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DESIGN AND ASSEMBLY PROCEDURES FOR LARGE-SIZED BIOHYBRID SCAFFOLDS AS PATCHES FOR MYOCARDIAL INFARCT IN A BIG ANIMAL MODEL

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Abstract

Objective: To assemble a biohybrid patch consisting of a large (5 cm X 5 cm) elastomer scaffold whose pores are filled with a self-assembling peptide (SAP) gel entrapping adipose stem cells (ASCs), to be used in a big animal model of myocardial infarction. The study focuses on the way to determine optimal procedures for incorporating the SAP solution and the cells in the patch in order to ensure cell colonization and a homogeneous cell distribution in the construct before implantation. The problems associated with the scale-up of the different procedures raised by the large size of the construct are discussed.

Materials and methods: Experiments were performed to settle different alternatives: incorporation of the SAP gel prior to cell seeding or simultaneous SAP-and-cells loading of the scaffold; surface seeding of cells or cell injection into the scaffold's pores; helping the seeded cells to disseminate throughout the scaffold prior to incubation by gentle shaking or by centrifugation. Immunocytochemistry techniques and confocal and scanning electron microscopies were employed in order to analyze and quantify the cell invasion and early distribution. Observed cell concentration and uniformity throughout the patch were taken as the indicators to settle the different alternative procedures.

Results: The peptide pre-loaded scaffold, seeded internally and smoothly shaken before incubation yielded the best results in terms of greater cell density and the most uniform distribution after 24 h of culture compared with the other methods. These procedures could be translated to obtain large biohybrid patches with success.

Conclusions: The results obtained after the different seeding methods permitted to establish an effective protocol for the assembly of large scaffold + SAPs gel + cells biohybrids for their subsequent implantation onto the infarcted myocardium of a preclinical big animal model progressing for clinical translation.

Keywords: scaffold, self-assembling peptide, cell seeding, myocardial patch, myocardial support and regeneration.

1. Introduction

Adult human hearts are able to functionally regenerate after cardiac infarction [1, 2] but this capacity seems limited when large areas of cardiac tissue are involved, resulting in substantial loss of ventricular mass, vascularization and contractibility. Proposed strategies to repair damaged cardiac tissue include cell transplantation directly at the injured site, or tissue engineering techniques to induce the regeneration of injured native tissue. Cell types investigated include cardiac stem cells, skeletal myoblasts, stem cells from bone marrow or mesenchymal stem cells from adipose tissue, and endothelial progenitor cells, among others, usually resulting in an improvement of heart contraction by paracryne effects (secretion of cytokines), increase of wall thickness and micro-vessel density (neovascularization) [3-6]. The main results reported with these procedures are however limited, not producing a complete restoration of the contractile function [7], owing to a poor cell retention and graft viability in the target site. Besides, a significant number of cells (more than 90%) die within the first days post-transplantation or disseminate to other tissues. A possible solution would be to encapsulate the transplanted cells in an injectable gel, but this has the drawback of the low mechanical properties of gels, which difficults their handling, localization and retention on a damaged moving tissue.

Alternative tissue engineering strategies [8, 9] combine cells with three-dimensional scaffolds or patches that host them and improve their survival, induce the formation of new blood vessels and extracellular matrix and at the same time mechanically assist the host tissue. The polymers employed to date to obtain scaffolds include collagen, gelatin, fibrin, hyaluronic acid and alginate [10-13] of natural origin, or the synthetic polylactide acid (PLA), polylactide-co-glycolic acid (PLGA), polycaprolactone (PCL), poly(ethylene glycol) (PEG), polypropylene (PP) and poly(glycerol sebacate) (PGS),

with different architectures and combined with a variety of cells [14-17]. As an example, Chachques *et al.* [18] implanted collagen sponges seeded with bone marrow cells onto the post-ischemic myocardial scar of a series of patients in a clinical feasibility study and observed an increase of the thickness of the infarct scar as well as a normalization of the cardiac wall stress. The main limitation of those scaffolds was the low mechanical characteristics of the materials employed to engineer the myocardial tissue and a too fast and complete bio-resorption of the material at mid-term.

The concept of the 7th FP project RECATABI, where this study is framed, is a biohybrid cardiac patch consisting in an elastomer polymer scaffold whose pores are filled with the self-assembling peptide (SAP) hydrogel RAD16-I, which encapsulates adipose-tissue derived stem cells (ASCs). A first series of these patches employed poly(ethyl acrylate), PEA, as matrix to develop scaffolds with interconnected spherical pores; this polymer is an elastomer compatible with the myocardial tissue in terms of mechanical properties, is easy to process, and has excellent biological performance [19].

SAPs are resorbable nanomaterials that mimic the structure of the extracellular matrix, promoting and modulating cell functions such as adhesion, proliferation and migration. Thanks to these properties, they have been used for a variety of applications *in vitro* with different types of cells (osteoblasts, embryonic stem cells, adult neural stem cells, endothelial cells,…) [20-22] and for regenerative strategies in animal models [23-26]. One commonly employed SAP, RAD16-I [18-20], is a hydrogel consisting of simple repeated sequences of RADA amino acids with alternating hydrophobic and hydrophilic lateral groups. They are injectable in aqueous solution, and form percolating β–sheet nanofibers when exposed to a salt solution or physiological media [21, 27, 28]. At low concentrations (0.15%-0.25%) the gel is soft and fragile, resulting in a poor manageability; on the contrary, at an elevated concentration (from 0.5 to 1%) the gel

becomes tough and impedes an adequate cellular ingrowth [21]. In the present proposal, the elastomer scaffold membrane provides the three-dimensional context and the mechanical integrity, whereas the peptide gel RAD16-I filling the scaffold's pores is expected to act as an encapsulating medium for the cells, improving their survival and retaining them inside the membrane, allowing permeability to cellular metabolites and wastes, and likely improving vascularization throughout the scaffold.

ASCs are a convenient cell source for cardiac regenerative purposes since they can be easily harvested from the patient, have an elevated proliferation rate *in vitro* and are non-immunogenic; besides, some studies and clinical trials have demonstrated their potential to improve ventricular function [29-32], which could lead to their use for cardiac clinical application [33,34]. Since the direct cell graft into infarcted myocardium has had poor results, with a rapid dissemination of cells to other sites and a low rate of cell survival of the effectively engrafted cells [35], the transplantation of the cells inside a physical support may help improve over those results: the scaffold can cover the damaged area, can protect the grafted cells by offering a cellular niche and thus prolong their survival and paracryne effect, and impede their migration from the site of interest. An increased localized activity of the cells could lead to wall thickening and neovasculogenesis, eventually improving the heart function.

In facing a preclinical study in a big animal model (sheep) additional factors that condition the design of the solution must be taken into account. The most relevant ones are the size of the patch, the number of the cells to be transplanted, and the timing of pre-implant seeding and *in vitro* culture of the cells in the patch. The dimension of the patch designed for this study (5x5 cm) is related to the size of the infarct model created by surgical procedure (coronary artery branch ligations) in adult sheep [19,36], and the number of cells to be transplanted and the pre-implant culture time have followed

previous experience [18]. Taken together, these circumstances impose stringent requirements on the design of the patch: it must be able to lodge those numbers of cells, these must seed and distribute uniformly across the patch in 24 h, and the whole patch must be rapidly vascularized throughout after implantation, to keep the seeded cells alive. In the present work we address these questions, related to the design and implementation of a biohybrid patch for use in an infarcted sheep heart.

2. Materials and methods

2.1. Preparation of the scaffolds

Scaffolds of polyethyl acrylate (PEA) with interconnected spherical pores were prepared following a porogen-template leaching method as described in [37, 38]. Briefly, poly(methyl methacrylate) microspheres (PMMA; Colacryl dp 300) of known size, 130 ± 20 μm, were sintered between two plates in order to obtain a porogen template. A monomer solution was prepared by mixing ethyl acrylate (EA; 99%, Sigma-Aldrich) with 2 wt% ethyleneglycol dimethacrylate (EGDMA; 98%, Sigma-Aldrich) as crosslinker and 1 wt% benzoine (98%, Scharlau) as initiator, stirred and injected into the porogen template. The filled template was then placed between two glass plates, polymerized under a UV source for 24 h, and post-polymerized in an oven at 90˚C for another 24 h. The template was removed by soxhlet extraction for 24 h with acetone (Scharlab). Afterwards, a gradual solvent exchange to water was performed to avoid the collapse of the obtained scaffolds due to the fast evaporation of acetone. Finally, the 1 mm-thick PEA scaffolds obtained were dried under vacuum at 40ºC until constant weight, and cut as small discs of 8 mm diameter for *in vitro* assays. The obtained samples were sterilized with a 25 kGy dose of gamma irradiation in a ^{60}Co source (Aragogamma, Barcelona, Spain) before use.

2.2. SAPs preparation and filling of the scaffolds

The self-assembling peptide (SAPs) RAD16-I solution (PuraMatrix™ 1% (w/v), BD Biosciences) was employed as a filler hydrogel in the PEA scaffolds' pores. The viscous stock solution was sonicated for 30 min at 25ºC applying 30 W in a Bandelin bath, diluted with water (extra pure, Scharlau) up to 0.3% (w/v) and vortexed (Elmi SkyLine) to ensure homogeneization. In one series of scaffolds, the SAP solution and the cells were simultaneously incorporated to the scaffolds; these will be hereafter called 1-step loaded scaffolds (Fig. 1a). In a second series of scaffolds (hereafter referred to as 2-steps loaded, Fig. 1b) the SAP was loaded as a 0.15% (w/v) solution with the help of some vacuum, as in [19, 39]; more precisely, the scaffold was placed (folded if necessary) in a large syringe, the aqueous SAPs solution was loaded, and the air removed. Next, maintaining the luer taper of the syringe sealed, the peptides solution was forced to penetrate in the scaffold by performing repeated strokes until it was completely wet.

2.3. Seeding of ASCs and pre-culture of the biohybrids

Adipose-tissue derived stem cells (ASCs) of subcutaneous fat tissue biopsies were obtained from the mediastinal fat tissue of female Ille de France sheep and isolated according to [12]. The adhered cells were incubated at 37ºC in a humidified atmosphere with 5% CO_2 in minimum essential medium alpha (α–MEM) supplemented with fetal bovine serum (FBS; 10%), L-Glutamine (2 mM), penicillin (10 U/ml), streptomycin (10 mg/ml), gentamicin (10 mg/ml) (all products from Gibco/Invitrogen) and plasmocin (5 µg/ml, Invivogen, ant-mp) to avoid mycoplasma contamination. The cells were allowed to proliferate in culture flasks until passage 6, then harvested by trypsinization (0.25%

Trypsin-EDTA, Gibco/Invitrogen) and resuspended in a 10% sucrose (Sigma-Aldrich) aqueous solution (instead of culture medium, to avoid the SAPs gelling upon their contact) at densities of 5 and $10 \cdot 10^6$ cells/ml to be used in the two series of scaffolds.

10^5 cells were seeded in each scaffold having a diameter of 8 mm in a 48-well tissue plate; this corresponds to a density of $2 \cdot 10^6$ cells/cm^3 of scaffold. In the 2-step loaded scaffolds, the 10^5 cells were seeded in a 20 μl aqueous droplet after the vacuum helped incorporation of the 0.15% (w/v) SAPs solution. For the 1-step loaded scaffolds, the initial 0.3% (w/v) SAPs solution was half diluted with the cells-sucrose suspension and 20 μl of the resulting 0.15% (w/v) SAPs solution containing the 10^5 cells was incorporated at once.

The cell seeding was performed by two different methods: in a subset of samples, the 20 μl cells (or cells+SAPs) droplet was seeded onto the upper-surface of the scaffolds, and in the other, the droplet was injected within the pores of the scaffolds making use of a Hamilton syringe.

After the encouraging results, in terms of better cells distribution within the pores, obtained in a previous work [19] with a dynamic seeding as compared to a static one. Two dynamic methods were compared here: half of the scaffolds were smoothly shaken (25 rpm, 30 min) after the seeding in a Titramax 101 shaker (Heidolph instruments, Germany) and the remaining were centrifuged (600 rpm, 5 min) in a centrifuge 5804 eppendorf device. Next, 300 μl more of culture medium were carefully added to each well to gel the peptides and entrap the cells, and cells were incubated at 37°C in a humidified atmosphere under 5% CO_2 for 24 h.

Each one of the 8 resulting experimental groups (1-step or 2-step loaded scaffolds, either seeded on their surfaces or by internal injection, and shaken or centrifuged before culture) consisted in three 8 mm-diameter replicas. They were subsequently

characterized to determine the optimal seeding procedure before the scale-up to the 5x5 cm^2 patches.

2.4. Biological characterization of the biohybrids

After 24 h culture, the biohybrids were processed for fluorescence staining. Samples were rinsed with 0.1 M phosphate buffered saline (PBS; pH 7.4) and fixed for 15 min in 4% paraformaldehyde (Panreac). After 30 min permeabilization with 10% fetal bovine serum and 0.1% triton X-100 in PBS, samples were incubated for 60 min in the selective F-actin stain Phalloidin Bodipy FL (Invitrogen) at a dilution of 1:200 in 0.1% BSA-PBS at room temperature and mouse monoclonal anti-vimentin (v6630, Sigma) overnight and incubated for 1 h with Alexa fluor goat anti-mouse 647 (A21236, Invitrogen)The samples were rinsed in PBS and then stained 5 min with 10 µg/mL DAPI (4',6-diamidino-2-phenylindole, Sigma, 1:5000). Afterwards, the samples were cryoprotected by immersion in 0.1 M PBS at pH 7.5 containing 30% sucrose and included in OCT. 50 µm-thick sections were obtained by using a cryostat (Leica, CM 1900), collected onto superfrost slides and rinsed with PBS. The slices were mounted on glass slides using Fluoromount-G™ (F4680, Sigma-Aldrich), and examined to collect fluorescent images under an epifluorescence (Leica DM6000) or confocal laser scanning microscope (CLSM; FV 1000, Olympus).

2.5. Image processing

For cell quantification, DAPI labelled cell nuclei were counted in three images taken under the fluorescence microscope before the scaffolds' cutting, and corresponding each to 0.004 cm^2 per each experimental group; the number of cell nuclei per unit area was obtained from these quantifications. To further evaluate the invasibility of the scaffolds

by the ASCs, the expression of F-actin (filamentous actin) in the cytoskeleton was quantified together with the DAPI labelling to determine the percentage of image area covered by cells. The mean cell surface area gave an idea of cells spreading and attachment in each case. All image processing and analysis were done using an in-house software developed under MATLAB R2006a (The MathWorks, Inc., Natick, MA).

2.6. Scale-up and characterization of large biohybrids

To set up the assembly of the biohybrids in the final dimensions following the best conditions found *in vitro*, large 5x5x0.1 cm^3 PEA scaffolds were firstly prepared following the fabrication methodology explained above. To validate such protocol for large patches and ensure a homogeneous and high porosity and pores' interconnections, bare scaffolds were examined by scanning electron microscopy (SEM) in a JSM 6300 (JEOL Ltd., Tokyo, Japan) device, previously sputter-coated with gold, at 15 kV of acceleration voltage and 15 mm of working distance. The scaffolds were fractured in liquid nitrogen in order to obtain surface and transversal images. The proposed SAPs injection protocol and their gelling inside the pores upon the addition of culture medium was checked in large scaffolds by congo red 0.1% (w/v) aqueous solution (Fischer Scientific) staining for 20 min followed by 30 min of rinsing with water and macroscopical observation.

The scaffold+SAPs+ASCs assembly protocol giving the best results in terms of invasion and homogeneous distribution of the cells in small biohybrids was translated to the large patches: the scaffolds were pre-loaded with the peptide solution, next seeded internally with a Hamilton syringe and smoothly shaken for 30 min at 80 rpm, and finally cultured for 24 h before analysis. After expansion and trypsinization, ASCs were resuspended in a 10% sucrose aqueous solution at a density of $40 \cdot 10^6$ cells/ml. $100 \cdot 10^6$

cells were seeded per SAPs pre-loaded scaffold, distributed in 50 uniformly spaced injections of 50 µl each.

After a 24 h culture, slices of the patches were analyzed by immunocytochemistry and confocal microscopy as described above, and by scanning electron microscopy in a SEM Hitachi S-4800 device. The samples were rinsed in 0.1 M PBS at pH 7.5 and fixed in a 2% paraformaldehyde and 2.5% gluteraldehyde solution. The samples for SEM were post-fixed with 1% OsO_4 (Aname, 19112) and dehydrated in serial ethanol (30, 50, 70, 96 and 100%); next, they were dried using liquid CO_2 (critical point values: 328ºC, 1100 psi; Autosambri 814, Rockville, MD, USA) and coated with gold to be observed.

2.7. Statistical analysis

All values were expressed as mean ± standard deviation (SD) and analyzed statistically using a two-tailed Student's t-test. The level of significance was set at $p<0.05$.

3. Results

3.1. Scale-up of the fabrication procedures to implantable large biohybrids

Fig. 2a and 2c shows macroscopical and SEM images, respectively, of 5x5 cm^2 PEA scaffolds, fabricated with the procedure employed previously for small scaffolds, but with special care on the uniform sintering of the porogen template and on the handling of the swollen scaffolds during the rinsing of the porogen. The obtained scaffolds showed interconnected spherical pores with pore diameters around 130 ± 20 µm, leading to a bulk porosity of 80.8±3.5%. They were flexible and adaptable to curved surfaces such as the myocardium.

The SAPs filling procedure employed for the smaller samples was also valid for the large scaffolds: their elastomeric nature allows their rolling and folding inside a syringe

and successive stroked of its piston force the viscous peptide solution to penetrate into the pores. Fig. 2d shows the SAPs filling in the scaffold's pores under cryoSEM, which appears as stretched-out fibers formed as water sublimates. The peptides solution successfully gels *in situ* within the pores of large scaffolds when in contact with culture medium, and the β-sheet structures stain red with congo red (Fig. 2b).

3.2. Cell viability and distribution after the different seeding procedures in small biohybrids

The effectiveness of the eight different seeding procedures chosen was studied after 24 h culture. Fig. 3 shows the distribution of cell nuclei (stained with DAPI) under the fluorescence microscope. In the 1-step loaded and internally seeded scaffolds, the cells were concentrated in the vicinities of the injection point, as was also the case in those seeded on top of the surface in one step; few cells were able to migrate through the scaffolds' pores and the non-attached leftover was lost. None of both dispersion methods (shaking or centrifugation before culture) helped to better distribute the cells within the scaffolds' pores. Contrarily, when cells were seeded after a SAPs pre-load (2-step loaded scaffolds), they were able to diffuse from the injection point, more when cells were seeded internally than on the surface; they invaded the whole available volume of the scaffolds. Both dynamic dispersion methods were equally effective in achieving rapid cell diffusion through the peptide filler.

Next, a more thorough study was undertaken of the following factors: *i*) the effect of the SAPs solution on the efficiency of cell seeding, *ii*) the seeding points of the cells, either internal or superficial, and *iii*) the dynamic conditions to enhance cell diffusion before incubation.

The 1-step and 2-step loaded scaffolds, both seeded in their core with a Hamilton syringe and shaken before incubation, were compared to understand the role of SAPs during the seeding. The confocal microscopy images of longitudinal slices of such scaffolds after the immunocytochemistries (Fig. 4) confirm that when SAPs and cells are incorporated simultaneously the cells are retained in the peptide solution at the site of injection (a, b, taken at the injection site), whereas, when cells are seeded following the prior SAPs loading, they invade the scaffold and distribute homogeneously throughout it (c, d, representative of the whole scaffold). Interestingly, once lodged within the pores (24 h), the cells seem to attach to the PEA hydrophobic surface rather than remain suspended in the SAPs hydrogel (see the detail, e, f). In 1-step loaded scaffolds the cells maintained a spherical morphology, whereas in 2-step loaded ones cells appeared more elongated, indicative of the adhesion to the material surface.

The viable cells of these 1-step and 2-step loaded scaffolds were next quantified by image analysis. The DAPI labelled cell nuclei yielded the number of cells per unit area, which increases 3-fold when the cells are incorporated after the peptide solution has been loaded in the inner pores, Fig. 5a. The F-actin (filamentous actin) staining with phalloidin allowed defining the area covered by cytoskeleton and . the mean cell surface area, calculated as the area covered by the cells divided through the number of cell nuclei (Fig. 5b). the first quantity was considerably larged in 2-step loaded scaffolds, although the cell area was approximately the same with both procedures analyzed, in spite of their rather different morphology.

Next, 2-step loaded scaffolds seeded either internally or on top of the surface, and shaken before incubation, were compared by immunocytochemistry and confocal microscopy to determine the depth to which cells diffuse through the peptide solution before being entrapped upon its gelation. The images of transversal cuts (Fig. 6) show

that only some of the cells seeded on the surface can penetrate, only 50 μm inwards the scaffold (a, b), whereas the rest are lost. By contrast, those injected internally are well distributed throughout the scaffold thickness (c, d).

In 2-step loaded scaffolds seeded internally no differences were found between the two dynamic (shaking and centrifugation) seeding protocols proposed, insofar as both helped to distribute sufficiently the cells (figure not shown). It must be remarked that the application of some cell-dispersing protocol before the SAPs gelling and consequent cell entrapment is crucial, as was demonstrated in [19] against a conventional (static) seeding.

3.3. Translation of the assembly protocol to large biohybrids for their implantation in a sheep model

The best results produced by the culture of small discs were those of the 2-step loaded scaffolds, seeded internally with a Hamilton syringe and smoothly shaken (or centrifuged) before incubation; such small format biohybrids hosted in their pores the highest and most uniformly distributed cell population. These same steps were then translated to the large scaffolds, but with doubled cell concentration, $4 \cdot 10^6$ cells/cm^3 of scaffold. Thus, $100 \cdot 10^6$ cells, suspended in 2.5 ml of sucrose supplemented aqueous medium, were incorporated to large 5x5x0.1 cm^3 scaffolds previously loaded with the SAPs solution, with 50 equally spaced injections of 50 μl each, which correspond to 1 internal injection per 0.5 cm^2 of external surface (as in the small discs, where they had proved to spread over such an area). Next, the biohybrids were shaken horizontally at 80 rpm for 30 min at 37ºC and incubated. After 1 day, they were analyzed to find out their state just before a hypothetical implantation in infarcted myocardium. Under confocal microscopy (Fig. 7) it was possible to observe that the ASCs were homogenously

distributed throughout the entire scaffold and tended to attach to the PEA trabecules establishing cell-cell contacts, rather than remaining suspended and isolated within the gel. Similar results were found by SEM (Fig. 8), although the preparation procedure is quite invasive and a non-negligible fraction of cells was lost from each slice: cells were accommodated on the available PEA three-dimensional struts establishing intimate cell-cell interactions.

4. Discussion

The purpose of the present study was to set a methodology to determine an effective way to build-up large implantable byohibrid patches, fixing the different choices available to combine their three components: scaffold, peptide gel, and cells. The variables under study were: the manner and sequence for the incorporation of the peptide solution and the cells, the way to seed the cells in the scaffold, and the subsequent dynamic conditions before their incubation to achieve the greatest invasion and most uniform cell distribution within the construct. The alternative choices were first studied in small-sized biohybrids, and the selected options were then scaled-up to establish a protocol to prepare and assemble the large biohybrid patches for their subsequent implantation in the infarcted sheep.

The need to have a large patch uniformly colonized by cells 24 h before surgery determined the main design options for the construct: the use of a filler hydrogel inside the pores of a patch with a highly regular pore architecture, produced by an opal-like porogenic template. It was argued that the large number of cells hosted in the patch could only be viable if the pore sizes of the scaffold allowed for a rapid ingrowth of microcapillaries when implanted, and it was judged that only a template-based manufacture of the scaffold, using a sintered template made of regular-sized spherical

microbeads, could ensure the necessary control over pore size and distribution. Other porogenic techniques lead to less controllable porous structures that cannot ensure perfect connectivity and size of the pores. Furthermore, it was advanced that a soft hydrogel filling the pores of the scaffold would constitute a medium improving cell spreading throughout the scaffold, thus permitting a fast and uniform cell colonization of the implantable structure. These basic hypotheses were confirmed by the results of our study. As several works have underlined [40-42], the microstructure of the scaffolds in tissue engineering is critical to ensure the hosting of a high enough cell density, allow cell migration, diffusion of nutrients and metabolites, new tissue growth and vascularization.

The SAPs solution proved to be an excellent diffusion medium for the cells, provided that it was incorporated within the scaffold's pores prior to the seeding (2-step loaded scaffolds). In the hours following the seeding, ASCs first migrate through the hydrogel and then attach to the PEA hydrophobic trabecules in an extended conformation with numerous cell-cell contacts. When cells were seeded simultaneously with the peptide in a specific location of the scaffold (1-step loaded scaffolds) the cells were only able to colonize the vicinities of the point where they had been seeded.

The internal seeding of the cells (injected with a Hamilton syringe) into the scaffold gave much better results than a seeding on top of the surface of the scaffold, because in this latter case those cells that have not been able to invade the three-dimensional structure (especially if they have been seeded simultaneously with the peptide solution) are dragged away when the culture medium is added, and are consequently lost. Before the gelling of the peptide solution upon the addition of culture medium, and the consequent entrapment of cells, the mechanical assistance to cell spread represented by shaking or centrifuging greatly contributed to the uniform distribution of the cells

throughout the construct. This had already been identified in a previous work [19] as advantageous when compared with a static seeding, and the results obtained here reveal that such mechanical assistance can be performed by any means available (shaker, centrifuge) without significant differences in its outcome, at least in the range of the parameters tested here.

These observations, gained from the experiments on small scaffolds samples, resulted in a biohybrid assembly method consisting in the following steps: 1) a pre-loading of the scaffold with the peptide solution with the help of syringe vacuum to force the viscous solution to penetrate into the pores of the hydrophobic scaffold, 2) the injection of the cell suspension within the filled scaffolds' pores, and 3) a smooth shaking of the biohybrid to distribute the cells before the peptide solution was gelled by adding culture medium, and 4) subsequent incubation of the construct.

Next, this assembly protocol was scaled-up to the needs of the large 5 X 5 cm^2 scaffolds intended as myocardial patches for the preclinical study.

The transition from the manufacture of small scaffolds to that of large format ones posed specific technical problems. Achieving a uniform sintering of the microbeads throughout the large porogen template is critical to guarantee the homogeneity of the porous structure of the ensuing scaffold, especially the pore interconnectivity. Furthermore, large scaffolds require a much more careful handling when swollen in solvents during the successive rinsings to obtain one-piece non-defective structures. A quality check to control these aspects had to be introduced, based on the inspection of SEM images of the structures obtained in different selected spots of the large patches.

As regards the cell seeding, these large scaffolds required multiple, uniformly distributed, injection sites with a Hamilton syringe. To scale-up the results obtained from the experiments on the smaller samples, the area of the large patch was divided

into units of the same area as the smaller samples, where it had been established that uniform cell distribution could be achieved in 24 h with the selected protocol. This resulted in a number of 50 injections uniformly spaced through the large scaffold. Once the cells were seeded, a mechanically assisted dispersion greatly helped the uniform colonization of the construct. Both methods here studied were equally effective with the small samples; nonetheless, in view of the technical limitations facing centrifugation of large biohybrids, the simpler shaker-assisted protocol was finally selected.

5. Conclusions

An effective protocol for assembling large biohybrid patches consisting in an elastomeric scaffold with a peptide-gel filling entrapping ASCs has been established. The alternative options in the way to achieve this goal have been settled with different experiments, and specific difficulties are discussed related to the fabrication of large uniform scaffolds with a high, interconnected porosity, their manipulation in the different stages, and the incorporation of the peptide solution and a high concentration of cells homogeneously distributed. The assembly protocol of these large biohybrid patches consisted in the incoporation of the peptide solution into the scaffold's pores, the injection of the cells in different selected locations, their dispersion by shaking prior to the gelling of the peptide hydrogel, and the incubation of the hybrid construct. This sequence ensures that after a 24 h *in vitro* culture such biohybrids host a large number of viable cells uniformly distributed throughout the large area of the patch, and are ready to be implanted in the infarcted myocardium.

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