Synthesis and Characterisation of Bismuth Oxide Nanoparticles using Hydrothermal Method: The Effect of Reactant Concentrations and application in radiotherapy

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Abstract. High atomic number (Z) of bismuth oxide nanoparticles (Bi2O3 NPs) has more cell penetration and less adverse effects than conventional radiosensitisers. In this work, 60 nm and 90 nm of Bi2O3 NPs were successfully synthesised using the hydrothermal method by varying the bismuth nitrate, Bi(NO3)3 concentration. The properties of Bi2O3 NPs were characterised to determine the phase presence, crystallinity, morphology, elements presence and size of nanoparticles. The as-synthesised Bi2O3 NPs was in monoclinic Bi2O3 phase (ICDD 98-008-5622). As the Bi(NO3)3 concentration increased, the particle size of Bi2O3 NPs decreased due to less ions diffusion per nuclei. The morphology observation showed that Bi2O3 NPs were in rods form. The produced Bi2O3 NPs were subjected to cytotoxicity analysis and radiotherapy. Bi2O3 NPs did not induce cytotoxicity in breast cancer (mcf-7) cell lines at concentration from 0.05 µM. The radiotherapy performance of the as-prepared Bi2O3 NPs was obtained by calculating the sensitisation enhancement ratio (SER). The optimum result was found for 60 nm Bi2O3 NPs with SER of 3.45.

1. Introduction

The National Cancer Registry of Malaysia (NCR) recorded 21,773 Malaysians being diagnosed with cancer and estimated that almost 10,000 cases are unregistered every year. One of the top 5 cancer in Malaysia is breast cancer [1]. In current clinical oncology practices, half of all cancer patients are treated with radiation
therapy either alone or in combination with other treatments [2]. In radiotherapy, the central pathways to increase the ratio of treatment efficacy to side effects are reversal of radiation resistance in tumour tissue, where the enhancement of radioresistance in healthy tissue increasing radiosensitisation in tumour tissue and better confinement of the deposited dose to the tumour volume [3]. Recent advances in nanotechnology have led to the development of novel nanomaterials and integrated nanodevices for cancer detection and screening, in vivo molecular and cellular imaging, and the delivery of therapeutics such as cancer cell killing radio-isotopes. An increasing number of studies have shown that the selective delivery of therapeutic agents into a tumour mass using nanoparticle platforms could improve the bioavailability of cytotoxic agents and minimize toxicity to normal tissues [4].

The most common radiosensitising approach is to exploit the increase photon absorption of high atomic number (Z) materials at kilovoltage (kV) photon energies [5]. Adopting this approach, therapeutic nanoparticles have been produced using silver (Z = 47), gadolinium (Z = 64) and gold (Z = 79). Recently, Bi₂O₃ NPs has received wide attention in the field of radiotherapy research based on its remarkable radiation dose enhancement under kilovoltage X-ray beams, which is significantly higher than the well-known gold radiosensitiser [6]. However, the study on Bi₂O₃ NPs in radiotherapy is still new. Several approaches have been employed to synthesis Bi₂O₃ NPs in materials science and engineering. Bi₂O₃ NPs has been synthesised via metallorganic Chemical Vapor Deposition (CVD) [7], oxidative metal vapor-phase deposition [8], and slow oxidation of Bi nanowires at 750 °C [9], etc. Although these methods have been proven to be successful in the synthesis of Bi₂O₃ NPs, the methods normally require high temperature heat treatment, long synthesis period, and post treatment, which is far from low cost and apparently require sophisticated equipments. Hence, a more simple synthesis method, which is precipitant-free, additive-free, and low cost, is sought after for the synthesis of Bi₂O₃ NPs. A hydrothermal method is a powerful method in synthesis of Bi₂O₃ NPs at a considerably low temperature, energy saving and cost effective benefits [10]. The hydrothermal method also has the advantages such as controllable particle size, morphology and the degree of crystallinity by simply changing the Bi(NO₃)₃ concentration.

Hence, in this study, Bi₂O₃ NPs were produced using hydrothermal method. The effect of bismuth nitrate, Bi(NO₃)₃ concentration was studied. The effect of size of Bi₂O₃ NPs in cytotoxicity study and as a radiosensitiser also was studied systematically.

2. Experimental Study

2.1 Synthesis of Bi₂O₃ NPs

Bi₂O₃ was synthesised using the hydrothermal method. In this synthesis, 0.05 M and 0.3 M of Bi(NO₃)₃·5H₂O was mixed with 3.0 mmol of Na₂SO₄ and dissolved in 40 ml distilled water, separately. The solution was stirred at room temperature for 45 min. Then, 18.0 mmol of NaOH dissolved in 40 ml of distilled water was added dropwise into the above solution with continuous stirring, which immediately resulted in the formation of yellowish precipitates in the container. The mixed solution was transferred into a Schott Bottle, sealed and subjected to hydrothermal reaction at 60°C. The samples were then allowed to cool to room temperature naturally. The as-formed yellow precipitates were washed with deionized water and ethanol several times, and finally dried in air at 80°C to obtain the final faint yellow Bi₂O₃ samples. The phase presence and crystallinity of the Bi₂O₃ were characterised by using XRD (Bruker AXS D8 Advance with Cu-Kα as a radiation source). The Bi₂O₃ morphology was examined by FESEM (ZEISS SUPRA 35VP), and analyzed using the ImageJ software (ImageJ, 2015) by counting 100 nanoparticles for particle size calculation.
2.2 Cytotoxicity analysis

For cytotoxicity analysis, firstly, 86 mM Bi₂O₃ NPs stock solution (each with 60 nm and 90 nm size) was diluted in media culture (DMEM) to a concentration of 0.05 µM. Secondly, DMEM containing mcf-7 cells in 25cm² flasks were rinsed with phosphate buffer saline (PBS) three times. 2 ml of trypsin was added into the flask for cell detachment from the surface of the flask and incubated for 5 minutes in 5% CO₂ incubator. 2 ml DMEM was added again to deactivate trypsin reaction. DMEM containing mcf-7 cells was transferred into conical tubes for centrifugation. After that, the cells were counted and 20µl of cells suspension containing 1 x 10⁴ cells were seeded in a 96-well plate (Thermo, USA). 3 wells from 96-well plate were left without cell for control. The plate was left overnight at 37°C in a humidified 5% CO₂ incubator. Next day, 100µl of prepared Bi₂O₃ with 500 µM concentration in DMEM was added into each well containing cells. Treated cells were incubated again in 5% CO₂ incubator for 24 hours. Then, the incubated treated cells were rinsed briefly with PBS three times. 90µl of DMEM was added again into each well followed by 10µl of PrestoBlue® reagent. The plate was incubated at 37 °C for 2 hours to increase sensitivity of Presto blue in order to detect live cells. Finally, the cell viability was measured using a microplate ELISA reader (Multiskan spectrum, USA). The work was performed in triplicate and the data was expressed as viability cell percentage using formula below.

\[
\text{Cell viability %} = \frac{(\text{Average absorbance sample} - \text{Absorbance blank})}{(\text{Average absorbance sample} - \text{Absorbance blank})} \times 100
\]

2.3 Cell irradiation

60 nm and 90 nm of prepared Bi₂O₃ with 0.05 µM concentration were added into each micro-vial containing Mcf-7 cells. Then, the cells were exposed to 6 MV photon beams with varying radiation dose from 0 to 10Gy. The LINAC machine was set-up to 100 cm source above the surface distance (SSD) with 10 x 10 cm² field. The irradiated cells were sub-cultured in 6-well plates and incubated in 5% CO₂ incubator for 5 days for clonogenic assay. Medium in 6-well plate was removed and rinsed with PBS. The cold methanol was added and left for 30 minutes to fix the cells on the plate. The methanol was then discarded and crystal violet was inserted and stained for 30 minutes. The plates were rinsed with distilled water and left drying before counting colonies. The colonies of the cell were counted and plot the survival fraction. Finally, the sensitisation enhancement ratio (SER) was calculated based on the survival fraction graft to measure lethal dose for loss of proliferative capacity to 50 % in the presence of Bi₂O₃ NPs. The SER formula as shown below.

\[
\text{Sensitiser enhancement ratio (SER)} = \frac{\text{Radiation dose alone (without NPs)}}{\text{Radiation dose with NPs}}
\]

3. Results and discussion

XRD patterns of the samples prepared using different concentration of bismuth nitrate, Bi(NO₃)₃ of 0.05 M and 0.3 M are shown in Figure 1. All diffraction peaks can be indexed as a pure monoclinic Bi₂O₃ phase, which matched with ICDD 98-008-5622. The strong and sharp XRD peaks indicated that the as-prepared Bi₂O₃ powders are in crystalline state. From Figure 1, the intensity of Bi₂O₃ decreased with increasing
Bi(NO₃)₃ concentration. In hydrothermal reaction, nuclei formed first followed by growth. During hydrothermal reaction at 60°C for 10 min, the amount of Bi(NO₃)₃ concentration that was mixed with Na₂SO₄ and NaOH directly affected the number of nuclei. After hydrothermally synthesised at 60°C, growth process would take place. Therefore, since number of nuclei increased with increasing Bi(NO₃)₃ concentration, less ions could diffuse per nuclei, thus particle size decreased. The average crystallite size of the as–synthesised samples were calculated using the Scherrer equation. From the calculation, the crystallite sizes are 29.6 and 23.2 nm for 0.05 M and 0.3 M of Bi(NO₃)₃, respectively.

**Figure 1.** XRD patterns of Bi₂O₃ NPs synthesised using hydrothermal method at 60 °C and 10 min with varying Bi(NO₃)₃ concentration: (a) 0.05 M, and (b) 0.3 M.

The morphology of the synthesized Bi₂O₃ NPs was observed using SEM as depicted in Figure 2. All samples are in rods form. When Bi(NO₃)₃.5H₂O and Na₂SO₄ were mixed in distilled water at room temperature, Bi₂O(OH)₂SO₄ nanowires formed, which played a role as a template in the following process of the reaction. By adding NaOH solution into the reaction system, OH⁻ ions reacted with Bi₂O(OH)₂SO₄ gradually to form Bi(OH)₃. When the Bi(OH)₃ was subjected to hydrothermal reaction at 60°C and 10 min reaction time, the mixture transformed into Bi₂O₃ nanorods. This process is similar to the formation of ZnO nanorods [11]. The diameters of particles measured using the ImageJ software are approximately 60 nm and 90 nm in diameter for samples prepared with 0.05 M and 0.3 M of Bi(NO₃)₃, respectively.

**Figure 2.** SEM images of Bi₂O₃ NPs synthesised using hydrothermal method at 60 °C and 10 min with varying Bi(NO₃)₃ concentration: (a) 0.05 M, and (b) 0.3 M.

The particle size dependence of Bi₂O₃ NPs on the cytotoxic response in mcf-7 cell lines is shown in Figure 3. A ratio between the nanoparticle-treated cell and untreated control cell was taken as the viabilities,
assuming the viability of untreated control was 100 %, despite the fact that normal cell metabolism processes still produced a small number of dead cells. The result showed that cell viability decreased as the particle size increased. The calculated cell viabilities were 100 %, 118 % and 96 % for control, 60 nm and 90 nm, respectively. Both Bi$_2$O$_3$ NPs sizes did not affect the cell viability of mcf-7 cell lines, indicated that both size were non-toxic. The study on cytotoxicity analysis of Bi$_2$O$_3$ NPs on mcf-7 cells revealed that 90 nm Bi$_2$O$_3$ NPs was more toxic to the mcf-7 cells than 60 nm Bi$_2$O$_3$ NPs. This happened because of the size of nanoparticles was found to play a critical role in both the rate and extent of cellular uptake. Larger nanoparticles exhibited higher cellular uptake, suggesting that larger nanoparticles more strongly induced apoptosis [12]. The cellular uptake of nanoparticles is closely associated with cellular response which is apoptosis. In general, as cellular uptake of nanoparticles increased, cell viability declined and apoptosis increased. Hence, the apoptotic features in the treated mcf-7 breast cancer cells including cells shrinkage indicated that mcf-7 breast cancer cells underwent cells death via apoptosis pathway.

The radiation enhancement effect of Bi$_2$O$_3$ NPs in mcf-7 cell lines was measured by clonogenic assay method (calculating the sensitisation enhancement ratio (SER)). Figure 4 shows the effect of Bi$_2$O$_3$ NPs on cell survival fraction at 6 MV photon energy with different radiation dose from 0 to 10 Gy. The calculated SER index are 4.36, 3.45 and 7.64 for control, 60 nm and 90 nm Bi$_2$O$_3$ NPs, respectively. This result indicated that the small size of nanoparticles is more internalised inside the cells and absorbed more ionizing radiation to damage the chromosomes. The internalization of Bi$_2$O$_3$ NPs could be via endocytosis that cross cell membranes and moved to vital component like DNA and mitochondria to generate ‘free radicals’ thus damage the cell. Several studies have demonstrated that AuNPs [13] and Bi$_2$S$_3$ NPs [14] were internalised by cells via endocytosis, and in some cases those nanoparticles have been found clustered around the membrane within the cytoplasm. Therefore, the NPs was expected to be internalized and engulfed inside the cells enhancing the radiation effect. This could be attributed to the fact that the inclusion of high electron density and high atomic number compounds in the cells was expected to generate high number of free radicals via both photoelectric and Compton interactions. These extra free radicals would increase the likelihood of DNA damage and subsequent cell death. The results suggested that 60 nm Bi$_2$O$_3$ NPs would be more beneficial as radiosensitiser.
4. Conclusion

In this work, Bi$_2$O$_3$ NPs were successfully synthesised by a simple and cost-effective hydrothermal method. The concentration of Bi(NO$_3$)$_3$ influenced the size of synthesised powders. Bi$_2$O$_3$ rods in monoclinic phase with diameter of 60 nm and 90 nm, were produced with varying Bi(NO$_3$)$_3$ concentration of 0.05 M and 0.3 M, respectively. Bi$_2$O$_3$ NPs did not induce cytotoxicity in breast cancer (mcf-7) cell lines at concentration from 0.05 µM. The radiotherapy performance of the as-prepared Bi$_2$O$_3$ NPs was obtained by calculating the sensitisation enhancement ratio (SER). The optimum result was found for 60 nm Bi$_2$O$_3$ NPs produced using 0.05 M Bi(NO$_3$)$_3$ concentration with SER of 3.45.

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