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Mineralized alginate hydrogels using marine carbonates for bone tissue engineering applications

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ABSTRACT

The search for an ideal bone tissue replacement has led to the development of new composite materials designed to simulate the complex inorganic/organic structure of bone. The present work is focused on the development of mineralized calcium alginate hydrogels by the addition of marine derived calcium carbonate biomineral particles. Following a novel approach, we were able to obtain calcium carbonate particles of high purity and complex micro and nanostructure dependent on the source material. Three different types of alginates were selected to develop inorganic/organic scaffolds in order to correlate alginate composition with scaffold properties and cell behavior. The incorporation of calcium carbonates into alginate networks was able to promote extracellular matrix mineralization and osteoblastic differentiation of mesenchymal stem cells when added at 7 mg/ml. We demonstrated that the selection of the alginate type and calcium carbonate origin is crucial to obtain adequate systems for bone tissue engineering as they modulate the mechanical properties and cell differentiation.

1. Introduction

Biominerals are biologically produced minerals that provide strength and defense to living organisms. They form endo- and exoskeletons and are the main structural components of the dentition system. Apatites are the most common biomineral in vertebrates whereas calcium carbonates ($CaCO_3$) are the major forms of biominerals in invertebrates (Gopinathan et al., 2014). The main structural tissue of the skeletal system in vertebrates is bone. The complex composition of bone, formed by an inorganic phase, hydroxyapatite (HAp), embedded in an organic phase, mainly collagen type I, is responsible for its bending, compression and elongation strength (Blitterswijk, Lindahl, Hubbell, Williams, & Cancedda, 2008).

The use of hydrogels as scaffolds for bone tissue engineering shows several advantages; mechanical stability, gradual degradation able to be coupled with tissue regeneration, possibility to be used for the repair of irregular shape defects using minimally invasive surgery and ability to carry small molecules and controlled release them through the intrinsic transport properties of the gel (Guarino, Galizia, Alvarez-Perez, Mensitieri, & Ambrosio, 2015; Kim, Kang, Mercado-Pagan, Maloney, & Yang, 2014; Pasqui, Torricelli, De Cagna, Fini, & Barbucci, 2014). Taking advantage of these properties, different strategies have been focused on the development of hydrogels useful for drug release and tissue replacement in a wide variety of biological systems as nucleus pulposus, central nervous system, cartilage and bone (El-Sherbiny & Yacoub, 2013; Gharat et al., 2018; Giordano et al., 2009; Reitmaier et al., 2012). One of the requirements for biomaterials bone integration is bioactivity, defined as the ability of a biomaterial to promote the formation of a hydroxyapatite layer (Magalhaes et al., 2013). Numerous authors have attempted to go a step further and mimic the natural bone mineralization by the incorporation of salt crystals or the nucleation and growth of inorganic minerals inside a polymeric matrix (Douglas Timothy, Pamula, & Leeuwenburgh Sander, 2013). This biomineralization process aims to obtain organic/inorganic hybrid hydrogels that resemble bone extracellular matrix (ECM) composition (Huh, Zhao, & Kim, 2015). Different polymers can be used for this approach such as; chitosan (Diaz-Dosque et al., 2008; Munro, Green, Dangerfield, & McGrath, 2011), alginate (Diaz-Dosque et al., 2008; Wang, Leng, Che, & Shao, 2010; Xie et al., 2010), κ-carragenan (Diaz-Dosque et al., 2008) or synthetic polymers (Cha, Kim, Kim, & Kong, 2011; Guarino et al., 2015;

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Pasqui et al., 2014; Phadke, Shih, & Varghese, 2012). These composites are characterized by a high biocompatibility and their ability to promote osteogenic differentiation of mesenchymal stem cells (Pasqui et al., 2014; Phadke et al., 2012).

Polysaccharides are polymers that can easily interact with water, cations and tissue components such as proteins. They form viscous solutions in water and, under some specific conditions, viscoelastic gels. The mechanical properties of those gels can be modulated by the ionic concentration of the solvent used and the molecular weight of the polymers selected (Park & Lakes, 2007; Rinaudo, 2008; Tiwari, 2010). Alginates are obtained from brown algae or produced by bacteria. They are biocompatible and non-immunogenic polysaccharides (Draget and Taylor, 2010) widely used in tissue engineering (Kuo & Ma, 2001), cell encapsulation (Draget and Taylor, 2010) and drug delivery (Laurienzo, 2010; Rinaudo, 2008; Tiwari, 2010; Zhang et al., 2008) applications. One of the main advantages of alginates is their ability to form stable hydrogels in mild conditions by the simple addition of divalent ions, such as Ca^{+2}, to the polymeric solution. The formation of these hydrogels is dependent on the pH, ion concentration and alginate composition (Hoffman, 2002; Kuo & Ma, 2001; Laurienzo, 2010; Pathak, Yun, Lee, & Paeng, 2010; Russo, Malinconico, & Santagata, 2007). The incorporation of HAp to alginate hydrogels has led to bioactive composite systems with excellent properties for bone tissue engineering (Gkioni, Leeuwenburgh, Douglas, Mikos, & Jansen, 2010).

Different minerals can be used as the inorganic component for the development of biomineralized hydrogels. Calcium phosphates are the most commonly used, due to their similarities with the composition of bone (Gkioni et al., 2010). Moreover, other biominerals as calcium carbonates have also been studied for bone tissue engineering applications. Nature derived calcium carbonate scaffolds, nanoparticles and granules have been explored in the last decade in the tissue engineering field. Calcium carbonate is the main component of mollusk shells. In a similar way to bone, shells are formed by an organic/inorganic composite with a complex macro and microscopic structure responsible for its exceptional mechanical properties (Alakpa et al., 2017; Li, Xin, Muller, & Estroff, 2009). Calcium carbonates possess better biodegradation rates than HAp while maintaining a suitable mechanical strength which make them appropriate for bone regeneration (Yang et al., 2016). Marine derived scaffolds, mainly formed by calcium carbonate, have been found to be biocompatible and osteoinductive, being able to produce a functional vascularized bone graft when implanted in vivo (Cui et al., 2007; Chen et al., 2004). More recently, the use of marine derived calcium carbonate particles has been analyzed for the control release of cytokines as BMP-2 (Adams, Mostafa, Schwartz, & Boyan, 2014; Shi et al., 2017).

Even though biomineralized hydrogels are able to resemble the composition of bone, natural and synthetic minerals show differences in porosity, thermal and structural properties (Sofronia, Baies, Anghel, Marinescu, & Tanasescu, 2014). Furthermore, these systems are not able to simulate the complex hierarchical architecture of bone at the nano and microscale. In this study, we propose the synthesis of novel biomimetic mineralized calcium alginate hydrogels by the addition of calcium carbonate biomineral microparticles obtained from mollusk shells. This approach should allow for taking advantage of both natural components; Alginate would form the biodegradable polymeric matrix while calcium carbonate would stimulate cell differentiation and mimic tissue nanostructure. The proposed systems are environmental-friendly and inexpensive giving a second use of the shells obtained as waste products of the canning industry and using a marine polysaccharide for the hydrogel network formation.

2. Materials and methods

2.1. Materials

Sodium alginates following the European Pharmacopeia purity criteria were supplied by Danisco (France). Three varieties were selected for the experiment; GRINDSTED® Alginate pH 150, GRINDSTED® Alginate pH 127 and GRINDSTED® Alginate pH 155. Anhydrous calcium chloride was purchased from Panreac (Spain).

2.2. Shells powder preparation

Particles of calcium carbonate were produced from residues of farmed mussels and oysters. Shells were pulverized to reduce their particle size and then calcined in an oven at 550 °C to remove the organic components of the shells. Initially, shells were crushed using a lab scale Jaw Crusher and, at a later stage, the resulting granules were washed several times, suspended in water and grinded down to particle sizes below 10 µm using a wet attrition mill at a rotation speed of 880 rpm. Mg-PSZ balls of 2 mm in diameter (partially stabilized zirconia with magnesia) were used as the grinding agent. The volume ratio of product/ball was 1/3 and the grind cycle used was 3 h for 120 g of sample. In between comminution, grinding media were washed with water and ethanol. The sterilization for the in vitro assays was carried out by autoclave (Raypa, Spain).

2.3. Marine $CaCO_3$ particle characterization

In order to corroborate the obtained powders were indeed, formed by calcium carbonate particles, their chemical composition was characterized by powder X-Ray diffraction (pXRD) on a PANalytical X'Pert Pro instrument (Cu kα1). Particle size distribution was analyzed by laser diffraction (Mastersizer X, Malvern Instruments, United Kingdom) while density and surface area of the powders were measured by helium pycnometry (Quantachrome Mod. PY2, USA) and nitrogen adsorption (Micromeritics ASAP 2000, USA) using the Brunauer-Emmett-Teller (BET) method, respectively. Void Fraction was estimated as a function of the real density (ρ_p) and the apparent density (ρ_b) as is shown in Eq. (1).

$$\varepsilon = 1 - \frac{\rho_b}{\rho_p} \quad (1)$$

Eq. (1): Void fraction quantification

Morphological assessments of the particles were carried out by scanning electron microscopy (SEM). Images were obtained after coating the samples with gold on a SEM Zeiss EVO LS 15 microscope.

2.4. Alginate characterization

2.4.1. ^1H nuclear magnetic resonance

In order to correlate the chemical composition of alginates with the properties of the developed hydrogels, the composition of the different alginates was analyzed by Nuclear Magnetic Resonance (NMR). Prior to the analysis, alginate samples were partially hydrolyzed following the methodology described by Andriamanantoanina and co-workers (Andriamanantoanina and Rinaudo, 2010). Briefly, GG (where G is α-L-guluronic acid) and MM (where M is β-D-mannuronic acid) block fractions of alginates were obtained after two separate processes of precipitation and concentration. The hydrolysis yield, expressed as mass percentage (%wt), was calculated by adding the weights of recovered purified GG and MM blocks and diving it by the initial sample weight. The M/G ratio of each alginate was analyzed by ^1H NMR as described elsewhere (Grasdalen, 1983; Grasdalen, Larsen, & Smidsroed, 1979). ^1H NMR experiments were performed at 85 °C on a 6 mg/ml alginate solution in D_2O (Sigma), spectra were recorded using a Bruker DRX 500 spectrometer (Germany) operating at 400.13 MHz. Calibration was performed using the signal of the residual protons of the solvent as a secondary reference.