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Electrospun Acellular Heart ECM for Cardiac Tissue Engineering

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ABSTRACT

Electrospun nanofiber is one of the promising alternatives for being used in tissue engineering and the drug delivery due to its controllable characteristics. However, choosing an appropriate biomaterial for regenerating a specific tissue plays a significant role in fabricating functional tissue-engineered constructs. The heart extracellular matrix (ECM)-derived electrospun nanofiber which mimics the physicochemical and structural characteristics of the cardiac tissue is advantageous to cardiac tissue engineering. In this study, the acellular calf heart ECM has been investigated as a potential biomaterial to be electrospun in a novel combination of poly vinyl pyrrolidone (PVP), gelatin (Gel) and polycaprolactone (PCL) for cardiac tissue engineering. The obtained fibers were aligned, uniform and bead free. After fabrication, the scaffolds were cross-linked in the glutaraldehyde vapor to become mechanically stronger and dissoluble in the aqueous environments. Considering the surface topography, biocompatibility, hydrophilicity, and mechanical properties, the fabricated hybrid electrospun ECM/PVP/Gel/PCL fibers can be proposed as a biomimetic scaffold for heart tissue engineering applications.

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1. Introduction

Since the myocardial infarction (MI) is one of the main causes of death around the world [1, 2], adopting a practical approach to regenerate infarcted tissue is a considerable concern of researchers [3, 4]. Among all suggested materials, electrospun nanofiber might be one of the best candidates for the cardiac regeneration due to having controllable physical and chemical characteristics, and high and well distributed porosity [5-7]. Moreover, having used natural and synthetic polymers, multiple types of electrospun micro/nanofibrous scaffolds have been fabricated for different applications in regenerative medicine and tissue engineering [8, 9]. All their characteristics cause electrospun nanofibers to mimic the cardiac

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extracellular matrix (ECM) more properly.

In addition to the technique, choosing suitable biomaterials to biomimetic the native ECM is essential. That's why, in previous studies, natural and synthetic biomaterials such as poly(e-caprolactone) (PCL) [10], gelatin (Gel) [11], polymethylglutarimide (PMGI) [12], PCL/Gel [13], collagen/elastin/gelatin/PCL [8], and collagen/elastin/PCL [14] were investigated specifically for the cardiac regeneration by the electrospinning approach.

PCL, as a biocompatible and biodegradable synthetic polymer, has had such different uses as scaffolds for the tissue regeneration, sutures in surgeries, biomedical devices for the bone fracture fixation, and the drug delivery system [10]. However, it has shown some important drawbacks including the slow degradation rate and low hydrophilicity, which limit its use [11]. To overcome this challenge, a mixture of PCL with other polymers have been widely used to enhance the physical and chemical properties of the electrospun scaffolds. Combining PCL and Poly(N-vinyl-2-pyrrolidone) (PVP), an excellent biocompatible and remarkable water-soluble polymer, is a great method to fabricate scaffolds, with the tunable fiber surface morphology and degradability, via simultaneous electrospinning [15].

Natural biomaterials mimic the native ECM. Since the percentage of natural proteins in the ECM of each tissue is specific according to the tissue's functions, the decellularized cardiac tissue would be a promising candidate for cardiac tissue engineering [16]. There are limited studies on using calf ECM as scaffolds for cardiac tissue engineering [17], however, due to their poor mechanical properties, there is still a need for ECM-based scaffolds with enhanced mechanical properties. Therefore, in this study, a novel combination of the acellular calf heart ECM,gelatin, PVP, and PCL was electrospun for being uses as a heart patch to regenerate cardiac tissue due to its biocompatibility, availability, and superior mechanical properties. Since, electrospinning the ECM solution was virtually impossible due to its low viscosity and the obtained scaffold lacked the proper mechanical strength and stretchability, we utilized its combination with Gel and PVP, which was soluble in water as well as organic solvents [18] and PCL solution [15, 19], to enhance the mechanical properties of scaffolds. To further mimic the anisotropy of the cardiac tissue, aligned Gel/PCL, PVP/Gel/PCL and ECM/PVP/Gel/PCL fibrous scaffolds were fabricated in the present study. After fabrication, the scaffolds were cross-linked using the glutaraldehyde vapor to improve their mechanical strength and prevent the scaffold dissolution in the culture media. Finally, the fabricated scaffolds were characterized in terms of morphology, hydrophilicity, mechanical properties, and biocompatibility.

2. Materials and methods

2.1. Materials

To prepare the polycaprolactone (PCL, Sigma-Aldrich, USA) solution, PCL with an average molecular weight of 80 kDa was dissolved in the mixture of chloroform (Merck Millipore, USA) and dimethylformamide (DMF, Merck Millipore, USA) with the ratio of 4:1. The calf heart was decellularized using sodium dodecyl sulfate (SDS, Sigma-Aldrich, USA) and triton X-100 (Bio Basic, USA). To solubilize the obtained ECM, hydrochloric acid (HCl, Merck Millipore, USA) and pepsin (Sigma-Aldrich,

USA) were used. Polyvinylpyrrolidone (PVP, Sigma-Aldrich, USA) and gelatin (Sigma-Aldrich, USA) were dissolved in 55 % acetic acid (Merck Millipore, USA). For the cell culture, Dulbecco's Modified Eagle Media (DMEM, Gibco™ by Life Technologies™, USA) and phosphate-buffered saline (PBS, Sigma-Aldrich, USA) were used.

2.2. Decellularized calf heart

The calf heart was frozen immediately after removing from the calf body. The heart was decellularized and characterized by the previously discussed method [20] with a slight alteration. Briefly, after removing its fat and vessels, it was cut into small pieces of about 2 mm of thickness. The pieces were rinsed with deionized (DI) water and then submerged in 1 wt % SDS in water and put in a shaker incubator at 37 °C for 6 days. Every 24 hours the SDS solution was changed with a fresh one. Afterwards, the remained tissue was stirred in 1 % (vol/vol) Triton X-100 for 30 min and subsequently in DI water for 24 hours. The obtained ECM was lyophilized for 24 hours. To solubilize the obtained matrix, it was stirred in 0.1 molar HCL in the presence of pepsin (the pepsin ratio to the matrix was 1:10) for 2 days. Afterwards, the solution was neutralized with 0.01 molar NaOH and lyophilized for 24 hours to obtain the ECM powder. The absence of cells in the decellularized heart tissue was confirmed by staining with hematoxylin and eosin (H & E).

2.3. Fabrication of scaffolds

10 % (w/v) PVP solutions in 55 % (v/v) acetic acid were prepared. Then a proper amount of gelatin was added to the solutions to obtain the final concentration of 10 % (w/v) gelatin. Afterwards, 10, 12, and 15 % (w/v) of the ECM was added to the PVP solutions and they were stirred for an hour. Separately, PCL (10 % w/v) was dissolved in the mixture of chloroform and DMF (4:1) to be electrospun from the opposite side of the collector.

After the solutions were prepared, they were loaded into the 5 mL syringes with blunt-tip 21-gauge needles and put into the syringe pumps. The process is schematically shown in Figure 1. The collector was covered with an aluminum foil (35 cm × 6 cm) and its spin rate was set at 2000 rpm. The fabricated electrospun scaffolds were put in a sealed desiccator containing aqueous glutaraldehyde (2.5 % v/v) at ambient temperature for 2 h to be crosslinked followed by being washed with distillated water several times. Preliminary studies showed samples with 15 % (w/v) ECM resulted in more uniform and bead-free fibers (data not shown), therefore, 15 % ECM was considered for the rest of characterization tests.

2.4. SEM

The scaffolds topography, and the alignment and diameter of fibers were studied by using the TESCAN, VEGA series 2007 scanning electrospinning microscopy (SEM). The samples were treated by a gold sputter coater before capturing the images. The average porosity and fiber diameters were calculated by using Digimizer software by randomly choosing at least 50 fibers on the SEM images of three different samples.

2.5. Fourier-transform infrared spectroscopy

Fourier transform infrared (FTIR) spectroscopy was conducted to corroborate the successful incorporation of the calf ECM into the electrospun PVP/Gel/PCL fibers. Samples were cut into squares (1 cm × 1 cm).

All spectra were attained exploiting a PerkinElmer, Spectrum Two series spectrometer. Spectra were presented between 4000 and 400 cm^{-1}.

2.6. Mechanical test

The mechanical properties of the electrospun scaffolds in a certain size (4 cm × 1 cm) were measured using a universal tensile testing instrument (Instron, model 3365) equipped with a 10 N load cell under the strain rate of 0.75/min. Tensile testing was performed under the ambient condition after the immersion (post-submersion, PS) in deionized water for 1 h at 37 °C. The samples were tested under tension in the direction of the fibers' alignment. At least three specimens were tested for each composition.

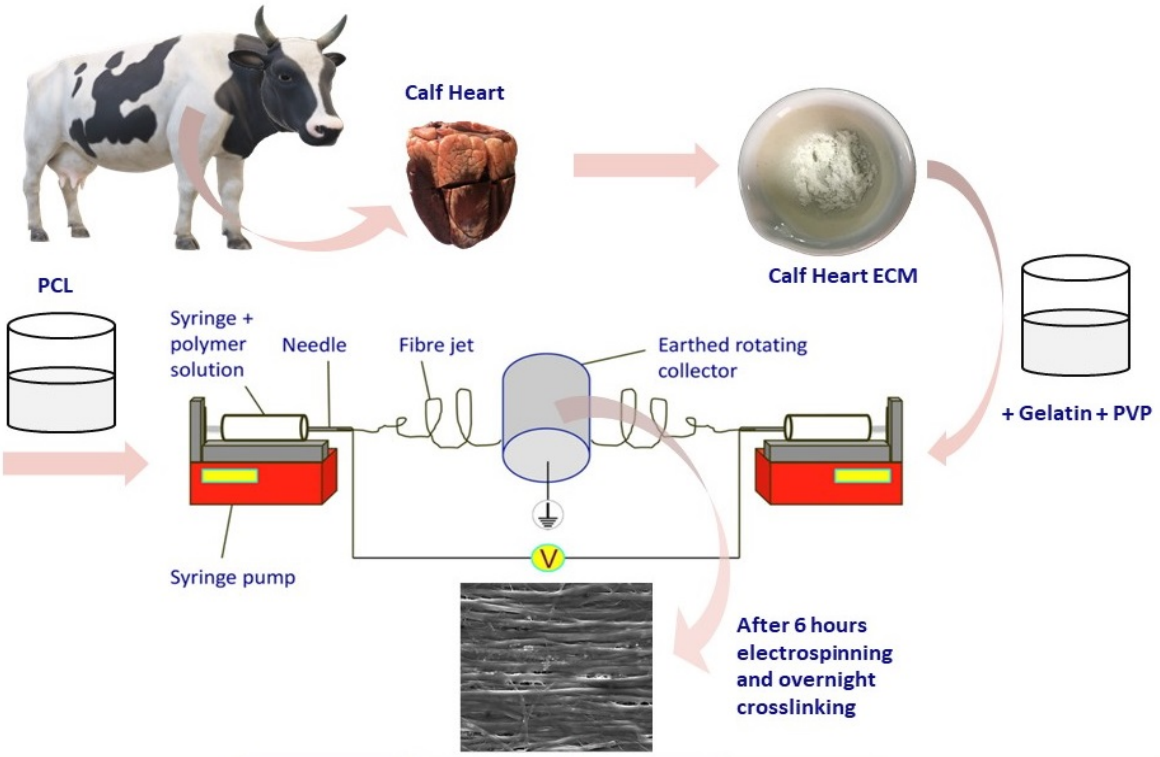

Figure 1. Schematic image of the electrospinning process. The calf heart tissue was decellularized using SDS and Triton X-100, and then proper amounts of it were dissolved in the 55 % acetic acid solution with 10 % PVP and 10 % gelatin. The final mixture was electrospun using a rotating collector at 2000 rpm. At the same time, a 10 % PCL solution in the mixture of chloroform and DMF (4:1) was electrospun from the opposite side of the collector. The obtained fibers were placed in a sealed desiccator containing 2.5 % glutaraldehyde aqueous solution to be crosslinked.

2.7. Contact angle

The hydrophilicity of the scaffolds was investigated by the measurement of the water contact angle using a digital camera. Several droplets of distilled water were randomly placed at different regions of each fiber. The wettability of the electrospun scaffolds was evaluated using Drop Shape Analyzer-DSA25 (Kruss, Germany) at room temperature via the sessile drop method. The measurement was carried out after subtending water drops for 10-15 s on the surface of scaffolds.

2.8. MTT assay

The viability of the cells on nanofibrous scaffolds was studied by MTT assay using the mesenchymal stem cells (MSCs) obtained

from the mouse bone marrow (second passage). After immersing the obtained scaffolds in 75 % ethanol for 2 h, the scaffolds were thoroughly rinsed using PBS at least for 3 times. Then, after 24 hours of treating the scaffolds with DMEM, MSCs were seeded onto them at a density of 4×10^3 cells/cm^2 in a 24 well plate. The same seeding density has been used for culturing MSCs in tissue culture polystyrene (TCP) as control samples. Afterwards, the cells on the scaffolds were incubated (at 37 °C and with 5 % CO_2) with the culture media containing DMEM with 10 % FBS .The cell viability was investigated on days 3 and 7 post seeding by MTT assay. For MTT assay, the culture medium of each well was replaced with 0.25 ml of the DMEM medium containing 5 mg/mL of MTT in PBS and then, the plate was incubated for 3 h at 37 °C and with 5 % CO_2. Afterwards, the medium in all wells were replaced with the 0.6 mL solution of isopropyl alcohol-hydrochloric acid and then incubated for 30 min. Finally, the absorbance of the medium at 560 nm was measured using a spectrophotometer (Bio Photometer, Eppendorf, Germany).

2.9. Statistical analysis

Statistical significance was analyzed using one-way ANOVA for more-than-two-group comparisons with one independent variable and two-way ANOVA for more-than-two-group comparisons with two independent variables. GraphPad Prism version 8 (GraphPad Software, La Jolla, CA) was used for such analysis.

3. Results and discussion

3.1. Characterization of acellular ECM

To evaluate the quality of the obtained acellular ECM, H & E staining was conducted to confirm the cells removal from the calf heart tissue. An efficient decellularization procedure removes all the cells with the minimal structural and chemical changes to the ECM. Figure 2A and 2B show the histological analysis of fresh and acellular tissues respectively. This result confirmed that the cells were completely removed from the tissue without significantly altering the ECM structure.

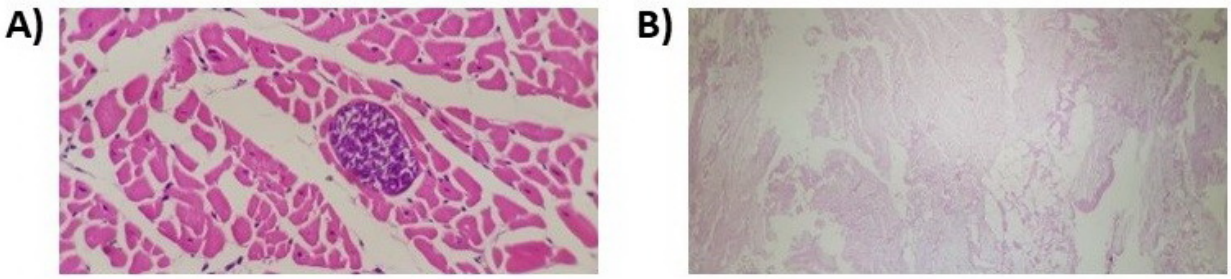

Figure 2. Histological analysis using H & E images confirmed that the decellularization procedure successfully removed all the cells.

3.2. Characterization of electrospun fibers

The decellularized ECM was in the form of a white powder after the second lyophilization step. It was simply dissolved in 55 % (v/v) acetic acid and blended with the PVP and Gel solution homogenously and was used for the fabrication of the electrospun scaffold. The morphology of the electrospun fibrous scaffolds was investigated by SEM and the fiber diameter was calculated by using Digimizer software. Figure 3 shows the SEM images of Gel/PCL, PVP/Gel/PCL and

ECM/PVP/Gel/PCL electrospun fibers. Using a solvent containing chloroform/methanol (4/1 in vol %) and optimal electrospinning parameters (listed in Table 1) resulted in successfully obtaining bead-free electrospun fibers in the entire range of blend composition.

Table 1
Electrospinning parameters.

Parameters	ECM/PVP/Gel	PCL
Voltage	30 kV	16 kV
Distance to the collector	16 cm	15 cm
Flow rate	0.4 ml/h	0.4 ml/h

As depicted by Figure 3, three-dimensional, aligned and bead free fibers were obtained showing the suitability of the applied conditions for electrospinning. The images showed that the addition of ECM and PVP did not adversely affect the fiber alignment in comparison with Gel/PCL fibers as the control sample. Previous studies showed that aligned electrospun nanofibers were able to mimic the anisotropic structure of myocardium and facilitate maturation of cardiomyocytes [21]. Figure 3B shows the microstructure of the three groups of electrospun scaffolds after the cross-linking reaction. Although glutaraldehyde can be cytotoxic at high concentrations, it is still a good choice for crosslinking electrospun fibers [22]. Therefore, it is widely used in tissue engineering at low concentrations followed by multiple washing steps without cytotoxicity effects [23, 24]. It could be noticed that the fibrous structure of the samples was maintained during the related chemical interactions.

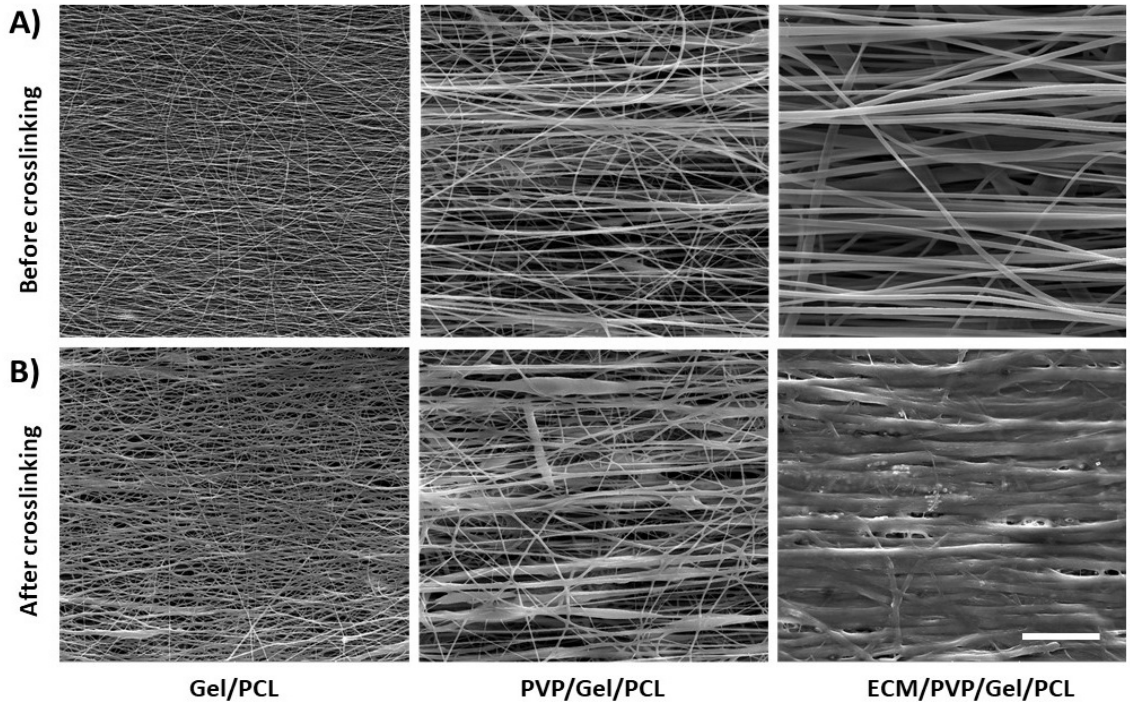

Figure 3. Scanning electron micrographs of the electrospun fibers consisting of different compositions, A) before and B) after cross-linking by the glutaraldehyde vapor. The scale bar is 5 μm.

The diameter of electrospun fibers is important as it can affect several properties of scaffolds including their mechanical strength, degradation rate, and pore size [9]. As shown in Figure 4, the average fiber diameter of Gel/PCL scaffolds was 112 ± 23 nm, which increased to 169 ± 41 and 870 ± 112 nm with the addition of PVP and ECM respectively. The native cardiac ECM has three fiber groups with distinct average diameters including nanoscale, 1 μ, and several micrometers. Each group has a specific role in creating the unique structure of the cardiac tissue. Among these fibers, nanoscale fibers with a diameter of tens to hundreds of nanometers play a significant role in surrounding individual cardiomyocytes and cell-ECM interactions [25]. In this work, the ECM/PVP/Gel/PCL fibers with 14.65 ± 2.45 % of porosity have larger diameters (870 ± 112 nm) than those of previous studies in which Gel/PCL fibers were obtained with 200-300 nm of diameter [26, 27]. However, it is still in the range of nanoscale and can serve as the native ECM nanofiber in wrapping individual cardiomyocytes and enabling the cell-ECM interactions. The relatively larger fiber diameter and less porosity also provide the scaffold with a higher mechanical strength and a more controlled degradation rate [9]. It should not be neglected that other parameters of the solution such as electrical conductivity can affect the fiber diameter. In this study, the addition of ECM solution needed a higher voltage to be electrospun, therefore, it could be concluded that the increase in ECM resulted in the decrease in the electrical conductivity of the solutions. Therefore, both viscosity and electrical conductivity of the solution have impacts on the fiber diameter. This is consistent with the result reported by Salles et al, which demonstrated that the electrical conductivity of the solution as well as its viscosity significantly affected the electrospun fiber diameter [28].

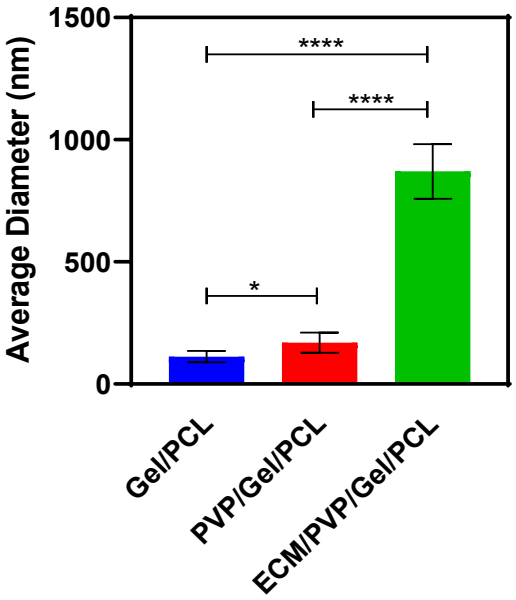

Figure 4. Average diameter of the fibers in three different groups of the samples. Error bars indicate the standard deviation (n = 20, * and **** indicate nonsignificant, $p < 0.1$ and $p < 0.0001$ respectively).

3.3. Fourier-transform infrared spectroscopy

The FTIR results demonstrated that the calf ECM was successfully incorporated into the electrospun PVP/Gel/PCL fibers. The spectra of ECM and ECM/PVP/Gel/PCL are shown in Figure 5. The locations of the characteristic peaks of the ECM spectrum that were contributed to the amide I bond at an absorbance of 1654 cm^{-1} and the amide II bond at an absorbance of 1541 cm^{-1} were also found virtually at the same site of the ECM/PVP/Gel/PCL fiber's spectrum. Based on the aforementioned analysis, it was concluded that the electrospinning process could properly integrate the calf ECM into the ECM/ PVP/Gel/PCL fibers. In addition, it can be inferred that no residue of glutaraldehyde

exists in the fibers as the ECM/ PVP/Gel/PCL electrospun fiber's spectrum does not show any observable peak at 1725 cm^{-1} that relates to the presence of the excess or non-reacted glutaraldehyde [29, 30].

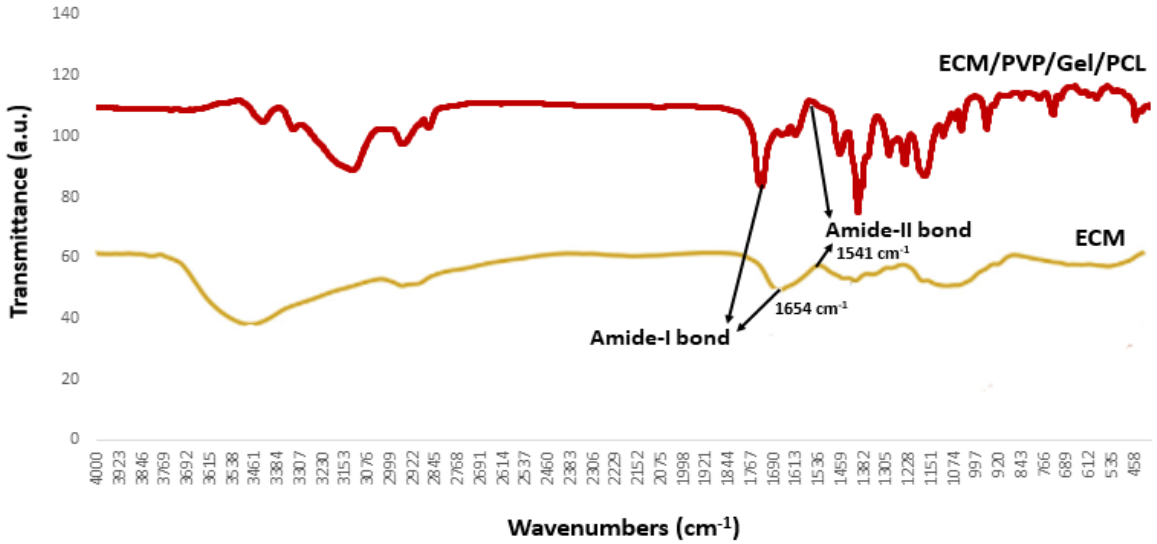

Figure 5. FTIR spectra of (red line) ECM/PVP/Gel/PCL and (yellow line) ECM.

3.4. Mechanical properties of electrospun scaffolds

To study the effect of ECM on the mechanical properties of electrospun scaffolds, the Young modulus and elongation at break of the samples were measured under tension in the direction of the fibers' alignment. Figure 6 shows the results of the mechanical tests of the fibrous scaffolds. These results have revealed that adding PVP to Gel/PCL has not significantly affected the Young modulus (Gel/PCL with 0.26 ± 0.06 MPa and PVP/Gel/PCL with 1.06 ± 0.03 MPa) while adding both PVP and ECM to the mixture has slightly increased the Young modulus of samples (4.5 ± 1.0 MPa, $p < 0.1$). However, in PVP/Gel/PCL and ECM/PVP/Gel/PCL samples the percentage of elongation at break significantly increased by adding only PVP or PVP and ECM to the Gel/PCL mixture. The elongation at break as an indicator of the fibers' resistance to the changes of shape without the crack formation is critical for the cardiac tissue because of the heart's stretches by expanding and contracting. The novel combination of ECM/PVP/Gel/PCL is more promising alternative in the cardiac tissue engineering compared to what used in previous studies in which the range of 4.9-6.4 % was obtained for the elongation at break from a combination of alginate, chitosan, and varying percentage of bovine ECM [17]. Therefore, since elasticity is a vital property for the cardiac scaffolds and patches to support the contractile heart tissue [31], the ECM/PVP/Gel/PCL scaffolds with the higher elongation at break (42 ± 3.8 %) can be more stretchable and suitable for contracting the heart tissue.

3.5. Contact angle measurement

In order to evaluate the surface hydrophilicity of the nanofiber scaffolds as a function of their compositions, the water contact angles on the Gel/PCL, PVP/Gel/PCL and ECM/PVP/Gel/PCL scaffolds were measured. As shown in Figure 7, adding PVP increased the hydrophilicity of Gel/PCL samples by up

to 16 % (the contact angel of the water drop was decreased about 16 %). Moreover, the addition of ECM could further decrease the contact angel of the water drop from 59˚ to about 40 ˚.

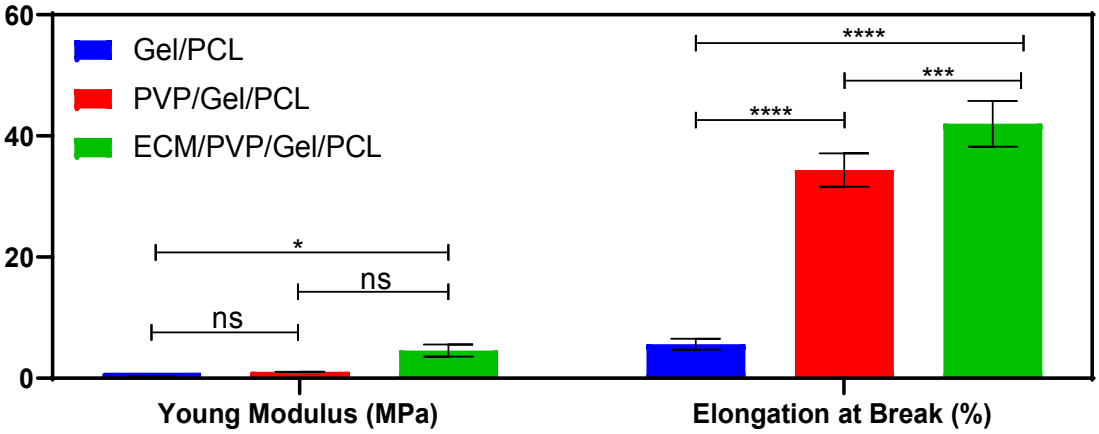

Figure 6. Mechanical properties of the fibrous scaffolds. Error bars indicate the standard deviation (n = 3, ns, *, **, ***, and **** indicate nonsignificant, $p < 0.1$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$ respectively).

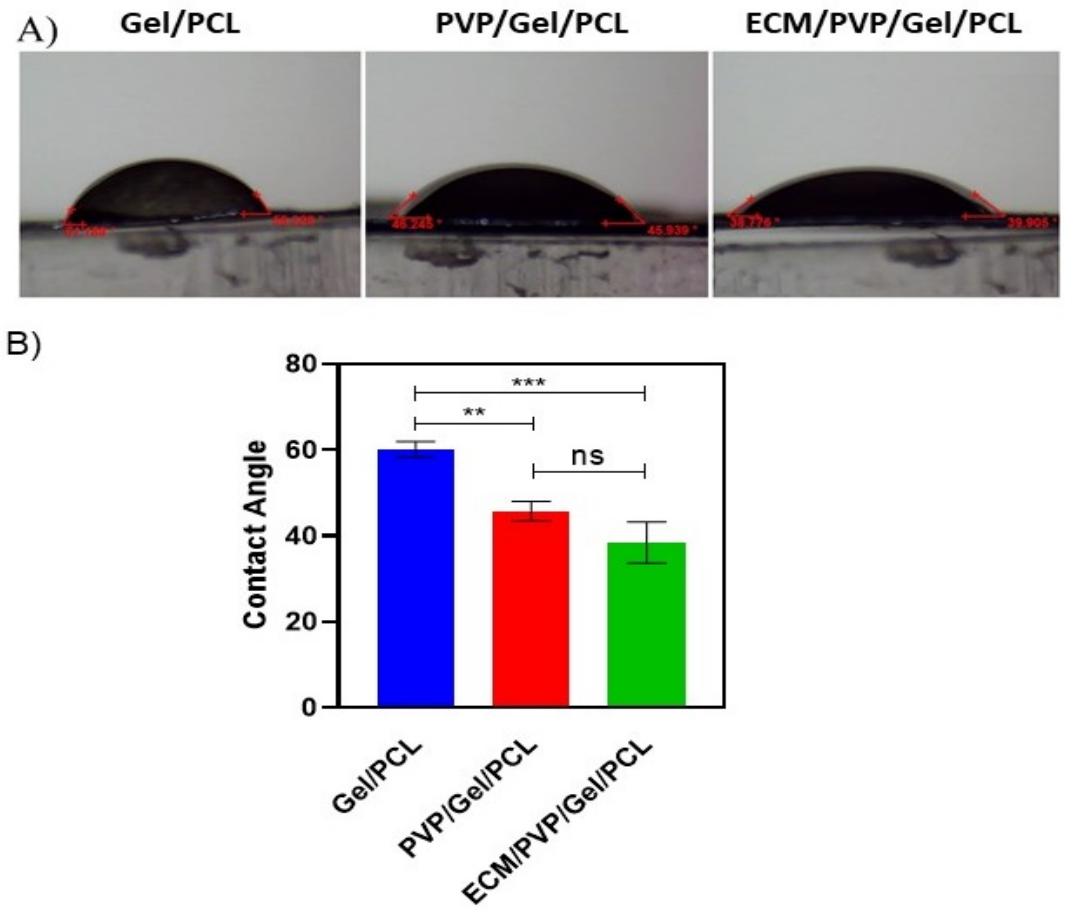

Figure 7. Water contact angel of the surface of the samples, A) the drop water images, B) the quantitative results of the average contact angel. Error bars indicate the standard deviation (n = 3, ns, **, and *** indicate nonsignificant, $p < 0.01$, and $p < 0.001$ respectively).

3.6. Viability analysis

The cell viability of the scaffolds was evaluated using MTT assay. After 7 days of culturing cells on three different groups of nano-fibrous scaffolds, the samples were investigated by MTT assay and the results have been shown in Figure 8. Those results demonstrated that on day 3, there was no significant difference between the cell viabilities of PVP/Gel/PCL and ECM/PVP/Gel/PCL scaffolds while the cell viabilities of both samples were significantly lower than that of the control sample (with $p < 0.001$ and $p < 0.1$ respectively). However, on day 7, no significant difference between the ECM/PVP/Gel/PCL and the control samples was observed while the cell viability of PVP/Gel/PCL scaffolds was still significantly lower than that of the control sample. It can be concluded that ECM has provided a higher number of cell binding sites and a more biomimetic environment for cells to attach and proliferate.

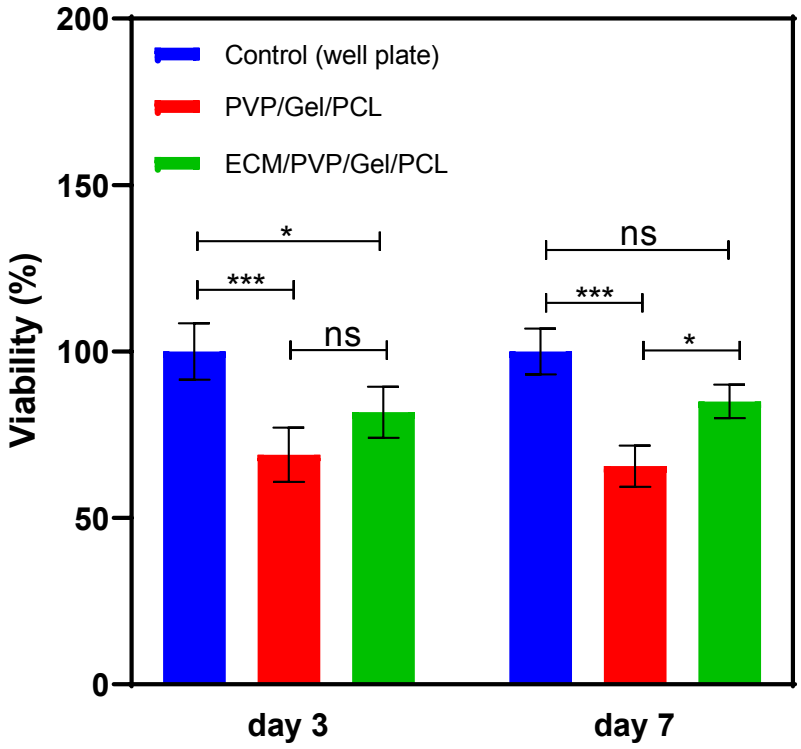

Figure 8. Cell viability on fibrous PCL scaffolds during a 7-day cell culturing period. Error bars indicate the standard deviation (n = 3, ns, *, and *** indicate nonsignificant, $p < 0.1$, and $p < 0.001$ respectively).

4. Conclusions

The myocardial infarction is still one of the main causes of death around the world. Since the current approaches for the MI treatment are not completely successful, there is still a substantial need for a more effective treatment strategy. The emergence of tissue engineering has opened a new door for developing an effective, powerful, and novel strategy for the MI treatment. The main goal of such studies is stimulating or enhancing the regeneration of the heart muscle tissues after infarction. Among the existing approaches, using electrospun nanofibrous scaffolds has attracted the researchers' attention due to the resemblance of electrospun nanofibrous scaffolds to the fibrous structure of the heart tissue. In the present study, we have

developed an electrospun nanofibrous scaffold using the acellular calf heart ECM in combination with Gel, PCL, and PVP. The results showed that the presence of the calf heart ECM enhanced the elasticity and hydrophilicity of the scaffold. Moreover, the more available cell binding cites of ECM increased the viability of cells cultured on the scaffolds to 80 %. The obtained nano-fibrous scaffolds were aligned, bead free and uniform. These results have confirmed that the acellular calf heart ECM in combination with gelatin, PVP, and PCL is a great potential biomaterial for developing electrospun scaffolds as basis for our future studies on fabricating a functional scaffold for the cardiac regeneration.

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