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Natural origin scaffolds with *in situ* pore forming capability for bone tissue engineering applications

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Abstract

This work describes the development of a biodegradable matrix, based on chitosan and starch, with the ability to form a porous structure *in situ* due to the attack by specific enzymes present in the human body (α-amylase and lysozyme). Scaffolds with three different compositions were developed: chitosan (C100) and chitosan/starch (CS80-20, CS60-40). Compressive test results showed that these materials exhibit very promising mechanical properties, namely a high modulus in both the dry and wet states. The compressive modulus in the dry state for C100 was 580 ± 33 MPa, CS80-20 (402 ± 62 MPa) and CS60-40 (337 ± 78 MPa). Degradation studies were performed using α-amylase and/or lysozyme at concentrations similar to those found in human serum, at 37 °C for up to 90 days. Scanning electron micrographs showed that enzymatic degradation caused a porous structure to be formed, indicating the potential of this methodology to obtain *in situ* forming scaffolds. In order to evaluate the biocompatibility of the scaffolds, extracts and direct contact tests were performed. Results with the MTT test showed that the extracts of the materials were clearly non-toxic to L929 fibroblast cells. Analysis of cell adhesion and morphology of seeded osteoblastic-like cells in direct contact tests showed that at day 7 the number of cells on CS80-20 and CS60-40 was noticeably higher than that on C100, which suggests that starch containing materials may promote cell adhesion and proliferation. This combination of properties seems to be a very promising approach to obtain scaffolds with gradual *in vivo* pore forming capability for bone tissue engineering applications.

Keywords: Natural origin scaffolds; Chitosan; Starch; Enzymes; *In situ* pore formation

1. Introduction

Tissue engineering has recently emerged as a new interdisciplinary science to repair injured body parts and restore their functions by using laboratory-grown tissues, materials and artificial implants. An ideal scaffold to be used for bone tissue engineering should possess characteristics of excellent biocompatibility, adequate pore size, controllable biodegradability and suitable mechanical properties [1–3]. The choice of the appropriate fabrication technique is critical because it can significantly influence the properties of the implant and its degradation characteristics. There is, therefore, an increasing need to look for new materials and methodologies to produce scaffolds for bone tissue engineering. One interesting possibility is to develop an *in vivo* responsive scaffold the properties of which may be regulated by the bone regeneration process, with gradual formation of pores *in situ* and consequent resorption. This hypothesis seems to be very promising due to the control of degradation *in situ* and the consequent pore formation,

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which allows the scaffold to have the required mechanical properties during the initial stage of implantation.

One of the present trends in implantable applications is for materials that are derived from nature. Natural origin materials have been demonstrated to promote healing at a faster rate and are expected to exhibit greater compatibility with human tissues. The combination of chitosan with other materials appears to be a common theme in various reports [4,5]. Chitosan is a linear copolymer of *N*-acetyl-D-glucosamine and D-glucosamine, and is a deacetylated derivative of chitin. The degree of deacetylation (DD) represents the proportion of D-glucosamine units with respect to the total number of units. DD is a structural parameter which influences physicochemical properties [6] such as solubility, crystallinity, swelling behaviour and biological properties [6], namely biodegradation by lysozyme [7,8], wound healing properties [9] and the enhancement of osteogenesis [10]. One interesting feature of chitosan is its cationic nature, resulting from primary amine groups, which allows it to form water–insoluble ionic complexes with a variety of polyanionic substances. It is normally insoluble in aqueous solution above pH 7. However, in weak acids (pH < 6), the free amino groups are protonated and the polymer becomes soluble. Chitosan has been used to induce extracellular matrix formation in tissue regenerative therapy [11]. The degradation of chitosan in the human body has been reported to be carried out by lysozyme [7,8]. The degradation kinetics appears to be inversely related to the degree of deacetylation [8,12]. Lysozyme, or muramidase, is an enzyme that catalyzes the hydrolysis of the peptidoglycan layer of bacterial cell walls. Human lysozyme is found in various body fluids in concentrations from 7 to 13 mg l^{-1} [13–15] in serum and from 450 to 1230 mg l^{-1} in tears [13,14], saliva [13,14] and other fluids, including those surrounding cartilage [16]. Following implantation of a biomaterial, neutrophils and monocyte-derived macrophages will be present around the foreign material in both the acute and chronic phases of inflammation. A number of enzymes, such as lysozyme, and reactive species will be released from these cells.

Biodegradable starch-based polymeric biomaterials have been studied and proposed for a wide range of biomedical applications. Starch is one of the most abundant naturally occurring polymers, presenting a combination of properties that is steadily increasing its use in several technologies. Starch is a natural polymer that presents excellent characteristics for applications in the biomaterials field, primarily low toxicity [17,18], biodegradability [19] and biocompability [20,21]. It is inexpensive and, above all, reusable. The main enzymes involved in starch degradation are α- and β-amylase, glucosidase and other debranching enzymes. Starch is hydrolyzed to glucose, maltose and dextrin. It is well known that salivary amylase is involved in the gastric and intestinal digestion of starch in food components. Amylase can also be found in human serum.

The aim of this work was to develop a biodegradable matrix, based in chitosan and native starch, that will form a porous structure *in vivo* by the preferential attack of the matrix by specific enzymes present in the human body (namely the α-amylase and lysozyme). The inclusion of an enzymatically degradable phase in biomaterials may constitute an interesting approach to obtain scaffolds with adequate mechanical properties and with a gradual *in situ* pore forming ability. Using this innovative methodology, the developed scaffolds can exhibit very promising mechanical properties, due to the absence of macroporosity during the initial stage of implantation. The porosity is developed *in situ* by enzymes present in human body.

In this work, chitosan/starch scaffolds were developed using a precipitation method. These systems were analyzed in terms of morphology, degradation behaviour and mechanical properties. This study also addressed the effect of leachables from developed scaffolds on the viability of mouse fibroblasts and the influence of the construct's surface on the morphology, adhesion and spreading of fibroblast and human osteoblasts.

2. Materials and methods

2.1. Materials

Chitosan with medium molecular weight and DD of 92% (determined by the titration method, as described in Ref. [22]) and native corn starch were purchased from Sigma (St. Louis, USA). Sodium hydroxide (NaOH) and sodium sulphate (Na_2SO_4) were supplied from Panreac (Barcelona, Spain). α-Amylase from *Bacillus amyloliquefaciens* was obtained by Genencor International, Inc. (Rochester, NY, USA) and egg white lysozyme was from Sigma (St. Louis, USA).

2.2. Scaffolds preparation

Finely ground chitosan powder was dissolved in acetic acid 1% (v/v) to obtain a 5% (w/v) clear solution (C100) without any particulate material. Because of the relatively high concentration, these solutions are quite viscous and consequently can be stirred only slightly. However, flow is still observed and it is still possible to inject such viscous solutions to fill out the moulds. Then, using the same procedure, other formulations were prepared with the following ratios: 80/20 chitosan/starch scaffolds (CS80-20) and 60/40 chitosan/starch scaffolds (CS60-40). The solutions were casted into moulds and frozen (−18 °C) overnight. To produce chitosan and chitosan/starch scaffolds, the solutions were immersed in a precipitation solution with containing 0.25 M NaOH and 0.375 M of Na_2SO_4 adapted from Tuzlakoglu et al. [23]. After precipitation, the samples were washed repeatedly with distilled water to remove excess of salts, dried at 37 °C and followed by successive washings for 5 days until no pH changes were detected.

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