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Hybrid 3D structure of poly(D,L-lactic acid) loaded with chitosan/chondroitin sulfate nanoparticles to be used as carriers for biomacromolecules in tissue engineering

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ABSTRACT

In the tissue engineering (TE) field, the concept of producing multifunctional scaffolds, capable not only of acting as templates for cell transplantation but also of delivering bioactive agents in a controlled manner, is an emerging strategy aimed to enhance tissue regeneration. In this work, a complex hybrid release system consisting in a three-dimensional (3D) structure based on poly(D,L-lactic acid) (PDLLA) impregnated with chitosan/chondroitin sulfate nanoparticles (NPs) was developed. The scaffolds were prepared by supercritical fluid foaming at 200 bar and 35 °C, and were then characterized by scanning electron microscopy (SEM) and micro-CT. SEM also allowed to assess the distribution of the NPs within the structure, showing that the particles could be found in different areas of the scaffold, indicating a homogeneous distribution within the 3D structure. Water uptake and weight loss measurements were also carried out and the results obtained demonstrated that weight loss was not significantly enhanced although the entrapment of the NPs in the 3D structure clearly enhances the swelling of the structure. Moreover, the hybrid porous biomaterial displayed adequate mechanical properties for cell adhesion and support. The possibility of using this scaffold as a multifunctional material was further evaluated by the incorporation of a model protein, bovine serum albumin (BSA), either directly into the PDLLA foam or in the NPs that were eventually included in the scaffold. The obtained results show that it is possible to achieve different release kinetics, suggesting that this system is a promising candidate for dual protein delivery system for TE applications.

1. Introduction

In tissue engineering (TE), a porous scaffold is typically required to act as a template for cell proliferation, differentiation and cell growth. Scaffolds may also act as controlled release devices that deliver growth factors (GFs) with rates matching the physiological need of the regenerating tissue. The process of new tissue formation may be augmented by the delivery of bioactive factors (e.g. GFs) that induce cells to behave in a specific manner [1]. The aim of targeted drug delivery is to selectively deliver drugs/bioactive agents to specific sites and to release them locally in order to induce a desired response of the target tissue, without affecting the surrounding tissues. Recently some studies [2–5] have reported the incorporation of drug or protein-loaded particles into TE scaffolds to form composites with the ability of delivering bioactive molecules that aid in tissue regeneration [6]. Ideally, the concept of delivering bioactive agents directly from the polymer matrix which is used as a template for cell transplanting is an attractive approach. However, the major challenge in developing drug delivery systems is to incorporate such biologically active guest species, without loss or change of activity, into a polymeric host. In the case of proteins, the major challenges in developing protein-encapsulated systems are [7,8]: (i) instability of encapsulated proteins; (ii) their incomplete release, and (iii) initial burst release. One of the major advantages of a sustained delivery approach is the possible reduction in the required cumulative dose, which is an advantage in order to avoid supraphysiological levels of proteins and to reduce the cost of the therapy [9]. Tissue morphogenesis and regeneration are typically driven by concomitant action of multiple factors, which can work

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synergistically on the same process, or can target different barriers to regeneration [10]. Temporal and spatial control of the release of such factors is crucial to a successful tissue repair and regeneration [11].

In this work, we propose a novel hybrid scaffold composed of a poly(D,L-lactic acid) (PDLLA) porous scaffold incorporating chitosan/chondroitin sulfate (CH/CS) nanoparticles (NPs) for ultimate application on the TE field.

PDLLA is a well known biodegradable material because of its excellent mechanical strength [12–14] and it has been commonly used for fabricating biocompatible and porous scaffolds [13,15,16]. However, PDLLA is a hydrophobic material that retards the diffusion of water molecules into its matrix, and so it takes a long time to degrade [12]. Common scaffold fabrication techniques include solvent casting/salt leaching [17], moulding/salt leaching [18], and gas foaming/salt leaching [19]. These conventional methods require the use of organic solvents and/or high processing temperatures, which can prohibit their use in the preparation of GF-loaded scaffolds. Additionally, some of the mentioned techniques might lead to the loss of fractions of the entrapped GF and also be harmful for cells and nearby tissues [20]. In order to overcome these limitations, carbon dioxide (CO_2) has been used as a plasticizer and foaming agent to form three-dimensional (3D) scaffolds [15]. Remarkable progress has been made in the application of dense gases to the processing of polymers over the last decade. Gas foaming technology [21], crosslinking reactions at high pressure CO_2 [22] and supercritical CO_2-water emulsion techniques [23] have been used to fabricate porous hydrogels or scaffolds from different kinds of polymers [24]. CO_2 is inexpensive, non-toxic and non-flammable and readily available in high purity from a variety of sources. Supercritical carbon dioxide ($scCO_2$) combines gas-like diffusivity with the liquid-like density, which makes it a unique medium for polymer synthesis and processing [15,22,24].

However, despite the obvious advantages, this technique presents some clear limitations. The control over internal scaffold architecture cannot approach that of, for instance, the 3D printing technique. Also the range of polymer types for which supercritical fluids (SCFs) are applicable might be limiting in some applications, particularly where high mechanical strength is required [11]. The ability to plasticize polymers at close to 37 °C and the inherently low solubility of proteins in $scCO_2$ provides a key processing advantage; thermal and solvent labile species can be processed easily whilst preserving protein structure and function [11,25].

Richardson et al. [26] also applied the gas foaming/salt leaching approach for the simultaneous encapsulation of two angiogenic GFs – vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) – in poly(lactide-co-glycolide) (PLG) foams for dual release, with tailored release profiles. In this case, PDGF was pre-encapsulated in PLG microspheres while VEGF was dispersed in an alginate powder, generating the different kinetics. The dual delivery of these two GFs promoted the formation of a vascular network *in vivo* to a greater extent than either of the two GF delivered alone [26]. The drawback of this strategy is the use of salt leaching step, which removes some of the incorporated bioactive material [11].

Several reports [1,27–36] have shown that SCF technology seems appropriate for the development of appropriate architectures for TE applications, with the inclusion of the biological cues for further stimulation of cell behaviour and enhancement of tissue regeneration. Some other studies [2–4,10,37–40] have already addressed the application of dual release systems for TE purposes. Encouraging resul1ts have been obtained in the enhancement of bone and cartilage regeneration, as well as improved vascularisation.

To our knowledge, no works have been published on the entrapment of NPs into polymeric sponges by gas foaming procedure. Chen et al. [41] reported the microencapsulation of pueranin NPs by PLA. In our study, CH/CS NPs previously developed in our group [42], which have shown to be able to release proteins in a controlled manner, were entrapped in the PDLLA foam. Bovine serum albumin (BSA) was used as model protein to assess the entrapment and *in vitro* release kinetics, in order to study the potential application of this PDLLA-NPs hybrid structure for dual protein release with distinct kinetics. The protein dispersed in the PDLLA polymer should be released at a faster pace, while the protein entrapped in the NPs should be delivered in slower fashion.

2. Experimental procedure

2.1. Materials

Low molecular weight (116 kDa) chitosan (CH) (448869), chondroitin sulfate (CS) (C9819), bovine serum albumin (BSA/A2153, pI 5.3), phosphate buffered saline tablets (PBS), Dulbecco's modified Eagle's medium low glucose, Dulbecco's modified Eagle's medium without phenol red and sodium bicarbonate were purchased from Sigma Aldrich (Germany). Fetal bovine serum (FBS) was purchased from Biochrom AG (Germany) and antibiotic–antimycotic (A/B) solution from Gibco (Spain). (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) MTS reagent was purchased from Promega (UK). Chitosan was further purified by the precipitation procedure [43], all the other chemicals were used with no further purification. After CH purification, the final deacetylation degree was 75–85%. PDLLA with an inherent viscosity of 1.87 dL/g was purchased from Purasorb. Carbon dioxide (99.998 mol%) was supplied by Air Liquide. MicroBCA protein detection kit was purchased from Pierce.

2.2. Preparation of the nanoparticles

Chitosan/chondroitin sulfate nanoparticles (CH/CS NPs) were prepared as described previously by Santo et al. [42]. Briefly, CH was dissolved in 1% (w/v) acetic acid while CS was dissolved in distilled water at room temperature, to obtain solutions of 6 mg/mL and 0.81–0.94 mg/mL, respectively, in order to reach final theoretical CH/CS ratio of 1/1 (w/w). The NPs suspension spontaneously occurred when the CH solution was added to the CS solution, under strong magnetic stirring at room temperature. To prepare the protein-loaded CH/CS NPs, BSA (pI 5.3) was dissolved in water and further mixed in the CS solution afterwards. Following a 10 min centrifugation at 11,500 × *g*, the supernatants were discarded and the NPs were isolated. The protein concentrations used for this study were such that allowed the preparation of NPs with 15% (w/w) of protein respective to the NP content.

2.3. Supercritical fluid foaming

The scaffolds were prepared by SCF foaming at 200 bar and 35 °C in a high pressure equipment especially designed for this purpose. In each experiment ca. 100 mg of PDLLA was loaded in a mould, which was placed inside the high pressure vessel. The vessel was heated in by means of an electric thin band heater (OGDEN, USA) connected to a temperature controller, which maintained the temperature within ±1 °C. Carbon dioxide was pumped into the vessel using a high pressure piston pump (P-200A Thar Technologies) until the operational pressure was attained. The pressure inside the vessel was measured with a pressure transducer. The system was closed for 30 min to allow the plasticization of the polymer. Afterwards the system was slowly depressurized (ca. 5 bar/min).

When BSA and/or the NPs were loaded in the 3D construct, they were previously suspended in ethanol and afterwards added to the