Original Article

Synthesis and Characterization of Zinc Oxide Nanoparticles Coated with Natural and Synthetic Polymer, its Antioxidant Status and Effect on Adult Zebrafish

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Abstract

Background: The application of zinc oxide (ZnO) nanoparticles in the field of photocatalysis, ultraviolet (UV) light attenuation as well as in cosmetics and paints has become popular in recent times. Although, ZnO is known to exert oxidative stress by production of reactive oxygen species (ROS) and can also cause cell killing at high doses.

Objectives: To overcome its toxicity and control the release of ROS, the present study enumerates the role of polymer coating on ZnO nanoparticles by using two types of polymer- a natural (chitosan, CTS) and a synthetic (polyethylene glycol, PEG).

Materials and Methods: The nanoparticles were synthesized and characterized using different tools like particle size analyzer for measurement of hydrodynamic diameter and surface charge and scanning electron microscopy (SEM) for surface morphology. The total antioxidant level as measured in ZnO, ZnO-CTS and ZnO-PEG and adult zebrafish was used for its in vivo toxicity study. Zebrafish was used as a model to mimic the situation of topical application of sunscreens containing nano ZnO. We have exposed the fishes to the nanoparticles and studied the toxicity as well as done the histology to study its effect on organs like eyes which were directly exposed to the nanoparticles suspended in water where the fishes were grown.

Results: The results demonstrated that CTS coating was to some extent protective for ROS generation compared to ZnO and PEG failed to give ROS protection. The effect of 5 mg/L of ZnO, ZnO-CTS and ZnO-PEG did not induce any toxicity to the adult zebrafishes and can be considered as a safe dose for usage.

Conclusions: We concluded that CTS can be a better option for coating ZnO to reduce its toxicity.

Key words: zinc oxide nanoparticles, chitosan coating, polyethylene glycol coating, zebrafish, histology

Introduction

Zinc oxide nanoparticle is a metal oxide nanoparticle which is used in different fields like sunscreens, sun protection fabrics, paints etc. These nanoparticles have a semiconductor nature and can exist in two crystal forms- zinc blende and wurtzite, wurtzite being more stable and after coating is widely used in cosmetics as sunscreen.[1] Coated and uncoated ZnO nanoparticles could attenuate ultraviolet (UV) light by reflection, absorption and scattering, and its special capacity is to absorb UV A.[2] Although the UV blocking efficacy depends on the size of the synthesized nanoparticles. The use of bare ZnO can possess some toxic effects as studied earlier.[3] The toxicity of bare ZnO nanoparticles can be reduced with some coating of macromolecules which are not hazardous for living beings as well as environment. To reduce the toxicity and also to prevent aggregation, we have synthesized ZnO nanoparticles and coated it with polymers of two types – polyethyleneglycol (PEG), which is a synthetic polymer and chitosan (CTS), which is a natural polymer. These polymers usually get

adhered to the surface of the metal oxide nanoparticles by a combination of chemical and electrostatic interactions, hydrogen bonding and van der Waals force.[4] The ZnO nanoparticles, PEG coated ZnO nanoparticles (ZnO-PEG) and chitosan coated nanoparticles (ZnO-CTS) were synthesized according to our previous work,[5] and characterized using different photophysical tools like zeta sizer for the measurement of hydrodynamic diameter and stability, scanning electron microscopy for the surface morphology visualization. The total antioxidant status was estimated and these nanoparticles were exposed to adult zebrafishes where the viability was observed. Adult zebrafish were taken as a model to study the effect of nanoparticles because we can expose the fishes to these nanoparticles and the skin penetration, effect on exposed organs like eye can be studied. Moreover, zebrafish can be easily maintained and we can dissolve the nanoparticles as per our desirable concentration in water, exposing the skin and eye to these nanoparticles, mimicking the condition of ZnO enriched sunscreen exposure to our skin. The histological studies were also done for the zebrafish after exposure to ZnO, ZnO-PEG and ZnO-CTS.

Materials and Methods

Materials

Zinc acetate dihydrate, sodium hydroxide, poly ethylene glycol and ascorbic acid were obtained from SRL, India; chitosan was obtained from HIMEDIA Laboratories; methylene blue (MB) was obtained from Merck, India; 1,1 diphenyl-2-picryl-hydrazyl (DPPH) was obtained from Sigma Chemicals, USA and other laboratory chemicals were purchased locally

Synthesis of zinc oxide nanoparticles and polymer coating

ZnO nanoparticles were synthesized as done previously.[5] Briefly zincacetate dehydrate (20 mM) was mixed with ethanol (50 mL) and post stirring sodium hydroxide (0.5 g) was added and further stirred for 2 h. A white precipitate was obtained which was centrifuged at 5000 rpm for 10 min to obtain a pellet which was washed further 2 times with distilled water. Calcination of this pellet was done at 300°C for another 1 h and the final white powder was made by crushing in mortar pestle which was used for characterization and a part of it was used for coating with PEG and CTS. ZnO nanoparticles were coated with CTS and PEG as done previously.[5] For characterization, a small amount of the synthesized nanoparticles were suspended in distilled water and sonicated twice (15 min each) with a cooling time of 5 min. All the above solutions were prepared freshly as and when required.

Characterization of the synthesized nanoparticles

The characterization was carried out according to Girigoswamiet al., (2015).[5] The hydrodynamic diameter of ZnO, ZnO-CTS and ZnO-PEG was determined using particle sizer (Malvern Nano ZS90), applying the principle of dynamic light scattering. The surface charge of the synthesized nanoparticles were also determined by zeta potential measurement (Malvern Nano ZS90), to find the stability of these nanoparticles. For the observation of surface morphology of ZnO nanoparticles, scanning electron microscopy was used (Quanta 200, FEI FESEM at 6.00 kV and high vacuum mode).

Total antioxidant activity of nanoparticles

The total antioxidant assay was determined by the method followed by Ramachandranet.al.[6] Standard ascorbic acid solution was prepared by dissolving 0.3526 g of ascorbic acid in 10 ml of distilled water (200μM). For DPPH solution preparation, 1.9 mg DPPH was dissolved in 30 ml of methanol (0.166 M) and kept in a dark for 15 min. 500 μL of each nanoparticle (100 mg/L) were taken in three test tubes. To that, 2 mL of DPPH was added and mixed well. For blank, 500 μL of methanol was taken, instead of nanoparticles. Ascorbic acid was taken as a positive control. In another two tubes, 500 μL & 250 μL of Ascorbic acid were added respectively. 250 μL methanol was added to the second tube of ascorbic acid to make the total sample volume 500 μL. Then 2 ml of DPPH was further added toeach tube and mixed well. All the tubes were kept in dark for 30 min to attend equilibrium. Immediately after removing from dark the absorbance was measured at 517 nm and the antioxidant activity was calculated using the following formula:[6]

$$\% \text{ inhibition} = [(A_0 - A_{30})/A_0]/100$$

Where A0 = absorbance at 0 min; A30 = absorbance at 30 min

Maintenance of adult zebrafish and toxicity study

Adult zebrafishes were exposed to different doses of ZnO and coated nanoparticles (1 mg/L, 5 mg/L & 10 mg/L) diluted in distilled water. The control fishes were maintained in only distilled water under similar conditions. For each dose and type of nanoparticles 10 fishes were exposed and another 10 fishes were taken for control group. The fishes were observed for viability for 7 days. The experiment was repeated twice.

Histology of nanoparticle exposed zebrafish

The histological studies were done according to Rahmaniet al.,2016.,[7] with slight modifications. The zebrafish after 24 h treatment with 5 mg/L of ZnO, ZnO-CTS and ZnO-PEG were placed in 4 % buffered formalin solution. The fishes were dehydrated after 1 h and inserted into paraffin wax. Microtome was used to make 5 µm slices of the zebrafish and were stained with haematoxylin and eosin using standard protocol.[8]

Results

Synthesis and characterization of nanoparticles

The nanoparticles ZnO, ZnO-CTS and ZnO-PEG were synthesized and characterized using particle sizer. The size and stability of the synthesized nanoparticles is shown in Table 1.

SEM image of ZnO nanoparticles

The surface morphology of synthesized ZnO nanoparticles and their size was found by SEM analysis. Figure 1 shows the SEM image of ZnO nanoparticles. The ZnO nanoparticles are shown to have an average size of 35 nm and the shape is spherical. The image also shows a uniform particle size of the ZnO nanoparticles.

Nanoparticle	Hydrodynamic diameter (nm)	Zeta Potential (mV)
ZnO	111	-5.2
ZnO -CTS	323	+27.3
ZnO -PEG	545	-15.1

Table 1: The hydrodynamic diameter and zeta potential of ZnO, ZnO-CTS and ZnO-PEG respectively.

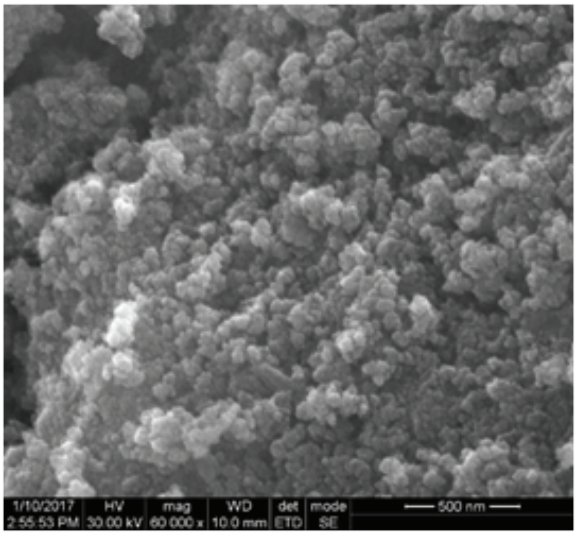


Figure 1: The scanning electron microscope image of ZnO nanoparticles.

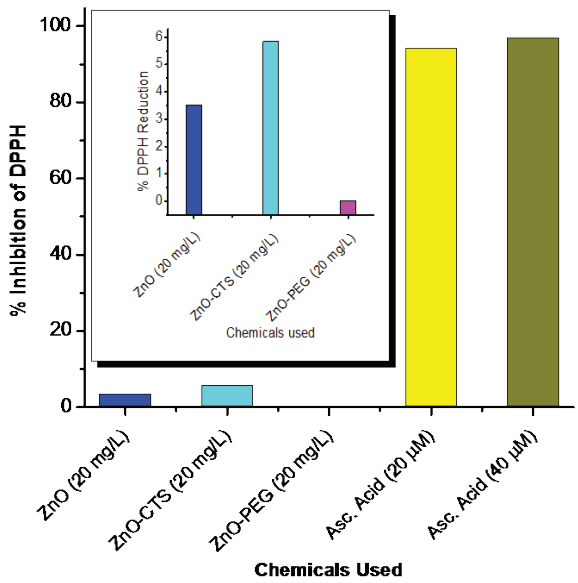


Figure 2: The radical scavenging activity of ZnO, ZnO-chitosan, ZnO-PEG & Ascorbic acid expressed as percent inhibition of DPPH

Total antioxidant status of the synthesized nanoparticles

The total antioxidant activity was determined for ZnO, ZnO-CTS and ZnO-PEG at a concentration of 20 mg/L for each. The radical scavenging ability was compared with ascorbic acid standard and it was found that ZnO nanoparticles exhibited very low antioxidant activity (3.53%). However, upon coating with chitosan the value increased by 1.65 fold, but no antioxidant activity was observed in case of ZnO-PEG (Figure 2). This result suggested that although, ZnO was a very poor radical scavenger, its scavenging ability was increased upon chitosan coating.

Effect of nanoparticle treatment inadult zebrafish

The ZnO, ZnO-chitosan and ZnO-PEG nanoparticles were treated to adult zebrafish at different concentrations (1 mg/L, 5 mg/L & 10 mg/L). The zebrafishes were monitored in different time intervals (24 h, 48 h, 72 h, 96 h and 120 h). All were found to be alive and for further studies the fishes were euthanized and fixed in 4 % formaldehyde. The morphology of eyes was observed by H&E staining under microscope at 400 X magnification (Figure 3).

Discussion

We have synthesized ZnO nanoparticles and coated them with two kinds of polymers-a natural polymer, CTS and a synthetic polymer, PEG. Chitosan can be classified as an aminopolysaccharide, obtained by

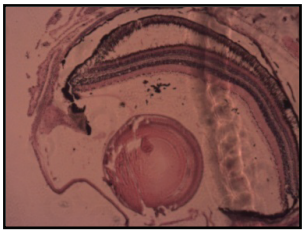
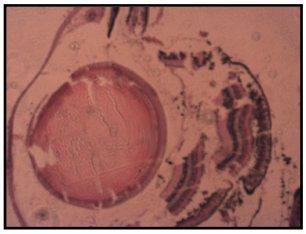
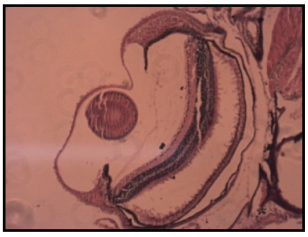
Dose	ZnO	ZnO-CTS	ZnO-PEG
5 mg/L			

Figure 3: The histology slides showing eyes of the zebrafishes treated with ZnO, ZnO-CTS, ZnO-PEG at 5 mg/L

the deacetylation of chitin (a major component of the cuticle of insects, the membrane of fungi, and the exoskeleton of arthropods). As the metal oxide coating involved subjecting to a solution at low pH where the chitosan tend to become soluble, it was important to control the cross-linking to the desired degree before such step.[9] Hence, a standardization of chitosan concentration for coating and final pH was essential for retaining the activity of ZnO post coating which we have successfully achieved earlier[5] and followed the same in this work. Advantages of natural polymer included biode- gradability, biocompatibility, easy availability, devoid of side effects and that they were economically cheap. Regarding synthetic polymers there are many kinds of them used for biomedical applications. The synthetic polymers include PEG, N-(2-hydroxy-propyl) methacrylamide (HPMA) copolymers, poly (vinyl pyrrolidone) (PVP), poly vinyl alcohol (PVA) ploy(ethyleneimine) (PEI), linear poly amidoamines and DIVEMA.[10] The advantage of synthetic polymer was that it was commercially available, low in cost and exhibited high solubilizing power.[11] These polymers were also biocompatible and biode-gradable in nature and reduced the toxicity and also prevented aggregation and agglomeration. Here we have used CTS and PEG for coating ZnO nano-particles. Our results showed that (Table 1) the hydrodynamic diameter of ZnO nanoparticles was lower and its stability was also less as shown by its low zeta potential, whereas on coating with polymers the hydrodynamic diameter increased which proves that the coating was done. Moreover the stability also increased upon CTS coating as well as PEG coating compared to bare ZnO because the magnitude of zeta potential has increased after coating with these polymers. A similar finding was observed in our previous study.[5] The surface morphology of the synthesized ZnO nanoparticles was perfectly uniform and spherical with an average particle diameter of 35 nm as seen from Figure1. Thus, the synthesis of the nanoparticles was done successfully. It is known that ZnO nanoparticles can exert oxidative stress,[12] we wanted to explore whether coating with CTS or PEG could affect the total antioxidant status of ZnO-CTS and ZnO-PEG. Our results showed that CTS coating could increase the total antioxidant level as measured by DPPH assay taking ascorbic acid as positive control by 1.65 fold compared to ZnO, whereas PEG coating decreased the total antioxidant level compared to only ZnO, showing its role as a contributor to oxidative stress (Figure 2). The percentage reduction of DPPH by ZnO, ZnO-CTS and ZnO-PEG was 3.53, 5.84 and 0.03 respectively at 20 mg/L concentration for all these nanoparticles. Thus, the results of total antioxidant level showed that CTS coating was favorable to reduce the oxidative stress exerted by ZnO nanoparticles to some extent compared to PEG coating.

Long-term effects of sunlight included different degenerative skin changes. The formation of actinic keratoses and skin cancer from epidermal cells were known examples. The dermal part of the skin played an important part in the photo aging process. The loss of the skin elasticity was ascribed especially to UVA producing reactive oxygen species (ROS) that activated different matrix metalloproteinases, which damage collagen and other dermal matrix proteins.[1] Zinc oxide (ZnO-nano) and titanium dioxide nanoparticles (20 to 30nm) were widely used in several topical skin care products, such as sunscreens. Sunscreens were used to protect the skin against the harmful effects of solar ultraviolet (UV) radiation. We have used adult zebrafish model to exploit the effect of the synthesized nanoparticles ZnO, ZnO-CTS and ZnO-PEG on skin and eyes of the fishes. This model mimics the condition of sunscreen exposure to skin and also their viability. Moreover, we can find out which polymer, natural or synthetic, is more biocompatible. In our previous study with zebrafish embryo, we have found that CTS coating was more efficient UV light scavenger as well as highly biocompatible compared to PEG coating on ZnO nanoparticles. It also showed that bare ZnO nanoparticles were toxic compared to the polymer coated ZnO, but better biocompatibility was shown for CTS coating with increased hatchability.[5] Many nanoparticles created toxicity in zebrafish[13,14] and the bioaccumulation of ZnO nanoparticles were observed in the gill, liver, intestine and brain of the fish. The toxicity was attributed to the increase in the ROS generation and

occurrence of oxidative stress. Researchers showed that the exposure of ZnO nanoparticles caused some neuro & behaviour changes in the adult fish.[15] In the present study, adult zebrafishes were exposed to different concentrations (1 mg/L, 5 mg/L & 10 mg/L) of ZnO, ZnO-CTS and ZnO-PEG. The results did not show any toxic effect of these nanoparticles at any dose on the viability of adult zebrafishes. They were alive and active up to a long period (we observed up to 2 months). The histology was done after exposure to 5 mg/L of ZnO, ZnO-CTS and ZnO-PEG and the results of the slides showing the eyeball was captured at 400 X magnification using inverted microscope and camera. The typical pictures are shown in Fig 3, which shows that there was no abnormality induced at this dose by any of these nanoparticles. A similar histology images of eyeball of zebrafishes are also observed by previous researchers.[16] Thus, this dose can be considered to be safe for topical application and our results predicted that the total antioxidant capacity was maximum for ZnO-CTS compared to ZnO and ZnO-PEG. Our previous study already supported that ZnO-CTS is a superior UV light scavenger, biocompatible and thus,we can propose that ZnO-CTS can be a better option to be used as a UV light scavenger.

Conclusion

ZnO in nano form is used in different cosmetic products and it may not be safe for the skin and eyes as it is known to produce oxidative stress. The application of ZnO must be screened for its biocompatibility as well as optimum dose should be identified to ensure minimum toxicity. To improve its biocompatibility we have coated the ZnO nanoparticles with two types of polymer CTS and PEG and studied their effect in adult zebrafish. These two polymers were selected based on their source of derivation-CTS from natural source and PEG was a synthetic polymer. The total antioxidant level when studied for ZnO, ZnO-CTS and ZnO-PEG indicated that the natural polymer coating prevented the oxidative stress to some extent, whereas the synthetic did not contribute for the oxidative stress prevention compared to only ZnO. Although, the effect on survival as well as histology of zebrafish did not show any toxic effect of ZnO, ZnO-CTS and ZnO-PEG at 5 mg/L exposure. Thus, we can conclude that ZnO-CTS can be accepted as a better option for coating compared to PEG.

Conflict of Interest

The authors declare no conflict of interest

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