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Chitosan and carboxymethyl-chitosan capping ligands: Effects on the nucleation and growth of hydroxyapatite nanoparticles for producing biocomposite membranes

Vitor C. Dumont, Alexandra A.P. Mansur, Sandhra M. Carvalho, Fernanda G.L. Medeiros Borsagli, Marivalda M. Pereira, Herman S. Mansur *

Center of Nanoscience, Nanotechnology and Innovation — CeNano²I, Department of Metallurgical and Materials Engineering, Federal University of Minas Gerais-UFMG, Brazil.

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ABSTRACT

Synthetic biomaterials based on calcium phosphates (CaP) have been widely studied for bone tissue reconstruction therapies, but no definitive solution that fulfills all of the required properties has been identified. Thus, this study reports the synthesis of composite membranes based on nanohydroxyapatite particles (nHA) embedded in chitosan (CHI) and *O*-carboxymethyl chitosan (CMC) matrices produced using a one-step co-precipitation method in water media. Biopolymers were used as capping ligands for simultaneously controlling the nucleation and growth of the nHA particles during the precipitation process and also to form the polymeric network of the biocomposites. The bionanocomposites were extensively characterized using light microscopy (LM), scanning and transmission electron microscopy (SEM/TEM), energy-dispersive X-ray spectroscopy (EDX), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), atomic force microscopy (AFM), X-ray micro-CT analysis (μCT), and MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) cell proliferation assays for cell cytotoxicity. The results demonstrated that the ligands used during the synthesis highly affected the composites produced, primarily due the changes in the mechanisms and kinetics of nucleation and growth of the HA particles at the nanoscale level. The SEM images revealed that the use of carboxyl-functionalized chitosan (CMC) ligands significantly reduced the average size of the HA nanoparticles and caused the formation of a narrower size distribution (90 ± 20 nm) compared to the HA nanoparticles produced with chitosan ligands (220 ± 50 nm). The same trend was verified by the AFM analysis, where the nHA particles were formed evenly dispersed in the polymer matrix. However, the CMC-based composites were more homogeneously distributed, which was endorsed by the images collected *via* X-ray micro-CT. The FTIR spectra and the XRD analysis indicated that nanosized hydroxyapatite was the predominant calcium phosphate phase produced during the co-precipitation aqueous process for both the chitosan and CMC biocomposites. These novel hybrid systems based on chitosan and chitosan-derivatives with nHA composites were non-cytotoxic to a human osteoblast-like model cell line (SAOS) according to MTT *in vitro* assays. Moreover, the CMC-nHA biocomposites revealed a striking improvement in the cell viability response compared to the CHI-nHA biocomposite, which was attributed to the much higher surface area caused by the refinement of the nanoparticles size. Thus, the results of this study demonstrate that these novel bionanocomposite membranes offer promising perspectives as biomaterials for potential repair and replacement of cartilage and bone tissues.

1. Introduction

Tissue engineering (TE) is emerging as a fascinating interdisciplinary area of research that combines several fields of science such as biology, pharmaceutics, medicine, engineering, chemistry and physics to develop biocompatible substitutes for the regeneration of diseased or damaged tissues using cells, molecules and biomaterials [1,2]. Among all of the tissues in the body, bone is the most extensively investigated for tissue engineering due to its high potential for regeneration. Bone graft materials have been used to repair bone fractures and other defects due to their osteoinductive and osteoconductive characteristics [3]. However, the risk of transmission of diseases, infections, chronic pain, possible immunogenicity, poor delivery, and increased surgical time and costs are limiting factors of bone grafts [4,5]. A variety of natural and synthetic biomaterials can be combined to manufacturing various composites that exhibit improved properties [6,7]. Nanocomposites incorporating biodegradable polymeric and bioactive ceramics

* Corresponding author at: Department of Metallurgical and Materials Engineering, Federal University of Minas Gerais, Av. Antônio Carlos, 6627-Escola de Engenharia, Bloco 2, Sala 2233, 31.270-901, Belo Horizonte, MG, Brazil.
E-mail address: hmansur@demet.ufmg.br (H.S. Mansur).

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have been considered as a strategy for tissue engineering and regeneration [5].

In recent years, chitosan (CHI) has been one of the most investigated materials for biomedical applications. Chitosan [poly-β(1 → 4)-2-amino-2-deoxy-D-glucose] is one of the most abundant polysaccharides available and is semi-processed from natural sources. Thus, it has been used in a wide range of applications in fields of biology, food nutrition, medicine, and pharmacy [8,9]. It exhibits interesting properties for applications in tissue engineering, such as its biocompatibility, biodegradability [10], antimicrobial and antioxidant activities [11–13].

The properties of chitosan can be improved by introducing chemical groups. In particular, its reactive amino, primary hydroxyl, and secondary hydroxyl functional groups can be used to induce chemical modifications under relatively mild reaction conditions [14]. Moreover, focusing on hard tissue engineering applications, chitosan-based biomaterials usually require low initial solubility followed by an appropriate designed biodegradation behavior of the system. Ideally, these biomaterials should mimic the properties and functionalities of the replaced tissue. Hence, the outer surface of the biomaterial is expected to be almost insoluble and the inner surface in contact with the supporting tissue would experience progressively biodegradation leading to the full resorption, when the regeneration process is completed [15].

Chitosan derivatives, such as carboxymethylated-chitosan (CMC), are water soluble under acidic, alkaline, and physiological conditions compared to chitosan that is soluble only in acidic solutions [16,17]. There are several types of carboxymethylated chitosan derivatives that can be prepared *via* different methods [18–20]. Its versatility allows for its use in many applications, such as ion chelating agents, drug delivery, antimicrobial agent, and biomedicine [13,20]. Muzzarelli [18] reported that chitosan does not bind significantly with calcium ions. On the contrary, CMC adsorbs calcium ions dependent on the proportion of protonation or whether the molecule is in a sodium salt form, which is considered a marked peculiarity of CMC compared to chitosan. By attracting calcium ions, it is expected that CMC can exert an important effect on the precipitation of biominerals [21] or on the growth of crystals either *in vitro* or *in vivo* [22,23]. Therefore, CMC is considered a viable alternative for the development of innovative materials aiming to facilitate the bone regeneration process.

In addition, other biomaterials, including both natural and synthetic biomaterials, can be combined to manufacture nanohybrids with designed properties to improve bioactivity and osteoinduction [6,7]. The use of nanostructured biomaterials in bone regeneration is inspired by the natural bone architecture. Bone possesses a complex organic–inorganic nanocomposite structure. The organic phase is primarily composed of type I collagen, which is arranged into nanofibers ranging from 50 to 500 nm in diameter [24]. The inorganic phase consists of non-stoichiometric hydroxyapatite (HA) crystals with lengths of approximately 100 nm, widths of 20 to 30 nm, and thicknesses of 3 to 6 nm, which are embedded between the collagen fibers [25,26]. Calcium phosphate (CaP) biomaterials are of special importance because they mimic the major inorganic component of bone, are bioactive and can form functional interfaces with adjacent bone tissue. Various forms of calcium phosphates, doped derivatives and other ceramic-based biomaterials have been widely investigated as substitutes in bone regeneration research [27–29]. Previous studies have focused on adding calcium phosphate nanoparticles to the polymeric matrix. In addition, regarding the biocompatibility of chitosan and calcium phosphate particles, the chitosan degrades rapidly and has a low mechanical strength, whereas calcium phosphate nanoparticles degrade slowly and have a high mechanical resistance [30]. These materials may bring together the intrinsic functionalities of inorganic nanoparticles and the biointerfaces offered by biomolecules and polymers of natural origin [14,31]. In recent years, several studies have investigated membrane/film composites based on hydroxyapatite and biopolymers prepared *via* many methods such as blending, biomimetic processes, *in situ* precipitation, and electrochemical deposition [32–34]. However, surprisingly, no reports were found in the consulted literature that have investigated the nucleation/growth of nanosized hydroxyapatite particles (nHA) tailored by the use of chitosan and CMC as the capping ligand during the co-precipitation process, followed by extensively characterizing the produced biocomposites.

Thus, this study demonstrated the effect of CHI and CMC ligands on the kinetics of the formation of nHA particles using a co-precipitation process in an aqueous media, and simultaneously producing bionanocomposite membranes offering great potential for biomedical applications in bone-tissue repair and replacement.

2. Experimental procedure

2.1. Materials

All of the reagents and precursors, sodium hydroxide (Merck, USA, ≥99%, NaOH), phosphoric acid (Sigma-Aldrich, USA, 85%, H_3PO_4), calcium hydroxide (Sigma-Aldrich, USA, ≥96%, $Ca(OH)_2$), hydrochloric acid (Sigma-Aldrich, USA, 36.5–38.0%, HCl), ammonium hydroxide (Synth, Brazil, 30%, NH_4OH), monochloroacetic acid (Sigma-Aldrich, USA, 99%, $ClCH_2COOH$), ethanol (Synth, Brazil, 99.8%, CH_3CH_2OH), and isopropanol (Sigma-Aldrich, USA, 99.5%, $(CH_3)_2CHOH$) were used as received. Chitosan powder (Aldrich, USA, MM = 310,000 to >375,000 $g \cdot mol^{-1}$, DD ≥ 75.0%, and viscosity 800–2000 cPoise) was used as the polymer ligand and as the precursor for the synthesis of carboxymethyl-chitosan (CMC) *via* chemical modification. Deionized water (Millipore Simplicity™) with a resistivity of 18 MΩ cm was used in the preparation of all solutions. All preparations and synthesis were performed at room temperature, 25 ± 2 °C, unless specified. Potassium bromide (Sigma-Aldrich, USA, ≥99%, KBr), suitable for spectroscopy, was used to prepare the FTIR pellets.

2.2. Synthesis and characterization of carboxymethyl-chitosan (CMC)

A similar method to that reported in the literature [16] was used to prepare CMC at room temperature. Briefly, approximately 3.0 g of chitosan powder were suspended in 70.8 mL of isopropanol. After 30 min of magnetic stirring, 12.24 g of NaOH were dissolved in 15.0 g of deionized water (60% in water), and 15 mL of isopropanol were added to the suspension and maintained under stirring for 1 h. Next, 14.4 g of a monochloroacetic acid/isopropanol solution (1:1 in mass) were added to the suspension. The reaction proceeded for 4 h under moderate magnetic stirring and was stopped by the addition of 100 mL of methanol. Then, the suspension was filtered, and the solid filtrate (Na-CMC) was washed with ethanol/water mixtures of increasing ethanol content (from 90% to 100%). The neutralization of any residual hydroxide was conducted by suspending 1.0 g of the Na-CMC salt in a solution of 80% ethanol/water (100 mL), adding hydrochloric acid (10 mL, 37%) and stirring for 30 min. Then, the suspension was filtered, and the solid filtrate was abundantly rinsed with ethanol and vacuum dried to favor the formation of the protonated CMC derivative (H-CMC).

2.3. Preparation of chitosan (CHI) and carboxymethyl-chitosan (CMC) films by solvent evaporation

CHI and CMC solutions (1%, *w*/*v*) were prepared by dispersing the polymer powder (1 g) in a 100-mL aqueous solution of phosphoric acid (0.6% *v*/*v*, pH = 1.7). The mixture was constantly stirred for 24 h until complete solubilization (pH ~ 2.0) leading to the formation of homogenous clear solutions. Next, the solutions were poured into plastic molds (polyethylene, round-plate shape, diameter = 65 mm) and allowed to dry for 96 h at room temperature.