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Biogenic terbium oxide nanoparticles as the vanguard against osteosarcoma

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ABSTRACT

The synthesis of inner transition metal nanoparticles via an ecofriendly route is quite difficult. This study, for the first time, reports synthesis of terbium oxide nanoparticles using fungus, *Fusarium oxysporum*. The biocompatible terbium oxide nanoparticles (Tb_2O_3 NPs) were synthesized by incubating Tb_4O_7 with the biomass of fungus *F. oxysporum*. Multiple physical characterization techniques, such as UV-visible and photoluminescence spectroscopy, TEM, SAED, and zeta-potential were used to confirm the synthesis, purity, optical and surface characteristics, crystallinity, size, shape, distribution, and stability of the nanoemulsion of Tb_2O_3 NPs. The Tb_2O_3 NPs were found to inhibit the propagation of MG-63 and Saos-2 cell-lines (IC_{50} value of 0.102 μg/mL) and remained non-toxic up to a concentration of 0.373 μg/mL toward primary osteoblasts. Cell viability decreased in a concentration-dependent manner upon exposure to 10 nm Tb_2O_3 NPs in the concentration range 0.023–0.373 μg/mL. Cell toxicity was evaluated by observing changes in cell morphology, cell viability, oxidative stress parameters, and FACS analysis. Morphological examinations of cells revealed cell shrinkage, nuclear condensation, and formation of apoptotic bodies. The level of ROS within the cells—an indicator of oxidative stress was significantly increased. The induction of apoptosis at concentrations ≤IC_{50} was corroborated by 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining (DNA damage and nuclear fragmentation). Flow-cytometric studies indicated that the response was dose dependent with a threshold effect.

1. Introduction

Cancer, a disorder leading to uncontrolled cell proliferation, is an outcome of a sequence of poorly understood molecular events that include enhanced invasive activity, uncontrolled cell proliferation, angiogenesis, dysregulation of apoptosis, subsequent local and distant metastases, and morphological and cellular transformations [1]. In osteosarcoma (osteogenic sarcoma)—a type of bone cancer—tumors are composed of osteoid-producing malignant cells, where some cells have fibromatoid ground substance and some predominant chondroid [2]. It is the most common primary malignant bone tumor after myeloma, which is still prevalent among adolescents and children despite dramatically improved prognosis in the last 30 years [3,4]. The treatment of osteosarcoma is very difficult due to very high chances of reoccurrence; therefore, an efficient chemoprevention therapy is imperative [5]. Methotrexate, doxorubicin, cisplatin, and ifosfamide are few drugs which have been proven to be effective in the treatment of osteosarcoma [6]. Being a new approach, nanotechnology asserts potential in the development of efficient anticancer drugs and utilizing targeted drug delivery [7]. Nanoparticles have the ability to overcome various limitations associated with heavy dosage, biodistribution of conventional chemotherapeutic agents, and the side effects of non-specific cytotoxic drugs on healthy tissues [8].

The mode of synthesis of nanoparticles plays an important role in their applications in biological systems. Chemically synthesized nanoparticles are highly suitable in terms of their properties but the use of toxic precursors in chemical synthesis limits their use in biomedical applications. In the quest for the synthesis of non-toxic, clean, and ecofriendly nanoparticles, green technology seems to be most promising. In this technology, biological entities such as microorganisms, plants, or enzymes (proteins) are used for the synthesis of stable nanoparticles [9,10]. The nanoparticles produced through green technology have many advantages such as greater surface area, higher catalytic activity, and provide better contact between the metal salt and enzyme [11,12]. In the last few years, the synthesis of luminescent lanthanide nanoparticles has attracted great attention. Rare earth ions (RE^{3+}),

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such as terbium, europium, and samarium, are well-known efficient luminescent groups [13]. Lanthanide compounds offer unique temporal and spectral properties such as long lifetime, sharp emission bands, and large stokes shifts, which make them particularly useful in time-resolved luminescence bioassays, wherein they can effectively be distinguished from the background noise [14,15]. The luminescence exhibited by rare earth ions is caused by well-defined transitions within 4f shells. Among various lanthanides, terbium and europium are most luminescent, but the development and applications of their luminescence are very limited [16]. In case of terbium, most of the work has been focused on its chemical synthesis by various methods and its use as powder phosphors in glasses, since it emits strong green luminescence corresponding to the $^5D_4$–$^7E_5$ transition. Although, no study was found that describes the biological synthesis of terbium NPs using microorganisms and their applications in nanomedicine.

The fungus, *F. oxysporum*, contains various extracellular enzymes, naphthoquinones [17–19] and anthraquinones [20], which possess redox properties to reduce the metal ions [21]. In biosynthesis of metallic nanoparticles, the fungal mycelium is exposed to a solution of metal salt, wherein the metal ions are reduced to nanoparticles by the action of metabolites and extracellular enzymes produced by the fungus. Recently, various reports have demonstrated the use of microorganisms in the synthesis of nanoparticles, e.g., extracellular synthesis of silver nanoparticles using fungus *Humicola* sp. [22] and *Nocardiopsis* sp. MBRC-1 [23], extracellular enzymatic reduction of MnO_2, selenite and ferric ions using *Trichoderma reesei*, [24] synthesis of platinum nanoparticles using sulfate-reducing bacteria, [25] biosynthesis of magnetite nanoparticles using *Fusarium oxysporum* and *Verticillium* sp. [26], synthesis of lead nanoparticles using *Aspergillus* sp. [27], and intracellular biosynthesis of gold nanoparticles using *Verticillium* sp. [28].

Nanoparticles have been used for several purposes such as in biolabelling, drug and gene delivery [29–30], in combating microbes [31], and in the treatment of cancer [32]. Recently, researchers have started to focus on the anticancer activity of lanthanide nanoparticles. In vitro studies suggest that the increase in ROS generation is an important factor in the process of DNA damage as well as in apoptosis [33]. Cytotoxicity studies of luminescent $TbPO_4$ doped with europium against human carcinoma HeLa cells have shown very low cytotoxicity, long luminescence lifetime, and high photostability [34]. It is also reported that terbium-doped gadolinium oxide and dysprosium oxide nanoparticles decrease the rate of proliferation of human bronchial epithelial cells (BEAS-2B) and mouse L929 cells [35]. In addition, it has been found that titanium oxide nanoparticles, upon exposure to the cells, increase ROS generation and decrease GSH in U2OS (Osteosarcoma) and SW13T3 (Chondrosarcoma) cell lines [36]. Another report suggests that Cemarium lanthanide inhibits the growth of HeLa and MCF-7 cells [37].

This study presents a simple, clean, ecofriendly, and non-toxic approach for the synthesis of terbium (heavy rare earth metal) nanoparticles using fungus, *Fusarium oxysporum*. The terbium nanoparticles were characterized by UV-Vis spectroscopy, transmission electron microscopy (TEM), fluorescence spectroscopy, selected area electron diffraction (SAED), and zeta potential analysis. The cytotoxicity of the terbium nanoparticles was tested on albino rats. The anticancer properties of biogenic terbium nanoparticles were evaluated on two human osteosarcoma cell lines, MG-63 and Saos-2, by MTT assay. The oxidative stress and apoptotic effects of the nanoparticles were confirmed by DCFDA and DAPI staining, respectively. Preliminary apoptosis caused by Tb_2O_3 NPs in MG-63 and Saos-2 cells was also confirmed by FACS analysis.

2. Materials and methods

2.1. Ethics statement

In vivo experiments involving rats were performed in accordance with the regulations of the Institutional Animal Ethical Committee of Integral University, Lucknow. The permit number/approval number for the work is IU/Biotech/project/CPSCEA/13/12.

2.2. Materials

Terbium oxide (Tb_4O_7) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and used as received. Microbiological media and ingredients were purchased from Himedia, India. Unless otherwise indicated, all solvents and chemicals were of analytical grade and used as obtained from Merck and Sigma–Aldrich (St. Louis, MO, USA).

2.3. Microorganism and growth

Fusarium oxysporum (NCIM No. 1008) was maintained on PDA slants (potato 20% w/v, dextrose 2% w/v, and agar 2% w/v) at 25 °C. The fermentation was carried out by inoculating a fungal mat of 1 cm diameter, from a 7-day-old PDA slant into 100 mL of liquid MGYP medium (0.3% w/v malt extract, 1.0% w/v glucose, 0.3% w/v, yeast extract, and 0.5% w/v peptone) in a 500 mL Erlenmeyer flask, followed by incubation at 26 ± 1 °C on a rotary shaker (200 rpm) for 96 h. The mycelium was collected by centrifugation (4500 × *g*, 20 min at 10 °C), washed extensively with distilled water under aseptic conditions, and used for further studies.

2.4. Extracellular synthesis of terbium nanoparticles

20 g (wet weight) of the mycelia was incubated in 100 mL of sterile distilled water containing 1 mM Tb_4O_7 in 500 mL Erlenmeyer flask for 96 h under shaking (200 rpm) at room temperature. The samples were collected at fixed time intervals and subjected to UV-Vis spectroscopy to trace the formation of nanoparticles. At the end of fermentation, the unbound proteins were removed by precipitation with 2 volumes of absolute ethanol and the nanoparticles were collected for further characterization.

2.5. Characterization of terbium nanoparticles

The Tb_2O_3 NPs synthesized using fungus *F. oxysporum* were characterized by UV-Vis spectroscopy, fluorescence spectroscopy, zeta-potential analysis, TEM, and SAED. UV-Vis spectroscopy measurements were performed on a Shimadzu dual-beam spectrophotometer (model UV-1601 PC) operated at a resolution of 1 nm. Fluorescence measurements were carried out on a time-correlated single-photon counting FLS90 Spectrofluorimeter (Edinburgh Instruments, Livingston, UK). Steady-state fluorescence measurements were carried out with a slit width of 2 nm for both the monochromators at a scan speed of 300 nm/min. Samples were excited at 340 nm and the emission spectra were recorded in the range of 350–700 nm. The resultant decay curves were analyzed by a multi-exponential iterative fitting program supplied by Edinburgh Instruments. Zeta-potential was measured using a Zetasizer Nano-ZS, Model ZEN3600 (Malvern Instrument Ltd., Malvern, UK). Transmission electron microscopy was performed by drying a drop of suspension of Tb_2O_3 NPs onto carbon-coated TEM copper grids followed by analysis on Tecnai™ G^2 Spirit BioTWIN, FEI, USA, operated at an accelerating voltage of 80 kV. Selected Area Electron Diffraction (SAED) patterns were recorded from ensembles of particles with an operating voltage of 120 kV, camera length of 80 cm and with the insertion of a 120 μm field limiting aperture.

2.6. Cell culture

The human osteosarcoma cell lines, Saos-2 and MG-63, were obtained from National Centre for Cell Science (NCCS), Pune, India, and primary rat osteoblasts were isolated from neonatal rat calvariae by enzymatic digestion. Saos-2, MG-63, and primary osteoblast were grown as monolayer in Mac Coy's, EMEM, and MEM media, respectively,