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Alginate based scaffolds for bone tissue engineering

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ABSTRACT

The design and production of scaffolds for bone tissue regeneration is yet unable to completely reproduce the native bone properties. In the present study new alginate microparticle and microfiber aggregated scaffolds were produced to be applied in this area of regenerative medicine.
The scaffolds' mechanical properties were characterized by thermo mechanical assays. Their morphological characteristics were evaluated by isothermal nitrogen adsorption and scanning electron microscopy. The density of both types of scaffolds was determined by helium pycnometry and mercury intrusion porosimetry. Furthermore, scaffolds' cytotoxic profiles were evaluated *in vitro* by seeding human osteoblast cells in their presence.
The results obtained showed that scaffolds have good mechanical and morphological properties compatible with their application as bone substitutes. Moreover, scaffold's biocompatibility was confirmed by the observation of cell adhesion and proliferation after 5 days of being seeded in their presence and by non-radioactive assays.

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1. Introduction

Bone is a metabolically active and highly organized tissue that provides a framework for the body, supports soft tissues, acts as attachment points for skeletal muscles, confers protection to the internal organs and serves as a reservoir of bone marrow stem cells [1]. Loss or deterioration of this tissue due to disease, trauma or surgical intervention often leads to premature disability and reduction of the quality of life of patients [2]. Currently, grafts from different sources have been used as bone substitutes. These include autografts, allografts and xenografts. Autografts are the clinically preferred grafting materials for bone repair, however, they have several drawbacks like limited bone supply, anatomical incompatibilities and are often associated with donor site morbidity. Nevertheless, the application of allografts and xenografts is limited due to disadvantages such as histoincompatibility and the possibility of pathogenic transmission [3].

To overcome these problems, bone tissue engineering (BTE) approaches are contributing for the development of different therapeutics, which include drug delivery systems, scaffolds, among others, to promote the host bone regeneration [4]. With this purpose different three dimensional (3D) constructs (scaffolds) have been produced to be used in BTE. Scaffolds should have different properties to be used in this area of tissue regeneration, including biocompatibility (good integration without host immune response), osteoconductivity (interconnected pores, allowing cell infiltration and neovascularization), biodegradability (degradation products must not be toxic) and bioactivity, promote osseointegration and stimulate bone ingrowth and differentiation [5].

Different methodologies such as freeze drying [6], salt leaching technique [7], gas foaming [8], rapid prototyping techniques [9], among others have been used for scaffolds' production [10]. However, none of the constructs produced so far completely reproduce the native bone properties [11].

Recently, a low-priced innovative methodology based on the random packing of microspheres with further aggregation by physical or thermal means has been used to produce 3D porous scaffolds [12–14]. These scaffolds could be used either in an acellular strategy (by implanting the scaffold without any seeded cells directly into the bone defect) or in a cellular strategy (creating a hybrid cell–material construct *in vitro*, followed by its implantation *in vivo*). Furthermore, these scaffolds can also be used as drug delivery systems, having a multifunctional purpose, giving support and being involved in bioactive agent release, enhancing their regenerative potential [15]. Natural and synthetic biomaterials, such as chitosan and poly (lactide-co-glycolide acid) (PLGA), have been used for the production of these types of scaffolds [14]. However, all the methods used until now to produce these kind of 3D matrices are extremely invasive and include, for example, the use of acetic acid, sinterization and freeze drying which will further affect the mechanical and the biological behavior of the scaffolds [14,16–18]. In this context, and trying to surpass all this disadvantages, our group developed a new and very

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simple method, based in the technique described before, to produce alginate microparticle and microfiber aggregated scaffolds.

The biopolymer selected being used in this research work was alginate that is a natural polysaccharide extracted from brown seaweeds and is composed of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues. In the presence of certain divalent cations (*e.g.*, Ca^{2+}, Sr^{2+} and Ba^{2+}), alginate has the ability to form stable hydrogels through ionic interaction between the cation and the carboxyl functional group of the G units of alginate [19]. This crosslinking process makes alginate insoluble in aqueous solutions, such as culture medium, enabling its use for 3D scaffolds' production [20].

In the present study, an easy, reproducible and low priced alginate microparticle (MP) and microfiber (MF) aggregated scaffolds were produced, from the best of our knowledge, for the first time with alginate through this aggregation method. Besides, these scaffolds have good mechanical properties conjugated with an adequate biological behavior. Due to their production process, these scaffolds consist of various independent components integrated in a unique scaffold, which makes the encapsulation of different drugs (growth factors) or/and cells into their structure through simple mixing easy. Furthermore, in the form of hydrogel, alginate has an adaptable shape that allows the filling of any bone defect when implanted *in vivo* [21].

2. Materials and methods

2.1. Materials

Alginic acid sodium salt (sodium alginate) (Mw 120,000–190,000 Da), calcium chloride ($CaCl_2$), Dulbecco's modified Eagle's medium nutrient mixture F-12 (DMEM-F12), penicillin G, streptomycin, trypsin, ethylenediaminetetraacetic acid (EDTA) and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich. Human osteoblast cells (CRL-11372) were purchased from the American Type culture collection (VA, USA). Fetal bovine serum (FBS) was purchased from Biochrom AG (Berlin, Germany) and L-glutamine, 3-(4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl) 2-(4-sulphofenyl)-2H-tetrazolium, inner salt (MTS) supplied by Promega.

2.2. Methods

2.2.1. Microparticle and microfiber production

MP and MF were produced by internal gelation according to a procedure previously described in the literature [22]. Sodium alginate was dissolved in distilled water at a concentration of 3% (w/v) and a solution of $CaCl_2$ (5% (w/v)) was used as gelation medium. MP were produced by using an electrospinning apparatus [23]. Briefly, the previously prepared alginate solution was loaded into a 10 mL plastic syringe attached with a needle of 23 gauge and the solution was extruded into 100 mL of gelation medium ($CaCl_2$) under stirring, at room temperature. The needle was connected to a high-voltage generator (CZE 1000R, Spellman, UK) at a voltage of 6 kV. The solution feed rate, 9 mL/h, was controlled through a syringe pump (KdScientific, KDS-100, Sigma) [23]. MF were obtained by loading 10 mL of sodium alginate solution into a syringe and continuously extruded through the needle into 100 mL of $CaCl_2$ solution. Then, the MP and MF were washed several times with distilled water and filtered, with a 0.22 μm filter.

2.2.2. Alginate aggregated scaffolds' production

The scaffolds studied were produced by an adaptation of the method previously described by Malafaya et al., 2005 [14]. In brief, this technique is based on the random packing of pre-fabricated MP or MF with further aggregation by physical means in order to create a 3D porous structure. The alginate-based scaffolds were press-fitted into a mold and dried at 37 °C for 3 days.

2.2.3. Swelling studies

The swelling properties of scaffolds were characterized in Tris buffer (pH 7.4). Both types of scaffolds were placed in a test tube containing 2 mL of Tris and allowed to swell at 37 °C. At predetermined intervals, the swollen scaffolds were removed and weighed. The wet weight of the swollen scaffolds was determined by blotting them with filter paper to absorb the excess of Tris. Then they were weighted and reimmersed into the swelling medium [24]. The swelling ratio was evaluated by using Eq. (1):

$$\text{Swelling ratio } (\%) = \frac{W_t - W_0}{W_0} \times 100 \quad (1)$$

where W_t is the final weight of scaffolds and W_0 is the initial weight of scaffolds.

2.2.4. Characterization of porosity and density

Scaffold's porosity was evaluated by mercury intrusion porosimetry (MIP) and isothermal nitrogen adsorption. MIP determinations were conducted in a Micromeritics (Model Poresizer 9320) and the percentage of porosity, total pore area and apparent (or skeletal) densities of the samples were determined. The pressures applied in the low pressure and high-pressure domains were from 0.1 to 33,000 psi, respectively. A contact angle of 130° and a surface tension of mercury of 485 mN/m were determined for the sample film.

The specific or BET (Brunauer–Emmett–Teller) surface area and the porosity of the scaffolds were determined by nitrogen adsorption experiments, using a Micrometrics ASAP 2000 analyzer. Nitrogen adsorption isotherms were determined in a range of relative pressures, P/P_0, from 0.002 to 0.989. The saturation pressure, P_0, was continuously registered in the course of the adsorption–desorption measurements. The average pore diameter, D_p, was calculated through Eq. (2):

$$D_p = \frac{4V_T}{A} \quad (2)$$

where V_T is the total volume of pores, and A is the BET surface area. Pore size distributions were also obtained from the desorption branch of the nitrogen sorption isotherms using BJH (Barrettoyner–Joyner–Halenda) analysis.

Scaffold's density was evaluated by helium pycnometry using an AccuPyc 1330, Micrometrics. Each measurement was preceded by five purges and five analytical runs were conducted with a fill pressure of 15 psi and an equilibration rate of 0.05 psi/min. The volume of the tested samples varied between 1.533 and 1.551 cm^3.

2.2.5. Mechanical properties

Thick specimens (15.20 × 7.45 × 1.10 mm) were analyzed by Dynamical Mechanical Thermal Analysis (DMTA). A Triton Tritec 2000 analyser was used in the Constrain Layer Damping mode, with a standard heating rate of 5 °C.min^{-1}, in multifrequency mode (1 and 10 Hz) and a displacement of 0.05 mm. The glass transition temperature (T_g) was determined as the peak in Tan δ (Tan δ = E″/E′) where E″ and E′ are the loss and storage modulus, respectively, derived from DMTA. The Thermo Gravimetric Analysis (TGA) was carried out in a SDT Q600 from Thermal Analysis at a 5 °C.min^{-1} heating rate and was performed in nitrogen atmosphere at a flow rate of 100 mL.min^{-1}.

The compression strength of the scaffolds was calculated with a Zwick® 1435 Materialprüfung (Ulm, Germany) in which the samples were uniaxially compressed until maximum load was achieved.

2.2.6. Scanning electron microscopy analysis

The scaffold's morphology with/without cells was analyzed by SEM. To evaluate cell adhesion and proliferation, human osteoblast cells were seeded over MP and MF aggregated scaffolds. After 3 days

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