

Bismuth oxide aqueous colloidal nanoparticles inhibit *Candida albicans* growth and biofilm formation

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Abstract: Multiresistance among microorganisms to common antimicrobials has become one of the most significant concerns in modern medicine. Nanomaterials are a new alternative to successfully treat the multiresistant microorganisms. Nanostructured materials are used in many fields, including biological sciences and medicine. Recently, it was demonstrated that the bactericidal activity of zero-valent bismuth colloidal nanoparticles inhibited the growth of *Streptococcus mutans*; however the antimycotic potential of bismuth nanostructured derivatives has not yet been studied. The main objective of this investigation was to analyze the fungicidal activity of bismuth oxide nanoparticles against *Candida albicans*, and their antibiofilm capabilities. Our results showed that aqueous colloidal bismuth oxide nanoparticles displayed antimicrobial activity against *C. albicans* growth (reducing colony size by 85%) and a complete inhibition of biofilm formation. These results are better than those obtained with chlorhexidine, nystatin, and terbinafine, the most effective oral antiseptic and commercial antifungal agents. In this work, we also compared the antimycotic activities of bulk bismuth oxide and bismuth nitrate, the precursor metallic salt. These results suggest that bismuth oxide colloidal nanoparticles could be a very interesting candidate as a fungicidal agent to be incorporated into an oral antiseptic. Additionally, we determined the minimum inhibitory concentration for the synthesized aqueous colloidal Bi₂O₃ nanoparticles.

Keywords: antimycotic agent, antifungal, cytotoxicity

Introduction

The occurrence of microorganisms living in cooperative, aggregated communities is called a biofilm. Biofilms can form on many kinds of surfaces and interfaces, including the human body.¹ The most common biofilms associated with human disease episodes are the fungi of the genus *Candida*, most notably *Candida albicans*, which causes both superficial and systemic disease. *C. albicans* biofilm is commonly found in children and elderly persons with a deficient immune system, and it is the etiological agent of oral moniliasis; the mortality of patients with invasive candidiasis can be as high as 40%.^{2,3} Fungal infections are difficult to eradicate, requiring high quantities and longtime exposure to an antimycotic agent to efficiently eliminate the etiological pathogen.⁴

Multiresistance among pathogen microorganisms has become one of the most important problems in current medicine.⁵ Since early reports in 1980, new evidence has suggested the emergence of multiresistant strains of *Candida*, suggesting that multiresistance is not an exclusive phenomenon of bacteria.^{6,7} The absence of new alternatives to efficiently treat these multiresistant microorganisms is a real problem,

and it is urgent to synthesize new broad spectrum drugs to fight antimicrobial resistance.

Typically, bismuth is found as bismuthinite (bismuth sulfide), bismite (bismuth oxide [Bi_2O_3]), and bismuthite (bismuth carbonate).⁸ In medicine, bismuth, in the form of bismuth subsalicylate, has been employed as an antidiarrheal to treat nausea, vomiting, and stomach pain.⁹

The chemistry of the binary oxides of bismuth is dominated by the +3 oxidation state. Most of the work on bismuth, concentrates on Bi_2O_3 . Bi_2O_3 shows a distinctive polymorphism, including the following solid state phases: α - Bi_2O_3 , β - Bi_2O_3 , γ - Bi_2O_3 , δ - Bi_2O_3 , and the recently characterized ϵ - Bi_2O_3 .¹⁰ The α - Bi_2O_3 is the most thermodynamically stable phase at room temperature and pressure. So, under standard reaction conditions in aqueous solutions, the α - Bi_2O_3 is formed, a poorly water-soluble specie that carries surface hydroxyl groups.¹¹ α - Bi_2O_3 is a basic oxide and its Bi-O bonds are predominantly ionic;¹² it is a p-type semiconductor material.¹⁰

Bi_2O_3 is a derivative of great technological importance, and it is used in the manufacture of glass and ceramic products, and also, as catalyst in the oxidation of hydrocarbons. It is widely used in applications, such as microelectronics, and sensor and optical technology.^{13,14}

Nanoparticles (NPs) have large surface areas, and therefore, they have increased interactions with biological targets. We recently demonstrated the antibacterial effectiveness of zero-valent bismuth NPs at inhibiting the growth of *Streptococcus mutans*;¹⁵ however, at the present time the antimycotic potential of bismuth nanostructured derivatives are not known. In this report, we present evidence of the antimycotic properties of Bi_2O_3 NPs against the growth of *C. albicans* and its capability to eliminate the fungal biofilm – the biocidal activity of Bi_2O_3 NPs was very similar to that obtained with chlorhexidine, a commonly used oral antiseptic and commercial antifungals as nystatin and terbinafine. Additionally, the *C. albicans* antifungal capacities of bulk Bi_2O_3 material and bismuth nitrate, the precursor salt used in the Bi_2O_3 colloidal NPs synthesis, are compared.

Materials and methods

Synthesis of Bi_2O_3 NP

For the synthesis of Bi_2O_3 nanoclusters (Bi_2O_3 NCs), the following chemical reagents were used: bismuth nitrate pentahydrate ($\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$), 98% (Sigma-Aldrich, St Louis, MO, USA); Bi_2O_3 , 99.999% trace metals basis (Sigma-Aldrich); potassium hydroxide (KOH), 85% (Meyer,

Tlahuac, Mexico City, Mexico); argon (Ar), 99.998% (Praxair Inc, Danbury, CT, USA); and deionized water, 18.2 Ω (Milli Q[®]; Thermo Fisher Scientific, Waltham, MA, USA). These chemical substances were used as they were received.

We used a typical preparation method for the production of the Bi_2O_3 NP colloids, as follows: First, all water to be used in the synthesis was boiled and after cooling to room temperature, was bubbled with argon gas for 15 minutes in order to prevent carbonation. Subsequently, 0.0274 g of $\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ were dissolved in 24.5 mL of the prepared water. This solution was heated, under vigorous magnetic stirring, at 80°C. Then, 0.5 mL of an aqueous solution of KOH 0.032 M were added to the $\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ solution. The final concentrations of bismuth and hydroxide ions were 2×10^{-3} M and 6×10^{-3} M, respectively. It is worth noting that during the entire process of dissolution and mixing of the reagents, a weak stream of argon was blowing over the surface of the reaction mixture. The newly synthesized yellowish Bi_2O_3 powder NPs were isolated by centrifugation at 14,000 rpm, for 30 minutes. Afterwards, they were washed with previously decarbonated water, three times. Finally, the powdered Bi_2O_3 NPs were once again resuspended in water and stored at room temperature for approximately 30 minutes, until their use. In order to prove the reproducibility of this synthesis method, these colloids were prepared in triplicate.

Characterization of Bi_2O_3 NPs

The needle-shaped morphology of Bi_2O_3 NPs was determined by transmission electron (TEM) micrographs obtained using a JEOL 1200-EXII (JEOL Ltd, Tokyo, Japan) of 40–120 KV. Single drops of the ethanol nanoparticle suspension were deposited onto 200 mesh copper grids coated with a carbon/collodion layer.

The Bi_2O_3 monoclinic phase identification was obtained from the X-ray diffraction pattern (PDF 41-1449 card), recorded on a Bruker D2-Phaser diffractometer (Bruker Corp, Billerica, MA, USA), using Cu K α radiation, 1.5406 Å (30 kV, 10 mA). The crystallite size, 77 nm, was determined using the Scherrer equation. From the X-ray diffraction pattern and the TEM images, we observed needle-shaped crystallites, which indicated a textured material (in the 120 direction) (Figure 1).

Antimycotic activity of Bi_2O_3 NPs against *C. albicans* growth

C. albicans (No 90029) was obtained from the American Type Culture Collection, (Manassas, VA, USA). The antifungal effect of Bi_2O_3 NPs on *C. albicans* growth was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Biotium Inc, Hayward, CA, USA),^{16,17} following

washed three times with phosphate-buffered saline (PBS), and 4',6-diamidino-2-phenylindole (DAPI) (Abcam Inc, Cambridge, UK) was added to stain the nucleus of cells.²² Cytotoxicity was interpreted as the presence of a degraded or amorphous nucleus, observing at 365 nm with an inverter Carl Zeiss microscope.

Results

Synthesis and characterization of Bi₂O₃ NPs

The colloidal dispersions of Bi₂O₃ NPs prepared from 2×10^{-3} M Bi(NO₃)₃ · 5H₂O and 6×10^{-3} M of KOH in deionized water yielded a fine beige powder with an approximate crystallite size of 77 nm. The X-ray diffraction pattern of the Bi₂O₃ NP powder showed the presence of α -phase nanocrystallites and the absence of other possible compounds (like carbonated elements) generated during their synthesis (Figure 1). The synthesized Bi₂O₃ NPs remained stable at room temperature for 15 days, and no visible trace of carbonation was observed, since the suspended particles in water kept their yellowish color. This established that the antimycotic and antibiofilm properties were associated only with the presence of Bi₂O₃ NPs.

Antimycotic activity of Bi₂O₃ NPs against *C. albicans* growth

To explore the possible antifungal activity of Bi₂O₃ NPs, their effect on *C. albicans* growth was determined. The results showed that Bi₂O₃ NPs reduced the number of fungi by 85%, in comparison with the control fungi grown in medium. At the same time, the treatment with 2% chlorhexidine and 1% terbinafine (inhibition controls) showed a 44% and 51% of reduction in the number of fungi, respectively, when compared with nontreated cells. The antifungal effect of Bi₂O₃ NPs was two times better when compared with the bulk Bi₂O₃ material (Figure 2), suggesting that nanostructured Bi₂O₃ is more effective as an antifungal agent.

Determination of minimal inhibitory concentration of Bi₂O₃ NPs

In order to characterize the antifungal activity of Bi₂O₃ NPs, we determined their MIC. The result obtained was 1.5 mM (Figure 3). This result is important to know as it represents the minimal quantity of Bi₂O₃ NPs that is required to effectively interfere with *C. albicans* growth.

Biofilm inhibitory activity of Bi₂O₃ NPs

In the previous experiment, we measured the antimycotic activity of Bi₂O₃ NPs. In order to analyze the possible biofilm inhibition of *C. albicans* by bismuth nanoclusters,

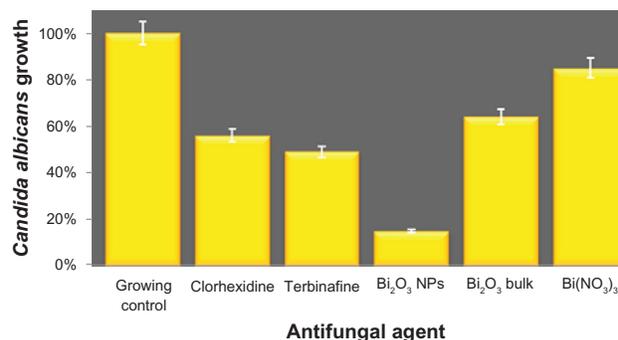


Figure 2 Antifungal activity of bismuth oxide nanoparticles against *Candida albicans* growth.

Notes: The y axis shows the optical density units of *C. albicans* growth. *C. albicans* culture without inhibitor was used as the growth control, whereas chlorhexidine 2%, terbinafine 1%, Bi₂O₃ (bulk material), and Bi(NO₃)₃ · 5H₂O were used as positive inhibition controls. For these experiments, we used a concentration of 2 mM of the respective bismuth species.

Abbreviations: Bi₂O₃, bismuth oxide; Bi(NO₃)₃ · 5H₂O, bismuth nitrate pentahydrate; NP, nanoparticle.

the antibiofilm activity of Bi₂O₃ NPs was determined by fluorescence microscopy. The results showed a complete inhibition of biofilm formation by chlorhexidine (Figure 4B), terbinafine (Figure 4C), and Bi₂O₃ NPs (Figure 4D), compared with the control (Figure 4A). The results did not change when Bi₂O₃ NPs were added at different postinoculation times – we tested the biofilm inhibitory activity at 6 and 18 hours postinoculation, obtaining similar results (data not shown). These data indicate that Bi₂O₃ NPs have an antibiofilm activity that is as effective as chlorhexidine and terbinafine.

Cytotoxicity of Bi₂O₃ NPs in culture cells, by fluorescence microscopy

The cytotoxic effect of Bi₂O₃ NPs was evaluated in monkey kidney (Vero) cells by fluorescence microscopy. The results

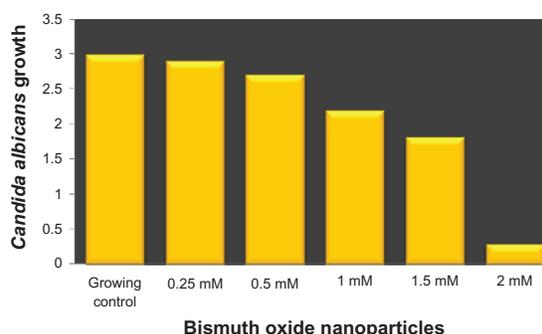


Figure 3 MIC of bismuth oxide nanoparticles against *Candida albicans* growth.

Notes: The y axis shows the optical density units of *C. albicans* growth; the x axis shows the different concentrations of Bi₂O₃ NPs analyzed. *C. albicans* culture without inhibitor was used as a growth control. Experiments were carried out three times and each time by triplicate. Average results were obtained from the three independent experiments.

Abbreviations: Bi₂O₃, bismuth oxide; MIC, minimal inhibitory concentration; NP, nanoparticle.

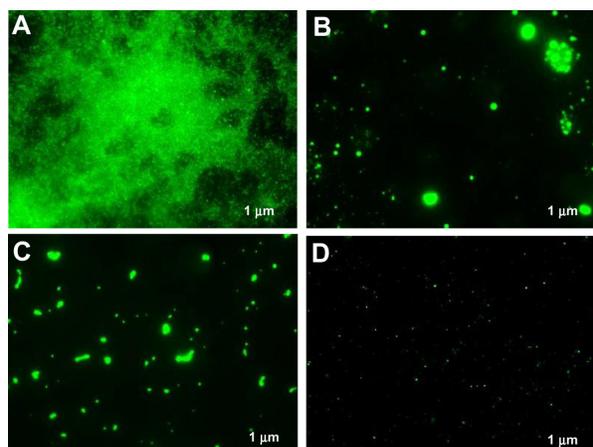


Figure 4 Inhibition of *Candida albicans* biofilm detected by fluorescence microscopy, after 24 hours. As a growth control, *C. albicans* was added to culture media; chlorhexidine 2% and terbinafine 1% were employed as positive inhibition controls. In these experiments, we used a concentration of 2 mM Bi_2O_3 NPs. (A) Growth control; (B) chlorhexidine; (C) terbinafine; and (D) Bi_2O_3 NPs.

Note: The bar indicates 1 μm .

Abbreviations: Bi_2O_3 , bismuth oxide; NP, nanoparticle.

showed that Bi_2O_3 NPs did not promote cytotoxic effects in the Vero cells at 24 hours of exposure compared with the cells without NP. The nuclei and, indeed, all the cells looked very similar in the presence or absence of Bi_2O_3 NPs (Figure 5). These results suggest the absence of cytotoxicity by Bi_2O_3 NPs, under our experimental conditions.

Discussion

In this study, we presented evidence of the antimycotic activity of Bi_2O_3 NPs. Their efficacy in inhibiting *C. albicans* growth was significantly better than that of chlorhexidine, nystatin, and terbinafine. These results indicate that Bi_2O_3 NPs are better antifungal agents than the most commonly used oral antiseptic and commercial antifungal agents. To be assured that the antifungal effect was due to the nanostructured Bi_2O_3 , this was compared with the antifungal

effect of bulk Bi_2O_3 . The Bi_2O_3 NPs were two times more effective than the polycrystalline Bi_2O_3 in inhibiting fungal growth. This result was very important because it demonstrated the effectiveness of the nanostructured material versus the same bulk compound. Bismuth salicylate, a typical molecular bismuth derivative, has been employed to treat fungal infections but was not shown to inhibit the fungal growth,²³ suggesting that it is the nanostructured particle of Bi_2O_3 that is conferred with the antimicrobial properties described earlier.¹⁵ These results suggest that Bi_2O_3 NPs could be very interesting antifungal agents. Previously, it was reported that zinc, titanium, and silver NPs have very good antifungal activity;^{24–26} however, it is not possible to establish any quantitative antibiofilm activity comparison, due to the type of published results.

Inorganic nanostructures have many applications in fields like the biological sciences and medicine. NPs have been applied as coating materials and in treatments and diagnosis.²⁷ NPs of titanium dioxide, silver, diamonds, iron oxides, carbon nanotubes, and biodegradable polymers have all been studied for their use in diagnosis and treatments. NPs of silver, copper oxides, and selenium have been reported to have antimicrobial activity.^{28–30} The advantages of inorganic NPs are their high surface-to-volume ratios, different shapes, many structural defects, (the presence of irregularities in the crystal lattice of the nanostructured bismuth oxide) and of course, their nanoscale size, which allows more active sites to interact with biological systems such as bacteria, fungi, and viruses. This is the most important difference between NPs and the typical organic molecular antimicrobial agents, and could minimize the risk of developing antimicrobial resistance.

The mechanism of the antimicrobial activity of inorganic NPs is not completely understood, and their precise mechanism of action against bacteria and fungi remains to be fully eluci-

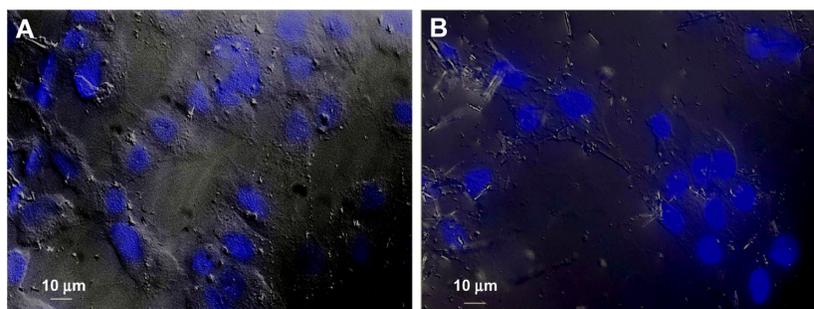


Figure 5 Cytotoxicity of bismuth oxide nanoparticles detected by fluorescence microscopy. The possible cytotoxic effect of bismuth oxide nanoparticles was evaluated in monkey kidney cells by staining the nuclei with DAPI and visualizing cell morphology with DIC. As positive control, Vero cells were used. (A) Positive control and (B) Vero cells, after interacting with bismuth oxide nanoparticles, for 24 hours.

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast microscopy.

dated. It has been shown that positive charges on the metal ion are critical for the antimicrobial activity, allowing for the electrostatic attraction between a negatively charged cell membrane and the positively charged NPs.³¹ It has also been reported that silver NPs can damage DNA, alter gene expression, and affect the membrane-bound respiratory enzymes.^{32–34}

As previously mentioned, Bi_2O_3 has a marked basic character, and when this compound is suspended in water, it tends to be associated with hydroxyl ions and water molecules on its surface, similar to titanium dioxide, zinc oxide, tin dioxide, and other metal oxides.^{35–39} When Bi_2O_3 is nanostructured, its ionic character is enhanced compared with the same bulk material, ie, it becomes a “harder” species with respect to its electrons (according to Pearson’s theory of hard and soft acids and bases).⁴⁰ As a consequence, when Bi_2O_3 is nanostructured, its dissociating character increases, as does the overall surface of the material. Therefore, the amount of OH^- ions and water molecules bound to the surfaces of the nanocrystallites is higher than in the same bulk material, as is sketched in (Figure 1D). Thus, Bi_2O_3 NPs dispersed in water are a chemical species with a highly negative surface electric potential; subsequently, these nanostructured species are strongly basic aggregation points, conferring them with a lethal character for the immersed *C. albicans* fungi. It has been reported that several microorganisms are very sensitive to alkaline media, among them, *C. albicans*.⁴¹ We suggest that this is the basis for the fungicidal character of Bi_2O_3 nanoclusters. Additionally, we cannot discard antifungal activity mechanisms involving free radicals, due to the presence of CO_2 in the culture media⁴² and the inherent $\alpha\text{-Bi}_2\text{O}_3$ NPs photocatalytic properties.⁴³ However, more experimental evidences are needed to support these hypotheses.

In parallel to their antifungal activity, the Bi_2O_3 NPs had the potential to interfere with biofilm formation of *C. albicans*. This antibiofilm activity of the Bi_2O_3 NPs was also studied. Surprisingly, the antibiofilm formation effect was complete in the media with the Bi_2O_3 NPs. This effect was unexpected since Bi_2O_3 NPs only reduced cell growth and did not completely inhibit it. To explain this observation, we suggest that 85% of cells were inactivated during inoculation with Bi_2O_3 NPs and that surviving cells were insufficient to build a biofilm. It may be that they went into planktonic and stress states due the presence of the Bi_2O_3 NPs, and it is possible that they were lost during the wash of dye excess. In the presence of chlorhexidine and Bi_2O_3 NPs, we only observed (via fluorescence microscopy) cellular debris on a dark background – mainly DNA of dead fungus with accumulates of dye. Morphologically, these dye accumulates clearly differed from fungal biofilm.

It is noteworthy that there are published reports of fungal growth inhibition, including inhibition of *C. albicans*, where inorganic NPs, such as diamond-, silver-, gold-, platinum-, and palladium-based NPs, were used as antifungal agents.⁴⁴ Particularly, silver (Ag) NPs have also been shown to inhibit yeast growth,³¹ and their antifungal activity against certain species of *Candida*⁴⁵ is well documented. These studies show evidence for the molecular mechanism of Ag NPs activity, whereby Ag NPs act on and inhibit a number of oxidative enzymes, such as yeast alcohol dehydrogenase, through the generation of reactive oxygen species.³¹ Ag NPs have been demonstrated to exhibit a desirable and promising antifungal effect in several studies, with no serious side effects to the host.⁴⁶ However, the results and the experimental conditions used in the aforementioned studies are not comparable with those of this research.

The possible cytotoxic effect of Bi_2O_3 NPs was explored in monkey kidney (Vero) cells by fluorescence microscopy. No change was detected in the morphology of the nuclei or, indeed, of all cells in the presence or absence of Bi_2O_3 NPs, suggesting the lack of cytotoxic effect after 24 hours of exposure. Earlier, the toxicity of metal oxide NPs, mainly Ag oxide (Ag_2O), zinc oxide (ZnO), and iron oxide (Fe_2O_3),⁴⁷ was reported. The possible toxicity of Bi_2O_3 NPs was analyzed using the same concentration of NPs as was employed in those antifungal assays. The preliminary results of this study reveal no cytotoxicity of colloidal Bi_2O_3 NPs, at least under the experimental conditions previously described. This lack of cytotoxicity can be attributed to the very small amount of inorganic nanomaterial that interacted with the Vero cells. The total Bi_2O_3 nanocluster mass and doses of 0.15 $\mu\text{g}/\text{mL}$ introduced were not enough to cause cell damage.

For particle toxicity, three factors are crucial: size, shape, and chemical composition. A reduction in the size of a nanosized particle results in an increase in the specific surface area of the nanostructured powder. Therefore more chemical species may attach to its surface, which enhances its reactivity and results in an increase in its toxic effects.^{48,49}

In this case, the colloidal Bi_2O_3 NPs had a relatively small size; however, the number of nanoclusters introduced into the Vero cell culture medium, was significantly low compared with the total loads of the other two derivatives of bismuth (Bi_2O_3 in bulk form and bismuth nitrate).

In this work we focused on the effectiveness of Bi_2O_3 NPs in inhibiting the growth of *C. albicans*. All together, the experimental data suggest that Bi_2O_3 NPs could be an interesting alternative to combat the fungal infections at the origin of biofilms. The property of Bi_2O_3 NPs could be used in

oral health, supporting the antifungal activity of oral antiseptics. Further experiments will be necessary to determine the possible toxicity of Bi₂O₃ NPs and analyze the genotoxicity (apoptosis, DNA fragmentation, DNA damage) in human fibroblasts cultures and analyze their potential use in humans.

In conclusion, Bi₂O₃ NPs of 77 nm average size have an antimycotic activity inhibiting the growth of *C. albicans*, as well as an antibiofilm activity. This behavior is different from the one of the corresponding bulk material; so, the observed activity is a size-dependent property, an explanation that was proposed first time. Additionally, our results suggest that the Bi₂O₃ NPs, under the experimental tested conditions and concentrations, do not exhibit cytotoxicity.

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Disclosure

The authors report no conflicts of interest in this work.

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