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Grid Polymeric Scaffolds with Polypeptide Gel Filling as Patches for Infarcted Tissue Regeneration

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Abstract— **Scaffolds of poly(ethyl acrylate) (PEA) with interconnected cylindrical orthogonal pores filled with a self-assembling peptide (SAP) gel are here proposed as patches for infarcted tissue regeneration. These combined systems aim to support cell therapy and meet further requirements posed by the application: the three-dimensional architecture of the elastomeric scaffold is expected to lodge the cells of interest in the damaged zone avoiding their death or migration, and at the same time conduct cell behavior and give mechanical support if necessary; the ECM-like polypeptide gel provides a cell-friendly aqueous microenvironment, facilitates diffusion of nutrients and cell wastes and is expected to improve the distribution and viability of the seeded cells within the pores and stimulate angiogenesis.**

I. Introduction

Some regenerative strategies demand combinations of complementary materials in order to fulfill multiple requirements; for instance, in cardiac or neural tissue engineering, not only the cells survival and integration with the native tissue are crucial, but also vascularization, and mechanical support of the infarcted non-contractile myocardial tissue in the first case or addressed axonal guidance in the latter. Hyaluronic acid (HA), fibrin and collagen gels have recently been employed as fillings or coatings in combination with different polymeric structures as potential candidates with regenerative purposes [1-6]. In a previous work [7], envisaging a variety of applications where combined systems could be useful, the authors developed a procedure to obtain from coatings of controlled thicknesses to fillings of HA gel in poly(ethyl acrylate) (PEA) scaffolds with cylindrical crossed pores, combining different solution concentrations and adsorption times, or varying the number of filling-drying-*in situ* cross-linking cycles.

In the extreme, if the gel completely fills the scaffold's pores, it may act as an encapsulating medium for the cells, improving their survival and hindering their migration under otherwise adverse circumstances; in the meantime it could incorporate and deliver active molecules such as drugs or growth factors in a controlled manner to conduct cells behavior or stimulate an angiogenic response, so that a bi- or multi-functional hybrid, vehicle of cells and molecules, could be assembled if required. This hypothesis was advanced as the basis of the RECATABI project [8], to support cardiac cell therapy by a combination of a polymeric scaffold and a cell-friendly nano-fibrillar hydrogel. In this work we propose as combined systems the same grid PEA scaffolds, which pores are filled with the commercial self-assembling peptide (SAP) hydrogel RAD16-I: the polymeric scaffold will provide the three-dimensional context to nurse the cells to be incorporated, mechanical support and/or structural guidance, and its soft gel filling will offer the necessary microenvironment and functionality at the cellular or molecular level. These polypeptides are multiple of the RADA sequence, with alternate hydrophobic and hydrophilic lateral groups. They are injectable in aqueous solution, and form β–sheet nanofibers percolating in a gel as a response to pH changes or elevations in the saline concentration when being injected *in vivo* or culture medium is added *in vitro* [9-11]. They have been found to promote the formation of capillaries [11,12], enhance survival of cardiac myocytes [13] and neural stem cells [14] and the formation of synapses [15] *in vitro*, which reinforces its potential benefit in the proposed applications. This work deals with the incorporation and gelation of the polypeptide to these typology of pores and the development of the combined systems obtained.

II. Materials and Methods

A. Fabrication of the grid PEA scaffolds

Scaffolds of polyethyl acrylate (PEA) with interconnected cylindrical orthogonal pores were obtained as described in [7,16,17]. Porogenic templates were previously obtained by sintering 8 layers of nylon fabrics with a nominal thread diameter of 150 μm and a mesh opening of 300 μm (SAATI S.A.). The monomeric mixture, consisting of ethyl acrylate (99%, Aldrich), 1 wt% of benzoin (98%, Scharlau) as photo-initiator and 2 wt% of ethyleneglycol dimethacrylate (98%, Aldrich) as cross-linker, was injected in a porogen template, polymerized for 24 h under a UV source, and post-polymerized for 24 h more in an oven at 90ºC. Next, the nylon templates were removed by dissolution in nitric acid (30%, Aldrich), which was afterwards slowly exchanged with water. The scaffolds obtained were cut to 8 mm-diameter samples, dried under vacuum and stored until use.

B. Incorporation of SAPs and in situ gelation

A 0.25% (w/v) aqueous solution of the self-assembling peptides RAD16-I (PuraMatrix™ 1% (w/v), BD Biosciences) was prepared and located in a syringe together with half the scaffolds. After removal of the air, the luer taper of the

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syringe was sealed and several movements of the piston were performed to force the viscous solution to penetrate in the pores of the hydrophobic PEA scaffolds. Next, the SAPs-filled scaffolds were placed in a Petri dish and a few droplets of phosphate buffered saline (PBS) solution were added to their boundary. The polypeptides were allowed to self-assembly for 30 min keeping the Petri dish closed to avoid their drying.

C. Characterization of the combined systems

The morphology of empty scaffolds was observed in a JSM 6300 (JEOL Ltd., Tokyo, Japan) scanning electron microscope (SEM), with the samples previously sputter-coated with gold, at 15 kV of acceleration voltage and 15 mm of working distance. The samples were previously fractured in liquid nitrogen to obtain transversal images. The experimental average pores diameter was determined with the free ImageJ software (National Institute of Mental Health, Bethesda, Maryland, USA).

The porosity of these scaffolds, π, defined as the pore volume fraction of the scaffolds:

$$\pi = V^{pore}/V = 1 - m/\rho V \quad (1)$$

was obtained through the density of PEA, ρ (1.13 g/cm^3 [18]), weight, m, and total volume of each scaffold (obtained from their measured linear dimensions), V. Assays were performed in triplicate. A Mettler AE 240 balance (Mettler-Toledo Inc., Columbus, OH, USA) was used for this purpose.

Tensile tests were performed in the longitudinal direction of 0.5 x 3 cm^2 scaffolds and control non-porous PEA films in a Microtest SCM3000 95 (Microtest S.A., Madrid, Spain) device at a strain rate of 0.2 mm/min until fracture. These measurements were also carried out in triplicate.

The incorporation of SAPs and their efficient *in situ* gelation once inside the pores was assessed by cryoSEM in a JSM5410 (JEOL Ltd., Tokyo, Japan) device equipped with a cryounit (Oxford CT 1500). Cross sections were previously obtained by immersion of the samples in liquid nitrogen. Water entrapped by the SAPs within the pores was sublimated at -70ºC in the cryogenic unit for 15 min in vacuum to observe the remaining structure. Then, the samples were gold-sputtered and observed at 15 kV and 15 mm of working distance.

Mechanical compression tests were performed on scaffolds bare and filled with SAPs before and after gelling, 5 replicas each, to find out any influence of the presence of the SAPs sol within the pores, or its gelling. Measurements were carried out in a Seiko EXSTAR TMA/ss6000 equipment (Seiko Instruments Inc., Chiba, Japan), from 0 to 150 g, at 10 g/min, at room temperature. The excess of SAPs on the surface of the scaffolds was carefully removed before measurements.

D. Cytotoxicity of the combined systems

Cytotoxicity experiments were carried out in this work as the first to assess the biological development of the proposed combined systems. L929 mouse fibroblasts (C34/An connective tissue, Sigma Aldrich) in its 10th passage were employed for this purpose. All scaffolds were sterilized previously with a 25 kGy dose of gamma irradiation in a ^{60}Co source (Aragogamma, Barcelona, Spain). 50 mg-pieces of scaffolds were employed, half of them filled with the SAPs solution and allowed to gel. A piece of latex disinfected with 70% ethanol, was employed as cytotoxic negative control (-C). All samples were preconditioned in Dulbecco's phosphate-buffered saline (DPBS; Sigma) 3 times for 20 min and twice for 1 h. Next, they were placed with 6 ml of culture medium in a Falcon tube each, and incubated at 37ºC for 24 h in a shaking bath at 60 rpm in order to obtain the extracts of the materials, to be later employed as culture medium of the cells. Culture medium was prepared with 1 g Dulbecco's modified Eagle medium 4.5 g/l glucose without red phenol dye (DMEM; Fisher), 10 g of fetal bovine serum (FBS; Invitrogen), 0.37 g $NaHCO_3$ and 1 ml of antibiotics (Penicillin/Streptomycin, Sigma), diluted with 100 ml of extra distilled milliQ water (Scharlau), its pH adjusted to 7.4 and filtrated.

In parallel, the L929 fibroblasts were seeded in a 96-well plate, 3500 cells/well and incubated in 200 µl of culture medium at 37ºC and 5% CO_2. After 1 day, the culture medium was replaced by the extracts of the materials or negative controls. In positive controls (+C), it was replaced by fresh culture medium.

To study cell proliferation the colorimetric MTS assay ((4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, Call titer 96 Aqueous One Solution cell proliferation assay Promega, USA) was performed after 1, 3, 7 and 14 days of incubation in the extracts without renewal. At each time, the extract (or culture medium in the case of the positive controls) of the corresponding wells was removed and the wells were rinsed with DPBS. The reactant solution (200 µL containing 1:5 volume ratio of MTS reagent/serum-free culture medium) was added to each well and incubated for 3 h with the reagent in the dark inside the incubator at 37ºC and 5% CO_2. Then, 100 µL aliquots were pipetted into new wells. Absorbance was measured at 492 nm with a Victor Multilabel Counter 1420 spectrophotometer (Perkin Elmer, Waltham, MA; USA), taking as reference MTS reagent solution incubated likewise in wells empty of cells.

III. Results and Discussion

The (cryo)SEM images of both empty and SAPs-filled PEA scaffolds in a frontal view and cross section are shown in Fig. 1. The empty ones (a, b) show cylindrical interconnected orthogonal pores, *i.e.*, cylindrical crossed pores in parallel planes and some cylindrical pores perpendicular to the previous ones due to interconnections between layers at different points during sinterization of the nylon layers. The different layers of fabrics can be clearly observed in the cross section image (a). The porosity of these scaffolds resulted in 76.4±6.1% and the effective average diameter of the pores is 149.08±17.02 µm, which coincides with the nominal diameter of the thread.

The tensile modulus of the grid PEA scaffolds, 0.04±0.02 MPa, was obtained as the average of the initial slope in the strain-stress plots of the different replicas. This value is proper for an elastomeric polymer. The so-called reduced

tensile modulus of a scaffold, E_R, normalized taking into account its measured porosity, is to follow

$$E_R = E(1-\pi)^2 \quad (2)$$

according to [19], where E is the modulus obtained for the bulk polymer, for PEA films in this case. Being this tensile modulus of 0.84±0.08 MPa, the equation fits well the tensile behavior of the scaffolds.

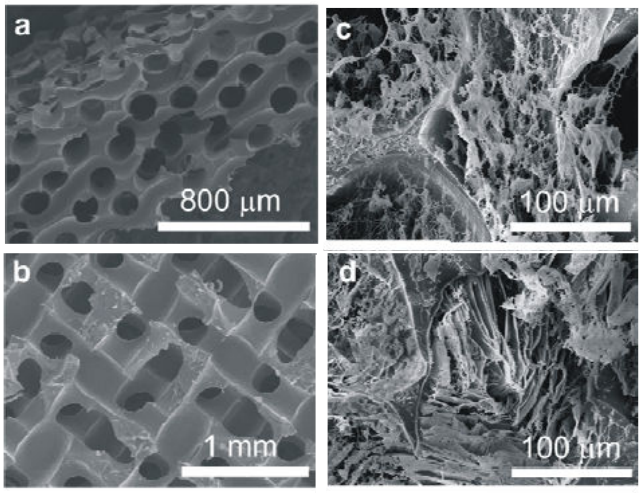

Figure 1. SEM images of the PEA scaffolds: (a) cross section, (b) surface. CryoSEM cross section images of the PEA scaffolds loaded with 0.25% SAPs solution (a) and (b) after gelling with PBS.

The *in vacuo* SAPs loading and *in situ* gelling procedure allowed to effectively fill the cylindrical orthogonal pores of these scaffolds with the peptide hydrogel, Fig. 1 (c, d), in spite of the relatively little interconnected structure (in comparison to sponge-like scaffolds, for example). Before gelling, the peptide solution leaves residual stretched-out fibers as its water sublimates in the cryogenic device. After gelation, the SAPs continuous network shows a honeycomb-like structure because of a different, more impeded, sublimation of its water.

The combined systems were characterized mechanically by compressive tests, to check any influence of the soft peptide filling on the different stages of the scaffolds compression. The stress-strain profiles of the bare scaffolds and those filled with the sol (non-self-assembled SAPs) or the gel (self-assembled SAPs), Fig. 2, show two different zones: an initial linear behavior extending up to 30% deformation and 30 kPa stress, the change of slope is abrupt, and the second linear zone occurs for strains greater than 70% at stresses above 200 kPa.

From both linear zones, the Young moduli, E_1 and E_2, were calculated as the slopes and are shown as insets in Fig. 2. Only the first elastic modulus increases slightly when the pores are filled with the aqueous peptide solution or gel. The second one does not seem to depend on the presence or not of the SAPs or their sol/gel state within the PEA scaffolds, its value remaining quite invariant. This behavior is likely due to the orthogonal structure of such scaffolds, which leaves solid PEA pillars that are compressed progressively from the very beginning; here the resistance of the polypeptide solution and even more that of the gel has an influence increasing the elastic modulus. The abrupt collapse of the pores leads to the second compressive linear behavior, now independent of the filling for it has been extruded, in any of its physical states, from the pores.

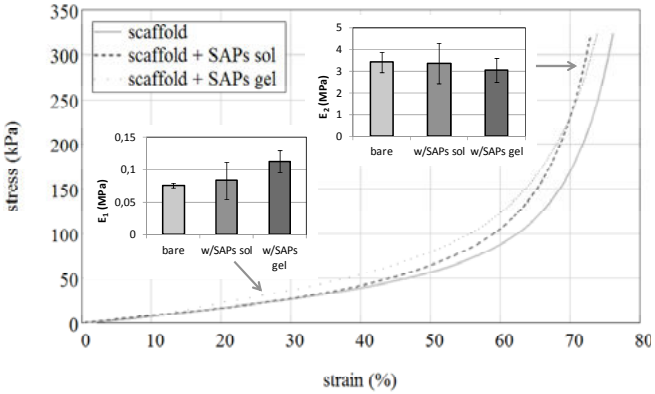

Figure 2. Stress-strain curves obtained from compressive experiments performed on bare, filled with SAPs (w/ SAPs sol) and filled with SAPs and gelled (w/ SAPs gel) scaffolds. Insets: Young moduli calculated in the first, E_1, and second, E_2, zone of the profiles.

Fig. 3 shows the absorbances after the MTS assay on fibroblasts cultured in the extracts of bare scaffolds, and filled with SAPs and gelled, together with positive (culture medium) and negative (latex) controls, for 1, 3, 7 and 14 days. Neither the scaffolds nor the combined systems are toxic for the cells; conversely, the negative control does show cytotoxic behaviour, as expected. The absorbances given by fibroblasts cultured in the scaffolds' extracts are very similar to those cultured in culture medium: low at short times, increase up to day 7, and decrease afterwards for lack of nutrients (the extracts are not renewed during the experiment).

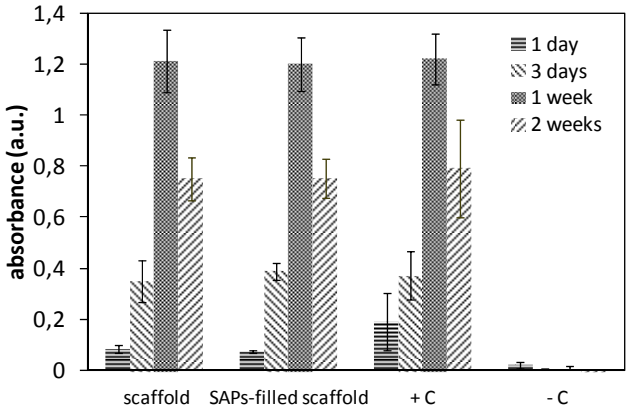

Figure 3. Citotoxicity analysis of bare scaffolds and combined systems. MTS test absorbance results for the proliferation of L929 fibroblasts in extracts of bare and SAP-filled PEA scaffolds, positive (culture medium) and negative (extract from latex) controls after 1, 3, 7 and 14 days of culture

IV. Conclusion

Scaffolds of polyethyl acrylate (PEA) with cylindrical orthogonal pores (approx. 150 µm-diameter, 76.4% porosity) were obtained by a template leaching technique. Their pores could be effectively filled with a SAPs aqueous solution, despite the low wettability of PEA and the relatively low

porosity and close and tortuous structure of these scaffolds, following an *in vacuo* loading. Once incorporated, the SAPs could be efficiently gelled *in situ* with PBS or culture medium. These combined systems could find application in advanced tissue engineering therapies requiring, apart from a cell-friendly gelly microenvironment, a larger-scale three-dimensional context with enhanced mechanical properties or a specific architecture to conduct cell fate.

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