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Deep eutectic choline chloride-calcium chloride as all-in-one system for sustainable and one-step synthesis of bioactive fluorapatite nanoparticles

Mohammad Karimi[a,*], Akbar Jodaei[b], Amirhosein Sadeghinik[b], Majid Rastegar Ramsheh[a], Tahereh Mohammadi Hafshejani[c], Mehdi Shamsi[c], Faranak Orand[d], Farahnaz Lotfi[d]

[a] Tracheal Diseases Research Center (TDRC), National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran
[b] Maham Tajhizat Parla Co., Chemistry & Chemical Engineering Research Center, Tehran, Iran
[c] Department of Nanotechnology and Advanced Materials, Materials and Energy Research Center, Karaj, Iran
[d] Department of Biomedical Engineering, Yazd Branch, Islamic Azad University, Yazd, Iran

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ABSTRACT

Fluorapatite (FA) nanoparticles were synthesized via an one-step and green pathway by employing the deep eutectic solvent based on choline chloride-$CaCl_2.6H_2O$. In an innovative approach, the eutectic solvent was chosen in such a way to act as solvent, reactant and template, simultaneously. The results obtained from characterization of the as-synthesized nanoparticles revealed the formation of FA nanoparticles having average crystal size of 26 nm, percent crystallinity of 99%, mean particle size of 30 nm, and high crystal, elemental, and structural purity. *In vitro* study was conducted by immersion of FA nanoparticles in SBF solution. The results obtained by SEM, EDS, FTIR, and Raman analysis confirmed the formation of bioactive apatite layer on surface of the nanoparticles after 7 days of immersion. The MTT assay and acridine orange staining test using hMSCs suggested the non-toxicity and osteogenic ability of as-synthesized FA nanoparticles. Based on the proposed synthesis mechanism, the deep eutectic system played a ternary role in synthesis of FA nanoparticles providing the synthesis environment, reactive calcium sites, and steric-electrostatic stabilization by which it can control the growth of as-formed FA particles in the nanometer size-range.

1. Introduction

In recent years, nanoceramic-based bioactive materials have proven to be promising candidates for many biomedical applications [1,2]. The nanobioceramics induce the bioactivity property in biomedical implants leading to the creation of a highly reactive surface for linking up with living tissue [3]. The link is known as bioactive bond that is created through formation of apatite layer at the intersection of the tissue and implant. The as-formed apatite along with adsorbed biological components forms a platform for osteoblasts to operate conveniently that subsequently provides the implant-tissue fixation [2,3].

Owing to their excellent biocompatibility, bioactivity and structural similarity to human bone constituents, fluorapatite (FA, $Ca_5(PO_4)_3F$)-based nanobioceramics are widely used in a variety of biomedical materials such as drug-delivery systems, dental implants, and bone substitutes [4–7]. FA is structurally and chemically similar to hydroxyapatite (HA); however, compared with HA, it shows better physicochemical properties such as the thermal stability, chemical stability, corrosion resistance and crystallization behavior [5,8]. In addition, FA shows promoted biological properties like bone cell proliferation, osteointegration and mineralization at physiological conditions. Thus, in recent years many studies have been reported on the synthesis, characterization, and biomedical application of FA-based nanomaterials [8–11].

To date various methods have been developed for synthesis of FA nanoceramics including sol-gel [9,12], co-precipitation [13,14], hydrothermal [4], solution combustion [15], mechanochemical synthesis [16,17], and neutralization method [18]. Despite the benefits of the foregoing methods, it seems that there is still a need to develop a rapid, simple, sustainable, and affordable synthesis method with the possibility of control over particulate properties of FA nanoceramics. This study aimed at providing a green and one-step method based on deep eutectic solvents for the synthesis of FA nanoparticles. Developing a facile, affordable, and green method for synthesis of FA nanoparticles having enhanced bioactivity is the main aspect of novelty in this study. In an innovative approach, the eutectic solvents have been selected in such a way to play a triple role: solvent, reagent and template (particle growth controller). In order to demonstrate the successful synthesis of

* Corresponding author.
E-mail addresses: m.karimi@merc.ac.ir, karimi.merc@gmail.com (M. Karimi).

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FA nanoparticles, the as-synthesized nanoparticles have been characterized by techniques of XRD, FESEM, EDS, TEM and FTIR. Also, the biocompatibility of the nanoparticles has been assessed by MTT test.

2. Experimental

2.1. Materials

All reagent grade chemicals were purchased from Sigma-Aldrich and used as-received without any purification.

2.2. Preparation of $CaCl_2{\cdot}6H_2O$-choline chloride deep eutectic solvent (DES)

The viscous and homogenous DES was prepared by simple mixing-heating of calcium chloride hexahydrate ($CaCl_2{\cdot}6H_2O$) and choline chloride ($C_5H_{14}ClNO$) salts in a molar ratio of 2:1 at 90 °C.

2.3. Synthesis of FA nanoparticles

FA nanoparticles were synthesized by a facile one-step method using the as-prepared DES. In a typical synthesis, 200 ml of the DES was preheated to 120 °C while stirring on a magnetic stirrer. Two concentrated aqueous solutions including 2.0 g of potassium phosphate tribasic (K_3PO_4) in 5 ml of deionized water and 0.12 gr ammonium fluoride (NH_4F) in 5 ml of deionized water were prepared separately and then were mixed together. The resulted aqueous solution was added dropwise to the eutectic system under vigorous stirring at 120 °C. The resulting clear solution became gradually opaque until after 20 min, the gel-like deposits were formed that eventually turned into solid milky deposits. After 90 min, the as-precipitated particles were rinsed five times with deionized water and then dried in an oven at 60 °C for 12 h.

2.4. Characterization of FA nanoparticles

The crystal properties of as-synthesized nanoparticles were studied by X-ray diffractometry (XRD) conducted on a Siemens D-500 X-Ray Diffractometer with CuKα radiation (λ = 1.5418 Å) operated at 40 kV and 30 mA. The analysis was performed on as-prepared powder over an angular range of 2θ = 20–60° at scan speed of 2° min^{-1} with step size of 0.02°.

The morphology of the nanoparticles were investigated by field-emission scanning electron microscopy (FESEM) performed on a Tescan Mira 3 LMU electron microscope. Prior to FESEM observation, FA nanopowders were dispersed in ethanol and sonicated for 20 min to minimize agglomeration. Then a drop of the suspension was placed on a microscope slide which was sputter-coated with gold.

The elemental composition of the nanoparticles was examined by energy dispersive X-ray spectroscopy (EDS) acquired on a Quantax 200 instrument. For this purpose, the whole of FESEM micrograph was scanned at magnification of 50 kx.

To analyze Ca/P molar ratio and F content of as-synthesized FA nanoparticles, inductively coupled plasma- optical emission spectroscopy (ICP-OES, Optima 8000) and F-selective electrode (using TISAB buffer) were employed. For analysis, 25 mg of FA powder was dissolved in 50 mg HCl (0.2 M) followed by addition of 25 mg deionized water and 100 mg trisodium citrate (0.2 M). NaF standard solution was used for calibration. It should be noted that the test was repeated three times.

The particle size of the nanoparticles was determined by transmission electron microscopy (TEM) obtained on a Philips CM 30 electron microscope. An aliquot of FA nanopowders was dispersed in ethanol and sonicated in an ultrasonic bath for 20 min to obtain a homogeneous suspension. Then a drop of as-prepared suspension was deposited on a carbon-coated copper grid. After complete evaporation of the ethanol solvent, the grid was introduced into the TEM chamber to be analyzed.

The surface functional groups were analyzed by Fourier transform infrared (FTIR) spectroscopy carried out on a PerkinElmer Spectrum 400 spectrophotometer. To this end, 2 mg of as-prepared FA powder was mixed with 200 mg of spectral-grade KBr, pressed as pellet and then analyzed.

2.5. In vitro bioactivity study

The *in vitro* bioactivity (apatite-forming ability) of the as-synthesized FA nanoparticles was investigated by soaking 20 mg of nanoparticles in 20 ml of simulated body fluid (SBF) solution which mimics the human blood plasma. The sample was stored in a sterile polyethylene container placed in a shaker at 37 °C for a time period of 7 days. The solution was renewed every 2 days. After 7 days, the soaked nanoparticles were filtered, washed several times with deonized water and dried at room temperature. The surface of the samples was analyzed by means of SEM-EDS (AIS2300C, Seron Technologies), FTIR (Spectrum 400, PerkinElmer), and Raman spectrometer (Senterra, Bruker) equipped with high-energy laser diodes at the wavelength of 785 nm. The experiment was reported three times. To compare the results, a control sample (in absence of FA nanoparticles) was used.

2.6. MTT cytotoxicity assay

The toxicity of the as-synthesized FA nanoparticles was examined by colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. To this purpose, the cells isolated from human bone marrow were cultured in 75 ml flasks using 12 ml of culture medium with high glucose content (DMEM-h, Gibco, Germany) along with 5 wt.% of fetal bovine serum (FBS, Gibco, Germany) and 1%wt. of antibiotic (Penicillin-Streptomycin, Gibco, Germany). The flasks then were incubated under the condition of 37 °C, 90% RH, and 5% CO_2. The culture medium was renewed every two days until cell density of 90% was achieved. Then, the cells were passaged, removed from the bottom of flask using 0.2% of triypsin-EDTA (Gibco, Germany) and counted using a Neubauer Chamber.

The hMSCs were seeded in a 96-well microplate (cell density of 1×10^4 cells per well) loaded by 500 µg FA nanoparticles (washed using 70 wt.% ethanol, sterilized under UV radiation for 20 min and autoclaved at 120 °C for 30 min). At various time intervals of 1, 3, 5 and 7 days, the supernatant was removed and washed with PBS. Then an amount of 200 µl of the culture medium containing 20 µl of MTT solution (Sigma Aldrich, USA) was added to each well. In the following, the samples were placed in an incubator at 37 °C, 98% RH, and 5% CO_2 for 4 h. The supernatant was removed and 100 µl of dimethyl sulfoxide (DMSO, Sigma Aldrich) was added to dissolve the Formosan crystals. Finally, the optical absorbance of the resulted blue-violet solution (directly related to the number of metabolically active cells) was measured by ELISA microplate reader (Sunrise-Tecan, Austria) at λ = 570 nm. A control sample (in absence of FA nanoparticles) was used for comparision.

2.7. Cell viability

Acridine orange staining test was performed to assess the cell viability on as-synthesized FA nanoparticles was assessed by. To this end, the dual fluorescent staining solution (1 µl) containing 100 µg/ml AO (Sigma Aldrich, USA) was added to the cell culture well which was subsequentely washed by PBS. Then, the well was monitored by fluorescent microscope (Leica 090-135002, Germany).

2.8. Alizarin red staining

To asses the bone differentiation of FA nanoparticles, alizarin red staining test was performed. The staining was done in 7 days of