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New bioactive bone-like microspheres with intrinsic magnetic properties obtained by bio-inspired mineralisation process

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ABSTRACT

A bio-inspired mineralisation process was investigated and applied to develop novel hybrid magnetic materials by heterogeneous nucleation of Fe^{2+}/Fe^{3+}-doped hydroxyapatite nanocrystals onto a biopolymeric matrix made of a Type I collagen-based recombinant peptide (RCP). The effect of the synthesis temperature on the phase composition, crystallinity and magnetic properties of the nucleated inorganic phase was studied. The as-obtained magnetic materials were then engineered, by using a water-in-oil emulsification process, into hybrid magnetic microspheres, which were stabilized by de-hydrothermal treatment yielding cross-linking of the macromolecular matrix.

Thorough investigation of the physicochemical, morphological and biological properties of the new hybrid microspheres, as induced by the presence of the inorganic nanophase and controlled iron substitution into hydroxyapatite lattice, revealed bone-like composition, good cytocompatibility, designed shape and size, and tailored magnetization. Such features are interesting and promising for application as new biomaterials with ability of remote activation and control by using external magnetic fields, for smart and personalized applications in medicine, particularly in bone tissue regeneration.

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1. Introduction

Nowadays, with the steady increase of age-related diseases, particularly bone tissue damage and degeneration which are among the most serious clinical problems, the scientific community is engaged in an intensive effort to investigate and develop new biomaterials with cell-instructive ability enabling safer and more effective approaches to trigger tissue neoformation and repair critical size bone defects [1,2]. To this purpose, the application of biologic factors is still controversial and would require the use of carriers able to release relevant drugs or growth factors in situ, along defined spatial and temporal profiles [3]. In this respect the development of bioactive materials with magnetic properties is raising a steadily increasing interest to boost bone tissue regeneration, due to the potential of remote activation on-demand by non-destructive external signals that can enable either direct stimulation of tissue repair in vivo, or the controlled and targeted release of therapeutic factors thus opening to more effective and personalized treatments [3–7]. However, cytotoxic effects related to the currently used magnetic materials (prevalently iron and gadolinium oxide nanoparticles) pose several concerns on their use so that complex engineering procedures are usually needed to reduce the direct exposition of such metal oxides to the body environment [8,9]. In this respect, the recent development of a biocompatible, bioresorbable superparamagnetic (Fe^{2+}/Fe^{3+})-doped hydroxyapatite nanophase (FeHA) prospectively opens to new smart and safe applications in nanomedicine and bone regeneration [10]. FeHA nanophase was obtained in the form of nanoparticles by modification of a typical neutralization method [11] and interesting properties were achieved: enhanced osteogenic ability [7,12], potential of controlled delivery of anticancer drugs [3], as well as ability to obtain magnetic cells by nanoparticles internalization that enables advanced cell therapies [7]. More recently, FeHA was also obtained by heterogeneous nucleation onto a collagen matrix [12], by using a bio-inspired assembling/mineralization process (herein reported as biomineralisation), which mimics the cascade of phenomena occurring in vivo during new bone tissue formation [13,14]. This process activates various physicochemical and morphological control mechanisms that constrain the inorganic phase to nucleate in specific loci of the collagen macromolecule, and limit the crystal growth to few nanometres with very low crystal ordering and specific orientation, similarly as occurring in the newly formed bone [14,15]. As a result of the above mentioned approach, the obtained hybrid construct exhibited high compositional and structural mimicry of bone tissue with excellent biocompatibility, osteogenic ability and intrinsic superparamagnetic properties [12].

The ability of natural polymers such as collagen to expose negatively charged functional groups enabling the link of Ca^{2+} ions and subsequent heterogeneous nucleation of apatite nanophases, opens to the

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development of hybrid biomimetic materials with tailored mineralization extent by using various macromolecular matrices [14]. In this respect, the present work aimed to translate the above mentioned biomineralisation process to a new collagen type I based recombinant peptide (RCP), to develop new hybrid magnetic microspheres by RCP assembling and simultaneous mineralisation with FeHA nanophase. RCP is a biocompatible and bioresorbable recombinant peptide mimicking human type I collagen (α I chain), comprising an increased amount of the cell adhesion and attachment arginine-glycine-aspartic (RGD) motif, relevant for cell adhesion and attachment [16]. Such inspired collagen designed as bio-polymer has already been investigated in respect to mineralisation with magnesium-doped hydroxyapatite [17], thus confirming the effectiveness of RCP in mediating bio-inspired mineralisation processes. Then, emulsification method was applied to produce hybrid magnetic microspheres and subsequently crosslinked by dehydrothermal treatment (DHT). The as-obtained microspheres are proposed as a new biomaterial to be applied in bone tissue engineering and potentially be remote activated by using magnetic field.

In particular, the present work investigates: (i) the mechanism of heterogeneous nucleation of the inorganic phase on the RCP matrix to develop new magnetic materials; (ii) the effect of the synthesis temperature on phase composition and magnetic properties; (iii) the effect of iron substitutions on the physicochemical and ultrastructural features of the new hybrids; (iv) the emulsification process, in respect to the achievement of hybrid magnetic microspheres with controlled phase composition, size and mineralization extent. Moreover, the present study is supported by preliminary assessment of cytocompatibility carried out on pre-osteoblast cells.

2. Experimental section

2.1. Raw materials

RCP, commercially available as Cellnest™, is characterised by molecular weight of 51.7 kDa and isoelectric point of ≈ 10.02, and was provided by Fujifilm Manufacturing Europe B.V. (The Netherlands). All the reactants used in the present study, i.e. calcium hydroxide ($Ca(OH)_2$, ≥95%), phosphoric acid (H_3PO_4, 85%), iron(II) chloride tetrahydrate ($FeCl_2.4H_2O$, ≥99%), iron(III) chloride hexahydrate ($FeCl_3.6H_2O$, 97%), sodium hydroxide (NaOH, ≥98%), 1,10-phenantroline ($C_{12}H_8N_2$, ≥99%), sulphuric acid (H_2SO_4, 99.999%), corn oil, acetone (≥99.9%), potassium bromide (KBr, ≥99%), phosphate buffered saline (PBS), dimethyl sulfoxide ($(CH_3)_2SO$, ≥99.9%), ethanol (≈96% (v/v)), were all obtained by Sigma Aldrich (St Louis, MO, USA), whereas nitric acid (HNO_3, 65%) was purchased from Titolchimica (Italy). αMEM w/o ascorbic acid, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Thermo Fisher Scientific (Waltham, MA, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), calcein acetoxymethyl (calcein AM) and ethidium homodimer-1 (EthD-1) were purchased from Invitrogen (Carlsbad, CA, USA). Ultrapure water (0.22 mS, 25 °C) was used in all the experiments.

2.2. Reaction of RCP with calcium and iron ions

The as-used divalent and trivalent ions (i.e. Ca^{2+}, Fe^{2+} and Fe^{3+}) in biomineralisation process were separately bound to RCP in aqueous media. Those reactions were prepared as a reference to be compared with biomineralised materials and to evaluate the involved chemical interactions. The amount of ions dispersed in the solution was defined on the basis of a 1:1 M ratio with the free carboxylic groups available in the RCP matrix, which are derived from aspartic and glutamic acid (1 mol of RCP corresponds to 57 acids) [16,18].

Firstly, 15.5 mg of $Ca(OH)_2$ was dispersed in 2 ml of deionized water at room temperature and 200 mg of RCP was dissolved in deionized water, in separated reaction vessel, at thermostated environment (i.e. 40 °C). Calcium solution was dropped into RCP solution under vigorous magnetic stirring and continuously mixed at room temperature for 30 min to obtain a solution henceforth coded as RCP/Ca.

Similar procedures were carried out to link Fe^{2+} and Fe^{3+} ions to RCP. Two solutions were prepared by dissolving 43 mg of $FeCl_2 \cdot 4H_2O$ and 29 mg of $FeCl_3 \cdot 6H_2O$ in 2 ml of deionized water and dropped in two reaction vessels containing the RCP solution, thus obtaining final materials henceforth coded as RCP/Fe2 and RCP/Fe3, respectively.

On the other hand, new reactions of RCP/Fe2 and RCP/Fe3 were performed and 0.1 M of NaOH was dropped to achieve the conditions of apatite crystallization (as obtained in the biomineralisation process), then the mixture was homogenized for 30 min. In all the as-mentioned reactions the final RCP concentration was 5 wt%. The resulting materials were freeze-dried at −40 °C under vacuum (≈0.1 bar) overnight, for future investigations.

2.3. Synthesis of hybrid mineralised materials

Mineralised materials were synthesised by heterogeneous nucleation of Fe-doped hydroxyapatite on RCP matrix. In detail, an aqueous solution of RCP (0.003 M) was prepared and thermostated at 40 °C. An acid solution of H_3PO_4 (1.2 M) was prepared and dropped in the RCP solution, then poured dropwise into a basic aqueous suspension of $Ca(OH)_2$ (0.8 M) also containing $FeCl_2 \cdot 4H_2O$ and $FeCl_3 \cdot 6H_2O$ as sources of Fe^{2+} and Fe^{3+} ions, to establish a Fe^{2+}/Fe^{3+} molar ratio of 3:2. The process was carried out at a fixed temperature and under magnetic stirring, to achieve a nominal mineralization extent equal to 40 wt%. The overall ion content was set to achieve $Ca/P_{mol} = 1.67$ and $Fe/Ca_{mol} = 0.2$ and the synthesis of materials were carried out at two different synthesis temperatures (i.e. 40 °C and 60 °C). The as-obtained materials are henceforth coded as RCPFeHA40 and RCPFeHA60, respectively.

Iron-free mineralised materials were synthesised at the same temperature conditions (i.e. 40 °C or 60 °C) and were used as a control in the chemical-physical characterisation, henceforth coded as RCPHA40 and RCPHA60, respectively.

By the end, the as-obtained hybrid mineralised slurries were used to produce the microspheres by the emulsification process, as described below, or to be freeze-dried at −40 °C under vacuum (≈0.1 bar) overnight, for further physicochemical investigations.

2.4. Production of microspheres by emulsification method

A water-in-oil emulsification process was established, to produce pure RCP and hybrid magnetic microspheres, starting from the previously obtained hybrid mineralised slurries (i.e. RCPFeHA40 and RCPFeHA60).

The emulsification process comprises three different stages, i.e. i) microspheres production in pre-warmed oil; ii) microspheres jellification and iii) oil phase washing. In detail, 20 g of hybrid mineralised slurry was dropped in 45 g of pre-warmed corn oil and kept under mechanical stirring for 20 min. The solution was cooled, until microspheres jellification, and dropped into a 300 mL of chilled acetone and kept under mechanical stirring for 5 min, then maintained for 1 h at room temperature, under mechanical stirring. The microspheres were left to sediment and acetone was carefully removed, then 300 ml of clean acetone was added and the microspheres were washed for 10 min. This step was repeated twice. The microspheres were filtered, dried overnight in an oven at 40 °C, and sieved to achieve a size distribution in the range 38 to 100 μm. For pure RCP microspheres, predetermined concentrated aqueous solution of RCP (≈5–36 wt%) was prepared and microspheres were obtained by the aforementioned method. DHT treatment was carried out by placing the dried microspheres in glass vials covered with aluminium foil and heated at 160 °C for 48 h under vacuum.

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