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ScienceDirect
Acta Biomaterialia 5 (2009) 3328–3336
Acta BIOMATERIALIA
www.elsevier.com/locate/actabiomat

Chitosan scaffolds incorporating lysozyme into CaP coatings produced by a biomimetic route: A novel concept for tissue engineering combining a self-regulated degradation system with in situ pore formation

Ana M. Martins, Rui C. Pereira, Isabel B. Leonor, Helena S. Azevedo, Rui L. Reis *

3B's Research Group—Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, 4806-909 Taipas, Guimarães, Portugal
IBB—Institute for Biotechnology and Bioengineering, PT Government Associated Laboratory, Guimarães, Portugal

Received 27 October 2008; received in revised form 25 March 2009; accepted 19 May 2009
Available online 27 May 2009

Abstract

This study describes an innovative self-regulated degrading material with gradual in situ pore formation ability for bone tissue engineering applications. This approach is based on the incorporation of the lysozyme enzyme into calcium phosphate (CaP) coatings, prepared on the surface of chitosan scaffolds by means of a biomimetic coating technique with the aim of controlling their degradation rate and subsequent formation of pores. However, because lysozyme has antibacterial properties, these coatings may act as a carrier for its sustained release, preventing infection upon implantation. In order to prove the concept of in situ pore formation, the coated scaffolds (with and without lysozyme) were incubated in two different solutions at different pH to simulate normal physiological conditions (pH 7.4) and inflammatory response (pH 5). The weight loss and morphology of the scaffolds was monitored over time. At pH 7.4, the scaffolds remained more stable than at pH 5. The scaffolds incubated at pH 5 showed a rapid decrease in their initial weight, and scanning electron microscopy imaging revealed the formation of a highly porous structure. Furthermore, evaluation of the activity of the incorporated lysozyme revealed that the enzyme was able to hydrolyse the peptidoglycan of the bacteria cell walls (as detected by the decrease in optical density with time), indicating that the enzyme remained active after being incorporated into the CaP coating.

Keywords: Chitosan; Cap coatings; Lysozyme; Degradation; In situ pore formation

1. Introduction

A novel concept in tissue engineering which proposes in situ pore-forming scaffolds has been reported previously [1]. Chitosan has been considered a promising material for tissue engineering applications. It is a linear copolymer of *N*-acetyl-D-glucosamine and D-glucosamine, derived from chitin by a deacetylation reaction. The degree of deacetylation (DD) measures the percentage of glucosamine units in the polymer chain and influences its physicochemical properties such as solubility, crystallinity and swelling behavior, and its biological properties [2], namely osteogenesis enhancement [3]. The degradation kinetics appear to be inversely related to the DD [4]. It has been demonstrated that chitosan is degraded in vitro and in vivo by lysozyme [4,5], an enzyme ubiquitous in the human body [6,7]. Lysozyme can attack cell wall polysaccharides of different bacterial species, especially Gram-positive bacteria, leading to rupture of the cell wall and killing of the micro-organism

* Corresponding author. Address: 3B's Research Group—Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, 4806-909 Taipas, Guimarães, Portugal. Tel.: +351 253 510902; fax: +351 253 510909.
E-mail address: rgreis@dep.uminho.pt (Rui L. Reis).

1742-7061/$ - see front matter © 2009 Published by Elsevier Ltd. on behalf of Acta Materialia Inc.
doi:10.1016/j.actbio.2009.05.027

[8,9]. This study proposes the incorporation of the lysozyme enzyme into calcium phosphate (CaP) coatings, prepared on the surface of chitosan scaffolds by means of a biomimetic coating technique, with the aim of controlling their degradation rate and subsequent formation of pores. Furthermore, since lysozyme has antibacterial properties, these coatings may act as a carrier for its sustained release, preventing infection upon implantation. An improvement in the osteoconductivity of implants has been achieved by coating their surfaces with CaP layers [10–12]. The biomimetic methodology for coating biomaterials with a bone-like apatite layer has been described in several publications [13–17]. This technique mimics natural biomineralization processes, involving controlled crystal nucleation and growth control over the phase of the mineral deposited [18]. The main advantage of the biomimetic methodology is the use of physiological conditions (pH 7.4 at 37 °C) simulating the manner in which apatite is formed in bone. Moreover, this technique allows the incorporation of proteins and bioactive agents into CaP coatings without compromising their activity [19–22]. An ideal material for the purpose of a temporary support for bone replacement should feature an adequate range of mechanical properties with convenient degradation kinetics, bone-bonding behavior and biocompatible performance [23].

In order to enhance the formation of pores in situ, chitosan scaffolds coated with CaP layers and lysozyme incorporated into the coatings were developed, presenting properties that promote degradation and osteoconductive potential. Lysozyme enzyme was incorporated into CaP coatings on the surface of chitosan scaffolds by means of a biomimetic route [13,14,22]. Moreover, CaP coatings enhance the osteoconductive properties of the chitosan scaffolds.

This study was designed to answer the following questions: Can a hydrolytic enzyme be incorporate at the surface of chitosan scaffolds without compromising its activity? Is it possible to develop a non-porous scaffold with in situ pore-forming ability? How many days does it takes to have a porous scaffold in situ fully interconnected simulating in vivo conditions?

2. Materials and methods

2.1. Materials

Chitosan with a DD of 92% and medium molecular weight was obtained from Sigma (St. Louis, USA). Bioglass 45S5 with composition 45SiO_2, 24.5CaO, 24.5Na_2O and 6.0P_2O_5 (in wt.%) was supplied by NovaMin Technology Inc. (Alachua, FL).

2.2. Preparation of chitosan scaffolds

Chitosan scaffolds were prepared as previously described by Martins et al. [1]. Briefly, chitosan was dissolved in acetic acid 1% (v/v) to obtain a 5% (w/v) solution. Then, the solution was cast into polyethylene moulds and frozen (−18 °C) overnight. After that, they were immersed in a precipitation solution (25% NaOH 1 M and 75% Na_2SO_4 0.5 M) overnight, adapted from the method developed by Tuzlakoglu et al. [24]. After precipitation, the samples were washed several times with distilled water and dried at 37 °C.

2.3. Preparation of CaP biomimetic coatings

The method of preparing the CaP coatings was based on the methodology previously developed by Abe et al. [13] and adapted by Reis et al. [14,15], consisting in impregnation of the materials with bioactive glass called Bioglass 45S5, followed by immersion in a simulated body fluid (SBF, 37 °C, pH 7.4), presenting ionic concentrations similar to human blood plasma. Prior to the coating process, chitosan scaffolds were sterilized by ethylene oxide. Bioglass 45S5 was sterilized by immersion in ethanol solution (70% v/v) and then dried inside the hood. All the subsequent work was performed under sterile conditions in a hood. Briefly, chitosan scaffolds were rolled in a wet bed of Bioglass 45S5. After that, the scaffolds were immersed in a 1.0 SBF solution, without (control) and with lysozyme (1 g L^{-1}) from chicken egg white (Fluka, USA), for 7 days at 37 °C, a period known as the nucleation stage, which allows CaP nuclei formation. After the nucleation stage, all samples were washed with distilled water and immersed in a concentrated 1.5 SBF solution for 3, 7 and 14 days at 37 °C, in order to enhance CaP nuclei growth. All the solutions were sterilized by filtration prior to use. After each time period, chitosan scaffolds were washed with distilled water and dried until surface characterization was performed. The morphology of the CaP coatings obtained was analysed by scanning electron microscopy (SEM; Leica Cambridge S-360, UK). Prior to microstructure analysis, specimens were coated with gold using a Fisons Instruments Coater (Polaron SC 502, UK). In order to characterize the crystalline/amorphous nature of the films, thin-film X-ray diffraction (TF-XRD; Philips X'Pert MPD, The Netherlands) was used. Data collection was performed by the 2θ scan method with 1° as the incident beam angle, using a Cu Kα X-ray line and a scan speed of 0.05° min^{-1} in 2θ. In addition, infrared analysis (FTIR) of the coatings was performed in a Perkin-Elmer spectrometer (Perkin-Elmer 1600 series equipment, USA) to examine the chemical structure of the CaP biomimetic coatings obtained. Coatings were scraped from the chitosan scaffolds, mixed with KBr (Riedel-de Haën, Germany) and then formed into a disc in a press. All spectra were obtained between 4400 and 450 cm^{-1} at a resolution of 2 cm^{-1}.

2.4. Degradation studies

In order to simulate in vivo conditions, degradation studies were carried out by incubating uncoated chitosan scaffolds and CaP-coated chitosan scaffolds, with and without incorporated lysozyme, in two different buffer solu-

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