International Journal of Biological Macromolecules 93 (2016) 1465–1478

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Glycol chitosan/nanohydroxyapatite biocomposites for potential bone tissue engineering and regenerative medicine

Vitor C. Dumont[a,b], Herman S. Mansur[a,b,*], Alexandra A.P. Mansur[a,b], Sandhra M. Carvalho[a,b], Nádia S.V. Capanema[a,b], Breno R. Barrioni[b]

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

ARTICLE INFO

Article history:
Received 27 January 2016
Received in revised form 7 April 2016
Accepted 12 April 2016
Available online 13 April 2016

Keywords:
Chitosan
Biocomposite
Glycol-chitosan
Nanoparticle
Hydroxyapatite nanoparticles

ABSTRACT

In the last few decades, research on biocomposite nanomaterials has grown exponentially due to the global demand for novel solutions in bone tissue engineering and repair. In the present study, it is reported the design and synthesis of biocomposites based on glycol chitosan (GLY-CHI) matrices incorporated with nano-hydroxyapatite particles (nHA) produced *via* an eco-friendly chemical colloidal process in water media followed by solvent casting and evaporation methods at room temperature. The structure, morphology, and crystallinity of the components and biocomposites were extensively characterized by light microscopy (LM), scanning electron microscopy (SEM), transmission electron microscopy (TEM), energy-dispersive X-ray spectroscopy (EDX), wavelength dispersive X-ray fluorescence spectroscopy (WD-XRF), X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), and X-ray micro-computed tomography analysis (μCT). Furthermore, cytotoxicity and cell viability tests were performed on three cell lines using a 3-(4,5-dimethylthiazol-2yl) 2,5-diphenyl tetrazolium bromide (MTT) assay, an alkaline phosphatase (ALP) activity test, and LIVE/DEAD® assays. The results demonstrated that the GLY-CHI ligand played a major role in the nucleation, growth and colloidal stabilization of calcium phosphate particles at nanoscale dimensions with a narrow distribution and average size of 74 ± 15 nm. The FTIR spectroscopy associated with the XRD results indicated that nanosized hydroxyapatite (nHA) was the predominant calcium phosphate phase produced in the colloidal processing route. In addition, the X-ray micro-CT analysis of the nanocomposite membranes showed that nHA particles were homogenously dispersed in the glycol-chitosan polymeric matrix. Moreover, according to the *in vitro* bioassays, the biocomposites showed an adequate cell viability response and non-cytotoxic behavior toward osteoblastic-like (SAOS) and embryonic cell lines (HEK293T). Finally, the results of osteogenic differentiation tests demonstrated that the nHA/GLY-CHI composites are osteoinductive for human bone marrow mesenchymal stem cells (HBMS), which can be envisioned for prospective use in tissue engineering (*e.g.*, bone, cartilage and periodontal) applications.

1. Introduction

Millions of patients worldwide are suffering from bone defects caused by accidents, violence, trauma, cancer, congenital deformity, surgical reconstruction, and bone-related diseases; however, no definitive ideal solution for bone tissue repair and replacement is available [1–6]. Despite undeniable advances in the recent decades in the field of biomaterials, the development of innovative bio-engineered nanomaterials that fulfill all of the requirements for bone tissue reconstruction represents an important challenge to overcome by the regenerative medicine researchers and professionals [1–6]. Essentially, the primary difficulty arises from the fact that natural bone possesses a very complex inorganic–organic hybrid structure that is well-organized in an intrinsically hierarchical architecture composed of nanocrystals of hydroxyapatite [HA, $Ca_{10}(PO_4)_6(OH)_2$] and fibrils of collagen proteins [7,8]. Thus, to mimic the structure and composition of living tissues, the strategy of combining different biomaterials, molecules, and cells to form nanostructured biocomposites and hybrids may be considered a very attractive alternative [1,7,9–11]. In general, hybrid composite

* 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 addresses: hmansur@demet.ufmg.br, herman.mansur2016@gmail.com (H.S. Mansur).

http://dx.doi.org/10.1016/j.ijbiomac.2016.04.030
0141-8130/

materials represent the combination of two or more distinct components, organic and inorganic, each with important contributions to the area of materials science and each with unique characteristics that result in improved properties of the whole system [12,13].

More recently, natural polymer-based composites, such as chitin, chitosan, and collagen, have gradually garnered more attention than synthetic polymer-based composites for tissue engineering bio-applications (*e.g.*, bone, cartilage and periodontal) primarily due to several aspects, such as abundance in nature, worldwide availability, biocompatibility, and environmental compatibility [1,3,14]. Among the many choices of polysaccharide-based biopolymers for developing composites for bio-applications, chitosan has increasingly been used because of its exceptional amalgamation of properties, such as natural biocompatibility, biodegradability, and low immunogenicity [15]. Chitosan (CHI) is a linear biopolymer commonly derived from the deacetylation of chitin formed by β-(1,4)-2-acetamido-2-deoxy-D-glucose and β-(1,4)-amino-2-deoxy-D-glucose units, and it has been widely investigated as the organic component of composites for bone-tissue engineering [1,15]. However, it is only reasonably soluble in acidic water solutions, mostly due to the protonation of amino groups ($R\text{-}NH_3^+$) with poor solubility above a pH of 6.5 (pKa = 6.5), such as under physiological conditions common to living animals [16,17]. Therefore, to broaden the range of solubility and to simultaneously add new properties to the polysaccharide backbone, chitosan derivatives, such as carboxymethyl chitosan (CMC) [18–20], PEGylated-chitosan [21,22] and glycol-chitosan (GLY-CHI) [23] have been recently synthesized and investigated for bio-medical and environmental applications. Glycol-based chitosan derivatives may be suitable candidates as water-soluble biopolymers for producing composite biomaterials because they are generally soluble over the entire pH range, *i.e.*, under acidic, neutral, alkaline and physiological conditions [24,25]. However, from the bone tissue engineering perspective, polymers (such as chitosan and its derivatives) possess low mechanical properties, which are usually not suitable as biomaterials for cortical bone implants [14]. Hence, ceramic-based biomaterials, such as calcium phosphate compounds, are of great interest in the field of bone tissue engineering for use as reinforcements of polymer-based composites. Hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) is considered one of the most stable crystalline forms of calcium phosphate, and it occurs as a major inorganic component in the bone (in the range from 60 to 65%) [14]. Consequently, HA has emerged as an important compound for artificial bone preparation because it stimulates osteoconduction as it is gradually replaced by the host bone after implantation for orthopedic replacements, especially in treatments for bone regeneration and dental implants [14,15,26]. Additionally, when HA is combined with biopolymers such as chitosan to form composites, it might be able to mimic several functions of natural bone-related tissues [15,26]. The inclusion of inorganic nanoparticles of calcium phosphates (CaP) into the biopolymer matrix has several objectives, such as improving the mechanical properties, biocompatibility, altering the degradation behavior and incorporating topographic features at the nanoscale that mimic the hybrid nanostructure of natural bone [27]. Current fabrication processes can synthesize hydroxyapatite particles within the nanometer range; however, they usually suffer from major aggregation, agglomeration and heterogeneity, mainly in size, shape, and surface electrostatic charge, which render them inappropriate for several biomedical applications [28]. Therefore, by fine-tuning the morphological and physicochemical characteristics of hydroxyapatite nanoparticles by strict control of the reaction conditions, the overall properties of the produced biocomposites can be significantly enhanced [29–31]. Unexpectedly, although there are a few reports published using glycol-chitosan for cancer diagnosis and treatment [24,32], pharmaceutical applications as drug nanocarriers [33] and gene delivery [34], no study was found in the consulted literature using glycol-chitosan combined with HA for producing nanocomposite membranes for potential periodontal repair and regeneration.

Thus, this study reports for the first time the one-pot synthesis and characterization of nanocomposites using glycol-chitosan simultaneously as the capping ligands and the biopolymeric matrix for the formation of nano-hydroxyapatite particles *via* aqueous colloidal chemistry. In addition, these biocomposites combining inorganic nHA with organic glycol-chitosan were extensively tested for preliminary cytocompatibility using MTT cell proliferation assay with three human cell cultures (osteoblastic-like, embryonic cells and human bone marrow mesenchymal stem cells) as well as using an alkaline phosphatase (ALP) activity test and a LIVE/DEAD® viability-cytotoxicity assay.

2. Experimental procedure

2.1. Materials

All of the reagents and precursors, phosphoric acid (Sigma–Aldrich, USA, 85%, H_3PO_4), calcium hydroxide (Sigma–Aldrich, USA, ≥96%, $Ca(OH)_2$), and ammonium hydroxide (Synth, Brazil, 30%, NH_4OH) were used as received. Glycol-chitosan powder (Sigma–Aldrich, St. Louis, MO, USA, PN# G7753; degree of polymerization = 2000 Mw–410 kDa; degree of deacetylation DD = 76.2%) was used as the polymer matrix. The schematic representation of the chemical structure of the glycol-chitosan biomolecule is depicted in Fig. 1. Deionized water (Millipore Simplicity™) with a resistivity of 18 MΩ cm was used in the preparation of all of the solutions. All of the syntheses and preparations were performed at room temperature (25 ± 2 °C) unless otherwise specified. Potassium bromide (Sigma–Aldrich, USA, ≥99%, KBr) suitable for spectroscopy was used to prepare the FTIR pellets.

2.2. Preparation of glycol-chitosan (GLY-CHI) films by solvent cast and evaporation

GLY-CHI solutions (1%, w/v) were prepared by dispersing the polymer powder (0.5 g) in a 50 mL aqueous solution of phosphoric acid (0.6% v/v). The mixture was placed under constant stirring for 24 h until complete solubilization occurred (pH ∼ 2.1). Then, the solutions were poured into plastic molds (polyethylene round plate, diameter = 65 mm) and were allowed to dry for 96 h at room temperature (Fig. 1S—Supplementary Material).

2.3. Preparation of biocomposite membranes by the colloidal process—solvent cast and evaporation

The synthesis of nHA particles using glycol chitosan as the ligand was adapted based on the procedure developed by our group [31]. Briefly, GLY-CHI solutions (1%, w/v) were prepared by dispersing the polymer powder (0.5 g) in a 40 mL aqueous solution of phosphoric acid (0.75% v/v). These acidic polymer solutions were referred to as "SOL_2". Approximately 0.55 g of $Ca(OH)_2$ powder was added to 10 mL of deionized water and vigorously stirred for 15 min. This calcium suspension was referred to as "SUS_1". In the sequence, "SUS_1" was added slowly to "SOL_2", leading to the immediate formation of the suspension mixture ("SUS_3"), which was magnetically stirred for 1 h. Next, the pH of "SUS_3" was measured and adjusted to 12.0 ± 0.2 with NH_4OH (1.0 mol L^{-1}), and the suspension was continuously stirred for 24 h (Eq. (1)).

$$6H_3PO_4\,(aq) - \text{biopolymer} + 10Ca(OH)_2\,(aq)$$