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Reinforced alginate/gelatin sponges functionalized by avidin/biotin binding strategy: a novel cardiac patch

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Abstract: Adequate mechanical properties to withstand the surgical procedure and decoration with bioactive molecules promoting tissue regeneration are crucial aspects in the development of successful matrices for cardiac tissue engineering. The aim of this work was the development of a novel cardiac patch based on a blend of alginate and gelatin, designed to combine the improvement of suture resistance with an effective growth factor immobilization. We defined the procedures to incorporate a poly(dioxanone) membrane within the alginate/gelatin sponges and to functionalize the biomaterial with insulin-like growth factor-1, using the avidin-biotin binding strategy. Morphological analysis of the reinforced scaffolds showed a porous structure and a good adhesion of the synthetic microporous membrane to the natural sponge. Infrared chemical imaging analysis demonstrated the efficacy of the chemical treatments performed for scaffold reinforcement and functionalization. A good hydrophilicity and an adequate permeability were shown by swelling and permeability tests. The inclusion of the synthetic membrane improved the viscoelastic properties, as measured by dynamic mechanical analysis, and suture retention force, under both dry and wet conditions. The in vitro and in vivo biological characterization showed that IGF-1 functionalization successfully enhanced cell adhesion and long term retention after implantation on the damaged myocardium, together with improved suturability by PDO reinforcement.

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Short Title: **A novel cardiac patch functionalized with biotinylated growth factor**

Abstract

Adequate mechanical properties to withstand the surgical procedure and decoration with bioactive molecules promoting tissue regeneration are crucial aspects in the development of successful matrices for cardiac tissue engineering. The aim of this work was the development of a novel cardiac patch based on a blend of alginate and gelatin, designed to combine the improvement of suture resistance with an effective growth factor immobilization. We defined the procedures to incorporate a poly(dioxanone) membrane within the alginate/gelatin sponges and to functionalize the biomaterial with insulin-like growth factor-1, using the avidin-biotin binding strategy. Morphological analysis of the reinforced scaffolds showed a porous structure and a good adhesion of the synthetic microporous membrane to the natural sponge. Infrared chemical imaging analysis demonstrated the efficacy of the chemical treatments performed for scaffold reinforcement and functionalization. A good hydrophilicity and an adequate permeability were shown by swelling and permeability tests. The inclusion of the synthetic membrane improved the viscoelastic properties, as measured by dynamic mechanical analysis, and suture retention force, under both dry and wet conditions. The *in vitro* and *in vivo* biological characterization showed that IGF-1 functionalization successfully enhanced cell adhesion and long term retention after implantation on the damaged myocardium, together with improved suturability by PDO reinforcement.

Keywords: polydioxanone, insulin-like growth factor 1, cardiac tissue engineering, suture retention

1. Introduction

Cardiovascular diseases represent the first cause of death and disability worldwide [1]. Cardiac cells are not able to efficiently replace the lost myocardium after infarction, ultimately leading to chronic cardiac dysfunction. End-stage heart failure can be currently treated by cardiac transplantation, but this procedure is limited by the shortage of organ donors [2]. Therefore, an increasing demand to develop new therapeutic strategies for patients affected by ischemic cardiomyopathy is still required. Over the past years, tissue engineering has emerged as a very promising strategy to treat the infarcted myocardium [3]. One of the most important requirements for the development of a successful tissue engineering solution is to mimic the natural tissue microenvironment by polymeric scaffolds able to provide a three-dimensional support promoting myocardial regeneration [4].

Among the different polymeric materials proposed for three-dimensional scaffold fabrication, polymers of natural origin are a promising choice, as they closely resemble the native environment of cardiac cells and are easily susceptible to *in vivo* degradation [5]. Collagen, gelatin, alginate and fibrin have been widely investigated as scaffold materials for myocardial repair. Although scaffolds based on natural polymers represent a promising option, if not appropriately reinforced they lack adequate mechanical strength to withstand the surgical procedures.

In addition to the appropriate selection of materials, another important aspect to achieve a successful cardiac tissue healing by tissue engineering is the "decoration" of the scaffolds with growth factors, which play an important role in promoting cell proliferation and differentiation [6]. However, an effective immobilization of growth factors is still a challenge, because chemical reagents and solvents used for conjugation can alter the biological properties of molecules, such as growth factors, that are easily susceptible to inactivation or denaturation. Moreover, loading methods are usually very expensive, as they require the use of large amount of growth factor to obtain only a partial immobilization.

The aim of this work was the development of a novel cardiac patch, able to improve suture resistance together with an effective growth factor immobilization.

The material used for scaffold preparation is a blend of alginate and gelatin, chosen to mimic the chemical composition and the interactions among components of the cardiac extracellular matrix (ECM) [7]. Adequate morphological, physicochemical, functional and biological properties of cross-linked alginate/gelatin sponges have been previously ascertained [8]. However, preliminary suturability tests, carried out on the alginate/gelatin scaffolds following a procedure described in literature [9], pointed out an inadequate resistance to sutures (unpublished data).

Therefore, in this work a poly(dioxanone) (PDO) membrane was introduced, as reinforcement, in the core of the alginate/gelatin sponges. PDO was chosen because provided with good flexibility, elasticity and biocompatibility and is commonly used as suture material [10]. The PDO membrane was prepared by phase inversion and the procedure for its inclusion in the core of the alginate/gelatin sponge was optimized.

The reinforced sponge was also subjected to functionalization by the avidin-biotin binding system. Avidin is a tetrameric glycoprotein found in egg white, with a specific and strong interaction (dissociation constant: 10^{15} M^{-1}) with biotin molecules [11]. The avidin-biotin bond is used to immobilize biomolecules on different surfaces, for several applications including chromatography, diagnostics, immunoassay, drug delivery [12]. Applications in the field of cell culture [13] and tissue engineering [14, 15] have been also investigated. However, to the best of our knowledge, the avidin-biotin binding strategy to produce functionalized three-dimensional preformed scaffolds for cardiac tissue engineering was never tested. Here the idea was to use, in the preparation of the scaffolds, a biotinylated gelatin, so that biotin is exposed on scaffold surface; avidin is then used as a bridge between biotin on scaffold surface and a biotinylated growth factor. Insulin-like growth factor-1 (IGF-1) was chosen for functionalization because of its ability to promote survival, homing, engraftment and differentiation of cardiac progenitor cells, as well as to improve revascularization [16-18].

The overall device concept is drafted in figure 1.

Developed prototypes underwent a complete characterization, including morphological, physicochemical, mechanical and functional analysis. *In vitro* cell culture tests were performed in order to evaluate the response of cardiac progenitor cells. Furthermore, *in vivo* tests were carried out in a small animal model.

2. Materials and methods

2.1 Materials

Alginic acid sodium salt (viscosity of 2% solution at 25°C ca. 250 cps), gelatin (type B from bovine skin), PDO, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), avidin, glutaraldehyde (GTA, 25% aqueous solution) and phosphate buffered saline (PBS) were supplied by SIGMA (St. Louis, MO). Biotinylated gelatin and biotinylated IGF-1 were provided by ibt-immunological and biochemical test systems GmbH (Binzwangen, DE). Hydrochloride solution, sodium hydroxide pellets, calcium chloride and dimethyl sulfoxide (DMSO) were from Carlo Erba Reagenti (Italy). All the other reagents were commercially available and used as received.

2.2 Preparation of PDO reinforced alginate/gelatin scaffold

A 10% w/v PDO solution in DMSO was prepared under constant stirring at 70°C. The solution was casted uniformly on a Teflon plate using a knife machine with a velocity of 1 m/min and a controllable height of the knife (set at 100 μm). The plate was immersed in bi-distilled water for 15 mins at room temperature, for phase inversion. The PDO membrane was subsequently removed from the plate and freeze-dried.

The following step was the modification with gelatin, in order to improve the adhesion with the alginate/gelatin layers. The procedure was adapted from a previous paper [19]. An alkaline hydrolysis treatment was performed on the PDO membrane, by immersion in a 0.025 M sodium hydroxide solution for 3 mins, at room temperature. During the reaction, the membrane was maintained

suspended inside the solution, in order to treat both surfaces. After hydrolysis, the membrane was immersed in 0.01 mol/L HCl for 2.5 mins, for the acidification of COO^- groups. The PDO membrane bearing carboxylic groups was immersed into a buffer solution (pH = 5) containing an EDC/NHS mixture, with a 3:1 M ratio, at 4 °C for 3 h. After several washings in deionized water to remove unreacted compounds, the PDO membrane was cut in a circular shape to fit the Petri dish and dipped in a 2% w/v solution of gelatin in bi-distilled water.

Alginate and gelatin were separately dissolved in bi-distilled water, at a concentration of 10% w/v, at 50°C. Adequate volumes of the two solutions were mixed to obtain a blend with a 20:80 weight ratio between alginate and gelatin, in order to mimic the composition of the cardiac ECM [7, 8].

Half volume of the blend was poured into a Petri dish and cooled for 30 mins at room temperature. The gelatin-treated PDO membrane was then positioned over the alginate/gelatin solution and the remaining blend was poured over the PDO membrane. The Petri dish was introduced into a freeze-drier. Freezing of the sample was performed at -25°C, followed by lyophilization using a ΔT of 10°C.

The obtained sponge was cross-linked by a double treatment: i) exposure to GTA vapors, for the cross-linking of gelatin and ii) immersion in a solution of calcium ions, for the ionic cross-linking of alginate, as described in a previous paper [8].

At the end of the cross-linking procedure, the scaffold was washed thrice with bi-distilled water, to remove traces of GTA and excess calcium ions, then freeze-dried again.

2.3 Scaffold functionalization

The first step of the functionalization procedure was the biotinylation of the scaffold, by dipping into a solution of biotinylated gelatin. Biotinylated gelatin was dissolved in bi-distilled water and a known volume of the solution was put in contact with the scaffold for 15 mins, until complete adsorption, in order to have 0.25 mg of biotinylated gelatin for each mg of gelatin in the scaffold.

After freeze-drying, biotinylated gelatin adsorbed on the scaffold was cross-linked by treatment with a solution of EDC in acetone/water 90/10 for 24 h. A quantity of EDC corresponding to 30% of

weight of biotinylated gelatin was used. The scaffold was washed thrice in bi-distilled water and then the freeze-drying process was repeated.

The second step was the treatment with avidin. Avidin was dissolved in PBS at a concentration of 0.5 mg/ml. The scaffold was immersed in 5 ml of avidin solution, until complete adsorption, washed thrice with bi-distilled water, to remove unlinked avidin, and then freeze-dried again.

The third step was the immobilization of biotinylated IGF-1 on the scaffold exposing avidin. The growth factor was dissolved in bi-distilled water and a known volume of the growth factor solution was adsorbed for 30 minutes at room temperature on the scaffold. For cell culture tests, two different IGF-1 concentrations were tested: 5.6 µg/ml and 10 µg/ml. Samples were then washed thrice with bi-distilled water to remove eventual unbound growth factor and the washing solutions were analysed spectrophotometrically in order to quantify the real amount of growth factor immobilized onto the scaffold surface.

A final freeze-drying process was performed on the functionalized scaffolds.

2.4 Morphological analysis

Morphological analysis of both PDO-reinforced sponge and PDO membrane was performed by scanning electron microscopy (SEM), using the microscope JSM 5600 (Jeol Ltd., Tokyo, Japan). SEM images were also analyzed by the ImageJ software (National Institutes of Health) to determine average pore dimension and porosity percentage. The percentage of porosity was calculated from the ratio between the total pore area and the total scaffold area.

2.5 Infrared Chemical Imaging analysis

Infrared analysis was performed with a Fourier transformed infrared (FT-IR) Spectrometer (Spectrum Spotlight 350 FT-NIR imaging system, Perkin Elmer, Waltham, MA, USA). The analysis was performed in attenuated total reflectance (ATR) mode. The penetration depth was less than 1 µm and the spectral resolution was 4 cm^{-1}.

Spectral images were acquired, using the infrared imaging system and analysed according to previously described procedures [8].

Near infrared (NIR) images in the range 7000-4000 cm^{-1} were also acquired in order to investigate the growth factor distribution.

2.6 Mechanical analysis

The characterization of the viscoelastic properties of the scaffolds was carried out using a dynamic mechanical analyser (DMA8000, Perkin-Elmer, Waltham, MA, USA), following the protocol described in our previous paper [8]. Before analysis, samples were equilibrated for 3 h in a solution simulating body fluids, at 37°C. Storage modulus (E'), loss modulus (E'') and tangent delta (tan δ) were evaluated.

2.7 Swelling test

The swelling properties of the scaffolds were evaluated exposing them to aqueous vapour at 37°C, following a standard procedure [8]. At appointed times, swelling percentage was evaluated according to the following equation: $(W_s - W_d)/W_d \times 100$, were W_d is the starting dry weight and W_s is the swollen weight.

2.8 Permeability test

A permeation cell was used to test the permeability of the scaffolds to bi-distilled water, inducing a flux through the scaffolds when subjected to a given pressure difference, as described in a previous paper [20].

The following parameters were calculated:

$L_p = \frac{J_v}{\Delta P}$ Hydraulic permeability ($cm^2 \times s/kg$)

$K = \mu \cdot S \cdot \Delta P$ Intrinsic permeability (from Darcy law, measured in μm^2)

where μ is the viscosity of the solvent and S is the thickness of the scaffold.

2.9 Suturability test

A test to evaluate the suturability of the produced scaffolds was performed following a procedure described in literature [9]. Briefly, samples were cut to obtain rectangular strips (length 40 mm, width 15 mm). A single 5–0 monofilament polyglyconate (Maxon^{TM}) suture was created 5 mm from the short edge of each sample and secured to a hook connected to a dynamometer (PCE group). An extension rate of 2 mm/s was used to pull the suture. Scaffolds were tested both in dry and in wet state, after equilibration for 15 mins in bi-distilled water. Suture retention force was considered to be the maximum force recorded by the dynamometer prior to pull-through of the suture.

2.10 In vitro study

2.10.1 Rat Cardiac progenitor Cells (rCPCs) isolation and culture

rCPCs were isolated from green Fluorescence positive (GFP^{pos}) rats [21] and cultured as previously described [18, 22]. rCPCs at passage 3 and 4 (P3-P4) were employed for the experiments carried on in this study.

2.10.2 DiI cell labeling

In order to detect rCPCs cultured on the scaffold, cells were stained before seeding with CellTracker CM-DiI (Invitrogen, C-7001) according to a previously described methodology [23].

2.10.3 Cell culture on PDO reinforced alginate/gelatin scaffolds

Alginate/gelatin scaffolds reinforced with PDO were cut to fit exactly the size of one well of 8 well chamber slides (BD, USA). Samples were sterilized by UV exposure for 15 min on each side, as we already did with alginate/gelatin films [7] and scaffolds [8]. UV irradiation is commonly used in literature for scaffold sterilization and a low risk of material properties alterations is reported for exposure times below 2 h [24]. After sample sterilization, DiI labeled rCPCs were counted and seeded at 45 × 10^3 cells/cm^2 density onto scaffolds containing 0.9 μg/cm^2 or 1.6 μg/cm^2 concentrations of

IGF-1. Cells cultured on scaffolds without IGF-1 were considered as control. Cell loaded membranes were evaluated 2, 7 and 14 days after cell plating. Quantification of cell adhesion was performed by "Image Pro Plus 4.0" software as previously described [23].

2.10.4 SEM analysis of cell seeding

In a subset of experiments, SEM analysis of rCPCs cultured for 14 days on alginate/gelatin scaffolds was performed. Briefly, after fixation with paraformaldehyde, samples were dehydrated and critically treated with 72 atm pCO_2 at 37 °C; the control and IGF-1 functionalized scaffolds were mounted on metal stubs and coated with gold to a thickness of 60 nm using a gold splutter and analyzed by SEM (Philips SEM 501, Eindhoven, The Netherlands).

2.11 In vivo study

In order to test the *in vivo* properties of functionalized alginate/gelatin scaffolds reinforced with PDO, experiments were performed on a cryoinjury (CI) rat model. In particular, suturability, cell adhesion to the scaffolds and myocardial remodeling were evaluated.

The study population consisted of male Wistar rats (Rattus norvegicus, Charles River, Italy) bred at the University of Parma departmental animal facility, weighing 230-280 g (BW).

The investigation was approved by the Veterinary Animal Care and Use Committee of the University of Parma and conformed to the National Ethical Guidelines (Italian Ministry of Health; D.L.vo 116, January 27, 1992) and the Guide for the Care and Use of Laboratory Animals (NIH publication no. 85–23, revised 1996).

In vivo studies in the CI model were performed employing alginate/gelatin scaffolds reinforced with PDO and functionalized with IGF-1 (0.9μg/cm^2). rCPC-seeded (Alginate/Gelatin/PDO + IGF-1 + rCPCs; n=4) or unseeded (Alginate/Gelatin/PDO + IGF-1; n=4) configurations were tested.

An additional group was represented by cryoinjured animals (Alginate/Gelatin/PDO + rCPCs; n=4) in which rCPC seeded alginate/gelatin scaffolds in the absence of IGF-1 were applied.

Cell seeding was performed as previously described in "*in vitro study*". After 2 days, alginate/Gelatin/PDO + rCPCs were surgically sutured to cover the damaged area of the rat hearts. Control groups (CTRL) were represented by animals subjected to myocardial damage without scaffolds application.

2.11.1 Surgical procedure

The surgical procedure and the subsequent macroscopic examination of the rat myocardium were performed according to a methodology well established in our laboratory and detailed in several publications [16, 17, 25].

Briefly, rats were anesthetized with a combination of ketamine (40 mg/kg, i.p, Imalgene, Merial, Milano, Italy) and Medetomidine Hydrochloride (0.15 mg/kg i.p., Domitor, Pfizer Italia S.r.l., Latina, Italy), and artificially ventilated (tidal volume: 8–9 µl/g; stroke rate: 165/min).

An incision was made at the left fourth intercostal space and cryoinjury was induced punching the epicardium by a 20-gauge copper needle freshly immersed in liquid nitrogen.

In each experimental group, scaffolds were sutured with 7-0 silk surgical thread in correspondence to the damaged area.

Sacrifice was performed at 10 days after CI. Rats were anesthetized as previously described and their Body Weight recorded.

The heart of anesthetized animals was arrested in diastole by injection of 5 ml of cadmium chloride (100 mmol/l iv) and briefly perfused at physiological mean arterial blood pressure with heparinized PBS, followed by perfusion with 10% of formalin solution.

2.11.2 Cardiac Anatomy

The heart was excised and fixed for 24 h in 10% formalin, and the right ventricle (RV) and the left ventricle (LV), including the septum, were separately weighed. The major cavitary axis of the LV was measured from the aortic valve to the apex under a stereomicroscope with a ruler calibrated exactly to 0.1 µm (2Biological Instruments). Subsequently, the LV was sliced in three 3 mm thick transverse sections corresponding respectively to the base, equatorial portion, and apex. On the

equatorial section, LV wall thickness and LV chamber diameter were measured using software for image analysis (Image Pro-plus, version 4.0; Media Cybernetics, USA). LV chamber volume was calculated according to the Dodge equation, which equalizes the ventricular cavity to an ellipsoid [26].

Finally, the basal, equatorial, and apical sections were embedded in paraffin, and 5-μm-thick sections were cut for morphometric and immunohistochemical studies.

2.11.3 Immunohistochemical analysis

The adhesion of GFP^{pos} rCPCs seeded on scaffolds was determined by immunohistochemistry.

GFP was detected by immunofluorescence. For this purpose, LV sections from different experimental groups were incubated with primary antibody (polyclonal goat anti-GFP, dilution 1:100, Abcam, UK). FITC-conjugated specific secondary antibody was used to detect the epitope. Nuclei were recognized by the blue fluorescence of 4',6-diamindine-2-phenyndole (DAPI, Sigma) staining. Immunostained sections were analyzed under a fluorescence microscope (Leica DMI6000B) in order to compute the number of GFP^{pos} rCPCs per mm^2 of myocardium.

2.12 Data management and statistics

The SPSS statistical package was used (SPSS, Chicago, IL, USA). Statistics of variables included mean ± standard error (S.E.M.), paired Student t-test, one- way analysis of variance (post-hoc analyses: Tukey test or Holm-Sidak test, when appropriate). Statistical significance was set at $p<0.05$, $p<0.01$ and $p<0.001$.

3. Results and Discussion

3.1 Morphological analysis

The aim of SEM analysis was to investigate the morphological properties of the produced scaffolds, especially in terms of porosity (pores dimension and interconnectivity), which can influence the interactions between scaffold and cells, as well as scaffold infiltration by nerves and capillaries and

the transport of nutrients necessary for cell metabolism. Moreover, morphological analysis was carried out to verify the adhesion of the PDO membrane to the alginate/gelatin layers, as well as to investigate any change in the alginate/gelatin sponge morphology after PDO inclusion.

SEM analysis was performed on PDO membrane and PDO reinforced alginate/gelatin sponges (figure 2). Images were acquired on sample surface (figure 2a, 2c) and on sample section (figure 2b, 2d).

As shown by SEM micrographs, the PDO membrane presented a microporous surface skin (figure 2a), while its section (figure 2b) was characterized by the presence of fingers, delimited by microporous walls. Fingers are normally obtained when a rapid phase inversion is carried out [27]. The alginate/gelatin sponge showed a porous structure, with pores of different sizes (figure 2c-d). According to the typical morphology of sponges obtained by freeze-drying [8], pores were characterized by a good interconnectivity. The presence of the PDO membrane did not alter the morphology of the two external layers of alginate/gelatin sponge (figure 2d) whose structure in not reinforced alginate/gelatin sponges was previously documented [8]. Moreover, PDO membrane properly adhered to the alginate/gelatin sponge, as no sites of detachment between the sponge and the synthetic membrane were observed by SEM.

Pore size and porosity percentage were quantified analyzing SEM images by Image J software. The PDO membrane had pores with an average diameter of 6 ± 1 μm and the porosity percentage, calculated from the ratio between pores area and total area, was 21.7 ± 3.3 %. Pores of PDO reinforced alginate/gelatin scaffold had an average diameter of 139 ± 37 μm and the porosity percentage was 55.1 ± 10.3 %. In a previous report on not reinforced alginate/gelatin scaffolds, we obtained pores with an average diameter of 198 ± 58 μm and a porosity percentage of 60 %, while the same parameters measured on decellularized myocardial tissue were, respectively, 56 ± 10 μm and 13% [8]. Thus, no significant variations in terms of porosity were observed with respect to not reinforced alginate/gelatin scaffolds. However, the reinforced scaffolds developed in this work showed a higher porosity with respect to the native tissue, which may exert a positive effect on promoting both cells colonization and nutrients availability.

3.2 Infrared Chemical Imaging analysis

Infrared analysis was performed on the scaffolds, at different steps of the preparation procedure, in order to verify the efficacy of the performed chemical treatments.

Infrared spectra were acquired for the PDO membrane, before and after the hydrolysis treatment, as well as after dipping in gelatin. As shown in figure 3a, PDO spectrum peaked at 1740 cm^{-1}, due to C=O. Basic hydrolysis of PDO determined the cleavage of the ester bonds, with the formation of COO^- groups (at 1600 cm^{-1}). After dipping in gelatin, the presence of the protein was shown by the typical absorption peaks due to Ammide I (at 1630 cm^{-1}) and Ammide II (at 1539 cm^{-1}).

In order to investigate the distribution of the protein on the membrane, the chemical map as a function of the ratio between the band at 1780-1714 cm^{-1} (due to PDO) and the band at 1714-1595 cm^{-1} (due to gelatin) was elaborated (figure 3b). The value of the band ratio (2.7) was almost constant on all the analysed surface, demonstrating a homogeneous distribution of gelatin.

Chemical Imaging analysis was also performed on a section of the reinforced scaffold, in a region close to the PDO membrane, to eventually point out changes induced by the presence of the synthetic membrane. The Chemical map is shown in figure 3c. The presence of the PDO membrane was demonstrated by a nearly 100 µm thick layer. The spectra acquired in this region were characterized by the presence of the typical PDO absorption band at 1740 cm^{-1}, while the spectra acquired in the region around the PDO membrane showed the typical absorption peaks of the two natural polymers: 1640 cm^{-1}, due to gelatin Ammide I; 1544 cm^{-1}, due to gelatin Ammide II and 1034 cm^{-1}, due alginate C-O-C (figure 3d). These results suggested that PDO did not alter the chemical properties of the natural polymer blend. In particular, the displacement of the typical absorption peaks, with respect to pure alginate (C-O-C = 1027 cm^{-1}) and pure gelatin (Ammide I = 1630 cm^{-1} and Ammide II = 1539 cm^{-1}), indicative of molecular interactions between the protein component and the polysaccharide component and previously observed in native sponges [8], were confirmed in PDO-reinforced sponges.

Chemical Imaging investigation in the near infrared region was performed to study the chemical modifications induced by dipping in biotinylated gelatin. NIR maps and spectra were acquired for alginate/gelatin/PDO sponges, before and after dipping (figure 4). All NIR spectra showed the presence of second overtone absorptions due to OH (5100 cm^{-1}), NH (4600 cm^{-1}) and CH (4378 cm^{-1}). In the spectra acquired after dipping, we observed an intensification of NH absorption and the presence of two additional bands (5800 cm^{-1} and 4800 cm^{-1}), which can be attributed to biotin. In order to investigate biotin distribution, the chemical map in function of biotin absorption peak (4800 cm^{-1}) was elaborated (figure 4c). Spectra acquired in different points of the chemical map showed the presence of biotin absorption peak, demonstrating a homogeneous distribution of biotinylated gelatin on the sponge (figure 4d).

Functionalized scaffolds were also characterized by chemical imaging analysis in μATR mode, at different steps of the functionalization procedure. Chemical maps were acquired for biotinylated scaffold, pure avidin, biontinylated scaffold treated with avidin and scaffold functionalized with the biotinylated growth factor. The medium spectra recorded for each of the previous mentioned chemical maps are collected in figure 3e-h. All the acquired spectra were similar, being protein the main component. In particular, avidin spectrum (figure 3f) showed a characteristic absorption peak at 1061 cm^{-1}, undetectable in the biotinylated scaffold (figure 3e) while present in the avidin treated scaffold (figure 3g), demonstrating the good outcome of this procedure. After growth factor binding, the avidin peak intensity decreased, because of the presence of the bioactive molecule (figure 3h).

3.3 Mechanical analysis

Mechanical properties of functionalized and not functionalized scaffolds were preliminary compared and no significant difference was found (data not shown). This result was expected, as there is no report in literature about the effect of growth factor immobilization on scaffold mechanical properties. Being not functionalized scaffolds significantly less expensive than the functionalized ones, complete investigation was performed on not functionalized scaffolds.

Results of the mechanical characterization, carried out by DMA, are collected in table 1.

As expected, increasing the oscillation frequency, an increase was observed for values of E' and E''. This result demonstrated that the stiffness of alginate/gelatin/PDO scaffolds increased with frequency increase. This stiffening involves both an increase in elasticity (E') and an increase in heat dissipation (E''). Values of tan δ did not show variations by increasing frequency, suggesting that E' and E'' increased with frequency at the same proportion. Values of E' were more than one order of magnitude higher than values of E'', with a tan δ equal to 0.04, and this result demonstrated the elastic behaviour of the developed scaffolds. In a previous paper, the same DMA analysis was carried out on not reinforced alginate/gelatin scaffolds and the values of E' and E'' were at least one order of magnitude smaller than those obtained in this work [8]. This result showed that the presence of the PDO membrane increased the stiffness of the alginate/gelatin scaffolds. Reinforced scaffolds resulted also stiffer than the native myocardium [8]. However, as reported in literature, a cardiac scaffold stiffer than the surrounding tissue can favour a positive outcome in terms of reduction of infarct expansion, attenuation of left ventricle remodeling, and amelioration of global left ventricle function [28].

3.4 Swelling test

Swelling test was performed to evaluate the ability of the obtained materials to absorb water, which is related to the hydrophilicity degree and depends on several factors, including the chemical properties of the materials used for scaffold preparation, the cross-linking degree, the interactions between components and the morphological properties of the developed scaffolds.

The swelling kinetics for the alginate/gelatin/PDO scaffolds and for the PDO membrane are reported in figure 5 and compared with that obtained for not reinforced alginate/gelatin scaffolds [8]. The PDO membrane showed a low swelling degree, with a plateau of around 10% after 96 hours, in agreement with the low hydrophilicity of the synthetic polymer [29]. For the alginate/gelatin/PDO scaffold, we observed a rapid water uptake during the first hours, then water absorption gradually proceeded, reaching a plateau of around 30% after 96 hours. Comparing these data with the swelling kinetics of

not reinforced alginate/gelatin scaffolds (swelling at plateau of around 67%, [8]), the presence of the PDO membrane induced a significant reduction in water absorption, although maintaining a good degree of hydrophilicity.

3.5 Permeability test

It is well known that one of the most important properties of the scaffolds for *in vitro* regeneration is their porosity and permeability, to provide cell growth nutrients transport and catabolites removal [30]. If cell culture is carried out in static conditions, nutrient transport can be of diffusive type, while with a cell culture in dynamic conditions based on the use of a bioreactor the transport might be of both diffusive and convective kind.

A permeability apparatus was used here to evaluate the hydraulic permeability (Lp), which is a quantitative index of the convective transport of a solution through a scaffold, and the coefficient K, called intrinsic permeability, which is dependent on the porosity of the scaffold. On the basis of the order of magnitude of the calculated K value, it is possible to evaluate the permeability degree of the membrane.

Permeability tests were performed on not reinforced alginate/gelatin sponges, PDO membrane and alginate/gelatin sponges reinforced with PDO. Calculated values of Lp and K are collected in table 2.

The obtained results showed that the introduction of the PDO membrane in the alginate/gelatin sponge produced a significant decrease of Lp and K values, with respect to not reinforced scaffold. Scaffold permeability, and in particular intrinsic permeability, is strictly connected to porosity and pore interconnectivity [31]. As discussed in the morphological analysis section, PDO membrane porosity was significantly lower than that of alginate/gelatin scaffold. Even if the presence of the PDO membrane did not reduce the overall porosity of the scaffold, representing only a 100 µm thick layer over a total scaffold thickness of around 4 mm, a significant impact on scaffold permeability was observed. However, values of permeability parameters obtained in PDO reinforced

alginate/gelatin scaffolds were comparable with those obtained for other scaffolds aiming at tissue engineering applications [32] and specifically applied to cardiac tissue [20].

3.6 Suturability test

Preformed three dimensional scaffolds for cardiac tissue engineering have to be implanted on the patient heart through a surgical procedure and their suture resistance is a fundamental parameter to avoid failures during implantation.

Results of suturability tests, performed on alginate/gelatin scaffolds with or without PDO reinforcement, under dry and wet conditions, are collected in table 3.

Obtained results showed that the introduction of the PDO membrane for scaffold reinforcement significantly increased the suture retention force of the alginate/gelatin scaffolds. This effect was particularly evident under wet conditions, where the assessment of suture retention force of not reinforced scaffolds was unfeasible due to a rapid rupture. Reference values of suture retention force for cardiovascular tissue are available in literature for human coronary artery (1.96 ± 1.1 N) and saphenous vein (1.92 ± 0.02 N) [33], while the suture retention force of a full thickness patch implanted in a human left ventricle was estimated equal to 0.61 ± 0.18 N [34]. After inclusion of the PDO membrane, values of suture retention force of our scaffolds were above these limits, both under dry and wet conditions. Therefore, the reinforced scaffolds developed in this work can be considered adequate for the aimed application.

3.7 In vitro study

Cell culture tests were performed on PDO reinforced alginate/gelatin scaffolds, functionalized with two different concentrations of biotinylated IGF-1 (0.9 μg/cm^2 and 1.6 μg/cm^2).

The evaluation of the presence of rCPCs two days after seeding revealed that higher concentrations of IGF-1 improved cell adhesion when compared to both IGF-1 free (2.67-fold increase) and 0.9μg/cm^2 IGF-1 functionalized (6.64-fold increase) scaffolds. On the other hand, low IGF-1 dose

better preserved long term cell adhesion since at 14 days a 3.18-fold increase in rCPC number was documented compared to 2 days cell culture. The increase in rCPCs adhesion produced by IGF-1 scaffold functionalization was also confirmed by SEM. On control scaffolds, round shaped cells weakly integrated with the membrane by few filopodia were observed (Figure 6a). Conversely, both doses of IGF-1 increased cell adhesion, promoted intercellular communication and full seeding of scaffolds by rCPCs (Figure 6b-c).

3.8 In vivo study

3.8.1 Cardiac Anatomy

Based on *in vitro* findings, further *in vivo* studies were performed only on scaffolds containing 0.9 μg of IGF-1/cm^2.

The alginate/gelatin/PDO scaffolds did not significantly affect rat body and cardiac weights 10 days after cryoinjury (CI, data not shown).

Application of alginate/gelatin/PDO scaffolds appeared to be able to reduce LV dilatation at 10 days after CI, although functionalization by IGF-1 did not result in an additive effect on this parameter. However, functionalized scaffolds seeded with rCPCs more effectively reduced LV dilatation, since a 1.33-fold decrease in chamber volume was measured at 10 days after surgical procedure compared to CTRL group (Table 4).

3.8.2 Immunohistochemical analysis

The evaluation of the number of GFP^{pos} rCPCs present on alginate/gelatin/PDO scaffolds (Fig. 7a-b), documented cells retention to the scaffold after 10 days from CI. Importantly, functionalization with IGF-1 significantly increased by 1.35-fold ($p=0.026$) the number of rCPCs within the alginate/gelatin/PDO scaffolds sutured to the damaged myocardium (Fig. 7c).

4. Conclusions

Here we report on a simple method to develop bioactive reinforced alginate/gelatin scaffolds. A microporous PDO membrane obtained by phase inversion was introduced within two alginate/gelatin sponge layers, producing a reinforced scaffold with improved mechanical properties and a resistance to suture adequate for surgical procedures. In addition to the introduction of the reinforcement membrane, the scaffolds were functionalized with biotinylated IGF-1, using the avidin-biotin binding strategy. *In vitro* and *in vivo* tests were performed on the produced prototypes. Overall, obtained results suggest that PDO reinforced alginate/gelatin sponges, decorated with biotinylated IGF-1, represent a promising system for promoting cardiac tissue regeneration.

In particular, for what concerns scaffold functionalization, both cell culture tests and *in vivo* tests showed that scaffold decoration with biotinylated IGF-1 promoted cell retention and reduced LV dilatation. These findings suggest that the avidin-biotin binding system, used for the first time in this work for the modification of three-dimensional preformed scaffolds for cardiac tissue engineering, is an effective strategy for scaffold decoration. Combining this strategy with the use of different bioactive molecules, it could be possible to create a complex microenvironment, controlling the endogenous regeneration of the injured myocardium.

With reference to scaffold reinforcement, results showed that the inclusion of the PDO membrane successfully improved the mechanical properties and the resistance to sutures of alginate/gelatin scaffolds. This achievement represents a significant step towards the clinical application of an engineered myocardial patch for the treatment of infarcted heart. However, the presence of the synthetic membrane partially reduced the hydrophilicity of the scaffold, as well as its permeability. Further investigations will aim at the design of different reinforcement structures, with a reduced impact on scaffold morphology and related properties, such as developing fiber-based support systems.

5. Acknowledgments

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Tables

	E' (MPa)	E'' (MPa)	Tan δ
1 Hz	7.67 ± 0.01	0.28 ± 0.01	0.04 ± 0.01
3.5 Hz	7.90 ± 0.01	0.31 ± 0.02	0.04 ± 0.01
10 Hz	7.99 ± 0.01	0.34 ± 0.03	0.04 ± 0.01

Table 1. Storage modulus (E'), loss modulus (E'') and tan delta of alginate/gelatin/PDO sponges, measured by DMA at three different frequencies (1, 3.5 and 10 Hz).

	Lp ($cm \times s^{-1} \times Pa^{-1}$)	K (μm^2)
Alginate/Gelatin	3.5×10^{-6}	2.5×10^{-2}
PDO	4.0×10^{-8}	1.1×10^{-4}
Alginate/Gelatin/PDO	6.5×10^{-7}	2.0×10^{-3}

Table 2. Values of Lp and K, calculated for alginate/gelatin sponge, PDO membrane, alginate/gelatin sponge reinforced with PDO.

	Suture retention force (N)	
	Dry Conditions	Wet conditions
Alginate/Gelatin	16.2 ± 0.3	n.d.
Alginate/Gelatin/PDO	24.4 ± 0.3	4.3 ± 1.3

Table 3. Values of suture retention force measured under dry and wet conditions for alginate/gelatin and alginate/gelatin/PDO scaffolds.

	Chamber Volume, mm^3 (Mean ± St.Dev.)
CTRL	360.01 ± 32.85
Alginate/Gelatin/PDO + rCPCs	278.78 ± 52.79
Alginate/Gelatin/PDO + IGF-1	296.28 ± 19.99
Alginate/Gelatin/PDO + IGF-1 + rCPCs	271.08 ± 72.78

Table 4. Measurements of Left Ventricular (LV) chamber volume in different experimental groups 10 days after cryoinjury. **CTRL**: cryoinjured rat in the absence of scaffolds; **IGF-1**: Insulin-like Growth Factor 1; **rCPCs**: rat Cardiac Progenitor Cells.

Figure Legend

Figure 1. Schematic representation of the alginate/gelatin cardiac patch developed in this work, comprising: (i) reinforcement with PDO membrane, for improved suture resistance; (ii) IGF-1 immobilization, through avidin-biotin binding strategy.

Figure 2. SEM micrographs of PDO membrane, (a) surface and (b) section; PDO reinforced alginate/gelatin scaffolds, (c) surface and (d) section.

Figure 3. (a) Infrared spectra of untreated PDO, hydrolysed PDO and gelatin functionalized PDO; (b) Chemical map of PDO membrane functionalized with gelatin, in function of the band ratio between the band at 1780-1714 cm^{-1} (due to PDO) and the band at 1714-1595 cm^{-1} (due to gelatin); (c) Chemical map of alginate/gelatin sponge reinforced with PDO; (d) Spectra from the chemical map in (c). (e-h) Infrared medium spectra of: (e) biotinylated scaffold; (f) avidin; (g) scaffold treated with avidin and (h) scaffold functionalized with biotinylated growth factor.

Figure 4. (a) NIR spectra of not functionalized alginate/gelatin/PDO sponge; (b) NIR spectra of alginate/gelatin/PDO sponge, after dipping in biotinylated gelatin; (c) NIR chemical map in function of biotin absorption peak at 4800 cm^{-1}; (d) spectra acquired in different areas of the NIR chemical map.

Figure 5. Swelling kinetic for PDO membrane and for alginate/gelatin/PDO scaffolds. Swelling test was performed by exposure to aqueous vapour. Results were compared with those obtained for not reinforced alginate/gelatin scaffolds [8].

Figure 6. SEM images of rCPCs two weeks after seeding on alginate/gelatin/PDO (**a**), alginate/gelatin/PDO + IGF-1 0.9 µg/cm^2 (**b**) and alginate/gelatin/PDO + IGF-1 1.6 µg/cm^2 (**c**). Scale bars: a=10µm, b and c=100µm.

Figure 7. Immunofluorescence detection of GFP^{pos} rCPCs (Green) on alginate/gelatin/PDO (**a**) and alginate/gelatin/PDO + IGF-1 (**b**) patches, 10 days after implantation on cryoinjured rat hearts. Nuclei are recognized by the blue fluorescence of DAPI. Scale Bars: **a** and **b** = 50µm. Bar graph in **c**

documents the increased number of GFP^{pos} rCPCs on IGF-1 functionalized alginate/gelatin/PDO patches. * $p<0.05$ vs alginate/gelatin/PDO.

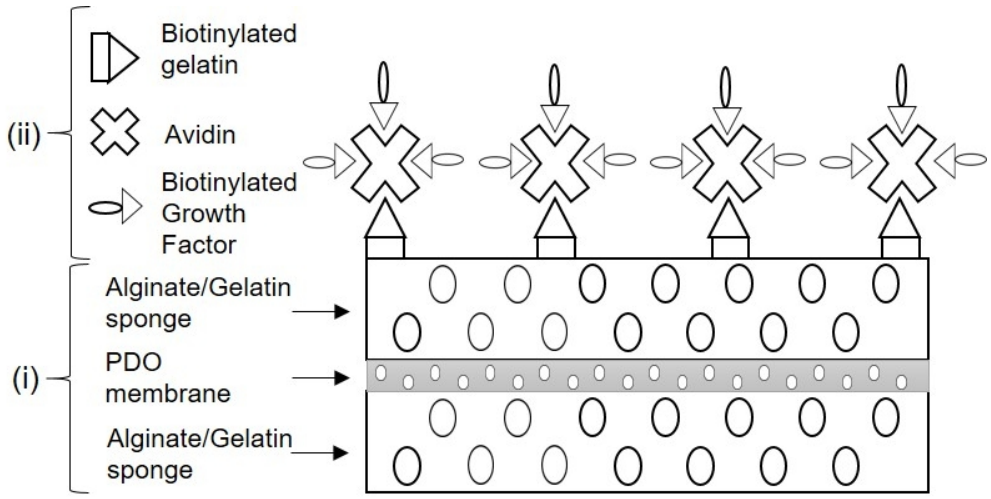

Figure 1. Schematic representation of the alginate/gelatin cardiac patch developed in this work, comprising: (i) reinforcement with PDO membrane, for improved suture resistance; (ii) IGF-1 immobilization, through avidin-biotin binding strategy.

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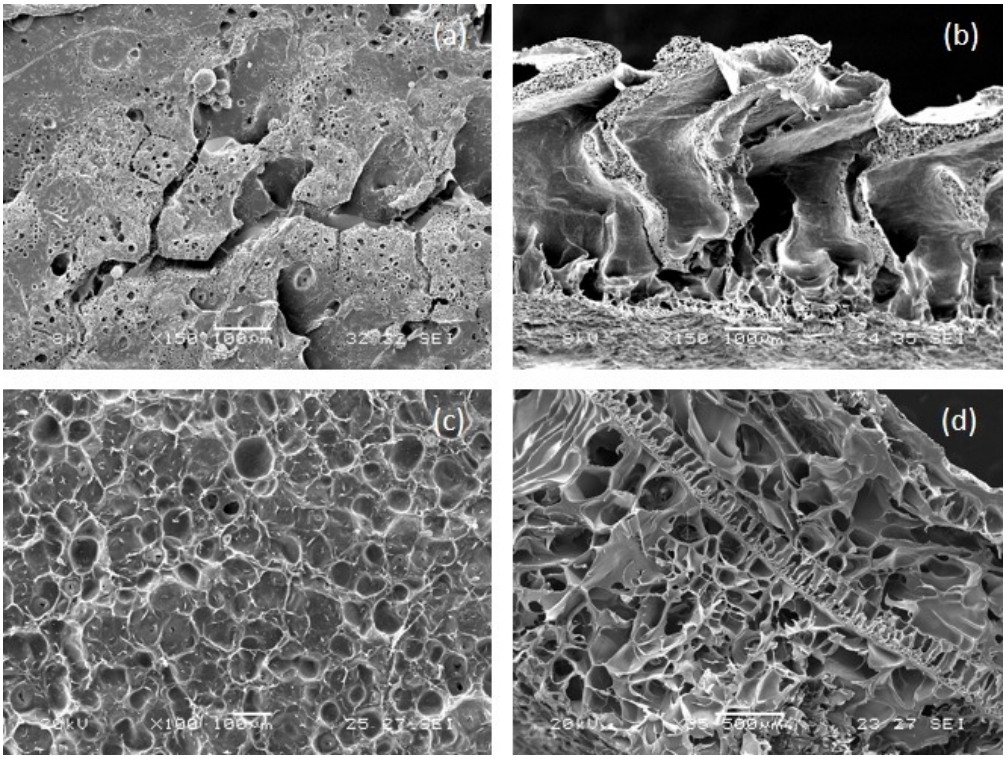

Figure 2. SEM micrographs of PDO membrane, (a) surface and (b) section; PDO reinforced alginate/gelatin scaffolds, (c) surface and (d) section.

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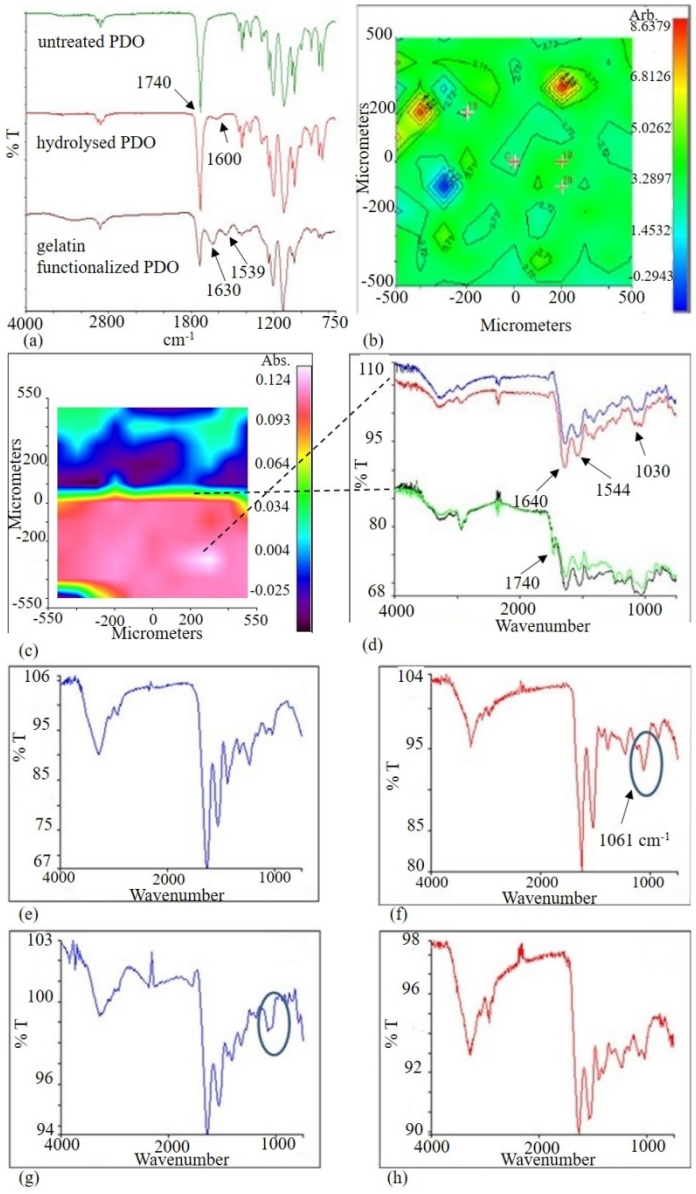

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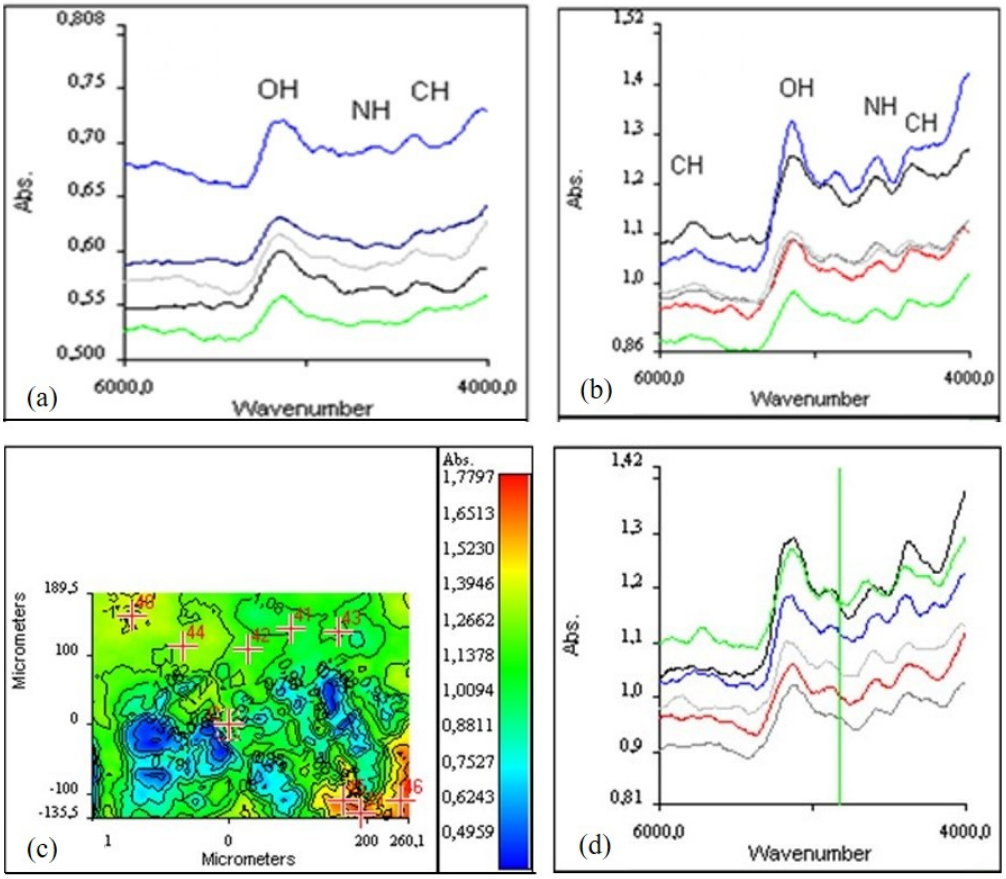

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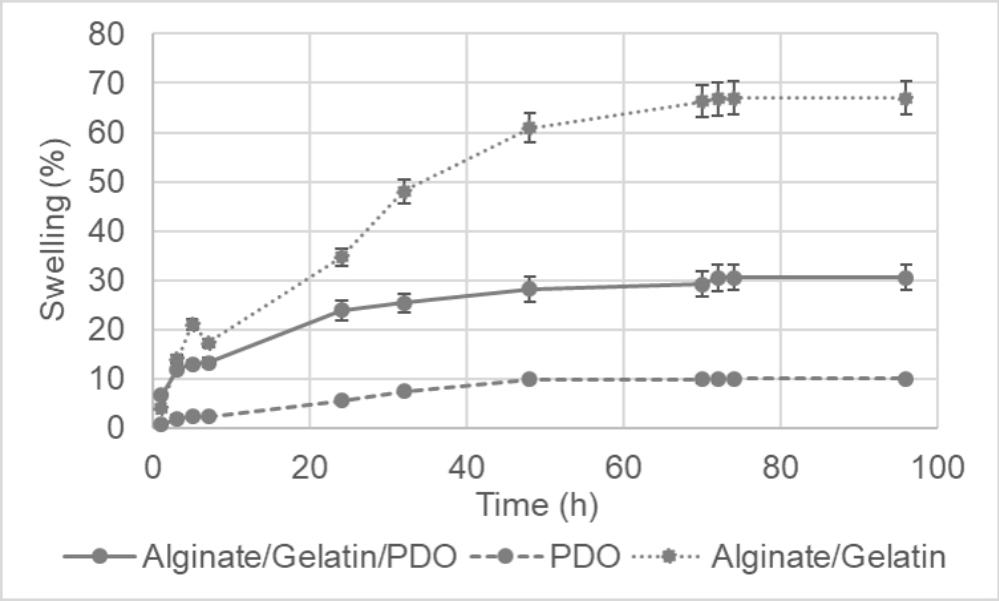

Figure 5. Swelling kinetic for PDO membrane and for alginate/gelatin/PDO scaffolds. Swelling test was performed by exposure to aqueous vapour. Results were compared with those obtained for not reinforced alginate/gelatin scaffolds [8].

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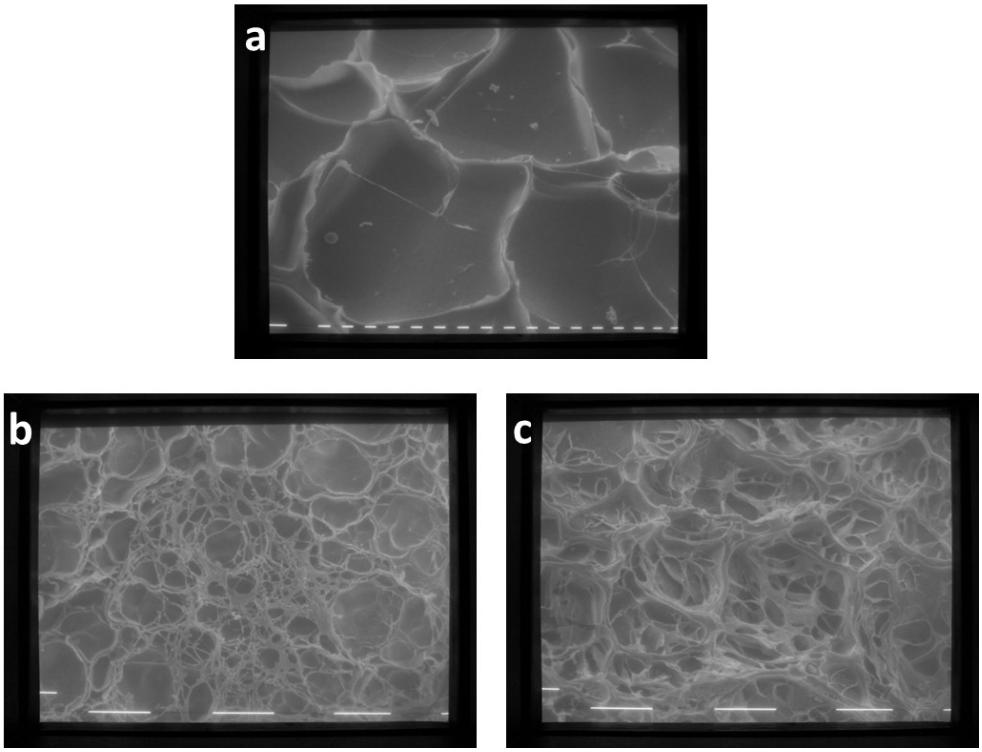

Figure 6. SEM images of rCPCs two weeks after seeding on alginate/gelatin/PDO (a), alginate/gelatin/PDO + IGF-1 0.9 µg/cm2 (b) and alginate/gelatin/PDO + IGF-1 1.6 µg/cm2 (c). Scale bars: a=10µm, b and c=100µm.

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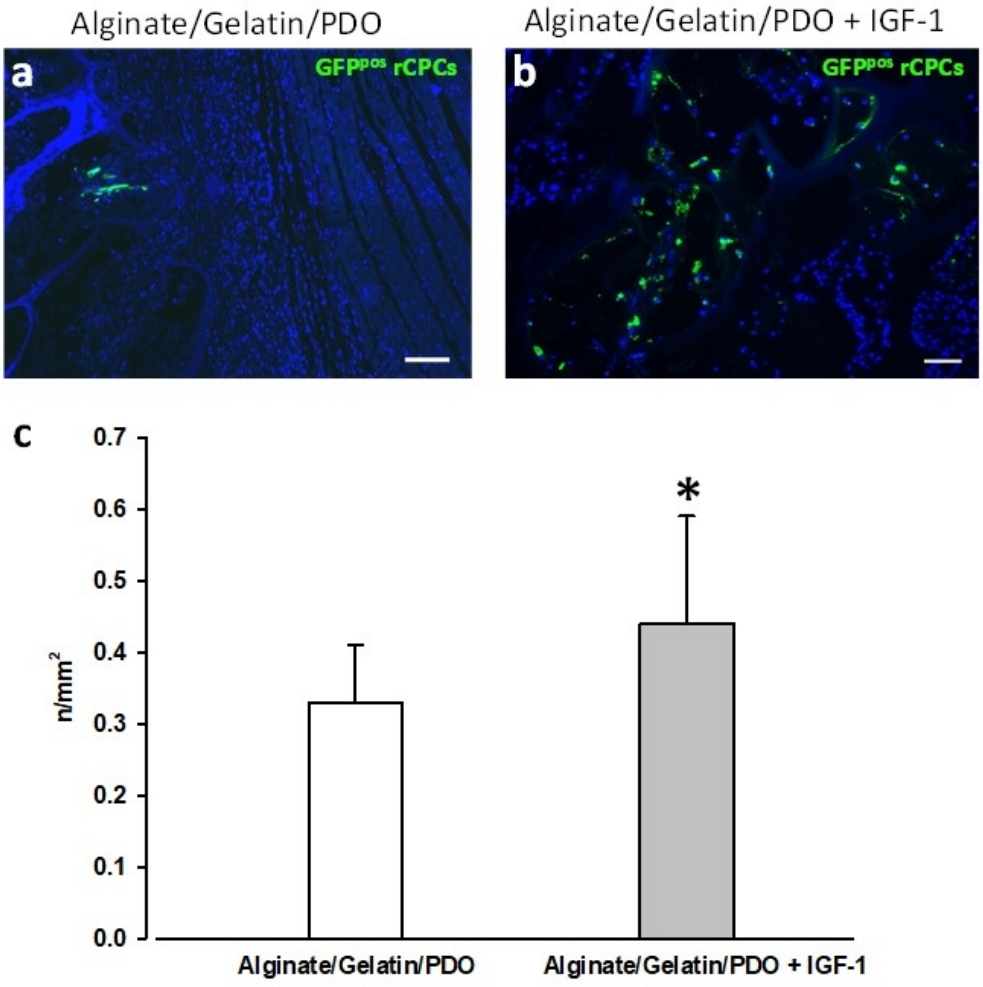

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