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Scaffolds for bone regeneration made of hydroxyapatite microspheres in a collagen matrix

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ABSTRACT

Biomimetic scaffolds with a structural and chemical composition similar to native bone tissue may be promising for bone tissue regeneration. In the present work hydroxyapatite mesoporous microspheres (mHA) were incorporated into collagen scaffolds containing an ordered interconnected macroporosity. The mHA were obtained by spray drying of a nano hydroxyapatite slurry prepared by the precipitation technique. X-ray diffraction (XRD) analysis revealed that the microspheres were composed only of hydroxyapatite (HA) phase, and energy-dispersive x-ray spectroscopy (EDS) analysis revealed the Ca/P ratio to be 1.69 which is near the value for pure HA. The obtained microspheres had an average diameter of 6 μm, a specific surface area of 40 m^2/g as measured by Brunauer-Emmett-Teller (BET) analysis, and Barrett-Joyner-Halenda (BJH) analysis showed a mesoporous structure with an average pore diameter of 16 nm. Collagen/HA-microsphere (Col/mHA) composite scaffolds were prepared by freeze-drying followed by dehydrothermal crosslinking. SEM observations of Col/mHA scaffolds revealed HA microspheres embedded within a porous collagen matrix with a pore size ranging from a few microns up to 200 μm, which was also confirmed by histological staining of sections of paraffin embedded scaffolds. The compressive modulus of the composite scaffold at low and high strain values was 1.7 and 2.8 times, respectively, that of pure collagen scaffolds. Cell proliferation measured by the MTT assay showed more than a 3-fold increase in cell number within the scaffolds after 15 days of culture for both pure collagen scaffolds and Col/mHA composite scaffolds. Attractive properties of this composite scaffold include the potential to load the microspheres for drug delivery and the controllability of the pore structure at various length scales.

1. Introduction

Bone is a composite organic–inorganic system with a complex multi-scale structure. Biomimetic scaffolds for use as bone implants should be designed with a structural and chemical composition similar to native bone tissue [1–3]. Under this perspective, ceramic/polymer composite scaffolds offer improved mechanical properties and biological activity compared to a ceramic or polymer material alone. Notably, calcium phosphate (CaP) ceramics such as hydroxyapatite (HA) and tricalcium phosphate (TCP) possess good osteoconductivity and osteoinductivity, and are commonly used in orthopaedic practice as filler materials for bone defects or as coatings on metal implants; whereas, natural and synthetic polymers, such as chitosan, collagen (Col), hyaluronic acid, and poly(lactic acid) (PLA) possess good biocompatibility and high elasticity [4,5]. Since natural bone tissue is primarily composed of hydroxyapatite and collagen, it is not surprising that Col/HA composites have been recognized as promising materials for use as bone implants [6–12]. The suitability and efficacy of these materials as bone graft substitutes largely depends on the structural features of the material such as the porosity, pore size, surface area, material strength, degradation properties and biocompatibility. Collagen has been used in tissue engineering and regenerative medicine for decades due to its biocompatibility, the ability to control the micro and macro structure of collagen scaffolds by different fabrication methods, the natural presence of ligands for cell attachment, the ability to perform various crosslinking reactions to affect the mechanical and degradation properties, and its potential use as a component in composite materials [13]. However, collagen on its own is not naturally osteoinductive and has low compressive strength; thus, hydroxyapatite complements collagen for bone regeneration applications by improving the mechanical properties, and providing osteoinductive and osteoconductive effects [9].

It is hypothesized that Col/HA composite materials provide the appropriate cellular microenvironment for bone regeneration by facilitating cell adhesion and migration, vascularization, and osteoblastic differentiation. The scale of the structural features of the material is a key consideration when designing a scaffold for bone growth, and prior work suggests that multi-scale structures, with defined features at the

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nano-, micro-, and macro-scales, contribute to the mechanical and biological properties of the substitute [10,14–18]. HA in the form of microspheres with a mesoporous structure provides a high surface area for protein adsorption and cell attachment, and when incorporated into a polymeric scaffold with tunable macropores may provide an ideal environment for cell infiltration, differentiation, and synthesis of bone extracellular matrix [19,20]. Furthermore, HA particle size has also been reported to affect cell response, and Liu et al. found that of two nano-sized HA, the smaller size had an increased intracellular uptake and resulted in reduced cell viability in vitro [16]. Hydroxyapatite in the form of microspheres has been investigated by many groups for biomedical application, specifically for their potential as local drug- or protein- delivery systems [21–32]. However, only few studies have investigated the incorporation of HA microspheres within a polymer matrix [20,32].

In this study we produced HA microspheres by spray drying and developed a porous composite scaffold, composed of collagen and HA microspheres by controlled freeze drying. This multiscale Col/mHA composite scaffold is suggested as an attractive material for human bone tissue engineering. Ongoing research is investigating the ability to load the microspheres for drug delivery and the controllability of the pore structure at various length scales.

2. Materials and methods

2.1. HA microsphere synthesis and characterization

Microspheres were synthesized using a spray drying method. The slurry for spray drying was obtained by precipitating HA nano particles from $Ca(NO_3)_2 \cdot 4H_2O$ and H_3PO_4 precursors with a Ca/P molar ratio of 1.67 [33]. After aging, the precipitated slurry underwent spray drying, with a flow rate of 2 l/h at an inlet pressure of 2 bar and chamber temperature of 200 °C. The obtained microspheres were calcined at 500 °C.

Transmission electron microscopy (HRTEM, Model Tecnai – Philips F30) analysis was carried out to investigate the particle size of the HA used for spray drying. The phase analysis, morphology, and size of HA microspheres were characterized by X-ray diffraction (XRD: D/max 2550 V, Rigaku), scanning electron microscopy (SEM: Zeiss-EVO) and particle size analyzer, respectively. Specific surface area and pore size of the microspheres were investigated by the Brunauer-Emmett-Teller (BET) and Barrett-Joyner-Halenda (BJH) methods using N2 adsorption/desorption isotherm (Quantachrome NOVA). Elemental analysis was performed using Energy-dispersive X-ray spectroscopy (EDS:Bruker) and chemical reactions between the HA microspheres and functional groups of collagen were evaluated using an attenuated total reflectance (ATR- FTIR: Perkin Elmer) spectrometer.

2.2. Composite scaffold fabrication and characterization

Porous scaffolds were fabricated by freeze-drying a 3% (wt/vol) suspension comprised of equal parts (by weight) of type I collagen (Opocrin SpA, Italy) and HA microspheres in a water solution of 0.3 (*w*/w) acetic acid. First the collagen was added and stirred until a homogeneous slurry was formed then the microspheres were added and the slurry was stirred for 2 more hours. The slurry was then transferred to plastic molds and freeze-dried to produce disk shaped scaffolds with 1 cm diameter and 2 mm thickness. Control scaffolds made only of collagen were fabricated using the same protocol. The formed scaffolds were then dehydrothermally (DHT) crosslinked (under vacuum) for 48 h at 120 °C.

The morphology of the scaffolds was studied using SEM, and the 3-dimensional structure was observed using microCT. Mechanical compression tests in PBS were performed on scaffolds with and without HA microspheres in order to evaluate the potential reinforcing role of the ceramic phase. Samples were compressed in displacement control until 75% of their initial thickness at a displacement rate of 0.01 mm/s after complete hydration in PBS. The compressive moduli at low and high strain value were calculated as the slopes of the initial (0–5% of strain) and final (70–75% of strain) linear part of the non-linear stress-strain curve for both Col/mHA composite scaffolds and collagen control scaffolds. Three samples were tested for each typology and the differences were considered statistically significant when the *p*-value calculated by *t*-test was <0.05.

2.3. Cell culture and cytocompatibility

2.3.1. 2-D (monolayer) cell viability study with and without mHA

Human osteosarcoma cell line, MG-63, cells were seeded onto 24-well plates at a density of 85,000 cells/cm^2 and the cells were allowed to attach directly to the cell culture plastic surface overnight. To determine if the presence of the microspheres affects the viability of cells growing in monolayer culture, a low (0.05 mg/cm^2), medium (0.5 mg/cm^2), and high (2 mg/cm^2) quantity of microspheres were added to the adherent cell cultures and cell viability was assessed, compared to cells growing in the absence of microspheres (control), after 2 days in culture using the Thiazolyl Blue Tetrazolium Blue (MTT) assay.

2.3.2. 3-D cell-seeded scaffold study (Col/mHA composite scaffolds and collagen alone control scaffolds)

Col/mHA composite scaffolds and control scaffolds made of collagen alone were placed in sterile phosphate buffered saline (PBS) overnight at 4 °C to hydrate the scaffolds. The following day the scaffolds were warmed to 37 °C then briefly placed on a sterile towel to remove excess PBS. Next the scaffolds were seeded on one side with a 50 μl suspension of 100,000 MG-63 cells within 24-well plates. After 10 min, 2 ml of warm culture medium was gently added to each well containing a cell-seeded scaffold. The plates were then placed in a 37 °C incubator with 5% CO_2 and the cells were cultured for 1, 3, 7, and 15 days, with the culture medium being change every 2 to 3 days. The MTT assay, and hematoxylin and eosin (H & E) staining were performed at each time point to assess cell viability, proliferation, morphology, and distribution. Statistical significance was determined using one-way ANOVA and Fisher's PLSD test.

3. Results and discussion

TEM of the precipitated HA used for spray drying revealed rod shaped particles in the size range of 20–40 nm (Fig. 1a). The XRD analysis of the microspheres revealed a pure HA phase (Fig. 1b) [25], and EDS spectra showed a Ca/P molar ratio of 1.69 (Fig. 1d), which is close to the stoichiometric value for pure hydroxyapatite. SEM reveal a perfectly spherical shape of the sprayed particles (Fig. 1c) and particle size analysis gave distribution values *D10*, *D50* and *D90* of 1, 6 and 13 μm, respectively (Fig. 2a). A typical adsorption–desorption graph of the microspheres is shown in Fig. 2b. BJH analysis showed the microspheres to be mesoporous with an average pore diameter of 16 nm (Inset Fig. 2b). The specific surface area calculated by the BET method was 40 m^2/g.

FTIR spectra of collagen only, hydroxyapatite microspheres, and the Col/mHA scaffold are shown in Fig. 3. The spectrum of the Col/mHA composite sample is characterized by IR absorption bands arising from HA and collagen. We observed the bands for collagen CO stretching at 1635 cm^{-1} for the amide I, and N—H deformation at 1545 cm^{-1} for the amide II. The phosphate bands are located between 900 and 1200 cm^{-1} in IR spectra. In the HA spectrum the typical stretching vibration bands of phosphoric groups at 1025 and 1047 cm^{-1} are detected. There are also CO3 bands located at 1445, 1414 and 876 cm^{-1} [34,35]. In the IR spectrum of Col/mHA, no shift was observed for the band corresponding to —COO— stretching indicating no chemical bonding between Ca^{2+} ions on the HA surface and —COO^- on collagen.

A picture with various examples of the fabricated scaffolds is shown in Fig. 4a. A representative 3-D microCT reconstruction of one of the disk shaped Col/mHA scaffolds is shown in Fig. 4b. The scaffold microstructure