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Synthesis of potential theranostic system consisting of methotrexate-immobilized (3-aminopropyl)trimethoxysilane coated α -Bi₂O₃ nanoparticles for cancer treatment

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Abstract

In this study we present the synthesis of a new theranostic system consisting of a core-shell structured nanoceramic-drug conjugate that can potentially combine chemotherapeutic, targeting, diagnostic and radio dose enhancing features in cancer treatment. The conjugate is made of α -Bi₂O₃ nanoparticles (NPs) which were first coated with (3-aminopropyl)trimethoxysilane (APTMS) to form a core-shell structure and then attached with methotrexate (MTX) through amidation between the amine moieties on the shell and the carboxylic acid groups on MTX. While α -Bi₂O₃ NPs with high effective atomic number can serve as both contrast agent and radiosensitiser in dose enhancement radiation therapy, MTX as an anti-cancer drug provides chemotherapeutic features and can selectively target cancer cells. The α -Bi₂O₃ NPs were firstly formed through a simple precipitation route, and were found through X-ray diffraction to be single phase. Fourier transform infrared spectroscopy and electron microscopy were then used to confirm the presence of a self-assembled layer of APTMS which aided in increasing their dispersibility compared to uncoated α -Bi₂O₃ NPs. Ultraviolet-visible spectroscopy was also used to show that 4% of the APTMS (mole ratio) has bound MTX leading to a conjugate with a size of 50 nm in diameter. The capability of α -Bi₂O₃ NPs to provide diagnostic features was proven with computed tomography (CT) scans and the degree of internalization of the uncoated, APTMS coated and MTX coated bismuth oxide NPs into 9L glioma cells was examined by flow cytometry analysis. Clonogenic assays exhibited a toxicity of 97% for the α -Bi₂O₃-APTMS-MTX conjugate, significantly higher than the initial components, which showed lower cancer cell death rates.

Keywords

bi₂o₃, cancer, nanoparticles, coated, trimethoxysilane, synthesis, potential, theranostic, aminopropyl, 3, immobilized, methotrexate, consisting, system, treatment

Disciplines

Engineering | Physical Sciences and Mathematics

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Synthesis of Potential Theranostic System Consisting of Methotrexate-Immobilized (3-Aminopropyl)trimethoxysilane Coated α -Bi₂O₃ Nanoparticles for Cancer Treatment

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Abstract

In this study we present the synthesis of a new theranostic system consisting of a core-shell structured nanoceramic-drug conjugate that can potentially combine chemotherapeutic, targeting, diagnostic and radio dose enhancing features in cancer treatment. The conjugate is made of α -Bi₂O₃ nanoparticles (NPs) which were first coated with (3-aminopropyl)trimethoxysilane (APTMS) to form a core-shell structure and then attached with methotrexate (MTX) through amidation between the amine moieties on the shell and the carboxylic acid groups on MTX. While α -Bi₂O₃ NPs with high effective atomic number can serve as both, contrast agent and radiosensitiser in dose enhancement radiation therapy, MTX as an anti-cancer drug provides chemotherapeutic features and can selectively target cancer cells. The α -Bi₂O₃ NPs were firstly formed through a simple precipitation route, which were found through X-ray diffraction to be single phase. Fourier transform infrared spectroscopy and electron microscopy were then used to confirm the presence of a self-assembled layer of APTMS which aided in increasing their dispersibility compared to uncoated α -Bi₂O₃ NPs. Ultraviolet-visible spectroscopy was also used to show that 4% of the APTMS (mole ratio) has bound MTX leading to a conjugate with a size of 50 nm in diameter. The capability of α -Bi₂O₃ NPs to provide diagnostic features was proven with computed tomography (CT) scans and the degree of internalization of the uncoated, APTMS coated and MTX coated bismuth oxide NPs into 9L glioma cells was examined by flow cytometry analysis. Clonogenic assays exhibited a toxicity of 97% for the α -Bi₂O₃-APTMS-MTX conjugate, significantly higher than the initial components, which showed less cancer cell death rates.

Keywords: nano-Bi₂O₃, methotrexate, dose enhancement, contrast agent, theranostic system

1. Introduction

At present, there is great concern with regard to malignant neoplasms, which are commonly known as cancer, being one of the leading causes of death worldwide. Various cancer therapies exist, such as surgery, chemotherapy, radiation therapy as well as individually applied methods like biological therapy, hormone therapy and photodynamic therapy.¹ New techniques showing promises and directly related to the focus of this work, is the development of theranostic systems that combine therapeutic, targeting and diagnostic agents on a single platform. Due to the ability to perfuse out of the bloodstream easily and the high tissue permeability, theranostic nanoparticle (NP) systems may be able to provide a high imaging sensitivity, precise targeting and a controlled drug release.² Typically, drug conjugates or complexes are used which combine the therapeutic effect of an anti-cancer drug with the ability of metal(oxide) NPs to be utilized for real-time imaging of the tumor. For instance, the combination of magnetite NPs which provide a contrast enhancement in magnetic resonance imaging, a self-assembled layer of (3-aminopropyl)trimethoxysilane (APTMS) and methotrexate (MTX) as a chemotherapeutic drug was shown to be selective in binding to tumor cells.³⁻⁵ MTX can target cancer cells whose surfaces are overexpressed by folate receptors and is one of the most widely applied drugs for the treatment of cancer in its various forms, such as breast cancer, head and neck cancer and carcinomas.^{3, 6, 7} Over the past several years, significant effort has been made to change the pharmacokinetic behaviour of MTX by linking it to macromolecular carrier systems. For instance, by conjugating MTX to microparticles that were made of gelatin or polyglutaraldehyde, the controlled drug release could be improved.^{8, 9} Another promising cancer treatment is based on radiation therapy. To enhance the radiosensitivity of the tumor in addition to the radiation beam, high atomic number (Z) materials such as gold or platinum NPs are introduced into cancer cells.¹⁰⁻¹⁴ Brown *et al.* provided a dose enhancement radiation on cancer cells by using tantalum pentoxide as radiosensitizer.¹⁵ Bismuth is the element with the highest atomic number ($Z=83$) that is reasonably cheap, available in great amounts and above all, biocompatible.¹⁶ Dependent on the synthesis method, bismuth oxide can scavenge reactive oxygen species (ROS) resulting in an increased proliferation of cells or generate ROS what in turn yields in a high cancer cell death rate.¹⁶ Therefore, bismuth compounds may be even more promising materials for dose enhancement radiotherapy compared with tantalum pentoxide. Bismuth compounds, such as bismuth subsalicylate and bismuth oxychloride are used in medicine or cosmetics, respectively.¹⁷⁻²⁰ Coated, non-single phase bismuth oxide NPs were investigated for biomedical application such as a contrast agent for X-ray imaging or in endodontic treatment.^{21, 22} Due to the adjustable cytotoxicity of bismuth oxide,¹⁶ the high effective Z and the ability to provide real-time imaging and dose enhancement, bismuth oxide NPs are a promising candidate for a theranostic system.

Our goal is to successfully combine a targeted chemotherapeutic treatment with diagnostic imaging capabilities based on computer tomography (CT) and an effective radiation cancer therapy using the high effective Z of employed ceramic NPs. For the first time, we report the synthesis and characterisation of single phase bismuth oxide NPs that were linked with MTX through APTMS (Fig. 1) in the form of a core-shell structure. MTX can be released from the linker by a cleavage of the amide bond at low pH in the presence of lysosomes which are typically found inside target cells.³

Since target cells provide a higher number of folate receptors in contrast to healthy cells, intoxication of latter ones can be reduced.

Fig. 1: Principle of post-synthetic grafting of dehydroxylated metal oxide NPs with aminosilanes and further linkage to methotrexate, with R₁= aminoalkyl group and R₂= alkyl group.

2. Experimental

2.1 Materials

The chemicals used include bismuth (III) nitrate pentahydrate ($\geq 98\%$), ammonium hydroxide (28–30%), (3-aminopropyl)trimethoxysilane (APTMS, $\geq 98\%$), toluene (anhydrous, 99.8%), methotrexate (MTX, $\geq 98\%$), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, $\geq 97\%$), *N*-hydroxysuccinimide (NHS, $\geq 98\%$), sodium hydroxide ($\geq 98\%$), crystal violet solution (2.3% crystal violet, 0.1% ammonium oxalate, 20% ethyl alcohol) and reagent-grade ethanol, all purchased from Sigma-Aldrich (Australia). Nitric acid (69%) was purchased from Merck (Australia), argon was purchased from Linde (Australia) and hydrochloric acid (36%) was purchased from UNIVAR (Australia). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Penicillin, Streptomycin, Hank's Balance Salt Solution (HBSS), Phosphate Buffered Saline (PBS) and trypsin ethylenediaminetetracetic acid (trypsin-EDTA) were purchased from Life Technologies (Australia).

2.2 Synthesis of $\alpha\text{-Bi}_2\text{O}_3$ Nanoparticles via Precipitation

The bismuth oxide nanoparticles (NPs) were synthesised from bismuth nitrate using the methodology outlined in the article published by M.M. Patil *et al.*²³ The amorphous precipitate was annealed in air in order to obtain bismuth oxide with an increased cancer cell death rate.¹⁶ First, 4 g (8.25 mmol) of bismuth (III) nitrate pentahydrate was dissolved in 40 mL of 69% nitric acid. Once fully dissolved, 80 mL of 30% ammonium hydroxide was added dropwise until a white precipitate was formed. This was then filtered out and washed with deionized water using a (Eppendorf, model 5702) centrifuge. The bismuth hydroxide precipitate was resuspended in 150 mL of deionized water and stirred at 100 °C for 5.5 h. Afterwards, the precipitate was removed by centrifugation, washed with deionized water and annealed in a (LABEC) tube furnace at 525 °C for 4 h under air atmosphere.

2.3 Coating of (3-Aminopropyl)trimethoxysilane on α -Bi₂O₃ Nanoparticles

First, 150 mg (0.32 mmol) of α -Bi₂O₃ NPs were suspended in 100 mL of anhydrous toluene and sonicated in a (Branson, model B1500R-MTH) sonication bath at 55 °C for 30 min under reflux and argon flow. Then, 1 mL (5.73 mmol) of APTMS was added dropwise to the homogenous and colloidal suspension of bismuth oxide NPs. The NPs were sonicated for 16 h at 55 °C and the resulting aminated particles were then isolated using the centrifuge. The product was washed with ethanol and deionized water and dried at 60 °C under vacuum for 24 h.

2.4 Attachment of Methotrexate to Aminosilane Coated α -Bi₂O₃ Nanoparticles

2.5 mL of an alkaline solution of 11 mM MTX and HBSS were mixed with 2.5 mL of an aqueous solution of 58.20 mg (0.37 mmol) of EDC and 8.63 mg (0.07 mmol) of NHS. The pH of the solution was adjusted to 8.5 by addition of 1.0 M sodium hydroxide solution and the mixture was allowed to react for 30 min. The resulting solution of MTX was then mixed with 5 mL of an aqueous suspension of 58.88 mg of aminosilane coated bismuth oxide NPs which was sonicated for 2 h at room temperature. The reaction mixture was incubated overnight at 37 °C in the dark. Following the immobilization of MTX, the particles were isolated using centrifugation (Heraeus, model Megafuge 1.0R) at 4 °C, washed with PBS and dried at 30 °C under vacuum for 48 h. The supernatant was collected and the amount of MTX was determined via UV-Vis spectroscopy at 372 nm.

2.5 X-Ray Diffraction (XRD)

Crystalline structures were investigated using an Enhanced Mini-Materials Analyzer (EMMA) X-Ray Diffractometer from GBC Scientific. The measurements were carried out at room temperature with Cu K-alpha radiation ($\lambda=1.5418$ Å) at 40 kV and 25 mA in the range of 20 to 80° at 2.00°/min with a step size of 0.02°. The mean crystalline size was determined using the Scherrer equation, where d is the average crystallite size, K is a constant (assuming that the particles are spherical, $K=0.89$), λ is the wavelength of the X-ray radiation, β is the full width at half maximum and θ is the Bragg angle of diffraction:

$$d = \frac{K \cdot \lambda}{\beta \cdot \cos(\theta)} \quad (1)$$

The lattice parameters were obtained via Rietveld refinement using the Marquardt least square method.

2.6 Fourier Transform Infrared (FTIR) Spectroscopy

Fourier transform infrared spectra were recorded on an IR Prestige 21 spectrometer from Shimadzu Corporation (Kyoto, Japan) over the range of 4000 to 600 cm^{-1} via attenuated total reflectance (MIRacle).

2.7 Ultraviolet-visible (UV-Vis) Spectroscopy

The absorbance was recorded on an UV-3600 spectrophotometer from Shimadzu Corporation (Kyoto, Japan) over the range of 800 to 200 nm by using quartz cuvettes. The unknown concentration of MTX was determined via a calibration curve using the Lambert-Beer law, where A is the absorbance, c is the concentration of the compound in solution, ϵ is the extinction coefficient and l is the path length of the sample:

$$A = c \cdot \epsilon \cdot l \quad (2)$$

2.8 Scanning Electron Microscopy (SEM), Scanning Transmission Electron Microscopy (STEM) and Energy Dispersive X-Ray Spectroscopy (EDS)

Particle sizes and core-shell structures of the uncoated and coated samples, as well as the chemical compositions were examined using the JEOL 7500 FESEM (Field Emission Scanning Electron Microscope) at the Electron Microscope Centre at the Australian Institute of Intelligent Materials (AIIM). The samples were prepared on carbon-coated copper grids. In order to acquire SEM images, an accelerating voltage of 16 kV with a working distance of 10 mm and a spot size of 10 were used. STEM images were taken with a 30 kV accelerating voltage at a working distance of 8 mm and spot size of 8. EDS spectra were obtained using a X-Flash 4010 10 mm^2 , 127 eV SDD energy dispersive X-ray detector (Bruker, Massachusetts, USA) with a working distance of 10 mm, accelerating voltage of 20 kV and a spot size of 13.

2.9 Computed Tomography (CT)

The anatomical contrast enhancement capability of $\alpha\text{-Bi}_2\text{O}_3$ NPs alone was demonstrated using CT. Suspensions with concentrations of $\alpha\text{-Bi}_2\text{O}_3$ ranging from 0 to 20 mg/mL were placed in 1.5 mL vials. The vials were imaged together using a Toshiba Asteion (model TSX-021A) whole body X-ray CT scanner with a 135 kV tube voltage, 200 mA tube current, 1 mm slice thickness and 93 mm field of view diameter. The CT images were obtained using the standard patient image reconstruction algorithms included with the scanner.

2.10 Cell Culture

Cellular experiments were carried out with GS-9L rat glioma cells derived from an *N*-nitrosomethylurea-induced tumor and were purchased from the European Collection of Cell Cultures (ECACC). Cell cultures were grown and maintained in a T75 cm^2 tissue culture flask (Falcon Franklin Lakes, New Jersey, USA) containing DMEM with L-Glutamine which was supplemented with 10% (v/v) FBS and 1% (v/v) Penicillin/Streptomycin at 37 °C and 5% (v/v) CO_2 .

2.11 Flow Cytometry Analysis

A LSRII (BD, Franklin Lakes, NJ, USA) Fluorescence Activated Cell Sorting (FACS) flow cytometer was used in order to determine the degree of internalization of the nanomaterials into the cultured 9L cells. First, 9L cells were seeded in T12.5 cm² tissue culture flasks, and after 2 days of incubation, a suspension of α -Bi₂O₃, α -Bi₂O₃-APTMS or α -Bi₂O₃-APTMS-MTX in PBS (without calcium or magnesium ions) was added to the cells to reach a final concentration of 50.00, 53.48 or 54.14 μ g/mL, respectively. After a further incubation of 24 h, the cells were removed from the tissue culture flask with 0.05% trypsin-EDTA and around 20,000 cells were transferred into 5 mL polystyrene flow tubes. Following this, the tubes were incubated for a further 2 h, and then analyzed with a flow rate of 60 μ L/min until 10,000 events were recorded. The degree of internalization of the nanomaterials was determined with FACSDiva software, assessing both forward and side scatter intensities. While the intensity of the forward-scattered light (FSC) is proportional to size and surface area of the cell and does not change with the degree of internalization, the side-scattered light (SSC) is proportional to the cell granularity and can be used to examine the cellular uptake.²⁴

2.12 24 h Cytotoxicity Assay

Clonogenic assays were performed to access the overall toxicity of the nanomaterials on 9L cancer cells. After the cells were brought to a confluence of 90%, suspensions of NPs or drugs were added to the cell culture medium for a total concentration of 50.00 (α -Bi₂O₃), 53.48 (α -Bi₂O₃-APTMS), 54.14 (α -Bi₂O₃-APTMS-MTX), 3.48 (APTMS) or 0.66 μ g/mL (MTX). After 24 h of exposure, the cells were trypsinated, plated at low density into 100 mm (BD Falcon) Petri dishes with 10 mL of media and incubated for 15 doubling times. Then, the plates were washed with 5 mL of HBSS (with calcium and magnesium ions) and stained with a solution of 25% crystal violet and 75% ethanol. Cell colonies were counted with the use of optical microscopy, provided that they were made of at least fifty healthy cells. The surviving fraction (SF) was obtained by comparing the plating efficiencies of the control (PE_c) and the treatment (PE_t):

$$SF = \frac{PE_t}{PE_c} \quad (3)$$

The plating efficiency is the ratio of counted cell colonies (*N*) and initial seeding number (*S*):¹⁵

$$PE = \frac{N}{S} \quad (4)$$

3. Results and Discussion

3.1 Characterization of the Nanomaterials

In order to determine which phase of Bi₂O₃ was synthesised and to investigate if the phase maintained single after coating with (3-aminopropyl)trimethoxysilane (APTMS) and further attachment

of methotrexate (MTX), X-ray diffraction was used. Fig. 2 displays the X-ray powder diffraction patterns of monoclinic $\alpha\text{-Bi}_2\text{O}_3$ (A), $\alpha\text{-Bi}_2\text{O}_3$ which was coated with APTMS (B) and the $\alpha\text{-Bi}_2\text{O}_3$ -APTMS-MTX conjugate (C). The diffraction pattern obtained for the uncoated $\alpha\text{-Bi}_2\text{O}_3$ nanoparticles (NPs) shows all major reflexions corresponding to crystal faces according to JCPDS card no. 00-027-0053. The calculated lattice parameters by Rietveld refinement were found to be $a=5.849$ Å, $b=8.166$ Å and $c=7.510$ Å. The coating of $\alpha\text{-Bi}_2\text{O}_3$ with APTMS and the additional linkage of MTX result in the preservation of crystalline phase. The particle sizes obtained from XRD data are 43 ± 6 nm for uncoated $\alpha\text{-Bi}_2\text{O}_3$, 37 ± 6 nm for the APTMS coated NPs and 54 ± 8 nm for $\alpha\text{-Bi}_2\text{O}_3$ -APTMS-MTX. No other characteristic reflections for $\alpha\text{-Bi}_2\text{O}_3$ -APTMS or $\alpha\text{-Bi}_2\text{O}_3$ -APTMS-MTX were detected.

Fig. 2: XRD patterns of (A) uncoated $\alpha\text{-Bi}_2\text{O}_3$ NPs, (B) APTMS coated $\alpha\text{-Bi}_2\text{O}_3$ NPs and (C) the $\alpha\text{-Bi}_2\text{O}_3$ -APTMS-MTX conjugate.

FTIR spectroscopy was used to confirm that both APTMS and MTX were successfully immobilized on bismuth oxide NPs. Fig. 3(A) shows the FTIR spectrum of uncoated $\alpha\text{-Bi}_2\text{O}_3$, without any characteristic bands. Due to the annealing at 525 °C for 4 h, no -OH groups on the NP surface were observed. According to Fruth *et al.*, characteristic absorption bands corresponding to stretching mode vibrations of each type of Bi–O exist in lower range of 800 to 200 cm^{-1} .²⁵ For $\alpha\text{-Bi}_2\text{O}_3$ NPs coated with APTMS (B), the bands at 3352 (stretching vibration) and 1562 cm^{-1} (bending vibration) indicate the presence of primary amine on the NP surface.^{26–30} Furthermore, the bands at 2933 (asymmetric stretching), 2831 (symmetric stretching), 1462 and 1394 cm^{-1} (scissoring, deformation) correspond to CH_2 groups.^{3, 26, 28–33} The band at 1323 cm^{-1} corresponds to stretching vibrations of the CN moieties.^{32, 33} Besides, bands corresponding to Si–O–Si groups (asymmetric stretching vibrations) at 1122 and 1050 cm^{-1} indicate the successful coating of APTMS on the surface of $\alpha\text{-Bi}_2\text{O}_3$.^{3, 34} The FTIR spectrum of the $\alpha\text{-Bi}_2\text{O}_3$ -APTMS-MTX conjugate (C) shows a characteristic band at 2928 cm^{-1} that corresponds

to asymmetric stretching vibrations of CH_2 groups.^{26, 28, 30-32} The formation of the amide bond was proven by the presence of a broad band at 3400 cm^{-1} which corresponds to stretching vibrations of N–H and a band at 1651 cm^{-1} that corresponds to C=O stretching vibration.^{26, 28, 30} The broad band at 3400 cm^{-1} may overlap in smaller wavenumbers with O–H stretching vibrations that correspond to carboxylic groups whose existence is also proven by a band at 937 cm^{-1} (bending vibration). The band at 1624 cm^{-1} corresponds to adsorbed water and the broad band at around 1500 cm^{-1} corresponds to aromatic C–C stretching (in ring).³⁰ The presence of C–N stretching vibrations which can be found in aromatic amines is confirmed by the band at 1325 cm^{-1} . Compared with the APTMS coated $\alpha\text{-Bi}_2\text{O}_3$, the intensities of the bands corresponding to siloxane groups at 1097 and 1050 cm^{-1} decreased.³⁴

Fig. 3: FTIR spectra of (A) uncoated $\alpha\text{-Bi}_2\text{O}_3$ NPs, (B) APTMS coated $\alpha\text{-Bi}_2\text{O}_3$ NPs and (C) the $\alpha\text{-Bi}_2\text{O}_3$ -APTMS-MTX conjugate.

Fig. 4 shows SEM and STEM images of uncoated $\alpha\text{-Bi}_2\text{O}_3$, APTMS coated $\alpha\text{-Bi}_2\text{O}_3$ and the $\alpha\text{-Bi}_2\text{O}_3$ -APTMS-MTX conjugate. The uncoated $\alpha\text{-Bi}_2\text{O}_3$ particles are uniform in size and mainly aggregated with aggregates bigger than 100 nm (A). There are plate-like crystals which are up to 70 nm long and approximately 40 nm wide (B). The APTMS coated $\alpha\text{-Bi}_2\text{O}_3$ NPs are both aggregated and non-aggregated (C, D). The shape of the non-aggregated particles is mainly plate-like with individual particle sizes of 30 to 100 nm , respectively. The edges of these particles are softer compared with uncoated $\alpha\text{-Bi}_2\text{O}_3$. The coating clearly enhances the dispersibility and stability of the particles, resulting in fewer aggregates that above all, are smaller in size. The SEM and STEM images of α -

Bi_2O_3 -APTMS-MTX (E, F) show more globular particles compared with APTMS coated $\alpha\text{-Bi}_2\text{O}_3$ NPs. The conjugate is still dispersed with an average size of 50 nm in diameter.

Fig. 4: SEM (A, B, C, E) and STEM (D, F) images of (A, B) uncoated $\alpha\text{-Bi}_2\text{O}_3$, (C, D) APTMS coated $\alpha\text{-Bi}_2\text{O}_3$ and (E, F) $\alpha\text{-Bi}_2\text{O}_3$ -APTMS-MTX.

3.2 Amount of Coated Silane and Attached MTX on $\alpha\text{-Bi}_2\text{O}_3$ Nanoparticles

The amount of coated silane on the surface of $\alpha\text{-Bi}_2\text{O}_3$ NPs was determined via EDS. The EDS spectra (Fig. S1) of $\alpha\text{-Bi}_2\text{O}_3$ and $\alpha\text{-Bi}_2\text{O}_3$ -APTMS both showed characteristic compositions of bismuth and silicon with an increased amount of up to $12.5 \pm 1.3\%$ (atomic) of Si for $\alpha\text{-Bi}_2\text{O}_3$ -APTMS. The quantity of bound MTX to $\alpha\text{-Bi}_2\text{O}_3$ -APTMS was determined via UV-Vis spectroscopy at 372 nm showing a total conversion of $5.8 \pm 0.6\%$ of MTX. This equals a mole ratio of $4.3 \pm 0.8\%$ of bound MTX and APTMS coated on $\alpha\text{-Bi}_2\text{O}_3$. The small conversion of MTX can be explained by the sterical hindrance of free amine moieties of APTMS. Due to the very bulky MTX and the comparatively small APTMS, the amount of free amine groups on APTMS decreases and thus, less MTX can be attached. In addition, APTMS is cross-linked with each other, which lowers the concentration of free amine groups as well.

3.3 *In Vitro* CT Imaging

The anatomical contrast enhancement capability of $\alpha\text{-Bi}_2\text{O}_3$ NPs alone was demonstrated using CT with the result CT number versus NP concentration shown in Fig. 5. The CT values in Hounsfield Units (HU) of each concentration were determined at two regions of interest along the central axis of the vial images (see red dashed line on the inset to Fig. 5); at the centre of the NP sediment (where

observable) and at the centre of the suspension height. The two ROIs were selected as a best and worst estimate of the deduced CT number for the corresponding suspension concentration. These data was plotted and fitted to deduce the relation between the CT value and concentration of NPs.

Fig. 5: Linear fitting of the CT value as a function of α -Bi₂O₃ NP concentration (equation of linear regression and R^2 value indicated). INSET: CT images of the α -Bi₂O₃ NPs with concentrations of 0 to 20 mg/mL that were imaged ~1 h after being placed on the patient couch. The red dashed line shown is the line along which the two CT number ROIs were selected that form the basis of the two plots.

Fig. 5 clearly indicates that the α -Bi₂O₃ NPs are excellent candidates for anatomical contrast enhancement agents. While the best case scenario plot is not a realistic estimate of expected result neither is the worst case scenario, yet the worst case values are clinically significant. The effective atomic number of the α -Bi₂O₃ NPs is so high that beam hardening effects were clearly visible in the reconstructed images. The corresponding deduced CT numbers are well outside the normal acceptable range for clinical scanners and were therefore a challenge to image by the current scanner. This challenge was unfortunately compounded by the proportion of the NP that fell out of suspension with time placed on the patient couch and highlights the need to reduce the size of the aggregates, which will be the focus of future work.

3.4 Cellular Uptake and Cytotoxicity of the Nanomaterials

In order to be suitable as a theranostic system, the synthesised nanoparticle-drug conjugate needs to exhibit an increased cellular uptake which was examined by comparing the mean side-scattered light (SSC) of the control without additives (Fig. 6(A)) with the mean SSC of the nanomaterials. The nanomaterials are mainly internalized with cellular uptakes of $324 \pm 16\%$ (α -Bi₂O₃, (B)), $432 \pm 22\%$ (α -

Bi₂O₃-APTMS, (C)) and 361±18% (α-Bi₂O₃-APTMS-MTX,(D)). The increased internalization of APTMS coated α-Bi₂O₃ NPs compared to uncoated NPs can be explained by the better dispersibility of the former, while the reduced cellular uptake of the nanoparticle-drug conjugate can be attributed to the increase in particle size due to the attachment of MTX and associated hindered cellular entry.

Fig. 6: The mean side-scattered light (SSC) as an indication of 9L cellular uptake after (A) no additives (control) and 24 h exposure of (B) α-Bi₂O₃ NPs (c=50.00 µg/mL), (C) α-Bi₂O₃-APTMS NPs (c=53.48 µg/mL) and (D) the α-Bi₂O₃-APTMS-MTX conjugate (c=54.14 µg/mL).

The clonogenic assays of the tested treatments are illustrated in Fig. 7 and show cytotoxicity for α-Bi₂O₃ (B) with a surviving fraction of 27±5%. The cytotoxicity can be explained by the high amount of oxygen with an atomic percentage of 52±3%, which was investigated via EDS, resulting in the generation of reactive oxygen species (ROS).¹⁶ The clonogenic assay of APTMS-coated α-Bi₂O₃ (C) reveals a surviving fraction of 30±5% which is higher compared with uncoated α-Bi₂O₃. This result may indicate a small shielding effect of APTMS (E) which shows a very low cytotoxicity with a high surviving fraction of 89±2%. The α-Bi₂O₃-APTMS-MTX conjugate (D) exhibits an increased cell death with a surviving fraction of 3±2%, proving that the attachment of MTX resulted in the desired effect of an enhanced cancer cell death rate. The observation is supported by the surviving fraction of 28±2% for pure MTX. The combination of MTX with its chemotherapeutic properties and the increased cell death rate attributed to α-Bi₂O₃ NPs apparently yielded in a maximum of cytotoxicity. The synergistic effect can possibly be explained by the two different mechanisms of induced cell death of the components: while MTX stops the metabolism of cancer cells, air-annealed α-Bi₂O₃ NPs generate ROS that in turn can damage the DNA.

Fig. 7: Cytotoxic assays of (A) untreated 9L cancer cells (control) and 24 h exposure of (B) α - Bi_2O_3 NPs ($c=50.00 \mu\text{g/mL}$), (C) α - Bi_2O_3 -APTMS NPs ($c=53.48 \mu\text{g/mL}$), (D) α - Bi_2O_3 -APTMS-MTX NPs ($c=54.14 \mu\text{g/mL}$), (E) APTMS ($c=3.48 \mu\text{g/mL}$) and (F) MTX ($c=0.66 \mu\text{g/mL}$) treated 9L cancer cells.

4. Conclusion

For the first time, a theranostic system was synthesised in which α - Bi_2O_3 nanoparticles (NPs) were linked with methotrexate (MTX) through (3-aminopropyl)trimethoxysilane (APTMS) via a precipitation and coating method. APTMS formed a self-assembled layer around the bismuth oxide NPs in form of a shell, to which MTX was covalently bound via amidation. The coating with APTMS led to a better dispersibility and stability of the monoclinic α - Bi_2O_3 NPs compared to uncoated α - Bi_2O_3 . The α - Bi_2O_3 -APTMS-MTX conjugate was small in size with a diameter of 50 nm and exhibited an increased cellular uptake. The clonogenic assays showed a cytotoxicity of cell treatments following the order $\text{APTMS} < \text{MTX} < \alpha\text{-Bi}_2\text{O}_3\text{-APTMS} < \alpha\text{-Bi}_2\text{O}_3 < \alpha\text{-Bi}_2\text{O}_3\text{-APTMS-MTX}$ and indicated a synergistic effect of the components of the nanoparticle-drug conjugate. In addition to its cytotoxicity, α - Bi_2O_3 with its high effective atomic number showed contrast enhancement capabilities and can also exhibit radiosensitising features in dose enhancement radiation therapy, which requires further investigation. MTX is a chemotherapeutic drug which can target cancer cells. In fact, a potential theranostic system was established which may improve the overall treatment procedure of cancer.

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