Colloids and Surfaces B: Biointerfaces 122 (2014) 209–215

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Synthesis, characterization and toxicological evaluation of iron oxide nanoparticles in human lung alveolar epithelial cells

Sourabh Dwivedi[a,b], Maqsood A. Siddiqui[a,b,*], Nida N. Farshori[c], Maqusood Ahamed[d], Javed Musarrat[a,b], Abdulaziz A. Al-Khedhairy[a,b]

[a] Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia
[b] Al-Jeraisy Chair for DNA Research, King Saud University, Riyadh, Saudi Arabia
[c] Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia
[d] King Abdullah Institute for Nanotechnology, King Saud University, Riyadh, Saudi Arabia

ARTICLE INFO

Article history:
Received 13 March 2014
Received in revised form 9 June 2014
Accepted 30 June 2014
Available online 7 July 2014

Keywords:
A-549 cells
Iron oxide nanoparticles
Cytotoxicity
Oxidative stress

ABSTRACT

The present investigation was aimed to characterize the synthesized iron oxide nanoparticles (Fe_3O_4-NPs) and to assess their cytotoxicity and oxidative stress in human lung alveolar epithelial cells (A-549). Fe_3O_4-NPs were characterized by X-ray diffraction, transmission electron microscopy, dynamic light scattering, and atomic force microscopy. The morphology of the Fe_3O_4-NPs was found to be variable with a size range of 36 nm. A-549 cells were exposed to Fe_3O_4-NPs (10–50 μg/ml concentrations) for 24 h. Post exposure, cytotoxicity assays (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, MTT; neutral red uptake, NRU; and cellular morphology) and oxidative stress (lipid peroxidation, LPO and glutathione, GSH) were evaluated. Further, intracellular reactive oxygen species (ROS) generation and mitochondrial membrane potential (MMP) were also studied. MTT and NRU assays revealed a concentration-dependent decrease in the cell viability of A-549 cells. Fe_3O_4-NPs exposed cells also altered the normal morphology of the cells. Furthermore, the cells showed significant induction of oxidative stress. This was confirmed by the increase in LPO and ROS generation, and the decrease in the GSH level and MMP. Our results demonstrated that Fe_3O_4-NPs induced cytotoxicity is likely to be mediated through the oxidative stress and ROS generation in A-549 cells.

1. Introduction

Metal oxide nanoparticles are widely being used in many industrial products, *i.e.*, catalysts, pigments, food additives, sun screens and cosmetics [1,2]. The applications and productions of these nanoparticles at large number have brought attention to their risk factors. It is well known that nanoparticles are being released during particle synthesis and handling of dry powders and liquid suspensions [3]. Toxicity of various metal oxide nanoparticles has also been reported *in vitro* [4–9] as well as *in vivo* [10–12] setups. Numerous studies indicate that metal oxide nanoparticles have the ability to generate reactive oxygen species (ROS) [13,14] and they are involved in the cytotoxicity due to their small size and large surface area [15]. Experimental evidences also showed that nanoparticles released by sprays and powders can potentially deposit in the respiratory system [16,17].

Iron oxide (Fe_3O_4) nanoparticles have been applied broadly to bioscience and clinical research for various purposes; the most common includes magnetic cell labeling [18,19], separation and tracking [20], for therapeutic purposes in hyperthermia [21], in drug delivery [22], and for diagnostic purposes, *e.g.*, as contrast agents for magnetic resonance imaging (MRI) [23]. Although the cytotoxic effects of iron oxide nanoparticles are known [24–26], the mechanism(s) of their induced cytotoxicity is not clearly understood. Since, human exposure to iron oxide may occur through the exposure routes of inhalation and ingestion at occupational settings. Therefore, the present investigation was aimed to understand the mechanism(s) of cell death induced by iron oxide nanoparticles in human lung alveolar epithelial cells (A-549) under *in vitro* conditions. These *in vitro* systems are cost-effective, rapid and reproducible with low or no ethical dubious [27,28]. In this study, firstly we have focused on the detailed synthesis and physicochemical characteristics of iron oxide nanoparticles; secondly, the effect

* Corresponding author at: Department of Zoology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia. Tel.: +966 14699532; fax: +966 542967835.
E-mail addresses: maqsoodahmads@gmail.com, masiddiqui@ksu.edu.sa (M.A. Siddiqui).

http://dx.doi.org/10.1016/j.colsurfb.2014.06.064
0927-7765/

of iron oxide nanoparticles on (i) cell viability, (ii) morphological alterations, (iii) oxidative stress markers, (iv) ROS generation, and (v) mitochondrial membrane potential (MMP) in A-549 cells.

2. Materials and methods

2.1. Cell culture

A-549 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.2% sodium bicarbonate, and antibiotic/antimycotic solution (100×, 1 ml/100 ml of medium, Invitrogen, Life Technologies, USA). The cells were maintained in 5% CO_2, 95% atmosphere at 37 °C. Cells were assessed for cell viability by trypan blue dye exclusion assay as described earlier [29] and batches showing more than 98% cell viability were used in the experiments.

2.2. Reagents and consumables

All other specified chemicals, culture mediums, reagents, and diagnostic kits were procured from Sigma Chemical Company Pvt. Ltd., St. Louis, MO, USA unless otherwise stated. Culture wares and other plastic consumables used in the study were procured from Nunc, Denmark.

2.3. Experimental design

A-549 cells were treated with various concentrations of Fe_3O_4-NPs (10–50 μg/ml) for 24 h. Cells were studied for the cytotoxicity by MTT and NRU assays and morphological alterations. Further, oxidative stress markers, *i.e.* lipid peroxidation (LPO), glutathione (GSH), reactive oxygen species (ROS) generation, and MMP were studied.

2.4. Synthesis of Fe_3O_4-NPs

Fe_3O_4-NPs were prepared by chemical co-precipitation of Fe^{2+} and Fe^{3+} ions in an alkaline solution followed by a treatment under hydrothermal conditions [30]. Briefly, $FeSO_4{\cdot}7H_2O$ (2.7 g) and $FeCl_3$ (5.7 g) were dissolved in 10 ml Mili-Q water. Then, these two solutions were thoroughly mixed and added to double volume of 10 M ammonium hydroxide with constant stirring at 28 °C. After that the dark black slurry of Fe_3O_4 particles was heated to 80 °C in a water bath for 30 min. Ion impurities such as chlorides and sulphates were removed by washing the particles several times with Mili-Q water. The particles were then dispersed in 10 ml Mili-Q water and sonicated for 20 min.

2.5. Characterization of Fe_3O_4-NPs

2.5.1. X-ray diffraction analysis of Fe_3O_4-NPs

Finely powdered sample of Fe_3O_4-NPs was analyzed using X'pert PRO analytical diffractometer (Almelo, The Netherlands) using CuKα radiation ($\lambda = 1.54056$ Å) in the range of $20^\circ \leq 2\theta \leq 80^\circ$ at 40 keV. In order to calculate the particle size (D) of Fe_3O_4-NPs, the Scherrer's equation ($D = 0.9\lambda/\beta\cos\theta$) has been used [31], where λ is the wavelength of X-ray, β is the broadening of diffraction line measured as half of its maximum intensity in radians and θ is the Bragg's diffraction angle. The average particle size of Fe_3O_4-NPs was estimated from the line width of the XRD peak.

2.5.2. Analysis of Fe_3O_4-NPs by transmission electron microscopy (TEM)

TEM analysis of Fe_3O_4-NPs was performed on a transmission electron microscope (Hitachi, H-7500, Japan) at an accelerating voltage of 90 kV. Samples were prepared by drop-coating Fe_3O_4-NPs solutions (1% Fe_3O_4-NPS) onto carbon-coated gold TEM grids. Film of Fe_3O_4-NPs sample on TEM grid was allowed to stand for 2 min. The extra solution was removed using a blotting paper and the grid was allowed to dry prior to measurement.

2.5.3. Atomic force microscopy (AFM) of Fe_3O_4-NPs

Fe_3O_4-NPs were examined using AFM (Veeco Instruments, USA). Analysis was performed by running the machine in non-contact tapping mode [32]. Characterization of Fe_3O_4-NPs was done by observing the patterns appeared on the surface topography and analyzing the AFM data. Tapping mode imaging was implemented in ambient air by oscillating the cantilever assembly at or near the cantilever's resonant frequency using a piezoelectric crystal. The topographical images were obtained in tapping mode at a resonance frequency of 218 kHz. Data were analyzed through WSXM software.

2.5.4. Dynamic light scattering

Fe_3O_4-NPs powder was suspended in deionized ultrapure water to obtain a concentration of 50 μg/ml, and was sonicated at 40 W for 15 min. Hydrodynamic particle size and Zeta potential (ζ) of Fe_3O_4-NPs in an aqueous suspension were determined by measuring the dynamic light scattering using of a ZetaSizer-HT (Malvern, UK).

2.6. Cytotoxicity of Fe_3O_4-NPs

2.6.1. MTT assay

Percent cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described by Siddiqui et al. [33]. Briefly, cells (1×10^4) were allowed to adhere for 24 h in CO_2 incubator at 37 °C in 96 well culture plates. After the exposure, MTT (5 mg/ml of stock in phosphate buffer saline, PBS) was added (10 μl/well in 100 μl of cell suspension), and plates were incubated for 4 h. The supernatant was discarded and 200 μl of DMSO were added to each well and mixed gently. The developed color was read at 550 nm. Untreated sets were also run under identical conditions and served as control.

2.6.2. Neutral red uptake (NRU) assay

NRU assay was carried out following the protocol described [34]. After the exposure, the medium was aspirated and cells were washed twice with PBS, and incubated for 3 h in a medium supplemented with neutral red (50 μg/ml). The medium was then washed off rapidly with a solution containing 0.5% formaldehyde and 1% calcium chloride. Cells were further incubated for 20 min at 37 °C in a mixture of acetic acid (1%) and ethanol (50%) to extract the dye. The plates were read at 550 nm. The values were compared with the control sets.

2.6.3. Morphological analysis by phase contrast microscope

Morphological changes in A-549 cells treated with Fe_3O_4-NPs were observed to determine the alterations induced by Fe_3O_4-NPs. The cell images were taken using an inverted phase contrast microscope at 20× magnification.

2.7. Lipid peroxidation (LPO)

Lipid peroxidation was performed using thiobarbituric acid-reactive substances (TBARS) protocol [35]. Briefly, after the exposure, A-549 cells were collected by centrifugation and sonicated in ice cold potassium chloride (1.15%) and were centrifuged for 10 min at 3000 × *g*. Resulting supernatant (1 ml) was added to 2 ml of thiobarbituric acid (TBA) reagent (15% TCA, 0.7% TBA and 0.25 N HCl) and was heated at 100 °C for 15 min in a boiling water