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Development and characterization of novel alginate-based hydrogels as vehicles for bone substitutes

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ABSTRACT

In this work three different hydrogels were developed to associate, as vehicles, with the synthetic bone substitute GR-HAP. One based on an alginate matrix (*Alg*); a second on a mixture of alginate and chitosan (*Alg*/*Ch*); and a third on alginate and hyaluronate (*Alg*/*HA*), using Ca^{2+} ions as cross-linking agents. The hydrogels, as well as the respective injectable bone substitutes (*IBSs*), were fully characterized from the physical–chemical point of view. Weight change studies proved that all hydrogels were able to swell and degrade within 72 h at pH 7.4 and 4.0, being *Alg*/*HA* the hydrogel with the highest degradation rate (80%). Rheology studies demonstrated that all hydrogels are non-Newtonian viscoelastic fluids, and injectability tests showed that *IBSs* presented low maximum extrusion forces, as well as quite stable average forces. In conclusion, the studied hydrogels present the necessary features to be successfully used as vehicles of GR-HAP, particularly the hydrogel *Alg*/*HA*.

1. Introduction

Every year, millions of patients, worldwide, are affected by bone defects caused by bone disorder or injury (Bostrom & Seigerman, 2005; Giannoudis, Dinopoulos, & Tsiridis, 2005; Vaccaro, 2002). In the last years, synthetic bone substitutes, mainly based on hydroxyapatite (HAP) and tricalcium phosphate, have been developed as treatment for those bone defects. These substitutes present several advantages against autografts or xenografts, namely, their unlimited availability and the fact that they eliminate the risk of disease transmission (Giannoudis et al., 2005; Moore, Graves, & Bain, 2001; Zimmermann & Moghaddam, 2011). To improve the chemical similarity between these bioceramics and bone inorganic part, glass-reinforced hydroxyapatite (GR-HAP) composites have been developed (Lopes, Knowles, Santos, Monteiro, & Olsen, 1999; Lopes, Knowles, & Santos, 2000; Salih, Georgiou, Knowles, & Olsen, 2001).

In order to potentiate the clinical application of the synthetic substitutes, they have also been developed in injectable form, by association with hydrogels. This approach presents some advantages, it can decrease the surgery time, allow a better fill of the bone defects and facilitate the substitute application in clinical situations with a difficult access to the defect. The hydrogel, working as a vehicle, should present a suitable viscosity to enable the bone substitute granules injectability (Couto, Hong, & Mano, 2009; Gaharwar, Dammu, Canter, Wu, & Schmidt, 2011; Larsson & Hannink, 2011; Liu et al., 2006; Oliveira et al., 2010).

Alginate is a polysaccharide produced mainly from brown seaweeds. It is a linear binary co-polymer composed of (1-4)-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues as monomers, constituting M-, G-, and MG-sequential block structures (Nunamaker, Purcell, & Kipke, 2007; Pawar & Edgar, 2012; Stevens, Qanadilo, Langer, & Prasad Shastri, 2004). This polymer has been widely used to produce biomedical hydrogels, mainly due to its biocompatibility, biodegradation, gel-forming ability through simple divalent cations (such as Ca^{2+}, Ba^{2+} and Sr^{2+}) addition and its very low cost (Lee & Mooney, 2011; Ueng et al., 2004; Ulery, Nair, & Laurencin, 2011). For pH values above alginate pK_a value (about 3.38 and 3.65 for M and G residues, respectively) it presents a polyanionic chemical structure, the monomers carboxylic groups are negatively charged (Andersen, Strand, Formo, Alsberg, & Christensen, 2012; Funami et al., 2009). Thus, the cations bind to them, preferentially toward the G-block rather than the

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M-block, forming a structure named as "egg-box" and providing the cross-linking of the polymeric chains (Davidovich-Pinhas & Bianco-Peled, 2010; Nunamaker et al., 2007; Pawar & Edgar, 2012). When G monomer proportion is higher than M monomer proportion, a strong brittle gel will be obtained. When M proportion is higher, the hydrogel will be weaker, but more flexible, because there are less junction zones between chains (Nunamaker et al., 2007; Pawar & Edgar, 2012; Sriamornsak, Thirawong, & Korkerd, 2007). Alginate hydrogels have already been used in bone tissue engineering as vehicles of HAP, allowing to properly fill the whole bone defect. To produce biomedical alginate hydrogels, Ca^{2+} ions are the most used crosslinking agents due to the mild reaction conditions compared to the cellular toxicity of both Ba^{2+} and Sr^{2+}. Moreover, for bone application this ion is preferred since it is the main extracellular matrix (ECM) ion. However, in biomedical applications, alginates have the main disadvantage of being non-biologically active, presenting a low cell adhesion (Andersen et al., 2012; Nair & Laurencin, 2007; Nunamaker et al., 2007; Ulery et al., 2011).

One way of improving the biological properties of alginate scaffolds, it is to ionically associate it to another polymer, namely a polycation as chitosan, forming a polyelectrolyte complex (Chen et al., 2009; Chunga et al., 2001). Thus, a complex between alginate and another polyanion, such as hyaluronic acid (HA), can also be formed using cations as intermediate agents (Oerther et al., 2000). Obviously, to ensure the complexes formation, the solution pH has to be controlled for the two polymers being charged (Dakhara & Anajwala, 2010).

In the present study, we developed three different alginate-based hydrogels for being used as vehicles of the GR-HAP bone substitute granules. One hydrogel was just composed by alginate cross-linked with Ca^{2+} ions, and other two hydrogels resulted from the association of chitosan and hyaluronic acid to alginate. The developed hydrogels were submitted to a physical–chemical characterization, as well as the respective injectable systems composed by each hydrogel and the GR-HAP granules.

2. Materials and methods

2.1. Materials

Alginic acid sodium salt from brown algae (bioreagent grade; low viscosity; 39% (w/w) of guluronic acid and 61% (w/w) of mannuronic acid), hyaluronic acid sodium salt from *Streptococcus equi* and calcium chloride hexahydrate ($CaCl_2{\cdot}6H_2O$, medical grade) were purchased from *Sigma* (USA). Chitosan HCl (medical grade) was purchased from *Heppe Medical Chitosan GmbH* (Germany). The hydroxyapatite powder was purchased from *Plasma Biotal* (UK).

2.2. Methods

2.2.1. Hydrogels preparation

2.2.1.1. Hydrogel Alg. Sodium alginate was dissolved overnight in deionized water at room temperature, in order to prepare a polymeric solution with a concentration of 7% (w/V) with pH 6. $CaCl_2{\cdot}6H_2O$ was also dissolved in deionized water to a concentration of 15 mg/mL. Finally, the $CaCl_2$ solution was added to the sodium alginate solution at a proportion of 1:4 (V_{CaCl_2} : $V_{\text{sodium alginate}}$).

2.2.1.2. Hydrogel Alg/Ch. Chitosan HCl was dissolved in deionized water to prepare a solution of 0.5% (w/V). Then, the $CaCl_2$ and sodium alginate solution were added to it, by this order, in the proportions of 1:4 (V_{CaCl_2} : $V_{\text{sodium alginate}}$) and 1:1 ($V_{\text{sodium alginate}}$: $V_{\text{chitosan HCl}}$), respectively.

2.2.1.3. Hydrogel Alg/HA. Sodium hyaluronate was dissolved overnight in deionized water, at 4 °C, to a concentration of 0.5% (w/V). This solution was added to the sodium alginate solution in a proportion of 1:1 ($V_{\text{sodium alginate}}$: $V_{\text{sodium hyaluronate}}$). Finally, to the solution of the two polymers the $CaCl_2$ solution was added in a proportion of 1:4 (V_{CaCl_2} : $V_{\text{sodium alginate}}$).

2.2.2. GR-HAP and injectable bone substitutes (IBSs) preparation

2.2.2.1. GR-HAP. GR-HAP was obtained by adding 2.5% (w/w) of glass (with the composition $65P_2O_5$–$15CaO$–$10CaF_2$–$10Na_2O$, mol%) to pure phase of prepared HAP mixed with microcrystalline cellulose. Then, discs were prepared by uniaxial pressing and heat treated at 600 °C to burn out the microcrystalline cellulose and then sintered at 1300 °C for 1 h. Finally the discs were milled and sieved to produce granules of a 500–1000 μm size range.

2.2.2.2. IBSs. In order to optimize the preparation of the three different *IBS*s, GR-HAP granules were simply mixed and aggregated with each one of the developed hydrogels until the desired consistency was achieved. For each system, the used apparent ideal proportions (w/w) of the bone substitute and each hydrogel were, respectively: *Alg_IBS* – 41% of hydrogel and 59% of GR-HAP; *Alg/Ch_IBS* – 47% of hydrogel and 53% of GR-HAP; *Alg/HA_IBS* – 48% of hydrogel and 52% of GR-HAP.

2.2.3. Scanning electron microscopy (SEM) analysis

On the case of the *IBS*s, the samples were firstly fixed with 1.5% (w/V) glutaraldehyde in 0.14 M sodium cacodylate buffer (pH 7.3). Afterwards, the samples were dehydrated using graded ethanol solutions from 50% (V/V) to 100% (V/V), followed by immersion in hexamethyldisilazanes (HMDS) solutions ranging from 50% (V/V) to 100% (V/V). The samples lasted 10 min in each ethanol and HMDS solution and overnight in 100% (V/V) HMDS. All the used reagents were purchased from *Sigma* (USA). Afterwards, the hydrogels and *IBS*s samples were mounted onto an aluminum stub and coated with gold/palladium using a *SPI Sputter Coater*. Finally, the samples morphology was analyzed using the equipment *FEI Quanta 400FEG SEM* (*FEI*, USA).

2.2.4. Fourier transform infrared – attenuated total reflectance (FTIR-ATR) spectroscopy analysis

The FTIR-ATR spectra, over the wavelength range of 1800–800 cm^{-1}, of the sodium alginate, chitosan HCl and sodium hyaluronate polymeric solutions, and of the three developed hydrogels were obtained by the FTIR spectrometer *FT/IR-4100* (*Jasco*, USA). FTIR-ATR analysis of the sodium alginate powder before and after autoclaving was also performed over the wavelength range of 2000–750 cm^{-1}.

2.2.5. Hydrogels swelling profile

Swelling studies were performed according to ASTM (American Society for Testing and Materials) F2900-11. For that, the hydrogels were immersed (in quadriplicate) in two different buffer solutions, phosphate buffered saline (PBS: pH 7.4, *Sigma*, USA) and potassium hydrogen phthalate (KHP: pH 4.0, *Sigma*, USA) as follows.

A sample of each gel was weighed before immersion in any of the solutions and it was designated as W_0. Afterwards, the samples were placed inside a previously weighed standard mesh and immersed in the buffers and incubated at 37 °C. The mesh was removed every minute for 5 min and left to dry until no drop was formed. The gel/mesh system was weighed and the mesh weight was subtracted to the system weight and designated as W_t. The weight change (%) for each sample at time t, was calculated using the following equation: $\textit{Weight change}\ (\%) = (W_t - W_0)/W_0 \times 100$.