



Bi₂O₃@folic acid nanoparticles: An effective sonosensitizer for melanoma sonodynamic therapy

M. Sarani^a, H. Haghighi^a, P. Tamaddon^b, M. Darroudi^c, A. Safari^d,
S.A. Dastgheib^{a,e}, N. Sattarahmady^{a,b,*}

^a Nanomedicine and Nanobiology Research Center, Shiraz University of Medical Sciences, Shiraz 7193635899, Iran

^b Department of Medical Physics, School of Medicine, Shiraz University of Medical Sciences, Shiraz 7134845794, Iran

^c Nuclear Medicine Research Center, Mashhad University of Medical Sciences, Mashhad 9176699199, Iran

^d Department of Radiology, School of Paramedical Science, Shiraz University of Medical Sciences, Shiraz, 7143918596, Iran

^e Department of Medical Genetics, School of Medicine, Shiraz University of Medical Sciences, Shiraz 7134845794, Iran

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ABSTRACT

Nanosized materials demonstrate considerable promise in sonodynamic therapy (SDT), particularly in addressing the limitations of conventional cancer treatments. This study focuses on the synthesis of bismuth oxide and folic acid (FA)-functionalized bismuth oxide NPs (FA-bismuth oxide NPs), using *Rheum turkestanicum* extracts as an eco-friendly method. Characterization of the synthesized NPs was performed using powder X-ray diffraction, field emission scanning electron microscopy, energy dispersive X-ray spectroscopy, Fourier-transform infrared spectroscopy, dynamic light scattering, zeta potential measurement, and UV-vis spectroscopy. Crystallite sizes of bismuth oxide and FA-bismuth oxide NPs were obtained as 43.42 and 57.90 nm, respectively, suggesting that FA incorporation facilitated particle growth. Additionally, FA modified the morphology of bismuth oxide NPs, transforming them from an amorphous structure to a conical-like shape in FA-bismuth oxide NPs. SDT effects of bismuth oxide and FA-bismuth oxide NPs on C540 (B16/F10) melanoma cells were evaluated by *in vitro* cytotoxicity assays, and reactive oxygen species (ROS) generation, glutathione degradation, apoptosis induction, mitochondrial membrane potential, cell migration suppression, and spheroid formation analyses. *In vivo* hemocompatibility in balb/c mice was also performed. Notably, FA-bismuth oxide NPs produced elevated level of ROS, leading to a marked reduction in intracellular glutathione level, and increased apoptosis via mitochondrial membrane potential decrement. The FA-bismuth oxide NPs also had *in vivo* hemocompatibility, and demonstrated *in vitro* enhanced therapeutic efficacy by significant inhibiting both cell migration and deep penetration in the spheroid. These findings established FA-bismuth oxide NPs as a highly effective sonosensitizer, offering potential advantages for SDT-based cancer treatments.

1. Introduction

Skin cancer is a common malignancy characterized by abnormal cell proliferation in the epidermis (Choi et al., 2020). Among its various types, melanoma accounts for a significant proportion of skin cancer-related deaths (He et al., 2023). This aggressive tumor

* Corresponding author at: Nanomedicine and Nanobiology Research Center, Shiraz University of Medical Sciences, Shiraz 7193635899, Iran.
E-mail addresses: sattarahmady@yahoo.com, nsattar@sums.ac.ir (N. Sattarahmady).

originates from melanocytes and may develop on previously normal skin, within a mole, or on mucosal surfaces such as the oral cavity and eyes (Canaparo et al., 2022a). Melanoma accounts for approximately 40 % of global skin cancer cases (Blasco-Chamarro and Farinas, 2023). Standard treatment options for melanoma include surgery, chemotherapy, immunotherapy, and radiation therapy. However, conventional radiotherapy and chemotherapy often lead to severe side effects and damage healthy tissues, emphasizing the need for alternative therapeutic approaches with higher efficacy and lower toxicity.

Ultrasound (US)-induced sonodynamic therapy (SDT) has emerged as a noninvasive strategy with deep penetration ability for treating solid tumors, including those located within deeper tissues (Zainudin et al., 2020). SDT involves ultrasonic cavitation, thermal effects, and reactive oxygen species (ROS) production, all of which contribute to cancer cell destruction (Sol et al., 2024). US's mechanical energy can activate sonosensitizers within the tumor microenvironment (TME) (Lin et al., 2020), eliciting enhanced anti-tumor effects. During SDT, non-inertial cavitation involves stable oscillation of microbubbles without collapse, allowing prolonged interaction with surrounding tissues (Canaparo et al., 2022b). In contrast, inertial cavitation occurs when microbubbles rapidly collapse, leading to the generation of localized high temperatures and pressures. These processes lead to the formation of ROS, which can induce oxidative stress, DNA damage, and apoptosis in cancer cells (Khavari et al., 2023). Factors such as hypoxia, the presence of antioxidant enzymes, and an immunosuppressive milieu within the TME reduce ROS accumulation and dampen SDT-induced cytotoxicity (Mendoza et al., 2025). Recent findings have shown that semiconductor-based sonosensitizers such as TiO_2 - (Perota et al., 2022; Haghighi et al., 2024), ZnO - (Liu et al., 2020), and Bi - (Wu et al., 2023) based ones can significantly increase ROS generation under US radiation, thereby improving SDT outcomes. Research has demonstrated that engineering bismuth oxide NPs with specific dopants or surface modifiers can significantly enhance their sonodynamic properties and overcome TME-induced resistance (Chen et al., 2024).

Bismuth oxide nanoparticles (NPs) have attracted attention due to their favorable biocompatibility and minimal systemic toxicity, tunable surface properties, synthesis ability in various sizes and morphologies, making them versatile for biomedical applications of tissue engineering, bioimaging, and cancer therapy (Nethravathi et al., 2023; Alqathami et al., 2016). These NPs represent anticancer, antibacterial, and antifungal activities, and exhibit promising radiosensitizing properties and selective toxicity toward cancer cells (Zainudin et al., 2020; Alqathami et al., 2016). Photocatalytic activity of bismuth oxide NPs has also been linked to enhanced ROS production, which plays a crucial role in oxidative stress-mediated cancer cell death (Dinic et al., 2022). This functionality positions bismuth oxide NPs as promising candidates for photodynamic therapy (PDT) and SDT. Bismuth oxide NPs can be synthesized in diverse sizes and shapes to enhance their physicochemical properties, opening new possibilities for advanced medical applications (Zainudin et al., 2020; Nethravathi et al., 2023; Alqathami et al., 2016).

Nanotechnology-based materials have gained traction in cancer diagnosis and therapy (Negahdary et al., 2020; Karimi et al., 2020; Nazari-Vanani et al., 2024; Morovat et al., 2025) due to their potential for sensing specific biomarkers and crucial role in diverse tumor treatment strategies, such as immunotherapy, targeted therapy, physical ablation, gene therapy, PDT, and SDT, alongside improved biocompatibility (Canaparo et al., 2022b; Alqathami et al., 2016; Karimi et al., 2025; Heli and Rahi, 2016). Despite the nanoscale size, NPs can encapsulate numerous small-molecule compounds, due to their large surface area, which allows for effective functionalization with various ligands, including small molecules, DNA or RNA strands, and peptides (Negahdary et al., 2020, 2019; Heli and Rahi, 2016). The multifunctional nature of NPs, combined with their minimal adverse reactions and superior efficacy, sets them apart from conventional therapeutic approaches. Although the production and use of nanosized particles have a long history, nanomedicine has been formally recognized as a multidisciplinary field, marking a significant evolution in contemporary medical practices.

The objectives of this research are: (i) synthesis of bismuth oxide NPs by a green synthesis method using *Rheum turkestanicum* extract; (ii) Synthesis of folic acid (FA)-functionalized bismuth oxide NPs (FA-bismuth oxide NPs); (iii) characterization of bismuth oxide NPs and FA-functionalized bismuth oxide NPs; (iv) assessing *in vitro* biocompatibility and selective cytotoxicity of NPs against C540 cancerous and NIH3T3 normal cells; (v) evaluation of sonosensitizing efficacy of NPs by measuring extracellular/intracellular ROS, intracellular GSH, mitochondrial membrane potential, apoptosis/necrosis, migration inhibition, and therapeutic performance in 3D spheroid models.

2. Methods and materials

2.1. Extraction of *Rheum turkestanicum*

Rheum turkestanicum was collected around Mashhad, Khorasan province (Iran). The plant identification was carried by herbarium of Zabol University (Iran). The wood of *Rheum turkestanicum* was first powdered, and a precisely weighed amount of the powder was mixed with distilled water in a 1:10 ratio (w/v). The mixture was stirred at 150 rpm for 18 h to facilitate the extraction process. Subsequently, the extract was filtered using a filter paper to remove insoluble residues. The resulting filtrate was collected and utilized for further use.

2.2. Synthesis of bismuth oxide NPs

For the synthesis of bismuth oxide NPs, the aqueous extract of *Rheum turkestanicum* was initially diluted with distilled water 2.5 times. The diluted solution was then heated in a water bath at 70 °C. A solution of bismuth nitrate ($\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$, Merck, Germany) was gradually added to the extract while stirring. The reaction mixture was stirred continuously for 3 h, followed by filter-separation of the sediment. The sediment was dried at 90 °C for 15 h. The dried sample was then subjected to calcination in a furnace at 500 °C for 2 h to obtain the final bismuth oxide NPs (supplementary material S1).

2.3. Synthesis of FA-functionalized bismuth oxide (FA-bismuth oxide) NPs

For bismuth oxide NPs functionalization, 0.1 g of the synthesized bismuth oxide NPs was dispersed in 50 mL of distilled water. Then, 2.5 mmol L⁻¹ FA and 0.5 mmol L⁻¹ sodium hydroxide (Merck, Germany) were added. The mixture was refluxed for 4 h and dried at 90 °C for 10 h. The final yellow powder (FA-bismuth oxide NPs, [supplementary material S1](#)) was collected for further characterization and use.

2.4. Characterization of NPs

The structure, morphology, and physicochemical properties of the synthesized NPs were characterized using various analytical techniques. The crystalline structure was determined by powder X-ray diffraction (PXRD), using a PANalytical X'Pert PRO MPD system (the Netherlands) with Cu-K α radiation. Optical properties were analyzed by UV-vis absorption spectroscopy using a Shimadzu UV-1800 double-beam spectrophotometer (Japan). Surface morphology and particle size were assessed by field emission scanning electron microscopy (FESEM), using a ZEISS Sigma 300 system (Germany), equipped with an OXFORD X-MaxN 80 EDX detector, operating at an accelerating voltage of 15 kV. Molecular composition of the samples was analyzed by a Fourier-transform infrared (FTIR) spectrometer of Bruker Tensor 27 (Germany). Hydrodynamic diameters and zeta potential of bismuth oxide and FA-bismuth oxide NPs in phosphate-buffered saline (PBS) and human serum (diluted with PBS in a 1:1 ratio) were measured by a SZ-100-Z2 HORIBA instrument (Japan). Also, long-term stability of FA-bismuth oxide NPs in Dulbecco's Modified Eagle Medium (DMEM) from Gibco (USA) containing 10 % fetal bovine serum (FBS) were evaluated by measuring hydrodynamic diameters.

2.5. In vitro hemolysis assay and serum stability

Fresh human blood samples were diluted with PBS at a 3:5 ratio. Bismuth oxide and FA-bismuth oxide NPs were prepared in PBS at five concentrations (10, 50, 100, 250, and 500 $\mu\text{g mL}^{-1}$). Each NPs solution (250 μL) was placed in a vial and incubated at 37 °C for 30 min. Deionized water and PBS were served as positive and negative controls, respectively. Next, 100 μL of diluted blood samples were added to each vial, and incubated at 37 °C for 2 h. Following incubation, the samples were centrifuged at 5000 rpm for 10 min to separate intact erythrocytes. The optical density (OD) of the supernatants was measured at 540 nm using a microplate reader of Biotek (USA) to determine the hemolysis rate (HR%) by [Eq. \(1\)](#):

$$\text{HR\%} = (\text{sample OD} - \text{negative control OD}) / (\text{positive control OD} - \text{negative control OD}) \quad (1)$$

2.6. Cytotoxicity and uptake assay

C540 (B16/F10) cell line derived from a mouse malignant melanoma cells, and NIH3T3 normal fibroblast cell line were obtained from Pasteur Institute of Iran. The cells were cultivated in DMEM, containing 10 % FBS and 1 % antibiotic solution containing penicillin and streptomycin from Gibco (USA). The cells were cultivated at a temperature of 37 °C in a humidified atmosphere, with 5 % CO₂ in an incubator.

The cells were treated with varying concentrations of bismuth oxide, FA-bismuth oxide NPs (10–500 $\mu\text{g mL}^{-1}$) and FA (without NPs) in DMEM for 24 h in triplicate. Following incubation, the culture medium was replaced with fresh DMEM, and 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg mL⁻¹) was added to each well. The cells were incubated for 4 h, after which 100 μL of dimethyl sulfoxide was added to dissolve the formazan crystals. OD of the wells' content was measured at 570 nm using a microplate ELISA reader of Biotek (USA). All assessments were performed in triplicate. Untreated cells served as a control with a 100 % viability. Cell survival was calculated using [Eq. \(2\)](#):

$$\text{Cell survival\%} = \text{OD of treated sample} / \text{OD of control} \quad (2)$$

C540 cells, at a density of 1.0×10^4 cells per well, were cultured in 96-well plates for 24 h to promote cell adhesion. Following this period, the cells were divided into different groups.

The cytotoxicity and growth inhibition effects of bismuth oxide and FA-bismuth oxide NPs on the cancer cells were assessed using the MTT assay within these groups. The treatment involved the treatment of 50 $\mu\text{g mL}^{-1}$ of bismuth oxide and FA-bismuth oxide NPs in the cells. In the US+ group, after a one-hour incubation, an ultrasonic device featuring an unfocused planar US transducer was placed in direct contact with the bottom of the cell culture plate. The model of the US device was a Novin P215 (Iran) with a maximum output of 12 W and an input power of 45 W. For US+ groups, the center of the device transducer was accurately positioned beneath the wells, with radiation conducted at a power intensity of 1.0 W cm⁻² for 5 min, utilizing a duty cycle of 50 % and a frequency of 1.0 MHz. After treatment, both US+ and US- cell groups were incubated overnight at 37 °C in a humidified atmosphere of 5 % CO₂.

For uptake evaluation, FA-bismuth oxide NPs were labeled with fluorescein isothiocyanate (FITC-FA-bismuth oxide NPs, 50 $\mu\text{g mL}^{-1}$) ([Pereira-Silva et al., 2024](#)), and introduced to seeded C540 (B16/F10) and NIH3T3 cells (1.0×10^4 cells per well) in a 96-well plate for 4 h. The cells were then washed twice with PBS, fixed with cold methanol, washed again with PBS, permeabilized using 0.1 % Triton X-100 for 15 min, washed with PBS, stained with 4',6-diamidino-2-phenylindole (DAPI, 10 $\mu\text{g mL}^{-1}$) for 15 min, and again washed with PBS. Subsequently, the wells were observed under an Olympus BX61 fluorescence microscope (Japan).

2.7. Cell-free ROS production ability of NPs

To evaluate the intrinsic capacity of bismuth oxide and FA-bismuth oxide NPs to generate ROS independently of cellular interactions, the fluorescent probe H₂DCF (2',7'-dichlorodihydrofluorescein) was employed. H₂DCF was synthesized via the deacetylation of H₂DCF-DA (2',7'-dichlorodihydrofluorescein diacetate). Subsequently, H₂DCF was incubated with bismuth oxide and FA-bismuth oxide NPs at a concentration of 50 µg mL⁻¹ to evaluate their ability to catalyze its oxidation into the highly fluorescent compound DCF (2',7'-dichlorofluorescein). A stock solution of H₂DCF-DA (1.0 mmol L⁻¹) was initially prepared by dissolving the compound in methanol, followed by adding 40 mL of a 100 mmol L⁻¹ NaOH solution. The mixture was stirred for 30 min at room temperature under light-protected conditions. Subsequently, to achieve a final H₂DCF concentration of 50 µmol L⁻¹, 150 mL of a 33 mmol L⁻¹ NaH₂PO₄ solution was introduced. For fluorescence measurements, 75 µL of the 50 µmol L⁻¹ H₂DCF solution was mixed with an equal volume (75 µL) of bismuth oxide or FA-bismuth oxide NPs (50 µg mL⁻¹) in a fluorescence cuvette. US radiation was then applied at a frequency of 1.0 MHz, with an output power density of 1.0 W cm⁻² and utilizing a duty cycle of 50 % for 5 min. Following US treatment, fluorescence intensities were recorded using a microplate reader of Biotek (USA), with phosphate-buffered saline (PBS), serving as a control.

In addition, singlet oxygen (¹O₂) generation by NPs (bismuth oxide or FA-bismuth oxide NPs) under US irradiation was detected by 1,3-diphenyl isobenzofuran (DPBF). Briefly, 20 µL of DPBF solution (10 mmol L⁻¹ in DMSO) was mixed with 3 mL of NPs solution (50 µg mL⁻¹). The mixture was irradiated by US at a power intensity of 1.0 W cm⁻² for 5 min, utilizing a duty cycle of 50 %, and a frequency of 1.0 MHz. After 15 min, the samples OD at 420 nm with or without US radiation were measured using a Rayleigh UV-2601 spectrophotometer (China).

The sonochemical (SC) activity of FA-bismuth oxide NPs under US radiation was assessed by monitoring oxidation of iodide ions to triiodide ions. Briefly, 1.7 mL of a degassed NPs dispersion (50 µg mL⁻¹) or DI water with or without US radiation (1.0 W cm⁻² for 5 min, a duty cycle of 50 % at 1.0 MHz), was mixed with a KI solution (0.4 mol L⁻¹ in 0.05 mol L⁻¹ NaOH), (NH₄)₆Mo₇O₂₄·4H₂O (0.00016 mol L⁻¹), and C₈H₅KO₄ (0.1 mol L⁻¹). The concentration of triiodide ions was determined at 350 nm using a Rayleigh UV-2601 spectrophotometer (China), and an extinction coefficient of 26400 mol L⁻¹ cm⁻¹. The experiment was performed in triplicate.

2.8. Intracellular ROS production ability of NPs

To investigate the intracellular ROS-generating potential of bismuth oxide and FA-bismuth oxide NPs, we utilized the cell-permeable probe H₂DCF-DA. In this assay, cells were incubated with 50 µmol L⁻¹ H₂DCF-DA for 30 min, followed by US radiation. The cells were then washed with PBS to remove extracellular DCF and lysed using a lysis buffer. Fluorescence intensities were measured at excitation and emission wavelengths of 485 and 520 nm, respectively, using a Biotek microplate reader (USA).

2.9. Intracellular GSH depletion of NPs

Cellular GSH levels were measured in control/US-, control/US+, FA-bismuth oxide/US-, and FA-bismuth oxide/US+ groups. C540 cells were treated according to Section 2.6. The cells were then lysed with a triton X-100 solution (0.5 %) at 4 °C for 20 min, centrifuged at 1200 rpm for 5 min, and supernatants were treated with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) (10 mmol L⁻¹) to generate a yellow derivative of 5'-thio-2-nitrobenzoic acid (TNB). TNB was quantified at 412 nm using a Rayleigh UV-2601 spectrophotometer (China).

2.10. Apoptotic assay

Apoptotic induction was evaluated through fluorescence-activated cell sorting (FACS) analysis using the annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) (Annexin V-FITC/PI) staining kit (BD Pharmingen, USA) based on the manufacture protocol. C540 cells were seeded in 12-well plates at a density of 1 × 10⁵ cells per well, and treated based on Section 2.6. Following a 24-h incubation, the cells were harvested, washed twice with cold PBS, and resuspended in 100 µL of 1 × binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4). Annexin V-FITC (5 µL, 50 µg mL⁻¹) and propidium iodide (5 µL, 50 µg mL⁻¹) were added to the cell suspension and incubated for 15 min at room temperature in the dark. After staining, 400 µL of binding buffer was added, and flow cytometric analysis was conducted using a BD FACSCalibur system (USA), with apoptosis rates determined based on fluorescence data acquired from 10,000 recorded events. Instrument settings were adjusted using unstained and single-stained controls; compensation was set to minimize spectral overlap between FL1 (FITC) and FL2 (PI). FlowJo software was used for data analysis. Cell populations were divided into four quadrants: Q1 (PI+ / FITC-, necrotic), Q2 (PI+ / FITC+, late apoptotic/necrotic), Q3 (PI- / FITC+, early apoptotic) and Q4 (PI- / FITC-, viable). Total cell death (sum of apoptotic and necrotic) was calculated as the sum of Q1 + Q2 + Q3.

2.11. Mitochondrial membrane potential (MMP) detection

The JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolylcarbocyanine iodide) assay was performed to determine changes in MMP and mitochondrial damage. C540 cells were seeded at a density of 1 × 10⁵ cells per well for 24 h, and treated based on Section 2.6. Following a 24-h incubation, the cells were washed with PBS, and incubated with a JC-1 staining solution (10 µmol L⁻¹) for 20 min at 37 °C. Then the cells were washed twice with the JC-1 staining buffer, and finally, cell images were captured using an Olympus BX61

fluorescence microscope (Japan). Carbonyl cyanide 3-chlorophenylhydrazone ($100 \mu\text{mol L}^{-1}$) was incubated with non-treated cells for 30 min, and used as a positive control after staining with JC-1.

2.12. Wound healing assay

To assess the migratory potential of C540 cells, we seeded them in 96-well plates at a density of 7×10^4 cells per well during their logarithmic growth phase. The plates were incubated at 37°C in a humid atmosphere containing 5 % CO_2 until the cells reached approximately 80 % confluence. A uniform scratch was then introduced at the center of each well using a 100- μL pipette tip, followed by the acquisition of optical images. The cells were subsequently treated with bismuth oxide NPs ($50 \mu\text{g mL}^{-1}$) and FA-bismuth oxide NPs ($50 \mu\text{g mL}^{-1}$), both subjected to US radiation. Cell migration into the wounded area was monitored via optical imaging under a Nikon Eclipse microscope (Japan) at 24 h post-treatment.

2.13. Spheroid formation

In a 96-well plate, 50 μL of a 2 % autoclaved agarose solution was added to each well, followed by the introduction of C540 cells at a density of 1×10^4 cells per well. Images were captured 72 h after cell seeding under controlled incubation conditions to observe spheroid formation. Following this incubation period, the cell medium was treated with bismuth oxide and FA-bismuth oxide NPs at a concentration of $50 \mu\text{g mL}^{-1}$, US radiation (one, two, and three cycles), and a combination of both treatments.

US radiation conducted at a power intensity of 1.0 W cm^{-2} for 5 min, utilizing a duty cycle of 50 % and a frequency of 1.0 MHz, and this radiation repeated in a multi-step process with one, two, and three cycles of exposure with one-hour interval between each cycle. Subsequently, all groups were incubated overnight at 37°C in a humidified atmosphere with 5 % CO_2 . Each cell group was prepared in triplicate. The relative viability of the spheroids was evaluated using a resazurin viability assay, which involved measuring the fluorescence intensity of resazurin at an excitation wavelength of 544 nm and an emission wavelength of 600 nm, employing a Biotek microplate reader (USA).

2.14. Deep penetration of FA-bismuth oxide NPs into spheroids

FA-bismuth oxide NPs were labeled with PI (PI-FA-bismuth oxide NPs) (Pereira-Silva et al., 2024), and their deep penetration capability into the spheroids was evaluated. The spheroids were treated with PI-FA-bismuth oxide NPs at a concentration of $50 \mu\text{g mL}^{-1}$, and US radiation (1.0 W cm^{-2} for 5 min, duty cycle of 50 % and at 1.0 MHz, one cycle) with a 2-h interval, and subsequently incubated for an additional 16 h. The spheroids were then washed twice with PBS, fixed with cold methanol, rewashed with PBS, permeabilized using 0.1 % Triton X-100 for 15 min, and washed with PBS. Nuclear staining was performed using DAPI ($5 \mu\text{g mL}^{-1}$), followed by washing with PBS. Finally, images of the spheroids were captured using an Olympus BX61 fluorescence microscope (Japan).

2.15. Statistics

The experimental results were presented as average values with their corresponding standard deviations. This ensured that a minimum of three measurements were taken to validate the reliability of the outcomes. The assessment of statistical significance was performed using GraphPad Prism 10 software, which incorporated the two-tailed Student's T-test. A p -value of less than 0.05 was deemed indicative of statistical significance.

2.16. In vivo evaluation of hemocompatibility

All the *in vivo* studies were followed according to the rules of the ethics of animal experiments of Alaa nanotechnology Lab (Iran). Inbred mice of male balb/c with an age of 6 weeks and weighting about 25–30 g were prepared, and the animals were settled at $24 \pm 2^\circ\text{C}$ and 40–70 % humidity with weekly cleaning of the floors. The animals were free to get water and standard food. A 12:12 light: dark cycle was followed in the mentioned animal vivarium.

For blood analysis, bismuth oxide NPs and FA-bismuth oxide NPs ($100 \mu\text{L}$, 2 mg mL^{-1}) were intravenously injected into four healthy Balb/c mice, and the mice were euthanized 5 days following the injection. Another three healthy Balb/c mice in good health served as a control group. Fresh blood samples (day 5) were collected before the mice were euthanatized for biochemistry analyses, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine, red blood cells (RBC), white blood cells (WBC), platelet, hemoglobin (HGB), hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red cell distribution width (RDW-CV).

3. Results and discussion

3.1. Characterization of NPs

PXRD spectra for bismuth oxide and FA-bismuth oxide NPs are shown in [supplementary material S2](#). The synthesized NPs exhibit distinct peaks at 2θ values of 24.6, 25.8, 28.0, 27.5, 28.0, 33.2, 35.5, 37.7, 40.2, 45.2, 46.4, 48.6, 52.4, 54.9, 57.9, and 63.6° ,

corresponding to the diffraction planes of (1 0 2), (0 0 2), (1 1 1), (1 2 0), (0 1 2), (1 2 2), (0 3 1), (1 1 2), (2 2 2), (0 4 0), (0 4 1), (1 0 4), (3 2 1), (2 4 1), (1 4 3), and (1 5 1), respectively. These peaks are characteristic of α - Bi_2O_3 , which has a monoclinic structure (JCPDS no. 41–1449) (Hou et al., 2013). The crystalline sizes of the NPs were also calculated using Scherrer's equation (Varshney et al., 2021),

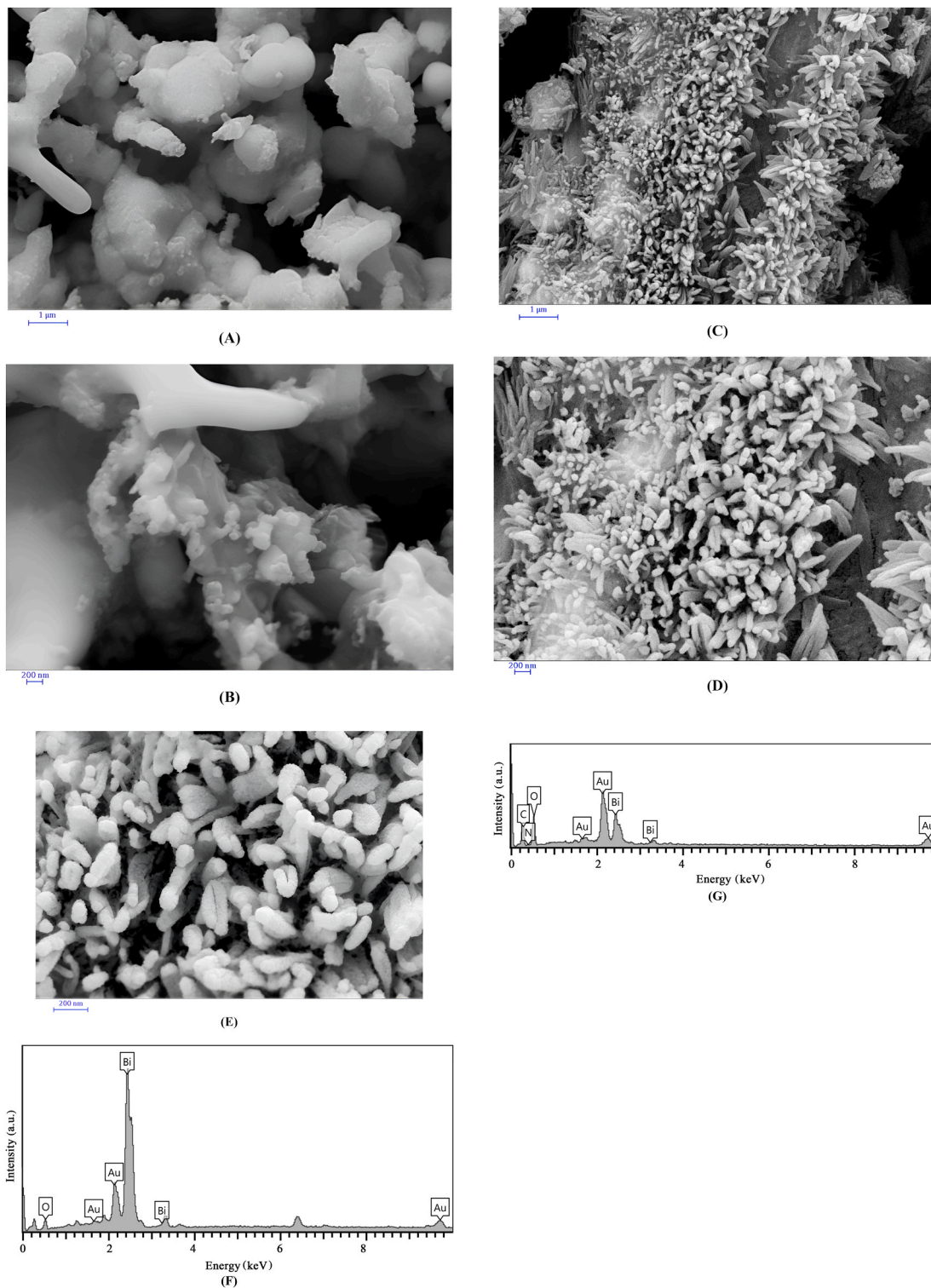


Fig. 1. (A-E) FESEM images of the synthesized bismuth oxide (A, B) and FA-bismuth oxide (C-E) NPs at two magnifications. (F, G) EDX spectra of bismuth oxide (F) and FA-bismuth oxide (G) NPs.

focusing on the (120) diffraction peaks of the NPs. The crystalline sizes for bismuth oxide and FA-bismuth oxide NPs were obtained as 43.4 and 57.9 nm, respectively.

Fig. 1 (A-E) depicts FESEM images of the synthesized bismuth oxide (A, B) and FA-bismuth oxide (C-E) NPs at two magnifications. Bismuth oxide NPs had an amorphous morphology and nonuniform size distribution. However, FA-bismuth oxide NPs had a rod-like shape in which each rod comprises survey small particles. It seems that the incorporation of FA modified the structural characteristics of FA-bismuth oxide NPs. Fig. 1 (F, G) presents EDX spectra of bismuth oxide and FA-bismuth oxide NPs. The bismuth oxide NPs spectrum confirms the presence of bismuth and oxygen. In contrast, the FA-bismuth oxide NPs spectrum indicates the presence of bismuth, carbon, oxygen, and nitrogen elements, confirming the successful incorporation of FA within the FA-bismuth oxide NPs structure.

The average hydrodynamic diameters (supplementary material S3) and zeta potentials (supplementary material S4) of bismuth oxide and FA-bismuth oxide NPs were measured in both PBS as well as human serum. Also, the average hydrodynamic diameters of FA-bismuth oxide NPs were measured in cell culture medium (DMEM+ 10 % FBS), for evaluation of the long-term stability. For bismuth oxide and FA-bismuth oxide NPs, hydrodynamic diameters of 236.8 and 246.7 nm, respectively were obtained in PBS, and 504.0 and 809.4 nm, respectively were measured in serum. Hydrodynamic diameter of 653.4 nm was obtained for FA-bismuth oxide NPs after one hour incubation in cell culture medium with $\cong 20$ % increment in the size after prolong incubation for 24 h. These sizes are suitable for penetrating and accumulating in the cancer cells since the typical C540 cells are about the micrometer scale (Cybulski et al., 2025). For bismuth oxide and FA-bismuth oxide NPs, zeta potentials of -12.5 and -20.6 mV, respectively were obtained in PBS, and 17.3 and -23.2 mV, respectively were measured in serum. These values of zeta potential ensured the good stability and dispersion of FA-bismuth oxide NPs in PBS and serum, and presence of FA induced negative shifts in zeta potential of bismuth oxide NPs. Both NPs underwent enlargement in size, and negativity in zeta potential due to adsorption of serum proteins and protein corona.

Supplementary material S5 represents UV-vis absorption spectra of bismuth oxide and FA-bismuth oxide NPs, and a 1.0 mg mL^{-1} FA dissolved in deionized water. The spectrum of bismuth oxide NPs showed an absorption peak at 230 nm related to electron transition in bismuth oxide from 4 f orbital of bismuth to 2p orbital of oxygen, which is consistent with previous reports (Varshney et al., 2021). The spectrum of FA showed a strong peak at 285 nm attributed to a $\pi \rightarrow \pi^*$ transition (El-Borady and El-Sayed, 2020). The FA-bismuth oxide NPs show a blue shift of this peak to 260 nm, compared to the FA spectrum, indicating a change in the FA environment. These results further confirmed the successful attachment of FA to bismuth oxide NPs. The band gap energy (E_g) of NPs was calculated using Eq. (3):

$$(\alpha h\nu)^2 = A (h\nu - E_g) \quad (3)$$

where α represents the absorption coefficient, $h\nu$ denotes the photon energy, and A is a constant value (independent of photon energy). E_g was obtained by the lines depicted in supplementary material S5 for bismuth oxide and FA-bismuth oxide NPs as 3.27 and 2.95 eV, respectively. This reduced band gap for FA-bismuth oxide NPs inhibits the rapid recombination of excited electrons and holes, thereby improving the quantum yield of ROS generation (Cao et al., 2019).

Supplementary material S6 shows FT-IR spectra for bismuth oxide and FA-bismuth oxide NPs, and FA. Bismuth oxide NPs spectrum exhibited a broad peak in the $3410\text{--}3480 \text{ cm}^{-1}$ range due to hydroxyl groups stretching of adsorbed water, two peaks at 431 and 520 cm^{-1} attributed to the Bi-O stretching vibration, and a peak at 640 cm^{-1} corresponding to the Bi-O-Bi bond vibrational stretching. The findings align with those reported elsewhere (Li et al., 2018), confirming the presence of $\alpha\text{-Bi}_2\text{O}_3$. FA-bismuth oxide NPs spectrum showed similar Bi-O and Bi-O-Bi stretching peaks at 475 and 580 , and 622 cm^{-1} , respectively. Furthermore, peaks in 1718 , 1621 , and 1396 cm^{-1} corresponded to FA, as they appeared in the pure FA spectrum.

Assessment of blood compatibility is an essential aspect in the evaluation of biomaterials, as their exposure to the blood environment has the potential to harm erythrocytes or result in thrombus formation. Evaluation of the compatibility of bismuth oxide and FA-bismuth oxide NPs with blood was conducted using an *in vitro* hemolytic assay (supplementary material S7). The assay demonstrated that the hemolysis percentage of both bismuth oxide and FA-bismuth oxide NPs was below 5 %, indicating their biocompatibility according to ISO/TR 7406 guidelines. These results suggest that the bismuth oxide and FA-bismuth oxide NPs do not significantly damage erythrocytes, making them safe for systemic use. However, further studies on toxicity are necessary to establish a comprehensive safety profile.

3.2. Cytotoxicity and radiosensitizing effects of NPs on C540 cells

To assess the biocompatibility and potential off-target toxicity of FA-bismuth oxide NPs, we investigated their effects on NIH3T3 normal fibroblast cells using the MTT assay. The viability of NIH3T3 cells remained high following 24-h exposure to FA-bismuth oxide NPs at concentrations of $10\text{--}500 \text{ }\mu\text{g mL}^{-1}$ (supplementary material S8).

Cytotoxicity of bismuth oxide and FA-bismuth oxide NPs was evaluated on C540 cells using the MTT assay at various concentrations ($10\text{--}500 \text{ }\mu\text{g mL}^{-1}$). C540 cells that received no treatment were considered as a control group. The results are shown in Fig. 2 (A). Statistical analyses (supplementary material S9) revealed a significant difference between the treatment and control groups (p -value < 0.0001). Notably, no significant toxicity was observed at concentrations below $50 \text{ }\mu\text{g mL}^{-1}$ for bismuth oxide NPs as well as $25 \text{ }\mu\text{g mL}^{-1}$ for FA-bismuth oxide NPs. Cell survival decreased as the concentration of bismuth oxide and FA-bismuth oxide NPs increased in a concentration-dependent manner. At $50 \text{ }\mu\text{g mL}^{-1}$, cell survival decreased to 55.6 % for bismuth oxide NPs and 39.8 % for FA-bismuth oxide NPs. IC_{50} values were calculated to be $72.6 \text{ }\mu\text{g mL}^{-1}$ for bismuth oxide NPs and $21.2 \text{ }\mu\text{g mL}^{-1}$ for FA-bismuth oxide NPs. Therefore, the presence of FA enhanced the cytotoxicity of bismuth oxide NPs, likely due to improved NPs

internalization via folate-mediated endocytosis. To evaluate the effect of free FA (without NPs) on C540 cells, the effect of different concentrations of FA (in equal amounts to FA-bismuth oxide NPs) on C540 cell toxicity (supplementary material S10). As an example, at $4.5 \mu\text{g mL}^{-1}$ FA (equal to the FA content of $50 \mu\text{g mL}^{-1}$ of FA-bismuth oxide NPs), cell survival decreased to 80.5 %. Considering the lack of FA receptor on the NIH3T3 normal fibroblast cells, and the results of toxicity of FA-bismuth oxide NPs on these cells (vide supra), it can be further confirmed that FA-bismuth oxide NPs induced cytotoxicity in C540 cells through their folate-receptors. At $50 \mu\text{g mL}^{-1}$ FA-bismuth oxide NPs maintained a cell viability of 92.4 % for NIH3T3 normal fibroblast cells (supplementary material S8), markedly higher than a 39.8 % viability observed for C540 cells (supplementary material S9). This indicated negligible FA-bismuth oxide NPs cytotoxicity toward the normal cells. This biocompatibility is likely attributed to the targeted behavior and reduced non-specific uptake of FA-bismuth oxide NPs to healthy cells. These findings supported the therapeutic index potential and biosafety of FA-bismuth oxide NPs for further translational developments. For further confirmation, cellular uptake of FITC-FA-bismuth oxide NPs in NIH3T3 and C540 cells was evaluated by fluorescence imaging (Supplementary material S12). In such the assays, nuclear staining using DAPI confirms cells localization, and FITC fluorescence signals imply NPs uptake by the cells. Based on the recorded images (Supplementary material S12), C540 cells showed strong intracellular green fluorescence indicating a high uptake of FA-bismuth oxide NPs into the C540 cells. However, NIH3T3 showed negligible FITC signal, and therefore, FA-bismuth oxide NPs had a negligible uptake into the NIH3T3 cells.

Various types of mammalian cell lines have represented different responsiveness toward bismuth oxide NPs (Abudayyak et al., 2017). An IC_{50} value of $200 \mu\text{g mL}^{-1}$ was reported for bismuth oxide NPs toward MCF7 cells (Ahamed et al., 2019). In this study, bismuth oxide NPs with a band gap of 2.82 eV induced ROS generation, lipid peroxidation, GSH depletion, decreased superoxide dismutase activity, and apoptosis via lowering MMP, along with a higher expression ratio of *bax/bcl-2* genes. Bismuth oxide NPs in another study represented IC_{50} values of $310 \mu\text{g mL}^{-1}$ toward a 2D monolayer of HCT116 cells and $> 1000 \mu\text{g mL}^{-1}$ for its 3D spheroid (Hu et al., 2025). At those conditions, bismuth oxide NPs induced anticancer effect by inducing apoptosis, inhibiting cell migration and

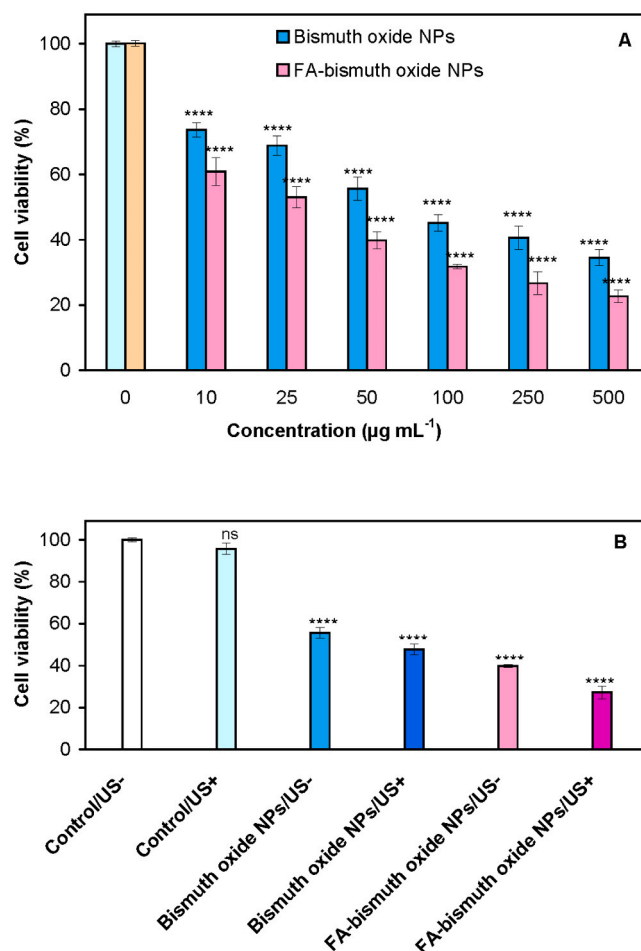


Fig. 2. *In vitro* viability percents of C540 cells. (A) Cytotoxicity of bismuth oxide and FA-bismuth oxide NPs at different concentrations of 0, 10, 25, 50, 100, 250 and $500 \mu\text{g mL}^{-1}$ without US irradiation. (B) Cytotoxicity of bismuth oxide and FA-bismuth oxide NPs at $50 \mu\text{g mL}^{-1}$ with or without US radiation. $n = 3$ and **** indicates very significant differences (p -value < 0.0001). Summary of the exact p -value are presented in supplementary materials S9 and S11.

mutation, cell cycle arrest at the G2 phase by targeting CDK2, and ROS production. Zhou et al. reported IC_{50} values of $48.93 \mu\text{g mL}^{-1}$ and $> 200 \mu\text{g mL}^{-1}$ for green synthesized bismuth oxide NPs against HCT116 and normal HK-2 cells, respectively (Zhou et al., 2022). The observed anticancer effect was related to apoptosis mediated by the regulation of the PI3K/AKT/mTOR signaling pathway. Bismuth oxide particles of mixed morphologies with a size ranging from 140 to 317 nm and a band gap of 3.11 eV represented an IC_{50} value of $47 \mu\text{g mL}^{-1}$ toward human A375 melanoma cells, and acted through ROS generation (Chaki Borrás et al., 2023). In another study, Anushya et al (Anushya et al., 2023). reported apoptosis induction in colorectal HCT116 cells by green synthesized bismuth oxide NPs in the former study, and cell shrinkage, nuclear fragmentation and chromatin condensation in lung A549 cells by reduced graphene oxide-bismuth oxide nanocomposite in the later one. Folate-functionalized nanomaterials, such as selenium NPs, combined with MAPK inhibitors in melanoma treatment, displayed a synergistic effect in reducing viability, cell cycle arrest, and apoptosis in BRAF mutant cancer cells with targeting through folate receptor (Bidkar et al., 2019). Another study showed that folate-functionalized photosensitizing agent carrying micelles exhibited potent anti-tumor activity toward B16F10 melanoma cells (Ma et al., 2019).

To examine the impact of bismuth oxide and FA-bismuth oxide NPs radiated with US, cells were cultured for 24 h in 96-well plates and divided into the groups: control/US- (neither US radiation nor NPs), control/US+ (US radiation without NPs), bismuth oxide NPs/US- (no radiation with bismuth oxide NPs), bismuth oxide NPs/US+ (US radiation with bismuth oxide NPs), FA-bismuth oxide/US- (no radiation with FA-bismuth oxide NPs), and FA-bismuth oxide/US+ (US radiation with FA-bismuth oxide NPs). The NPs concentration in the related groups was $50 \mu\text{g mL}^{-1}$. The results are presented in Fig. 2 (B) and supplementary material S11, showing a significant decrease in cell survival in bismuth oxide/US+ and FA-bismuth oxide/US+ groups. This indicates that US radiation enhanced the cytotoxicity of NPs in a manner that FA-bismuth oxide NPs showed higher cell killing activity (27.2 %), compared to bismuth oxide NPs (47.8 %) under US radiation. Therefore, FA improved the cytotoxicity of bismuth oxide NPs when combined with US radiation, and FA-bismuth oxide NPs are an effective sonosensitizer.

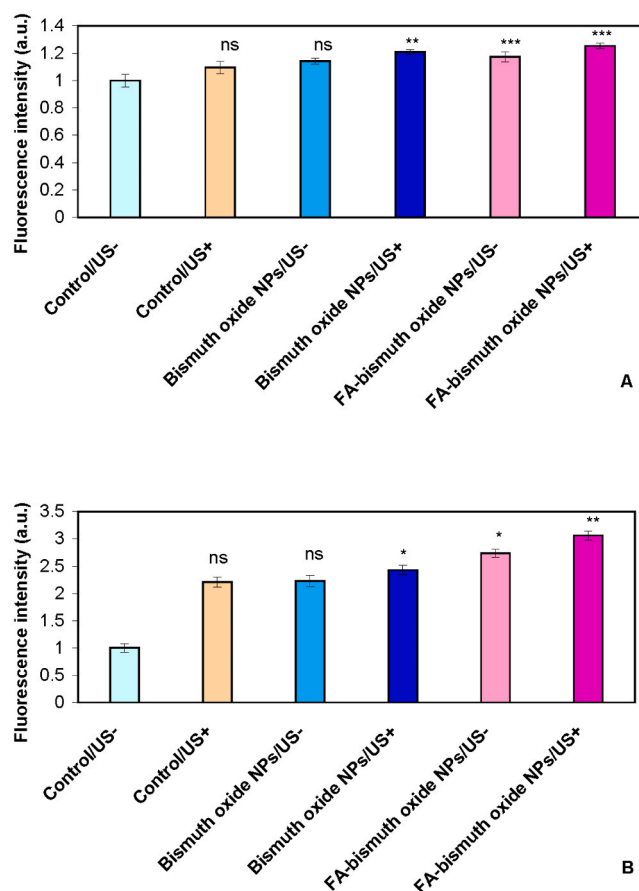


Fig. 3. Relative F.I. values of H_2DCF in bismuth oxide and FA-bismuth oxide NPs at $50 \mu\text{g mL}^{-1}$ with or without US radiation in acellular (cell-free, A) and intracellular (B) conditions. $n = 3$, ns indicates non-significant differences, and *, ** and *** indicate significant (p -value < 0.05), very significant (p -value < 0.01), and high significant (p -value < 0.001) differences, respectively. Summary of the exact p -value are presented in supplementary material S11.

3.3. ROS generation and intracellular GSH degradation assays

A cellular generation of ROS by bismuth oxide and FA-bismuth oxide NPs at a concentration of $50 \mu\text{g mL}^{-1}$, either in the absence or presence of US radiation under cellular conditions, was assessed utilizing the H_2DCF assay. Fig. 3 (A) and [supplementary material S11](#) represent relative fluorescence intensity (F.I.) values of H_2DCF in the control, bismuth oxide NPs, and FA-bismuth oxide NPs groups upon US radiation. The findings revealed that F.I., as indicative of ROS generation levels, for the FA-bismuth oxide/US- group (1.17-fold), produced a higher level of ROS than the bismuth oxide/US- group (1.14-fold), compare to control. US radiation further enhanced ROS generation in the FA-bismuth oxide/US+ group compared to bismuth oxide/US+, indicating that the combination of US and FA-bismuth oxide NPs was more effective in generating ROS.

DPBF was employed as a singlet oxygen probe to detect the generation ability of $^1\text{O}_2$ by bismuth oxide and FA-bismuth oxide NPs upon US radiation. The results of the assay are displayed in [supplementary material S13](#). DPBF was oxidized upon US irradiation of both bismuth oxide and FA-bismuth oxide NPs, with a higher rate attained for FA-bismuth oxide NPs, and therefore prominent ability of the latter NPs to generate $^1\text{O}_2$.

Iodide oxidation was used for SC efficiency evaluation. Hydroxyl radicals generated during US radiation oxidized the iodide ions, and initiated a cascade of chemical reactions that produce triiodide ions. The results of this assay are presented in [supplementary material S13](#) indicating the SC potential of FA-bismuth oxide NPs under US radiation. The SC efficiency serve as an indirect assay of US-induced cavitation activity of FA-bismuth oxide NPs.

Intracellular generation of ROS by bismuth oxide and FA-bismuth oxide NPs at a concentration of $50 \mu\text{g mL}^{-1}$, either in the absence or presence of US radiation in C540 cells, was assessed utilizing the H_2DCF assay. Fig. 3 (B) and [supplementary material S11](#) represent the results, indicating F.I. for the FA-bismuth oxide/US- group (2.7-fold) was higher than the bismuth oxide/US- group (2.2-fold). The combination with US radiation further increased ROS levels in the FA-bismuth oxide/US+ group (3.1-fold) compared to bismuth oxide/US+ (2.4-fold), suggesting enhanced cytotoxicity. Therefore, the sonosensitizer had a significant impact on both extracellular and intracellular ROS generation in agreement with the findings of the MTT assay.

Bismuth oxide NPs function as a sonocatalyst, and when subjected to US waves, their electrons transition from the valence band to the conduction band, resulting in the formation of electron-hole pairs. The generated electrons cause the creation of superoxide radicals, while the holes give rise to hydroxyl radicals. On the other hand, FA-bismuth oxide NPs with a lowered band gap produce higher electron-hole pairs, subsequently higher radicals ([Haghighi et al., 2024](#)).

The mitochondria are the primary target for damaging by ROS. Mitochondrial damage can be monitored by measuring the intracellular GSH level. Intracellular GSH levels were quantified using DTNB/TNB conversion, and the results are presented in [supplementary material S14](#). Based on the results, control/US+, FA-bismuth oxide/US-, and FA-bismuth oxide/US+ groups showed negligible, 59.2 % and 82.7 % reduction in GSH level, respectively. Therefore, US radiation alone had minimal disruption of the intracellular redox balance, and FA-bismuth oxide had the potential to induce mitochondria damage upon US radiation.

3.4. Apoptotic assay

The Annexin V-FITC/PI staining method is utilized to distinguish between viable cells, early apoptotic cells, and those undergoing late apoptosis or necrosis. During the initial phases of apoptosis, phosphatidylserine moves to the outer layer of the cell membrane, disrupting its symmetry as it binds to annexin V, a calcium-dependent protein that binds phospholipids. This binding results in a characteristic green fluorescence signal. Simultaneously, PI staining enables to detection of late apoptotic or necrotic cells by producing a red fluorescence signal, which correlates with morphological changes and a loss of membrane integrity.

[Supplementary material S15](#) illustrates dot plots of FL1 versus FL2 derived from the flow cytometric analysis of C540 cells, conducted 24 h post-treatment with bismuth oxide and FA-bismuth oxide NPs without and with US radiation. Distribution of various stages of necrosis, late apoptosis, early apoptosis, and viable cells is also depicted in [supplementary material S15](#). These findings indicated that the FA-bismuth oxide/US+ group demonstrated that a significant 80.2 % of the cells underwent death (sum of apoptotic and necrotic) in contrast to the bismuth oxide NPs/US-, bismuth oxide NPs/US+, and FA-bismuth oxide NPs/US- groups, which showed cell death percentages of 61.1 %, 73.6 %, and 73.8 %, respectively. This suggests that US radiation in combination with FA-bismuth oxide NPs enhanced apoptosis in C540 cells. The results obtained from the MTT assay and ROS production confirmed those obtained for cell apoptosis. FA-bismuth oxide NPs, elevate ROS levels, and thereby promote increased cell apoptosis.

3.5. MMP detection

For evaluation the effect of FA-bismuth oxide on MMP, the JC-1 assay was performed, and the obtained results are shown in the [supplementary material S16](#). Control/US- and control/US+ groups showed strong red stain, FA-bismuth oxide/US- group showed transition from red to green fluorescence, and FA-bismuth oxide/US+ showed strong green fluorescence. Decrement in the fluorescence intensity of red stain, compared to green one in FA-bismuth oxide/US+ group, indicated mitochondrial depolarization of C540 cells. These results in combination with those obtained for singlet oxygen generation detection suggested that FA-bismuth oxide/US+ treatment led to $^1\text{O}_2$ generation in the cells mitochondria and MMP decrement.

3.6. Wound healing assay

Cell migration is essential for processes like embryonic development, wound healing, angiogenesis, and tumor metastasis. The actin

cytoskeleton primarily facilitates this migration, while microtubules modulate the directional movement of fibroblasts and cancer cells (Kauanova et al., 2021). The effect of bismuth oxide and FA-bismuth oxide NPs without and with US radiation on cell migration was assessed using a wound healing assay. Photographic evidence from different experimental groups is presented in [supplementary material S17](#). The cells in the bismuth oxide NPs/US+, FA-bismuth oxide NPs/US-, and FA-bismuth oxide NPs/US+ groups exhibited limited movement into the wound area after 24 h, in comparison to the control/US- group. Notably, bismuth oxide NPs without US radiation did not yield any significant results, compared to the controls, and could not inhibit cell migration. However, the presence of FA in FA-bismuth oxide NPs contributed to reduced migration, likely due to the enhanced NPs internalization that improved therapeutic efficiency. Also, the combination effect of US radiation with bismuth oxide and FA-bismuth oxide NPs inhibited migration efficiently, as illustrated in [supplementary material S17](#).

3.7. Spheroid formation

The limitations of 2D cell culture to mimic the challenges of NPs in targeting specific cell populations have led to the use of 3D models like tumor spheroids. Spheroids effectively replicate key features of solid tumors, including tissue heterogeneity, cellular mechanics, nutrients, and oxygen gradients, providing a more realistic environment for studying tumor growth and drug responses (Ahmed-Cox et al., 2022). The reduced spheroid proliferation compared to 2D cell culture results to decreased cytotoxic effects. In 2D cultures, cells experienced stable oxygen levels, while spheroids limited oxygen diffusion, resulting in lower ROS production (Pereira et al., 2017). [Fig. 4](#) and [supplementary material S11](#) show the impact of bismuth oxide and FA-bismuth oxide NPs without or with US radiation on spheroid viability. These results indicated that the bismuth oxide NPs/US- and FA-bismuth oxide NPs/US- groups resulted in 6.2 % and 15.6 % reduction in viability, respectively. However, US radiation led to further reductions of 14.8 % and 34.6 %. The combination of US radiation and bismuth oxide and FA-bismuth oxide NPs effects resulted in enhanced cytotoxicity in the 3D spheroid model. To enhance the therapeutic index in FA-bismuth oxide NPs/US+ group, US radiation cycles were prolonged for two and three cycles, and the results ([Fig. 4](#)) showed viability reduction to 19.6 and 14.1 %, respectively.

Additionally, it is worth mentioning that FA also played a key role in decreasing survival rates, which aligned with the findings from other experiments. Similar behavior of spheroids, including factors such as hypoxia, modified metabolic processes, and the impact of the extracellular matrix, closely resembles the *in vivo* condition. This validated the accurate assessment of the potential of therapeutic efficiency of bismuth oxide and FA-bismuth oxide NPs in conjunction with US radiation for probable clinical applications.

Images captured from the deep penetration ability of PI-FA-bismuth oxide NPs into spheroids are shown in [supplementary material S18](#). In the images, red fluorescence dots originated from PI-FA-bismuth oxide that diffused throughout the spheroid, while the area of spheroid was blue staining with DAPI. This distribution suggests that FA-bismuth oxide NPs have a desirable capability to penetrate inside the melanoma spheroids.

A summary of outcomes of SDT using FA-bismuth oxide NPs is presented in [supplementary material S19](#), and compared with other bismuth oxide-based sonosensitizers. Compared to already reported Bi₂O₃-based sonosensitizers, which predominantly feature complex heterostructures such as ellipsoidal heterojunctions, floral assemblies, and mesoporous nanospheres, FA-bismuth oxide NPs presented a distinct morphological profile of uniform conical/rod-like structures. This well-defined architecture may facilitate enhanced cellular internalization. Regarding surface engineering, while prior systems employ coatings such as polydopamine, mPEG-SH, or PVP to improve the colloidal stability or modulate electronic properties. The functionalization of FA-bismuth oxide NPs with FA confers active targeting specificity toward folate receptor-overexpressing malignancies, a direct strategy compared to passive or non-

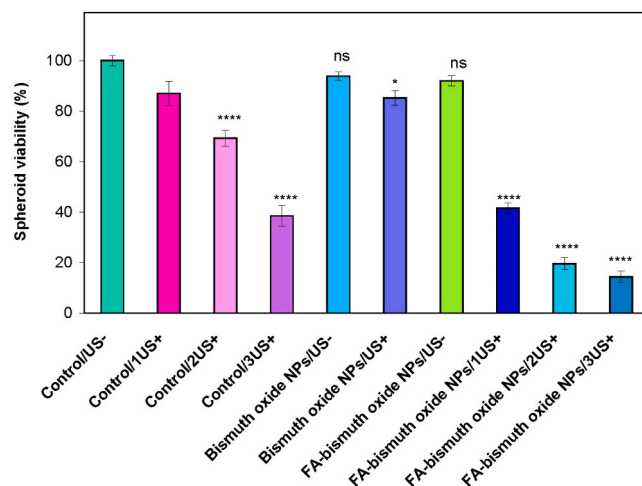


Fig. 4. Spheroid viability percents of C540 cells after treatment with bismuth oxide and FA-bismuth oxide NPs at $50 \mu\text{g mL}^{-1}$ with or without US radiation. $n = 3$ and ns and ** indicate non-significant and very significant (p -value < 0.01) differences, respectively. Summary of the exact p -value are presented in [supplementary material S11](#).

specific polymer modifications.

The principal advantages of FA-bismuth oxide NPs are evident in its synthetic route and biological activity. In contrast to the multi-step hydrothermal, coprecipitation, or sol-gel methods used for synthesis, FA-bismuth oxide NPs were synthesized via a single-step green protocol using *R. turkestanicum* extract, offering significant benefits in sustainability and biocompatibility. Although its ROS generation and apoptosis pathway mechanism are not explicitly detailed, its potent sonodynamic efficacy was demonstrated by a significant inhibition of migration and 3D spheroid growth in C540 melanoma models. This targeted activity against a clinically aggressive cancer type, validated in both monolayer and spheroid cultures. It underscores a promising anti-metastatic potential not as prominently featured in the earlier systems, which were largely evaluated in murine breast cancer (4T1) models. Therefore, FA-bismuth oxide NPs emerged as a targeted, eco-friendly, and synthetically efficient sonosensitizer with compelling preclinical performance in a distinct therapeutic context.

Recent *in vivo* studies provided clear evidence that engineered bismuth-based nanomaterials can actively modulate TME and overcome TME-mediated resistance to therapy. Chen et al. showed that Bi₂O₃ NPs within the Au-Bi₂O₃ nanoheterojunction effectively depleted overexpressed GSH in TME, disrupting its redox balance and weakening antioxidant defenses. This GSH consumption synergized with US-induced ROS and photothermal heat generation to induce immunogenic cell death (ICD), thereby reversing the TME's immunosuppressive state and promoting immune cell infiltration for systemic antitumor immunity (Chen et al., 2024). Yang et al. developed PEG-modified BiVO₄ nanorods as a sonosensitizer that modulated tumor hypoxia and enhanced US-triggered ROS production, providing in-tumor evidence that bismuth-based NPs can restore SDT efficacy in oxygen-deficient environments (Yang et al., 2022). Zhao et al. showed that Bi₂Te₃@PEG superstructures produced effective second near-infrared photothermal ablation in U14 tumor-bearing mice. Treated animals showed tumor removal without systemic toxicity (stable body weight, normal organ histology and blood parameters), supporting a safe, and imaging-guided photothermal therapy (Zhao et al., 2021). In Shi's et al. study, bismuth oxycarbonate nanosheets markedly increased radiotherapy efficacy *in vivo* and raised tumor inhibition from ~49.9 % to ~90.8 % under low-dose X-ray. Their high oxygen content and abundant surface oxygen vacancies alleviated tumor hypoxia and generated a burst of ROS upon irradiation by overcoming radioresistance and inducing tumor apoptosis (Shi et al., 2024). Zhu et al. showed that Au@Bi₂Te₃-PEG nanozymes relieved tumor hypoxia, improving radiotherapy outcomes and promoting ICD. TME reshaping included raised intratumoral oxygenation and reduced hypoxia-related cytokines, which correlated with increased CD4 + and CD8 + T-cell infiltration, higher cytokine levels, and inhibition of both primary and distant (abscopal effect) tumors (Zhu et al., 2023). Taken together, these *in vivo* reports substantiated that rationally designed bismuth-based NPs can relieve hypoxia, and increase ROS generation, under enable effective multimodal therapy, thereby addressing key limitations of SDT and related modalities in animal tumor models.

3.8. *In vivo* hemocompatibility

To investigate the hemocompatibility of bismuth oxide NPs and FA-bismuth oxide NPs, blood biochemical analysis and whole blood cell analysis were conducted on day 5, following the intravenous injection of these NPs to assess the long-term safety in mice. The results are summarized in [supplementary material S20](#). The results indicated that the administrated NPs did not cause substantial hepatotoxicity, as liver enzyme levels (AST, ALT) remained to those of control animals. Renal markers (creatinine, BUN) showed only minor, non-substantial alterations, which may reflect transient renal handling and excretion of the NPs via the urinary tract. Negligible changes in blood parameters suggested that the NPs did not provoke notable hematological toxicity or systemic inflammatory responses.

3.9. Practical applications and future research prospects

FA-bismuth oxide NPs as a combination of folate-receptor and high-Z bismuth would be a promising sonosensitizer for localized tumor SDT. Folate mediates cancerous cells uptake for accumulation in folate-receptor-overexpressing tumors, and bismuth provides intrinsic X-ray/CT absorption for theranostics applications and image-guided treatments. Enhanced cytotoxicity with repeated US in 3D spheroids supports localized intertumoral delivery or repeated noninvasive US sessions to improve therapeutic index potential. While MMP loss and Annexin V/PI data indicate apoptosis, a limitation of this study is the lack of direct confirmation of apoptotic pathway components (e.g., western blots for Bax, Bcl-2, cleaved caspase-9 and caspase-3), and would be addressed in future investigations. Some evidences demonstrated a clear connection between MMP loss induced by bismuth-based nanostructures, and activation of apoptotic pathways in cancer cells. For example, Bi-Ag@PVP NPs combined with 808-nm light caused MMP loss and ROS production in HepG2 cells, combined by downregulation of the anti-apoptotic protein bcl-2 and upregulation of cleaved caspase-3 and caspase-8 with mitochondrial-mediated apoptosis (Zhou et al., 2021). X-ray irradiation of Pt@Bi₂Se₃-reduced graphene oxide nano-assembly increased ROS and induced MMP loss in 4T1 tumor cells, and initiated the intrinsic apoptotic pathway via bcl-2 down-regulation caspase-3 activation (Tan et al., 2025). Also, bismuth oxide/reduced graphene oxide nanocomposites induced apoptosis in NRK52E and HepG2 cells through oxidative stress, as evidenced by increased ROS, depleted GSH, caspase-3 activation, chromatin condensation, and cell cycle arrest (Lateef et al., 2024). These findings support that MMP lose is a recognized precursor to mitochondrial-mediated apoptosis; however, for precise detection of apoptosis pathway, direct detection of key apoptotic proteins is needed.

For translation, comprehensive *in vivo* studies are needed include longitudinal tumor control and survival, pharmacokinetics, clearance, immune responses, long-term organ accumulation, and comparisons with existing sensitizers. Optimization of NPs size, shape, FA density, and coatings, plus co-loading with drugs, photosensitizers, or immunomodulators, can boost tumor penetration and

multimodal efficacy. Finally, scalable synthesis, good laboratory practice (GLP) toxicology, and early-phase clinical trials in folate-receptor-positive cancers are essential for clinical adoption.

4. Conclusions

Synthesis of bismuth oxide and FA-bismuth oxide NPs was performed using a *Rheum turkestanicum* extract, and their employment as nano-sonosensitizers for SDT targeting melanoma cancer cells was subsequently demonstrated. The integration of FA into bismuth oxide resulted in structural modifications, while the application of the SDT method enhanced various properties, including but not limited to cell death (i.e., apoptosis) and ROS in C540 cells. The results of the cell assay demonstrated that the FA-bismuth oxide/US+ group exhibited considerable effectiveness in eradicating cancer cells, with significant GSH degradation, apoptosis via mitochondrial membrane potential decrement, and inhibition of both cellular migration and 3D cell viability. Future research directions may include substituting FA with its derivatives, investigating different morphologies of bismuth nanostructures, utilizing PDT or combined SDT-PDT strategies for cancer treatment with FA-bismuth oxide NPs, and furthering the application of SDT with FA-bismuth oxide NPs in animal models.

CRediT authorship contribution statement

M. Sarani: Writing – original draft, Investigation, Formal analysis, Data curation. **Sattarahmady Naghmeh:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **S.A. Dastgheib:** Resources, Methodology, Funding acquisition, Formal analysis. **A. Safari:** Validation, Methodology, Conceptualization. **M. Darroudi:** Validation, Conceptualization. **P. Tamaddon:** Writing – review & editing, Validation. **H. Haghighi:** Investigation, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.eti.2025.104741](https://doi.org/10.1016/j.eti.2025.104741).

Data availability

Data will be made available on request.

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