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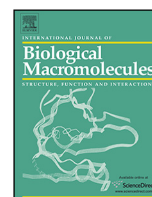

Biologically active and biomimetic dual gelatin scaffolds for tissue engineering

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ABSTRACT

We have designed, developed and optimized Genipin cross-linked 3D gelatin scaffolds that were biologically active and biomimetic, show a dual activity both for growth factor and cell delivery. Type B gelatin powder was dissolved in DI water. 100 mg of genipin was dissolved in 10 ml of DI water. Three genipin concentrations were prepared: 0.1%, 0.2% and 0.3% (w/v). Solutions were mixed at 40 °C and under stirring and then left crosslinking for 72 h. Scaffolds were obtained by punching 8 mm-cylinders into ethanol 70% solution for 10 min and then freeze-drying. Scaffolds were biologically, biomechanically and morphologically evaluated. Cell adhesion and morphology of D1-Mesenchymal stem cells (MSCs) and L-929 fibroblast was studied. Vascular endothelial grwoth factor (VEGF) and Sonic hedgehog (SHH) were used as model proteins. Swelling ratio increased and youngís module decreased along with the concentration of genipin. All scaffolds were biocompatible according to the toxicity test. MSC and L-929 cell adhesion improved in 0.2% of genipin, obtaining better results with MSCs. VEGF and SHH were released from the gels. This preliminary study suggest that the biologically active and dual gelatin scaffolds may be used for tissue engineering approaches like bone regeneration.

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1. Introduction

Since Hauschka and Koningsberg [1] showed the influence of interstitial collagen in myoblast behavior in 1966, the theory that extracellular matrix (ECM) serves only as a structural support of tissues began to change. After more than a decade, in 1977, Hay [2] published a paper that did glimpse of the presence of receptors on the cellular surface able to interact with specifics molecules of the ECM. The extracellular matrix is a complex structure composed by a mixture of proteoglycans, glycosaminoglycans, hyaluronan, elastin and glycoproteins where collagen is the most abundant [3] becoming more than one-third by weight protein of the body [4]. Nowadays, is totally acepted that process like cell adhesion, migration, growth, differenciation and apoptosis are regulated by interactions between cells and ECM. Furthermore, there are molecules such as cytoquines and growth factors that also influence cell fate.

There are many medical situations including burn or bone loss due to a trauma or a tumor rejection, in which tissue is critically damaged being unable to regenerate itself. Tissue engineering and regenerative medicine aim to develop biocompatible and biodegradable systems suitable to support therapeutic cells, deliver growth factors in a controlled manner and serve as temporary replacement of damaged ECM allowing the colonization of host cells. To address this, the biocompatibility and bioactivity of the selecting material is an initial critical issue [5,6]. Collagen has a complex hierarchical conformation within the ECM. Cell adhesive ligands such as Arginine-Glycine-Aspartic acid (RGD) aminoacid triplets are abundant in collagen and play vital roles for cellular attachment via integrin-mediated binding to ECM. Although there is an absorbable collagen sponge with rhBMP2 (INFUSE® Bone Graft) approved by United States Food and Drug Administration (FDA) for treating acute, open tibial fractures, collagen by itself, due to its animal origin may trigger an immune response with consequent rejection of the scaffold. Gelatin is a protein derived from the hydrolysis of collagen considered as a generally regarded as safe material by the FDA [7]. Besides having the characteristics of col-

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lagen, including biodegradability and cell adhesion capacity, it can be dissolved in water increasing its biocompatibility. Depending on the extraction and manufacturing method of collagen proteins, the resultant gelatin has different isoelectric points (pI). Type A gelatin derived from acid-cured tissue has a pI of 7.0–9.0 whereas Type B derived from lime-cured tissue has a pI of 4.7–5.2 becoming a good biomaterial to retain and deliver growth factors [8]. All of these characteristics make gelatin an attractive material to be used in tissue engineering. However, since gelatin is a biopolymer that is liquid at higher temperatures than 30°, polymerization is needed to use it as a scaffold.

Genipin (Gnp), which is extracted from *Gardenia jasmoides* fruits, is an interesting alternative to other more classic and toxic crosslinkers such as glutaraldehyde (GA) [9–11]. Implants cross-linked with Gnp show a trend towards reduced inflammation [12,13] and enhanced scaffolds stability [14]. Recently, it has been reported that Gnp exerts anti-inflammatory, anti-oxidative [15] and anti-cancer effects [16]. There are different pathways through Gnp polymerizes gelatin chain. One of the possible reactions happens when Gnp molecule is covalently attached through its ester group to any amino group present in gelatin. Another reaction occurs when a primary amino group reacts spontaneously with Gnp forming a nitrogen-iridoid [17,18] and in turn, two of those iridoid can dimerize by a radical reaction [19,20]. All of the reactions are inter or intramolecular (Fig. 1).

In the present study, we have designed, developed and optimized Gnp cross-linked three-dimensional (3D) gelatin scaffolds. We have carried out a morphological and biomechanical characterization of the scaffolds and evaluate their "dual" potential, that is, their cell adhesion capacity and growth factor release potential. For this last objective, we have studied the release of two relevant angionenic growth factors: vascular endothelial growth factor (VEGF) and sonic hedgehog (SHH) from the Gnp-gelatin scaffolds.

2. Material and methods

2.1. Materials

Type B gelatin powder with a strength of ≈225 bloom number, Heparin sodium salt from porcine intestinal mucosa with ≥180 USP units/mg, Cell Counting Kit-8 (CCK-8) for quantitation of viable cell number in adhesion and cytotoxicity assays, triton X-100, bovine serum albumin and glycerol were purchased from Sigma-Aldrich, Spain. Trypsin, Hoechst 33258 and Alexa Fluor 488-Phalliodin were supplied by Life Technologies, Spain. Gnp was purchased from Wako Pure Chemical Industries, Ltd, Japan. For cytotoxicity and adhesion assays Mouse L-929 fibroblasts and mouse D1 ORL UVA MSCs, DMEM 30-2002 and EMEM 30-2003 culture mediums, horse serum and fetal bovine serum were purchased from ATCC, Spain. Formaldehyde 3.7–4.0% (w/v) buffered to pH = 7 and stabilized with methanol was supplied by Panreac, Spain. Collagenase P was purchased from ROCHE, Spain. Finally, rhVEGF was purchased from Agrenvec, Spain and rhShh was purchased from R&D Systems, Spain. VEGF ELISA kit and SHH ELISA kit were supplied by Peprotech and R&D Systems, respectively.

2.2. Preparation of hydrogels

Type B gelatin powder with strength of ≈225 bloom number was dissolved in DI water for 45 min at a temperature of 40 °C under constant agitation. At the same time, a Gnp solution was prepared by dissolving 100 mg of Gnp in 10 ml of DI water. Once the gelatin solution was homogeneous the required amounts of the solution of Gnp were added to reach final concentrations of 0.1% (GEN_01), 0.2% (GEN_02) and 0.3% (GEN_03) (w/v). Three solutions were mixed at 40 °C and under stirring until the color turned green (approximately 15 min). Later, 5 ml of each solution were put into 100 mm petri dishes and were left crosslinking during 72 h at room temperature. After crosslinking, the scaffolds were punched and cylinders of 8 mm in diameter were plunged into ethanol 70% solution during 10 min. Afterwards, scaffolds were washed twice in PBS and freeze-dried.

2.3. Swelling and mechanical properties

Swelling ratios were determined using a gravimetric method; Swelling ratio (S) = (Ws-Wo)/Wo where Ws is the weight of wet scaffold and Wo is the weight of dry scaffold.

After weigh dry scaffolds they were plunged into PBS during 24 h at real temperature under constant shaking (300 rpm). Thereafter, wet scaffolds were weighed again and swelling ratio was calculated. Immediately before weighing excess surface water was carefully removed with a filter paper.

Mechanical properties were evaluated and calculated following the protocol described by Acosta Santamaría [21]. The experiments implemented were uniaxial Unconfined and Confined static compression tests. Both tests were performed in immersion in PBS so first dry scaffolds were rehydrated with PBS during 24 h and then they were punched to a diameter of 6 mm. The elastic modulus and poissioní s ratio (ν), which describes the lateral expansion during axial compression, being defined as the ratio of lateral and axial strains [21], were determined with a precision of 0.0001N and 0.001 mm in force and displacement, respectively using an Instron Microtester 5548 machine. Six replicate per scaffold type were performed.

In case of permeability, which describes the movement of fluid through the scaffold and dependent on the pore size and the impedance, another uniaxial confined compression test in immersion in PBS was performed but in this case the bottom of the chamber was composed by a permeable surface. Thus the fluid was forced to go through it. The protocol described by Poveda-Reyes [22] was followed; $k = h/(Ha \cdot \tau \cdot \pi 2)$ where h is the thickness of the sample, Ha is the aggregated modulus and τ is the relaxion time.

2.4. Superficial and cross-section analysis

To study the surface, internal structure, and the arrangement and sizes pores of the scaffolds, scanning electron microscopy analysis was performed. Freeze dry scaffolds were adhered with carbon adhesive tape to SEM stub. Then, Gold metallization was made in a metallizing Emitech K550x and samples were visualized on a Hitachi S4800. At the same time, in order to see the real structure of scaffolds, another scaffolds were rehydrated with PBS for 24 h. Such as genipin is capable of emitting fluorescence at a particular wavelength, a fluorescence and phase contrast Axio1 microscope (Carl Zeiss, Germany) was use.

2.5. Cytotoxicity

The toxicity of hydrogels was evaluated by two methods following the "Biological evaluation of medical devices guideline (ISO 10993): cytotoxicity on extracts and cytotoxicity by direct contact". For two assays L-929 fibroblast were used culturing in EMEM 30-2003 growth medium supplemented with horse serum (10% v/v). To assess the cytotoxicity produced by extracts released from scaffolds, 5×10^3 cells per well were cultivated in a 96 wells plate for 24 h at 37 °C and 5% of CO2. At the same time 8 mm freeze dried hydrogels were plunged into complete culture medium during 24 h at 37 °C under continuous soft shaking with the aim of obtain the extractants. After this time, cell culture of fibroblasts was substituted by the hydrogel's extractants complete medium

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