International Journal of Biological Macromolecules xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Micro- and nano-hydroxyapatite as active reinforcement for soft biocomposites

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ARTICLE INFO

Article history:
Received 5 June 2014
Received in revised form 15 July 2014
Accepted 25 July 2014
Available online xxx

Keywords:
Biocomposites
Pectin
Hydroxyapatite
Hydrogels

ABSTRACT

Pectin-based biocomposite hydrogels were produced by internal gelation, using different hydroxyapatite (HA) powders from commercial source or synthesized by the wet chemical method. HA possesses the double functionality of cross-linking agent and inorganic reinforcement. The mineralogical composition, grain size, specific surface area and microstructure of the hydroxyapatite powders are shown to strongly influence the properties of the biocomposites. Specifically, the grain size and specific surface area of the HA powders are strictly correlated to the gelling time and rheological properties of the hydrogels at room temperature. Pectin pH is also significant for the formation of ionic cross-links and therefore for the hydrogels stability at higher temperatures.

The obtained results point out that micrometric-size hydroxyapatite can be proposed for applications which require rapid gelling kinetics and improved mechanical properties; conversely the nanometric hydroxyapatite synthesized in the present work seems the best choice to obtain homogeneous hydrogels with more easily controlled gelling kinetics.

1. Introduction

In the last decade, biocomposite hydrogels, i.e. composite biomaterials made of an organic polymeric matrix and an inorganic reinforcement, have been developed as scaffolds or delivery systems in tissue engineering.

Tissue engineering aims to heal damaged tissues by the use of implantable or injectable systems able to release bioactive agents, such as drugs, proteins, genes or cells, as well as to guide and support the regeneration of the host tissues [1–4]. Hydrogels represent a versatile class of biomaterials with attractive properties for tissue engineering, including biocompatibility, controlled biodegradability and easy manufacturing of the constructs in desired shapes [5,6].

Biocomposites have been proposed for tissue engineering either to improve the mechanical performances of the hydrogels [7,8] or to mimic specific biological structures like bones [9,10].

Natural polymers have an advantageous role in the production of biocomposites, since they derive from or mimic the native extracellular matrix secreted by the cells. Among these polymers, collagen, chitosan and alginate are the typical biomaterials employed to produce natural-based biocomposites [9–17].

Concerning the inorganic reinforcing phase, tricalcium-phospates and hydroxyapatite have been widely employed as reinforcement, due to their bioactive and osteoconductive properties [18–21,15,22,23]. The size of the inorganic reinforcement has a central role in the determination of the final biocomposite [24]. In particular, nano-sized fillers can drastically improve the properties of the conventional micrometric biocomposites, due to higher surface/volume ratio, better dispersion and the possibility of incorporating smaller amounts of fillers in the polymeric matrix [25,26].

Since natural bone is composed of nano-sized hydroxyapatite crystals with needle-like morphology, several research groups synthesized and employed hydroxyapatite with a bone-like nanometric structure [27–30].

In some cases, micro-structured hydroxyapatite particles were used to improve the mechanical properties of the biocomposites [13,31,32].

In such context, the present work concerns the preparation and characterization of a novel biocomposite made of pectin and hydroxyapatite with different microstructures.

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http://dx.doi.org/10.1016/j.ijbiomac.2014.07.050
0141-8130/

Pectin is a natural polysaccharide showing appealing properties for biomedical applications. In recent works [33–36], it was exploited especially for cell immobilization, due to good biocompatibility and to the possibility of forming hydrogels without the use of harmful reagents.

Pectin can form gels in the presence of calcium ions [33–38]; therefore, hydroxyapatite was selected both as cross-linking agent and as reinforcement of the biocomposite. The limited water solubility of hydroxyapatite allows obtaining a certain amount of free calcium ions which can be involved in the gelling process while the remaining insoluble part can act as reinforcement of the biocomposite.

The use of hydroxyapatite as a reactive compound in the polymeric matrix, in addition of being a mere reinforcement phase, is a promising strategy that can improve the connection and the permanence of the inorganic phase in the polymeric matrix, which is a critical issue to obtain stable biocomposites.

The aim of this work is to evaluate how the properties of pectin hydrogels are affected by the composition, grain size, microstructure, specific surface area and possible presence of impurities of the hydroxyapatite powders. The investigation of such parameters is carried out to gain specific control over the gelling kinetics, pH and rheological properties of the biocomposite hydrogels, to tailor the precise requirements of tissue engineering and regenerative medicine applications.

2. Materials and methods

2.1. Materials

Three different hydroxyapatite powders were considered in the present work as inorganic second phase to be added to the organic pectin matrix. The first powder, labeled as M-HA, was provided by Eurocoating SpA (Italy) and nominally consists of pure hydroxyapatite granules, in the range 20–40 μm; the second one, labeled as m-HA, was acquired by Fluidinova SA (Portugal) (nanoXIM Hap201, 2.5 μm; crystalline phase: pure hydroxyapatite; chemical analysis: Ca/P ratio = 1.66–1.72; particle size: $d_{0.1}$ = 1.4 μm, $d_{0.5}$ = 2.2 μm, $d_{0.9}$ = 3.6); the third one, labeled as n-HA, was synthesized by the wet chemical method as reported below, starting from reagent grade chemicals (calcium hydroxide, orthophosphoric acid, ammonium hydroxide) purchased from Sigma Aldrich.

Pectin CU701 lot 250, with galacturonic acid content = 85%, degree of esterification (DE) = 35%, Mw = 358,360, Mn = 149,471 and d = 2.4, was kindly provided by Herbstreith & Fox (Neuemberg, Germany) and was used as received. The galcturonic acid content and degree of esterification were provided by the supplier, while the molecular weight of pectin solutions was determined using a GPC system consisting of a Waters 1515 isocratic HPLC pump with a differential refractive index detector (Waters 2414). The mobile phase (0.1 M $NaNO_3$) was eluted through Waters Ultrahydrogel ™ 1000, 500 and 250 columns with a flow rate of 0.8 mL/min at 25 °C. Samples were prepared by dissolving 2.4% (w/v) pectin solutions in 0.1 M $NaNO_3$ for a final pectin concentration of 0.2% (w/v). After filtration through RC syringe filters (0.45 μm porosity), 200 μL of the solutions were injected in the GPC apparatus.

2.2. Synthesis and characterization of n-HA powder

Ammonium hydroxide solution (30 wt%) was slowly added to 240 ml of H_3PO_4 solution (0.25 M, pH = 1.67), until reaching pH = 8.5. Then, 100 ml of a milky $Ca(OH)_2$ suspension (prepared adding 20 g of $Ca(OH)_2$ to 0.500 l of bidistilled water) was dropped into the solution, checking continuously the pH, which reached a final value of 8.88. The white suspension was maintained under vigorous stirring overnight; then, the suspension was aged at 25 °C for 24 h and at 75 °C for further 24 h. After filtration, the white powders were washed several times with distilled water and dried at 75 °C for 24 h. At this stage, the powder was named "as prepared" or n-$HA_{(RT)}$. After drying, n-$HA_{(RT)}$ powder was calcined in air at 650 °C (heating rate = 10 °C/min) for 30 min and the material was further labeled as n-$HA_{(650\,°C)}$.

The synthesized n-$HA_{(RT)}$ and n-$HA_{(650\,°C)}$ powders were subjected to physical and microstructural characterization by using complementary techniques.

The powder morphology was observed by a Jeol JSM 5500 Scanning Electron Microscope (SEM), operating at 20 kV.

The synthesized powders, n-$HA_{(RT)}$ and n-$HA_{(650\,°C)}$, were analyzed also by Transmission Electron Microscopy (TEM) (Philips, CM12). In this case the powder was mixed with ethanol and a drop of the suspension (100 μl) was dripped over the sample holder.

XRD analysis was performed on a Rigaku D Max powder diffractometer, in Bragg-Brentano configuration, using Cu Kα radiation, in the range 2Θ = 10–60°, with step 0.05° and 5 s counting time.

The chemical analysis was carried out by Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES), using a Spectro Ciros instrument. The sample was dissolved into 5 wt% HNO_3 solution and the measurement was repeated three times. Sigma Aldrich pure 99.99% hydroxyapatite standard and Fluka multi-element standard – sol.IV were used for the quantitative analysis of Ca–P and for the other chemical elements, respectively.

For a better comparison of the three HA powders used in the present work, also M-HA and m-HA powders were subjected to SEM, XRD and ICP characterization, using the same procedures described for the n-HA powder.

FT-IR spectra were recorded on a Thermo Optics Avatar 330 instrument, in transmission mode in the range 4000–400 cm^{-1} using KBr pellets (64 scans, 4 cm^{-1} resolution).

Solid state NMR analyses were carried out with a Bruker 300WB instrument applying a carrier frequency of 300.13 MHz (^{1}H). Powder samples were packed in 4 mm zirconia rotors, which were spun at 9.5 kHz. ^{31}P SP MAS spectra were recorded at 121.49 MHz, with a $\pi/2$ pulse length of 3.6 μs and proton decoupling, recycle delay 300 s, acquiring 16 scans. The CP MAS experiment was run with contact time of 5 ms, recording 100 scans. Ammonium dihydrogen phosphate $(NH_4)H_2PO_4$ was used as secondary reference. ^{1}H MAS spectra were performed at 300.13 MHz, 5 μs $\pi/2$ pulse length, recycle delay 5 s, averaging 16 scans. Ethanol was used as secondary reference.

N_2-physisorption measurements were performed with a Micromeritics ASAP 2010 instrument. The samples were degassed below 1.3 Pa prior to the analysis. The Specific Surface Area (SSA) of the samples was evaluated with the BET equation within the relative pressure range: $0.05 \leq P/P_0 \leq 0.33$. BJH model was used for calculating the pore size distribution.

2.3. Preparation and characterization of the composite gels

HA-containing pectin composites were prepared with M-HA, m-HA, n-$HA_{(RT)}$ and n-$HA_{(650\,°C)}$ hydroxyapatite powders by mixing pectin solutions at pH 3.2 ± 0.1 (native pH) and pH 3.7 ± 0.1 (adjusted with 5 M NaOH) with 0.1% (w/v) hydroxyapatite suspensions. In brief, HA powders were dispersed in dH_20 and stirred vigorously for 10 min, and then the suspension was added to a 6% (w/v) pectin aqueous solution, diluting it to a 3% (w/v) final concentration. The mixture was gently stirred for 3 min to allow the homogeneous dispersion of HA in pectin solution prior to casting the gels in cylindrical molds (Ø = 2 cm). The hydrogels were left at room temperature for 24 h to allow for complete gelling, then they were characterized as follows.

Please cite this article in press as: F. Munarin, et al., Int. J. Biol. Macromol. (2014), http://dx.doi.org/10.1016/j.ijbiomac.2014.07.050