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Bilayered chitosan-based scaffolds for osteochondral tissue engineering: Influence of hydroxyapatite on *in vitro* cytotoxicity and dynamic bioactivity studies in a specific double-chamber bioreactor

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Abstract

Osteochondral tissue engineering presents a current research challenge due to the necessity of combining both bone and cartilage tissue engineering principles. In the present study, bilayered chitosan-based scaffolds are developed based on the optimization of both polymeric and composite scaffolds. A particle aggregation methodology is proposed in order to achieve an improved integrative bone–cartilage interface needed for this application, since any discontinuity is likely to cause long-term device failure. Cytotoxicity was evaluated by the MTS assay with the L929 fibroblast cell line for different conditions. Surprisingly, in composite scaffolds using unsintered hydroxyapatite, cytotoxicity was observed *in vitro*. This work reports the investigation that was conducted to overcome and explain this behaviour. It is suggest that the uptake of divalent cations may induce the cytotoxic behaviour. Sintered hydroxyapatite was consequently used and showed no cytotoxicity when compared to the controls. Microcomputed tomography (micro-CT) was carried out to accurately quantify porosity, interconnectivity, ceramic content, particle and pore sizes. The results showed that the developed scaffolds are highly interconnected and present the ideal pore size range to be morphometrically suitable for the proposed applications. Dynamical mechanical analysis (DMA) demonstrated that the scaffolds are mechanically stable in the wet state even under dynamic compression. The obtained elastic modulus was, respectively, 4.21 ± 1.04, 7.98 ± 1.77 and 6.26 ± 1.04 MPa at 1 Hz frequency for polymeric, composite and bilayered scaffolds. Bioactivity studies using both a simulated body fluid (SBF) and a simulated synovial fluid (SSF) were conducted in order to assure that the polymeric component for chondrogenic part would not mineralize, as confirmed by scanning electron microscopy (SEM), inductively coupled plasma-optical emission spectroscopy (ICP) and energy-dispersive spectroscopy (EDS) for different immersion periods. The assays were carried out also under dynamic conditions using, for this purpose, a specifically designed double-chamber bioreactor, aiming at a future osteochondral application. It was concluded that chitosan-based bilayered scaffolds produced by particle aggregation overcome any risk of delamination of both polymeric and composite parts designed, respectively, for chondrogenic and osteogenic components that are mechanically stable. Moreover, the proposed bilayered scaffolds could serve as alternative, biocompatible and safe biodegradable scaffolds for osteochondral tissue engineering applications.

Keywords: Osteochondral; Tissue engineering; Scaffolds; Particle aggregation; Chitosan

1. Introduction

Osteochondral defects are lesions of the articular cartilage where the underlying bone tissue is also damaged. Currently, osteochondral defects are mostly treated by (i) osteochondral autograft transfer, taken from an outer

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region of the joint [1]; (ii) filling the lesion with autologous, precultured chondrocytes (autologous chondrocyte transplantation, ACT) [2]; or (iii) matrix-induced autologous chondrocyte implantation [3]. Although some studies have achieved success in repairing small cartilage defects, no accepted method for complete repair of osteochondral defects exists [4]. Large osteochondral defects are associated with mechanical instability and are accepted indications for surgical intervention to prevent development of degenerative joint disease. Ideally, a large osteochondral defect should be repaired with a graft that can provide mechanical stability and allow early postoperative function under physiological loading conditions [5]. In addition, the graft should integrate with the host tissue both structurally and functionally, since any discontinuity can incite long-term device failure [6].

The requirements for an osteochondral graft could potentially be met by using a tissue-engineered osteochondral (bone–cartilage) composite of predefined size and shape, generated *in vitro* using autologous cells. Such a graft would provide mechanical stability from the time of implantation, minimize donor site morbidity by using cell expansion techniques, and eliminate complications related to the use of allografts and/or mechanical devices. The bone region of the engineered osteochondral composite may further help anchor the graft within the defect, since a bone-to-bone interface integrates better and faster than a cartilage-to-cartilage interface [6].

The overall objective of tissue engineering is the restoration of normal tissue function. Ideally, lost or damaged tissue should be replaced by an engineered graft that can re-establish the appropriate structure, composition, cell signalling and function(s) of the native tissue [7]. The clinical utility of tissue engineering likely depends on our ability to replicate the site-specific properties of the tissue being replaced across different size scales and provide the continuity and strength of the interface with the neighbouring host tissues [8]. Therefore, for an osteochondral defect, one should consider the need for simultaneous regeneration of both cartilage and subchondral bone, making osteochondral tissue engineering a challenge to present-day research since it necessitates the combining of both bone and cartilage tissue engineering principles.

Several possible strategies for developing hybrid constructs for osteochondral tissue engineering have been published [5,9–11], namely: (i) cell culturing is performed independently on two sides, which are integrated before implantation; (ii) two different cell sources are seeded in the two sides of a single- or double-phase scaffold, and cultivated in a special bioreactor with two separated chambers; and ideally (iii) common progenitor cells are seeded in the two sides of a biphasic scaffold that contains different differentiation agents and then cultivated in a bioreactor with one or two chambers. For these, several bioreactors have been described, as reviewed [8,12] and proposed [13] by different authors. Bioreactor systems can provide the technological means to reveal fundamental mechanisms of cell function in a three-dimensional (3-D) environment and the potential to improve the quality of engineered tissues, providing environmental control, biochemical and mechanical cues. Several bioreactor systems, such as rotating bioreactors and perfused cartridges or chambers, have been used. Nevertheless, we believe that double-chamber bioreactors [12,13] consisting of one chamber for the culture of the chondrogenic part and another chamber for the osteogenic part, seems to be the most suitable for osteochondral applications. The double-chamber bioreactor should allow for the culture of chondrocytes, osteoblasts or common progenitor cells in two different cell culture media.

Many tissue engineering scaffolding strategies dealing with osteochondral repair engage the design of bilayered scaffolds that could regenerate both cartilage and subchondral bone involving different combinations of materials, morphologies and properties in both parts of the scaffold. Nevertheless, some researchers suggest that osteochondral defects could be regenerated from single-layer scaffolds by seeding autologous chondrocytes at the top of the 3-D scaffold to create a cell-scaffold construct for *in vivo* implantation [14,15] or that complex tissue grafts could be engineering with gradients of molecular, structural and functional properties. Several strategies with single-layer materials can be followed, as recently reviewed by Mano and Reis [9]. Nevertheless, it is more widely accepted that a bilayered structure would be more challenging to produce but more suitable for regenerating an osteochondral defect able to incorporate/induce different types of cells in a favourable environment requiring different chemical surroundings and mechanical requirements, leading to the growth of two different tissues, with different biological requirements.

Bearing this in mind, different approaches have been reported. Swieszkowski et al. [4] proposed two different systems using bone marrow-derived mesenchymal cells cultured in chondrogenically and osteogenically favourable conditions. The biphasic scaffolds consist of fibrin and polycaprolactone (PCL) and PCL and PCL/β-tricalcium phosphate (TCP), where the PCL-based scaffolds are fabricated via fused deposition modelling (rapid prototyping system). Both phases are fabricated and seeded separately, then integrated into a single construct by using fibrin glue. Their results demonstrate the potential of the porous PCL and PCL-TCP scaffolds in promoting bone healing. Scotti et al. [16] reported a biphasic composite made of an expanded chondrocytes–fibrin glue gel composite with a calcium phosphate scaffold. This paper focused mainly on the problem of the neocartilage-to-bone integration, so no cells were used in the osteogenic part of the biphasic scaffold. Hence, the bilayered construct showed a triphasic structure, with a cartilaginous zone, an intermediate zone with the chondrocytes–fibrin glue complex penetrating the calcium phosphate and a zone free from cells, made of the remaining part of the calcium phosphate scaffold [16].

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