In vitro antioxidant activity of synthesized BSA conjugated manganese dioxide nanoparticles

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Received: 7 April 2020 / Accepted: 21 August 2020 / Published online: 31 August 2020
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Abstract

Nanozymes represent the class of nanomaterials exhibiting enzyme mimicking properties. Biocompatible nanozymes with the ability to imitate antioxidant enzymes hold tremendous possibility for development of therapeutics in various disorders related to stress. In the present work we synthesized BSA templated MnO_2 nanoparticles. Synthesized nanoparticles was found to mimic antioxidant enzymes viz, superoxide dismutase, catalase and peroxidase. Additionally, it also showed oxidase mimicking activity. Nanoparticles were assessed for cytotoxicity by MTT assay in mammalian cells by treating human embryonic kidney cells (HEK 293) at varying doses and time period. Morphological variations induced after treatment of HEK 293 with nanoparticles was analyzed by transmission electron microscopy. Nanoparticles at 50 $\mu\text{g}/\text{mL}$ concentration were found to significantly increase viability of HEK 293 cells compared to control due to their inherent antioxidant property. But at higher doses nanoparticles induced toxicity. This may be due to the intrinsic oxidase mimicking activity of the nanoparticles which suggest that at higher doses nanoparticle has cross over role as pro-oxidant. Additionally, there was no visible morphological alteration in TEM micrograph of treated cells. The results indicated that the BSA template MnO_2 particles act as pro-oxidant at higher doses due to its oxidase mimicking property however, at an optimum dose it functions as therapeutic under stress related conditions due to its anti-oxidant property.

Keywords Manganese dioxide · Bovine serum albumin · Antioxidant · Nanozymes · Reactive oxygen species · Pro-oxidant

1 Introduction

Biological enzymes are macromolecules that catalyse biochemical reactions in natural systems, basically by lowering activation energy, resulting in enhancement of the reaction kinetics. However, there are limited applications of these biological enzymes due to stability issues, high cost of synthesis and purification. As a substitute to these natural enzymes, researchers focus on utilization of chemical molecules such as cyclodextrins, metal-complexes, porphyrins, polymeric and supramolecular ligands that exhibit high catalytic power [1, 2]. Conversely, there are issues regarding their catalytic efficiency and biocompatibility that limits its application [3]. Since last decade, artificial

enzymes are being investigated as nontoxic, stable and cost-effective solution to chemical and natural enzymes. Breslow coined the term “artificial enzymes” for enzyme mimics that replicate the essential principles of natural enzymes with alternate materials [4]. Pasquato and co-workers initially coined the term “nanozymes” to explain the thiol monolayer covered goldclusters that exhibited ribonuclease mimicking activity [5]. Nanomaterials based enzymes or nanozymes have found wide applications in fields of biosensing, cancer diagnostics, therapy, immunoassays, stem cell growth, neuroprotection and pollutant removal [6, 7]. A number of nanomaterials such as palladium and gold nanoparticles, fullerene derivatives, rare earth nanoparticles and ferromagnetic nanoparticles,

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were found to exhibit enzyme-like activity [5, 8–12]. Stimuli-responsive MnO_2 (manganese dioxide) based nanomaterials have been used for cancer diagnosis and treatments [13, 14]. Albumin template MnO_2 nanomaterials have been used for tumor imaging and elimination where they acted as pH/ H_2O_2 stimuli-responsive nanoplatforms [15]. Albumin template nanoparticles have also been studied as potential therapeutic agents [16].

Reactive oxygen species (ROS) are formed when oxygen is reduced incompletely. It includes species like superoxide (O_2^-), hydroxyl radical ($\text{HO}^\cdot$) and hydrogen peroxide (H_2O_2). These species are highly reactive, hence are thought to mediate oxygen toxicity. At basal levels they serve as intracellular signalling molecules and are essential for normal physiological process [17]. A healthy cell controls excess ROS by antioxidant defence system that includes enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and small molecule antioxidants like vitamins C and E, glutathione, etc. [18]. However, when the antioxidant system is not able to control excess ROS, it results in oxidative stress. Several pathological conditions like diabetes, cancer, cardiovascular and neurodegenerative diseases, ageing etc. result from damaged biomolecules such as DNA, proteins and lipids are caused by oxidative stress [19–21]. Hence, it is imperative to maintain redox equilibrium in a cell to combat oxidative stress.

An inherent function of nanozymes is that they possess antioxidant mimicking properties and hence have the potential to protect cells from reactive oxygen mediated damage in cases where the antioxidant system of cell fails [22]. Enzymes like heme peroxidase, oxidases, CAT or SOD mimicking nanomaterials have been reported earlier [6, 11, 23–25]. SOD scavenges superoxide anions and CAT catalyses decomposition of excess H_2O_2 to water and oxygen. Peroxidase enzymes detoxify free radicals (e.g., glutathione peroxidase) and defend the cells against invading pathogens (e.g., myeloperoxidase). Oxidase enzymes catalyzes oxidation of substrate by oxygen and produces water or hydrogen peroxide [3]. Recently, Mn_3O_4 nanoparticles were reported to mimic SOD, CAT and GPx enzymes. These nanoparticles were able to protect the cells from neurotoxin 1-methyl-4-phenylpyridinium (MPP+), that induce neurodegenerative disease i.e. Parkinson's, as it interferes with mitochondrial oxidative phosphorylation [26]. It is thus essential to develop a nanoparticle that can mimic all the major antioxidant enzymes (SOD, CAT and GPx) due to the differential affinity of the enzymes towards ROS [22].

The present study, thus aimed to synthesize bovine serum albumin (BSA) fabricated manganese dioxide nanoparticles (BSA- MnO_2 NPs), characterize and investigate its enzyme mimicking property. Various analytical characterization methods were used for physicochemical analysis of synthesized NP. Additionally, a number of in vitro assays

(SOD, catalase, peroxidase) were performed for determining the antioxidant property of BSA- MnO_2 NPs. Moreover, we also analysed the in vitro viability assay of the synthesized NPs on human embryonic kidney (HEK 293) cells.

2 Methodology

2.1 Preparation of BSA- MnO_2 NPs

The BSA- MnO_2 NPs were synthesized according to Liu and colleagues with slight modifications [12]. Briefly, 25 mg BSA was dissolved in 10 mL of 0.01 M Phosphate buffer saline (PBS), and then 50 μL of the precursor manganese salt (100 mM) was added. The reaction mixture was allowed to stir for 2 min at room temperature. 770 μL of Sodium Hydroxide (NaOH) (1 M) was then added and again stirred for 6 h. The solution was thereafter dialyzed in double distilled water for 48 h in order to remove unreacted salts and centrifuged at 18,500 rpm for 30 min. The obtained NPs were suspended in PBS and stored at 4 °C for future use.

For optimization of synthesis protocol, we used three salts of manganese (manganese chloride, manganese acetate and manganese sulphate) as precursors. For analysing the variation in size and morphology of BSA- MnO_2 nanoparticles, we varied BSA concentration (2.5 mg/mL, 5 mg/mL, 7.5 mg/mL and 10 mg/mL) and characterized the synthesized nanoparticles.

2.2 Instrumentation

Various analytical characterizations were performed to analyse the optical and physio-chemical nature of synthesized nanoparticles. UV–visible spectra were recorded from 200 to 600 nm in UV1800 Shimadzu Spectrophotometer. Fluorescence spectra were recorded in RF-5301PC Shimadzu spectrofluorophotometer. Size and morphology of nanoparticles was observed with Transmission electron microscope (TEM) (JEOL-JEM-2100F) operated at 200 kV and Scanning electron microscope (SEM) (ZEISS, GERMANY EV-40) equipped with a tungsten filament. Dynamic Light Scattering (DLS) was used to determine the hydrodynamic size (R_h) of nanoparticles. Experiment was performed at room temperature (25 °C) at $\theta = 90^\circ$ and laser wavelength 647 nm. R_h was calculated using the Stokes–Einstein equation (Photochor), details of which can be referred from Priyadarshini and Rawat [27]. Zeta potential was determined on a ZEECOM instrument (USA), operating in the range of –200 to 200 mV. Experiment was performed at 25 °C, the sample pH was 6.7 and 100 particles were analysed. Elemental composition was analysed by using energy dispersive X-ray (EDX-200). Fourier transform infrared

spectroscopy (FTIR) was performed on Agilent Cary FTIR Spectrophotometer in the range of 400–4000 cm^{-1} .

2.3 Assessment of antioxidant potential of BSA-MnO₂ NPs

The BSA-MnO₂ NPs were assessed for various antioxidant enzyme activities. Oxidase, peroxidase and superoxide dismutase assays were performed on UV1800 Shimadzu spectrophotometer. Catalase assay was conducted on fluorescence spectrophotometer.

2.3.1 Peroxidase mimicking activity

Peroxidase mimicking activity of BSA-MnO₂ was investigated using *o*-phenylenediamine (OPD) as a chromogenic substrate with hydrogen peroxide. Upon oxidation, the substrate turns yellow in color. To 0.2 M acetate buffer (pH = 4.0), different concentration of nanoparticle (25, 50, 100, 150, 200, 250, 300 $\mu\text{g/mL}$), 20 mM OPD and 1 mM H₂O₂ was added and the absorbance slope was recorded at 450 nm for 3 min [12].

$$\text{Inhibition of NBT reduction (\%)} = \left[(A_{\text{sample}} - A_{\text{control}}) / A_{\text{control}} \right] * 100 \quad (1)$$

2.3.2 Oxidase mimicking activity

Oxidase mimicking activity was investigated using its chromogenic substrate, 3,3',5,5'-Tetramethylbenzidine (TMB). On oxidation, the substrate turns blue in color. Different concentration of nanoparticles were added (final concentration in reaction volume 25, 50, 100, 150, 200, 250, 300 $\mu\text{g/mL}$) to citrate buffer (0.2 M, pH = 4.0) respectively, followed by 20 mM TMB. The absorbance slope was recorded at 652 nm for 2 min [12].

2.3.3 Catalase mimicking activity

H₂O₂ decomposition by BSA-MnO₂ was studied using a fluorescent probe i.e. terephthalic acid. It reacts with hydroxyl radical to form a highly fluorescent compound, 2-hydroxyterephthalic acid. 10 mM H₂O₂ with 0.5 mM terephthalic acid and different concentration of nanoparticles (final concentration in reaction volume 25, 50, 100, 150, 200, 250, 300 $\mu\text{g/mL}$) were added to 100 mM sodium acetate buffer (pH = 5.0) and incubated in UV light (365 nm) for 20 min. The solution was then centrifuged and fluorescence was measured using fluorescence spectrophotometer.

2.3.4 Superoxide dismutase mimicking activity

Superoxide dismutase activity of BSA-MnO₂ NPs was evaluated by measuring the inhibition of photoreduction of nitro blue tetrazolium (NBT), a method described by Beyer and Fridovich [28]. The superoxide source was created by reduction of photochemically excited riboflavin to a semiquinone, which donates an electron to oxygen. The superoxide readily reacts with NBT to form a purple formazan product that can be measured at 550 nm. A superoxide scavenger prevents the conversion of NBT to formazan [29]. 0.01 M methionine, 1 mM riboflavin and 2 mM NBT was prepared in 50 mM phosphate buffer (pH = 7.8). Care was taken to prevent light exposure to riboflavin. 0.1 mL nanoparticle (final concentration in reaction volume 25, 50, 100, 150, 200, 250, 300 $\mu\text{g/mL}$) was added to 2.5 mL methionine followed by 0.1 mL NBT and 0.3 mL riboflavin. The reaction mixture was incubated for 15 min in light and absorbance recorded at 550 nm. Control set was prepared, where nanoparticle was replaced with PBS. Percentage inhibition of NBT reduction by different concentration of nanoparticles was calculated as per Eq. 1

2.4 Determination of enzyme activity

Enzyme activity is a measure of catalytic ability and is calculated by estimation of decrease in substrate concentration or increase in product concentration after a defined time period [30]. Alternatively, enzyme activity refers to moles of product formed per unit time that is equivalent to rate $\times$ reaction volume. Moles of product formed was calculated using Beer Lambert's law ($C = A/\epsilon L$) for each concentration of NP used. ϵ was taken as $3.9 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for TMB product [31] and $16,700 \text{ M}^{-1} \text{ cm}^{-1}$ for OPD product [32]. SOD activity was measured as "unit SOD". As One unit SOD is the amount of SOD required to cause 50% inhibition of NBT photoreduction rate, it was calculated as $(1/50) * \% \text{ inhibition}$ [33].

2.5 In vitro toxicity analysis of BSA-MnO₂ nanoparticles

2.5.1 Cell culture

Human embryonic kidney (HEK 293) cell line was used for the study, which was procured from National Centre for Cell Science, Department of Biotechnology, Pune, India. For the cell culture, RPMI-1640 medium supplemented with 10% (v/v) Fetal bovine serum (FBS), antibiotics (streptomycin 10 $\mu\text{g/mL}$, penicillin 100 U/mL) was used.

2.5.2 Cytotoxicity (MTT) assay

BSA-MnO₂ NPs was tested for any potential toxicity towards the HEK 293 cell lines. In brief, HEK 293 were seeded at cell density of 5×10^3 cells/well in 96 well plate and incubated at 37 °C and 5% CO₂. On adherence of cells to the plate, nanoparticles were treated at varying concentration (50, 100, 200 and 400 µg/mL) for 24, 48 and 72 h respectively. After completion of respective time periods, 30 µL of 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 70 µL fresh media was added to the well and incubated for 4 h. The media was removed and replenished with 100 µL Dimethyl Sulfoxide (DMSO). Absorbance was recorded in BIORAD ELISA plate reader at 570 nm. Percentage cell viability was determined using the formula in Eq. 2.

$$\% \text{ Cell Viability} = \frac{\text{Mean Absorbance(Treated Sample)}}{\text{Mean Absorbance(Control Sample)}} \times 100. \quad (2)$$

2.5.3 Electron microscopic analysis of treated HEK 293 cells

To ascertain the internalization and subcellular localization of NPs, treated cells were subjected to TEM analysis. For this, cells were treated with 100 µg/mL of BSA-MnO₂ NPs and incubated for 24 h. On completion of treatment period, cells were trypsinized, fixed with 2.5% glutaraldehyde for 30 min, post-fixed using 1% osmic acid and 0.1 M PBS was added. Cells were thereafter dehydrated in ethanol and were embedded in Epon 812 and sections were done using Ultramicrotome (Leica Ultracut-UCT) and visualized under on JEOL-JEM-2100F transmission electron microscope after staining with uranyl acetate.

3 Result and discussion

3.1 Optimization of BSA-MnO₂ NPs synthesis

The BSA-MnO₂ NPs synthesized in the present study involve oxidation of Mn²⁺ ions and BSA was used as the template for reaction [12]. Formation of NPs was inferred from the change in colour of reaction mixture from colourless to brownish orange color on addition of NaOH to the solution. UV–visible absorption spectra showed peaks at 280 and 335 nm corresponding to BSA and MnO₂ NPs respectively, confirming the formation of BSA-MnO₂ NPs (Fig. 1a). Insert shows a magnified image showing the peak at 335 nm [34]. In the absence of NaOH, no NP formation was observed which signifies the requirement of an alkaline pH for synthesis of BSA-MnO₂ NPs.

For optimization studies, we used three Mn precursor salts, among which NPs synthesized using MnSO₄ showed maximum intensity with a distinct SPR peak. MnSO₄ was therefore chosen as the optimized salt for synthesis of NPs. Additionally, we varied the initial concentration of BSA to optimize the size and shape of synthesized NPs. Figure 1b reflects that variation of initial BSA concentration plays an integral role in the corresponding size and shape of NPs. BSA-MnO₂ NPs synthesized using 7.5 mg/mL BSA showed maximum intensity in UV–visible absorption spectra. Larger sized particles with irregular morphology were formed at 2.5, 5 and 10 mg/mL BSA concentrations while monodispersed and spherical NPs were synthesized at 7.5 mg/mL BSA concentration. This was evident from the TEM micrographs shown in Fig. 2. The initial studies thus signify that the functional groups of BSA assist in the reduction and subsequent stabilization of formed MnO₂ NPs which was also confirmed from FTIR analysis. Amino acids moieties present in BSA also show a strong interaction affinity to Mn²⁺ ions. The present synthesis process is an example of protein-NP interaction, depicting the importance of primary, secondary and tertiary protein structure in NP formation [12].

Fig. 1 **a** UV–visible spectra of BSA-MnO₂ nanoparticles synthesized from different manganese salts, **b** optical densities of nanoparticles synthesized with 2.5, 5, 7.5 and 10 mg/mL BSA at 335 nm

Fig. 2 TEM images of BSA-MnO₂ NPs synthesized at varying concentration of BSA
a 2.5 mg/mL, **b** 5 mg/mL, **c** 7.5 mg/mL, **d** 10 mg/mL

3.2 Characterization of synthesized BSA-MnO₂ nanoparticles

TEM images suggest the formation of monodispersed and homogenous NPs in the range of 4–20 nm with most of the particles in the size range of 10–12 nm (Fig. 2c). FTIR analysis was performed to determine the nature and purity of synthesized BSA-MnO₂ NPs

(Fig. 3). The presence of a small peak at 1000 cm⁻¹ corresponds to –S=O bond which might appear due to the sulphate salt used in synthesis process [35]. BSA-MnO₂ NPs show peak at 2927 cm⁻¹ and 1400 cm⁻¹ which represents the –OH stretching band and appear because of the use of NaOH in the synthesis process. Mn–O vibration is attributed to the presence of small peak at 890 and 782 cm⁻¹ suggesting the α-MnO₂ conformation of

Fig. 3 Graph showing the FTIR spectra of BSA and BSA-MnO₂ nanoparticles

NPs [36]. The additional peaks at 1650 and 1548 cm^{-1} is contributed from C=O vibrations which corresponds to BSA-MnO_2 NPs synthesized by Si et al. [37]. The FTIR spectra thus further confirm the formation of stabilized BSA-MnO_2 NPs.

Scanning electron microscopy analysis suggested the crystalline nature of NPs. However, the particles showed aggregated surface morphology which may be due to thin film formation prior to SEM analysis (Fig. 4a). Scanning Electron Microscope-Energy Dispersive X-ray spectroscopy (SEM-EDX) analysis further confirms the formation of BSA-MnO_2 NPs evident by the appearance of strong signals for Mn and O (Fig. 4b). Elemental analysis showed the presence of manganese (25.4%), carbon (25.38%), nitrogen (6.99%), copper (0.48%) and oxygen (41.74%). The presence of carbon is because of the use of carbon tape in SEM-EDX analysis. Presence of nitrogen in the BSA-MnO_2 NPs was in accordance with Liu et al. [12]. DLS analysis signified that the average hydrodynamic radius (R_h) of the particles to be 79.8 nm and the zeta potential was observed to be in the range of -40 to -60 mV (Fig. 4c and d), which suggests the aqueous stability of formed BSA-MnO_2 NPs.

3.3 Analysis of anti-oxidant property of BSA-MnO_2 NPs

Antioxidant enzymes exert a protective role, preventing the damages by free radicals generated by various biochemical reactions in the body. Humans possess an endogenous antioxidant system comprising of free radical scavenging enzymes such as catalase, superoxide dismutase, glutathione reductase and peroxidase [3]. Previous studies suggest that inorganic nanoparticles such as cerium oxide and manganese dioxide scavenge free radicals and prevent the in vitro system from oxidative damage [12, 38]. These nanoparticles, form a novel group of enzymes called nanozymes. Nanozymes can either be antioxidants or pro-oxidants depending on their ability to scavenge or generate free radicals respectively. In order to divulge the potential role of the synthesized BSA-MnO_2 NPs, we performed a series of antioxidant assays as described below.

On assaying for peroxidase activity, the particles were observed to show a positive peroxidase activity evident by oxidation of *o*-phenylenediamine substrate. A change in colour of the assay mixture to yellow, along with an increase in optical absorbance of substrate was found with increasing BSA-MnO_2 NPs concentration. The high efficiency of peroxidase like activity was depicted by

Fig. 4 **a** SEM micrograph of as-synthesized BSA-MnO_2 nanoparticles, **b** EDX analysis of particles showing the presence of Mn and O peaks confirming the formation of MnO_2 particles, **c** DLS analysis showing the hydrodynamic radius of particles, **d** Zeta potential histogram of the particles

change in colour within a minute of adding NPs to the assay mixture, the intensity of which was found to increase with concentration of NPs. The control set did not show any change in colour nor was there any change in the optical absorbance (Fig. 5a). Gao and et al. showed that iron nanoparticles exhibit peroxidase like activity, with smaller size exhibiting higher activity than the bigger ones. Iron oxide NPs of different sizes (30, 50 and 300 nm) were first reported for peroxidase activity [11]. Similarly, V_2O_5 nanowires have been recently reported to show an exceptional peroxidase activity with increasing particle concentration [39]. Other nanomaterials like nanostructured layered double hydroxide (LDH), ruthenium and gold nanoparticles have also been reported to show peroxidase-like activity [40–42].

Likewise, the NPs were found to show oxidase activity observed by change in colour of the reaction mixture on addition of NPs, within 30 s. The intensity of blue colour was found to increase with NPs concentration as can be seen in Fig. 5b. Moreover, the oxidase substrate TMB showed increase in absorbance with increase in concentration of BSA- MnO_2 nanoparticle (Fig. 5b). The results confirmed that the synthesized nanoparticles have oxidase-like activity. These results are in accordance with Liu and colleagues who reported the oxidase like activity of MnO_2 NPs [12]. In yet another study, oxidase like activity of MnO_2 nanowires was used for developing immunoassay platform for sulfate reducing bacteria. The resultant nanowire showed superior detection performance compared to HRP-based enzyme-linked immunosorbent assay (ELISA) [43]. Several other nanomaterials have been shown to mimic oxidase enzyme-like activity [41, 44]. Nanomaterials like gold nanoparticles, gold platinum nanostructure

and Fe–Pt alloy nanoparticles have been shown to exhibit increasing oxidase activity with increase in concentration [45–47]. Variation in size, shape and stabilizing substrate is known to affect the property of NPs. Studies signify that dextran coated cerium NPs have oxidase mimicking property while naked cerium NPs synthesized by a similar method lack oxidase activity, however showed SOD and catalase like property [23, 48].

Further, the BSA- MnO_2 NPs showed catalase like activity which is in accordance with the study reported by Liu and colleagues [12]. This shows that MnO_2 NPs possess the ability of catalysing the decomposition of H_2O_2 with the formation of water and oxygen and thus reduce the $OH^\cdot$ radical in a concentration dependent manner. For this, terephthalic acid was used as the fluorescence probe that reacts with $OH^\cdot$ ions to form highly fluorescent 2-hydroxyterephthalic acid. A gradual decrease in fluorescence intensity of 2-hydroxyterephthalic acid was found with increasing NPs concentration (Fig. 6) signifying the ability of the BSA- MnO_2 NPs, to reduce $OH^\cdot$ production. Reports show that several types of nanoparticles exhibit catalase like activities. Concentration dependent increase in the catalase mimicking activity of cerium oxide [49] and manganese dioxide [12] have been reported. Other nanoparticles such as gold nanoparticles (AuNPs), iron oxides, and cobalt oxide nanoparticles also show catalase like activity [50–52].

Besides catalase, the BSA- MnO_2 NPs showed SOD like activity as well. As can be seen in Fig. 7, the percent inhibition of NBT reduction was enhanced with increase in NP concentration. This suggests that the NPs could scavenge superoxide radicals in a concentration dependent manner, so that the radicals were unable to reduce NBT to

Fig. 5 UV-visible absorption spectra showing **a** peroxidase and **b** oxidase like-activity of BSA- MnO_2 NPs

Fig. 6 Catalase like activity of BSA-MnO₂ NPs evident by decrease in fluorescence intensity with increasing NPs concentration

formazan. The SOD mimicking activity of the synthesized nanoparticles is in accordance with the enzyme mimicking activities of MnO₂ NPs discussed above. Recent study reported that nanoceria mimics SOD [53]. The increase in SOD activity with increase in concentration of nanoceria has been reported [54]. Apart from manganese and cerium nanoparticle, apo-ferritin encapsulated platinum nanoparticles (PtNPs) have been shown to mimic SOD [55].

Furthermore, the enzyme activity was determined for SOD, oxidase and peroxidase by using Beer-Lambert's law as described in material section. The results have been

summarized in Table 1. However, we were not able to calculate catalase activity as the assay uses the fluorescent probe, terephthalic acid which on binding with hydroxide ions generate fluorescence. Since, Beer-Lambert's law can't be used for fluorescence studies, we were not able to determine the enzyme activity. Decrease in fluorescence intensity was used as an estimate for the same.

3.4 In-vitro cytotoxicity profiling of BSA-MnO₂ NPs

The synthesized NPs show antioxidant activities as has been described in the previous sections. In vitro antioxidant property of NPs, instigated us to analyse the cytoprotective role of the synthesized NPs on HEK 293 cells, which is a normal human embryonic kidney cell line. MTT assay was used to analyse the cell proliferative activity,

Table 1 Peroxidase, oxidase and SOD enzyme like activity of BSA-MnO₂ NPs

BSA-MnO ₂ NP (µg/ml)	Peroxidase activity (µmol product/min)	Oxidase activity (µmol product/min)	SOD activity (unit SOD)
50	0.005	0.188	0.048
100	0.023	0.339	0.104
150	0.076	0.497	0.309
200	0.142	0.680	0.397
250	0.202	0.792	0.565
300	0.237	0.962	0.705

Fig. 7 Graph showing an increase in percentage inhibition of NBT reduction with increasing NPs concentration suggesting SOD mimicking property

which suggests the BSA-MnO₂ NPs to be potentially non-toxic. Cellular response to increasing concentration of MnO₂ NPs is shown in Fig. 8. Results showed no significant cell death within 24 h of treatment in doses from 50 to 150 µg/mL whereas a significant cell death was noticed on cells treated with 200 µg/mL. Upon 48 h of treatment, significant decrease in percent viability was observed at 150 and 200 µg/mL concentration. After 72 h, percentage viability of cells increased significantly at 50 µg/mL, while decreased significantly at higher doses. 66.5% cell viability was observed for the maximum concentration of NPs studied i.e. 200 µg/mL. The MTT assay thus suggests a significant higher concentration of MnO₂ NPs induced toxicity after exposure time of 72 h. Thus, despite the nanoparticle acting as an antioxidant at lower doses, in higher doses it caused the generation of ROS rather than quenching it and thus could significantly decrease % viability of cells. This may be due to oxidase mimicking activity of nanoparticles as the enzyme do not require hydrogen peroxide, rather it can produce H₂O₂ (or superoxide in some cases) [3].

Electron micrograph was analysed to confirm the internalization of the NPs into the cells. The MnO₂ particles

Fig. 8 Percentage viability of HEK 293 cells upon treatment with synthesized nanoparticles (* $p < 0.05$ significant; ** $p < 0.01$ very significant; *** $p < 0.001$ highly significant)

were entrapped by the cells (Fig. 9). A comparative analysis of untreated and treated cells suggest no significant change in cell morphology or nuclear damage. This signifies that though the MnO₂ NPs were taken up by the cells but were potentially non-toxic. Figure 9b and c show the treated cells wherein the nanoparticles are observed to be localized in the cell interior. We observed that the NPs were internalized in the lysosomes and no cellular damage was observed. Non-toxicity of MnO₂ cells towards HEK 293 cell lines might be due to the acidic nature of lysosome. As lysosomal vesicles are known to be acidic with pH of 4, that can lead to degradation of BSA-MnO₂ NPs [56], resulting in the release of free Mn²⁺ ions. Mn²⁺ are known to possess a very high affinity towards H₂O₂ and O₂⁻ free radicals. Hence, the Mn²⁺ ions can bind to them thereby preventing excess ROS level and thus have a protective effect [57, 58]. This is in accordance with the antioxidant property of as-synthesized BSA-MnO₂ NPs and thus explains the non-toxic nature towards HEK 293 cell lines at lower doses.

The results signified that the synthesized BSA-MnO₂ NPs exhibit both pro and antioxidant activity as they were found positive for oxidase, peroxidase, catalase like activity respectively. These results were in accordance with Liu et al. [12]. In addition, we also found a positive SOD mimicking activity by the synthesized NPs. SOD enzyme activity suggested an increment in SOD activity with increasing concentration of NPs. This confirmed the dual-property of antioxidant and pro-oxidant role of synthesized particles, which was further elucidated by in vitro assay (MTT and TEM) on normal HEK 293 cells. The dose-dependent invitro response of NPs, suggest that by simple modulation of the BSA-MnO₂ NPs concentration, it may be used as therapeutic and diagnosis substrate. Graphical illustration in Scheme 1 represents the basic reaction of pro- and antioxidants catalysed by BSA-MnO₂ NPs and the generalized reaction product formed. NPs of iron and copper were known to have pro-oxidant activity wherein they had the ability to generate free radicals in the living body and lead to oxidative stress. Various toxicological

Fig. 9 TEM micrograph of **a** Control HEK 293 cells (L-Lysosome, M-Mitochondria, ER-Endoplasmic reticulum, N-Nucleus, NM-Nuclear membrane, CM-Cell membrane) **b, c** nanoparticle treated cells. The internalized particles have been depicted by red circle in the image

Scheme 1 Graphical representation of the catalysed reactions and the generalized end-products formed

studies involving Mn NPs report it to be non-toxic nature at lower doses but showed toxicity at higher doses, which is in accordance with the *in vitro* experiments observed in the present study. MnO_2 NPs treated on human neuronal cells SH-SY5Y showed decline in mitochondrial membrane potential, increase in lipid peroxidation, SOD, CAT and decrease in glutathione in dose and time dependent manner. An increase in caspase 3 activity, DNA strand break (comet assay), and apoptotic cells was observed at 60 $\mu\text{g/mL}$ of the nanoparticles at 48 h [59]. The effect of manganese oxide nanoparticle in PC-12 cells of 24 h treatment did not significantly change the cell morphology whereas silver nanoparticle of the same concentration produced cell shrinkage with irregular membrane borders. Mitochondrial reduction activity in the cells was of moderate toxicity as compared to the silver nanoparticle of similar dose [60]. However, there was a significant increase in ROS with increase in dose. But, these studies are limited to *in vitro* conditions and require *in vivo* investigation before coming to any conclusive evidence on toxicity of MnO_2 NPs. *In vivo* oral treatment of Wistar rats with Mn NPs for 28 days showed DNA damage in leukocytes, micronuclei and chromosomal aberration in bone marrow cells, acetylcholinesterase inhibition in blood and significant alterations in biochemical enzymes at higher doses (300 and 1000 mg/kg bw). However, there was no or minimal toxicity at low dose (30 mg/kg bw) [61]. Daily intraperitoneal administration of MnO_2 NPs (10 mg/kg bw) for 50 days to BALB/c mice significantly reduced liver and kidney functions and elevated glucose and bilirubin serum levels [62]. MnO_2 NPs also showed reproductive toxicity at 100 mg/kg

on subcutaneous administration in male rats once a week for 4 weeks [63].

On the other hand, MnO_2 NPs have shown to work as nanozymes and improve conditions as discussed previously in the introduction section. It can thus be suggested that size, morphology and surface functionalization has an essential role in devising the subsequent property and nature of NPs. Optimizing the dose and fabricating MnO_2 NPs with appropriate stabilizing agent, can result in generation of NPs with high anti-oxidant activity which can be used in biomedical applications after determination of their *in vivo* toxicity.

4 Conclusion

The BSA- MnO_2 NPs synthesized were in the size range of 10–12 nm and of α - MnO_2 conformation. A comprehensive study signified that the MnO_2 NPs exhibited antioxidant (SOD, Catalase, Peroxidase) activity along with oxidase activity. SOD mimicking activity of the NPs to inhibit the NBT reduction to formazan enhanced with increasing NP concentration. The NPs also mimicked catalase by preventing OH^- production from H_2O_2 . Furthermore, oxidase and peroxidase mimicking activity increased with concentration of NPs, which was also evident from the color change of their respective chromogenic substrates. Oxidase mimicking activity of the synthesized NPs signifies their pro-oxidant ability which makes the NPs toxic at high dose (200 $\mu\text{g/mL}$) in cell proliferation assay. However the NPs were able to increase the cell viability at a low dose (50 $\mu\text{g/mL}$). The results confirms that BSA template MnO_2 NPs mimic oxidase, peroxidase and catalase enzymes. Further, SOD mimicking property and *in vitro* assay of NP in HEK 293 cells, revealed the non-toxic concentration and toxicity at higher concentration. Moreover, the NP showed *in vitro* anti-oxidative nature at optimum dose. This allows optimization of the doses for subsequent *in-vivo* experiments. It can thus be concluded that though the nanoparticle is toxic at higher doses, at an optimum dose, it has enormous potential in biomedical application where it can act as antioxidant nanozymes to protect mammalian cells from oxidative stress. The results presented here insights into the promising therapeutic applications of NPs in inflammatory, ocular, neurodegenerative, gastrointestinal, autoimmune, renal, musculoskeletal, cardiovascular, pulmonary diseases and in tumours and cancers.

Acknowledgements The authors thank Advanced Instrument Research Facility (AIRF), Jawaharlal Nehru University, New Delhi for Analytical Characterization. SP is grateful to Council of Scientific and Industrial Research (CSIR) for providing fellowship (Sanction No. 09/263(1038)/2014-EMR-I) for performing the study. EP is grateful

to Science and Engineering Board for fellowship under National Post-doctorate Scheme (Grant number PDF/2017/000024).

Compliance with ethical standards

Conflict of interest The authors declare here no conflict of interest.

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