Original Article

Decorated ultrathin bismuth selenide nanosheets as targeted theranostic agents for in vivo imaging guided cancer radiation therapy

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An efficient radiotherapeutic agent is synthesized using ultrathin two-dimensional 30-nm-wide and 2-nm-thick Bi$_2$Se$_3$ nanosheets (NSs) as a radiosensitizer. Chitosan (CS) and RGD peptide are employed to enhance the radiotherapy efficiency and biocompatibility. The Bi$_2$Se$_3$-CS-RGD NSs exhibit excellent targeting ability to αvβ3 integrin-overexpressing cancer cells and potent radiosensitization efficiency with high stability. Detailed in vitro experiments show that the Bi$_2$Se$_3$-CS-RGD NSs enhance the sensitivity of HeLa cells to X-ray-induced cell death by inhibiting TrxR activities and activating downstream reactive oxygen species-mediated signaling pathways. In vivo experiments using intravenous or intratumor injection demonstrate that the Bi$_2$Se$_3$-CS-RGD NSs are more efficient tumor growth inhibitors compared to bare Bi$_2$Se$_3$ NSs. The multifunctionality of the NSs enables the use of photoacoustic imaging and magnetic resonance imaging to examine their targeting ability and therapeutic effects, respectively. In addition, the RGD-decorated Bi$_2$Se$_3$ NSs show much better in vivo biocompatibility and can be efficiently expelled from the body after 48 h post injection. This study reveals an effective and safe theranostic agent for next-generation cancer radiotherapy.

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Introduction

In addition to surgery and chemotherapy, radiotherapy is an important treatment in cancer therapy that is suitable for breast, cervical, lung and brain cancers. Despite recent efforts, radiotherapy fails to fully eradicate tumors due to inevitable damage to surrounding healthy tissues and the radiation insensitivity of some tumors. Hence, various chemical radiosensitizers have been suggested to accentuate the effects of radiation therapy. For example, electron-affinic nitroaromatic compounds as hypoxic-specific cytotoxins, and pyrimidines substituted with bromine or iodine can be incorporated into DNA to enhance free radical damage. Moreover, it has been reported that pentoxifylline can improve tumor oxygenation and radiation responses. However, few radiosensitizers are available on the market due to the difficulty and high cost of large-scale production; thus, development of new radiotherapy agents is of scientific and clinical interest.

In recent years, high-Z (high atomic number) nanomaterials with enhanced photovoltaic and Compton effects have aroused interest, especially from the perspective of enhancing the therapeutic efficiency and specificity of radiotherapy. High-Z elements with larger X-ray interaction cross-sections than light elements (for example, H, O, N and C) could increase the energy deposition and radiolytic hydrolysis in the vicinity of the materials. Several high-Z materials, such as germanium nanoparticles (NPs), iron oxide NPs, lanthanide-based compounds, iodine, gold-based NPs and nuclear-targeting gadolinium-based NPs, have recently been used as adjuvants to enhance cellular radiosensitivity. However, few are efficient in in vivo cancer radiotherapy. The ideal radiotherapeutic nanoagent should have high radiosensitization and radio-stability, suitable size and proper surface functionalization to prolong the circulation lifetime and facilitate efficient tumor targeting. The additional imaging ability rendered by the agent can provide real-time information to guide procedures, monitor the therapeutic response, and treat diseases with greater specificity and sensitivity.

Selenium (Se) is an essential and unique trace element that provides effective prevention against serious diseases, such as cancers, inflammation and cardiovascular disease, and may therefore open up new horizons in disease treatment. Selenium nanoparticles (SeNPs) can be employed in nanocarriers for drug delivery and therapeutic agents due to their excellent bioavailability, low toxicity and significant radiosensitization effects with X-ray irradiation. Selenadiazole derivatives and Se-containing Ru complexes have been designed to combine radiotherapy and chemotherapy in vitro and in vivo due to their surface plasmon resonance effect and high refractive index, which facilitate light absorption. As new responsive materials for use in combination radiotherapy and chemotherapy, diselenide-containing polymers...
nanomaterials, such as Bi$_2$S$_3$ nanodots, Bi$_2$S$_3$ nanorods and Bi$_2$Se$_3$ is larger than those of I, Au, and Pt. Therefore, several Bi-based (PAI) and photothermal therapy, the large X-ray attenuation coefficients of Se elements for low-energy X-rays (μ = 48.18 cm$^2$ g$^{-1}$ at 20 keV) highlight their potential in X-ray computed tomography (CT) imaging, whereas sulfides do not exhibit this beneficial effect. These examples clearly illustrate the huge potential of selenide in chemo-/radiotherapy and in multimodal imaging-guided synergistic therapy against cancer. Notably, the valence of selenium may not influence the treatment effect for cancer.

Bi is a high-Z element (Z = 83), and its X-ray attenuation coefficient is larger than those of I, Au, and Pt. Therefore, several Bi-based nanomaterials, such as Bi$_2$S$_3$ nanodots, Bi$_2$S$_3$ nanorods and Bi$_2$Se$_3$ NPs with different morphologies, have been proposed as X-ray CT contrast agents and radio-/photothermal therapy sensitizing agents. Compared with orthorhombic Bi$_2$S$_3$, the topological insulator Bi$_2$Se$_3$ has a two-dimensional layered rhombohedral structure with a unique surface electronic state and has aroused increasing interest in biomedical applications due to its multifunctionality and good biocompatibility. Notably, Bi$_2$Se$_3$ nanostructures can release vital oxygen-free NaHSe solution (0.667 mol l$^{-1}$) at 160 °C. The reaction mixture immediately turned dark due to the formation of Bi$_2$Se$_3$ NSs. The reaction proceeded for 10 min before cooling to room temperature. The Bi$_2$Se$_3$-CS-RGD NSs were combined by electrostatic interaction. The Cs-RGD complex is combined with the Bi$_2$Se$_3$ NSs by electrostatic interaction. The Bi$_2$Se$_3$-CS-RGD NSs demonstrate good stability in the biological environment even under X-ray irradiation. Detailed in vitro experiments are conducted to investigate the anticancer mechanism of the NS-based X-ray radiotherapy. In vivo experiments are also performed to evaluate the applicability of imaging-guided radiotherapy, radiosensitization effects, and in vivo toxicity of the Bi$_2$Se$_3$ and Bi$_2$Se$_3$-CS-RGD NSs by either intravenous or intratumor injection. To simulate real clinical conditions, all the radiation experiments are performed using an Elekta Precise linear accelerator (6 MeV energy, 5 cm penetration, 100 MU) in a hospital.

MATERIALS AND METHODS

Synthesis of Bi$_2$Se$_3$-CS-RGD NSs

The Bi$_2$Se$_3$ NSs were synthesized according to a previously published method. Briefly, the Bi$_2$Se$_3$ NSs were prepared by reacting Bi(NO$_3$)$_3$·5H$_2$O with a NaHSe solution. The solution contained ethylene glycol (EG) (32.5 ml), Bi(NO$_3$)$_3$·5H$_2$O (0.226 g) and polyvinylpyrrolidone (PVP) (0.5 g) as a surfactant. The reaction proceeded by rapid injection of the freshly synthesized oxygen-free NaHSe solution (0.667 mol l$^{-1}$, 1.048 ml) at 160 °C. The reaction mixture immediately turned dark due to the formation of Bi$_2$Se$_3$ NSs. The reaction proceeded for 10 min before cooling to room temperature. The Bi$_2$Se$_3$-CS NSs were synthesized by conjugating RGD to CS via the formation of amido linkages in the N-hydroxysuccinimide (NHS)/1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride (EDC) reaction system. The CS and Bi$_2$Se$_3$ NSs were combined by electrostatic interaction. The crude product was purified by dialysis in Milli-Q water for 24 h until no Bi could be detected from the outer solution using ICP-AES (OPTIMA 2100 DV, Waltham, MA USA).

In this work, decorated ultrathin Bi$_2$Se$_3$-CS-RGD NSs (30 nm wide and 2 nm thick) are designed and produced as a targeted theranostic agent for in vivo imaging-guided cancer radiotherapy. The design and synthesis protocol of the agent are shown in Scheme 1. Briefly, the RGD peptide, which has an excellent targeted ability to $\alpha$-$\beta$3 integrin, is conjugated with chitosan (CS) by forming the amido linkage, and the CS-RGD complex is combined with the Bi$_2$Se$_3$ NSs by electrostatic interaction. The Bi$_2$Se$_3$-CS-RGD NSs demonstrate good stability in the biological environment even under X-ray irradiation. Detailed in vitro experiments are conducted to investigate the anticancer mechanism of the NS-based X-ray radiotherapy. In vivo experiments are also performed to evaluate the applicability of imaging-guided radiotherapy, radiosensitization effects, and in vivo toxicity of the Bi$_2$Se$_3$ and Bi$_2$Se$_3$-CS-RGD NSs by either intravenous or intratumor injection. To simulate real clinical conditions, all the radiation experiments are performed using an Elekta Precise linear accelerator (6 MeV energy, 5 cm penetration, 100 MU) in a hospital.

Scheme 1 Schematic illustration of the rational design of Bi$_2$Se$_3$-CS-RGD NSs as a targeted theranostic agent for in vivo imaging-guided cancer radiation therapy.
Characterization
The morphology of the samples was examined by transmission electron microscopy (Hitachi H-7650, 80 kV, Tokyo, Japan) and high-resolution transmission electron microscopy (JEOL 2010, 200 kV, Tokyo, Japan). Atomic force microscopy (AFM) was conducted on drop-cast flakes on Si/SiO2 substrates using an MFP-3D-S atomic force microscope (Asylum Research, USA) with the AC mode (tapping mode) in air. Powder X-ray diffraction (XRD) was conducted using an MSAL XD-2 X-ray diffractometer. A Zetasizer Nano-ZS particle analyzer (Malvern Instruments Limited) (Malvin City, UK) was used. The chemical composition was determined by Fourier transform infrared spectroscopy (FT-IR, Equinox 55, Bruker, Billerica, MA, USA) and UV–vis–NIR spectrophotometry.

Determination of the NS-grafted RGD peptide content
The Bi2Se3-CS-RGD NSs were removed from the reaction solution by centrifugation. The supernatant was collected, and the residual peptides were determined using a BCA assay kit. The conjugated content of RGD peptide (μg mg⁻¹ NSs) was determined by comparing the difference with the amount added.

Cell culture and in vitro cytotoxicity test
The human cervical carcinoma cell line HeLa, Siha, Caski and human melanoma A375 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and Ect1/E6E7 human normal cervical endothelial cells were obtained from the Sunzhou BeNa Culture Collection. The HeLa, Siha, Caski and A375 cells were grown in DMEM, and the Ect1/E6E7 cells were cultured in MEM/NEAA medium supplemented with fetal bovine serum (10%), 100 units per ml of penicillin, and 50 units per ml of streptomycin at 37 °C in a humidified incubator under 5% CO2. The cell viability (2 × 10⁴ cells per ml) after treatment with different concentrations of Bi2Se3 NSs, Bi2Se3-CS NSs, Bi2Se3-CS-RGD NSs and X-ray for 72 h was determined using an MTT assay.⁴⁴

Clonogenic assay
The HeLa cells were seeded on six-well plates at 2000 cells per ml (2 ml) and allowed to attach for 24 h. After incubation for 6 h with different concentrations of the Bi2Se3-CS-RGD NSs, the cells were exposed to different X-ray dosages and incubated at 37 °C for 7 days. The cells were fixed with 4.0% paraformaldehyde (vol/vol) for 10 min and stained with 0.5% crystal violet (wt/vol) for 20 min. The survival fraction of the clones was used to evaluate the effects of different treatments.

Cellular uptake and intracellular trafficking of NSs
The in vitro cellular uptake and localization of the Bi2Se3-CS-RGD NSs were determined by using coumarin-6-loaded Bi2Se3-CS-RGD NSs. Solution of 5 mg ml⁻¹ coumarin-6 and CS were refluxed with ethanol. The products were purified by gel elution to remove unreacted coumarin-6, and the Bi2Se3 NS solution was added to the CS-conjugated biocarcinogen under constant stirring for 24 h in the dark to form the coumarin-6-loaded Bi2Se3-CS NSs. For RGD conjugation, the RGD was activated with EDC, NHS was added dropwise to the coumarin-6-loaded Bi2Se3-CS-RGD NSs, and the solution was stirred for 24 h at room temperature. After the reaction, the mixture was dialyzed against Milli-Q water for 24 h, and the coumarin-6-loaded Bi2Se3-CS-RGD NSs were obtained.

HeLa and Ect1/E6E7 cells were seeded on six-well plates at a density of 2 × 10⁵ cells per ml (2 ml) and incubated for 24 h. The medium in each well was replaced with non-phenol red DMEM. After 2 h, the cells were incubated with 160 μg ml⁻¹ coumarin-6-loaded Bi2Se3-CS-RGD NSs for various durations at 37 °C in a CO2 incubator. A total of 100 μl of the medium was removed from the 96-well plates. The concentration was measured using the standard-curve method based on the specific absorbance of coumarin-6 with the excitation and emission wavelengths set at 430 and 485 nm, respectively, in the same medium. The cellular uptake efficiency was expressed as the percentage of Bi2Se3-CS-RGD NSs adsorbed to the amount added.

The lysosomal marker Lyso-tracker was used to trace the intracellular localization of coumarin-6-loaded Bi2Se3-CS-RGD NSs in the HeLa cells. The HeLa cells were incubated on 2-cm cell culture dishes with 80 nM Lyso-tracker for 2 h and 1 μg ml⁻¹ H33342 for 15 min. After rinsing with phosphate-buffered saline (PBS) three times, the cells were incubated with 160 μg ml⁻¹ coumarin-6-loaded Bi2Se3-CS-RGD NSs for various durations and observed by fluorescence microscopy (EVOSFL auto, Life Technologies, 20×, Beijing, China).

RGD competition assay
The Bi2Se3-CS-RGD NSs and excess RGD competed for binding integrin on HeLa cells. The HeLa cells (8 × 10⁴ cells ml⁻¹) were cultivated on 96-well plates and incubated for 24 h. Different concentrations of RGD (0–0.5 mg ml⁻¹) were treated for 2 h at 37 °C in a CO2 incubator and then incubated with 160 μg ml⁻¹ coumarin-6-loaded Bi2Se3-CS-RGD NSs for 6 h. The cells were rinsed with PBS three times and lysed. A fluorescence microplate reader (SpectraMax M5, Silicon Valley, Sunnyvale, CA, USA) with excitation and emission wavelengths of 430 and 485 nm, respectively, was used to measure the internalized Bi2Se3-CS-RGD NSs. The HeLa cells (2 × 10⁴ cells per ml) were incubated with different concentrations of RGD and 160 μg ml⁻¹ Bi2Se3-CS-RGD NSs for different durations and then exposed to X-ray (8 Gy). After 24 h, the cell viability was determined using an MTT assay.

Flow cytometric analysis of the cell cycle distribution
The effects of the Bi2Se3-CS-RGD NSs and X-ray irradiation on the cell cycle distribution were determined by flow cytometric analysis. The DNA histogram represents the percentages of cells in the G0/G1, S and G2/M phases, and the hypodiploid DNA content in the apoptotic cells was quantified by analyzing the sub-G1 peak.⁵⁶

Caspase activity assay
The activation of caspases was examined by measuring the fluorescence intensity with specific caspase-3, -8 and -9 substrates. Briefly, HeLa cells with 40 μg ml⁻¹ Bi2Se3-CS-RGD NSs and X-ray (8 Gy) treatment for 72 h were incubated with lysis buffer (Beyotime) to obtain the total cellular proteins. The protein concentrations were determined using a BCA assay. A total of 100 μg of the cytosolic protein and caspase substrates (caspase-3, -8 and -9) were added to the 96-well plates at 37 °C for 2 h. The caspase activity was examined using a fluorescence microplate reader with excitation and emission wavelengths of 380 and 460 nm, respectively. The fluorescence intensity was monitored to evaluate the caspase activity.⁵⁵

Change of mitochondrial morphology
The HeLa cells were cultured on 2 cm glass-bottom dishes for 24 h. They were treated with the Bi2Se3-CS-RGD NSs (40 μg ml⁻¹) for 6 h and then irradiated with X-ray (8 Gy). After incubation for 12 h, the cell monolayer was rinsed with ice-cold PBS 3 times. The cell mitochondria and nuclei were stained with 100 nM Mito-tracker for 2 h and 1 μg ml⁻¹ H33342 for 15 min. Changes in the mitochondrial morphology were observed by fluorescence microscopy (EVOSFL auto, Life Technologies, 100×).

Examination of the TrxR activity
A Thioreredoxin Reductase Assay Kit (Cayman, Ann Arbor, MI, USA) was used to determine the inhibition of TrxR in the HeLa cells after different treatments. In this assay, TrxR catalyzes the reduction of 5,5-dithiobis (2-nitrobenzoic) acid (DTNB) with NADPH to 5-thio-2-nitrobenzoic acid (TNB⁻), generating a strong yellow color. Briefly, 60 μg of cytosolic protein was added to 96-well plates and incubated with NADPH and DTNB for 30 min. The reaction was monitored at 410 nm using a microplate reader (MD VERSA max, Silicon Valley), and the TrxR activity was expressed as % of the control.

Detection of singlet oxygen in vitro
The traditional chemical probe of 1,3-diphenylisobenzofuran (DPBF) was used to identify the generation of O₂⁻, which could react with O₂ and cause a decrease in the absorption intensity of the DPBF band centered at 410 nm. One milliliter of ethanol solution with DPBF (2 mM) was mixed with 1 ml of Bi2Se3-CS-RGD NSs at a specified concentration in the dark for 2 h to reach the

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adsorption/desorption equilibrium prior to the testing. Then, the mixed solution was exposed to different dosages of X-ray, and the absorbance at 410 nm was monitored using a UV–vis spectrophotometer. In the cell model, the HeLa cells (4 × 10⁴ cells per ml) were treated with the Bi₂Se₃-CS-RGD NSs (40 μg ml⁻¹) for 4 ~ 5 h followed by incubation with DPBF (0.1 mM final concentration) for 2 h and irradiation with X-ray (8 Gy). The generation of ¹O₂ was measured by detecting the absorbance of DPBF at 410 nm every 5 min.

Determination of intracellular reactive oxygen species (ROS) generation
The effects of the Bi₂Se₃-CS-RGD NSs and radiation on intracellular superoxide generation in the HeLa cells were examined using the dihydroethidium (DHE) fluorescence probe. Briefly, the HeLa cells (2 × 10⁵ cells per ml) were treated with the Bi₂Se₃-CS-RGD NSs for 6 h. Then, the cells were treated with X-ray (8 Gy) and incubated with DHE at a final concentration of 10 μM at 37 °C for 30 min. The intracellular reactive oxygen species (ROS) level was measured as the fluorescence intensity of DHE (excitation and emission wavelengths of 300 and 600 nm, respectively). Fluorescence images were acquired from the HeLa cells to examine whether the combined radiotherapy induced variations in ROS. The cell-free model was the same as that described above except that the cultured HeLa cells were replaced by PBS.

ABTS radical scavenging activity
The antioxidant activity was assessed using an ABTS assay according to the requirement of the Total Antioxidant Capacity Assay Kit. The ABTS⁺ radical was formed by the same volume of ABTS and oxidant solution after incubation at room temperature in darkness for 12 ~ 18 h. The radical was diluted with PBS (pH = 7.4) to obtain an absorbance value of 0.700 ± 0.0200 at 734 nm. Under dark conditions, 190 μl of the ABTS⁺ radical solution was added to 10 μl of the Bi₂Se₃ NSs, Bi₂Se₃-CS NSs and Bi₂Se₃-CS-RGD NSs at different concentrations, and the absorbance at 734 nm was measured using a microplate reader.

Western blot analysis
The effects on the expression levels of proteins associated with different signaling pathways were determined by Western blot analysis.⁵⁶

Tumor model
Female Balb/c nude mice (3~4 weeks old and weighing 14~15 g) were purchased from Beijing HFK Bioscience (Beijing, China) and used under protocols approved by the Jinan University Laboratory Animal Center. The animals acclimated to the environment for 10 days before treatment. HeLa cells (1 × 10⁷ cells ml⁻¹) suspended in 100 μl of PBS were subcutaneously injected into the back of each mouse to develop the tumor. All the procedures were in compliance with the animal ethics committee guidelines.

In vivo photoacoustic imaging
The Balb/c nude mice bearing HeLa tumors (7~8 mm) were intravenously and intratumorally injected with the Bi₂Se₃ NSs (1 mg ml⁻¹, 0.1 ml) and Bi₂Se₃-CS-RGD NSs (1 mg ml⁻¹, 0.1 ml), respectively. Photoacoustic imaging was performed using a photoacoustic computed tomography scanner (Endra Nexus 128, Ann Arbor, MI, USA), and 808 nm was selected as the laser wavelength with an average of 30 pulses. Anesthesia was administered with pentobarbital (2%, wt/vol, 30 μl), and a water heating system maintained the water temperature at 37.5 °C to keep the mice comfortable. Photoacoustic images were obtained at different time intervals.⁴⁷

Biodistribution
The Bi₂Se₃-CS-RGD NSs and Bi₂Se₃ NSs were injected into the Balb/c nude mice (1 mg ml⁻¹, 0.1 ml) intravenously and intratumorally, respectively. At 12, 24, 36 and 48 h post injection, the mice were killed, and the tissues were excised, weighed, and digested in HNO₃ and HClO₄ (3:1) for 180 °C. The homogenized tissue lysates were dried using heat, diluted with ultrapure water, and filtered. The Bi and Se contents were determined by inductively coupled plasma mass spectrometry.
In vivo assessment of the radiation therapy effect

When the tumor grew to 5–6 mm in diameter, the mice were randomly divided into eight groups (three mice per group): G1: saline, as a control group; G2: X-ray; G3: Bi$_2$Se$_3$-CS-RGD NSs (intravenous); G4: Bi$_2$Se$_3$-CS-RGD NSs (intratumoral); G5: Bi$_2$Se$_3$ NSs (intravenous) + X-ray; G6: Bi$_2$Se$_3$ NSs (intratumoral) + X-ray; G7: Bi$_2$Se$_3$-CS-RGD NSs (intravenous) + X-ray; and G8: Bi$_2$Se$_3$-CS-RGD NSs (intratumoral) + X-ray. A PBS solution of Bi$_2$Se$_3$ NSs (1 mg ml$^{-1}$, 0.1 ml) was intravenously injected into each tumor in the G5 mice for 6–8 h and intratumorally injected into each tumor of the G6 mice for 0.5 h. A PBS solution of Bi$_2$Se$_3$-CS-RGD NSs (1 mg ml$^{-1}$, 0.1 ml) was intravenously injected into each tumor of the G3 and G7 mice for 6–8 h and intratumorally injected into each tumor of the G4 and G8 mice for 0.5 h. A total of 100 μl of saline was injected into each tumor in G1. After the injections, the G2, G5, G6, G7 and G8 mice were anesthetized using pentobarbital and underwent X-ray irradiation (4 Gy and 6 MeV energy, 5 cm penetration, 100 MU) for 100 cm from the source using a 2-cm-thick lead plate with holes of appropriate size for protection. After one day, the process was repeated nine times to achieve 40 Gy of exposure. During the treatment, the body weight of the mice and size of the tumors were measured daily for 21 days, and the tumor volume was calculated as the width$^2$×length/2.

In vivo magnetic resonance (MR) imaging

After 21 days, the mice were anesthetized with 2% pentobarbital (40 μl) and placed under magnetic resonance imaging (MRI) study conditions. Imaging analysis was performed using the Imaging software, and a quantification analysis of the MR signals was performed using the MR images. The T$_2$-weighted MR images were obtained using a 1.5 T Signa HDxt superconductor clinical MR system (GE medical, Milwaukee, WI, USA) using the following parameters: TR 2620 ms; TE 82.3 ms; slice thickness 2.0 mm; slice spacing 0.2 mm; matrix 256×192; and FOV 5 cm×5 cm.

Hematological and histological analyses

After the experiments, blood samples (~1 ml) were collected from the eyes of the nude mice before sacrifice and centrifuged to obtain the serum (~500 μl),...
which was sent to the Blood Test Center of Guangzhou Overseas Chinese Hospital for blood panel analysis and blood chemistry assays. The tumor tissue and main organs (heart, liver, spleen, lung and kidney) were stripped and weighed. A paraffin section was made and stained with hematoxylin and eosin (H&E) for further observation of the pathological changes caused by different treatments. The percentage of the relative tumor growth ratio was calculated as \( \frac{V_2 - V_0}{V_1 - V_0} \times 100\% \).

**Statistical analysis**

All the experiments were conducted in at least triplicate and repeated three times; the results are expressed as the mean ± s.d. The differences between the control and experimental groups were analyzed by a two-tailed Student’s t-test. Differences with \( P < 0.05 \) (*) or \( P < 0.01 \) (**) were considered statistically significant.

**RESULTS AND DISCUSSION**

**Targeted design and characterization of Bi2Se3-CS-RGD NSs**

As shown in the transmission electron microscopy images in Figures 1a and b, the Bi2Se3-CS-RGD NSs have a sheet-like morphology with an average diameter of ~30 nm. The high-resolution transmission electron microscopy image presented in Figure 1c shows hexagonal lattice fringes with 0.21 nm spacing corresponding to the (110) plane of the Bi2Se3 crystal.48 The AFM image in Figure 1d shows hexagonal lattice fringes with 0.21 nm spacing corresponding to the (110) plane of the Bi2Se3 crystal.48 The AFM image in Figure 1d shows hexagonal lattice fringes with 0.21 nm spacing corresponding to the (110) plane of the Bi2Se3 crystal.48

The peaks at 1398, 1155 and 1072 cm\(^{-1}\) in the Bi2Se3-CS-RGD NS spectrum correspond to the stretching of C-H, C-O-C, and C-O-H, respectively, characteristic of the biopolymer CS.49 The two amide bands I and II at 1650 and 1556 cm\(^{-1}\) correspond to the interaction between the amino group and Se atoms on the surface of the Bi2Se3 NSs. The peaks at 1398, 1155 and 1072 cm\(^{-1}\) correspond to the stretching of C-H, C-O-C, and C-O-H, respectively, characteristic of CS.49 The two amide bands I and II at 1650 and 1556 cm\(^{-1}\), respectively, in the spectrum of the Bi2Se3-CS-RGD NSs confirm the formation of CO-NH- between RGD and CS. The Fourier transform infrared spectroscopy results corroborate the surface conjugation of CS and RGD onto the Bi2Se3 surface.

The presence of RGD peptides is verified by a protein staining BCA assay and UV-vis-NIR spectroscopy. The absorption spectrum in Figure 1h shows higher absorbance at 560 nm due to the presence of proteins in the NPs, which is confirmed by the change in the particle color (inset photos). The coupling amount of RGD peptide is 20.54 \( \mu \text{g mg}^{-1} \). In this nanosystem, the positive charge from the amine groups of CS facilitates internalization in the tumor cells through endocytosis. Moreover, RGD peptide surface decoration enhances the recognition of the NSs by the integrin that is overexpressed in the cancer cell membrane, thus increasing the selectivity between the cancer and normal cells.

The stability of the Bi2Se3-CS-RGD NSs is examined. It has been observed that naked Bi2Se3 NSs are oxidized extensively in PBS buffer solution (pH = 7.4) after exposure to ambient conditions (room temperature) for one month, and the color changes from black to brown before precipitation of red amorphous Se sediment.41
In comparison, Supplementary Figure S2 (Supplementary) shows that the Bi2Se3-CS-RGD NSs have excellent stability in PBS for more than 30 days at 4 °C and in DMEM containing 10% FBS, simulating physiological conditions, for 72 h.

The stability of the Bi2Se3-CS-RGD NSs under X-ray irradiation is assessed because it influences the efficiency in radiosensitization applications.50 As shown in Supplementary Figure S3 (Supplementary), the morphology and crystal structure of the Bi2Se3-CS-RGD NSs are preserved after X-ray (8 Gy, 6 MeV) irradiation for as long as 2 min due to the strong chemical bonds in the Bi2Se3-CS-RGD NSs (Se-Bi-Se-Bi-Se).

Enhanced cancer radiotherapy

Although radiation therapy is widely adopted in the treatment of cervical cancer, the clinical success rate is unsatisfactory. This result is partly associated with the resistance to radiotherapy caused by hypoxic cells in the tumor microenvironment. In this study, the Bi2Se3-CS-RGD NSs exhibit a radiotherapy-enhancing effect of X-ray irradiation on cervical cancer. The MTT assay shows that after treatment for 72 h, the Bi2Se3 NSs, Bi2Se3-CS NSs, and Bi2Se3-CS-RGD NSs (Figures 2a and b) and X-ray radiation (Figure 2c) alone show no significant inhibition of HeLa and Etc1/E6E7 cells. It has been reported that high-dose irradiation increases the cytotoxic effects on tumors.51 In this study, exposure to 8 Gy X-ray only inhibits HeLa cell growth by 20% (Figure 2c), thus demonstrating the radiation resistance of HeLa cells. By contrast, the combined treatment with the Bi2Se3-CS-RGD NSs and X-ray radiation results in higher growth inhibition of HeLa cells in a dose-dependent manner (Figure 2d). The NSs and X-ray also show synergistic effects on other cell lines, including Siha and Caski human cervical carcinoma cells and A375 human melanoma cells (Supplementary Figure S4). The combination indexes (CIs) of the co-treatment are 0.41 (8 Gy: 80 μg ml⁻¹) and 0.47 (8 Gy: 40 μg ml⁻¹), as shown in Supplementary Figure S5 (Supplementary), confirming the synergistic effects of X-ray and the NSs. In comparison, the combined treatment shows no toxicity in the Etc1/E6E7 human normal cervical endothelial cells, confirming the good selectivity of the Bi2Se3-CS-RGD NSs as a radiosensitizer between cancer and normal cells.

The clonogenic assay is used to evaluate the anticancer effects of NSs and X-ray on the cell population by examining the inhibitory effects of the combined treatment on the colony formation of HeLa cells. As shown in Figure 2e, the survival fractions of HeLa cells treated...
with X-ray (2 Gy) and NSs (8 μg ml⁻¹) alone are 89.7% and 91.9%, respectively, and this fraction is reduced to 48.1% after the combined treatment. When the X-ray dose is increased, the survival fraction of the HeLa cells decreases to 24.9% and 12.4%. The microscopic images show that the NSs enhance the radiation-induced inhibition by X-ray of cancer cell colony formation (Figure 2f and Supplementary Figure S6), demonstrating that the combined treatment with Bi₂Se₃-CS-RGD NSs and X-ray reduces cell colony formation in a dose-dependent manner.

**Cellular uptake and trafficking**

The cellular uptake efficacy is an important regulator in controlling and enhancing the selective cellular uptake of nanomedicine by the cancer cells. The conjugation of RGD peptides enables antineoplastic drugs to recognize the αβ³ integrin receptor, which is abundantly expressed on cervical cells but not on normal tissues, enabling the selectivity. In this study, to verify the selectivity of the Bi₂Se₃-CS-RGD NSs between cancer and normal cells, the cellular uptake of the NSs by the cancer (HeLa) and normal (Etc1/E6E7) cells is monitored via the Bi₂Se₃-CS-RGD NSs and X-ray reduces cell colony formation in a dose-dependent manner.

**Mechanism of NS-enhanced X-ray radiotherapy**

Generally, the major mechanisms of cell death induced by anticancer drugs are apoptosis, cell cycle arrest, and/or a combination of these two modes by regulating different signaling pathways. Flow cytometry is utilized to investigate radiosensitization to X-ray of HeLa cells via the Bi₂Se₃-CS-RGD NSs. The DNA histograms in Figure 4a demonstrate that NSs alone cause G0/G1 phase arrest in the HeLa cells in a dose-dependent manner, and X-ray exposure (8 Gy) induces slight G0/G1 arrest. However, the combination of NSs and X-ray enhances cell apoptosis and cell cycle arrest. For instance, the sub-G₁ peak of HeLa cells treated with 80 μg ml⁻¹ of the NSs is 10.3%, and it increases to 38.9% after co-treatment with X-ray. The results demonstrate the important role of cell apoptosis in the radiosensitization effects of the Bi₂Se₃-CS-RGD NSs.

Caspases are a family of cystein proteases that play important roles in cell apoptosis. Caspase-3 acts as a central regulator of cell apoptosis, and caspase-8 and caspase-9 are the initiators of death receptor-mediated and mitochondria-mediated apoptotic pathways, respectively. To investigate the signaling pathways in Bi₂Se₃-CS-RGD NS-enhanced radiation-induced apoptosis, a fluorometric assay is applied to monitor the activation of caspase-3, -8 and -9. As shown in Figure 4b, the Bi₂Se₃-CS-RGD NSs cause activation of caspase-3, caspase-8, and caspase-9 in the HeLa cells after co-treatment with X-ray, indicating that both the death receptor-mediated and mitochondria-mediated pathways are involved in apoptosis.
activity of caspase-9 is higher than that of caspase-8, suggesting the more important role of mitochondria in caspase-mediated apoptosis.

Mitochondria are the energy factory and are vital in different cellular activities. However, a variety of factors can affect the structure and function of mitochondria and further induce cell apoptosis. Therefore, the mitochondrial fragmentation and activation of caspase-9-dependent apoptosis are assessed to confirm mitochondrial dysfunction resulting from the Bi2Se3-CS-RGD NSs with radiotherapy. As shown in Figure 4c, Mito-tracker red is used to label the mitochondria, which are present as red threadlike filaments in healthy HeLa cells. The combined treatment induces mitochondria fragmentation and release of constituents into the cytosol, but no significant change is observed for the cells exposed to X-ray or the NSs alone. These results demonstrate the important contribution of mitochondria to the X-ray radiosensitization effects of the Bi2Se3-CS-RGD NSs.

Activation of ROS-mediated signaling pathways

Mitochondria are the major source of intracellular free radicals in mammalian cells. Therefore, mitochondrial fragmentation induced by the Bi2Se3-CS-RGD NSs may trigger ROS overproduction in cancer cells. TrxR, an NADPH-dependent selenoenzyme, is vital to the intracellular redox balance and regulation of apoptosis signaling, and it has been shown that TrxR inhibition can produce ROS-dependent DNA damage responsible for cell growth inhibition. Moreover, radiation-induced DNA damage is related to the inhibition of TrxR activity. Here, the combined effects of Bi2Se3-CS-RGD NSs and X-ray irradiation on the TrxR activity in HeLa cells are studied. As shown in Figure 5a, the NSs and X-ray irradiation alone inhibit the TrxR activity slightly to 98.02% and 85.41%, respectively. However, the TrxR activity diminishes markedly to ~53.21% in the HeLa cells after the combined treatment. The western blot analysis results also show that the Bi2Se3-CS-RGD NSs in combination with X-ray inhibit the expression level of TrxR and Trx in HeLa cells (Figure 5b), providing evidence that decreased expression of TrxR and Trx may contribute to the anticancer efficacy of radiotherapy sensitization by destroying the intracellular redox balance and triggering cell apoptosis.

Reactive oxygen species (ROS) are highly reactive ions and free radicals, including hydrogen peroxide (H2O2), superoxide (O2−), hydroxyl radical (·OH), and singlet oxygen (1O2); these ROS play a vital role in physiology. Production and enhancement of ROS generation under high-energy radiation is an important factor in cancer radiotherapy. Dihydroethidium (DHE) is a fluorescence probe used to detect the intracellular superoxide anion (O2−), which is the initiator of the free radical chain reaction. Upon receiving high-energy X-ray radiation, high-Z NPs can enhance the O2− conversion to generate O2·−/HO2·, OH·, and 1O2; 1O2 is the most prominent and can damage DNA and induce cellular toxicity. Here, ROS generation is investigated in cells treated with the Bi2Se3-CS-RGD NSs and X-ray using the DHE fluorescence probe. In the cell-free model (Supplementary Figure S7A) and cell model (Supplementary Figure S8A, B), X-ray (8 Gy) alone slightly elevates ROS generation, but ROS generation declines sharply after exposure to the Bi2Se3-CS-RGD NSs alone in a dose-dependent manner (Supplementary Figure S7B). It is possible that the Bi2Se3-CS-RGD NSs are capable of scavenging free radicals, and the total antioxidant activity of the Bi2Se3-CS-RGD NSs is determined by the ABTS⋅− scavenging assay. The detailed time course analysis shown in Supplementary Figure S8C shows that the NSs scavenge the ABTS⋅− in a time- and dose-dependent manner.
dependent manner. It has been reported that $^{1}\text{O}_2$ is formed in irradiated solutions only in the presence of NPs.\textsuperscript{51} The combined treatment reduces the intracellular ROS level compared with the control group, but the level is slightly higher than that for the treatment with Bi$_2$Se$_3$-CS-RGD NSs alone. We supposed that the NSs become a new source of radiation and emit high energy through a secondary electron effect, enhancing the $^{1}\text{O}_2$ conversion to generate $^{1}\text{O}_2$ in aqueous suspensions due to X-ray absorption. To confirm this hypothesis, the $^{1}\text{O}_2$ generation of the NSs is studied using DPBF as the probe molecule in ethanol. As shown in Figures 6a and b, the absorption intensities at ~ 410 nm for NSs gradually decrease with increasing X-ray irradiation dose and NS concentration. From the calculated curves (Figure 6c), there is no absorption of DPBF in the NSs without irradiation within 60 min; once irradiated by X-rays, the NSs present the maximal efficiency in generating $^{1}\text{O}_2$. These results are corroborated by the fluorescence imaging (Figure 6d). Therefore, the NSs enhance the radiosensitivity of HeLa cells to X-ray by producing $^{1}\text{O}_2$, inhibiting the TrxR activity and causing an intracellular reactive oxygen species imbalance.

**Activation of intracellular apoptotic signaling pathways**

During radiotherapy, radiation-induced DNA damage can lead to cell death by activating different cellular events,\textsuperscript{62} and DNA damage can cause cell death by activating downstream signaling pathways such as p53, MAPKs and AKT.\textsuperscript{63} We investigate the expression and phosphorylation of the DNA damage-related proteins in the treated cells, including histone and p53. The combined treatment elevates the phosphorylated p53 compared to X-ray or Bi$_2$Se$_3$-CS-RGD NS treatment alone, but the total p53 expression remains unchanged (Figure 7a). Moreover, phosphorylation of histone at the Ser 139 site, an important marker of DNA double-stranded break, is upregulated in response to the combined treatment, indicating the involvement of the DNA damage-mediated p53 pathway in cell apoptosis.

Cancer cells have the ability to repair DNA double strand breaks in radiation-induced lesions and reverse drug-induced cell damage.\textsuperscript{64} Therefore, the expression levels of the DNA repair proteins VEGFR 2 and MGMT, the recovery proteins of cancer cells after radiation, are studied. As shown in Figure 7b, X-ray irradiation up-regulates VEGFR 2 and MGMT, whereas the Bi$_2$Se$_3$-CS-RGD NSs and combination treatment reduce the expression levels, inhibiting the self-repair capability of the HeLa cells. Mitogen-activated protein kinases (MAPKs), including p38, ERK, and JNK, and AKT kinase are important regulators of cell proliferation, growth, and apoptosis in response to drug treatment. The combined treatment is found to produce different effects on the phosphorylation status of MAPKs and AKT in HeLa cells (Figure 7c); phosphorylation of the pro-apoptotic kinase p38 displays a trend of upregulation, whereas that of the anti-apoptotic kinases AKT and ERK is suppressed and that of JNK is not affected. It can be inferred that the Bi$_2$Se$_3$-CS-RGD NSs enhance X-ray-induced DNA damage and suppress the cell self-repair capability via the p53, MAPKs and AKT signaling pathways (Figure 7d).

**In vivo imaging and biodistribution investigations**

In PAI, which offers high spatial resolution, the optical energy absorbed by light-absorbing tissues or contrast agents with thermal expansion creates reflected ultrasound signals.\textsuperscript{65,66} PAI has the advantage of deep tissue penetration as a result of the ultrasonic wave produced by the pulsed laser\textsuperscript{67,68} and can be utilized to investigate tumor-targeting effects. The bare Bi$_2$Se$_3$ NSs and Bi$_2$Se$_3$-CS-RGD NSs are injected intravenously (1 mg ml$^{-1}$, 0.1 ml) into tumor-bearing nude mice, and the PA images are acquired 2–18 h post injection. As shown in Figure 8a, gradually increasing PA signals of the Bi$_2$Se$_3$-CS-RGD NSs are detected from the tumor tissue, and the tumor outline becomes increasingly clear during the first 6 h (PA signals $\sim$ 271 au, Supplementary Figure S9A), indicating time-dependent accumulation of the NSs at the tumor. The PA signal then decreases, suggesting clearance of the NSs from the cancer cells. A post-injection time point

| a | NSs | 8 Gy |    |    |    |
|---|-----|------|----|----|----|
|   | p-p53 |    |    |    | 53 KD |
|   | p53   |    |    |    | 53 KD |
|   | p-Histone (Ser139) |    |    |    | 15 KD |
|   | $\beta$-actin |    |    |    | 42 KD |
| b | NSs | 8 Gy |    |    |    |
|   | VEGFR2 |    |    |    | 210 KD |
|   | MGMT |    |    |    | 22 KD |
|   | $\beta$-actin |    |    |    | 42 KD |

**Figure 7 Activation of intracellular apoptotic signaling pathways by Bi$_2$Se$_3$-CS-RGD NSs and radiation.** (a) Bi$_2$Se$_3$-CS-RGD NSs enhances radiation-induced activation of the p53 signaling pathway. (b) Bi$_2$Se$_3$-CS-RGD NSs down-regulates the expression of VEGFR2 and MGMT in HeLa cells. (c) Effects of X-ray and Bi$_2$Se$_3$-CS-RGD NSs on the expression levels of correlotive protein of MAPKs and AKT pathways in HeLa cells. (d) Proposed signaling pathways accounting for cells apoptosis induced by radiation and Bi$_2$Se$_3$-CS-RGD NSs.
of 6 h is selected for the subsequent X-ray radiosensitization experiments. The tumor-targeting effects by intratumoral injection are also compared. As shown in Figure 8b, the PA signal from the tumor site lasts up to 6 h after intratumoral injection of the Bi$_2$Se$_3$-CS-RGD NSs at the same concentration as intravenous injection. The PA images of the bare Bi$_2$Se$_3$ NSs are shown in Figures 8c and d. After intravenous injection of the Bi$_2$Se$_3$ NSs, the largest PA signal intensity is only $\sim$193 au and is weaker than that of the Bi$_2$Se$_3$-CS-RGD NSs. The corresponding time point for the largest PA signal is 12 h, longer than that of the Bi$_2$Se$_3$-CS-RGD NSs (Supplementary Figure S9A). After intratumoral injection of the Bi$_2$Se$_3$ NSs, the PA signal intensity from the tumor area declines rapidly, and faint signals can be detected at 6 h post-injection (Supplementary Figure S9B). These results demonstrate that the RGD-decorated Bi$_2$Se$_3$ NSs have more efficient tumor-targeting ability and have longer residence time than the bare Bi$_2$Se$_3$ NSs.

The biodistribution and clearance properties of nanomaterials are crucial for the rational design of nanomedicine in advanced cancer therapy. To explore the biodistribution profile of the Bi$_2$Se$_3$-CS-RGD NSs in vivo, inductively coupled plasma mass spectrometry is employed to determine the concentrations of Bi and Se in tumor tissues and main organs at 0, 12, 24, 36 and 48 h after injection of the NSs into HeLa tumor-bearing nude mice. As shown in Figure 9a, the original amounts of Bi in the different organs of the
mice are 0.032 μg g⁻¹ (heart), 0.25 μg g⁻¹ (liver), 0.15 μg g⁻¹ (spleen), 0.12 μg g⁻¹ (lung), 0.14 μg g⁻¹ (kidney) and 0.036 μg g⁻¹ (tumor). After injection of the NSs, they aggregate mainly in the liver, spleen and kidney, and the lung and heart show relatively low Bi concentrations. Moreover, high concentrations of Bi are detected in the tumor due to the active targeting effect attributed to the RGD peptides. The Bi concentrations decrease from 35.64 μg g⁻¹ (liver), 18.25 μg g⁻¹ (spleen), 6.79 μg g⁻¹ (kidney) and 7.16 μg g⁻¹ (tumor) at 12 h to 4.66 μg g⁻¹ (liver), 2.69 μg g⁻¹ (spleen), 1.05 μg g⁻¹ (kidney) and 1.58 μg g⁻¹ (tumor) at 48 h after intravenous injection. The Se biodistribution in different tissues and tumors of the nude mice before injection of the Bi₂Se₃-CS-RGD NSs are also measured. As shown in Figure 9b, the original amounts of Se in the different organs of the mice are 0.21 μg g⁻¹ (heart), 1.13 μg g⁻¹ (liver), 0.61 μg g⁻¹ (spleen), 0.49 μg g⁻¹ (lung), 0.30 μg g⁻¹ (kidney) and 0.49 μg g⁻¹ (tumor). At 12 h post-injection, Se shows the highest accumulation in the liver, spleen, kidney and tumor, with concentrations of 6.02, 3.12, 1.31 and 1.34 μg g⁻¹, respectively, and decreases to 1.98, 0.92, 0.45 and 0.53 μg g⁻¹ at 48 h, respectively. To investigate the clearance of the NSs, the time-dependent residual amounts of Bi and Se in the mice are calculated by summing the amount of Bi and Se in all the tissues from the injected Bi₂Se₃-CS-RGD NSs (Figure 9c). The initial total amounts of Bi and Se are 100.1 and 39.45 μg, respectively. As expected, the Bi concentrations decrease to 68.62 μg after 12 h and to 16.09 μg after 48 h, and similar behavior is observed for Se. The residual amount is 9.44 μg after 12 h and decreases to 2.11 μg after 48 h. Both the biodistribution and clearance efficiency of Bi and Se after intratumoral injection are similar to those after intravenous injection, and much larger concentrations are present at the tumor (Figures 9d–f). The liver and spleen are the dominant organs participating in the metabolism of the NSs, and their cancer-targeting effects and subsequent excretion are verified experimentally.

The biodistribution and clearance of the bare Bi₂Se₃ NSs in vivo are also investigated (Supplementary Figure S10). Compared to the Bi₂Se₃-CS-RGD NSs, the bare Bi₂Se₃ NSs show similar clearance profiles, but there is much less accumulation in the tumor, confirming that RGD decoration leads to a more efficient tumor targeting ability of the Bi₂Se₃ NSs.

**In vivo radiosensitization effects**

The in vivo cancer radiosensitization activity of the Bi₂Se₃ NSs and Bi₂Se₃-CS-RGD NSs is studied using HeLa tumor-bearing nude mice. Based on the PAI results, the samples are injected intravenously 6 h in advance and intratumorally within 0.5 h before injection with 1 mg ml⁻¹ and 0.1 ml doses. The mice are irradiated by X-ray at a dose of 4 Gy using a 2-cm-thick lead plate with appropriate holes to protect other parts of the body from radiation. The changes in the tumor volume and body weight are determined for 21 days post injection (Figure 10). In the control groups treated with the Bi₂Se₃-CS-RGD NSs alone, the tumor volume increases with time, and X-ray irradiation alone inhibits tumor growth from 2.55 to 1.78 cm³. When the mice are treated with both X-ray and Bi₂Se₃ NSs by intravenous and intratumoral injection, the tumor volume decreases to 1.48 cm³ (G5) and 1.08 cm³ (G6), respectively. In comparison, better therapeutic effects are achieved from the X-ray and Bi₂Se₃-CS-RGD co-treatment groups, in which intravenous and intratumoral injection reduce the tumor volume to 1.20 cm³ (G7) and 0.79 cm³ (G8), respectively. Therefore, the in vivo radiotherapy efficacy of Bi₂Se₃ NSs is enhanced by targeted modification with RGD peptide. The excellent active targeting ability of the Bi₂Se₃-CS-RGD NSs results in accumulation of Bi in the tumor, acting as a radiosensitizer to inhibit tumor growth. In addition, the X-ray treatment reduces the body weight of the treated mice, but no decline is observed for the NS groups, suggesting that the Bi₂Se₃-CS-RGD NSs have low in vivo toxicity.

MRI is a powerful nondestructive diagnostic tool that provides high spatial resolution in soft tissues and morphological details and multifunctional information about lesions. This technique is employed in this study to confirm the radiosensitization properties of the Bi₂Se₃-CS-RGD NSs. The T₂-weighted MR images of HeLa tumors using an EG 1.5 T clinical MR scanner equipped with a small animal imaging...
coil are shown in Figure 11a. After 21 days, darkening effects are observed in the tumors in the Bi$_2$Se$_3$-CS-RGD NSs and X-ray co-treated groups, indicating a high tumor-active targeting effect. The Slow ADC and Fast ADC signals reflect the necrotic degree in the tumor area and blood flow in the tumor vessels. The Bi$_2$Se$_3$ NSs + X-ray co-treatment groups (G5, G6) show little changes in Slow ADC (Figure 11b) and Fast ADC (Figure 11c) compared with the control group. However, a stronger Slow ADC signal and a weaker Fast ADC signal at the tumor sites can be observed in the Bi$_2$Se$_3$-CS-RGD NSs combined with X-ray treatment groups (G7, G8). This effect is confirmed by quantifying the relative signal intensity (Figures 11d and e), revealing that the Bi$_2$Se$_3$-CS-RGD NSs reduce the cancer cell activity and density more than bare Bi$_2$Se$_3$ NSs after the co-treatment, consistent with the pathological H&E staining results (Figure 10d). Overall, these results indicate that the Bi$_2$Se$_3$-CS-RGD NSs possess unambiguous advantages over the bare Bi$_2$Se$_3$ NSs in radiotherapy of cervical cancer due to conjugation of the cancer-targeting RGD peptide.

To further assess the possible in vivo toxicity, the mice in different treatment groups are sacrificed and subjected to hematological analysis after 21 days. As shown in Figure 12a, compared to healthy nude mice, the HeLa xenograft nude mice show reduced plasma glucose (GLU), increased low-density lipoprotein (LDLC), and acute liver and renal dysfunction, as manifested by decreases in the total protein (TP) and uric acid (UA) and increases in aspartate aminotransferase (AST) and blood urea nitrogen (BUN). The Bi$_2$Se$_3$-CS-RGD NSs combined with X-ray treatment does not cause hepatic damage compared to healthy mice, as shown by the hepatic function markers (TP, AST). The renal function markers (UA and BUN) also recover to the levels of normal mice after the combined treatment. However, treatment of the nude mice with Bi$_2$Se$_3$ NSs results in toxicity to the liver, as reflected by the changes in the blood biochemical values, especially AST. Therefore, these results demonstrate that RGD conjugation is an effective strategy to reduce the in vivo toxicity of Bi$_2$Se$_3$ NSs. Histological analysis of the major organs, such as the heart, liver, spleen, lung and kidney, is performed on the treatment groups. As shown in Figure 12b, several dark spots are observed on the liver after Bi$_2$Se$_3$ NS treatment, which was due to the cellular uptake of liver macrophage through phagocytosis and accumulation in the liver sinus (Supplementary Figure S11). No other notable organ damage or inflammatory lesions can be detected. In the Bi$_2$Se$_3$-CS-RGD NS treatment, no dark spots could be detected in the hepatic sinusoid, verifying its selectivity. These results show that covalent conjugation of RGD improves Bi$_2$Se$_3$ NS accumulation in tumor tissues and reduces the in vivo toxicity, making Bi$_2$Se$_3$-CS-RGD NSs safe in cancer radiotherapy.

**CONCLUSION**

Ultrathin Bi$_2$Se$_3$-CS-RGD NSs with excellent tumor targeting ability and potent radiosensitization efficiency are constructed for imaging-
guided cancer radiotherapy. The Bi$_2$Se$_3$-CS-RGD NSs with X-ray irradiation inhibit HeLa cell growth by inducing G0/G1 cycle arrest and mitochondria-mediated intrinsic cell apoptosis. The Bi$_2$Se$_3$-CS-RGD NSs enhance the sensitivity of cancer cells to X-ray-induced apoptosis by inhibiting TrxR and activating downstream ROS-mediated signaling pathways to regulate cell apoptosis. In the nude mice model, the RGD coating enables the Bi$_2$Se$_3$ NSs to rapidly aggregate in the tumor regions, enabling efficient PAI of the entire tumor to facilitate radiotherapy of cervical cancer. Nondestructive MR imaging demonstrates increased tumor tissue necrosis, reduced blood flow in the tumor vessels, and reduced cancer cell activity and density after the combined Bi$_2$Se$_3$-CS-RGD NSs and X-ray treatment, which is more efficient than bare Bi$_2$Se$_3$ NSs alone. The Bi$_2$Se$_3$ NSs after RGD decoration show much better in vivo biocompatibility and are efficiently expelled from the body after 48 h post-injection. No evident damage or inflammatory lesions is observed in the major organs, including the heart, liver, spleen, lung and kidney, in the hematological and histological analyses. The Bi$_2$Se$_3$ NSs with RGD modification have great potential as an effective and safe in vivo theranostic agent for next-generation cancer radiotherapy.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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Supplementary Information

For

Decorated ultrathin bismuth selenide nanosheets as targeted theranostic agents for in vivo imaging-guided cancer radiation therapy

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Figure S1 (A) TEM image. (B) Magnified TEM image of the Bi$_2$Se$_3$ NSs. (C) Size distribution of Bi$_2$Se$_3$ NSs, Bi$_2$Se$_3$-CS NSs and Bi$_2$Se$_3$-CS-RGD NSs measured by Malvern Instruments Limited.

Figure S2 Stability of (A) Bi$_2$Se$_3$ NSs and (B) Bi$_2$Se$_3$-CS-RGD NSs in PBS solution at 4 °C. (C) Stability of Bi$_2$Se$_3$ NSs and Bi$_2$Se$_3$-CS-RGD NSs dispersed in DMEM (10% FBS) incubated at 37 °C.
**Figure S3** (A) TEM, (B) SAED pattern, and (C) HR-TEM image of Bi$_2$Se$_3$-CS-RGD NSs after X-ray (8 Gy, 6 MeV) irradiation for 2 min.

**Figure S4** Bi$_2$Se$_3$-CS-RGD NSs sensitizing various human cancer cells to X-ray: (A) A375, (B) Siha and (C) Caski cells were treated with Bi$_2$Se$_3$-CS-RGD NSs for 6 h and then exposed with or without X-ray (8 Gy). The cells were cultured for another 72 h and examined by MTT assay. Values expressed are means SD of triplicates. A significant difference between the treatment and control groups is indicated by *$P$* < 0.05.
**Figure S5** CI corresponding to different ratio of X-ray and Bi\textsubscript{2}Se\textsubscript{3}-CS-RGD NSs in the combined treatment of HeLa cells.

**Figure S6** Colony formation of HeLa cells after co-treatment with Bi\textsubscript{2}Se\textsubscript{3}-CS-RGD NSs (0~8 µg/mL) and radiation (0~8 Gy).
Figure S7 (A) Effects of Bi$_2$Se$_3$-CS-RGD NSs (40 µg/mL) with or without radiation (8 Gy) on ROS generation in cell free model. (B) Role of intracellular ROS in HeLa cells induced by different concentrations of Bi$_2$Se$_3$-CS-RGD NSs.

Figure S8 Changes in ROS generation induced by cotreatment of Bi$_2$Se$_3$-CS-RGD NSs and X-ray radiation. (A) HeLa cells pretreated with 40 µg/mL Bi$_2$Se$_3$-CS-RGD NSs for 6 h were treated with 8 Gy of X-ray radiation and were finally incubated with 10 µM DHE for 30 min. (B) Fluorescence imaging of ROS generation in HeLa cells after the incubation of Bi$_2$Se$_3$-CS-RGD NSs (40 µg/mL) and X-ray (8 Gy) for indicated times using a DHE probe. (C) Time- and dose-dependent in vitro antioxidant activities of Bi$_2$Se$_3$ NSs, Bi$_2$Se$_3$-CS NSs and Bi$_2$Se$_3$-CS-RGD NSs determined by ABTS$^+$ assay. Values expressed are means ± SD of triplicates.
Figure S9 The intensity of photoacoustic signals in tumor sites at different time points post-injection of Bi$_2$Se$_3$ NSs and Bi$_2$Se$_3$-CS-RGD NSs with (A) intravenous injection and (B) intratumoral injection, respectively.

Figure S10 In vivo biodistribution and clearance of the Bi$_2$Se$_3$ NSs in different organs. Se concentrations at different time points of 0, 12, 24, and 48 h after (A) intravenous injection and (C) intratumoral injection, respectively. The residual amounts and corresponding residual ratios of Se plotted versus time (B) intravenous injection and (D) intratumoral injection of the Bi$_2$Se$_3$ NSs.
**Figure S11** Magnified H&E stained images of liver collected from G5 and G6. Bi$_2$Se$_3$ NSs was uptook by liver macrophage through phagocytosis and circled by a yellow dashed line. G5: Bi$_2$Se$_3$ NSs (intravenous) + X-ray; G6: Bi$_2$Se$_3$ NSs (intratumoral) + X-ray.