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Synthetisation Temperature-Dependent Cytotoxicity of Bismuth Oxide Nanoparticles in Vitro

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ABSTRACT

Bismuth oxide nanoparticles (Bi_2O_3 NPs) have gained a spot in the development of novel molecular probes for *in vivo* biomedical imaging. It exists in six polymorphic forms and each of them exerts with different stabilities according to its synthetisation temperature. The aim of this preliminary study is to determine effect of different synthetiation temperatures on cellular viability *in vitro*. One hundred µg/ml Bi_2O_3 NPs synthesised at 60, 90 and 120°C were characterised using scanning electron microscope (SEM) and their cytotoxicity was evaluated using cell viability assay (MTT assay) upon 24 hours exposure to Chang liver cells. Images captured by SEM showed an average diameter of 300 nm monoclinic-shaped with high crystalline formation of all three Bi_2O_3 NPs. MTT assay revealed increase in liver cell viability as the synthetisation temperature of Bi_2O_3 NPs increase. The outcomes suggested that synthetisation temperature of Bi_2O_3 NPs plays a role in cellular viability, hence predictive to the biocompatibility of these nanoparticles to be applied as in vivo radiographic contrast medium.

Keywords: Bismuth oxide nanoparticles (Bi_2O_3 NPs), cell viability, cytotoxicity, synthetisation temperature

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INTRODUCTION

Fast growing nanotechnology is one of the most propitious areas in biomedical research besides other areas ranging from electronics and aerospace engineering to environmental restoration. Regardless its promising potential to improve diagnosis

and treatment of intractable diseases, its safety consideration to human health is not parallel with the growth of the nanomaterial development (Singh et al., 2009). Longer years might be needed to fully realise its potential and make it possible for clinical trial. Nanoparticles are the particles which have been technologically engineered to nanoscale dimensions ranging from 1 to 100 nm (Wilczewska et al., 2012). Due to novel physico-chemical properties contributing to its nanoscale structure, nanoparticles may exert different properties compared to its bulk counterparts and may be a causative factor to adverse biological effects in human. The impacts of nanomaterials on human health are still inadequately elucidated despite its emerging manufacturing and usage for medical purposes (Fu et al., 2014).

Nanomaterials with high atomic number (Z) such as gold (AuNPs) and iron oxide nanoparticles (IONPs) have been discovered to induce dose and contrast enhancement in improving theranostics efficacy. However, investigations on other promising and cheaper nanoparticles with high Z number such as bismuth-based nanoparticles are still insufficient and poorly understood (Alqathami et al., 2013). Bismuth (Bi) compound is generally safe. Bi has been used in various medical settings, such as in treating syphilis, tumours, gastrointestinal disorders, eliminating *Helicobacter pylori* in peptic ulcer treatment and reduction of cisplatin-induced renal toxicity (Luo et al., 2012; Türkez, Geyikoğlu, & Keleş, 2005). Bismuth oxide nanoparticles (Bi_2O_3 NPs) have recently drawn tremendous attention in bioimaging, X-ray radiosensitising and biomolecular detection. It can be possibly exploited as dose and contrast enhancement in medical imaging due to its high atomic number (Z = 83), which is theoretically higher than gold (Z = 79).

There are several ways in synthesizing Bi_2O_3 NPs such as through co-precipitation, chemical vapour deposition, microwave-assisted and sol-gel method (Drache, Roussel, & Wignacourt, 2007). Despite these techniques, hydrothermally synthetisation has been proven as the best method in synthesising Bi_2O_3 NPs powder by providing high degree of crystallinity, high purity, narrow particle size distribution and uniform morphology. Bi_2O_3 NPs are known to exist in six polymorphic forms, denoted by α-Bi_2O_3, β-Bi_2O_3, γ-Bi_2O_3, δ-Bi_2O_3, ε-Bi_2O_3 and ö-Bi_2O_3 with monoclinic, tetragonal, body-centred cubic, face-centred cubic, orthorhombic and triclinic shape respectively (Jiang et al., 2015). All of them are high-temperature metastable, except for α-Bi_2O_3 (low-temperature stable), while δ-Bi_2O_3 is high-temperature stable. Bi_2O_3 NPs exerts stability distinctively at various temperatures; hence, synthetisation-temperature effect must be comprehensively evaluated to determine whether this factor plays a role in contributing cellular toxicity.

Toxicity of Bi_2O_3 NPs has promoted serious concerns among researchers due to its nanoscale size, high surface area and various fabrication modification techniques (Luo et al., 2012). The aim of this study is to elucidate the viability of cells *in vitro* with Bi_2O_3 NPs synthesised at different temperatures. To date, this has been no study done to find the relationship between Bi_2O_3 NPs synthesised at different temperatures with possible toxicity associated. This paper focuses on *in vitro* cytotoxicity investigation of Bi_2O_3 synthesised at 60, 90 and 120°C by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay for 24 hours.

MATERIALS AND METHOD

Bismuth Oxide Nanoparticles and Reagents

Bismuth oxide nanoparticles (Bi_2O_3 NPs) of three different synthetisation temperatures (60, 90, and 120°C) were obtained in dry powder form from Nano-Biotechnology Research and Innovation (NanoBRI), Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia, Pulau Pinang, Malaysia. HeLa [Chang Liver] (ATCC® CCL13™) cell lines are from American Type Culture Collection (ATCC), Minimum Essential Media (MEM), 100 X Penicillin-Streptomycin are from BioWest. Foetal Bovine Serum and 1X TrypLE express enzyme are from Gibco. Phosphate buffer saline (PBS) tablets, dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) are from Sigma-Aldrich.

Bismuth Oxide Nanoparticles Preparation and Characterisation

All Bi_2O_3 NPs in dry powder form were suspended in serum-free Minimum Essential Media (MEM) at 1 mg/ml concentration. The suspensions were vortexed at 800 rpm for 20 minutes at room temperature to form homogenous suspension before they were being filtered through a 0.22 µm hydrophilic polysulphonic membrane syringe bacterial filter. The stock solutions of all three Bi_2O_3 NPs were then diluted to 100 µg/ml working solution in order to perform cytotoxicity assay.

The particles size and structure of Bi_2O_3 NPs were visualised using Quanta™ 450 FEG (Scanning Electron Microscope) at the Microscopy Imaging Centre, Faculty of Pharmacy, Universiti Teknologi MARA, Puncak Alam. Bi_2O_3 NPs powders were placed on sample holders for the images to be taken. SEM images of Bi_2O_3 NPs were obtained at 5000 x magnification power, 10 kV acceleration voltage, and 2 Pa pressure.

Cell culture. HeLa [Chang Liver] (ATCC® CCL13™) cells were purchased from American Type Culture Collection prior to the study. The cells were maintained in Minimum Essential Media (MEM) (Gibco, USA), 10% Foetal Bovine Serum (Gibco, USA) and 1% Penicillin-Streptomycin (Gibco, USA). Cells were cultured in T-75 flask (Corning, USA) with 37°C humidified atmosphere of 5% CO_2 and 95% air for 3 days or until reached 80% confluency prior to treatment with Bi_2O_3 NPs. Confluent cells were detached from the culture flask by using 1X TrypLE (Gibco, Denmark) express enzyme for 5 minutes. Five ml of complete media was added to the flask and fairly mixed with TrypLE express enzyme and the cells were then centrifuged at 4000 rpm for 5 minutes. Cells suspension was counted using Vi-CELL™ Cell Counter (Beckman Coulter, USA) and seeded at a density of 1 x 104 cells per well in 96-well microplate (Corning, USA).

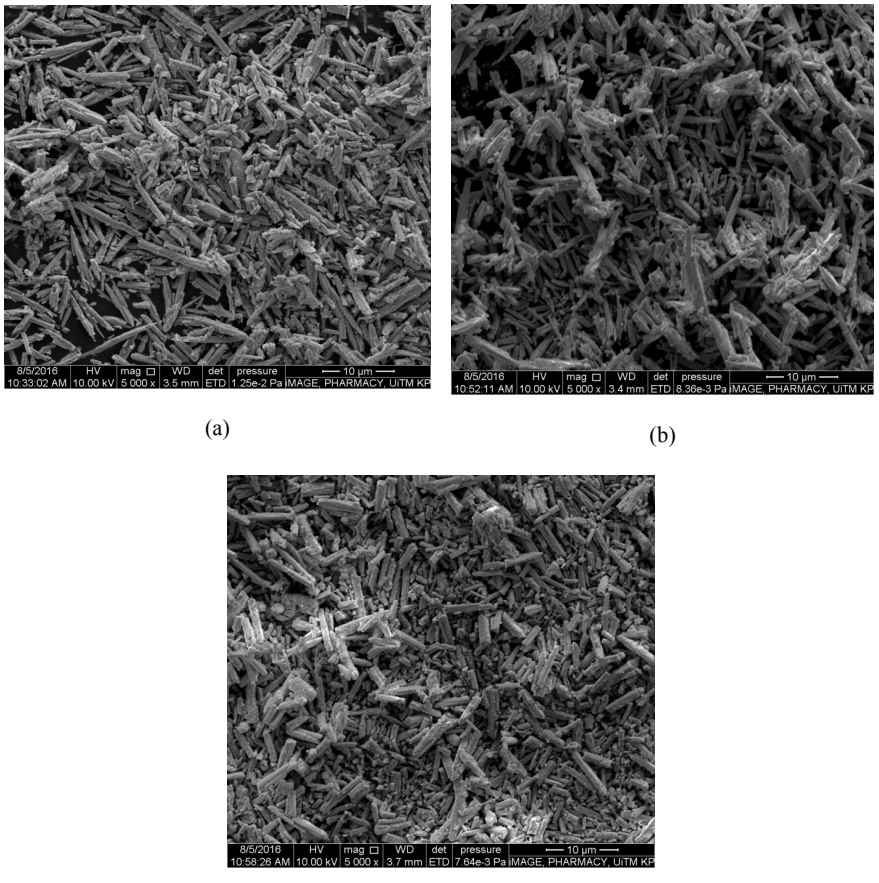
24 hours Cell Viability Assay. Bi_2O_3 NPs synthesised at 60, 90, and 120°C were used to investigate *in vitro* cytotoxicity. Seeded cells in 96-well microplate were incubated for 24 hours (37°C, 5% CO2 and 95% air) before exposed to 100 µl of 100 µg/ml of all Bi_2O_3 NPs for 24 hours' treatment period (37°C, 5 % CO_2 and 95% air). After the incubation period, cells viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) colorimetric assay to elucidate preliminary toxicity of Bi_2O_3 NPs. 50 µl of MTT solution was added to each well and incubated for 4 hours (37°C, 5% CO_2 and 95% air). MTT solution was discarded and 200 µl of Dimethyl Sulfoxide (DMSO) was then added in each well. The fluorescence intensity was measured at 550 nm by using Tecan F200 Infinite 200-TWT Microplate Reader.

Statistical analysis. Statistical significance of data obtained in this study was determined by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. Significance of data was designated as $p < 0.05$.

RESULTS

Bismuth Oxide Nanoparticles Characterisation Using Scanning Electron Microscope (SEM)
SEM micrographs [Figures 1(A), (B) and (C)] showed the morphology of Bi_2O_3 NPs synthesised at 60, 90 and 120°C, respectively. The average size of Bi_2O_3 NPs measured by SEM was 300 nm.



(a) (b)

(c)

Figure 1. The SEM micrographs of Bi_2O_3 NPs synthesised at: (a) 60°C; (b) 90°C; and (c) 120°C, respectively

Cell Viability of Various Bismuth Oxide Nanoparticles *in Vitro*

Cell viability assay using MTT-based colorimetric method was used to portray the cytotoxicity profiling of 100 µg/ml Bi_2O_3 NPs synthesised at 60, 90 and 120°C, respectively, in Chang liver cells following 24 hours exposure (see Figure 2). MTT results were based on the mitochondrial activity in Chang liver cells upon exposure to Bi_2O_3 NPs. Non-treated cells labelled Cx was used as the negative control.

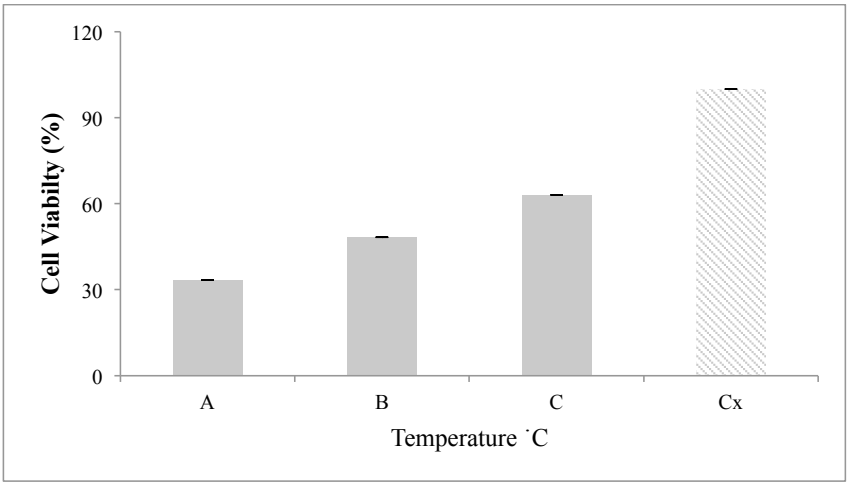


Figure 2. Cell viability percentage of Chang liver cells after 24 hours of exposure with 100 µg/ml Bi_2O_3 NPs synthesized at A- 60°C, B- 90°C, and C- 120°C, respectively. Each value was expressed as means ± SEM, $n = 4$. All the percentages of cell viability were statistically significant difference ($p < 0.05$), as compared to control group

DISCUSSION

Bismuth (Bi) is relatively non-toxic although it exists as one of the elements in the heavy metals group and found sparsely in the environment as co-metal in copper, lead and tin ores (Von Recklinghausen et al., 2008). Recent advancement in nanotechnology has developed several promising colloidal systems which are able to actuate as imaging contrast media with longer circulation period and less harmful excretion route. Conventional contrast media, like iodine and gadolinium, are known to have short imaging time, reduced performance and efficacy (Hainfeld et al., 2008). With further alarming concern in iodine-based contrast media regarding its toxicity which could highly danger iodine-intolerance patient, several nanoparticulated contrast media have been fabricated such as gold nanoparticles, iron oxide nanoparticles, iron-platinum alloy nanoparticles and bismuth oxide nanoparticles (Bi_2O_3 NPs) as alternative contrast enhancing agents. However, among these nanoparticulated contrast media, Bi_2O_3 NPs have gained special attention due to their high x-ray attenuation coefficient, high atomic number, low toxicity and are reasonably cheap.

Although Bi has been used in various medical applications, especially in treating gastrointestinal disorders, bismuth is found to be able to accumulate in various cell types including neuron, kidney and Leydig cells due to excessive ingestion and chronic use

(Stoltenberg & Danscher, 2000; Urgast et al., 2012; Stoltenberg et al., 2000). Therefore, to date, little is known about the potential cytotoxicity of Bi in human cells. The toxicity of Bi compound is associated with the release of Bi ions (Bi^{3+}), but it is relatively low because it is the least toxic among other heavy metals (Luo et al., 2012). Unlike its bulk compound, nano-sized Bi_2O_3 may induce toxicity due to its unique physicochemical properties. For example, silver nanoparticles were reported to be more toxic than silver ions (Christen, Capelle, & Fent, 2013; Johnston et al., 2010). Changes in the physicochemical properties play a major role in NPs performance. Small-sized NPs are able to penetrate into tissues and cells, hence making it easier for the detection of histopathological, cellular and molecular changes of diseases. NPs have a bigger total surface area to volume ratio, which enhances its surface activity (Hillegass et al., 2010; Mohanraj & Chen, 2007).

Bi_2O_3 NPs can be synthesised using multifarious methods and apparently hydrothermal method was suggested as advantageous in synthesising the nanostructures of Bi. It is known that there are six polymorphs of Bi_2O_3 as described. Each exhibits wide diversity of phases, which is either stable or not stable at a moderate or high temperature (Drache, Roussel, & Wignacourt, 2007). This study was designed to characterise and investigate the preliminary toxicity of Bi_2O_3 NPs synthesised at different temperatures (60, 90 and 120°C) using human Chang liver cells. Liver is the major organ for detoxification, and introduction of NPs in the body is susceptible to accumulate in this organ. Accumulation of gold nanoparticles ranging from 10 to 50 nm was found to be accumulated in the liver and spleen at 24 hours post injection (Jong, Hagens, Krystek, & Burger, 2008; Sonavane, Tomoda, & Makino, 2008). Since no study has been done to outline the Bi_2O_3 NPs temperature-synthesise effect, Chang liver cells are suitable to be used as a model structure for hepatotoxicity study.

The SEM micrographs revealed that Bi_2O_3 NPs synthesised at the highest temperature of 120°C showed slightly brittle looking particles with no differences of shapes and sizes observed at 60 and 90°C. This result indicates that Bi_2O_3 NPs synthesised at high temperature may be less harmful to Chang liver cells. All Bi_2O_3 NPs have an average diameter of 300 nm and can be ascribed as monoclinic-shaped with high crystalline formation. The transformation of bismuth polymorphic forms are highly affected by its fabrication method such as electrical conductivity or thermal expansion (Koto, 1994).

MTT colorimetric assay detects the cytotoxicity of nanoparticles by reflecting the level of damage on mitochondria function by measuring mitochondria activity enzyme and integrity of the cell membrane. From the MTT cell viability histogram, a similar trend was observed as in the SEM outcomes, where Bi_2O_3 NPs synthesised at the highest temperature of 120°C have the least toxicity effects (63% cell viability, 37% cell death) in Chang liver cells, indicating biocompatibility. This result shows an indirect proportional relationship between Bi_2O_3 NPs synthetisation temperatures and the release of bismuth ions which induces toxicity in cells. Bi_2O_3 NPs synthesised at 60°C exhibits the highest toxicity effect (33% cell viability, 67% cell death), followed by Bi_2O_3 NPs synthesised at 90°C (48% cell viability, 52% cell death). However, it is difficult to weight the nanotoxicity of Bi_2O_3 NPs in human cells based on only one parameter. Therefore, an extended research is warranted to further explore the actual toxicity

mechanism of Bi_2O_3 NPs inducing damage to human cells. Previously, *in vivo* nanotoxicity studies showed exposure to 100 µg/l of colloidal bismuth subnitrate induced liver damage and cerebellar disruption (Stoltenberg et al., 2001; Türkez et al., 2005). Meanwhile, Von Recklinghausen et al. (2008) found that bismuth citrate and bismuth glutathione are not toxic to human hepatocytes and lymphocyte even at high concentrations, which contradicted with 4 µM methybismuth compound toxicity. Ribeiro et al. (2009) reported a similar finding of non-toxic 100 µg/ml bismuth oxide via single-cell gel assay. It can be postulated that toxicity of bismuth compound may be different and highly influenced by its fabrication method.

CONCLUSION

The preliminary cytotoxicity of Bi_2O_3 NPs synthesised at different temperatures (60, 90 and 120°C) in Chang liver cells was evaluated using MTT assay based on the viability percentage of the cells. The outcomes of this study suggested that the biocompatibility of Bi_2O_3 NPs increases with temperature. Therefore, this study may provide early information to biosafety of Bi_2O_3 NPs with temperature. However, further research with extended parameters is needed to further justify Bi_2O_3 NPs synthetisation temperature effects on cellular toxicity.

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