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Cerium oxide nanocluster based nanobiosensor for ROS detection

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ABSTRACT:

The present study demonstrates the synthesis of cerium oxide nanoclusters (CeO_2-NC) and its application towards detection of reactive oxygen species (ROS). ROS are generated due to exogenous exposure to oxidants, car smoke, pollutants etc. as well as endogenously as a byproduct of metabolism that can cause cellular damage. Although, protective antioxidant molecules and antioxidant enzymes are present to combat these oxidative damages *in vivo*, the overload of oxidants may lead to escape this combat pathway and expose us with a chronic low dose of oxidants creating oxidative stress. Thus, detection of ROS in biological fluids may lead us to combat the possible damage caused by the oxidant. Our synthesized nanoclusters are of extremely low size, highly stable and have a very high fluorescent property. Moreover, CeO_2 nanostructures have been known to exhibit dual property; to act as a prooxidant as well as a free radical scavenger. We have utilized these properties to design a nanobiosensor which can detect ROS with high sensitivity. We have used hydrogen peroxide (H_2O_2) as a ROS generator and ascorbic acid as a free radical scavenger to react with the synthesized CeO_2-NC and monitored the fluorescence intensity change. Our CeO_2-NC exerted oxidants thereby increased the fluorescence intensity with H_2O_2 and decreased fluorescence with ascorbic acid. The fold increase or decrease in fluorescence intensity was linear. Thus, with the results obtained we can propose that CeO_2-NC can act as an ROS nanobiosensor.

1. Introduction

Cerium is a rare earth metal in lanthanide series with atomic number 58. The rare earth metals mostly exist in the trivalent state but cerium (electron configuration $[Xe]_4f_5^1d_6^1s^2$) can exhibit both the +3 (Ce^{3+} = $[Xe]_4f_1$) and +4 (Ce^{4+} = [Xe]) oxidation states (Dahle and Arai, 2015; Korsvik et al., 2007). The Ce^{3+} salts in the form of nitrate, acetate, chloride, etc. have been used traditionally by humans because of their antiemetic, bacteriostatic, bactericidal, immunomodulating and antitumor activity (Jakupec et al., 2005; Ji et al., 2000). Cerium oxide nanoparticles (CeO_2-NPs) have excellent catalytic activities that are derived from Ce^{4+} and Ce^{3+} oxidation state. Since, the Ce atom exists in both trivalent (Ce^{3+}) and more stable tetravalent (Ce^{4+}) state, it allows the nanoparticles to store and release oxygen when present in the form of CeO_2 (Skorodumova et al., 2002). It arises as a result of redox reactions where there is a loss of oxygen and/or its electrons, alternating between CeO_2 and CeO_{2-x}.CeO_2-NPs are recently found to mimic the multi-enzyme activities like superoxide dismutase and catalase. In biological fields such as biology, nanomedicine, bioscaffolding and tissue engineering CeO_2-NPs has emerged as an effective nanomaterial (Karakoti et al., 2010; Das et al., 2013). CeO_2-NPs have also been found to be radioprotective in normal cells like MRC-5 normal cells (Abdi Goushbolagh et al., 2018).

Free radicals, such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$) are highly reactive metabolites of oxygen known as reactive oxygen species (ROS). In the intracellular and extracellular environment antioxidants exists both in enzymatic and non-enzymatic forms. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPx) are the antioxiant enzymes and glutathione is an antioxidant molecule which scavenges free radicals (Nimse and Pal, 2015). Although some of the oxidants escape these defense systems and we are continuously exposed to a low dose of oxidative stress that give rise to different response to damaging agents (Bose (Girigoswami) et al., 2003; Bose (Girigoswami) and Ghosh, 2005; Bose (Girigoswami) et al., 2005; Ghosh et al., 2012). The early detection of oxidants in the body is warranted for preventing the damage

Abbreviations: ROS, Reactive oxygen species; CeO_2-NC, cerium oxide nanoclusters; H_2O_2, hydrogen peroxide; CeO_2-NPs, Cerium oxide nanoparticles; SOD, Superoxide dismutase; CAT, catalase; GSHPx, glutathione peroxidase; DPPH, 2,2-diphenyl-1-picryl-hydrazyl-hydrate; DLS, dynamic light scattering; SEM, scanning electron microscopy; BSA, bovine serum albumin

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caused due to oxidative stress. The ROS is generated in several diseased conditions in our body like cardiovascular diseases, Alzheimer's disease and other neurological diseases (Breiger et al., 2012). There is also a significant role of ROS present in the complications associated with insulin resistance, type II diabetes, obesity and comorbidity conditions, inflammation and infections etc. (Alfadda and Sallam, 2012).

There are many methods for the detection of ROS like spin trapping (Finkelstein et al., 1980), DPPH (2,2-diphenyl-1-picrylhydrazyl) assay (Oh et al., 2010)etc. Most of these methods either involve sophisticated instruments or require expensive chemicals (Dikalov et al., 2007). Nanoparticles are nowadays being extensively used for different applications including biosensing. Metal oxides possess very important role in biological science, analytical chemistry and environmental science (Ansari et al., 2010) due to the development of nanobiosensors based on semiconductor nanostructures. For fabrication of cytochrome c and hemoglobin biosensors, respectively nanostructured SnO_2 film was deposited on a substrate and was used as an electrode (Topoglidis et al., 2003). The further reduction in size of the nanoparticles leads to the formation of nanoclusters (usually below 10 nm) which show typical quantum size behaviour at room temperature. These nanoclusters are sometimes coined as 'artificial big atoms' and there is a variation in the band gap of semiconductor clusters with size that alters the magnetic and optical properties (Weigend and Ahlrichs, 2010; Liu et al., 2014). In the present study, we have engineered cerium oxide nanoclusters (CeO_2—NCs) by tuning the size and capping them with BSA. The synthesized CeO_2-NC was characterized using the different instrumentation techniques. Dynamic light scattering (DLS) was utilized for hydrodynamic diameter and stability detection, UV— visible spectrophotometry was used find the absorption of light, fluorescence spectroscopy was used to monitor the fluorescence property as well the ROS sensor application, scanning electron microscopy was done to investigate the surface morphology, X ray diffraction spectroscopy and FTIR was executed to find the structural properties of the synthesized nanoclusters. The results of our study indicated that the CeO_2—NCs are highly stable, highly fluorescent, detects ROS in a linear manner and cost effective.

2. Materials and methods

2.1. Materials

Cerium nitrate was purchased from SPECTROCHEM Pvt. Ltd. Mumbai, India. BSA (bovine serum albumin), ammonia solution & ascorbic acid were bought from Sisco Research Laboratories (SRL), India, hydrogen peroxide (H_2O_2) was procured from Fisher Scientific, Mumbai, India and other chemicals were procured locally.

2.2. Synthesis of cerium oxide nanocluster (CeO_2-NC)

Synthesis of cerium oxide nanocluster (CeO_2-NC) was done according to Liu et al. (2014) by precipitation method with slight modification by using BSA as capping and stabilizing agent. 0.05 g of cerium nitrate was dissolved in 5 mL of distilled water using magnetic stirrer for 10 min. 5% BSA (5 mL) was gradually added in the solution while in stirring condition. The solution was kept under stirring for further 10 min and to it 0.5 mL of ammonia solution was added dropwise till the color changed. The solution was kept for stabilization under stirring condition for an additional 3 h, followed by purification using dialysis membrane against water (Haribabu V et al., 2019). The as synthesized CeO_2-NC was characterized using dynamic light scattering (DLS), UV–visible spectrophotometry, fluorescence spectroscopy and scanning electron microscopy (SEM).

2.3. Characterization of the synthesized CeO_2-NC

The synthesized CeO_2-NC at an amount of 0.1 mL was added to 2.9 mL of deionized water and was analyzed using UV–visible spectrophotometry, fluorescence spectroscopy, DLS measurements and X-ray diffraction (XRD) according to Girigoswami et al. (2015). Shimadzu (Japan) UV-1800 spectrophotometer was used for the recording of UV–visible spectral data and the absorbance spectra from 400 nm–600 nm were measured taking water as reference. For studying the fluorescent properties of the synthesized CeO_2-NC, Jasco FMP-825 spectrofluorimeter was used. The samples were excited at 515 nm (the excitation maxima of the CeO_2-NC) and emission was recorded from 535 nm to 650 nm. The hydrodynamic diameter of the CeO_2-NC was recorded using Malvern Nano ZS90 particle size analyzer. The CeO_2—NCs were lyophilized and the powder XRD was done. The radiation source of the diffractometer was CuKα emitting wavelength 1.54 Å and the 2θ range of the data collected was from 10° to 100°, with a step size of 0.05°. SEM image was used for the structural confirmation of synthesized CeO_2-NC. The liquid sample was filtered using 200 μm filter and was dropped in a dirt free solid surface (Al foil) and air dried in a dirt free atmosphere for SEM analysis. This dried sample template was analyzed using FESEM according to Akhtar et al. (2017). The IR spectrum was recorded using attenuated reflectance in transmission mode using Bruker, Alpha –T FTIR, according to Sharmiladevi et al. (2017).

2.4. Spectrofluorimetric titration of CeO_2—NCs with different concentrations of H_2O_2

Hydrogen peroxide yields ROS which can be detected using the nanoclusters. A stock solution of 80 mM H_2O_2 was prepared in distilled water. Six test tubes were taken and to each 100 μL CeO_2-NC was added. To make the final volume up to 3.0 mL, H_2O_2 was added at different concentrations from test tube no. 2–6 yielding a final concentration of H_2O_2 to be 5 mM, 10 mM, 16 mM, 21 mM and 26 mM respectively. Test tube number 1 was taken as blank which did not contain H_2O_2. The samples were incubated in dark for 30 min for equilibrium. The fluorescence emission spectrum was monitored after excitation at 515 nm, and emission from 535 nm to 650 nm.

2.5. Spectrofluorimetric titration of CeO_2—NCs with different concentrations of ascorbic acid

Ascorbic acid scavenges ROS which can be taken as a positive control to detect ROS using the nanoclusters. Ascorbic acid stock solution of 1 mM was prepared. Six test tubes were taken and to each 100 μL CeO_2-NC was added. To make the final volume up to 3.0 mL, ascorbic acid was added at different concentrations from test tube no. 2–6 yielding a final concentration of ascorbic acid to be 10 μM, 20 μM, 50 μM, 100 μM and 500 μM respectively. Test tube number 1 was taken as blank which did not contain ascorbic acid. The samples were incubated in dark for 30 min for equilibrium. The fluorescence emission spectrum was monitored after excitation at 515 nm, and emission from 535 nm to 650 nm.

3. Results

3.1. Characterization of CeO_2-NC

The synthesized CeO_2-NC was characterized physicochemically using DLS, UV–visible spectrophotometry, spectrofluorimetry and SEM. The size distribution of the CeO_2-NC was determined using dynamic light scattering (DLS) analysis and shown in Fig. 1 (A). The major scattering peak obtained at 10.65 nm indicated the hydrodynamic diameter of the nanoclusters. The picture of the CeO_2-NC (Fig. 1 (A)) shows the color of the nanoclusters to be brown. The surface charge and stability of the CeO_2-NC was determined using zeta potential analysis. The zeta potential distribution was found to be −27.9 mV which indicated that the nanoclusters were highly stable (Fig. 1 (B)). The optical

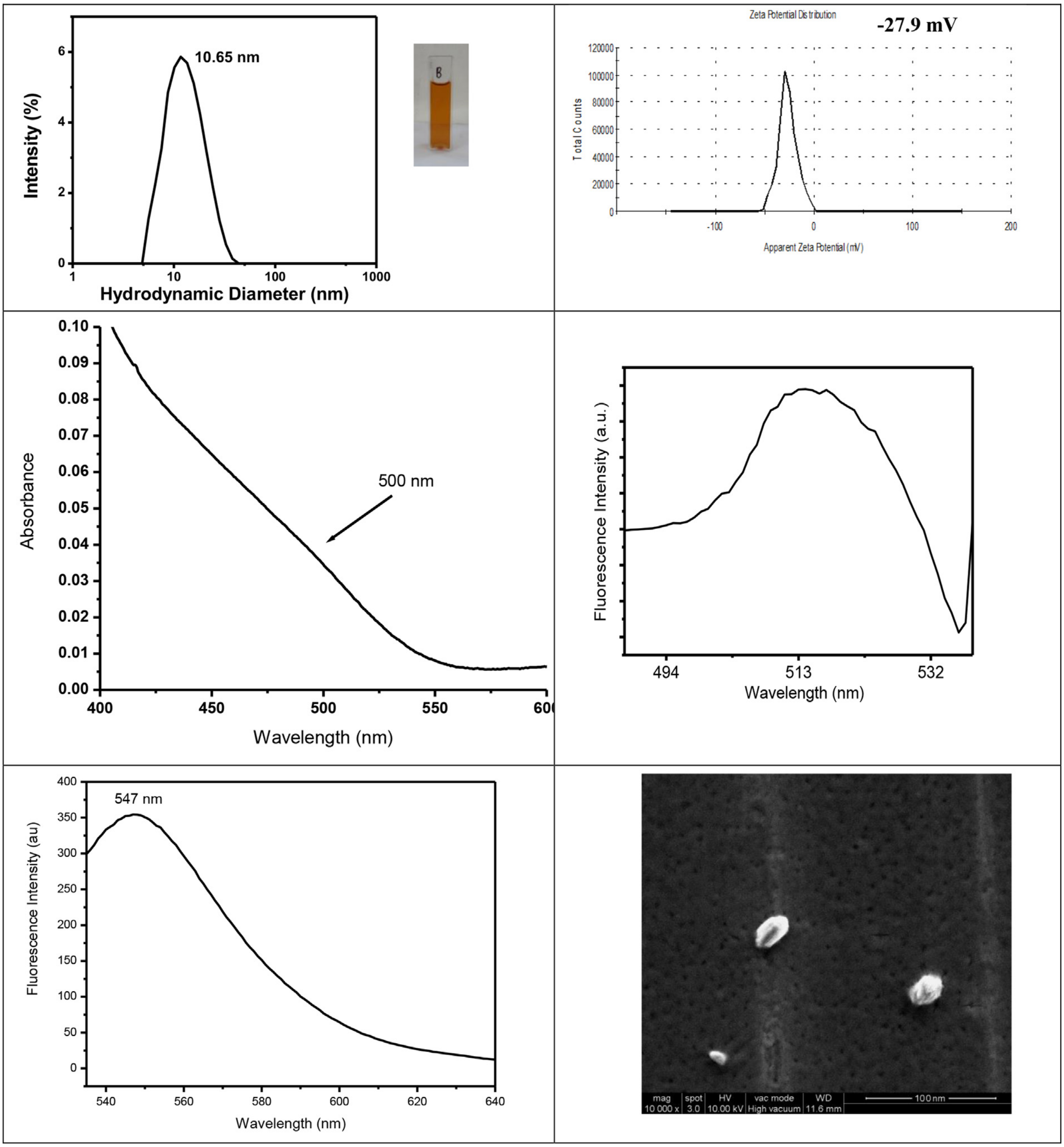

Fig. 1. (A) The hydrodynamic diameter of the synthesized CeO_2—NCs. The picture of the nanoclusters also shows a brown color (B) Zeta potential of the synthesized CeO_2—NCs (C) UV–visible spectrum of the synthesized CeO_2-NC (D) Fluorescence excitation spectrum of the synthesized CeO_2-NC (E) Fluorescence emission spectrum of the synthesized CeO_2—NCs after exciting at 515 nm (F) SEM image of synthesized CeO_2—NCs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

absorbance spectrum of CeO_2-NC was recorded between 400 and 600 nm. The broad shoulder peak was found around 500 nm (Fig. 1 (C)) and to obtain a specific absorption peak the study was extended to fluorescence excitation spectrum. The fluorescence excitation spectrum of CeO_2-NC was recorded between 480 and 535 nm. The distinct peak was found at 515 nm (Fig. 1 (D)) which showed that the nanoclusters absorb light at 515 nm. To estimate the fluorescent nature of the synthesized nanoclusters, we have recorded the emission spectra for the CeO_2-NC after excitation at 515 nm and emission from 535 nm to 600 nm. The emission peak is found at 547 nm (Fig. 1 (E)) and the intensity of fluorescence is very high showing that only 100 μL of sample in 3 mL reaction volume can give very high fluorescence which is desirable to be used as a sensor. The visualization of the surface morphology of the synthesized CeO_2-NC was done using SEM. The SEM image is shown in Fig. 1 (F) depicting a smooth surface morphology and a clear ellipsoidal shape and the size corroborates with our finding using DLS. The structural properties were obtained by X-ray diffractometry where the nanoclusters were lyophilized and made into powder form for analysis. The XRD spectrum of CeO_2-NC is shown in Fig. 2, which clearly indicated the hkl peaks of CeO_2 as (211), (200), (220), (311) and (222). To further exploit the nature of bonds present in the synthesized nanoclusters, IR spectroscopy was done using attenuated reflectance and the spectrum is shown in Fig. 3. The Ce—O bond stretching is observed at the peak corresponding to 550 cm^{-1}.

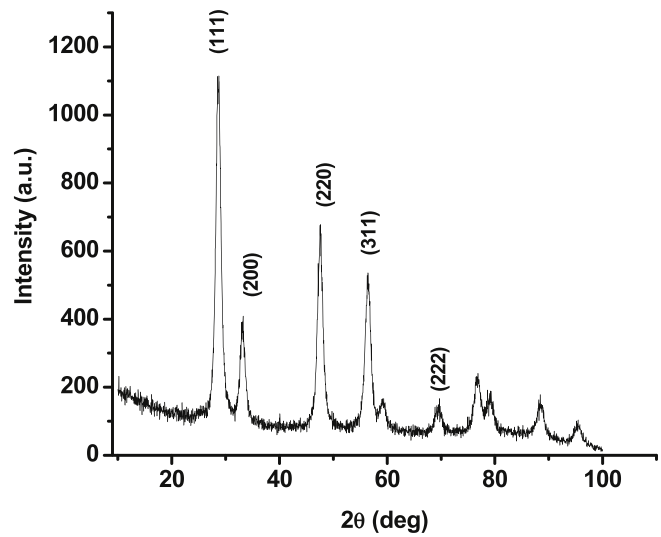

Fig. 2. The XRD spectrum of synthesized CeO_2—NCs after lyophilization.

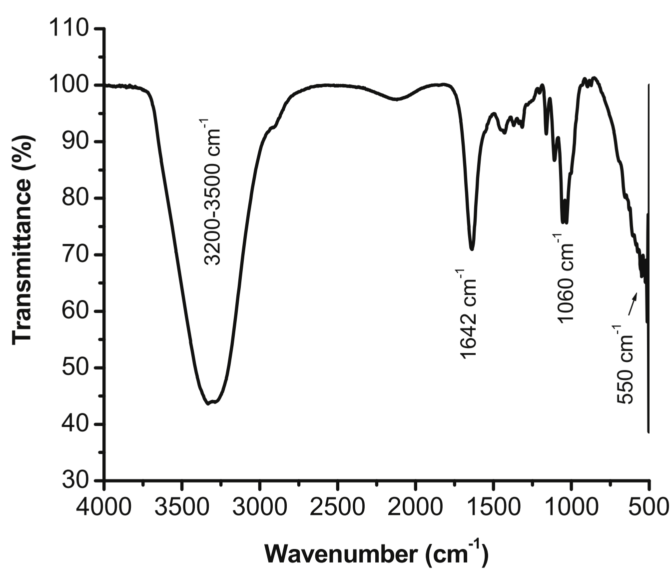

Fig. 3. The FTIR spectrum of the synthesized CeO_2—NCs.

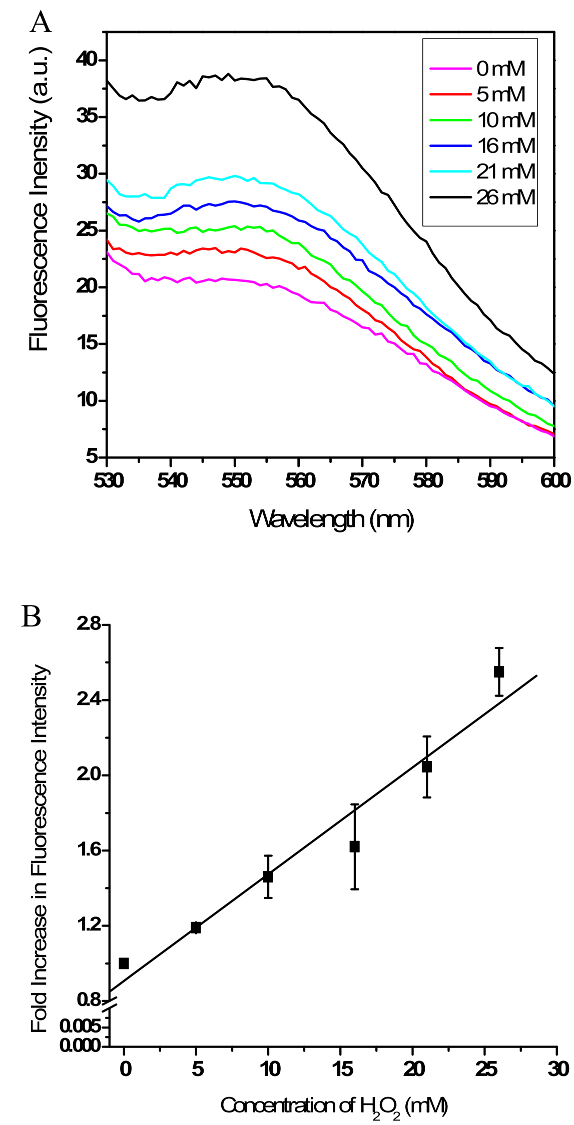

Fig. 4. (A) The increase in fluorescence intensity of CeO_2—NCs after addition of different amount of H_2O_2. (B) The fold increase in fluorescence intensity with increase in H_2O_2 concentration.

3.2. Effect of synthesized CeO_2-NC on hydrogen peroxide (H_2O_2)

The fluorescent property was explored to sense ROS using CeO_2-NC and H_2O_2 as a source of ROS. CeO_2 is known to have radical scavenging activity or radical exerting activity depending on the surface ratio of Ce^{3+}/Ce^{4+} present in the nanocluster. There was an increase in fluorescent intensity after addition of increasing concentrations of H_2O_2 (Fig. 4 (A)). The fold increase in the fluorescence intensity was plotted against the increase in concentration which showed a linear relationship (Fig. 4 (B)). The experiments were repeated three times. This linear relation of fold increment in fluorescence with increase in H_2O_2 concentration revealed that these nanoclusters can be used to sense ROS.

3.3. Effect of CeO_2-NC on the radical scavenging activity of ascorbic acid

Since ascorbic acid is a radical scavenger we have used it as a positive control. The effect of CeO_2—NCs was studied using different concentrations of ascorbic acid and it has been found that there is a decrease in fluorescence with increasing concentration (Fig. 5 (A)). This decrease in fluorescence intensity may be attributed to the free radical exerted by Ce^{4+} being scavenged by the ascorbic acid. The fold decrease in fluorescence intensity was plotted and a linear graph was obtained (Fig. 5 (B)) showing that the decrease in fluorescence can be used to sense the amount of ascorbic acid.

4. Discussion

Nanostruture based biosensors have been used in the diagnosis of various biomarkers related to different diseases (Metkar and Girigoswami, 2019; Akhtar and Girigoswami, 2019). Europium doped cerium oxide nanoparticle has been found to ameliorate the ischemic injury in intestine by reducing ROS (Gubernatorova E.O. et al., 2017). The redox activity of cerium oxide has also been exhibited against dopamine representing its applicability towards different diseases *in vivo* (Hayat A et al., 2014). Our aim was *in vitro* detection of ROS using the redox properties of Cerium oxide nanoclusters (CeO_2-NC) that were synthesized by precipitation method using BSA as capping and stabilizing agent. The synthesized CeO_2-NC was characterized using the different instrumentation techniques. The absorption maximum as measured using UV visible spectrophotometry was nearly 500 nm showing a broad shoulder peak (Fig. 1 (C)). Further, the fluorescence excitation spectrum was analyzed to obtain the accurate absorption

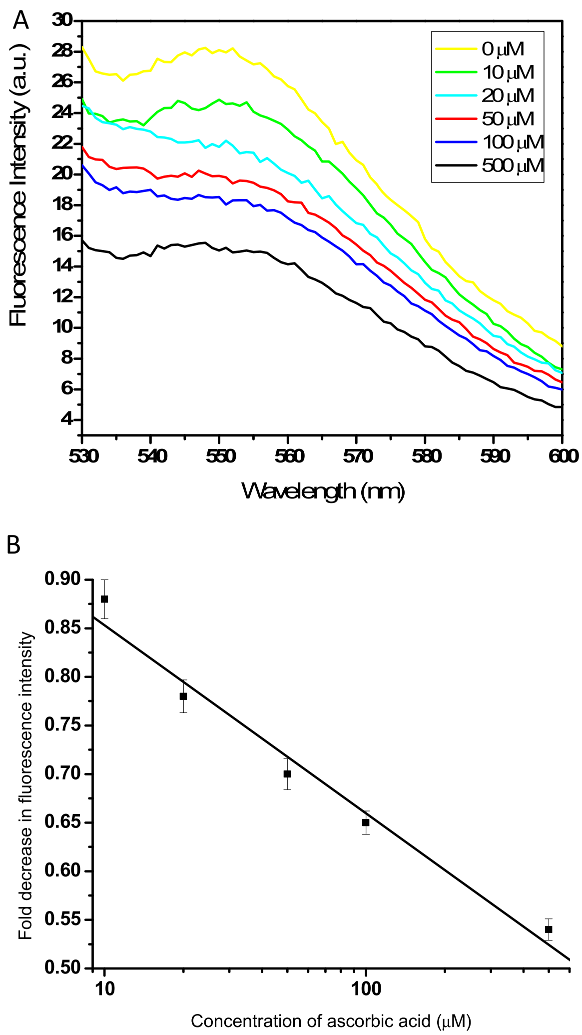

Fig. 5. (A) The decrease in fluorescence intensity of CeO_2—NCs after addition of different amount of ascorbic acid. (B) The fold decrease in fluorescence intensity with increase in ascorbic acid concentration.

peak of the nanoclusters and was obtained at 515 nm (Fig. 1 (D)). After exciting the nanoclusters at 515 nm, a distinct fluorescence emission peak was obtained at 547 nm (Fig. 1(E)) showing the high fluorescence property of the nanoclusters. Dynamic light scattering (DLS) was used to measure the size and stability of a sample from scattered light in very short period of time. The hydrodynamic diameter of the nanoclusters was found nearly 10 nm (Fig. 1(A)) which corroborates with the size defined for nanoclusters. Further the cluster size was confirmed by SEM (Fig. 1 (F)). The SEM image showed the ellipsoidal shape of the clusters. The reduction in size of a nanocluster makes it very highly reactive due to enhancement of surface area to volume ratio compared to nanoparticles. This may be the reason that the nanoclusters exhibited very high fluorescence. Researchers have shown to synthesize cerium oxide nanoclusters at the range of 2 nm (Inoue et al., 1999). The structural analysis was executed with the lyophilized powder of the CeO_2—NCs. The XRD analysis (Fig. 2) showed that the nanoclusters represented the peaks at the hkl values in coherence with the JCPDS pattern of CeO_2 (JCPDS No. 75–0076) (Priyanka et al., 2014). To further analyze the functional groups present in the synthesized nanoclusters attenuated reflectance was done. The FTIR spectra of the synthesized CeO_2—NCs (Fig. 3) showed a peak at 550 cm^{-1} indicating the stretching of Ce—O bond (Chelliah et al., 2012). The broad peak located between 3200 cm^{-1}– 3500 cm^{-1} corresponded to the O—H stretching vibration. The peak at 1060 cm^{-1} and 1642 cm^{-1} indicted the Ce—OH stretching vibrations and C=C stretching vibrations respectively (Wu et al., 2002). Thus, the XRD and FTIR spectrum data confirmed the presence of CeO_2 in the synthesized nanoclusters. Any synthesis method can be successful if the particles so synthesized are stable. Zeta potential analysis was employed to determine the surface charge of the nanoparticles in solution (colloids). Any nanoparticle possesses a charge on its surface which can attract the ions of opposite charge that make a thin layer around the surface of the nanoparticle. The value of zeta potential ranges typically from +100 mV to −100 mV and the nanoparticles having zeta potential values greater than +25 mV and less than −25 mV are considered to be highly stable (Zhang et al., 2008). In our study the synthesized CeO_2—NCs showed a zeta potential of −27.9 mV (Fig. 1 (B)) which confirmed that they were highly stable.

Reactive oxygen species (ROS) are defined as highly reactive metabolites of oxygen which include superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$) (Ozcan and Ogun, 2015). These ROS are very short lived and they have a lifetime of nanoseconds to seconds in the biological system. The lifetime of ROS depends on their reactivity and the cellular antioxidant level. Previous reports exist stating that rare earths like gadolinium, europium, terbium, neodymium and erbium doped titania nanoparticles can upregulate the cellular reactive oxygen species after exposure to X radiation (Townley and Wakefield, 2014). ROS can be detected by various methods such as spin trapping, assay of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity respectively. In DPPH assay, the alcoholic DPPH solution gets reduced by the hydrogen donated by the antioxidant leading to the formation of the non-radical, DPPH–H by the reaction (Oh et al., 2010). The drawback for this method was the chemical is very expensive and lacks photostability. Spin trapping technique to detect ROS (Finkelstein et al., 1980) also needs sophisticated instrumentation and can be time consuming (Dikalov et al., 2007). Fluorescence spectrophotometry is a type of electromagnetic spectroscopy that analyses the fluorescence emitted from a sample with high sensitivity. Our aim was to detect the ROS generated *ex vivo* in biological fluids. We explored this fluorescence property of our synthesized CeO_2—NCs to sense ROS, as CeO_2—NCs have a radical scavenging as well as radical exerting property. Size tuned CeO_2-NPs were previously been used for detection of ascorbic acid at very low concentrations (Krishnan et al., 2016). We extrapolated our study, using nanoclusters which gave more sensitivity towards ROS detection at a low concentration due to its ultrasmall size compared to the nanoparticles. By adding H_2O_2, we observed that as the concentration of H_2O_2 increased, the fluorescence intensity increased and, by adding ascorbic acid it was found that as the concentration of ascorbic acid increased, the fluorescence intensity decreased. Moreover, the relationship between concentration and fold increase in fluorescence intensity was found to be linear. The nanoclusters were also found to be highly sensitive to be used as ROS detectors and thus, the synthesized CeO_2—NCs can be used as a sensor for rapid detection of ROS, *ex vivo*.

The thermodynamic efficiency of redox cycling between the Ce^{3+} and the Ce^{4+} on the surface of CeO_2-NC induces both enzyme-mimetic and ROS scavenging properties along with radical exerting capacity (Korsvik et al., 2007; Deshpande et al., 2005; Reed et al., 2014). The surface of CeO_2-NC contains a mixture of Ce^{3+} and Ce^{4+}. The change in oxidation states is controlled by the availability of oxygen. Therefore, the absolute diameter of CeO_2-NC varies when there is a loss of oxygen atom and there is an increase in the Ce^{3+} on the surface of the CeO_2-NC. This alteration of oxidation state has ability to generate the ROS and it can work as an amplifier to increase the sensitivity of CeO_2-NC as ROS sensor.

5. Conclusion

CeO_2-NPs are well known to exert dual effect towards ROS generation or ROS scavenging depending on the Ce^{+3}/Ce^{+4} ratio present at the surface of the nanoparticle. In the present study we have brought down the size of CeO_2 to nanocluster dimension and stabilized the structure with BSA as a capping agent. BSA is used because it is biocompatible and does not interfere by exerting or scavenging any ROS. The nanoclusters showed high fluorescence at a very small concentration. This fluorescence was found to increase in a linear fashion when an increasing concentration of H_2O_2 was added. To prove that the increase in fluorescence is due to the radical exerting effect of the nanoclusters, we have used ascorbic acid as a positive control. With addition of ascorbic acid the fluorescence intensity decreased with increase in concentration of ascorbic acid. The decrease was linear and showed that the synthesized nanoclusters can be used for sensing ROS produced by H_2O_2. A nanobiosensor which is cost effective, stable and has rapid detection time is thus, proposed in this study. Our study can be extrapolated by testing for ROS generated by other sources.

Conflicts of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101124.

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