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Radiation Dose Enhancement Using Bi2S3 Nanoparticles in Cultured Mouse PC3 Prostate and B16 Melanoma Cells

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Abstract

Gold nanoparticles (Au NPs) have been extensively investigated as contrast and dose enhancing agents. Bismuth sulfide (Bi_2S_3)-NPs have recently been investigated as contrast agents in radiology. In this study the dose enhancing effects of Bi_2S_3-NPs on radiated mouse PC3 prostate and B16 melanoma cells were examined. Equimolar concentrations of both Au and Bi_2S_3-NPs displayed equal dose enhancement with B16 cells, while the latter provided higher values with PC3 cells. At, equimolar concentrations there are less Bi atoms compared to Au in their respective NPs. Both NPs at comparable concentrations (0-1 mM) elicited similar cytotoxicity in PC3 mouse prostate cells. This study demonstrates that the less expensive Bi_2S_3 NPs are a viable alternative to Au NPs as a dose enhancing agent in clinical applications.

Keywords

Dose enhancement, Bi_2S_3 nanoparticles, Gold nanoparticles, PC3 mouse prostate cancer cells, B16 mouse melanoma cells

Introduction

The chief aim of radiotherapy is to maximize the radiation dose delivered to tumours and at the same time minimize the level of damage to the surrounding healthy tissues by reducing the dose they receive. The methods used to achieve this aim have mainly focused on employing the latest technologies and computers, such as intensity modulated radiotherapy (IMRT) and volumetric modulated arc therapy (VMAT) [1,2]. A complementary approach for potentially achieving this aim involves the localisation of high atomic number (Z) elements and compounds at the target tumour tissue prior to irradiation. The presence of high Z atoms in the target leads to an increase in the photoelectric effect cross section and the generation of low energy free radicals (photoelectrons and Auger electrons) [3-5]. These low energy free radicals have high linear energy transfer (LET) values, [6] and as a result can cause DNA damage via the generation of reactive oxygen species (ROS). Moreover, it has been recently reported that metallic nanoparticles sensitize the DNA molecules chemically and make them more sensitive to radiation damage [7]. If the cell sustains sufficient DNA damage it can impair its ability to replicate and/or activate cell death pathways. This line of investigation started with the use of iodine-based compounds of the type normally used as contrast agents for imaging in radiology [4]. Subsequently, this was fully studied

using SQ20B human head and neck squamous carcinoma cells, and the levels of anticipated dose enhancement were formulated [8]. Recently, metallic nanoparticles (NPs) have been used for this purpose. NPs possess several advantages over iodine in that they (i) have a higher Z number and therefore a higher probability of photoelectric interactions, which depends approximately on Z [3]; (ii) accumulate more readily and to a greater extent in tumour tissue, [9-11] and (iii) sensitize the DNA molecules [7]. One of most commonly used metallic NPs for dose enhancement is gold (Au) [12-14]. Au has a high atomic number (79) and density (19.32 g/cm^3), this leads to higher interaction and scattering probabilities with radiations, higher absorption via photoelectric (which depends strongly on the atomic number of the target (Z^3)), and higher Compton interactions due to the high density of its atoms. Au NPs also have a low relative toxicity compared to other metallic NPs [15]. Dose enhancement has been observed by inclusion of Au NPs with low energy X-ray beams (of the levels obtained from typical X-ray tubes) and to a much lower extent with higher energy beams (of the type generated by linear accelerators used in radiotherapy) [11-13]. Detailed descriptions of the outcomes of various studies have been recently reported by McMahon et al. [16] as well as full theoretical studies of such methods for radiation dose enhancement [17]. Few studies have been documented in the literature describing alternatives to Au NPs [18]. Among these alternatives are bismuth nanoparticles, including Bi_2O_3 NPs [19]. While that earlier study was based on the use of phantoms, [19] the current one is based on *in vitro* investigation of the radiation effects of Bi_2S_3 NPs.

Bismuth-based materials have a number of potential advantages over gold. Bismuth has a higher Z [Bi-83 and Au-79], resulting in a higher cross section for radiation absorption via the photoelectric effect, which depends strongly on the target atom's Z value. It also has a slightly higher density of free electrons than does Au, hence increasing the probability for Compton scattering, which involves outer shell electrons. Beside its enhancement of energy absorption, Bi NPs have been shown to increase the nuclear damage by inducing the chemical radio-sensitivity of DNA [7]. Bi is one of the least expensive heavy metal-based NPs and is significantly cheaper than Au. It has a low level of toxicity and has been used in biological applications, [20] and pharmaceuticals formulations for the treatment of gastrointestinal disorders, [21] eradication of *Helicobacter pylori* in peptic ulcers, [22-24] syphilis, tumours, [25] and the reduction of cisplatin-induced renal toxicity [26]. Even when exposed to the highest dose of bismuth subsalicylate used to treat stomach disorders (~4.2 g BSS/kg) the plasma concentrations of salicylate were found to be well below toxic levels [27].

In this study, we conducted a head-to-head comparison of Bi_2S_3 and Au NPs and demonstrated that the level of radiation dose enhancement inflicted by the former is higher than that reported for the latter. These studies were conducted using two mice tumour cell lines *in vitro,* which were exposed to X-ray beams in the kilovoltage range.

Materials and Methods

Preparation of Bismuth Sulfide NPs

Spherical Bi_2S_3 NPs (3-5 nm in diameter) were synthesized as previously described [28]. Briefly, bismuth neodecanoate (7.2 g) was dissolved in octadecane (80 mL) and oleic acid (40 mL) with sonication. This mixture was heated at 165°C under Ar for 20 min before thioacetamine (0.75 g) dissolved in oleylamine (7 mL) was added. This mixture was heated at 110°C under Ar and vigorously stirred. The reaction mixture turned dark brown immediately after the injection of thioacetamine. After 1 min, the mixture was cooled to room temperature and washed with ethanol (ratio ethanol to Bi_2S_3 NPs is 2:1). The NPs were isolated via centrifugation at 12,000 g for 3 min.

The NP pellet was redispersed in dichloromethane (DCM) (10 mL), and ethanol (20 mL) was added to re-precipitate the NPs, which were isolated via centrifugation (1 min, 12,000 g). The Bi_2S_3 NPs (~300 mg) were redispersed in DCM (200 mL) and polyvinylpyrrolidone (PVP, M_w 8,000, 2 g) was added. The mixture was heated at 70°C for 4 h, cooled to room temperature, and the solvent was removed in vacuo. The NPs were suspended in Milli-Q water (50 mL). They were dialyzed (MWCO 10 kDa) against Milli-Q water for 24 h, before the NPs were isolated via freeze-drying.

Preparation of Bi_2S_3 solutions

The Bi_2S_3 NPs were suspended in Roswell Park Memorial Institute 1640 medium (RPMI; Invitrogen, Carlsbad, California) and passed through a 0.22 μm hydrophilic polysulfonic membrane (Sartorius, Göttingen, Germany) before use. The resulting stock was diluted with complete medium in order to obtain the required concentrations as listed in the results section.

Preparation of AuNP solutions

Spherical Au NPs (AuroVist™ 1.9 nm) were purchased from Nanoprobes Inc. (Yaphank, New York, USA). They were washed with RPMI and then being passed through a 0.22 μm hydrophilic polysulfonic membrane. The resulting stock was diluted with complete medium in order to obtain the required concentrations.

Determination of elemental Bi and Au concentrations

The amount of elemental Au in the commercial Au NPs (AuroVist™, Nanoprobe Inc., 1.9 nm diameter) was quoted by the supplier to be 75 wt%. Therefore, 1 g of Au NPs used in this study contains 0.75 g of elemental Au. For the Bi_2S_3 NPs, the amount of Bi_2S_3 present was determined via thermal gravimetric analysis (TGA).

Cell culture techniques

Mouse prostate (PC3) and B16 melanoma cells were cultured and maintained in RPMI containing 10% (v/v) heat-inactivated foetal bovine serum (FBS; Bovogen Biologicals, Melbourne, Australia) at 37°C in a humidified 5% CO_2 incubator.

Time course of the effect of NPs on PC3 cell viability

PC3 cells were seeded (10^4 cells/well) in 96 well plates, cultured for 24 h, and washed with RPMI prior to being exposed

to Au and Bi_2S_3 NPs (0-2 mM) suspended in RPMI plus 10% FBS for up to 48 h. The cytotoxicity of the nanoparticles was assessed using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega Corp, Madison, Wisconsin) [29]. Cell viability was measured using the PrestoBlue cell viability reagent (Invitrogen) according to the manufacturer's instructions. Finally, the Optical absorption was measured at 490 nm on a CLARIOstar® plate reader (BMG LABTECH, Mornington, Australia). Experiments were performed in triplicate with 4 replicates for each treatment at each time point. Data is expressed as the percentage of the viable cell population for that treatment relative to that of the untreated controls at that time point, which was expressed as 100%.

Irradiation of cells

PC3 mouse prostate and B16 breast cancer cells (10^4 cells/well) were added to a 96 well plate and allowed to adhere for 24 h. On the next day the cells were exposed to different concentrations of Au and Bi_2S_3 NPs (0-1 mM) for 24 h prior to being irradiated with kilovoltage beams from 80 kV superficial radiotherapy beams at William Buckland Radiotherapy Centre (Alfred Hospital, Melbourne, Australia). The cells were irradiated as described previously [18]. Using various radiation doses (0-7 Gy). A special holder for the 96 cell plate was used to fulfill the full scatter conditions required to satisfy dose delivery conditions and the procedure for dose optimisation documented by Claridge et al. [30] was followed. Dose distribution was confirmed with radiochromic film (GafChromic film EBT; International Specialty Products, Wayne, New Jersey) in phantoms holding the plates to insure that the cells in the wells were exposed to a uniform radiation dose.

Dose enhancement effect of NPs on cell viability

PC3 mouse prostate and B16 breast cancer cells were seeded (10^4 cells/well) and cultured for 24 h in 96 well plates prior to being exposed to X-rays (0-7 Gy) in the presence of Bi_2S_3 or Au NPs (0-1 mM). Cell number was measured 24 h post-treatment using the Prestoblue reagent according to the manufacturer's instruction. Briefly, 10 µL of the reagent was added directly in each well and then incubated for 20 min at 37°C. Absorbance was then measured at 490 nm on a CLARIOstar® plate reader. In each experiment the number of surviving cells was determined by comparing the absorbance to that recorded against a standard curve of known cell concentrations.

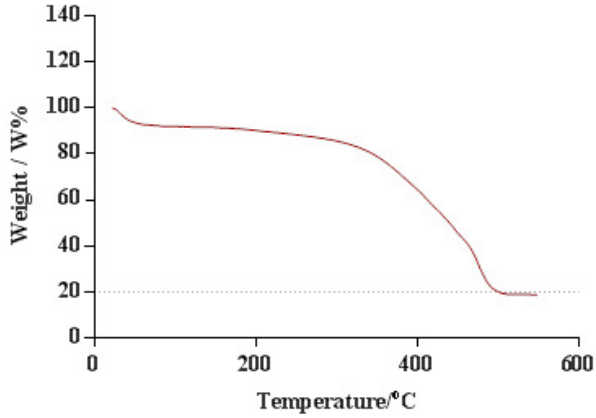

Figure 1: TGA curve of PVP- Bi2S3 Nanoparticles. The weight loss of 80% was ascribed to the PVP on the surface of the nanoparticles.

Result

Determination of elemental Bi and Au concentrations

The amount of Bi_2S_3 present in the prepared NPs was determined via thermal gravimetric analysis (TGA) to be 20 wt%, with the remainder being the stabilising PVP coating. The amount of elemental Bi in Bi_2S_3 is 81% by weight. Therefore, in each gram of Bi_2S_3 NPs used in this study there was 0.16 g of elemental Bi.

Cytotoxicity of Au and Bi2S3 NPs

The effect of Au and Bi_2S_3 NPs on the viability of mouse PC3 cells was investigated to compare their cytotoxic effects in order to determine the maximum concentration at which the NPs were not cytotoxic. The effect of 48 h exposure to different concentrations of NPs on cell viability can be seen in Figure 2. The absorbance of the cultures treated with different concentrations of NPs were expressed as a ratio of that of the untreated controls (no NPs) at the same time point, which were given a value of 100%. It was observed that both NPs were only cytotoxic at concentrations of 2 mM or greater. The NPs at 0.5 mM elicited a hormetic effect on both cell lines [31], as it appears that at all time points the cell viability is >100%. This is because the number of cells in these treated cultures is greater than that of the untreated controls at the same time point.

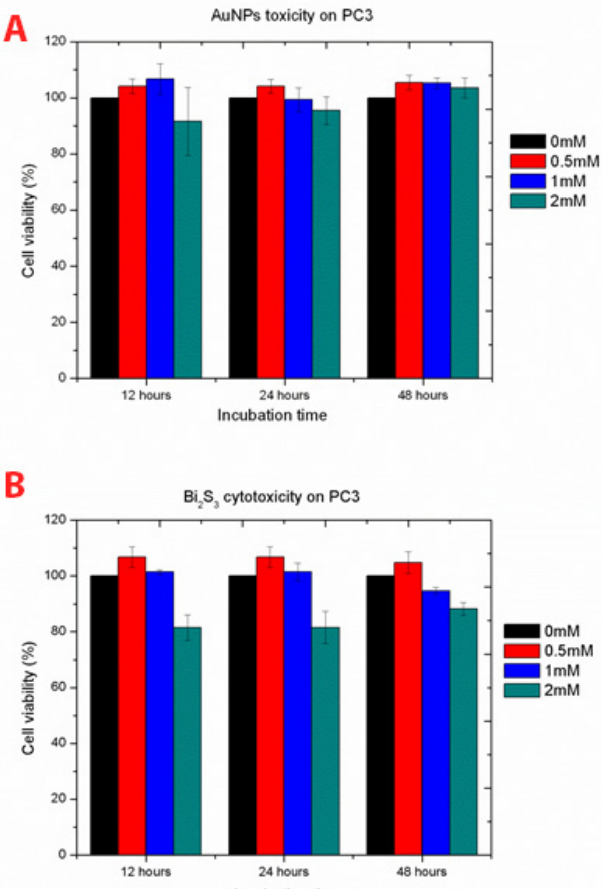

Figure 2: (A) Cytotoxicity of AuNPs and **(B)** Bi2S3 NPs in PC3 cells.

Dose enhancement

The effect of Au and Bi_2S_3 NPs on the viability of PC3 and B16 cells was investigated after they were irradiated with 80 KVp X-rays. This was to determine the dose enhancement factor (DEF) of these NPs. The effects of different concentrations of Au and Bi_2S_3 NPs on the survival curves for irradiated of PC3 and B16 cells are shown in Figures 3 and 4, respectively. The NPs significantly decreased the survival of these irradiated cells. The cytotoxic effect of Bi_2S_3 compared to Au NPs on enhancing the X-ray dose was slightly higher in PC3 cells and but was similar in B16 cells.

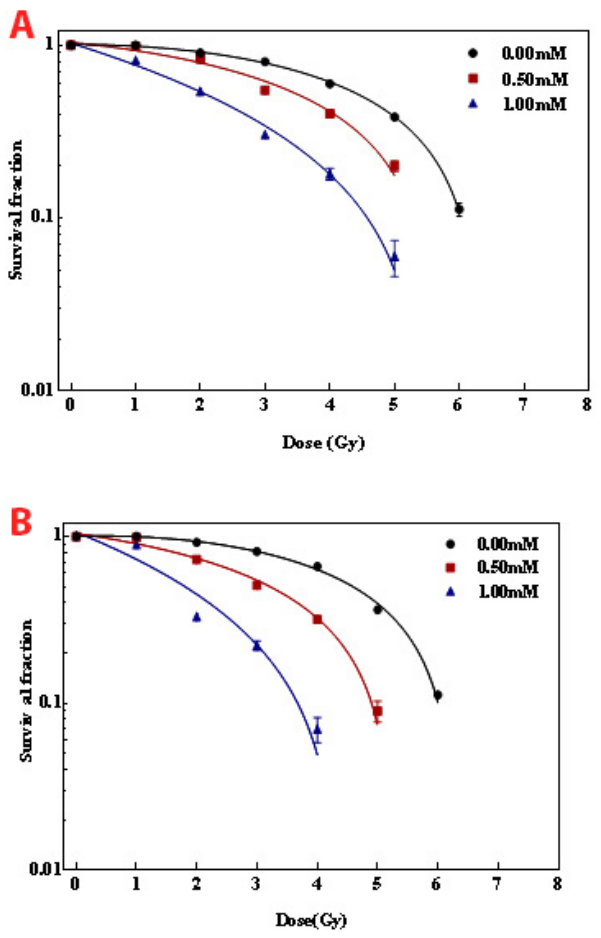

Figure 3: (A) PC3 cells survival curves with Au nanoparticles and without using kilovoltage x-rays. **(B)** Similar conditions using Bi2S3 NPs.

The dose enhancement factor (DEF) is determined from the ratio of the doses required to decrease cell survival to 80% in the presence or absence of added NPs [18]. The DEF for Au and Bi_2S_3 NPs in both cell lines were extrapolated from the survival curves (Figures 3 & 4) and is shown in Table 1. The DEF was shown to be concentration dependent, and was higher in those cells treated with 1 mM NPs compared to those treated with 0.5 mM. PC3 cells were more sensitive to dose enhancement than were B16 cells under all conditions tested. Following irradiation, the Bi_2S_3 NPs generated a higher DEF than did Au NPs at the same concentration. The results suggest that Bi_2S_3 NPs would be more beneficial as a dose enhancer that Au NPs, and as it is cheaper to manufacture, it may be a viable alternative to use in clinical applications.

Discussion

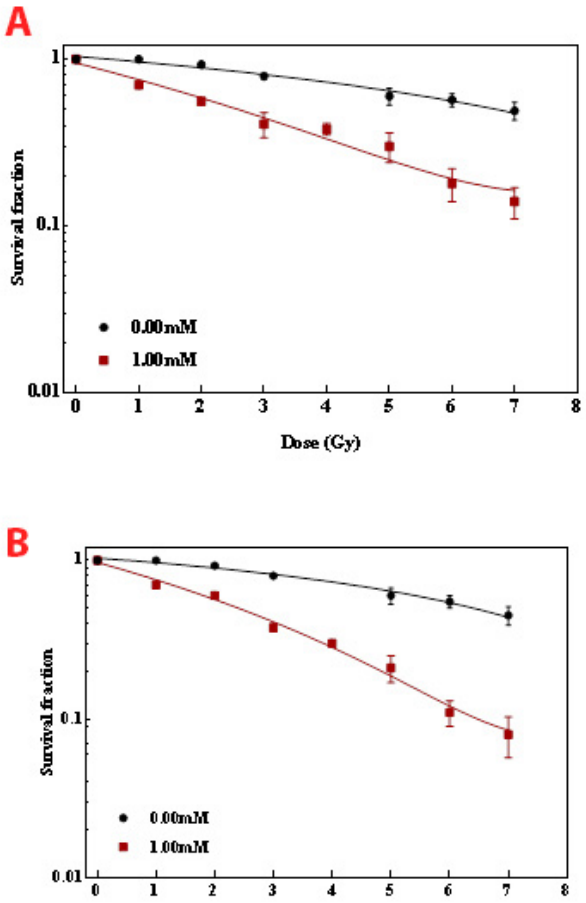

Figure 4: (A) B16 cells survival curves withAu nanoparticles. and without using kilovotage x-rays. **(B)** Similar conditions using Bi2S3 NPs.

Table 1: Comparison of the NP dose enhancement factors at 80% cell survival in irradiated mouse PC3 and B16 cell lines.

Radiation	Type of cells	NPs concentration (mM)			
		Au		Bi_2S_3	
		0.5	1	0.5	1
80KVp	PC3	1.45	2.65	1.6	3.07
	B16	_	2.13	_	2.24

Both the Au and Bi_2S_3 NPs are coated in a stabilising layer (also known as passivating layer) that prevents the NPs from irreversibly aggregating which allows them to be easily dispersed in aqueous solutions. For Au NPs this stabilising coating is composed of (1-mercaptoundec-11-yl) tetraethylene glycol. Whereas for the Bi_2S_3 NPs a PVP coating was used. The interaction of radiation with this organic coating is negligible and can be ignored in dose enhancement calculations. However, to directly compare the contribution of elemental Au and Bi it is important to consider the weight percentage contribution of the organic coating and any other elements in calculations. For Au NPs, the amount of elemental gold present is 75 wt% (as determined by the supplier), with the remaining 25 wt% attributed to the organic coating. The amount of Bi_2S_3 in the Bi_2S_3 NPs was determined by TGA

(Figure 1), which revealed two main regions of mass loss: (a) <80°C due to water loss, and (b) 300-500°C, due to the degradation and loss of the organic PVP coating. The remaining 20 wt% at 500°C can be attributed to the Bi_2S_3 present in the sample. As the compound Bi_2S_3 contains 81 wt% elemental Bi, the total amount of elemental bismuth in these NPs was calculated to be 16 wt%. Therefore, when comparing these NPs to determine their dose enhancement, the actual amount of elemental Au or Bi should be compared. For Au and Bi_2S_3 NPs, the concentrations of the whole element and compound with their coatings should be taken into account with respect to their molarity. However, in the perspective of radiation, the significant factor is the percentage of Au and Bi atoms.

Another factor which could have an effect on dose enhancement is the size of the NPs. The smaller the nanoparticle, the larger is the proportion of atoms on its surface, and therefore a larger surface to volume ratio which influences the cross section to radiations and also affects its uptake and internalization within the cell [9].

In order to compare the dose enhancement effect of Bi_2S_3 NPs to that of Au NPs we used two tumour cell lines (mouse PC3 prostate and B16 melanoma cells). Both tumour cells were chosen because they can form both primary and secondary tumours *in vivo*, and as such are a good model to use to investigate the dose enhancement effect of NPs.

This study indicated that at 0.5 mM both Au and Bi_2S_3 NPs elicited a hormetic effect at 12, 24 and 48 h. While 1 mM Au NPs also enhanced cell growth over the 48 h period, 1 mM Bi_2S_3 NPs did not (Figure 2B). At 2 mM, Au NPs were shown to elicit a slight cytotoxic effect on these cells (15% at 12 h) but by 48 h the cultures appeared to have fully recovered. On the other hand Bi_2S_3 only displayed a hormetic effect at 0.5 mM, but at higher concentrations it was shown to be cytotoxic. At 2 mM Bi_2S_3 NPs caused a 20% reduction in the number of cells in culture after 12 h, which was similar to that seen for Au NPs, but by 48 h these cells had not recovered unlike those treated with the gold nanoparticles.

Having observed the dose enhancement effect of the NPs on both cell lines grown *in vitro*, these experiments need to be repeated under *in vivo* conditions using laboratory animals. The advantage of using cultured cell monolayers is that the cells are exposed to NPs in the culture media. These *in vitro* models do not take into account issues found *in vivo* such as tumour circulation, ensuring the concentration of NPs remains high, or the uptake of these nanoparticles into the cancer cells. Shielding effects due to structures such as bone, are not seen in monolayer cell cultures, which can have an impact on the effectiveness of X-rays in killing tumour cells.

Several studies have demonstrated that Au [32] and Bi_2S_3 [28] NPs are internalized by cells via endocytosis, and in some cases the former have been found '*clustered*' around the membrane within the cytoplasm [18, 33]. Therefore, the NPs are expected to be internalized and enclosed inside the cells enhancing the radiation effect. The survival curve of those cells treated with either NPs was steeper than the untreated controls and showed that the nanoparticles enhanced the cytotoxic effects of the X-rays. This shift of the curve to the left is a measure of the dose enhancement caused by the inclusion of the NPs into the cells prior to them being exposed to the X-rays.

This can be attributed to the fact that the inclusion of high electron density and high atomic number compounds in the cells is expected to generate high numbers of free radicals via both photoelectric and Compton interactions. These extra free radicals would increase the likelihood of DNA damage and subsequent cell death [7]. More importantly, the Bi_2S_3 NPs used in this study only contained 16 wt% Bi (from Bi_2S_3), which generated a similar dose enhancement effect to that of Au NPs (75 wt% Au) in B16 cells. A significant finding was that Bi_2S_3 NPs generated a greater dose enhancement effect to that of Au NPs in irradiated PC3 cells. The results suggest that Bi_2S_3 NPs would a cheaper viable alternative to the use of Au NPs as effective dose enhancing agents when used in clinical applications.

Conclusion

The results from this study suggest that Bi_2S_3 NPs containing ~16 wt% elemental Bi have a similar, or even slightly higher radiological effect, to that of Au NPs which are comprised of 75 wt% elemental Au at the same concentration when taking into account their surface coatings. Bi_2S_3 NPs have been shown to be suitable contrast agents for radiologic imaging and this work proves their value in radio-sensitisation, in particular, dose enhancement at the kilovoltage range of X-ray energies. Therefore, they can be considered as valuable theranostic agents that can improve image quality and at the same time enhance the effects of radiation on the target tissue. Another advantage of Bi_2S_3 NPs as theranostic agents is their proven long blood circulation, rendering them suitable agents for lengthy therapeutic and diagnostic radiation-based procedures.

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