Facile Synthesis of Zn-Doped Bi$_2$O$_3$ Nanoparticles and Their Selective Cytotoxicity toward Cancer Cells

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ABSTRACT: Bismuth (III) oxide nanoparticles (Bi$_2$O$_3$ NPs) have shown great potential for biomedical applications because of their tunable physiochemical properties. In this work, pure and Zn-doped (1 and 3 mol %) Bi$_2$O$_3$ NPs were synthesized by a facile chemical route and their cytotoxicity was examined in cancer cells and normal cells. The X-ray diffraction results show that the tetragonal phase of β-Bi$_2$O$_3$ remains unchanged after Zn-doping. Transmission electron microscopy and scanning electron microscopy images depicted that prepared particles were spherical with smooth surfaces and the homogeneous distribution of Zn in Bi$_2$O$_3$ with high-quality lattice fringes without distortion. Photoluminescence spectra revealed that intensity of Bi$_2$O$_3$ NPs decreases with increasing level of Zn-doping. Biological data showed that Zn-doped Bi$_2$O$_3$ NPs induce higher cytotoxicity to human lung (A549) and liver (HepG2) cancer cells as compared to pure Bi$_2$O$_3$ NPs, and cytotoxic intensity increases with increasing concentration of Zn-doping. Mechanistic data indicated that Zn-doped Bi$_2$O$_3$ NPs induce cytotoxicity in both types of cancer cells through the generation of reactive oxygen species and caspase-3 activation. On the other hand, biocompatibility of Zn-doped Bi$_2$O$_3$ NPs in normal cells (primary rat hepatocytes) was greater than that of pure Bi$_2$O$_3$ NPs and biocompatibility improves with increasing level of Zn-doping. Altogether, this is the first report highlighting the role of Zn-doping in the anticancer activity of Bi$_2$O$_3$ NPs. This study warrants further research on the antitumor activity of Zn-doped Bi$_2$O$_3$ NPs in suitable in vivo models.

1. INTRODUCTION

Bismuth (Bi) is known as one of the least toxic heavy metals for the human body and it has a long history in medicine due to its antibacterial activity.\textsuperscript{1} Several bismuth-based medical formulations such as ranitidine bismuth citrate and bismuth subsalicylate are utilized for the treatment of gastrointestinal problems.\textsuperscript{2} Moreover, organic-based bismuth compounds have antitumor effects.\textsuperscript{3,4} Although organic-based bismuth complexes have potential anticancer activity, they can exert adverse health effects to humans.\textsuperscript{5,6}

Currently, bismuth (III) oxide nanoparticles (Bi$_2$O$_3$ NPs) have received great attention for their applications in chemical, electrical, optical, engineering, and biomedicine due to their excellent physicochemical properties including high stability, high surface area, desirable catalytic activity, low toxicity, and cost-effectiveness.\textsuperscript{7} The ease of controlling the physicochemical properties of Bi$_2$O$_3$ NPs during synthesis can open new opportunities for its application in medicine.\textsuperscript{8} A recent study suggested that Bi$_2$O$_3$ NPs can be used as a radiosensitizer in cancer radiotherapy.\textsuperscript{9} Some studies also observed that Bi$_2$O$_3$ NPs exert toxicity to cancer cells by intracellular reactive oxygen species (ROS) generation.\textsuperscript{10,11}

A higher intracellular level of ROS is associated with induction of apoptosis, and cancer cells can be killed by ROS-generating agents.\textsuperscript{12−14} A low-to-moderate level of ROS is essential for cellular function and survival. However, excessive ROS production can lead to oxidative DNA damage, lipid peroxidation, and apoptosis.\textsuperscript{15,16} Comparatively, the elevated level of ROS is found in cancer cells than those of their normal counterparts providing a promising strategy to target cancer cells selectively. For example, ZnO NPs and heterocyclic organobismuth (III) kill cancer cells selectively through the ROS pathway.\textsuperscript{4,17}

Research on anticancer potential of Bi$_2$O$_3$ NPs is still in the infancy stage because of their significant side effects.\textsuperscript{18} Hence, there is a need to prepare Bi$_2$O$_3$ NPs with improved anticancer activity and excellent biocompatibility. Keeping this point in mind, we synthesized pure and Zn-doped Bi$_2$O$_3$ NPs with...
higher toxicity in cancer cells and better biocompatibility to normal cells. Pure and Zn-doped (1 and 3 mol %) Bi$_2$O$_3$ NPs were prepared by a facile chemical method. Synthesized pure Bi$_2$O$_3$ NPs and Zn-doped Bi$_2$O$_3$ NPs were characterized by X-ray diffraction (XRD), field emission scanning electron microscopy (FESEM), field emission transmission electron microscopy (FETEM), energy-dispersive X-ray spectroscopy (EDS), photoluminescence (PL), and dynamic light scattering (DLS). Anticancer efficacy of pure and Zn-doped Bi$_2$O$_3$ NPs was studied in two different cancer cell lines: human lung cancer (A549) and liver cancer (HepG2) cells. Biocompatibility of prepared NPs was examined in primary rat hepatocytes. The possible mechanism of antitumor activity of prepared NPs was explored through ROS generation and caspase-3 (apoptotic marker) activation.

2. RESULTS AND DISCUSSION

2.1. XRD Analysis. XRD spectra of pure and Zn-doped Bi$_2$O$_3$ (1 and 3%) are presented in Figure 1. The major peaks of all the NPs at 2$\theta$ values of 25.96, 28.19, 30.52, 32.95, 41.52, 46.50, 47.12, 51.48, 54.46, 55.73, and 57.96 corresponded to the diffractions of the (210), (201), (211), (002), (220), (212), (222), (400), (123), (203), (421), and (402) planes of the tetragonal structure of $\beta$-Bi$_2$O$_3$ (JCPDS no. 27-0050). The absence of Zn peaks in Zn-doped Bi$_2$O$_3$ NPs could be due to a small amount of Zn$^{2+}$ concentration (1 and 3%) as well as the smaller ionic radius of Zn$^{2+}$ (0.074 nm) as compared to Bi$^{3+}$ (0.117 nm).

The crystallite size of prepared samples was determined for the (201) diffraction of Bi$_2$O$_3$ using the Scherrer equation. The crystallite size of pure Bi$_2$O$_3$ 1% Zn–Bi$_2$O$_3$, and 3% Zn–Bi$_2$O$_3$ particles was 67, 63, and 54 nm, respectively (Table 1). The reduction in size upon Zn-doping was because of the smaller ionic radius of Zn$^{2+}$ (0.074 nm) compared to Bi$^{3+}$ (0.117 nm). The crystallite size of prepared samples was determined for the (201) diffraction of Bi$_2$O$_3$ using the Scherrer equation. The crystallite size of prepared samples was determined for the (201) diffraction of Bi$_2$O$_3$ using the Scherrer equation.

2.2. TEM Study. Microstructure, morphology, and particle size of prepared samples were characterized by FETEM. Figure 2A–B depicts the representative transmission electron microscopy (TEM) images of pure and 3% Zn–Bi$_2$O$_3$ NPs, respectively. These TEM images display that prepared NPs possess spherical morphology with some degrees of agglomeration. The shape of Bi$_2$O$_3$ NPs remains the same but particle size decreases after Zn-doping (71–51 nm) (Table 1), which is in agreement with size estimated from the Scherrer equation. The tetragonal structure of Bi$_2$O$_3$ NPs is further confirmed by the visible lattice fringes in the high-resolution TEM images (Figure 2C,D). These images demonstrate the presence of both Bi$_2$O$_3$ and Zn with high-quality lattice fringes without distortion. The interplanar spacing of the lattice of Bi$_2$O$_3$ NPs and 3% Zn–Bi$_2$O$_3$ NPs is 0.325 nm and 0.321 nm, respectively, which relates to the (201) plane of the tetragonal phase of Bi$_2$O$_3$, whereas the lattice fringe of Zn has an interplanar spacing of 0.233 nm which corresponds to the (101) plane of the cubic Zn crystallographic structure. TEM machine-generated EDS spectra of 3% Zn–Bi$_2$O$_3$ NPs showed the presence of Bi, Zn, and O peaks without a contamination peak (Figure 3). The presence of Cu and C peaks was due to the use of carbon-coated copper TEM grid.

2.3. SEM Study. Morphology, elemental composition, and elemental mapping of pure and Zn-doped Bi$_2$O$_3$ NPs were further observed by FESEM. Figure 4A–C represents the

| Table 1. Physicochemical Characterization of Pure and Zn-Doped Bi$_2$O$_3$ NPs |
|-----------------------------|----------------|----------------|
| parameters                  | Bi$_2$O$_3$    | 1% Zn–Bi$_2$O$_3$ | 3% Zn–Bi$_2$O$_3$ |
| XRD size (nm)               | 67            | 63             | 54            |
| TEM size (nm)               | 71            | 64             | 51            |
| SEM size (nm)               | 70            | 63             | 52            |
| hydrodynamic size (nm)$^a$  | deionized water | 247 ± 23        | 215 ± 13       | 188 ± 15    |
|                            | culture media  | 211 ± 17        | 197 ± 18       | 167 ± 11    |
|                            | zeta potential (mV)$^a$ | 15 ± 4          | 19 ± 5         | 25 ± 4      |
|                            | deionized water | 18 ± 3          | 22 ± 6         | 27 ± 3      |

$^a$Data presented mean ± SD of triplicates ($n = 3$).
typical scanning electron microscopy (SEM) images of pure Bi$_2$O$_3$, 1% Zn–Bi$_2$O$_3$, and 3% Zn–Bi$_2$O$_3$ NPs, respectively. In agreement with TEM, these images also suggested spherical morphology, and particle size of Bi$_2$O$_3$ was found to be gradually decreased with the increase in Zn-doping (71–51 nm) (Table 1). The smaller particle size of semiconductor NPs after metal ion-doping would give rise to a higher surface area, which is generally beneficial for increased cytotoxicity to cancer cells\textsuperscript{19,22} SEM machine-generated EDS spectra of 3% Zn–Bi$_2$O$_3$ NPs showed the stoichiometric presence Bi (87.46%), O (9.67%), and Zn (2.91%) (Figure 4D). The SEM elemental mapping of 3% Zn–Bi$_2$O$_3$ NPs further confirmed the uniform distribution of Zn in Bi$_2$O$_3$ (Figure 5).

2.4. PL Study. The room-temperature PL spectra of pure and Zn-doped (1 and 3%) Bi$_2$O$_3$ NPs with a 330 nm as an excitation wavelength are given in Figure 6. The visible
emission peaks at 431, 451, and 472 nm are observed for all the prepared NPs. However, intensity of Zn-doped Bi2O3 NPs decreases with increasing Zn concentrations. Lower intensity of 3% Zn−Bi2O3 NPs suggested the effective migration of charge carriers (electron and holes) from the inner part of NPs to the surface so that they can participate in surface redox reactions.21,23 These phenomena are useful in photocatalysis and biomedicine.12,24

2.5. DLS Study. It is crucial to explore the aqueous behavior of prepared NPs before their cytotoxicity investigations. In this study, hydrodynamic size and zeta potential of pure Bi2O3, 1% Zn−Bi2O3, and 3% Zn−Bi2O3 NPs were measured in deionized water and complete culture medium (DMEM + 10% FBS). Results showed that the hydrodynamic size of pure and Zn-doped Bi2O3 NPs in deionized water and culture medium was 3–5 times higher than the primary size of nanopowder calculated from XRD and TEM (Table 1). Higher hydrodynamic size could be due to the tendency of NPs to agglomerate in an aqueous suspension. Such a phenomenon was also reported by other investigators.11,25,26 The zeta potential results demonstrated that the particle surface charge of pure and Zn-doped Bi2O3 NPs was ranging from 15 to 25 mV in deionized water and 18–27 in culture medium. These

Figure 4. SEM characterization. (A–C) SEM images of pure Bi2O3, 1% Zn−Bi2O3, and 3% Zn−Bi2O3 NPs, respectively. (D) EDS spectra of 3% Zn−Bi2O3 NPs.

Figure 5. SEM elemental mapping of 3% Zn−Bi2O3 NPs. (A) SEM image and (B) bismuth, (C) oxygen, and (D) zinc mapping.

Figure 6. Photoluminescence spectra of pure and Zn-doped Bi2O3 NPs.
values suggested that prepared NPs were fairly stable in an aqueous suspension. Generally, the zeta potential value of NPs around 30 mV (positive or negative) showed excellent colloidal stability.\textsuperscript{17,27} The positive surface charge of NPs under the physiological condition as reported in the present study provides an encouraging environment for their interaction with cancer cells as they hold negative surface charges.\textsuperscript{17}

2.6. Cytotoxicity Study. Recent studies highlight the importance of metal-based NPs in biomedical applications including antimicrobial and anticancer activity.\textsuperscript{28–30} Moreover, metal ion-doping can further improve the biomedical application of metal oxide NPs through the tailoring of properties.\textsuperscript{22,31} For example, Miri et al. observed that the increase in Ni-doping for Ce\textsubscript{2}O NPs increased the cytotoxicity in colon cancer cells (HT-29).\textsuperscript{32} Our previous study also showed that Ag-doping increases the cytotoxicity of TiO\textsubscript{2} NPs in cancer cells and improves their biocompatibility in normal cells.\textsuperscript{19}

In the present study, cytotoxicity of pure Bi\textsubscript{2}O\textsubscript{3}, 1\% Zn–Bi\textsubscript{2}O\textsubscript{3}, and 3\% Zn–Bi\textsubscript{2}O\textsubscript{3} NPs was examined in two types of cancer cells (human lung cancer A549 and human liver cancer HepG2) along with the noncancerous normal cells (primary rat hepatocytes). Cells were exposed for 24 h to different concentrations (0–400 \(\mu\)g/mL) of pure and Zn-doped Bi\textsubscript{2}O\textsubscript{3} NPs and cytotoxicity was examined by MTT assay. Figure 7A,B shows that all three NPs (Bi\textsubscript{2}O\textsubscript{3}, 1\% Zn–Bi\textsubscript{2}O\textsubscript{3}, and 3\% Zn–Bi\textsubscript{2}O\textsubscript{3}) induced dose-dependent cytotoxicity in both types of cancer cells (A549 and HepG2). Moreover, Zn-doped Bi\textsubscript{2}O\textsubscript{3} NPs induce higher cytotoxicity in comparison with pure Bi\textsubscript{2}O\textsubscript{3} NPs, and cytotoxicity increased with increasing concentration of Zn-doping. IC\textsubscript{50}’s of A549 cells for pure Bi\textsubscript{2}O\textsubscript{3}, 1\% Zn–Bi\textsubscript{2}O\textsubscript{3}, and 3\% Zn–Bi\textsubscript{2}O\textsubscript{3} NPs were 205, 110, and 63 \(\mu\)g/mL, respectively. Besides, IC\textsubscript{50}’s of HepG2 cells for pure Bi\textsubscript{2}O\textsubscript{3}, 1\% Zn–Bi\textsubscript{2}O\textsubscript{3}, and 3\% Zn–Bi\textsubscript{2}O\textsubscript{3} NPs were 223, 120, and 73 \(\mu\)g/mL, respectively (Table 2).

\begin{table}[h]
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\textbf{IC\textsubscript{50} (\(\mu\)g/mL)} & \textbf{Bi\textsubscript{2}O\textsubscript{3}} & \textbf{1\% Zn–Bi\textsubscript{2}O\textsubscript{3}} & \textbf{3\% Zn–Bi\textsubscript{2}O\textsubscript{3}} \\
\hline
A549 cells & 205 & 110 & 63 \\
HepG2 cells & 223 & 120 & 73 \\
primary rat hepatocytes & 533 & 1136 & 2303 \\
\hline
\end{tabular}
\caption{IC\textsubscript{50}’s (\(\mu\)g/mL) for Different Cells against Pure and Zn-Doped Bi\textsubscript{2}O\textsubscript{3} NPs}
\end{table}

The anticancer activity of bismuth-based NPs was also reported by other investigators. For example, a recent study observed that bismuth lipophilic (BisBAL) NPs exhibit significant cytotoxicity to breast cancer MCF-7 cells and relatively low toxicity to noncancerous MCF-10A cells.\textsuperscript{33} Ouyang and co-workers also found that compared to cisplatin, bismuth-based complex (Bi\textsubscript{2}Cl\textsubscript{3}) showed better anticancer activity against tumor cells and lower toxicity to normal cells.\textsuperscript{34}

The Bi is considered as one of the least toxic and biologically less-reactive heavy metals, which is more appropriate for biomedical applications in comparison with other metals such as silver.\textsuperscript{7} Hence, cytotoxicity of pure and Zn-doped Bi\textsubscript{2}O\textsubscript{3} NPs was further examined in noncancerous primary rat hepatocytes. Figure 8 demonstrates that all three prepared NPs did not exert much toxicity to primary rat hepatocytes. Moreover, Zn-doping further improves the biocompatibility of Bi\textsubscript{2}O\textsubscript{3} NPs in primary rat hepatocytes. The IC\textsubscript{50}’s of primary rat hepatocytes for pure Bi\textsubscript{2}O\textsubscript{3}, 1\% Zn–Bi\textsubscript{2}O\textsubscript{3}, and 3\% Zn–Bi\textsubscript{2}O\textsubscript{3} NPs were 533, 1136, and 2303 \(\mu\)g/mL, respectively. These results suggested that Bi\textsubscript{2}O\textsubscript{3} NPs selectively induced cytotoxicity in cancer cells while not affecting the normal cells. Moreover, Zn-doping increases the cytotoxicity of Bi\textsubscript{2}O\textsubscript{3} NPs in cancer cells and improves their biocompatibility in normal cells. High toxicity of Bi(OH)\textsubscript{3} and Bi\textsubscript{2}O\textsubscript{3} NPs in gliosarcoma 9L cells and human breast cancer MCF-7 cells and low toxicity in normal cells were also reported by Bogusz et al.\textsuperscript{35}

2.7. Possible Mechanisms of Anticancer Activity of Zn-Doped Bi\textsubscript{2}O\textsubscript{3} Nanoparticles. Earlier studies have shown that ZnO NPs have inherent potential of killing cancer cells through ROS while sparing the normal cells.\textsuperscript{37,38} Some previous reports also demonstrated that Bi-based nanoscale materials selectively induce cytotoxicity to cancer cells via ROS without much affecting the normal cells.\textsuperscript{33,35} ROS-induced oxidative stress has been also suggested as one of the potential mechanisms of selective cytotoxicity of cancer cells by other
Hence, we further explored the potential mechanisms of anticancer activity and biocompatibility of Zn-doped Bi2O3 NPs. Intracellular generation of ROS in cancer cells (A459 and HepG2) and primary rat hepatocytes following exposure to moderate concentration of (50 μg/mL) of 3% Zn–Bi2O3 NPs for 24 h was examined. Figure 9A shows that 3% Zn–Bi2O3 NPs significantly induce ROS generation in both types of cancer (A459 and HepG2) cells. Interestingly, 3% Zn–Bi2O3 NPs did not generate ROS in primary rat hepatocytes. Intracellular ROS generation seems the potential mechanisms of anticancer activity of Zn–Bi2O3 NPs. Zn-doping brings two important changes in the physicochemical properties of Bi2O3 NPs, which plays an important role in the anticancer activity of Zn–Bi2O3 NPs. First, Zn-doping decreases the particle size of Bi2O3 NPs. The ROS-generating potential of NPs increases with decreasing particle size. Second, PL study indicated that the intensity of PL spectra of Zn-doped Bi2O3 NPs decreases with increasing Zn2+ ion concentration. Lower intensity of 3% Zn–Bi2O3 NPs suggested the effective migration of charge carriers (electron and holes) from the inner part of NPs to the surface so that they can participate in surface redox reactions. This is a favorable condition for intracellular generation of reactive oxygen species (ROS), which is useful for various applications including photocatalysis, antibacterial activity, and anticancer activity. In the present study, in vitro results demonstrated that Zn-doped Bi2O3 NPs exert higher cytotoxicity to cancer cells than those of pure Bi2O3 NPs and cytotoxicity increased with increasing doping concentration of Zn2+ ions.

There are increasing evidences that NPs induced apoptosis in cancer cells through the activation of caspases. Caspase-3 enzyme activity of cancer cells (A459 and HepG2) and primary rat hepatocytes was assessed following exposure to 50 μg/mL of 3% Zn–Bi2O3 NPs for 24 h. Figure 9B demonstrates that 3% Zn–Bi2O3 NPs significantly activate caspase-3 enzyme in both cancer cells. Interestingly, 3% Zn–Bi2O3 NPs did not affect the activity of caspase-3 enzyme in primary rat hepatocytes. Altogether, Zn-doped Bi2O3 NPs exerted cytotoxicity in cancer cells via ROS generation and caspase-3 activation. Potential mechanisms of selective cytotoxicity of Zn-doped Bi2O3 NPs in cancer and normal cells are depicted in Figure 10.

**3. CONCLUSIONS**

Pure and Zn-doped (1 and 3%) Bi2O3 NPs were synthesized by a facile chemical method. XRD spectra show that the tetragonal phase of β-Bi2O3 NPs did not change after Zn-doping. HR-TEM and SEM mapping demonstrated the homogeneous distribution of Zn in Bi2O3 with high-quality lattice fringes with no distortion. PL study revealed that the intensity of Bi2O3 NPs decreases with increasing level of Zn-doping. Cytotoxicity studies demonstrated that Zn-doped Bi2O3 NPs induce higher toxicity to cancer cells (A459 and HepG2) than those of pure Bi2O3 NPs, and intensity of toxicity increases with increasing concentration of Zn-doping. Mechanistic study indicated that Zn-doped Bi2O3 NPs induce toxicity in cancer cells by ROS generation and caspase-3 activation. On the other hand, biocompatibility of Zn-doped Bi2O3 NPs in normal cells (primary rat hepatocytes) was greater than that of pure Bi2O3 NPs. Our data provide an alternative strategy for cancer therapy using Zn-doped Bi2O3 NPs. This study warrants further research on antitumor activity of Zn-doped Bi2O3 NPs in suitable in vivo models.

**4. MATERIALS AND METHODS**

**4.1. Synthesis of Pure and Zn-Doped Bi2O3 NPs.** Bismuth nitrate (Bi2(NO3)3·5H2O), sodium hydroxide (NaOH), and zinc acetate (Zn(CH3COO)2·2H2O) were used as starting materials. All the chemicals were of analytical grade and used as received from Sigma-Aldrich (St. Louis, MO, USA). Zinc-doped (1 and 3 mol %) Bi2O3 was prepared by dissolving 1 M bismuth nitrate into 50 mL of deionized water. Then, the stoichiometric amount of zinc acetate dissolved in 50 mL of deionized water was added under magnetic stirring.
Moreover, 0.1 M NaOH dissolved in 50 mL of deionized water was added dropwise to the abovementioned solution under stirring. This mixture solution was magnetically stirred for 5 h at 80 °C until a light-yellow precipitate was appeared. Then, the precipitate was washed several times with deionized water and filtered. The precipitate was washed in an oven at 100 °C for 2 h and further annealed at 500 °C for 3 h in a muffle furnace to get Zn-doped (1 and 2 mol %) Bi₂O₃ NPs. The same protocol was applied for the preparation pure Bi₂O₃ NPs without adding zinc acetate. A schematic diagram of Zn-doped Bi₂O₃ NP preparation is provided in Figure 11.

**Figure 11.** Schematic diagram of Zn-doped Bi₂O₃ NP preparation.

### 4.2. Characterization.

The purity of phase and crystalline nature of prepared pure and Zn-doped (1 and 3%) Bi₂O₃ NPs were assessed by X-ray diffraction (XRD) (PanAnalytic X′Pert Pro, Malvern Instruments, UK) with Cu-Kα radiation (λ = 0.15405 nm, at 45 kV and 40 mA). Structural characterization was carried out by FETEM (JEM-2100F, JEOL, Inc., Tokyo, Japan). In brief, stock suspension of NPs (1 mg/mL in deionized water) further diluted into an appropriate working suspension (50 μg/mL in deionized water). This suspension was sonicated for 15 min at 40 W in a water bath sonicator (Cole-Parmer, Vernon Hills, IL, USA). Then, a drop of working suspension of NPs was poured onto TEM grid and air-dried, and the TEM measurements were carried out.

Elemental composition assessed by EDS. Surface morphology and elemental mapping were assessed by FESEM (JSM-7600F, JEOL, Inc.). The photoluminescence spectra were observed using a fluorescence spectrophotometer (Hitachi F-4600).

Aqueous behavior of prepared NPs in deionized water and complete culture medium (DMEM + FBS) was carried out by dynamic light scattering (DLS) (ZetaSizer, Nano-HT, Malvern Instruments). In brief, NPs were suspended in deionized water and culture medium at a concentration of 400 μg/mL and incubated for 24 h at 37 °C. Then, suspensions were sonicated for 15 min at 40 W in a water bath sonicator (Cole-Parmer) and the DLS measurements were carried out.

### 4.3. Cell Culture.

The A549 and HepG2 cell lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA, USA). Primary rat hepatocytes were isolated by collagenase perfusion methods as described earlier. Cells were cultured in Dulbecco’s modified eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA, USA) or Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) supplemented with 100 μg/mL of streptomycin (Invitrogen), 100 U/mL of penicillin (Invitrogen), and 10% fetal bovine serum (FBS, Invitrogen). Cells were maintained in a humidified incubator (5% CO₂ supply at 37 °C). At 80–85% confluence, cells were harvested with trypsin (Invitrogen) and subcultured.

### 4.4. Preparation of Stock Solution of Pure and Zn-Doped Bi₂O₃ NPs and Exposure Protocol.

Stock suspension (1 mg/mL) and different dilutions (1—400 μg/mL) of pure and Zn-doped (1 and 3%) Bi₂O₃ NPs were prepared in complete culture medium (DMEM + 10% FBS). Different dilutions were sonicated in a water bath for 15 min at 40 W to avoid agglomeration of NPs before exposure to cells. For cytotoxicity endpoint assay, cells were treated for 24 h to various concentrations (0, 1, 5, 10, 25, 50, 100, 200, and 400 μg/mL) of pure and Zn-doped (1 and 3%) Bi₂O₃ NPs. For ROS and caspase-3 enzyme assays, cells were exposed for 24 h to a moderate concentration of 3% Zn—Bi₂O₃ NPs (50 μg/mL). Hydrogen peroxide (H₂O₂) (200 μM) was used as the positive control in ROS and caspase-3 enzyme assays. Cells without NPs served as the negative control.

### 4.5. Cytotoxicity Assay.

Cell viability was examined by MTT assay with some modifications. The MTT assay is based on the principle of the ability of mitochondria of living cells to reduce MTT salt into blue formazan crystals. These crystals dissolved in acidified isopropanol and absorbance was recorded at 570 nm using a microplate reader (Synergy-HT, BioTek, Vinnoski, VT, USA).

The probe 2,7-dichlorofluorescin diacetate (DCFH-DA) was applied to assess the intracellular generation of ROS after exposure to 50 μg/mL of 3% Zn—Bi₂O₃ NPs for 24 h. The DCGH-DA is a cell-permeable nonfluorescent dye, converted into highly fluorescent DCF when oxidized by intracellular ROS. The fluorescence of DCF was measured at the 485 nm excitation and the 520 nm emission using a microplate reader (Synergy-HT, BioTek).

The cell extract was prepared for caspase-3 enzyme assay. In brief, cells were cultured in 75 cm² culture flask and exposed to 50 μg/mL of 3%Zn—Bi₂O₃ NPs for 24 h. Then, cells were harvested in ice-cold phosphate buffer saline by scraping and washed with PBS at 4 °C. Cell pellets were further lysed in cell lysis buffer [1× 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1% Triton, 2.5 mM sodium pyrophosphate]. After centrifugation (15,000 g × 15 min at 4 °C), the supernatant (cell extract) was stored at 4 °C for further experiment. The fluorometric assay of the caspase-3 enzyme was examined using the 7-amido-4-trilluoromethylcoumarin (AFC) standard. Protein content in the cell extract was quantified using Bradford’s protocol which was adapted to measure the protein level.

### 4.6. Statistical Analysis.

One-way analysis of variance (ANOVA) followed by Dennett’s multiple comparison tests was used for statistical analysis of biological results. The p < 0.05 was ascribed as statistically significant. All the biological quantitative data are presented as mean ± SD of three independent experiments (n = 3).
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Notes
The authors declare no competing financial interest.

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