Toxicology Research

PAPER

Cite this: *Toxicol. Res.*, 2015, **4**, 1037

Biocompatibility studies on lanthanum oxide nanoparticles

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Received 8th November 2014,
Accepted 27th April 2015

DOI: 10.1039/c4tx00198b

www.rsc.org/toxicology

Lanthanum oxide nanoparticles (LONP), a rare earth metal oxide, have unique properties that make them a suitable candidate for several biomedical applications. We investigated certain key *in vitro* and *in vivo* biocompatibility endpoints on LONP. LONP were cytotoxic in *in vitro* assays and predominantly exerted their action *via* release of reactive oxygen species. These nanoparticles were neither irritants nor sensitizers in a rabbit model. LONP extracts did not exert any acute systemic toxicity effects in mice. On the other hand LONP exerted toxicity to the liver following oral administration, suggesting that these particles are absorbed from the gastrointestinal (GI) tract and deposited in the hepatobiliary system. LONP did not induce any mutation in the Ames test both in the presence or absence of S-9. These observations provide a base line biocompatibility and toxicity data on LONP. The current findings will also be useful in defining standards for nanoparticle containing devices.

Introduction

Lanthanum oxide nanoparticles (LONP), a rare earth metal oxide, have unique properties that make them a suitable candidate for several biomedical applications. Probes coated with LONP are being developed as implantable sensors of various molecules such as glucose, phosphate and uric acid.[1] In combination with other elements, lanthanum oxide (La_2O_3) is being developed as an optical sensing system for measuring variations in human body temperature.[2] Because of its paramagnetic properties, it is also being developed for the magnetic field controlled targeted release of drugs within the body. La_2O_3 suppresses bacteria,[3] viruses,[4] and fluorescence dyes,[5] selectively binds to several proteins,[6] suppresses calcium channels[7] and has light emitting properties.[8] While it is important to note that there are several potential applications of this nanoparticle in medical device technologies, no biocompatibility data are currently available on LONP to assure their safety.

In this manuscript, we investigated biocompatibility of LONP in line with well established ISO 10993 standards.[9] Specifically we investigated cytotoxicity, irritation, sensitization, acute systemic toxicity and mutagenicity potential of LONP, which are critical biocompatibility indicators for any medical devices.[9] Several guidance documents on the safety evaluation of nanomaterials to be used in food/feed, cosmetics and medical devices have been published by European Food Safety Authority (EFSA), Scientific Committee on Consumer Safety (SCCS) and Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) as Scientific Committees of the European Commission. While International Organization for Standardization (ISO) ISO 10993 standards do not give specific guidance on testing the biocompatibility of materials in the nanoscale range, we adopted these broad principles and combined them with the usual techniques employed in nanotoxicology to evaluate the biocompatibility of LONP. It should be noted that ISO is currently developing guidance on evaluating medical devices containing nanoparticles (ISO/NP TR 10993-22 under development) and our results may feed into this process. Also our results will be helpful in evaluating the safety of medical device technologies using LONP as a raw material. It should be noted that there is an increasing interest in biocompatibility of nanomaterials.[10–12] This work will further add to the knowledge base of biocompatibility of nanomaterials.

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Experimental section

Materials and methods

Characterization of lanthanum oxide particles. La_2O_3 bulk and nanoparticles were purchased from Ottokemi, Mumbai, India and Nanoshel USA, respectively. The certificate of analysis from the manufacturer indicated the purity as 99.99%. The list of impurities were Fe_2O_3, CaO, SiO_2, Cl and others, at concentrations of 0.002, 0.002, 0.01, 0.001 and 0.004 ppm, respectively. These impurities were unlikely to affect the results obtained. The morphology and size of the particles were carried out using a High-Resolution Transmission Electron Microscope (HRTEM-JEOL 3010). The surface charge and the hydrodynamic diameter of the La_2O_3 particles were measured by using a Dynamic Light Scattering analyzer (Nanopartica analyzer SZ-100). The zeta potential of the samples was measured at 25 °C and 37 °C to characterize the particles at room temperature and at physiological temperature.

Cytotoxicity. Cytotoxicity was assessed by the direct contact method. We used the MTT assay (from the battery of cytotoxicity assays described in ISO 10993, part 5)[9,13] to assess the cytotoxicity of LONP. In addition we used assays that reflect other pathways of cytotoxicity such as the LDH assay (membrane damage), Caspase 3/7 (apoptosis), adenosine triphosphate levels (ATP, metabolic competency of cells) and malondialdehyde levels (MDA, measure of lipid peroxidation). We also conducted these cytotoxicity assays in the presence of 25 μM ascorbic acid to evaluate whether or not the cytotoxic effects seen were mediated by the release of reactive oxygen species (ROS).

Balb/c 3T3 (clone A31) mouse fibroblast cells originally were obtained from American Type Culture Collection and are routinely used in our laboratory for cytotoxicity studies. These cells are cultured in culture flasks containing Dulbecco's modified Eagle medium (DMEM) supplemented with 10% newborn calf serum, 4 mM glutamine and penicillin/streptomycin at 37 ± 1 °C under an atmosphere of 5% CO_2. A total of 1×10^5 cells per mL were treated with LONP or La_2O_3 bulk materials in 96-well plates and various endpoints assessed as given in Tables 1 and 2. Commercially available kits were used to evaluate the MTT assay (Sigma Aldrich, UK), LDH assay (Roche Applied Sciences, Germany), ATP levels (Sigma Aldrich, UK) and Caspase 3/7 (Promega BioSciences, USA). Lipid peroxidation was measured in cultures treated with various concentrations of LONP quantify the amount of oxidative damage to phospholipid membranes. For this experiment, the cells were seeded at 5×10^7 cells per T25 flask. At the end of 24 hours treatment, 200 μL of the medium supernatant was taken for lipid peroxidation measurements, and the cultures were washed thrice with PBS. The cells were then trypsinized and counted. Around 10^6 cells were also taken for cellular lipid peroxidation measurements. The lipid peroxide samples (MDA) were stabilised by adding butylated hydroxytoluene and thiobarbiturate to each sample. The samples were then heated to 95 °C for 1 hour and absorbance was measured at 540 nm. A similar experiment was repeated with 25 μM ascorbic acid pretreatment. The lipid peroxidation experiments were repeated thrice.

Irritation. All animal studies were carried out following the approval from the Institutional Animal Ethics Committee. A skin irritation test was carried out in rabbits as described in OECD 404. Briefly 0.5 g of LONP was applied to the skin of the rabbit for 4 hours and the skin was observed for 14 days for signs of irritation and corrosion.[14] Furthermore, an intracutaneous test in rabbits was also carried out using polar (saline) and non-polar (cotton seed oil) extracts (0.2 g mL^{-1}) prepared at 121 °C for 1 hour.[12,13] Rabbits were injected intracutaneously with 0.1 mL of the extract and the injected site was

Table 1 Experimental design for cytotoxicity endpoints

	Pretreatment	Treatment[a]	Concentrations[b]	Endpoints tested
Group 1	—	La_2O_3 nanoparticles	0, 20, 60, 100, 200, 400, 600, 800, and 1000 μg mL^{-1}	1. LDH
Group 2	—	La_2O_3 bulk	0, 20, 60, 100, 200, 400, 600, 800 and 1000 μg mL^{-1}	2. MTT assay
Group 3	25 μM ascorbic acid[c]	La_2O_3 nanoparticles	0, 40, 80, 400, 800 and 1000 μg mL^{-1}	3. ATP levels 4. Caspase 3/7 activation

[a] Treatment was for 24 hours at 37 °C in a humidified atmosphere of 5% CO_2. [b] Concentrations for various groups were selected based on preliminary trials. [c] Pretreatment was for 3 hours and continued during the 24 hours treatment with test articles.

Table 2 Experimental design for lipid peroxidation experiments

	Pretreatment	Treatment[a]	Concentrations[b]	Endpoints tested
Experiment 1	—	La_2O_3 nanoparticles	0, 40, 80, 400, 800 and 1000 μg mL^{-1}	Lipid peroxidation
Experiment 2	25 μM ascorbic acid[c]	La_2O_3 nanoparticles	0, 40, 80, 400, 800 and 1000 μg mL^{-1}	

[a] Treatment was for 24 hours at 37 °C in a humidified atmosphere of 5% CO_2. [b] Concentrations for various groups were selected based on preliminary trials. [c] Pretreatment was for 3 hours and continued during the 24 hours treatment with the test article.

Table 3 Positive control chemicals used in the Ames test

Assay	Mutagen	Solvent	Concentration/plate (μg)	*Salmonella* strains
Absence of S-9	Sodium azide	Water	1	TA100,TA1535
	2 Nitrofluorene	DMSO	10	TA98
	9 Aminoacridine	Ethanol	50	TA1537
	Mytomycin C	Water	0.5	TA102
Presence of S-9	Benzo(*a*)pyrene	DMSO	5	TA98,TA100
			10	TA102,TA1535 & TA1537

observed for 72 hours. Solvents were also injected which served as negative controls.

Sensitization. Both Beuhler's (LONP) and guinea pig maximization tests (GPMT; saline and cotton seed oil extracts of LONP; 0.2 g mL^{-1}; 121 °C for 1 hour) were used to evaluate type 4 sensitization reactions. Cinnamic aldehyde served as positive controls.[15,16]

Acute toxicity. Acute systemic toxicity studies were carried out, using saline (IV route) and cotton seed oil (IP route) extracts (0.2 g mL^{-1}; 121 °C for 1 hour), in mice.[17] Also the acute oral toxicity study of LONP was performed in mice. Initially two mice were treated with 300 mg per kg body weight and observed for 14 days. Even though there was no mortality, animals showed some signs of discomfort and lethargy. Therefore they were tested with two additional lower doses (50 and 5 mg per kg body weight). Blood samples were taken from these animals at days 7 and 14 for hematology and biochemistry. Gross and histopathology were also carried out. Higher doses of 2000 mg kg^{-1} were also tested.

Ames test. The genotoxicity of LONP (extracts as well as the nanoparticle itself) was assessed using five strains of the bacterium *Salmonella Typhimurium* (TA98, TA100, TA102, TA1535 and TA1537), following the procedures described by Maron and Ames.[18,19] Appropriate positive control chemicals were used as shown in Table 3.

Results and discussion

Characterization of lanthanum oxide particles

The morphology and size of the La_2O_3 particles were measured by TEM. The morphology of the nanoparticles was spherical and the mean diameter was approximately 40 nm (see Fig. 1a). The bulk material seems to be agglomerated and irregular in shape. The mean size was found to be approximately 600–800 nm in diameter (Fig. 1b).

Furthermore, we also measured the hydrodynamic sizes of the particles. The observed mean sizes of the particles are 44 nm and 748 nm for nano and bulk, respectively; and their corresponding size distribution is given in Fig. 2a and b.

The results of the zeta potential suggest that the surfaces of both particles were positively charged at both temperatures. The determined values are 7.1 mV (at 25 °C) and 8.1 mV (at 37 °C) for nanoparticles and 8.2 mV (at 25 °C) and 10.1 mV (at 37 °C) for bulk particles (Fig. 3).

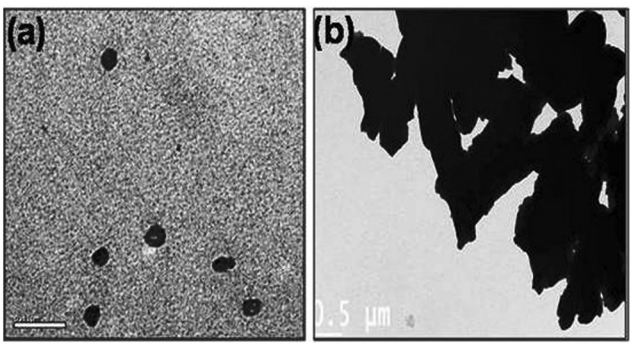

Fig. 1 TEM image of lanthanum oxide (a) nanoparticles (b) bulk.

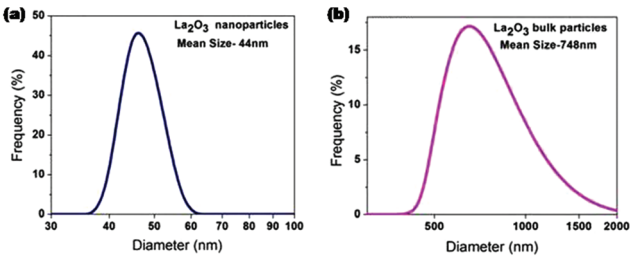

Fig. 2 Hydrodynamic size distribution of lanthanum oxide (a) nanoparticles (b) bulk.

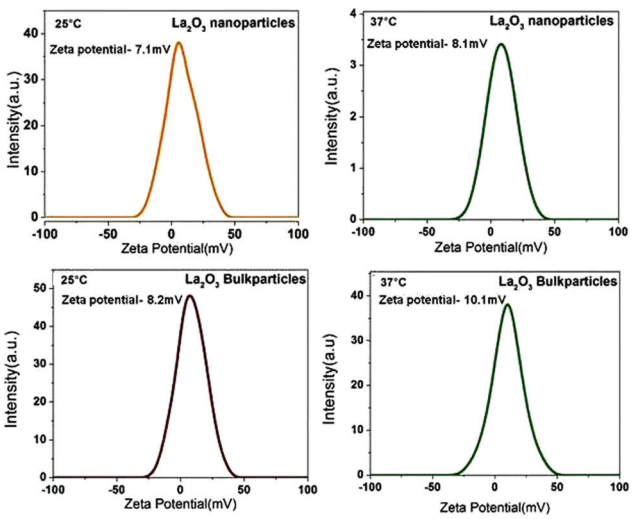

Fig. 3 Zeta potentials of La_2O_3 nanoparticles and bulk materials at 25 °C and 37 °C.

Cytotoxicity

Treatment of Balb/c 3T3 cells with LONP resulted in a statistically significant increase in LDH release at concentrations of 400 μg mL^{-1} (around 38%) and above (around 65%), compared to that in the untreated cultures (around 4%). No such increases in LDH release were observed in cultures treated with bulk materials, when tested up to 1000 μg mL^{-1} (Fig. 4a). Pre-treatment of cells with 25 μM ascorbic acid also resulted in significant increase in LDH release compared to concurrent untreated controls at 400 μg mL^{-1} and above, however the magnitude of response was less compared to the corresponding concentrations without pre-treatment. With ascorbic acid pre-treatment, the percentages of LDH release at 400, 800 and 1000 μg mL^{-1} were approximately 23, 24 and 38%, respectively (Fig. 5a). Cultures treated with both LONP and bulk materials show reduction in cell viabilities as measured by the MTT assay. Nanoparticles showed statistically significant decrease in cell viability at 100 μg mL^{-1} (cell viability of approximately 52%) and above. With the bulk material, statistically significant cytotoxicity was observed at 800 and 1000 μg mL^{-1} (Fig. 4b). Interestingly, pre-treatment of cultures with 25 μM ascorbic acid eliminated the cytotoxic capacity of LONP over the entire range of concentrations tested (Fig. 5b). Treatment of cultures with LONP resulted in a small but statistically significant decrease in cellular ATP at 100 μg mL^{-1} and above. No such decrease in ATP levels were observed in cultures treated with bulk materials (Fig. 4c). Similar to the MTT assay results, pre-treatment of cultures with 25 μM ascorbic acid also completely stopped the cellular ATP reductions observed with nanoparticles over the entire range tested (Fig. 5c). Treatment of cultures with LONP resulted in activation of Caspase 3/7 at 20 μg mL^{-1} and above. The activation of Caspase was dose dependent up to 100 μg mL^{-1} and then saturated at the same level. The La_2O_3 bulk material also activated Caspase 3/7, but at higher concentrations (800 and 1000 μg mL^{-1}). The activation of Caspase 3/7 was prevented with pre-treatment of cells with ascorbic acid (see Fig. 4d and 5d).

A lipid peroxidation product (MDA) was measured in culture medium as well as in cells following treatment with LONP. Treatment of cultures with LONP resulted in a significant release of MDA in culture medium at concentrations of 400 μg mL^{-1} and above, whereas cellular MDA levels showed a significant release at 40 μg mL^{-1} and above. Pretreatment of cultures with 25 μM ascorbic acid resulted in a significant protection against the production of lipid peroxidation (Table 4).

Irritation

Neither the skin irritation test (with LONP) nor the intracutaneous test (with extracts) showed any signs of irritation in rabbits.

Sensitization

Neither the Beuhler's test (with LONP) nor GPMT (with extracts) showed any signs of sensitization in guinea pigs. Positive control chemicals gave a clear positive response.

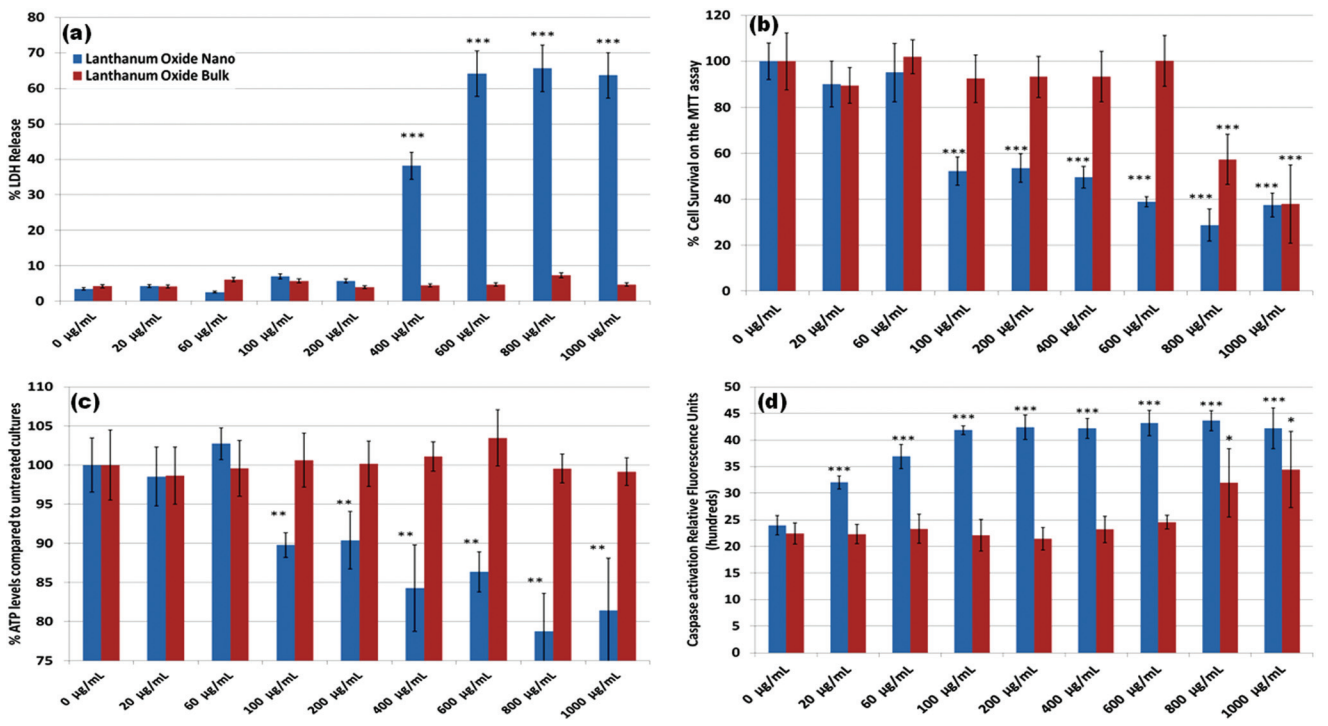

Fig. 4 Comparison of various cytotoxicity (LDH release, MTT and ATP levels) and apoptosis endpoints between La_2O_3 nanoparticles and La_2O_3 bulk particles. *P < 0.05; **P < 0.01 and ***P < 0.001 compared to 0 μg mL^{-1}.

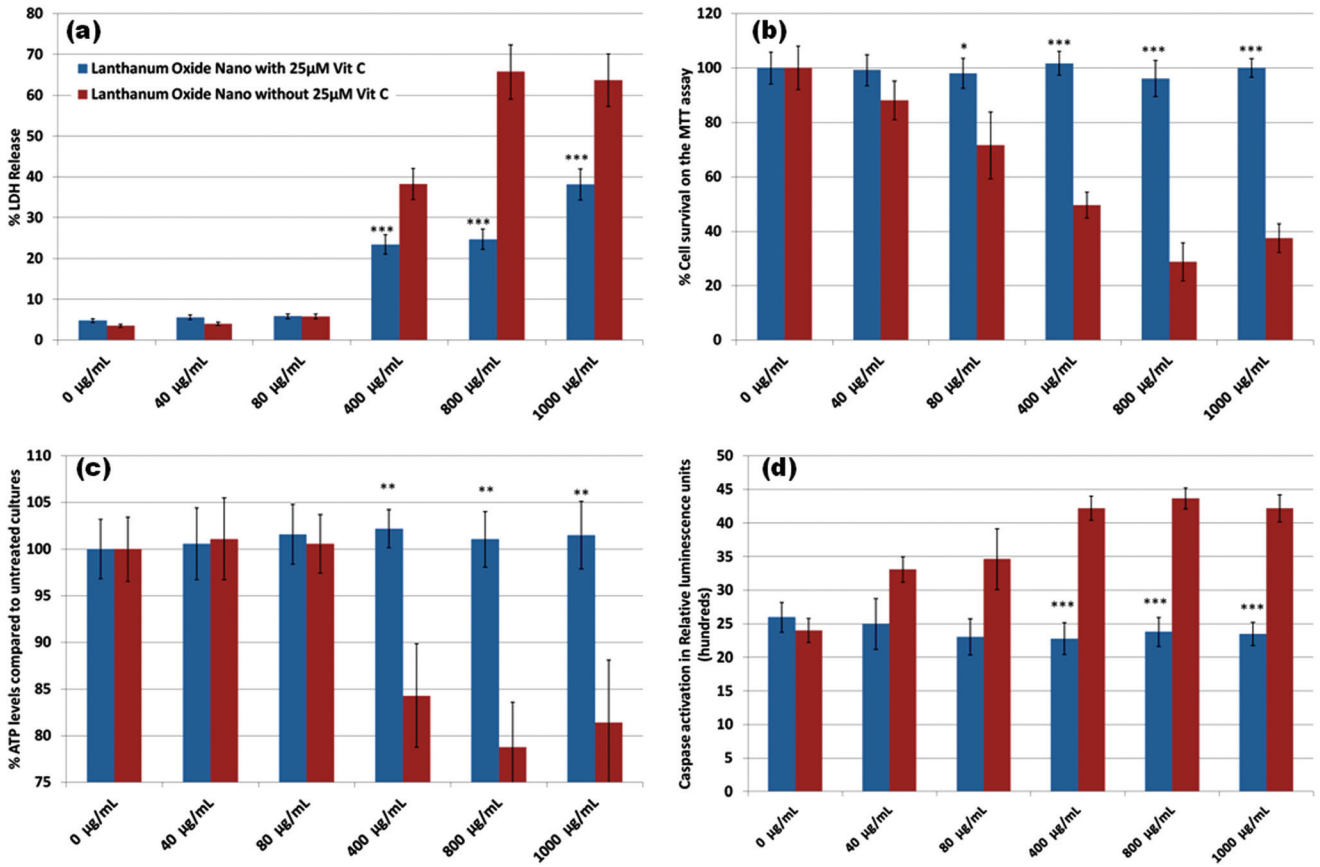

Fig. 5 Comparison of various cytotoxicity and apoptosis endpoints between La_2O_3 nanoparticles and La_2O_3 bulk particles in the presence of 25 μM ascorbic acid. *$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$ compared to the corresponding concentration in the absence of ascorbic acid.

Table 4 Lipid peroxidation levels (as measured by optical densities of MDA-TBA adducts) in cells and culture medium following treatment with La_2O_3 nanoparticles

Concentration (μg mL^{-1})	La_2O_3 nanoparticles[a]		La_2O_3 nanoparticles pre-treated with 25 μM ascorbic acid[b]	
	Culture medium[c]	Cellular[c]	Culture medium[c]	Cellular[c]
0	0.033 ± 0.030	0.043 ± 0.032	0.037 ± 0.008	0.052 ± 0.021
40	0.029 ± 0.011	0.193 ± 0.070***	0.031 ± 0.019	0.045 ± 0.020
80	0.056 ± 0.016	0.306 ± 0.187***	0.053 ± 0.009	0.042 ± 0.021
400	0.113 ± 0.009***	0.550 ± 0.166***	0.040 ± 0.019	0.053 ± 0.032
800	0.106 ± 0.013***	0.743 ± 0.218***	0.177 ± 0.056***	0.050 ± 0.024
1000	0.183 ± 0.046***	0.670 ± 0.104***	0.174 ± 0.038***	0.251 ± 0.055***

[a] Treatment was for 24 hours at 37 °C in a humidified atmosphere of 5% CO_2. [b] Pretreatment with ascorbic acid was for 3 hours and continued during the 24 hours treatment with the test article. [c] Lipid peroxidation products from 0.2 mL of culture medium or 10^6 cells. ***$P < 0.001$ compared to 0 μg mL^{-1}.

Acute toxicity

The injection of saline(IV) or cotton seed oil (IP) extracts of LONP did not show any signs of acute systemic toxicity in mice.

In the acute oral toxicity study, two animals treated with 300 mg kg^{-1} of LONP did not show any mortality at the end of the 14 day observation period. Therefore a higher dose of 2000 mg kg^{-1} was tested in two more animals. Even at this dose, no mortality was observed. Animals treated with 5 or 50 mg kg^{-1} showed lethargy and weakness. Gross pathology showed enlarged hyperemic liver in all animals. Histopathology showed several areas of necrosis, infiltration with inflammatory cells, Kupffer cell hyperplasia and sinusoidal distension (see Fig. 6). Alanine transaminase (ALT) and aspartate aminotransferase (AST) were moderately elevated.

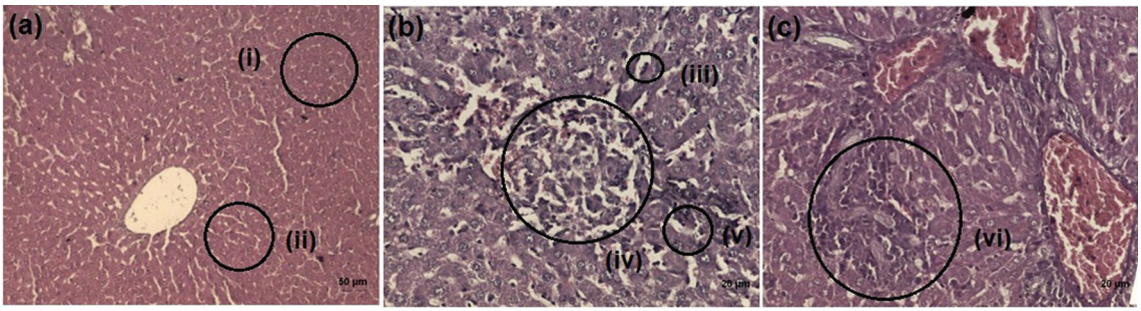

Fig. 6 Optical image of the liver cells (a) control animal – (i) normal hepatic cells, (ii) normal sinusoidal space; (b & c) La_2O_3 nanoparticles treated animal – (iii) Kupffer cell, (iv) necrosis, (v) sinusoidal distension, (vi) mononuclear cell infiltration.

Ames test

Positive mutagens used in the present study resulted in a positive mutagenic response by inducing a multi-fold increase in the number of His+ revertant colonies over the negative control. The revertant colony frequencies in the solvent controls fell within the historical negative control ranges. Saline or cotton seed oil extracts of LONP did not show any mutagenic response in the Ames test both with and without S-9. Similarly, LONP also did not show any mutagenic response when tested up to 1000 µg per plate (both with and without S-9). Higher doses resulted in severe cytotoxicity.

Discussion

An LONP is being developed as a potential ingredient in many medical device technologies by different groups around the world and this is the first report on its biocompatibility. In this manuscript we investigated key biocompatibility tests that will be required for all classes of medical devices. It should be noted that ISO 10993 standard is not specifically designed for assessing devices containing nanoparticles. Nanoparticles may have a different toxicity profile because of their small size and relatively high surface area, making them highly reactive. A new standard, ISO 10993 part 33 is being developed for this purpose. The finding from this manuscript would be helpful in developing standards for nanoparticle containing devices.

Cytotoxicity studies by direct contact showed severe cytotoxicity to Balb/c 3T3 cells assessed by MTT, LDH release, ATP levels and Caspase 3/7 activation assay. By using an antioxidant, we were able to reduce this cytotoxic response, suggesting that cytotoxicity may in part be due to the release of ROS. The same phenomenon was observed with MDA levels. Even though, LONP was cytotoxic by itself, when used in a medical device technology, it would be firmly bound to some material and the magnitude of cytotoxic response would be considerably less. Cytotoxicity of LONP is caused predominantly by ROS.[20] The ROS generated may react with the cell membrane and intracellular proteins and cause cytotoxicity.[21,22]

Two *in vivo* local tolerance tests were carried out. Neither the skin irritation test (with LONP) nor the intracutaneous test (with extracts) showed any signs of irritation in rabbits. Also, the sensitization studies of Beuhler's (with LONP) and GPMT (with extracts) were completely negative. These *in vivo* dermal local tolerance results indicate that LONP is not penetrating the skin in sufficient quantities to produce local cytotoxicity in skin cells.

The acute systemic toxicity study using polar and non-polar extracts did not show any signs of toxicity. It is understandable that LONP are insoluble in both solvents and do not release lanthanum ions. However, oral administration of LONP resulted in liver toxicity suggesting that LONP was absorbed from the gastro intestinal tract *via* the portal system. This is contrary to other lanthanum salts that are not readily absorbed.[23] It is interesting to speculate that the small size of the LONP somehow causes transport to the portal veins and gets deposited in the liver, where it produces localised cytotoxicity. This could be due to the direct physical damage caused by these nanoparticles or *via* the release of ROS. Understanding the antioxidant status of the liver is of paramount importance to understand its mechanisms; and this work is in progress.

The response of the liver to chemical exposure depends on the intensity of the insults, the cell population affected, and the duration of the chemical exposure. A single oral dose of LONP resulted in severe hepatobiliary damage as seen in histopathology. There were large areas of necrosis especially around the portal vein suggesting that LONP induced severe necrosis and inflammation to the hepatocytes surrounding these particles. These particles were not cleared rapidly as this damage persisted even after 14 days. ALT and AST were elevated even at the end of 14 days. These findings suggest that LONP induced nonspecific toxicity to the hepatobiliary system following oral administration. This is similar to the observations by Kumari *et al.*, 2014[21] who showed similar changes in the liver following cerium oxide nano and micro particles. Furthermore, subacute and chronic studies are required to understand the possible long term toxicity of LONP.

LONP did not show any mutation by the Ames test both in the presence and absence of S-9. It is commonly believed that

nanoparticles are not easily taken in by prokaryocytes and therefore the Ames test may not be of value in assessing the genotoxicity of nanoparticles.[24] On the contrary, there are several published literature suggesting that nanoparticles are internalised by bacteria and other prokaryotic cells.[25–27] Therefore the notion that nanoparticles will generally be negative in the Ames test would require further investigations. Furthermore a battery of mutagenicity assays are required to understand the genetic toxicity potential of LONP.

In conclusion, we have generated some key *in vitro* and *in vivo* biocompatibility data on LONP in anticipation of potential use of this nanoparticle in medical device technologies. Like many other nanoparticles, LONP also showed non-specific cytotoxicity, most probably due to its size effect and by releasing ROS. One unexpected finding from this study is that LONP is rapidly absorbed from the gastrointestinal tract and gets deposited in the liver, where it produces persistent nonspecific hepatoxicity for up to two weeks. Furthermore repeated dose toxicity studies are underway to understand more about its uptake.

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