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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 youngs 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.

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 collagen, 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

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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.

1.1. 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,

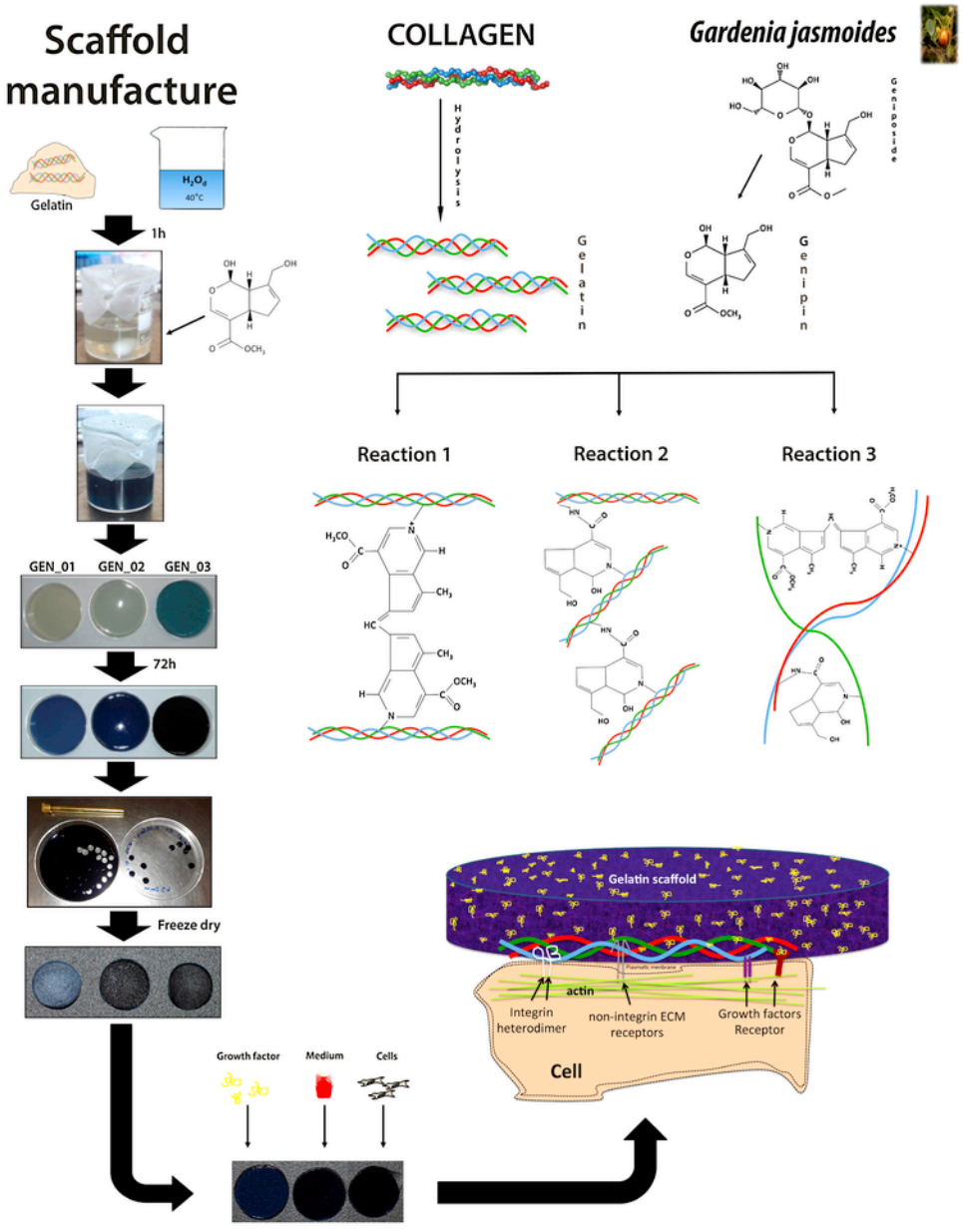

Fig. 1. On the left side of the figure scaffold manufacture process is shown. They can provide three possible reactions in the crosslinking process of scaffolds. The first one occurs when Genipin molecule is covalently attached through its ester group to any amino group present in gelatin (reaction 2). Another reaction occurs when a primary amino group reacts spontaneously with Genipin forming a nitrogen-iridoid and in turn, two of those iridoid can dimerize by a radical reaction (reaction 1). All of the reactions are inter or intramolecular (reaction 3).

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.

1.2. 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.

1.3. 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 poissións 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.

1.4. 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.

1.5. 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 leaving culturing during 24 h more. Then, the metabolic activity of seeded cells was measured making CCK-8 assay following kit guideline (10 μl of kit reagent in 90 μl of complete culture medium per well). The absorbance was measured with Tecan Infinite M2000. On the other hand, it was evaluated the cytotoxicity caused by cell-scaffold direct contact. 35×10^3 fibroblasts per well were cultivated in a 24 wells plate at 37 °C and 5% of CO2. At the same time, dry scaffolds were plunged into complete growth medium during the same time and same conditions. After 24 h, rehydrated scaffolds were put on top of cells (one scaffolds per well) allowing the direct contact between cells and scaffolds. After other 24 h, the scaffolds were removed and two wash with PBS were made to take off died cells. Then metabolic activity of seeded cells was measured making CCK-8 assay following kit guideline (35 μl of kit reagent in 315 μl of complete culture medium per well). The absorbance was measured with Tecan Infinite M2000.

To evaluate the results in both assays, it was considered a 100% cell viability the absorbance value obtained for the wells of cells that were not contacted gels or gels extractants medium (control sample).

1.6. Cell adhesion

Cell adhesion assay was made concurrently with L-929 fibroblasts and D1 mesenchymal stem cells in 24 wells plates. For that assay, in order to cover the entire of the well surface hydrogels with plate wells size were needed. Therefore, a larger diameter punches were used in gels manufacture process. At first hydrogels were rehydrated in complete culture medium inside the incubator for 24 h. Thereafter, hydrogels were put in plate, one per well, being careful for not to let bubbles under the gels. In case of GEN_01 scaffolds, after rehydrate and before put in the 24 wells plate, they had to be punched again to fit the size of the well. 20.000 cells were seeded over the scaffolds. After 4 h, the scaffolds were washed twice with PBS to remove no adherent cells. Then, they were degraded with collagenase P (0.5 mg/ml) and free cells were counted. At the same time, other scaffolds suffered the same process of hydration, seeding and washing but they were degraded after 12 h. 2D culture was used as a control in both cases, 4 and 12 h.

On the other hand, some of scaffolds were used to examine the morphology of adherent cells. After 4 and 12 h, they were washed twice to remove no adherent cells. Subsequently hydrogels with cells were fixes immersing in formaldehyde at 3.7% for 12 min. Following fixation, cell membranes were treated with triton-X100 0.1% (w/v)

and bovine serum albumin 1% (w/v) solution in order to modify its permeability. Two new washes with PBS was performed before add Alexa Fluor 488 Phalloindin to died actin microfilaments. After 40 min hydrogels were washed another twice. Hoestch 33258 was used to die DNA. Finally the samples were washed last twice washed with PBS and left immersed in DPBS at 4 °C overnight. Samples were then analyzed by confocal microscopy.

1.7. Growth factors release

Growth factor release assays were made with GEN_02 scaffolds. It is known that some proteins like VEGF and SHH have heparin binding sites in their sequences [23,24]. As already seen in the case of VEGF, the incorporation of this glycosaminoglycan into scaffold could capture growth factors retaining their release profile [25]. In order to modified and control the deliver of growth factors from gelatin scaffolds, a low amount of heparin was incorporated to some of hydrogels. The heparin was added to gelatin solution before crosslinking. Protein LoBind eppendorf tubes were used (one tube per hydrogel) to avoid as far as possible the adhesion of the growth factors to plastic. rhVEGF was reconstituted in PBS to a final concentration of 50 ng/μl and rhSHH was reconstituted in PBS to a final concentration of 100 ng/μl. 20 μl of PBS were put in the bottom of the tubes in order to create humidity inside each tube. After get dry hydrogels into the tube, 300 ng of growth factor were added over each dry hydrogel and they left incubating during 12 h. Then, two washes of 5 min with 1 ml of PBS were made under shaking to remove unbound protein. Thereafter, 1 ml of PBS was place into the tubes. Samples were taken at 12, 24, 48, 72, 96, 144, 192, 240, and 336 h replacing the milliliter of PBS for another one. The samples were frozen at −80 °C. After 14 days a PBS solution with collagenase P (0.5 mg/ml) was added to degrade the scaffolds and determine the amount of not released growth factor. During all experiment, tubes were under orbital shaking (40 rpm) at 37 °C. Growth factors were measured using commercially available Quantikine colorimetric sandwich ELISA kit.

2. Results and discussion

2.1. Morphology and superficial analysis

Following the manufacturing process, three different scaffolds were obtained. Gnp alone is colorless but when is crosslinked to an amino group forming the heterocyclic compound, appears as a blue pigment. This characteristic was noticed in the scaffolds manufacture where the intensity of blue color was increased along with Gnp concentration. Once the scaffolds were lyophilized, SEM microscopy revealed an apparent absence of pores (Fig. 3A). However, high water holding capacity and the high speed to grasp prove otherwise. Gnp is excited at 590 nm wavelengths and emits red fluorescence at 630 nm wavelengths. Rehydrating the scaffolds and analyzing through fluorescence microscopy it could verify the existence of a fluffy structure with pores ranging from 20 μm to over 100 μm (Fig. 3B).

2.2. Swelling and mechanical properties

After weighing lyophilized and rehydrated scaffolds it was shown that swelling ratio decreased with the concentration of Gnp, being GEN_01, which had the greatest ability to raise water with S of 9.07. GEN_02 and 03 had a significantly lower capacity than GEN_01 being 6.92 and 6.04 respectively. These values demonstrate the high capacity of the scaffolds to capture and retain water, ranging between 90 and 60% of the total weight. Our results corroborated the theory that more amount of Gnp in scaffolds implies less capacity of water retention [26].

Once the scaffolds were hydrated, mechanical tests were made. Unlike swelling ratio, the youngs module increased along with concentration of Gnp both in confined and non-confined tests. However, permeability decreased and Poisson distribution remained constant in three scaffolds (Fig. 2).

2.3. Cytotoxicity

The toxicity assays demonstrated the absence of cytotoxicity of all scaffolds and allowed both qualitative and quantitative assessment of cytotoxicity. According to the ISO 10993 to consider scaffold non-cytotoxic cell viability must be more than 70% compared to viability of the control sample. In the case of test on extracts, all scaffolds exceeded the 100% of cell viability being GEN_03 the one showing the best results (110%) (Fig. 4A). In the case of direct contact assay, the results showed lower cell viability, ranging from 69.7% of GEN_02 to 74% of GEN_01 and GEN_03 (Fig. 4B). These values are close to the limit stipulated by the ISO. However, after analyzing the scaffolds that had been in contact with the cells, it was found that most of fibroblasts were attached to the scaffold. As a consequence, we can resolve that cells were not dead but instead were adhered to the scaffold.

2.4. Cell adhesion

Following the rehydration of the scaffolds with complete medium, D1-MSCs and L-929 fibroblasts were seeded. No differences in cell adhesion were found at 4 h. However, at 12 h, cell adhesion improved only in GEN_02 (no differences with control) being significantly higher than cell viability observed in the other two scaffolds (Fig. 5B). In the case of L-929 fibroblasts, cells adhesion at 4 h was significantly lower than with MSCs. At 12 h, cells adhered better to the GEN_02 scaffold than to the GEN_01 and GEN_03 (though no significant differences were found among three types). In all the scaffolds the adhesion was significantly lower than in control (2D) (Fig. 6A). At the same time, some of the scaffolds that were cultured with the cells were fixed after 4 and 12 h for subsequent staining of actin filaments and cell cores. It was found that both fibroblasts and MSCs maintained a normal morphology (Fig. 5A). Other scaffolds were left growing for 72 h for subsequent fixing and cell staining. It was found that both cell phenotypes were able to completely grow over the scaffold, showing a normal conformation and cytoskeletal reorganization (Figs. 5C, 6B).

Scaffold	Young (kPa)	Poisson (ν)	Ha (kPa)	Permeability (m^4/N·s)	Swelling
GEN_01	51.69	0,48	404.16	2,11E-14	9.07
GEN_02	81.16	0,48	512.05	6,04E-15	6.92
GEN_03	90.29	0,479	780.12	4,32E-15	6.04

Fig. 2. Swelling and biomechanic properties of scaffolds. The experiments implemented were uniaxial Unconfined and Confined static compression tests and were made using an Instron Microtester 5548 machine.

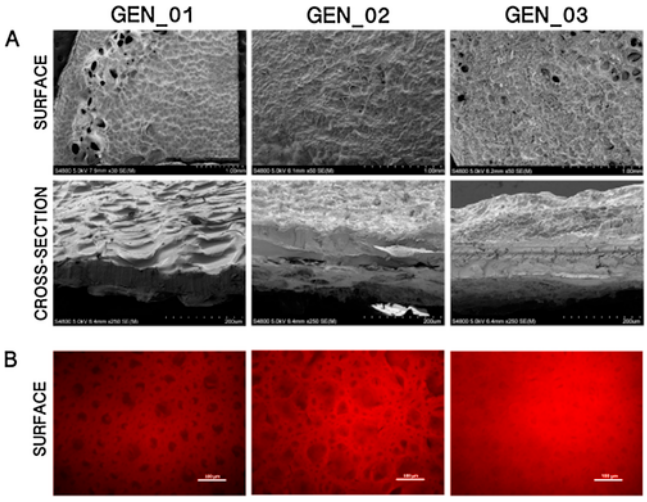

Fig. 3. Surface and cross-section SEM analysis of lyophilized scaffolds (A) and surface UV analysis of rehydrated scaffolds (B).

Cell adhesion and behavior is principally conditioned by two different processes that in turn are closely related. One of them is focal adhesion mediated through ECM molecules and membrane integrins. The second is the one mediated by mechanosensing. RGD sequences present in gelatin are one of the most frequently used focal adhesion sequences for material biofunctionalization. Some studies demonstrate that alginate modification with RGD, a biomaterial without any cues for cell adhesion, leads to a biopolymer suitable for MSCs and Fibroblast adhesion [27,28]. The differences in cell adhesion found in our study could be explained by the range of adhesion sequences and elastic modulus of the scaffolds. Mechanical properties of a biomaterial can markedly influence cell fate. It is well known that cell commitment and differentiation is compromised by the substrate stiffness modifying the expression of cell integrins [29], a process related to the mechanosensing process. The latter occurs when the motor proteins that are connected to the cell cytoskeleton interact actively with extracellular matrix [30]. Depending on the ECM these unions forces are different triggering the activation of intracellular signaling pathways and thus activate or inhibit gene expression [31].

Furthermore, it is known that cells respond in different ways depending on their phenotype and differentiation stage [32,33]. Our results corroborate the theory that not only the scaffold and its cues presentation are important to obtain a good cell adhesion but also the type of cells line used. Analyzing the results, it seems that GEN_02 scaffold is the best candidate to be used as vehicle and delivery system of MSCs to the damage location. Furthermore, unlike the case of MSCs, fibroblasts do not have great affinity for the GEN_02 scaffold. Our results also suggest that each cell phenotype may have a different behavior on our scaffolds. This could favor the invasion by mesenchymal stem cells of the host while reducing the attraction of fibroblasts becoming a good provisional matrix for cell migration.

2.5. Growth factors release

VEGF, a growth factor involved in angiogenic process and SHH, a morphogen principally involved in development of the embryo, were used for growth factors release studies from the GEN_02 scaffolds. We also fabricated GEN_02 scaffolds incorporating fractioned heparin to determine the influence of the heparin on protein delivery.

We first studied the encapsulation efficiency (EE), which is the amount of protein loaded on the scaffold after two washings assuming that the 100% was the total protein detected (TPD); EE = (TPD – unload protein)/TPD * 100. Regarding the release of VEGF, it was found that the scaffolds without heparin (GEN_02H0) showed higher encapsulation efficiency, than scaffolds with heparin (GEN_02H50), being of 91.59% and 62.13% respectively. Comparing the amount of encapsulated protein in both scaffolds, it was lower in GEN_02H50 (132.80 ng) than in GEN_02H0 (210.80 ng). In addition, it was found an initial burst release of over than half of total encapsulated growth factor (51.25% in the case of GEN_02H0 and 59.7% in GEN_02H50) during the first 24 h. In GEN_02H0 scaffolds, a constant release of 7,5 ng (±) of VEGF per day over the next 13 days was obtained. Thus, almost of VEGF loaded was released (94%). However, in the case of GEN_02H50, after the initial burst release, the delivery only reached less than 1 ng per day until the end of the study. In fact, after

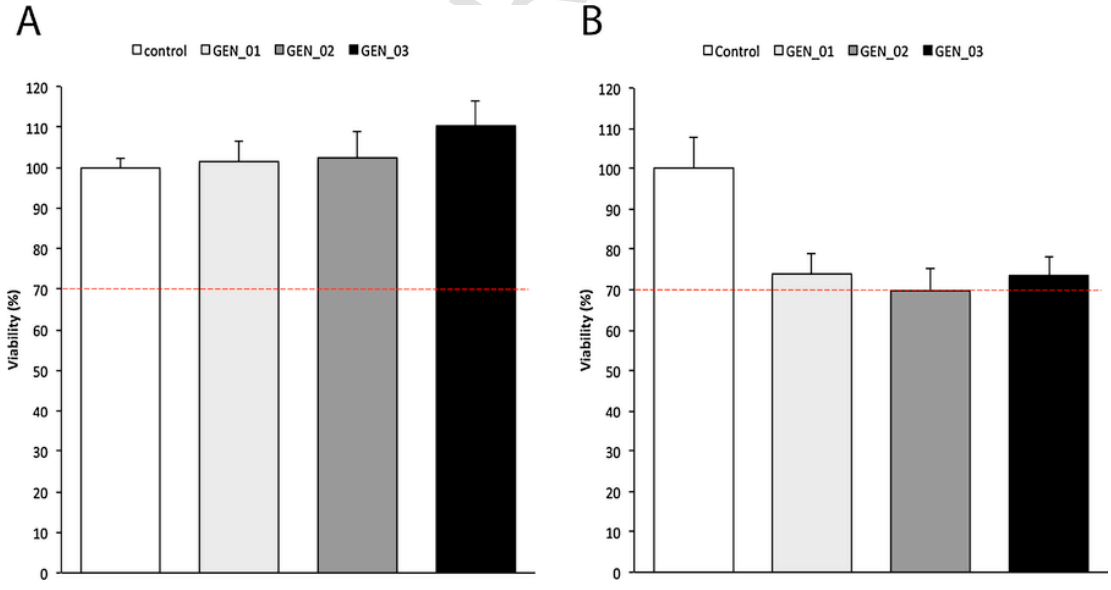

Fig. 4. Evaluation of cytotoxicity of scaffolds. Both cytotoxicity on extracts (A) and cytotoxicity by direct contact (B) were made following "Biological evaluation of medical devices guideline (ISO 10993)". For two assays L-929 fibroblast were used.

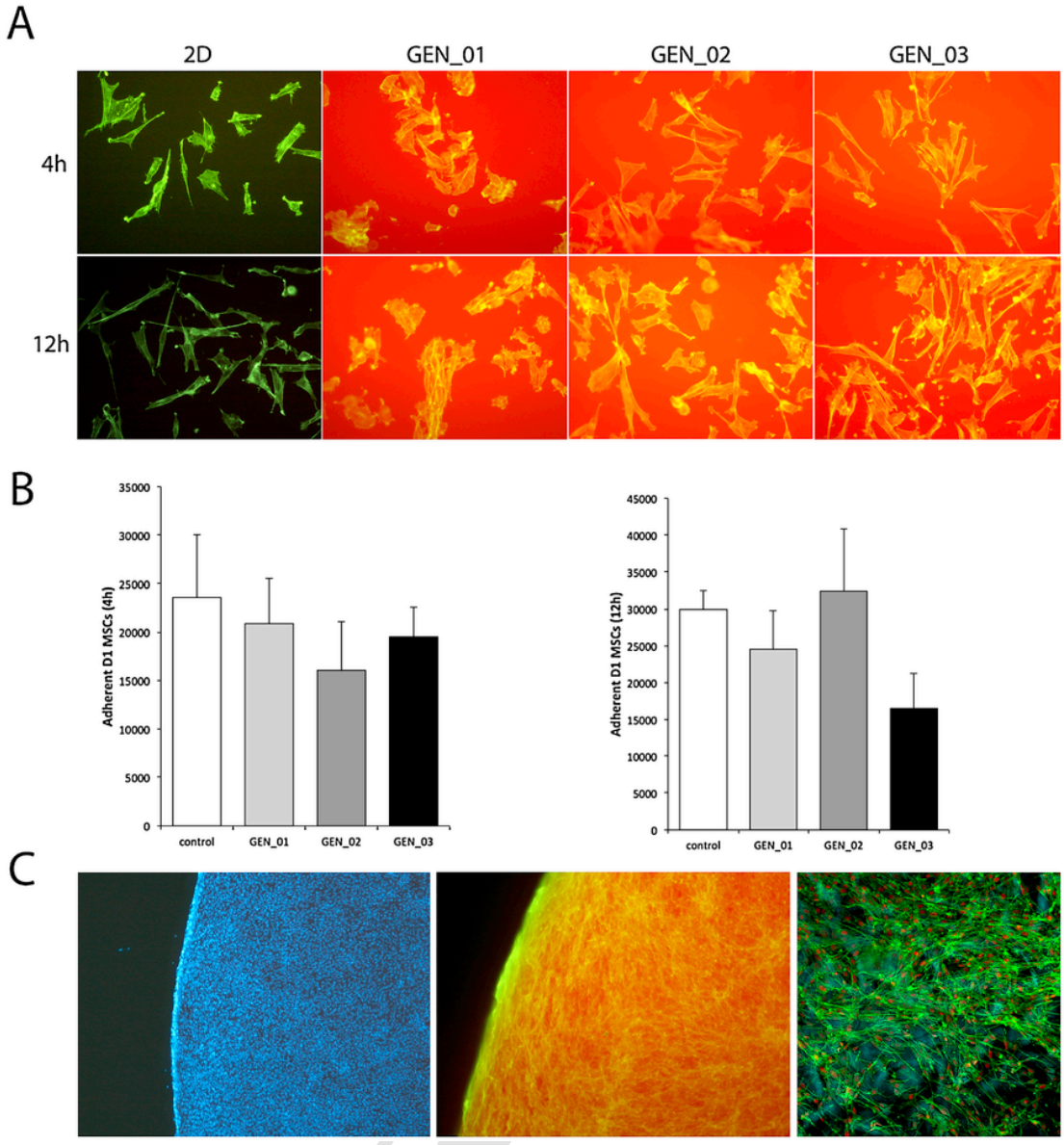

Fig. 5. Cytosqueleton organization of D1-MSCs adhered on three different scaffolds at 4 and 12 h after seeding (A) and at confluence (C). Cells were stained with Alexa fluor 488 for phalloidin for F-actin. The red background color is the fluorescence emitted by the genipin of the scaffolds. Adherent cells were counted at same time points (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

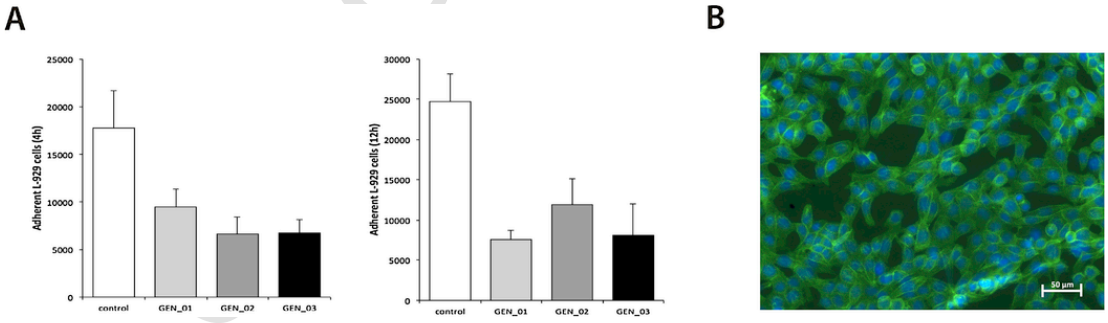

Fig. 6. Amount of L-929 fibroblast adhered at 4 and 12 h after seeding (A). Confocal images of those cells at confluence on scaffolds stained with Alexa fluor 488 for phalloidin for F-actin (green) and Hoechst (red) for nucleus (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

two weeks nearly 69% of total loaded VEGF was released (Fig. 7A, B).

In the case of SHH, the GEN_02H0 scaffolds showed higher encapsulation efficiency (73,68%) than GEN_02H50 (63,46%) scaffolds but if the total amount of encapsulated protein is analyzed, the scaffold with heparin captured more than double protein (45,28 ng/GEN_02H0 scaffold versus 110,27 ng/GEN_02H50 scaffold). In both types of scaffolds, a significant burst effect was observed in the first 24 h, being lower in case of GEN_02H0 (82,21% and 94,48%). Then, SHH delivery was almost null in both scaffolds. It can be observed that in both cases (VEGF and SHH) the amount of non-encapsulated protein was more than 4 times higher in GEN_02H0 than in GEN_02H50 (Fig. 7C, D).

It is well known that due to physical and chemical properties of gelatin, it is a suitable delivery vehicle for the release of proteins and other bioactive molecules. As mentioned above, the type B gelatin used for this study was derived from lime-cured tissue with consequent isoelectric point of 4.7–5.2. By contrast, the isoelectric points of both VEGF and SHH are above 7 (7.6 and 8.9 respectively). This implies that at physiologic pH the scaffold and growth factors have opposite charges thereby facilitating bonding by electrostatic charges. The incorporation of heparin into the scaffold may affect its charge thereby decreasing the encapsulation capacity of the device. However, it is known that heparin has binding sites for growth factors attachment [34] and as demonstrated Sven Knaack et.al, incorporation of heparin into scaffolds modified the molecules release kinetic [25]. Furthermore, heparin is a glycosaminoglycan found in mast cells, it is released only into the vasculature at sites of tissue injury and protects growth factors for its degradations. VEGF and SHH are both heparin-binding proteins, having domains to adhere to heparin [23,24,35]. In case of VEGF, after 14 days, an amount of almost 4-fold higher was trapped without released in GEN_02H50 (48,74 ng) compared to GEN_02H0 (13,14 ng) (Fig. 7A). However, in the case of SHH, after 14 days, there was hardly any protein in both scaffolds (5 ng in GEN_02H0 and 1,84 ng in GEN_02H50), though, the protein uptake capacity of GEN_02H50 was more than twice the capacity of GEN_02H0 (110,23 ng and 45,29 ng respectively) (Fig. 7C).

When a tissue such as bone is injured, a sequential cascade of biological processes begins. Traditionally clinical research has focused on angiogenesis processes and endothelial cells. But it should not be overlooked that the first phase that triggers after tissue damage is inflammation followed or even overlaid by angiogenesis [36]. Within all cells of the immune system, macrophages have been identified as one of the fundamental actors in the control of inflammatory angiogenesis and vascular remodeling. It appears that both macrophages present in damaged tissue as well as circulating monocytes participate in neovascularization and arteriogenesis in a model of ischemia [37,38]. VEGF, which has a chemotactic function of cells such as macrophages, could be involved in these processes [39]. At the same time of inflammation phase, it is important to start promoting vessel formation by upregulating the expression of proangiogenic molecules like VEGF. It is a fact that VEGF is a potent angiogenic growth factor, which promotes the proliferation and survival os endothelial cells. In addition, there are studies that have shown that spatiotemporal control of VEGF delivery from hydrogels enhances angiogenesis [40]. SHH protein is a morphogen, which has been found to be precursor and activator of GLI genes. SHH activates pathways that promote the synthesis of factors such as VEGF and BMPs. SHH protein promotes the proliferation, migration and VEGF production of endothelial progenitor cells from bone marrow [41] and induced angiogenesis of endothelial cells by Rho kinase dependent signal [42]. SHH synthesis and expression has been observed in the early phase of bone fracture repair, specifically two days post fracture [43]. Eva Dohle et al. compared the effects of VEGF and SHH in cultures of outgrowth endothelial cells (OEC) and in co-cultures of primary osteoblasts and OEC. Their results showed that SHH, unlike VEGF, promotes tube-like structures and interconnected net formations in early phase (24 h) of the treatment. Nevertheless, at day 14 there were no differences between both proteins. In addition, SHH favored

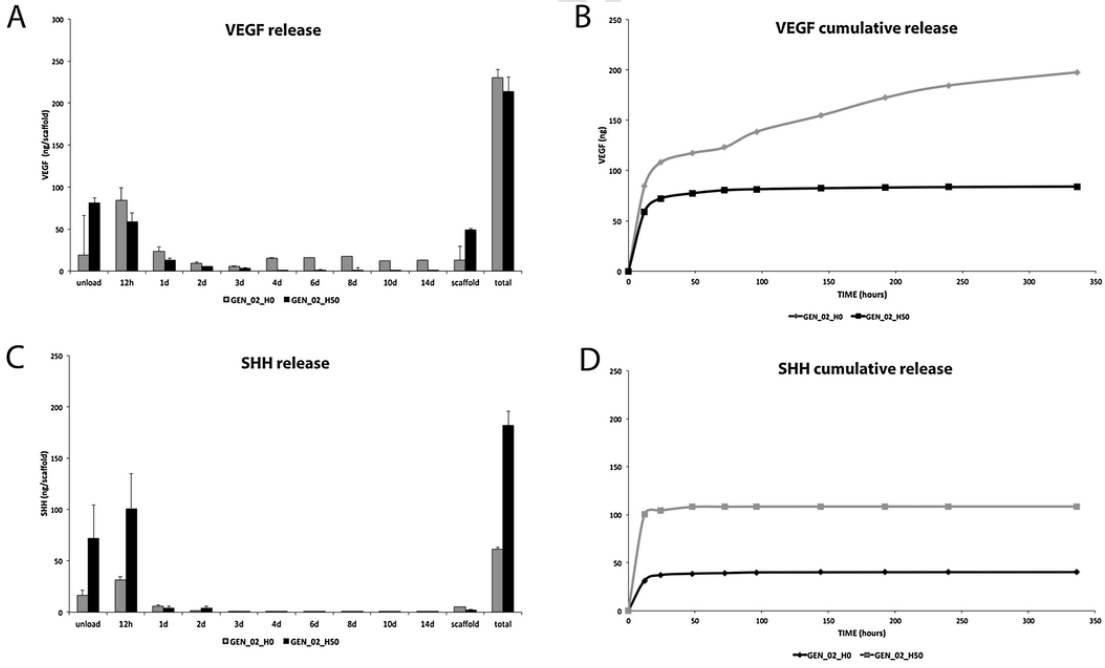

Fig. 7. VEGF and SHH release at different time points, protein amount remaining into the scaffolds after 14 days, the total protein detected during assays (A, C) and their cumulative release from GEN_02H0 and GEN_02H50 scaffolds (B, D).

several factors involved in vessel maturation and stabilization in vitro [44].

As seen in other studies, the release of VEGF in combination with another growth factor may enhance angiogenesis and tissue regeneration [45]. In view of studies indicating the expression of SHH the in the first two days after bone fracture, which activates pathways that promote the synthesis of BMPs and VEGF, and the importance of VEGF in both the inflammation and revascularization phase, one would expect that the profile given in GEN_02 of early release of SHH and followed by sustained release of VEGF would be appropriate to promote and establish a long-lasting and stable vascularization.

3. Conclusions

We fabricated and characterized three different scaffolds using gelatin and different concentrations of genipin (Gnp). Regardless of the Gnp concentration, all scaffolds showed similar Poissoṅs module but an increase in the Young's elastic modulus and a decrease of swelling ratio. None of scaffolds showed signs of cytotoxicity. L-929 fibroblasts showed significantly lower affinity for the all scaffolds. GEN_02 scaffolds showed improved D1 mesenchymal stem cells adhesion capacity. GEN_02 demonstrated its ability to retain and release VEGF at a constant speed for two weeks after an initial burst release in the first 24 h. On the other hand, GEN_02 showed SHH low retention capacity, facilitating its near total release within the first 24 h. These results highlight the importance of the physicochemical properties of both gelatin and growth factors in modifying their release profile.

The present study supports the use and evaluation of the gelatin-genipin scaffolds in future in vivo studies to determine their safety and bone regeneration capacity.

Conflicts of interest

The authors declare that they have no conflict of interest.

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References

[1] S.D. Hauschka, I.R. Konigsberg, The influence of collagen on the development of muscle clones, Proc. Natl. Acad. Sci. U. S. A. 55 (1966) 119–126.
[2] E.D. Hay, Interaction between the cell surface and extracellular matrix in corneal development, Soc. Gen. Physiol. Ser. 32 (1977) 115–137.
[3] Y.C. Lin, F.J. Tan, K.G. Marra, S.S. Jan, D.C. Liu, Synthesis and characterization of collagen/hyaluronan/chitosan composite sponges for potential biomedical applications, Acta Biomater. 5 (2009) 2591–2600.
[4] M.G. Patino, M.E. Neiders, S. Andreana, B. Noble, R.E. Cohen, Collagen: an overview, Implant Dent. 11 (2002) 280–285.
[5] J.M. Anderson, A. Rodriguez, D.T. Chang, Foreign body reaction to biomaterials, Semin. Immunol. 20 (2008) 86–100.
[6] P. Roach, D. Eglin, K. Rohde, C.C. Perry, Modern biomaterials: a review – bulk properties and implications of surface modifications, J. Mater. Sci-Mater. 18 (2007) 1263–1277.
[7] A.O. Elzoghby, W.M. Samy, N.A. Elgindy, Protein-based nanocarriers as promising drug and gene delivery systems, J. Control. Release 161 (2012) 38–49.
[8] S. Young, M. Wong, Y. Tabata, A. Mikos, Gelatin as a delivery vehicle for the controlled release of bioactive molecules, J. Control. Release 109 (2005) 256–274.
[9] C. Nishi, N. Nakajima, Y. Ikada, In vitro evaluation of cytotoxicity of diepoxy compounds used for biomaterial modification, J. Biomed. Mater. Res. 29 (1995) 829–834.
[10] H.W. Sung, R.N. Huang, L.L. Huang, C.C. Tsai, In vitro evaluation of cytotoxicity of a naturally occurring cross-linking reagent for biological tissue fixation, J. Biomater. Sci. Polym. 10 (1999) 63–78.
[11] B. Manickam, R. Sreedharan, M. Elumalai, 'Genipin' – the natural water soluble cross-linking agent and its importance in the modified drug delivery systems: an overview, Curr. Drug Delivery 11 (2014) 139–145.
[12] S. Shindo, Y. Hosokawa, I. Hosokawa, K. Ozaki, T. Matsuo, Genipin inhibits MMP-1 and MMP-3 release from TNF-a-stimulated human periodontal ligament cells, Biochimie 117 (2014) 391–395.
[13] K.N. Nam, Y.S. Choi, H.J. Jung, G.H. Park, S.K. Moon, K.H. Cho, C. Kang, I. Kang, M.S. Oh, E.H. Lee, Genipin inhibits the inflammatory response of rat brain microglial cells, Int. Immunopharmacol. 10 (2010) 493–499.
[14] E.V. Dare, M. Griffith, P. Poitras, J.A. Kaupp, S.D. Waldman, D.J. Carlsson, G. Dervin, C. Mayoux, M.T. Hincke, Genipin cross-linked fibrin hydrogels for in vitro human articular cartilage tissue-engineered regeneration, Cells Tissues Organs 6 (2009) 313–325.
[15] Y. Koriyama, K. Chiba, M. Yamazaki, H. Suzuki, K. Muramoto, S. Kato, Long-acting genipin derivative protects retinal ganglion cells from oxidative stress models in vitro and in vivo through the Nrf2/antioxidant response element signaling pathway, J. Neurochem. 115 (2010) 79–91.
[16] E.S. Kim, C.S. Jeong, A. Moon, Genipin a constituent of Gardenia jasminoides Ellis, induces apoptosis and inhibits invasion in MDA-MB-231 breast cancer cells, Oncol. Rep. 27 (2012) 567–572.
[17] M. Butler, Y.F. Ng, P. Pudney, Mechanism and kinetics of the crosslinking reaction between biopolymers containing primary amine groups and genipin, J. Polym. Sci. A Polym. Chem. 41 (2003) 3941–3953.
[18] L. Solorio, C. Zwolinski, A.W. Lund, M.J. Farrell, J.P. Stegemann, Gelatin microspheres crosslinked with genipin for local delivery of growth factors, J. Tissue. Eng. Regen. Med. 4 (2010) 514–523.
[19] H.W. Sung, D.M. Huang, W.H. Chang, R.N. Huang, J.C. Hsu, Evaluation of gelatin hydrogel crosslinked with various crosslinking agents as bioadhesives: in vitro study, J. Biomed. Mater. Res. 46 (1999) 520–530.
[20] Y.J. Hwang, J. Larsen, T.B. Krasieva, J.G. Lyubovitsky, Effect of genipin crosslinking on the optical spectral properties and structures of collagen hydrogels, ACS Appl. Mater. Interfaces 3 (2011) 2579–2584.
[21] V.A. Acosta Santamaría, J.M. García Aznar, I. Ochoa, M. Doblare, Effect of sample pre-contact on the experimental evaluation of cartilage mechanical properties, Exp. Mech. 53 (2013) 911–917.
[22] S. Poveda-Reyes, T.C. Gamboa-Martínez, S. Manzano, M.H. Doweidar, J.L. Gómez Ribelles, I. Ochoa, G. Gallego Ferrer, Engineering interpenetrating polymer networks of poly(2-Hydroxyethyl acrylate) as ex vivo platforms for articular cartilage regeneration, Int. J. Polym. Mater. 64 (2015) 745–754.
[23] W. Zhao, S.A. McCallum, Z. Xiao, F. Zhang, R.J. Linhardt, Binding affinities of vascular endothelial growth factor (VEGF) for heparin-derived oligosaccharides, Biosci. Rep. 32 (2012) 71–81.
[24] F. Zhang, J.S. McLellan, A.M. Ayala, D.J. Leahy, R.J. Linhardt, Kinetic and structural studies on interactions between heparin or heparan sulfate and proteins of the hedgehog signaling pathway, Biochemistry 46 (2007) 3933–3941.
[25] S. Knaack, A. Lode, B. Hoyer, A. Rosen-Wolff, A. Gabrielyan, I. Roeder, M. Gelinsky, Heparin modification of a biomimetic bone matrix for controlled release of VEGF, J. Biomed. Mater. Res. A 10 (2014) 3500–3511.
[26] L. Gao, H. Gan, Z. Meng, R. Gu, Z. Wu, L. Zhang, X. Zhu, W. Sun, J. Li, Y. Zheng, G. Dou, Effects of genipin cross-linking of chitosan hydrogels on cellular adhesion and viability, Colloids Surf. B: Biointerfaces 117 (2014) 398–405.
[27] A. Garate, E. Santos, J.L. Pedraz, R.M. Hernández, G. Orive, Evaluation of different RGD ligand densities in the development of cell-based drug delivery systems, J. Drug. Target 23 (2015) 806–812.
[28] A. Garate, J. Ciriza, J.G. Casado, R. Blazquez, J.L. Pedraz, G. Orive, R.M. Hernandez, Assessment of the behavior of mesenchymal stem cells immobilized in biomimetic alginate microcapsules, Mol. Pharm. 12 (2015) 3953–3962.
[29] J.C. Friedland, M.H. Lee, D. Boettiger, Mechanically activated integrin switch controls alpha5beta1 function, Science 323 (5914) (2009) 642–644.
[30] N. Huebsch, D.J. Mooney, Mechanically activated integrin switch controls alpha5beta1 function, Nature 462 (7272) (2009) 426432.
[31] F. Guilak, D.M. Cohen, B.T. Estes, J.M. Gimble, W. Liedtke, C.S. Chen, Control of stem cell fate by physical interactions with the extracellular matrix, Cell. Stem. Cell 5 (2009) 17–26.
[32] S.X. Hsiong, P. Carampin, H.J. Kong, K.Y. Lee, D.J. Mooney, Differentiation stage alters matrix control of stem cells, J. Biomed. Mater. Res. A 82 (2008) 145–156.

[33] S.X. Hsiong, N. Huebsch, C. Fischbach, H.J. Kong, D.J. Mooney, Integrin-adhesion ligand bond formation of preosteoblasts and stem cells in three-dimensional RGD presenting matrices, Biomacromolecules 7 (2008) 1843–1851.
[34] Heparin vs. heparan sulfates, in: H. Edward Conrad (Ed.), Heparin-binding Proteins, Academic Press, San Diego, 1998, pp. 1–6.
[35] S.C. Chang, B. Mulloy, A.I. Magee, J.R. Couchman, Two distinct sites in sonic Hedgehog combine for heparan sulfate interactions and cell signaling functions, J. Biol. Chem. 286 (2011) 44391–44402.
[36] K. Schmidt-Bleek, B.J. Kwee, D.J. Mooney, G.N. Duda, Boon and bane of inflammation in bone tissue regeneration and its link with angiogenesis, Tissue Eng. Part B Rev. 21 (2015) 354–363.
[37] E. Khmelewski, A. Becker, T. Meinertz, W.D. Ito, Tissue resident cells play a dominant role in arteriogenesis and concomitant macrophage accumulation, Circ. Res. 283 (2004) 56–64.
[38] M. Heil, T. Ziegelhoeffer, F. Pipp, S. Kostin, S. Martin, M. Clauss, W. Schaper, Blood monocyte concentration is critical for enhancement of collateral artery growth, Am. J. Physiol. Heart Circ. Physiol. 283 (2002) 2411–2419.
[39] J. McLaren, A. Prentice, D.S. Charnock-Jones, S.A. Millican, K.H. Muller, A.M. Sharkey, S.K. Smith, Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids, J. Clin. Invest. 98 (1996) 482–489.
[40] E.A. Silva, D.J. Mooney, Spatiotemporal control of vascular endothelial growth factor delivery from injectable hydrogels enhances angiogenesis, J. Thromb. Haemost. 5 (2007) 590–598.
[41] J.R. Fu, W.L. Liu, J.F. Zhou, H.Y. Sun, H.Z. Xu, L. Luo, H. Zhang, Y.F. Zhou, Sonic hedgehog protein promotes bone marrow-derived endothelial progenitor cell proliferation, migration and VEGF production via PI 3-kinase/Akt signaling pathways, Acta. Pharmacol. Sin. 27 (2006) 685–693.
[42] M.A. Renault, J. Roncalli, J. Tongers, T. Thorne, E. Klyachko, S. Misener, O.V. Volpert, S. Mehta, A. Burg, C. Luedemann, G. Qin, R. Kishore, D.W. Losordo, Sonic hedgehog induces angiogenesis via Rho kinase-dependent signaling in endothelial cells, J. Mol. Cell. Cardiol. 49 (2010) 490–498.
[43] T. Miyaji, T. Nakase, M. Iwasaki, K. Kuriyama, N. Tamai, C. Higuchi, A. Myoui, T. Tomita, H. Yoshikawa, Expression and distribution of transcripts for sonic hedgehog in the early phase of fracture repair, Histochem. Cell. Biol. 119 (2003) 233–237.
[44] E. Dohle, S. Fuchs, M. Kolbe, A. Hofmann, H. Schmidt, C.J. Kirkpatrick, Comparative study assessing effects of sonic hedgehog and VEGF in a human co-culture model for bone vascularisation strategies, Eur. Cell. Mater. 21 (2011) 144–156.
[45] C. Borselli, H. Storrie, F. Benesch-Lee, D. Shvartsman, C. Cezar, J.W. Lichtman, H.H. Vandenburgh, D.J. Mooney, Functional muscle regeneration with combined delivery of angiogenesis and myogenesis factors, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 3287–3292.