RGD-pectin microfiber patches for guiding muscle tissue regeneration

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Abstract

Among different scaffolding options, opportunely arranged microscaled fibers offer an attractive 3D architecture for encapsulating cells in degradable hydrogel microfibers appears as particularly attractive strategy. Hydrogel patches, in fact, offer a highly hydrated environment, allow easy incorporation of biologically active molecules, and can easily adapt to implantation site. In addition, microfiber architecture is intrinsically porous and can improve mass transport, vascularization, and cell survival after grafting. Anionic polysaccharides, as pectin or the more popular alginate, represent a particularly promising choice for the fabrication of cell-laden patches, due to their extremely mild gelation in the presence of divalent ions and widely accepted biocompatibility. In this study, to combine the favorable properties of hydrogel and fibrous architecture, a simple coaxial flow wet-spinning system was used to prepare cell-laden, 3D fibrous patches using RGD-modified pectin. Rapid fabrication of coherent self-standing patches, with diameter in the range of 100–200 μm and high cell density, was possible by accurate choice of pectin and calcium ions concentrations. Cells were homogeneously dispersed throughout the microfibers and remained highly viable for up to 2 weeks, when the initial stage of myotubes formation was observed. Modified-pectin microfibers appear as promising scaffold to support muscle tissue regeneration, due to their inherent porosity, the favorable cell–material interaction, and the possibility to guide cell alignment toward a functional tissue.

Keywords

Cell encapsulation, Cell–matrix interactions, Hydrogel Microfibers, Muscle Tissue Engineering, Pectin Hydrogels

1. Introduction

In regenerative medicine and tissue engineering (TE), the use of hydrogels as 3D platforms to support cellular proliferation and differentiation guiding a functional tissue regrowth has been emerged as an extremely promising solution[1]. Naturally occurring hydrogels, in particular, present several attractive properties; the first of which is they are able to closely resemble some properties of the extracellular matrix (ECM) of native tissues by retaining a large amount of water and providing functional bioactive moieties. Equally important, theirability to assemble under mild conditions makes them ideal structures for cell entrapment and 3D cell culture [2].

Several naturally derived biomaterials have been widely employed and investigated to form hydrogels for TE. Some of them, such as hyaluronic acid and collagen, can be found in native tissue as ECM components [3,4], whereas others (e.g., alginate, chitosan, and pectin) can be extracted from abundant natural sources [5-8]. In particular, pectin collective indicates a family of anionic polysaccharides found as structural components in plants. They have been long employed as gelling and emulsifying agents in the food industry, but have been only recently introduced as hydrogels for biomedical applications [9]. Pectin is a complex structural polysaccharide, but its characteristic and quantitatively predominant structure is a linear chain of partially methyl-esterified α-1-4-D-galacturonic acid. The fraction of methylesterified subunits defines the degree of esterification (DE) of pectin and depends on its sourcing and processing. When less than 50% of its galacturonan units are esterified, pectin is classified as lowmethoxyl (LM), and opportunely concentrated solution can be crosslinked in an hydrogel network in the presence of divalent cations (e.g., Ca2+). As for the significantly more widespread sodium alginate, both diffusion and internal gelation can be achieved and were successfully employed to prepare pectin hydrogels, and microspheres, in particular, for cell encapsulation and delivery [10,11]. Moreover, pectin presents the additional advantage of an interesting degradation profile under simulated physiological conditions, which can be tuned for the desired application[10].

Due to its advantageous properties, pectin has been used in a variety of pharmacological and medical applications. This polysaccharide is able to positively interact with the mucus of the gastrointestinal tract, and it has been used as a colon-specific drug delivery system[12]. Moreover, pectin can be useful in decreasing cholesterol and glucose absorption,13 and its potential inhibitory role in cancer cell metastasis, invasion, and survival has been recently explored[14,15]. Regarding TE and regenerative medicine applications, pectin has found large employment in the development of drug delivery systems and hydrocolloidal coatings for wound healing, which have been already commercialized [9,16].

Here, pectin gelation properties were used to prepare hydrogel fibers using a simple wet-spinning technique, to combine the above-mentioned advantages of pectin with a macroporous 3D structure amenable to support myocardial tissue regeneration, resembling a cardiac patch. This structure, in its simplest form, consists in a multiple layer of randomly oriented cardiomyocytes usually embedded inside hydrogels[17]. In particular, the use of hydrogel microfiber has been presented as interesting solution to stimulate cells organization in a functional tissue. In this context, Zhang et al have first demonstrated the possibility to encapsulate cardiomyocytes inside peptide microfibers by a simple extrusion process[18]. A microfluidic system and 3D-printing technique were later used to obtain functional constructs for cardiac tissue regeneration[19,20]. Both the cited studies presented the combination of hydrogel microfibers and cardiomyocytes to obtain a cardiac patch able to support cell proliferation and contractile activity. However, in the majority of the scientific literature, cardiac tissue regeneration has been investigated only inside a single hydrogel microfibers structure. Our interest is to support cardiac tissue regeneration inside a self-standing cellularized hydrogel scaffold fabricated in a one-step procedure using a simple wet-spinning system. At this aim, we produced RGD-modified pectin microfibrous scaffold, and we used skeletal myoblasts (C2C12) to test its suitability as ECM-mimicking environment. Being the myocardial tissue mainly

composed by muscle fibers,21 C2C12 were widely used as a preliminary model for in vitro studies [22,23]. Here, we selected them to investigate cell proliferation and arrangement inside a deformable hydrogel network made of modified pectin, aiming at stimulate muscle tissue regeneration.

2. Materials and Methods

2.1. Materials

All chemicals, if not otherwise stated, were purchased from SigmaAldrich and used without further treatment.

2.2. Synthesis and characterization of RGD-pectin

LM citrus pectin (Classic CU701—DE = 36%) was kindly provided by Herbstreith & Fox (Neuenbürg, Germany) and used to produce pectin microfibers. Pectin was biofunctionalized by covalently grafting the integrin-binding peptide (glycine)4-arginine-glycineaspartic acid-serine-proline (RGD, molecular weight = 758.74 g/ mol—GenScript) using aqueous carbodiimide chemistry as previously described.11 In brief, pectin was dissolved at 1% w/v in a freshly prepared 2-(N-morpholino)ethanesulfonic acid buffer solution (MES buffer: 0.1 M MES, 0.3 M sodium chloride, pH 6) and stirred overnight at 4C. N-Hydroxyl-sulfosuccinimide (Sulfo-NHS; Pierce) and 1-ethyl-(dimethylaminopropyl)-carbodiimide (EDC; 48.42 mg/g pectin) were sequentially added at a molar ratio of 1:2, followed by 40 mg of RGD peptide per gram of pectin. A control sample was prepared without the addition of peptide (RGD-blank). The reaction was quenched after 20 hr of stirring at 4 °C with hydroxylamine (18 mg/g pectin), and the solution was dialyzed in decreasing salt solutions of deionized water and NaCl (seven steps: 30–25–20–15–10–5–0 g of NaCl) for 3 days at 4C (dialysis membrane cutoff = 2000). Then, the solution was mixed with activated charcoal (0.5 g/g of pectin), stirred for 1 hr, and centrifuged for 10 min at 4000 rpm three times. The RGD-pectin solution was then sterile-filtered through 0.22 μm filter membranes, lyophilized, and stored at 20 °C until further use.

The amount of grafted RGD was quantified by a ultraviolet (UV) absorbance assay in the 200–300 nm region using a spectrophotometer (NanoDrop 2000, ThermoFischer Scientific[11]. Absorbance readings were converted into the concentration of RGD using a calibration curve obtained from serially diluted RGD solutions mixed with RGD-blank (1 wt % in ultrapure water) solutions as standards. The spectra were normalized against the 1 wt % in ultrapure water RGD-blank solution.

Rheological characterization of pectin and RGD-grafted pectin solutions was performed using a Anton Paar Rheometer equipped with a cone/plate geometry (cone diameter = 50 mm and cone angle = 1). During the viscosimetry test, a Peltier plate was used to condition the solutions at 25C, and the steady shear measurements were performed using a stress range of 0–100 Pa.

2.3. Production of pectin microfibers

Pectin microfibers were produced using a wet-spinning system previously developed for alginate processing,24 and schematized here in Figure S1.

In brief, the system is composed of a centrifugal pump (Sicce Syncra 0.5–700 L/hr, max head 1.2 m) that circulates at a flow rate of 3850 ml/min a $CaCl_2$ cross-linking solution (50–150–300 mM) in a silicon rubber tubing (internal diameter = 5 mm, thickness = 1 mm) flow loop. Pectin or RGD-pectin Dulbecco's Modified Eagle Medium (DMEM)-based solutions (hereafter

referred as pect and RGD-pect, respectively) are injected through a 24G needle into the CaCl2 flow at different flow rates (30–50–70 mm/hr, with 1 mm/hr corresponding to 0.3 ml/hr) using a syringe pump. To optimize the fabrication process and evaluate the influence of solution parameter, the pectin concentration was varied from 0.75 to 3% w/v. The coaxial flow draws the pectin solutions, and Ca2+ ions cross-linking causes its solidification, resulting in a continuous fiber that whips, bends, and spontaneously assembles in a fibrous patch collected in a strainer. After collection, the patch is post-treated in a 150 mM CaCl2 solution for 10 min to further consolidate the 3D fibers structure. Then, microfibrous constructs were accurately rinsed in complete medium to remove any possible excess of Ca2+ ions. The microfiber morphology was investigated by optical microscope (Olympus) and by a scanning electron microscope (SEM; Stereoscan 360—Cambridge Instruments) for hydrated and dehydrated samples, respectively. Quantitative analyses on fiber diameter were performed on acquired images, using the image-processing software ImageJ, based on 30 different measures in random points from at least three different images (see Figure S2 for the detailed method).

2.4. Evaluation of microfibers stability

Patches stability and swelling properties were investigated by immersing samples (n = 3 per time point, diameter = 4 mm) in 1 ml of complete cell culture medium (DMEM supplemented with 10% v/v fetal bovine serum [FBS], 1% v/v penicillin/streptomycin, glutamine 2 mM, and 1% v/v non-essential amino acids) and incubating them at 37 °C, for up to 3 weeks. The swelling kinetic was investigated by weighting samples in their hydrated state at established time points (i.e., 1, 3, and 5 hr, and 1, 2, 7, 14, and 21 days). The weight variation (ΔW%) curve was constructed by relating the weight of the swollen samples at the considered time points (wt) to the initial weight of samples after fabrication process (W_0), using the following equation, and reporting the average weight variation (ΔW%) in function of time.

$$\Delta W\% = \frac{w_t - w_0}{w_0} \cdot 100$$

The microfibrous samples were collected at each time point and freeze-dried to allow their complete dehydration. The gel solid fraction (G_f) was then calculated as the ratio between the weight of the sample after swelling and dehydration (W_f), to the initial weight of the dehydrated sample (W_i), following the equation:

$$G_f = \frac{w_f}{w_i} \cdot 100$$

2.5. Embedding of myoblasts in RGD-pectin microfibers

Murine myoblast cell line (C2C12, ATCC), here used as a preliminary model, was expanded and cultured in DMEM supplemented with 10% v/v FBS, 1% v/v penicillin/streptomycin, glutamine 2 mM, and 1% v/v non-essential amino acids. Cells were detached from flasks and carefully mixed with pectin solution, which pH had been adjusted at a value of 6, at a density of 8 * 10^6 cells per ml. The suspension was then injected in the $CaCl_2$ flow loop to obtain the encapsulation of myoblasts inside hydrogel microfibers. Cell-laden microfibers patches were then post-treated in high molar $CaCl_2$ solution (150 mM) for 10 min, rinsed in culture medium, and punched in 4-mm circular samples. Microfibers samples were maintained at 37 °C under a 5% v/v CO_2 humidified atmosphere for different periods of time, according to the aim of the experiment.

2.6. Assessment of cell viability inside microfibers

The assessment of cell metabolic activity inside microfibers was carried out by resazurin assay, according to a previous protocol.25 After 3, 7, and 14 days of culture, microfibers samples (three samples for each time point and condition) were incubated at 37 °C in 20% v/v resazurin solution. After 2 hr, 150 µl/sample were transferred to a 96 well plate, and fluorescence (560/590 nm) was measured by a microplate spectrophotometer (Genios™ Microplate Reader, Tecan). Fluorescence was normalized to the number of viable cells per each sample that were quantified as previously described[25]. In brief, cells were released from microfibers by pectin dissolution with 0.25% v/v trypsin/50 mM EDTA in phosphate buffered saline (PBS) for 5 min at 37 °C. Afterward, cells were counted in a Neubauer chamber, and their viability was assessed using the trypan blue exclusion test.

2.7. Assessment of cell morphology inside microfibers by immunostaining

At culture days 3, 7, and 14, microfibers samples were washed with tris buffered saline (TBS)-$CaCl_2$ (7.5 mM), fixed with 4% v/v paraformaldehyde (PFA) in TBS-$CaCl_2$ for 20 min at room temperature and then washed three times in TBS-$CaCl_2$ (5 min each washing). For immunostaining assay, microfibers were permeabilized with 0.2% v/v Triton X-100 for 10 min and blocked with bovine serum albumin (BSA; 1.5% w/v) solution in TBS-$CaCl_2$ for 1 hr. Microfibers samples were then incubated for 45 min with Phalloidin Fluorescein Isothiocyanate Labeled 1 mg/ml (diluted 1:100) in TBS-$CaCl_2$, 1.5% BSA for the staining of filamentous actin, followed by three washing with TBS$CaCl_2$ (5 min each washing). The staining of nucleic acid was performed by Hoechst solution (diluted 1:1000) in TBS-$CaCl_2$, 1.5% BSA for 15 min. Finally, samples were washed three times with TBS-$CaCl_2$ and kept protected from light at 4 °C until they were analyzed by fluorescent microscopy (Zeiss Axioplan).

2.8. Evaluation of myotubes formation

After 3 days of culture, the complete culture medium was switched to a differentiation medium by lowering the FBS content (5 vol %) and keeping unvaried the remaining components to promote cell fusion and the development of myotubes. At culture days 3, 7, and 14, microfibers samples were fixed as described earlier and incubated overnight at 4C in a dark humidified chamber with mouse antisarcomeric myosin (MHC, 1:100) in TBS-$CaCl_2$, 1.5% BSA, followed by 2 hrs of incubation with anti-mouse secondary antibody (1:100) and Hoechst solution (1:1000) for 15 min. Between each staining, three washing with TBS-$CaCl_2$ were performed. Finally, samples were analyzed by confocal microscopy (Fluoview, Olympus).

2.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc., version 8). Data are expressed as mean ± standard deviation (SD). Datasets tested for normality show a normal distribution. Unpaired Student's t test and one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test were adopted to estimate statistical significance between two and more group comparisons, respectively. Differences between groups were considered statistically significant when $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3. Results

3.1. Pectin microfibers production and characterization

The wet-spinning system previously developed to process alginate24 was here validated for the production of pectin hydrogel microfibers. During preliminary tests, it was verified that the pH of pectin solution must be adjusted around 6 before the fabrication of microfibers, because it influences the formation of the gel, and, in particular, the dissociation of pectin carboxyl groups involved in the gelation mechanism of pectin[26] (Figure S3).

The effect of different parameters was then evaluated to obtain homogeneous microfibers with diameter in the range 100–200 µm, assembled in a coherent structure. First, the polymer concentration was considered, and a range of 0.75–2% w/v was investigated. All the tested values allowed the formation of microfibers which cohesiveness in a stable structure depended on the molarity of $CaCl_2$ solution that circulated inside the circuit and caused the cross-linking of pectin. The use of a high molar solution of $CaCl_2$ (300 mM) resulted in the formation of non-cohesive fibers, mostly likely due to a too fast saturation of the ion-binding sites presented on pectin chains. Similarly, non-cohesive scaffolds were observed also using a 50 mM $CaCl_2$ solution. In this case, the amount of binding ions appeared inadequate for cross-linking and consolidation of the fiber structure. Accordingly, the 150 mM solution was selected as the most suitable for obtaining a good level of fibers cohesiveness and used in further experiments.

From the morphological characterization presented in Figure 1a, pectin microfibers appeared as continuous structures randomly assembled in a self-standing patch. Fibers diameter was strongly influenced by pectin solution flow rate and, as reasonably expected, increased when the feed was raised (Figure 1b). The intermediate value of 50 mm/hr allowed the formation of microfibers with suitable diameter that assembled in a cohesive patch in about 10 min, which resulted a compatible time for cell survival during an encapsulation process.

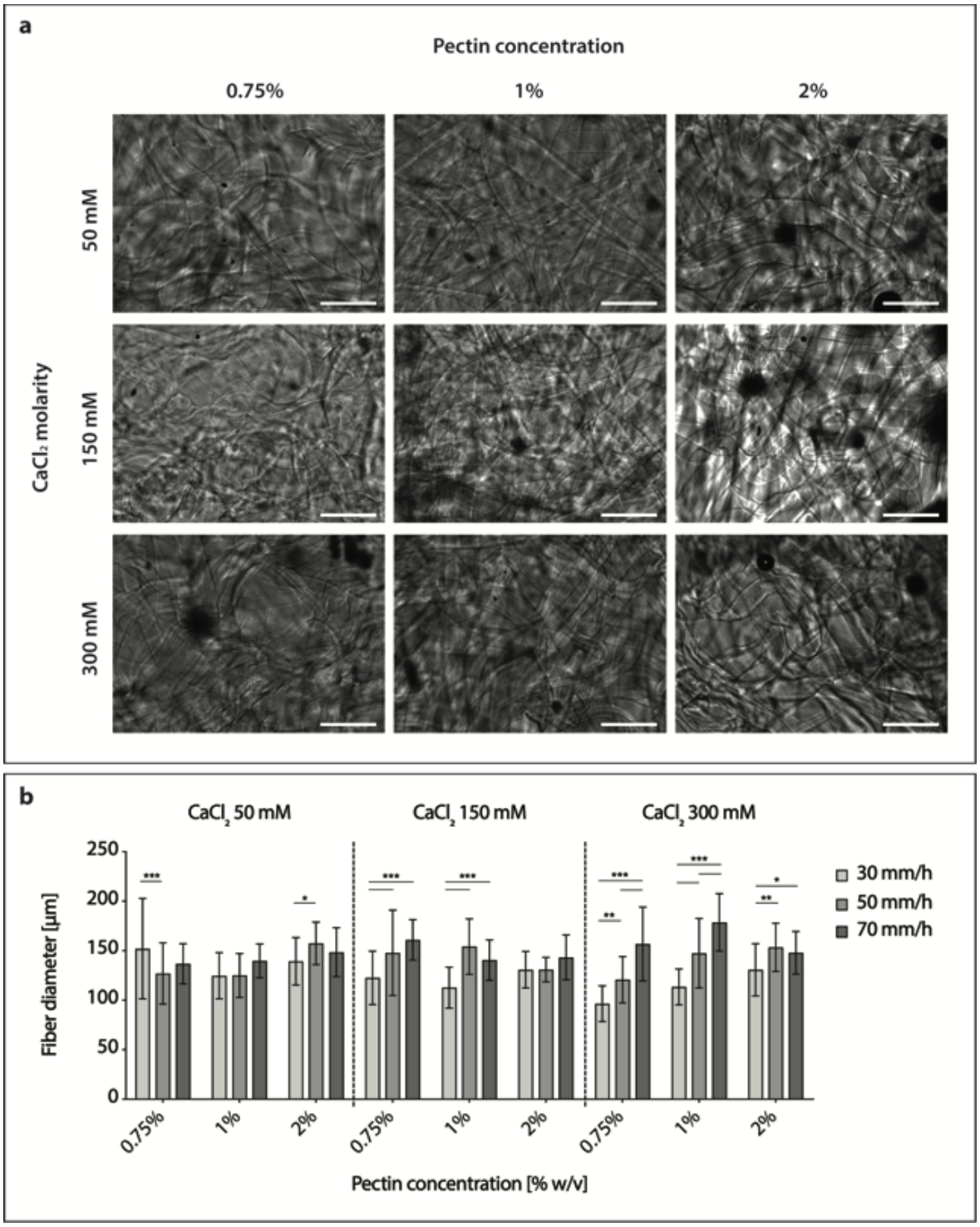

Figure 1 (a) Qualitative influences of CaCl2 molarity and pectin concentration on the morphology of microfibers produced using the wetspinning system and a polymer solution flow rate of 50 mm/hr (scale bars = 500 µm—representative optical images are reported, and each condition was tested in triplicate). (b) Effect of solution and process parameters on fibers dimensions (n = 3)

3.2. Functionalized RGD-pect microfibers

Pectin was functionalized with an RGD containing peptide, and the UV spectrum (data presented in Figure S4) of RGD-pect (normalized against RGD-blank) showed the presence of a characteristic peak around 230 nm, indicating that the peptide was effectively grafted to the polymer. The quantity of RGD coupled to pectin was 29.16 mg RGD per gram of pectin (a coupling yield of 58%), which corresponds to an RGD final concentration of 384 µM.

The pectin solutions were further characterized by viscosimetry test (Figure 2), because the viscosity of the polymer solution has been shown to be a crucial parameter in the microfibers fabrication process[27]. Both tested solutions (i.e., pect and RGD-pect) exhibited a shear-thinning behavior, associated with a decrease of viscosity in response to an increase of shear rate. However, the introduction of RGD-coupling caused a decrease in pectin viscosity, which did not affect the formation of smooth microfibers, but impaired the cohesiveness of the final scaffold. For this reason, after further optimization, RGD-pect concentration was raised from 1.2 to 2.4% (w/v) for fiber preparation in the wet-spinning system, as it resulted in a favorable balance between the ion-binding sites presented on modified pectin chains and the quantity of ions involved during both the fiber formation and scaffold consolidation step with 150 mM $CaCl_2$ solution. As already tested in previous works [24,27], after the fabrication process, microfibers were immersed in a high molar $CaCl_2$ solution (150 mM) to consolidate the cross-linking among fibers and allow the formation of self-standing, manageable scaffold.

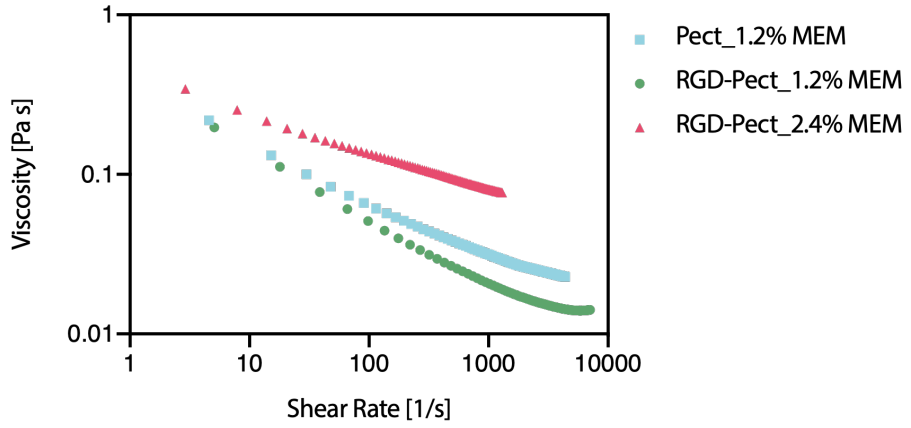

Figure 2- Viscosity as a function of shear rate for pectin-based solution. Comparison between unmodified and modified pectin at different concentrations at 25C (n = 3)

The morphology of hydrated and dehydrated fibers was investigated using an optical electron microscope and SEM, respectively (Figure 3a–d). Before and after pectin modification, no significant differences were observed in fibers morphology attributable to the coupling of RGD sequences. The assembled fibrous network showed an open porous structure that appeared more compact in RGD-pect samples. This should be correlated with the fact that the use of a 2.4% RGD-pectin solution determined the formation of fibers with an average diameter of 202 ± 24 µm, which is slightly bigger than the mean dimension observed in non-modified pectin fibers (154 ± 28 µm) obtained with a 1.2% pectin solution.

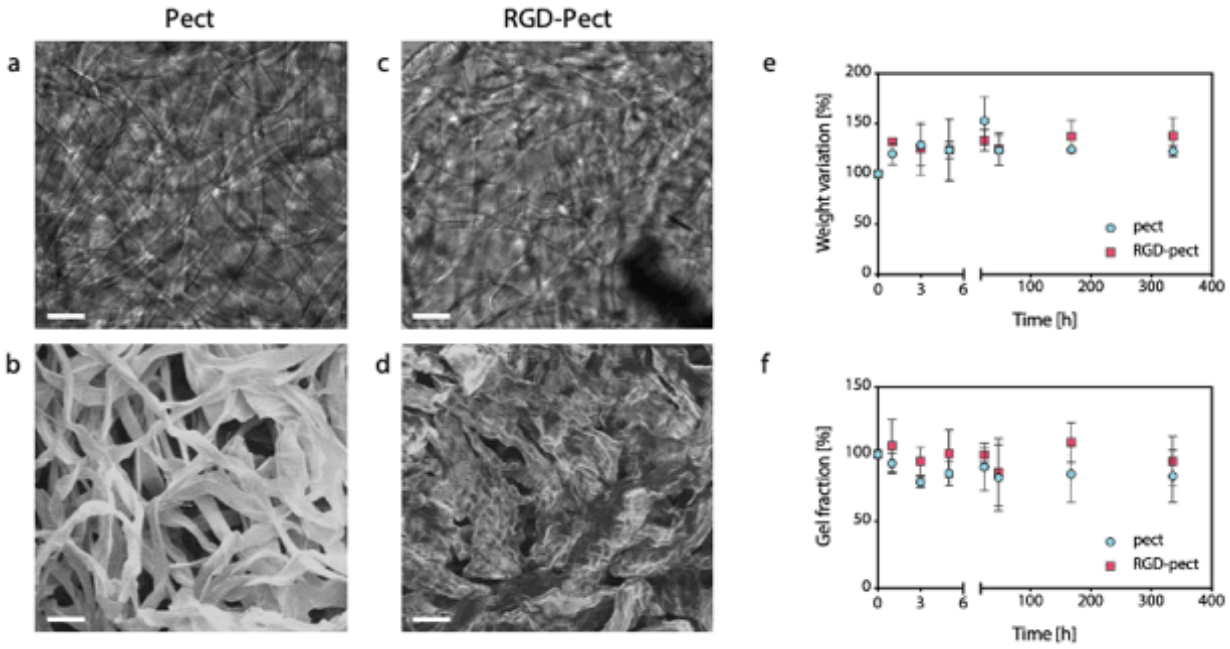

Figure 3 - Optical (a,c) and scanning electron microscopy images (b,d) of pectin microfibers before and after RGD-grafting modification (scale bars = 200 μm). (e) Percentage weight variation of microfibrous samples (pect vs. RGD-pect) after incubation in complete culture medium at 37C, and (f) gel fraction values of corresponding dehydrated samples (n = 3 per time point)

The stability of microfiber samples produced with the two formulations (i.e., pect and RGD-pect) in complete culture medium up to 21 days showed no statistically differences between their percentage weight variation (Figure 3e). Both samples showed an initial phase of swelling (up to 20–30%) due to the absorption of fluid, and then remained stable. A similar trend was observed in the gel fraction values (Figure 3f), where the residual fraction of polymer was measured along the incubation time.

During the first 5 hr, a 10% of weight loss was registered for both the formulations. A hypothesis was that the shortest and barely attached fibers were lost in the first hours of incubation, and the consistent part of the scaffold remained compact for the remaining time, proving a good stability of the microfibers in physiological-like conditions.

C2C12 cells were successfully embedded inside pect and RGDpect microfibers at high cell density ($8 * 10^6$ cells/ml) using the wet-spinning system developed. Cells became homogeneously distributed inside microfibers along the whole extension of the microfibrous scaffold. To validate the fabrication process and assess cell survival inside pectin microfibers, after 3, 7, and 14 days of culture, the viability and metabolic activity of entrapped cells were evaluated (Figure 4a). The majority of cells remained viable (around 90%) at each time point. Interestingly, a decrease in the percentage of viable cells was observed in pect microfibers at day 14, probably due to outward migration of cells for the lack of binding sites. The overall metabolic activity of cells embedded in RGDpect microfibers increased during the culture period, whereas cells entrapped in non-modified microfibers showed a decrease in the metabolic activity signal. A dual explanation can be addressed: on the one side, the decrease in fluorescence signal could be related to the decrease in the number of entrapped cells and, on the other side, could be caused by an effective decrease in the metabolic activity of the embedded cells due to the less favorable embedding matrix.

Actin filaments and nuclei of entrapped cells were stained to investigate cell disposition inside microfibers as a function of maturation time. From the immunofluorescence images reported in Figure 4b, it is possible to appreciate how RGD-pect

hydrogel matrix influenced cells behavior. In this type of microfibers, cells were able to spread and interact with the embedding matrix from the first days of culture. At day 7, cells started to elongate along RGD-pect microfibers axis, achieving a good level of alignment at day 14. On the contrary, cells embedded in non-modified pectin microfibers exhibited a round shape during the culture time, for being embedded in a soft hydrogel matrix that lacks adhesion sites.

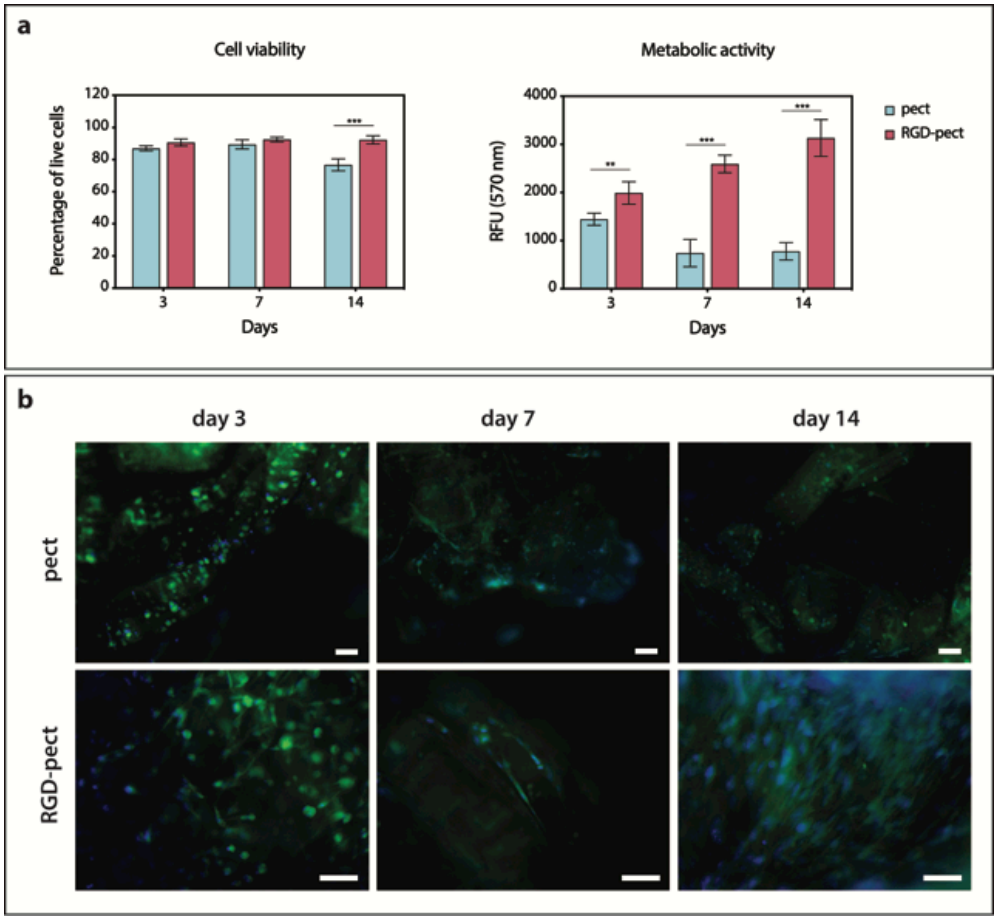

Figure 4 (a) Embedded C2C12 were characterized after 3, 7, and 14 days of culture in terms of cell viability and metabolic activity. A comparison between cells behavior inside pectin and RGD-pectin microfibers was carried out (n = 3). (b) Immunofluorescence micrographs of embedded C2C12 in pectin and RGD-pectin microfibers. Staining: nuclei in blue and actin filaments in green (scale bars = 200 μm, n = 3)

To better understand cell behavior and evaluate possible myotubes formation inside RGD-pect microfibers, C2C12 were cultured up to 14 days, inducing a differentiation process at day 3 by lowering the concentration of FBS from 10 to 5 vol %. A qualitative investigation was performed by staining myosin and nuclei to evaluate the presence of multinucleated cells containing myosin, indicative of myotube formation (Figure 5). Myosin was detected in both control and differentiated samples, showing how the RGD-pect hydrogel matrix resulted in a suitable environment for myoblast proliferation. As observed in previous investigation performed using alginate, cells tended to elongate inside microfibers assuming a preferential alignment. Interestingly, multinucleated cells were detected at day 14 (highlighted by arrows in Figure 5), after

the differentiation process was induced, confirming the ability of myoblasts to differentiate in myotubes inside RGD-pect microfibers.

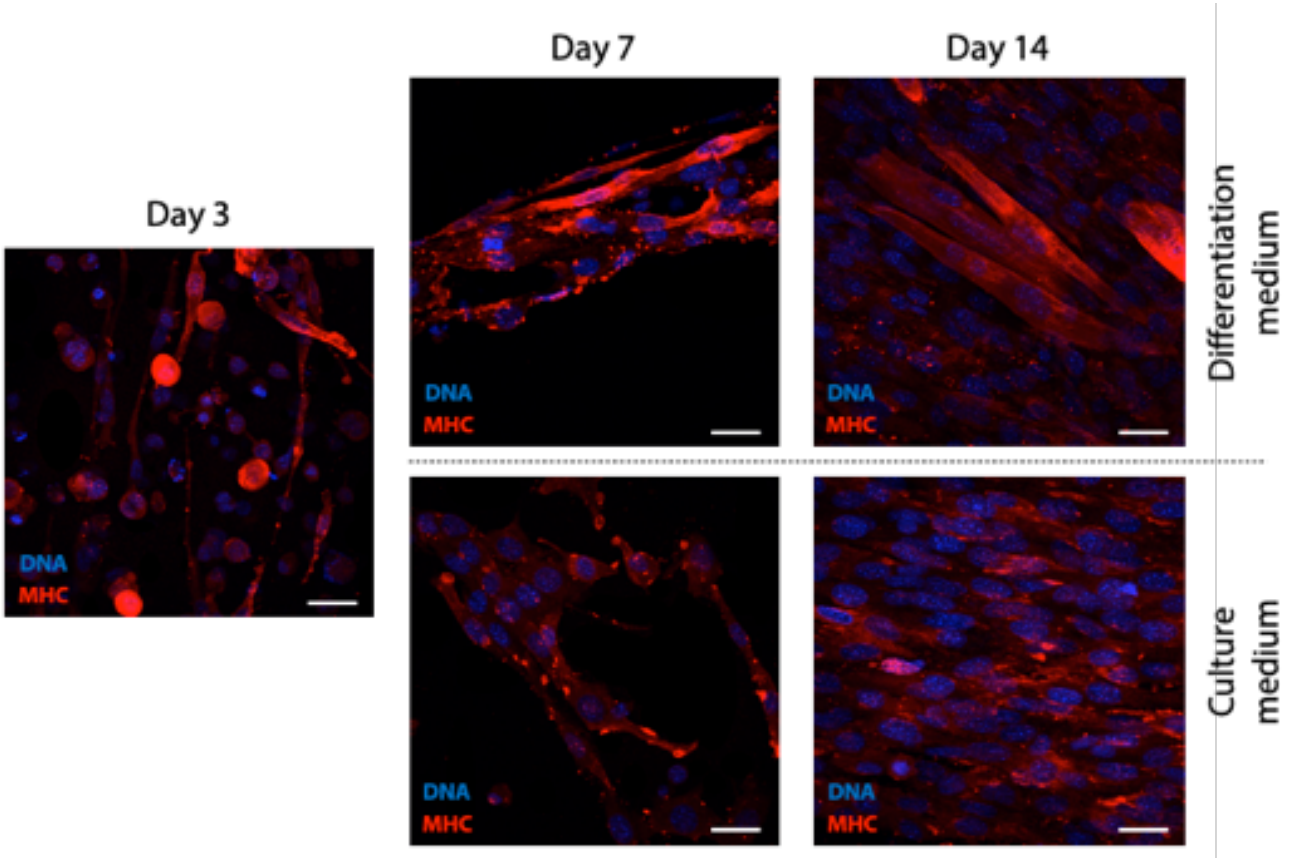

Figure 5 - Immunofluorescence micrographs of C2C12 cells embedded in RGD-pectin microfibers. After 3 days of culture, the culture medium was switched to differentiation medium, and the organization of myosin (MHC in red and nuclei in blue) was investigated at days 7 and 14 (scale bars = 20 µm - the experiment was completed in triplicate). Arrows indicate the multinucleated cells observed at day 14 in the differentiated samples.

4. Discussion

The versatility of the wet-spinning system previously developed and tested for the production of cell-laden alginate microfibers[24,27] was here proved by successful production of cell-laden pectin microfibers. The ability of pectin to cross-link under mild conditions together with its favorable biomimicry properties makes this polysaccharide a promising scaffolding material,[28] here studied for the production of microfibrous patches aiming to enhance a functional cells arrangement and stimulate muscle tissue regrowth.

Pectin gelation mechanism is affected by both intrinsic and extrinsic parameters, as resumed by Fraeye et al.(26). Among extrinsic differentiated samples parameters, the pH of polymer solution resulted crucial in the formation of hydrogel microfibers. In fact, pectin solution pH regulates the dissociation of pectin carboxyl groups and pectin gelation process as a consequence. In the cross-linking mechanism, the interaction between pectin carboxyl groups and calcium ions occurs when the carboxyl groups present a negative charge; that is, they are dissociated. To reach this favorable condition and increase the affinity of carboxyl groups for divalent cations, the pH of pectin solution must be close to 4.5, being the pKa of galacturonic acid monomer around 3.5 [(26)]. Therefore, pectin solution with a pH of 5–6 allowed to obtain homogeneous fibers that assembled in a cohesive patch. Although this value is not physiological, the pH has not been increased much above 6, because, at high pH, pectin undergoes a depolymerization process that determines a decrease in its molecular weight altering the crosslinking mechanism with divalent cation [(10,26,29)].

Despite its benefits, it is well known that unmodified pectin does not present functional adhesion sites for integrin-mediated cell interaction.11 To overcome this limitation and promote a better cell adhesion inside hydrogel microfibers, pectin was successfully modified with RGD peptides. The chemical grafting allowed to obtain an optimal RGD concentration, similar to the one present on biological ECM derivatives[(30)]. However, the introduction of RGD-peptides along pectin chains determines a change in the chemico-physical properties of the hydrogel precursor material, such as the decrease in DE, molecular weight, and intrinsic polymer viscosity.10 These modifications resulted in a consistent decrease in microfibers cohesiveness after their fabrication, leading to the production of single-dispersed fibers instead of handling scaffold. Although a low DE normally encourages the formation of pectin gels due to an increase in the carboxyl groups presented on pectin chain, the DE reduction is associated with a molecular weight decrease that hinders gel formation[(26)]. The optimization of pectin concentration and cross-linking bath molarity allowed the production of cohesive microfibrous scaffold also with the RGD-modified pectin. The modification of these two important parameters together with the introduction of a $CaCl_2$ post-treatment[(27)] allowed to find the right balance between divalent cations and available pectin carboxyl groups and promote hydrogel fibers formation and their inter-crosslinking during the post-treatment with high molar $CaCl_2$ solution (150 mM).

Scaffold cohesiveness and stability were positively proved up to 21 days in a complete cell culture medium (Figure 3e,f). A different behavior was observed when pect and RGD-pect microfibers were immersed in PBS at 37C: a complete dissolution of hydrogel fibers was reached in 1 hr (data not presented). PBS is a saline solution which composition let to the formation of monovalent ions (i.e., K^+ and Na^+) that increase pectin solubility in an aqueous environment, due to the exchange of these ions with Ca^{2+} in the cross-linking sites. This phenomenon determines an electrostatic repulsion between carboxyl groups that causes polymer chains relaxation and consequent hydrogel swelling and dissolution. Moreover, calcium ions have a strong affinity with the calcium phosphates of PBS, which induces a pH variation speeding up the egg-box structure disintegration and pectin hydrogel solubilization[(31,32)]. Fast dissolution of pectin in PBS is in accordance with results reported in other studies and depends on material dimension. For example, bulk hydrogels of pectin are stable in PBS for 4 days [(9)],

whereas pectin microspheres endure for 12 hr[10]. This is a further demonstration that the arrangement of microfibers creates a porosity structure highly perfused by the aqueous surrounding medium. Despite the high porosity, pectin microfibers dissolution in culture medium is prevented, because the adverse role of monovalent ions is counterbalanced by the presence of divalent ions (i.e., Ca2+, Mg2+), aminoacids, and proteins, and the pH is stabilized by HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) around a physiological value of 7.4.

From the comparison between pect and RGD-pect swelling profiles, no significant differences emerged, even if the morphological appearance of dehydrated samples resulted different (Figure 3). The porosity of RGD-pect scaffold appears lower than the one observed in pect scaffold due to the higher dimensions of RGD-modified microfibers. Possibly, the decrease in molecular weight and viscosity after polymer modification promotes high water absorption at microfibers level compensating for the water accumulation that can occur in a more porous microfibers architecture [33]. This phenomenon resulted in a comparable water absorption by pect and RGD-pect scaffold at a macroscopic level.

The arrangement of microfibers is of great interest in creating cellladen constructs with favorable open porosity that facilitate the diffusion of oxygen and nutrients. The proposed fabrication system allowed the embedding of a considerable number of myoblasts (i.e., 8 106 cells/ml), which resulted in homogeneous distribution throughout the entire 3D microfibers structure. As already demonstrated, the presence of RGD sequences increases and stimulates the metabolic activity of C2C12 cells that are able to adhere and interact with the surrounding hydrogel matrix[34]. Moreover, as it resulted evident for pect samples, the absence of RGD anchor points facilitates a consistent escape of cells from hydrogel constructs, underlining the relevance of such chemical modification to provide adequate chemical signal for cell proliferation. The confinement of cells inside an anisotropic RGD-modified structure (i.e., cylindrical microfibers) has been proved to be a successful strategy for the fabrication of biomimetic constructs. In fact, different studies have demonstrated that an improved cells organization and proliferation stimulate cells to secrete a self-synthetize ECM network rich in fibronectin and collagen. This network contributes to reinforce and stabilize the hydrogel construct itself [27,35-37].

In cardiac tissue regeneration, C2C12 myoblasts are used as interesting and well-studied cell model due to their ability to differentiate in myotubes and simulate cardiac tissue regrowth [38,39]. Moreover, they were successfully employed in different clinical trials for the functional recovery of myocardial tissue [22,23].

In the present study, the use of a differentiation medium has been proved to be an adequate strategy to induce myotube formation inside RGD-pect microfibers. Myosin staining (i.e., immunostaining of sarcomeric myosin) allowed to appreciate the myotubes maturation in multinucleated structures and their alignment inside microfibers, even if the typical muscle strips were not detected (Figure 5). This last observation let us to presume that the myotubes differentiation is still in an initial phase. Similar results were reported when C2C12 myoblasts were cultured into bulk hydrogel[40] or on RGD-functionalized substrates[41]. The first study reported a random orientation of myotubes inside a 3D bulk hydrogel, whereas the second one highlighted the ability of C2C12 cells to differentiate onto microgrooved-modified polymer surface upon their detachment observed after 8 days of culture. The RGD-pect microfibrous construct here presented can overcome both the limitations of the cited studies, favoring a long cells culture inside a 3D functionalized environment and encouraging cells alignment along a preferential direction.

While further investigations are needed, the modified-pectin microfibrous scaffold here developed stands up as a favorable microenvironment for complex tissue maturation and a promising approach for muscle tissue regeneration.

5. Conclusions

Processing pectin using a simple, economic wet-spinning system allows to obtain homogeneous cell-laden microfibers arranged in a selfstanding, cohesive and manageable 3D patch. The introduction of cell-interactive integrin-binding motifs among pectin chains contributes to support cells proliferation mimicking a physiological extra-cellular matrix environment. The deformable pectin network encouraged cellular activities and the preliminary differentiation of embedded myoblasts. Moreover, the microfibrous cellularized structure could maximize the interactions with host tissue upon implantation by offering an open porous structure and a high surface-to-volume ratio. Collectively, the presented results point out modified-pectin microfibers as promising scaffold to support muscle tissue regeneration.

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Figure S1 - Schematic representation of the setup used for microfibers fabrication. The system employed allowed the production of microfibers assembled in a patch structure.

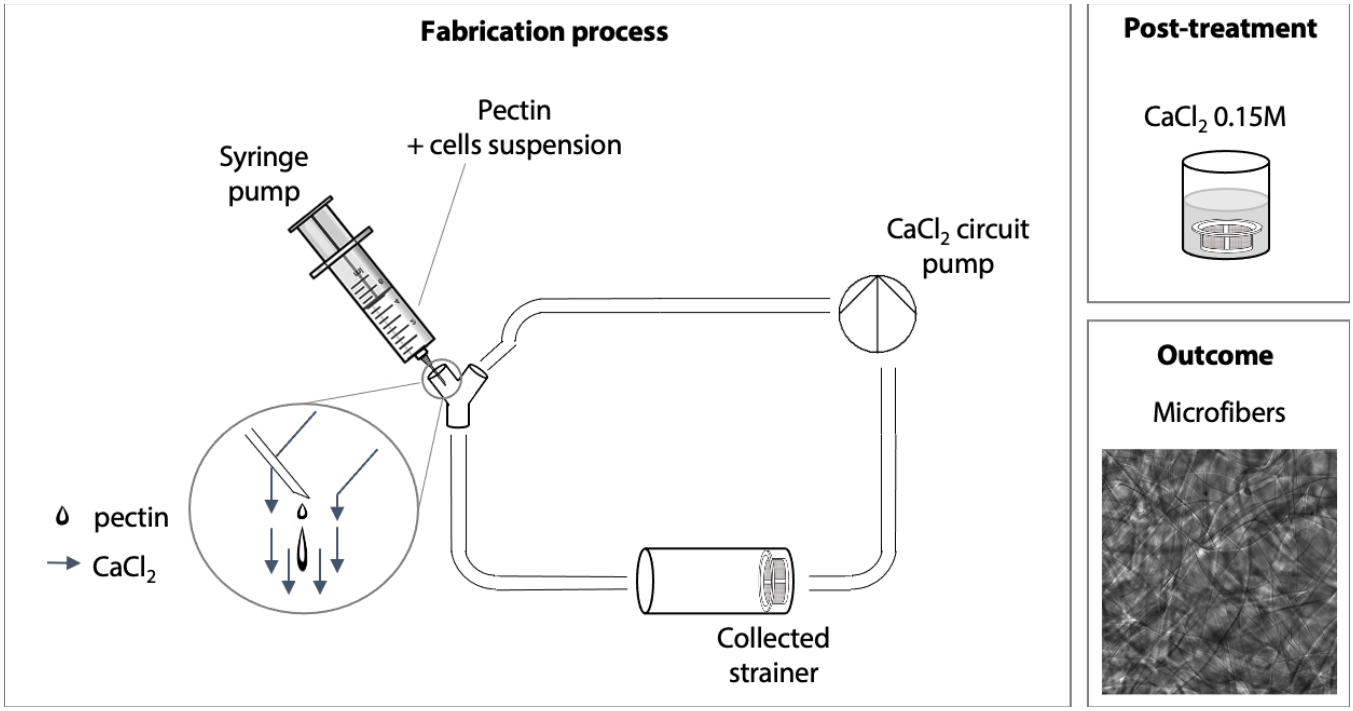

Figure S2 - For the analysis of fiber diameter, the software ImageJ was employed as follows: 30 different measures in random points (yellow line) were taken from three different images of microfibers acquired by optical microscope. The measurements were then expressed as mean value ± standard deviation and compared in Figure 1b for the set of parameters (i.e., pectin concentration, $CaCl_2$ molarity, and pectin solution flow rate) investigated during the optimization process.

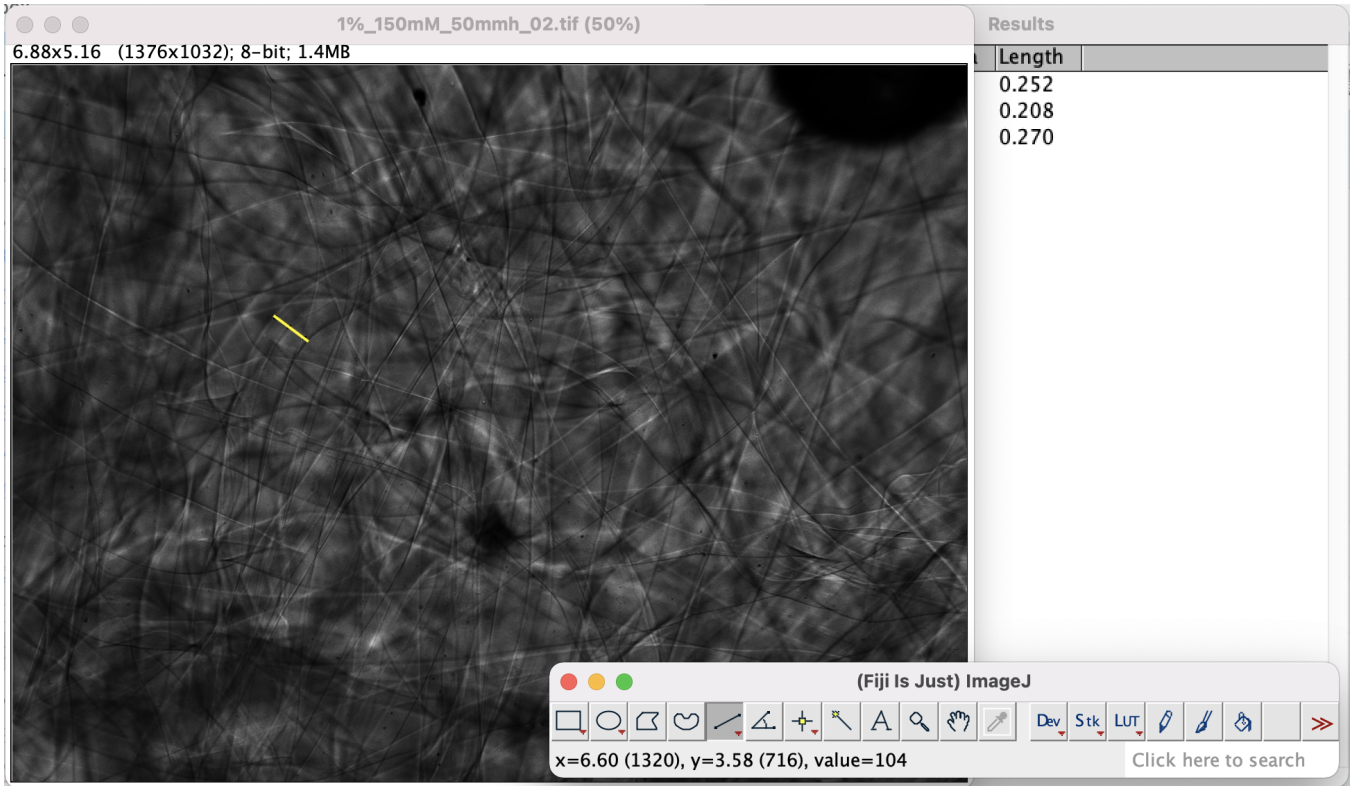

Figure S3 - Effect of pH variation of hydrogel precursor solution on pectin microfibers formation. The upper row reports the results obtained with a pH 6 pectin solution: homogeneous microfibers assembled in a cohesive, self-standing patch. In the second row, unsuitable microfibers obtained using a pH 3.8 pectin solution are shown. Process parameters are constant: 1.2% w/v pectin concentration, 50 mm/hr pectin solution flow rate, and 150 mM $CaCl_2$ cross-linking solution molarity.

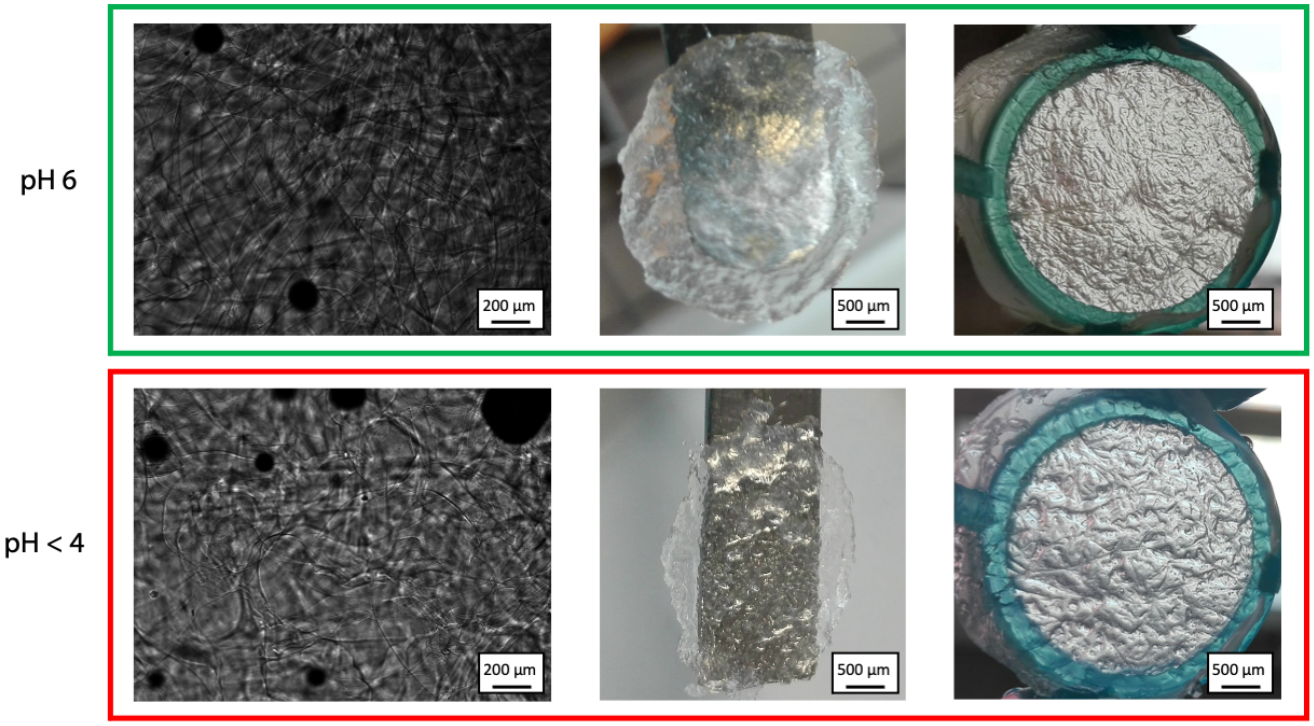

Figure S4 (a) UV spectra of the different 1-wt % BLK-pect solutions mixed with known RGD quantities and the 1-wt % RGD-pect solution and (b) the calibration curve obtained using the endpoint readings at 230 nm.