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A biological method for in-situ synthesis of hydroxyapatite-coated magnetite nanoparticles using *Enterobacter aerogenes*: Characterization and acute toxicity assessments☆

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ABSTRACT

Hydroxyapatite (HA)-coated magnetite nanoparticles (MNPs) are being widely investigated for various applications in medical engineering and wastewater treatment. In this work, the MNPs were thoroughly coated by bacterial synthesized HA nanoparticles during biomineralization process using *Enterobacter aerogenes*. The resulting bacterial-induced precipitate was then calcined at 600 °C and investigated with respect to structural characteristics, particle size and magnetic strength by XRD, FT-IR, SEM, EDS, TEM and VSM analyses. The effects of MNPs and HA-coated MNPs (HA-MNPs) on the viability of human MCF-7 cell lines were also investigated via mitochondrial activity test (MTT) and lactate dehydrogenase (LDH) assays.

The powder characterization results showed appropriate structural properties for HA-MNPs samples. The particles diameter size of the MNPs and HA-MNPs were in the range of 3–25 nm and 20–80 nm, respectively. The biologically-synthesized HA-MNPs formed a stable suspension in water while keeping their magnetic property. The saturation magnetization (Ms) of HA-MNPs was measured at ~10 emu g^{-1} which was in good agreement with the structural composition of this sample. Finally, the results of the cell lines viability indicated that coating of toxic MNPs via biomineralization was a promising approach in order to synthesize bio-compatible magnetic nanoparticles with suitable physical and chemical structural characteristics. The toxicity level of MNPs was reduced by 10 fold when coated by bacterial-synthesized HA.

1. Introduction

The performance of nanoparticle materials for biomedical applications is often assessed by their suitable magnetic saturation, narrow particle size, and low toxicity effects [1]. Magnetite nanoparticles (Fe3O4, MNPs) are among the nanoparticles that have been widely used in biomedical and environmental engineering. In addition, human cells exposure to nanoparticles-contained-products raises safety and health concerns that needs to be further investigated. Previous studies showed that exposing MNPs to the living tissue can contribute to a homeostasis imbalance in human body [1–4]. Uptake of MNPs by eukaryotic cells can potentially generate oxidative stress caused by reactive oxygen species (ROS) and apoptotic bodies leading to oxidative DNA damage and cellular toxicity effects such as impaired mitochondrial function, lysosomal swelling and rupture and membrane leakage of lactate dehydrogenase enzymes (LDH) [2,3].

To cope with this problem various biomaterials such as hydroxyapatite ($Ca_5(PO_4)_3(OH)_2$, HA) have been used for bioactive coating of MNPs in order to increase their biocompatibility. HA is the main inorganic component of bone and teeth matrix of mammals. Hydroxyapatite-coated magnetite nanoparticles (HA-MNPs), owing to their nontoxic behaviors, have been used in several biomedical applications such as targeted drug delivery [5], chemo-hyperthermia treatment of bone and lung cancers [6,7], and enhancing the proliferation of osteoblast cells leading to more osteo-calcification [8]. Other applications of HA-MNPs include utilization as recyclable composite nanoparticles for heavy metals removal [9], fabrication of biosensors [10], photocatalysts [11] and oxidation catalysts [12].

In all previous studies, HA-MNPs were produced via chemical methods. In recent years, there has been an increasing interest toward the coating and synthesis of various nanoparticles via biological route [13–16]. To the best of our knowledge, there is no report based on the biological synthesis of HA-MNPs through which MNPs are coated during bacterial synthesis of HA. Despite chemical approaches which may suffer from using and/or releasing toxic chemicals, high temperature and pressure as well as requirement for precise control of physical

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parameters such as temperature and pH during production process, biological methods are environmentally-benign processes with capacity to catalyze reactions in aqueous media under mild condition [13–18]. In addition, another restriction in chemical methods is lack of sufficient adhesion property of MNPs as a nucleation template for the HA nanoparticles. Therefore, several studies investigated utilization of stabilizing materials to improve the adhesive property of MNPs for efficient coating process. However, poor affinity and adhesion property even after using stabilizing agent was also reported in aqueous media [19].

In this study, based on the results formerly reported [18,20], it has been hypothesized that MNPs with positive surface charges are adsorbed and trapped on the plasma membrane of a mobile, peritrichous, alkaline phosphatase (ALP) positive bacterial strain such as *Enterobacter aerogenes* with negative surface charge and biomineralized by the plasma membrane-bond ALP enzymes. In previous work [18], this bacterial strain synthesized nanostructured HA with unique in vivo properties such as high biocompatibility and biodegradability. Therefore, the main objective of the present work was to develop a novel biological method for coating of MNPs by HA in order to increase biocompatibility of the resultant nanoparticles. The bacterial synthesized powder samples were particularly investigated with respect to structural phase, morphology, particle size and magnetic strength using appropriate characterization methods. Furthermore, the effects of bio-coating of MNPs by HA on in vitro cytotoxicity of the prepared nanoparticles were particularly investigated through mitochondrial activity test (MTT) and lactate dehydrogenase (LDH) assay.

2. Experimental

2.1. Bacterial synthesis of HA and coating of MNPs

A mineralization solution consisting of 25 mM of β-glycerophosphate disodium salt dehydrate (Sigma-Aldrich, USA), 13 mM of calcium chloride (Merck, Germany), 20 mM of trisodium citrate (Merck, Germany) in 25 mM Tris buffer (Across, Belgium) was prepared for synthesis of bacterial HA [18]. The pH of the solution was adjusted to 8.6 by dropwise addition of 1 M HCl solution and then sterilized by autoclaving at 121 °C for 20 min.

For synthesis of HA-MNPs, 0.3 g of MNPs (purchased from Nanostructured & Amorphous Materials Inc., USA) with the original particle size ranging from 20 to 30 nm was sonicated (duty cycle 0.5, 50 Hz) in 50 mL of mineralization solution using an ultrasonic processor (UP400S, 400 watt, Heilscher, Germany) for 70 s, prior to addition to the 950 mL of mineralization solution.

Inoculum was prepared by culturing *Enterobacter aerogenes* PTCC 1221 (obtained from Persian Type Culture Collection (PTCC, Tehran, Iran)) as an ALP positive strain [21] in the sterile nutrient broth (Quelab, Canada) at 30 °C overnight. Inoculum (wet cell biomass) was collected by centrifuging at 2500*g* for 10 min and added to each mineralization solution at 4% (v/v).

Each inoculated mineralization solutions were agitated at 24 °C under aerobic jar test (JLT6, Velp, Italy) with a rotation speed of 230 rpm. At the end of sedimentation process, the bacterial suspensions were filtered through filter paper (Whatman no. 42, Sigma-Aldrich, USA) and dried at 45 °C for 12 h followed by calcination at 600 °C for 2 h to remove the organic content according to the previous works [14,18].

2.2. Powder characterization

The structural phase composition of the powder samples i.e. MNPs, bacterial synthesized HA-MNPs and bacterial synthesized HA were characterized by X-ray diffraction (XRD; D8 Advance, Bruker, Germany) using Cu Kα radiation at 35 kV and 30 mA. The XRD patterns were characterized by XRD evaluation software (DiffracPlus V1.01, Bruker, Germany). Fourier transform infrared spectroscopy (FTIR; TENSOR 27, Bruker, Germany) was performed to examine the chemical functional groups of the samples. Morphology and elemental compositions of the samples were investigated using scanning electron microscopy (SEM) coupled with energy dispersive X-ray spectroscopy (EDX) analyses (Vega II XMU, Tescan, Czech Republic) at 20 kV. Transmission electron microscopy (TEM, LEO-912AB-OMEGA, Carl Zeiss, Germany) was applied to investigate the particle size and morphology of the samples. The diameter size distribution (DSD) of the particles was calculated by ImageJ software (Ver. 1.410, National Institute of Health, Bethesda, MD, USA) using TEM images. To investigate magnetic properties of MNPs before and after coating with bacterial HA, the magnetic properties of the samples were obtained using a vibrating sample magnetometer (VSM, MDKB, Iran) in an applied coercive field of ±10,000 Oe and frequency of 60 Hz at room temperature.

2.3. In vitro cytotoxicity assays

Cytolysis or membrane leakage and cytotoxicity assessments using LDH and MTT assay kits (Thermo Fisher Scientific, NH, USA) were carried out by exposing human MCF-7 cell lines breast cancer (purchased from the Pastor Cell Line Bank, Iran) to various concentrations of MNPs and HA-MNPs in the range of 0.1–1000 μg/mL. These analyses were used for detection of apoptosis or cell death process in the presence of nanoparticles when cell membranes are no longer intact [22,23]. The LDH and MTT tests were performed with the same density of 10^4 MCF-7 cell lines/well, cultured in 96-well plates over 48 h before the cytotoxicity assays reach to ~100% confluence. The cells were washed using phosphate-buffered saline (PBS) and incubated for 4 h at different concentrations (0.1–1000 μg/mL) of MNPs and HA-MNPs dispersed in Dulbecco's modified Eagle's medium (DMEM) at 37 °C. After 4 h incubation of the cell lines with the nanoparticles and addition of LDH substrate, the supernatant was removed and the absorbance was measured at 492 nm within 5 min of incubation using an infinite 200 Microplate Reader (Tecan, Mannedorf, Switzerland). Next, the cells were washed and incubated with MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for the same period of time (4 h) and then dissolved in dimethyl sulfoxide (DMSO). The metabolic activity of the cells was determined by measuring the absorbance of released formazan content at 550 nm using the microplate reader. In order to calculate the viability (%) of the cell lines, all results were normalized to positive and negative controls i.e. cell culture medium 100% viability and Triton X-100, respectively. Because lysed cells release LDH, the viability of the cell lines for LDH assay was calculated by detecting the absorbance of extracellular LDH content and the controls (+ and −). All data were expressed as mean values ± standard deviation of triplicate experiments and statistical significance was accepted at a level of $p < 0.05$ [22,23].

3. Results and discussion

3.1. Characterization of structural phase and chemical functional groups

Bacterial HA coating on MNPs was completed within 9 days of experiment and keep running the experiment up to 14 days showed no significant effect on the sediment production. The amount of calcined HA-MNPs and HA powders produced from the respective mineralization solutions were measured at 1.279 ± 0.032 g/L and 0.813 ± 0.019 g/L after calcination. The HA production in the presence of MNPs was increased by 20% when compared to the synthesis of pure bacterial HA at identical condition excluding the amount of MNPs used. In recent years, many studies have investigated the utilization of magnetic nanoparticle as a career for enzyme immobilization through direct or indirect binding. Most of these studies showed long term retained activity and increased stability of various enzymes. Therefore, higher production of HA-MNPs might be attributed to the positive