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Additional Information

Peptide gel in a scaffold as a composite matrix for endothelial cells

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 # Equal contribution

Abstract

The performance of a composite environment with human umbilical vein endothelial cells (HUVECs) has been studied to provide an *in vitro* proof of concept of their potential of being easily vascularized. These cells were seeded in 1 mm-thick scaffolds whose pores had been filled with a self-assembling peptide gel, seeking to improve cell adhesion and viability of these very sensitive cells. The combination of the synthetic elastomer poly(ethyl acrylate), PEA, scaffold and the RAD16-I peptide gel provides cells with a friendly ECM-like environment inside a mechanically resistant structure. Immunocytochemistry, flow cytometry and scanning electron microscopy were used to evaluate the cell cultures. The presence of the self-assembling peptide filling the pores of the scaffolds resulted in a truly 3D nano-scale context mimicking the extracellular matrix environment, and led to increased cells survival, proliferation as well as developed cell-cell contacts. The combined system consisting of PEA scaffolds and RAD16-I, is a very interesting approach as seems to enhance endothelization, which is the first milestone to achieve vascularized constructs.

Keywords: scaffolds, self-assembling peptide, HUVEC, flow cytometry, endothelial markers

INTRODUCTION

The aim of tissue engineering is the development of artificial ways to assist tissue and organ recovery from degeneration or injury by a combination of cells, biomaterials and/or bioactive factors. Thick scaffolds are difficult to vascularize, and diffusion in them must be improved in order to ensure cell viability: oxygen and nutrients diffusion in scaffolds guarantee viable engineered tissues for thicknesses not greater than 100 microns.[1] Attempts to improve over this situation have been undertaken, such as adding channels to the scaffolds,[2] culturing the scaffolds in bioreactors with forced medium flow to favour the medium exchange and renewal,[3-5] or adding chemical compounds that can improve diffusion.[6]

The study here presented is a step towards the goal of designing easily vascularizable scaffolds with good mechanical properties. Human umbilical vein endothelial cells, HUVECs, were studied on different synthetic platforms. Acrylate copolymers with different degrees of hydrophilicity were produced by varying the number of –OH groups in the side chain of the polymers, and HUVECs were seeded on them to analyze on plane substrates the influence of surface chemistry and hydrophobicity on HUVEC cell adhesion. The best-performing composition (that of poly(ethyl acrylate), PEA) was next chosen to produce scaffolds with regularly interconnected spherical pores. PEA is a hydrophobic polymer that was shown in previous studies to behave very well *in vivo* with osteoblasts,[7] dental pulp stem cells,[8] neural cells,[9] keratocytes,[10] chondrocytes,[11] endothelial cells[12] and human embryonic stem cells,[13] as well as *in vivo* in rats.[14] The pores of PEA scaffolds were filled with a self-assembling peptide (SAP) gel, which is capable of forming fibrillar structures in the range of nanometres.[15,16] The system combining the acrylate scaffold and the SAP gel was presented in[17,18] where it was used with fibroblasts and adipose stem cells (ASCs) from the abdominal area, leading to dramatically increased cell seeding efficiency and proliferation as compared with bare scaffolds.

Synthetic SAP is a relatively new group of materials capable of providing a 3D environment in the scale of cells, and presents some advantages over other ECM-like proteins from animal origin. As laboratory-synthesized materials, there is no risk of illness transmission and there is a greater homogeneity among batches of the product. Moreover, chemical modifications like binding growth factors,[19] or short-sequence motifs of the basement membrane [20] can be introduced in the peptide sequence. This family of peptides has been reported to be non-immunogenic.[21] In our work unmodified RAD16-I was employed. This SAP has been previously employed with mouse embryonic stem cells[22] and fibroblasts,[23] also to obtain hepatocyte-like spheroid clusters[24] and to maintain functional hepatocytes,[25] with positive results.

HUVECs have been here employed because they have been extensively studied in therapeutic approaches to promote vascularized tissue growth *in vitro* and *in vivo*;[26,27] the development of prevascularized constructs to increase the probability of *in vivo* rapid vascularization has been pursued in.[28-30] Surface markers related with the occurrence of such process in the 2D substrates and the 3D structures have been here analyzed, as well as the effect of the SAP environment on the cell adhesion, proliferation, migration and formation of tubular structures by these cells.

MATERIALS AND METHODS

Planar substrates preparation

Planar copolymer substrates were obtained by radical polymerization of monomer mixtures consisting of either ethyl acrylate (EA; 99%, Sigma-Aldrich), 2-hydroxyethyl acrylate (HEA; 96% Sigma-Aldrich) or 50/50 EA/HEA mixtures with 2%wt ethyleneglycol dimethyl acrylate (EGDMA; 98%, Sigma-Aldrich) as crosslinker and 1%wt benzoin as initiator (Scharlab). The monomer mixtures were injected between two glass plates separated 1 mm, kept for 8 h in a UV oven for polymerization to take place, and next post-polymerized at 90ºC for 24 h. Finally, films (referred to

as 2D samples) were rinsed in boiling ethanol for 24 h for residual reactants extraction, changing the ethanol every 8 h, next dried under vacuum during 24 h and 24 h extra under vacuum and 40ºC.

Scaffolds preparation

Scaffolds (also referred to as 3D PEA) were obtained by injection of the EA-based monomer mixture previously described into a template obtained by sintering poly(methyl methacrylate) microspheres (PMMA; Colacryl dp 300, Lucite) as reported in.[31] The porogen templates soaked in monomer solutions were placed between glass plates and UV-polymerized for 24 h followed by a post-polymerization at 90ºC for another 24 h. The PMMA template was eliminated through dissolution in acetone in a soxhlet extractor during 8 h for 4 consecutive days, with daily acetone renewal. Next, acetone was progressively exchanged with water, the scaffolds were then dried at room conditions, under vacuum for 24 h and finally under vacuum at 40ºC for another 24 h.

Materials conditioning and sterilization

1 mm-thick films and scaffolds were punched into 5 or 8 mm-diameter discs, and sterilized with a 25 kGy dose of gamma irradiation in a ^{60}Co source (Aragogamma, Barcelona, Spain). Prior to their use the scaffolds were washed twice with either water, in the case of the scaffolds to be combined with the peptide, or with DPBS (Dulbecco's Phosphate Buffer Solution, Sigma-Aldrich) in the case of scaffolds to be used bare.

Incorporation of the self-assembling peptide into the scaffolds

Scaffolds were combined with the RAD16-I self-assembling peptide (SAP) (PuraMatrix™ 1, BD Biosciences) to fill the pores and provide a three-dimensional environment to the cells. Prior to its use the SAP solution was placed in a bath sonicator (Bandelin) for 30 minutes at 30 W. Then, a solution of 0.15% (w/v) was prepared by diluting the stock solution with water (extra pure,

Scharlau) and homogenized with a vortex. In order to incorporate the SAP to the scaffold micropores, the scaffolds were placed in a syringe together with the peptide solution, the air was removed and then the syringe was sealed with a luer sealer and five strokes of about 4 mL were applied to ensure the penetration of the solution into the scaffolds' pores throughout their thickness. Once completely filled with the peptide solution, the scaffolds were transferred into a new well plate. These composites will be referred to as PEA-SAP. Two experimental groups were established: one without SAP (PEA group), and one with SAP (PEA-SAP group). 2D films were employed as a control group.

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Gibco (C-003-5C, Spain). Cells in its 4^{th} passage were cultured in Medium 200 (Gibco, Life technologies, Spain) supplemented with foetal bovine serum (2% v/v), hydrocortisone (1 µg/ml), human epidermal growth factor (10 ng/ml), basic fibroblast growth factor (3 ng/ml) and heparin (10 µg/ml).

Briefly, HUVECs were grown in flasks (T75); when the culture reached 80% confluence, cells were tripsinized with trypsin-EDTA (Gibco, Life Technologies) after a phosphate buffer saline (PBS) rinse. Trypsin neutralizer solution (Gibco, Life Technologies, R-002-100) was added to stop the trypsin effect. After centrifugation (180 x g for 7 min), cells were counted and resuspended to be seeded at a density of 40.000 cells per film and 400.000 cells per scaffold, in a drop of 10 µL and 40 µL, respectively.

HUVECs were seeded in two series of PEA scaffolds: in one series, the pores had been previously filled with non-gelled 0.15 % (w/v) SAP solution, and in the other they had been filled with PBS instead. Both bare and filled scaffolds were cultured at 37°C in a humidified atmosphere under 5% CO_2 for 1, 3 and 7 days.

The seeded materials (films and scaffolds) were first incubated for 30 min in a shaker inside the incubator without the addition of more culture medium, in order to optimize the initial cell adhesion

to the materials. Next, the medium was completed to 400 μL per well (48-well plate). The culture medium was renewed every day.

Cell viability assay

In order to study the cell seeding efficiency and proliferation of the cells, a set of films were seeded at a density of 15.000 cells/cm^2, and a colorimetric MTS ((4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; Cell titer 96 Aqueous One Solution cell proliferation assay Promega, USA) assay was followed. Concisely, at the selected culture times (1, 3 and 7 days), samples were placed in new wells and washed twice with PBS. The cell viability test was performed following manufacturer's instructions: 200 μL of the 1:5 MTS reagent: phenol red free DMEM (Gibco) solution was added per well and incubated 3 h in the dark in the incubator. Next, 100 μL aliquots were transferred into new wells and read with a Victor Multilabel Counter 1420 spectrophotometer (Perkin Elmer, Waltham, MA; USA) at 490 nm. Three replicates per material and time were measured in duplicate.

Flow cytometry

At the selected culture times (1 and 7 days), flow cytometry scans were performed to evaluate the expression of monoclonal antibody PECAM-1, platelet/endothelial cell adhesion molecule (anti-CD31, Chemicon) and polyclonal VE Cadherin antibody (anti-CD144, Abcam), in cellular PEA scaffolds with and without the peptide gel in the pores and 2D PEA substrates (these latter were used as controls to compare). Briefly, once cells were trypsinized and blocked for 30 min with PBS with 1% BSA (PBSA), multi-colour staining was performed by incubating the cells with mouse monoclonal anti-human CD31 (1:100) and rabbit anti-human CD144 (1:100) for 30 min at 4°C. Then, samples were washed with PBS and incubated 30 min with Alexa 488 and Alexa 647 (Jackson Immunoresearch, 1:200) diluted with PBSA in the dark at 4ºC. Finally, the cells (n=10.000

per group) were washed twice with PBS and scanned in a flow cytometer (FC500, Beckman Coulter). Species-specific IgG isotype controls were used. Samples were analyzed by using RXP software.

By means of imaging flow cytometry, detailed images of every individual cell were obtained of PEA and PEA-SAP cultured scaffolds in order to quantify the mean intensity of each marker after 7 culture days. Hence, after incubation with primary and secondary antibodies, cellular scaffolds were trypsinized, and the cells extracted for imaging flow cytometry were diluted to 5 x 10^5 in 70 µL of FACS Buffer (buffered saline solution containing BSA 1%), according to the manufacturer's instructions. Images were acquired using an ImageStream (Amnis Corporation, USA) imaging flow cytometer with a blue 488 nm laser, and a bright field lamp at 60x magnification. Classifiers were set to eliminate cell debris and clusters prior to data acquisition based on low and high bright field areas, respectively. After acquisition, a compensation matrix was applied to all data aiming to correct spectral overlap. All analyses were completed on a population of spectrally compensated, single cells and by using IDEAS (Image data exploration and Analysis Software, Amnis Corporation).

Morphological characterization by SEM

Scanning electron microscopy (SEM) images were used to monitor the morphology features of HUVECs cultured in scaffolds with SAP, and the peptide within scaffolds pores without cells but incubated in culture medium in the same conditions. After culture, the substrates were immersed in 2.5% glutaraldehyde (Aname, Spain) in 0.1 M PB for 1 h at 37ºC; then cells were post-fixed with osmium tetroxide (OsO_4) for 1 h, and washed three times with Milli-Q water. Dehydration was performed by immersing the samples in increasing concentrations of ethanol in MilliQ water solutions (30%, 50%, 70%, 96%, and 100% ethanol). The dehydrated cells were critical-point dried

by using an Autosambri 814 device (Rockville, MD, USA), and sputter coated with gold (surfaces and sections) before observation (Hitachi S-4800) at 10 kV and 15 mm of working distance.

Immunocytochemistry

In an alternative set of tests, HUVECs cultured on the different materials were washed with PBS after 14 days of culture and fixed for 20 min in 4% paraformaldehyde. At that point cells were permeabilized for 60 min with PBS containing 0.1% Triton-X100 and 10% FBS, and incubated with anti-CD31 (1:100) and anti-CD144 (1:100) overnight at 4ºC in a humidified chamber. After three rinses with PBS, cultured scaffolds were incubated 1 h with the secondary antibody: goat anti-mouse Alexa 488 and goat anti-rabbit Alexa 647. The nuclei and F-actin filaments were counterstained with DAPI (Sigma, 1:5000) and phalloidin–FITC (Gibco, Life Technologies), during 10 min and 1 h, respectively. After three additional PBS rinses, immunoreactive cultured cells were observed. Next, 100 μm-thick sections were obtained by using a cryostat (Leica, CM 1900) and collected onto superfrost slides. Sections were observed under an Olympus FV1000 confocal microscope.

Statistics analysis

The results are expressed as mean ± standard deviation from at least three replicates. Data were analyzed pair wise with ANOVA test with Statgraphics Centurion XVI.I. Significance was assigned at p-values<0.05. Statistically significant differences are noted in the results.

RESULTS

HUVECs viability and proliferation on films with varying hydrophilicity

MTS results show great differences in terms of metabolic activity with the degree of hydrophilicity of the 2D substrate (Fig. 1); the best results of adhesion and proliferation were obtained on PEA

films, which is the most hydrophobic of the investigated materials. Significant differences were also observed between the other two materials: the 50/50 copolymer seems to favour a better initial adhesion than PHEA, and despite a decrease in absorbance after 3 days, it increases later on; contrastingly, the absorbance of cells cultured on PHEA does not change during the first 3 days of culture, and slightly increases at day 7. From these results, the material with the best performance in terms of cell viability, PEA, was selected for the next experiments.

Morphology of PEA scaffolds bare and combined with RAD16-I gel in the pores

The observation of bare scaffolds under SEM revealed a porous structure with a great porosity and very good interconnected pores and pore sizes around 90 microns (Fig. 2 A and B). Fig 2 C shows that the pores of the scaffold were uniformly filled with the peptide solution (as the arrows indicate), and that under culture conditions (*i.e.*, 37ºC in culture medium in an incubator), the gel filling remains stable after at least 7 days (Fig 2 C-F), showing the characteristic nanofibrillar network appearance of the self-assembled peptide.

Expression of cell surface markers and organization of cell cytoskeleton on PEA films and scaffolds

The expression in cultured PEA substrates of the cell adhesion molecule CD31, which localizes in the cell membrane and cell junctions, and the cell–cell adhesion glycoprotein VE-cadherin was monitored by flow cytometry and is shown in Fig. 3. After 1 day of culture, the expression of CD31 in 3D PEA (bare or with SAP) was lower than on planar (2D) PEA substrates. At day 7, this marker decreased in the cultures on 2D PEA and in bare scaffolds and controls, whereas it increased in cultured 3D PEA scaffolds combined with the SAP.

The expression of VE-cadherin was much higher in PEA scaffolds with SAP gel than in the other materials, after 1 and 7 days. For both 2D and 3D bare PEA, an increase in the expression of such

marker with culture time was observed, to a lesser extent, though, than in 3D PEA with the SAP filling.

Cell-cell interactions in 3D constructs were further explored by double-labelling and employing image flow cytometry (Amnis) in order to analyse the co-expression of VE-cadherin and CD31. As presented in Figure 4 A, an elevated expression of both markers was found in the scaffolds with the gel filling (70.4%) when compared with those bare (63.0%). No co-localizations were detected of both surface receptors (data not shown).

Representative images of the obtained fluorescence histograms, which show the frequency of occurrence of different fluorescence intensities, are presented for PEA-SAP (Figure 4 B); explanatory images of representative stained cells cultured in PEA-SAP are displayed in Figure 4 C. These pictures evidence that the CD31 marker is present in a greater fraction of the cells' surface than VE-cadherin (endothelial specific cell-cell adhesion molecule), which appears in relatively smaller areas.

HUVECs distribution throughout the scaffolds and expression of endothelial markers

Seeking to characterize the HUVECs distribution in PEA scaffolds from a morphological point of view, samples were stained with phalloidin and DAPI. Figure 5 displays representative CLSM images of stained cytoskeletons and nuclei of HUVECs in the studied 3D scaffolds (PEA in A, B; PEA-SAP in C, D). These images reveal that cells were properly adhered and able to grow in the scaffolds' pores, but showing slightly different features. In the case of bare PEA scaffolds, cells are located following the scaffolds' trabeculae (Fig. 5 A, B), start to establish cell-cell connections and adopt circular dispositions. In the PEA-SAP scaffold, more cells seem to occupy the space of the pores, a more organized distribution of actin filaments was observed (Fig. 5 C, D), and more intimate cell-cell interactions were established in the scaffolds pores.

The expression of CD31 and VE-cadherin was assessed by immunocytochemistry and observed at higher magnification under confocal laser microscopy (Fig. 6). After selected times, HUVECs cultured in both materials were able to express both markers at cell-cell contact regions, along the cell boundary in contact with their neighbours and also with the scaffold surface. CD31 and VE-cadherin were localized in a broad area between cells nuclei, which represents the cell-cell contact zone overlapping. More immunopositive cells were observed in the case of PEA-SAP (Fig. 6 C, D) than in bare PEA scaffolds (Fig. 6 A, B).

Morphological characterization of HUVECs cultured in PEA-SAP scaffolds

For PEA-SAP, SEM images show a rounded cell morphology after 1 day of culture, but how at day 7 cells already coat the inner surfaces of the pores, adopting more extended and elongated morphologies (Fig. 7). Despite the presence of the self-assembling peptide, cells are also capable to adhere to the PEA surface. Cells tend to extend adopting circular dispositions in the pores (arrows in Fig. 7 B), not only following the geometry of the scaffold but also approaching neighbouring cells.

DISCUSSION

Questions raised by the combination of self-assembling peptides and PEA scaffolds with different degrees of porosity and morphology were previously studied.[17,32] PEA scaffolds with the peptide showed higher cell density and better distribution with fibroblasts and ASCs. In the present study, the potential of these relatively thick composite scaffolds to hold HUVECs cultures and be prevascularized before implantation was undertaken.

Initial cell cultures on 2D substrates showed an outstanding cell adhesion and proliferation on PEA compared with more hydrophilic (co)polymers P(EA-co-HEA) 50/50 and PHEA. The conformation acquired by ECM proteins when adsorbed onto synthetic surfaces is a major factor for the

biological performance of biomaterials.[33-35] The extent to which the adsorbed conformations of proteins alter the natural exposure of relevant active sites along the molecule depends on the nature of the protein-material interactions;[36] here the presence of hydrophilic or hydrophobic functionalities at the material surface, their density, and their topological distribution is of relevance. Very hydrophilic surfaces are preferentially covered by a layer of water molecules, thus hindering stable attachment of proteins. The effect of different adsorbed ECM proteins on HUVEC fate in culture has been studied on different surfaces,[37,38] and the nanotopography induced by alternating hydrophilic and hydrophobic domains in copolymer surfaces influences HUVEC density in culture.[39] The hydrophobicity and distribution of polar/non-polar groups of PEA has been previously reported to facilitate cell adhesion and spreading; indeed, laminin and fibronectin were found to form a fibrillar protein network that promotes cell attachment,[8,40,41] and this may be explanatory for the different cell densities of neural cells[42] and of HUVEC[12] on acrylate surfaces of different hydrophilicity.

In the 3D scaffolds cell invasion of the inner regions was observed for both bare and gel-filled PEA scaffolds following seeding; cells were distributed throughout the scaffold thickness with a spread cytoplasm. This result indicates that nutrient diffusion through the scaffolds is enough for cell survival. Due to their porosity, scaffolds can accommodate more cells, and facilitate their capacity to spread, migrate and colonize in comparison with 2D structures; thus, the cell density attained after 10 days of culture was higher in 3D scaffolds, as a greater amount of cells could be lodged within the pores after this time than on a flat substrate.

The stability of the RAD16-I gel is remarkable: it has been reported that it is capable to resist pH variations and temperatures up to 90ºC.[43] Moreover, in some cell culture applications, this gel has been employed as a scaffold itself [20,21,24] to the point of enduring at least 33 days under culture conditions.[23] The data obtained here (Figure 2) prove that, despite self-assembling *in situ* within the pores, the peptide solution formed a stable network throughout the scaffold, which remained

integral at least 7 days under culture conditions followed by the aggressive treatments involving the fixation, post-fixation and fracture prior to observation of scaffolds' sections under SEM. The relevant processes ensuring early cell adhesion and survival take place in a time shorter than 7 days, so the stability of the peptide network in this period guarantees its biological efficacy.

The presence of a complex 3D environment, as is the scaffold combined with the peptide gel, proved to be beneficial for CD31 expression and VE-cadherin: a significant increase in the number of cells expressing this marker was observed. Since VE-cadherin is a protein expressed in endothelial cell connections, the fact that the amount of positive cells increases is a good indicator for this system. The up-regulation of endothelial markers may be a sign of preserved potential of HUVECs to form interconnected capillary-like structures in PEA-SAP scaffolds, which could stimulate vasculogenesis.

The flow cytometry imaging allowed the follow-up of the spatial distribution and localization of the markers: there was no statistically significant co-localization of the markers expression, which is in accordance with previously published works.[44] It served as a verification of the proper stain of the cultured samples. The presence of the peptide increased the fraction of cells co-expressing both factors, which is to state that the number of junctions between endothelial cells increases when they are cultured in the scaffold-peptide context. This indicates that the PEA-SAP scaffolds are suitable systems to promote endothelial cell-cell contacts.

Cell adhesion, spreading and migration processes are known to depend on the cytoskeleton development and the morphological organization of the ECM.[45,46] As shown in Figure 5, clear differences in actin filaments are observed. Cells growing in PEA scaffolds display extended actin filaments, while those cultured in the PEA-SAP system tend to spread: on day 10 HUVECs coated ions. These changes in the systems' performance can be an effect of the peptide structural featurecompletely the scaffolds and revealed a more flattened morphology when compared to those in bare PEA ones. These results confirm that these cells adhere, proliferate and spread very well on

the PEA hydrophobic surface of the scaffolds; moreover, incorporating a self-assembling peptide within the pores results in a greater cell-cell and cell-material interacts (fibres in the same dimension scale of ECM fibres), and its ability to retain water up to 99.5% w/v,[47] which is an environment more alike to the growing conditions that cells have *in vivo*. Altogether these results are promising for the development of vascularizable precultured constructs possessing good biological and mechanical properties.

CONCLUSIONS

The combination of different materials providing a micrometric 3D scaffold and a nanometric 3D structure (self-assembling peptide) has a synergistic effect, enhancing their biological performance. HUVECs exhibit a better adhesion, survival, proliferation and interaction (cell-cell connections) when cultured in PEA scaffolds combined with SAP, than in bare ones. The expression of surface markers increased with the introduction of the self-assembling peptide, showing that this combination can enhance the endothelization process in the scaffolds pores. The obtained results corroborate that SAP represent a powerful tool for tissue engineering applications in combined 3D structures, as it creates an ECM-like permissive microenvironment for nutrients and gases diffusion, which favours cell migration and colonization and the endothelialisation of the construct, which is the first step to induce angiogenesis.

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Príncipe Felipe Research Center (Valencia, Spain) is thanked for the support and advice in flow cytometry experiments.

FIGURE CAPTIONS

Figure 1: MTS results of HUVECs cultured on PEA, a 50/50 %wt. P(EA-co-HEA) copolymer and PHEA films for 1, 3 and 7 days. Statistically significant differences inter and intragroup, unless noted otherwise (*).

Figure 2: SEM images of sections of bare scaffolds (A and D), and scaffolds filled with the SAP gel after 1 day in culture conditions (B and E), and 7 days (C and F). Arrows outline the presence of the peptide within the pores.

Figure 3: Flow cytometry analysis of CD31 (A) and VE-Cadherin (B) expression of HUVECs cultured on 2D, 3D bare PEA (PEA w/o SAP), and PEA scaffolds filled with SAP (PEA-SAP).

Figure 4: Population analysis of the percentage of HUVECs co-expressing CD31 and VE-Cadherin when cultured in 3D PEA and PEA-SAP (A). ImageStream fluorescence histograms of the fluorescence intensity for HUVECs stained with CD31-AF488 and VE-AF555 (B). Cell representative images including SSC (side scatter, blue, Ch01), CD31-AF488 (green, Ch03), VE-Cadherin- AF555 (red, Ch04) and bright field (grey, Ch05); the same single cell is shown in the row of images (C).

Figure 5: CLSM images showing the distribution of actin cytoskeleton (green) and nuclei (blue) of HUVECs in 3D PEA (A, B) and PEA-SAP (C, D) scaffolds after 10 days of culture. Asterisks indicate cell-cell contacts with circular dispositions. Scale bar = 100 μm (A, C) and 50 μm (B, D).

Figure 6: Images of cultured scaffolds stained against CD-31 (green) obtained by CLSM after 10 days of culture: (A, B) are bare PEA scaffolds and (C, D) are PEA-SAP scaffolds. HUVECs show an intimate contact in the PEA-SAP group (see white arrows in D). Scale bar = 100 μm (A, C) and 50 μm (B, D).

Figure 7: SEM images of the surfaces of scaffolds filled with SAP and cultured with HUVECs for 1 (A) and 7 days (B).

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