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Bimolecular based heparin and self-assembling hydrogel for tissue engineering applications

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ABSTRACT

One major goal of tissue engineering is to develop new biomaterials that are similar structurally and functionally to the extracellular matrix (ECM) to mimic natural cell environments. Recently, different types of biomaterials have been developed for tissue engineering applications. Among them, self-assembling peptides are attractive candidates to create artificial cellular niches, because their nanoscale network and biomechanical properties are similar to those of the natural ECM. Here, we describe the development of a new biomaterial for tissue engineering composed by a simple combination of the self-assembling peptide RAD16-I and heparin sodium salt. As a consequence of the presence of heparin moieties the material acquired enhances the capacity of specific binding and release of growth factors (GFs) with heparin binding affinity such as $VEGF_{165}$. Promising results were obtained in the vascular tissue engineering area, where the new composite material supported the development of tubular-like structures within a three dimensional (3D) culture model. Moreover, the new scaffold enhances the cell survival and chondrogenic commitment of adipose-derived stem cells (ADSC). Interestingly, the expression of specific markers of mature cartilage tissue including collagen type II was confirmed by western blot and real-time PCR. Furthermore, positive staining for proteoglycans (PGs) indicated the synthesis of cartilage tissue ECM components. Finally, the constructs did not mineralize and exhibited mechanical properties of a tissue undergoing chondrogenesis. Altogether, these results suggest that the new composite is a promising "easy to prepare" material for different reparative and regenerative applications.

1. Introduction

Nowadays, self-assembling peptides have been widely used as scaffolds for tissue engineering applications due to their similarity to the natural extracellular matrix (ECM) in terms of mechanical properties and nanoscale network. These peptides self-assemble under physiological conditions into a network of interweaving nanofibers of around 10-nm diameter, forming a hydrogel scaffold with pores sizes of 50–200 nm and over 99% water content [1]. Moreover, mechanical properties can be modulated by changing the peptide sequence and concentration [2,3] and can be defined as "non-instructive" from the point of view of cell receptor recognition/activation [1]. Several *in vitro* studies have shown their ability to support cell attachment, growth, maintenance and differentiation of a variety of mammalian cells [4–11]. Other characteristics of self-assembling peptides are the ease of synthesis, injectability, biocompatibility, biodegradability and options for the incorporation of bioactive motifs or molecules [4,12]. In addition, drugs and GFs can be covalently or non-covalently coupled to the self-assembling peptides [13], depending on the molecular decoration of the scaffold with specific binding sites. As an example of this strategy, self-assembling peptides have been designed containing a heparin binding domain (HBD) to obtain a strong binding affinity for heparin, which can also bind a wide variety of heparin-binding GFs including VEGF165 or FGFβ [13–16]. Thus, heparin is a class of glycosaminoglycan with growth factor binding affinity sequestering GFs and localizing of their activity. As a consequence, heparin protects them from degradation and in some cases enhances their binding to cell surface receptors [17]. Similar strategies have been used with other types of materials where

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heparin has been covalently linked to polymers such as alginate and collagen or entrapped within chitosan [16,18,19].

In the present work, we report the development of a new injectable nanofiber scaffold with growth factor binding affinity. The material is formed by the simple combination of the commercially available self-assembling peptide RAD16-I (Puramatrix™), which confers the three-dimensional (3D) environment and heparin moieties, which allow the binding of GFs. RAD16-I peptide is soluble in water and self-assembles into nanofibers hydrogels by changing the ionic strength and/or the pH of the solution forming a soft hydrogel. The driving force of the self-assembling process is driven by weak non-covalent interactions including hydrogen bonds, ionic bonds, electrostatic interactions, van der Waals interactions, etc. This brings a unique opportunity to embed cells in a truly 3D matrix during the self-assembling process. Importantly, this nanofiber network promotes cell–cell and cell–matrix interactions allowing cells to freely grow, proliferate, migrate and differentiate under specific experimental conditions [1,20,21].

In order to evaluate the functionality and potentiality of this approach, the new biomaterial was evaluated in two different tissue engineering strategies: vascular tissue formation and chondrogenic differentiation. Thus, the purpose of the present study is to characterize the versatility of the new material rather than to describe the complexity of either vasculogenesis or chondrogenesis.

Currently, vascularization of the engineered tissues is one of the major challenges in tissue engineering. Regardless of the specific approach, this is an essential issue because any tissue engineered construct that involves living cells needs an adequate blood supply for cell survival in tissues of macroscopic dimensions [16,22,23]. Strategies that have been designed in order to solve this issue are mainly divided in two groups: (1) pre-vascularization of the engineered construct *in vitro* and (2) promotion of rapid vascularization after transplantation by the host's own system mainly focused on the delivery of angiogenic growth factors [22]. Independent of the strategy used, the generation of functional vessels has been reported using different cell sources including mature endothelial cells (ECs) [24–27], and a wide spectrum of stem cells such as bone marrow, adipose derived or mesenchymal stem cells [28]. In terms of scaffolds, several types of biomaterials have been developed; among them self-assembling peptides are gaining importance in vascular tissue engineering due to their application as drug delivery systems for angiogenic growth factors [13]. This motivated the potential use of the new material developed in this work in vascular tissue engineering applications.

Owing to adult articular cartilage having a limited capacity of regeneration; conventional methods for cartilage defects include cell-based therapies such as autologous chondrocyte implantation [29]. However, chondrocytes are available in very limited quantities and rapidly dedifferentiate in their *ex vivo* expansion [30]. Therefore, many efforts have been made looking for new cell sources. Mesenchymal Stem Cells (MSCs) are a population of multipotent cells able to differentiate into chondrogenic, osteogenic and adipogenic lineages [31]. They can be isolated from the bone marrow, muscle and other sources; particularly the adipose tissue provides an abundant supply of cells and non-invasive surgery. Many studies have demonstrated the potential of MSCs to differentiate into bone and cartilage [32]. For these reasons, MSCs from the adipose tissue were selected to test the chondrogenic potential of the new bicomponent hydrogel. In addition to cell sources, the microenvironment has a crucial role in the determination of cells to the chondrogenic lineage. Therefore, efforts have been focused on tissue engineering applications using biomimetic scaffolds to improve the conventional treatments for cartilage injury [33]. In particular, the advantages of the new biomaterial developed in this work include: the commercial availability of both components, which eliminates the need of synthesis as compared to previous studies [13–15]; and the availability of clinical grade components, which enables its future use in *in vivo* studies.

2. Materials and methods

2.1. Material characterization

2.1.1. Sample preparation for staining

RAD–Heparin composites were prepared combining 95 μL of RAD16-I 0.5% (w/v) and 5 μL of heparin sodium salt solution (H3149, Sigma) in a concentration range between 0.01% and 1% (w/v), control RAD16-I samples were prepared with a final concentration of 0.5% (w/v). 100 μL of each type of sample were loaded into a cell culture insert (PICM-1250, Millipore) previously placed into a 6-well culture plate, and 500 μL of PBS were added under the insert to star the self-assembling process. Samples were let for 30 min at room temperature to allow the gelation process. Once this time elapsed, 200 μL of PBS were added in the inner wall of the insert letting it slowly slide to the gel and 2.5 mL of PBS were added outside the insert.

2.1.2. Toluidine blue staining

Toluidine blue staining was performed to study the presence of highly negative charges provided by the heparin molecules. Samples were incubated with toluidine blue 0.05% (w/w) in water during 20 min and then washed several times with distilled water. Finally the samples were analyzed under a stereoscopic microscope (Nikon SMZ660).

2.1.3. Congo red staining

Congo red staining was performed to study the presence of the β-sheet structure characteristic of the self-assembling peptide RAD16-I. Thus, a wide range of blending ratios of mg RAD16-I/mg Heparin was analyzed by visual inspection, from very low to very high quantities of heparin. Samples were incubated with 0.1% (w/v) Congo red (75768, Sigma) in water for 5 min and washed several times with PBS. Finally the samples were analyzed for the presence of Congo red staining under a Stereoscopic microscope (Nikon SMZ660).

2.1.4. Scanning Electron Microscopy (SEM)

Briefly, samples were fixed in 5% (w/v) glutaraldehyde and dehydrated in successive ethanol washes. Once dehydrated, samples were dried using a CO_2 critical point dryer (Polaron, CPD Jumbo E-3100). This methodology has been reported to enable the observation of a three-dimensional nanofiber network comprising peptide amphiphile gels [16,34,35]. Then, dried samples were subsequently sputter-coated with gold (approximate thickness 15–20 nm). Finally, samples were examined under SEM (NovaNano SEM 230 model, FEI, The Netherlands) at an accelerating voltage of 5 kV.

2.1.5. Circular Dichroism (CD)

CD spectroscopy was performed on a JASCO-715 spectropolarimeter equipped with a Peltier system. RAD16-I samples were diluted from a peptide stock solution (1% (w/v), 5.38 mM) in deionized water to a final concentration of 100 μM. RAD–Heparin composites were prepared using different blending ratios and diluted in deionized water to a final concentration of 100 μM RAD16-I. CD data were acquired in a range of 195–250 nm at a band width of 1 nm, scan speed of 20 nm/s and path length 1 mm.

2.1.6. Growth factor release quantification using ELISA

RAD–Heparin composite gels were prepared combining 95 μL of RAD16-I 0.5% (w/v) and 5 μL of 0.01% (w/v) Heparin (H3149,

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