_2O_2 sensing property. In one such attempt, Zhang *et al*. have shown that nanoceria-gold composite can be used as a highly sensitive nonenzymatic electrochemical sensor for H_2O_2.[82] They carried out sensing test by cyclic voltammetry and showed that screen-printed carbon electrode coated with nanoceria-gold composite exhibited an electrocatalytic response to H_2O_2. This method could detect H_2O_2 concentration as low as 5 μM with a sensitivity of 27.1 $\mu A\ mM^{-1}\ cm^{-2}$.

2. Glucose detection

Due to its nontoxic nature, inertness, and electrically conductive nature, nanoceria has been shown to be an excellent electrode material. Therefore, using its electrochemical redox couple property, mediator-less glucose sensors have

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been created. Interconversion between Ce^{+3} and Ce^{+4} allows easier electron flow between and even interface with the active sites of the GOx and electrode surface.[83,84] Saha *et al*. have used a Pt-coated glass plate for deposition of nanoceria, which was further modified with GOx exploiting electrostatic interaction between negatively charged enzyme and positively charged nanoceria at neutral *p*H.[85] It was shown that this electrode resulted a linear response to glucose oxidation and exhibited low K_m value (1.01 mM), suggesting enhanced enzyme affinity to glucose. Ansari *et al*., reported an electrochemical approach by physically adsorbed GOx on nanoceria-gold composite.[86] This electrochemical method showed a linear response to glucose detection in the range of 0.5 to 4 g/l with the detection limit of 12 μM and shelf-life of 12 days. Jiao *et al*. have exploited the peroxidaselike activity of nanoceria for the sensitive and low cost method for the development of glucose detection.[87] It was found that this method could detect glucose from 0.5–10 mmol/l with a limit of detection of 0.1 mmol/l. Owing to the excellent physicochemical properties of nanoceria, a paper-based bioassay method has been developed for the sensing of glucose. In this method, nanoceria and GOx were coimmobilized on a filter paper through silanization.[88] Upon addition of glucose, enzymatically produced H_2O_2 produces a visual color change of nanoceria present on the paper from white to dark orange. With a detection limit of 0.5 mM and linear range up to 100 mM, this method is fully reversible and can be reused for at least ten consecutive cycles without any significant loss of activity.

3. Oxidase-coupled methods

Except GOx, other oxidase enzymes have also been coupled with nanoceria for glucose detection on a biosensing platform. Kang *et al*. have reported on the synthesis of a conductive nanocomposite consisting of immobilized oxidase enzyme in the pores of nanoceria-carbon for an efficient electrochemical biosensing of glucose.[89] Similarly, cholesterol oxidase (ChOx) has also been immobilized on nanoceria deposited on the indium-tin-oxide coated glass substrate.[90] The obtained low Km (2 mM) values indicated the enhanced ChOx affinity to cholesterol even after immobilization.[91] Such attempts could be used for glucose or other biomolecule biosensing without any functionalization. Nafion-coated graphene decorated nanoceria has been shown to sense dopamine by electrochemical impedance spectroscopy.[92] It was shown that the response of the composite toward dopamine showed a lower oxidation potential (0.23 V) and high oxidation current. This biosensor exhibited linear response from 10 to 780 μM with a detection limit of 1 μM. Nesakumar *et al*. have developed an electrochemical biosensor to detect lactate for diagnosis purposes.[93] They fabricated a nicotinamide adenine dinucleotide/lactate dehydrogenase/nanoceria/glassy carbon electrode bioelectrode for sensing lactate in human blood samples. The electrochemical studies were carried out using cyclic voltammetry and the linearity in detection was found in the range of 0.2–2 mM within the response time of 4 s. Since nanoceria have been shown as excellent scavenger of free radicals, they are used for biosensing ROS. In one attempt by Neal *et al*., a biosensing platform was developed using gold electrode coated with a SAM of mercapto dodecanoic acid (MDDA) as substrate.[94] Nanoceria was further conjugated to MDDA using its carboxylic acid terminus. The constructed biosensor was used to detect the concentration (in μM) of H_2O_2 by cyclic voltammetry and chronoamperometric testing in phosphate buffer saline and potassium hexacyanoferrate.

4. Nanoceria in immunoassays

Owing to the *p*H dependent antioxidant property, nanoceria have been used for selective protection of normal cells/tissues during cancer radio and chemotherapy. In this context, it was found that at neutral *p*H nanoceria acts as antioxidant; however, at acidic *p*H as an oxidant. Exploiting this property of nanoceria, single-reagent immunoassays have been developed for the sensitive detection of biomolecules and cells, thus eliminating the need for secondary antibodies, conjugated enzymes (peroxidase), and H_2O_2. Perez and coworkers have developed immunoassay-based detection of cancer cells and cancer biomarkers using nanoceria (Fig. 3).[36,95] They used dextran (10 kDa) or polyacrylic acid (1.8 kDa) coated nanoceria exhibiting oxidase-like activity. Nanoceria exhibited size and coating dependent activity in slightly acidic *p*H. Nanoceria were further modified by folate receptor, which facilitated the binding to cancerous cells over-expressing folate receptors. This detection method was applied to lung carcinoma cells (A549 cells), which express folate receptors, and normal cardiomyocytes (H9c2) which lack the expression. It was found that A549 cells were able to oxidize TMB and produce the color, whereas H9c2 did not produce any color. This observation suggests that nanoceria could be tailored to be used as a novel agent for the detection of biomolecules and cells, like any regular immunoassay. Perez and coworkers further improved the nanoceria-based immunoassays by modifying the method which could be used at neutral *p*H conditions.[36] In this method, they replaced the TMB with Ampliflu, which oxidizes to resorufin (fluorescent) by weak oxidase activity; however, the strong oxidase activity of nanoceria triggers complete oxidation to the resazurin (nonfluorescent). Nanoceria (at neutral *p*H) exhibit mild oxidase activity; therefore, it would partially oxidize Ampliflu to the fluorescent resorufin. Nanoceria modified with antifolate receptor antibody was used to detect folate receptors expressed on cancer cells using protein G. Similarly, other groups have reported to the targeted delivery of nanoceria after monoclonal antibody conjugation. Cimini *et al*. have used PEG-coated and antibody conjugated nanoceria (Aβ-nanoceria) for selective delivering to Aβ aggregates to modulate neuronal survival pathways.[96] It was found that Aβ-nanoceria could rescue the neurons from free radical-mediated damage by modulating the brain-

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derived neurotrophic factor signaling pathway. Thus, the nanoceria-based immunological assays are faster and cheaper detection methods than traditional enzyme-linked immunosorbent assay (ELISA) and therefore are expected to be used in point-of-care devices and molecular diagnostics (Fig. 1).

B. Therapeutic applications

1. Drug delivery

Recently, nanomaterials based drug delivery methods have shown tremendous potential use in clinics. Due to the pharmacological potential, nanoceria have been shown as a better therapeutic agent. Recently, Qu and coworkers have developed a multifunctional nanoceria-based drug delivery system by modifying it with β-cyclodextrin and ferrocene-functionalized mesoporous silica nanoparticles.[97,98] It was shown that after uptake into lung carcinoma cells (A549) through acidic lysosomes, the ferrocenyl moieties were oxidized to ferrocenium ions by nanoceria, which ultimately triggers the drug release. Further, the cytotoxicity of nanoceria at acidic *p*H shows synergistic anticancer with anticancer drugs. In another report, nanoceria-based, H_2O_2 responsive drug delivery system has been synthesized by Qu *et al*. for the neuroprotection and treatment of neurodegenerative disorders.[99] Although metal chelators have been developed and used as a therapeutic agent for Alzheimer's disease, their use is limited due to poor blood–brain barrier permeability. Therefore, the metal chelators were loaded in a phenylboronic acid-functionalized silica nanocarrier layered with glucose-coated nanoceria through covalent reaction between phenylboronic acid and glucose to form cyclic boronate moieties. This cyclic boronate moieties can be broken when exposed to H_2O_2, thus leading to the release of nanoceria and the drug molecules. The combination of an antiaggregation property of metal chelator and antioxidant nature of nanoceria in one system could effectively inhibit the Aβ aggregation, free radical generation, and thus protect the cells from the toxicity induced by Aβ aggregation.

2. Anti-invasive properties

It is well known that tumor–stroma interactions play significant role in metastasis. Additionally, stroma signaling is mainly mediated by myofibroblasts, which are mediated by tumor-derived transforming growth factor β1 (TGFβ1).[100] TGFβ1 initiates the formation of ROS and expression of alpha-smooth muscle actin, which is a biomarker for myofibroblastic cells. Thus, it is expected that antioxidants prevent the formation of myofibroblasts; however, the same antioxidants are shown to induce aggressive behavior of tumor cells. Therefore, in an attempt by Alili *et al*., it has been shown that nanoceria (antioxidant) downregulate the expression of alpha-smooth muscle actin positive myofibroblast cells and the invasion of tumor cells.[100] It was also seen that the nontoxic concentration of nanoceria to normal cells showed cytotoxicity to the squamous tumor cells. It has also been seen that nanoceria could decrease the intrinsic invasive property of cultured squamous tumor cells even in the absence of any myofibroblast stimulation. Therefore, based on these data, it can be concluded that nanoceria has the ability to modulate the tumor environment to inhibit the invasion properties of tumor cells/tissues.[101]

3. Radiation protection and radio-sensitization

Radiation exposure to cells/tissues is known to generate free radicals, which is a major limitation in current cancer treatment. It becomes more important as nearly half of all cancer patients receive radiation treatment at some point of their treatment. Due to radiation therapy, free radicals are generated in excess, which lead to several side effects such as fatigue, nausea, and dermatitis. To mitigate these effects, few radiation adjuvants are available (e.g., Amifostine), which have shown radioprotective effects. These clinical adjuvants are associated with nausea and hypotension.[99,102] Several reports have shown that, at least in their initial studies, pretreatment of nanoceria to the cancer cells/tissues prior to the radiation treatment have shown decreases in cell death and damage to normal cells than cancerous cells. Mechanistically, it was found that the exposure of nanoceria scavenges the free radicals, thereby inhibiting caspase 3 and 7 in irradiated lung fibroblasts cell culture models. Nanoceria treatment has also been found to increase the SOD enzyme expression up to twofold in human colon cells in culture.[103–105] Additionally, the acidic *p*H in cancerous cells further promotes the formation of free radicals after nanoceria exposure, due the oxidase activity, which synergistically act with the radiation treatment leading to the enhanced cell death.[106] Recently, it has been shown in pancreatic cells that nanoceria lead to free radical generation after radiation treatment. Arguably, acidic *p*H is known to inhibit the intrinsic catalaselike activity of nanoceria, which could be correlated with the accumulation of more H_2O_2 at acidic *p*H, probably due to the conversion of highly unstable superoxide radicals into H_2O_2.[101] Therefore, the dual role of nanoceria, as antioxidant in normal cells and as oxidant in cancerous cells, could significantly impact the life of cancer patients.

4. Cell growth and tissue engineering

Cell growth and tissue proliferation is an indispensable part of rapid and effective wound healing, which involves a coordinated cellular response of fibroblasts, keratinocytes, and vascular endothelial cells.[107] Therefore, a universal cell growth promoting agent is needed, which could accelerate the wound healing process. In this quest, topical application of nanoceria has been shown as an excellent wound healing agent in mice. Mechanistically, nanoceria accelerates the proliferation and migration of fibroblasts, keratinocytes, and vascular endothelial cells. It was also found that nanoceria penetrated in the wound could reduce the level of free radicals, thus reducing the oxidative damage to cells and biomolecules, suggesting its potential use as topical agent for

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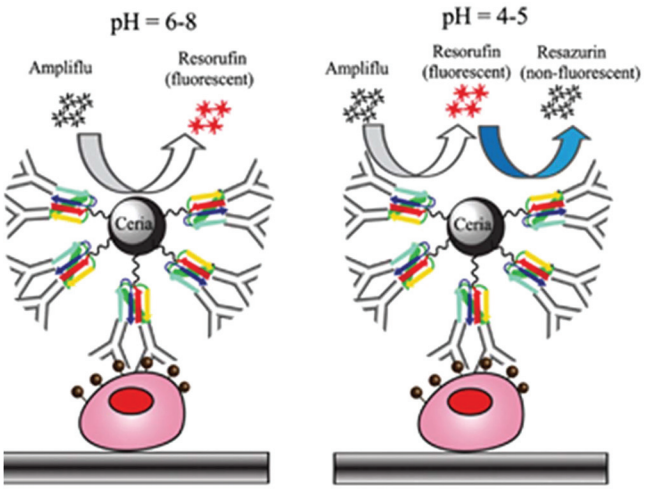


FIG. 3. Nanoceria-based ELISA assay. Nanoceria-based ELISA assay operated at acidic (pH = 4–5) and neutral (pH = 6–8) pH range. Reprinted with permission from Asati *et al*., Anal. Chem. 83, 2547 (2011). Copyright 2011 American Chemical Society.

wound healing.[108] Ponnurangam *et al*. have shown that nanoceria could be used for modulating the development of engineered cartilage and combating the deleterious effects of interleukin-1α.[109] It was also observed that nanoceria was biocompatible with bovine chondrocytes up to fairly high concentration, which lead to the improved compressive mechanical properties and biochemical composition of engineering cartilages. It was further revealed that chondrocytes exposed to nanoceria have increased levels of proline, procollagen, and glycogen than the untreated cells. Since the harsh inflammatory environment is known to be associated with a major challenge to articular cartilage repair, nanoceria was found to inhibit the inflammatory responses induced by interleukin-1α. Exploiting the interconversion of Ce^{+3} to Ce^{+4} has been used for tissue engineering applications. One such attempt was made by Naganuma *et al*., where they studied the influence of valence states of nanoceria on the proliferation of human mesenchymal stem cells and osteoblastlike cells (MG63) grown on poly-L-lactide scaffold surfaces functionalized with nanoceria having dominant Ce^{+3} and Ce^{+4} regions.[110] It was observed that different surface oxidation states of nanoceria responded differently to the cell types and promoted or inhibited the cell growth, proliferation, migration, and adhesion behavior. Cell morphologies were further correlated with the valance states of nanoceria, and the type of cell–material interaction was found responsible for the cell proliferation to be triggered. This study suggested that cell–material interactions can be controlled by modulating the valence states of the nanomaterials; thus, cell growth and proliferation can also be tuned as per the need.

IV. FUTURE OF NANOCERIA IN MEDICINES

Owing to the biocompatibility and redox-dependent properties, the development and application of nanoceria in medicine is on the rise. During last decade, nanoceria have been discovered as potential treatment modality for many types of cancers, ocular diseases, neurodegenerative diseases, chronic inflammation, ischemic cardiomyopathy, endometriosis, and diabetes.[8,11,100,111–114] Nanoceria have shown promising results in both *in vitro* and *in vivo* experimental models. Recently, the emphasis has been given to the use of nanoceria in cancer and ocular diseases such as macular degeneration, retinoblastoma, glaucoma, diabetic retinopathy, etc.[115–117] The observed medicinal potential can be explained on the basis of two reasons, ability to act as regenerative antioxidant and pH dependent tenability from pro-oxidant to antioxidant.[118] It has been suggested that at acidic pH (4–5), the antioxidant ability of nanoceria is inhibited, whereas pro-oxidant property is revealed, which leads to the generation of hydroxyl radical. One study reported that at acidic pH, nanoceria produces hydroxyl radical without the use of external H_2O_2, and the cellular H_2O_2 concentration is enough to generate these radicals, which lead to the cancer cell death.[111] Additionally, in ocular diseases, the autoregenerative property (due to easy interconversion between Ce^{+3} to Ce^{+4}) provides an ability to act as an autoregenerative antioxidant, thus continuously removing the free radicals from cell cytoplasm under the physiological condition. Based on the demonstrated site and cell specific activity, nanoceria can be considered as a potential candidate for personalized nanomedicine such as precision oncology and ocular diseases treatments in near future.

V. CONCLUSION AND FUTURE DIRECTIONS

It is clear from the studies summarized in this review that nanoceria is an emerging biological enzyme mimicking material. The superoxide and hydrogen peroxide are the two major radical generating systems in living organisms and are scavenged by SOD and catalase enzymes, respectively; nanoceria is known to exhibit SOD-like and catalaselike activity. The peroxidaselike activity of nanoceria has also been explored to use them in biosensing and other immunoassays. Further, these enzymes like properties of nanoceria are largely dependent on chemical components of biologically relevant buffers, culture media, and pH. Although the current literature of nanoceria about its enzymelike properties and interaction at nanobio interface is motivating, many questions still remain unanswered and thus encourage further research. More characterization of nanoceria under *in vivo* experimental condition will answer the intriguing question about the formation of protein corona and the interaction with other biomolecules, and cationic and anionic species present, predominantly in living organisms. This will also riposte the mechanism of action of catalytic activity of nanoceria *in vivo*. Undoubtedly, in coming years, research on nanoceria will continue to expand at the interface of bionano, biomimetics, and bioinspired applications.

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