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Original Article

Reinforcement of a Decellularized Extracellular Matrix-Derived Hydrogel Using Nanofibers for Cardiac Tissue Engineering

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ABSTRACT

The role of heart disease in increasing worldwide death and the limited availability of organs for transplantation have encouraged multiple strategies to fabricate functional and implantable constructs. One of these strategies is to develop a biologically similar heart tissue scaffold, in which two types of fiber and hydrogel are commonly used. Toward this goal, taking advantage of both hydrogels properties and fibers features with excellent mechanical properties can be considered as a promising method. The purpose of this study was to develop a fiber/hydrogel composite of gelatin, poly-caprolactone (PCL), cardiac extracellular matrix (ECM), and chitosan. The fibrous scaffolds of PCL and gelatin were characterized by SEM, water drop contact angle test, FTIR, and mechanical tests. The results showed that the average diameter of nanofibers, hydrophilicity and mechanical properties of the fibrous scaffolds increased with increasing the gelatin content in the spinning solution. Furthermore, the results of mechanical tests indicated that by integrating fibers with gelatin to PCL mass ratio of 2 in the hydrogel of chitosan and ECM with a mass ratio equal to 1, we obtained a construct with similar mechanical properties to native heart tissue, which may be proposed as an appropriate scaffold for heart tissue engineering.

Key words: Cardiac tissue engineering, Composite scaffold, Extracellular matrix, Mechanical properties, Nanofiber

Introduction

Today, cardiovascular has a key factor in most of human deaths annually. Given the shortage of organ donors, cardiac tissue engineering has been emerged as a promising approach for cardiac regeneration using biomimetic scaffolds and cardiomyocytes (Jafarkhani *et al.,* 2018; Hu *et al.,* 2019). An interesting method in cardiac tissue engineering is to develop functional

biomimetic 3D constructs incorporating biocompatible and biodegradable nanofibers and hydrogels with suitable mechanical strength (Ying *et al.,* 2011).

An ideal biomaterial for cardiac tissue regeneration should imitate the micro-structure of the native tissue, presents an appropriate mechanical strength, and has electroactive properties to direct cardiac cell behaviors. Up to now, development of such functional biomaterial is still a big challenge (Pomeroy *et al.,* 2019).

Recently, some success has been achieved in developing nano-fibrous biomaterials with morphological similarity to the cardiac ECM. (Ismail *et al.,* 2018; Ahn *et al.,* 2018; Su *et al.,* 2016). Using electrospinning, biomimetic nano-fibrous constructs with tunable properties can be developed easily by changing biomaterials composition and processing factors (*Yang et al.,* 2016). Numerous kinds of fibrous scaffolds have been fabricated as heart muscle patches. A rigid and non-elastic fibrous patch may prevent cardiac tissue from its normal motion. To overcome this challenge and provide a 3D environment for cells, hydrogels are usually employed as a bed to surround the fibers and control different growth factors release (Ying *et al.,* 2011).

PCL is an interesting synthetic polymer which can be easily processed to achieve an implantable scaffold (Siddiqui *et al.,* 2018). However, the use of PCL alone as a scaffold material is limited due to its hydrophobic properties and poor cell attachment (Kucinska-Lipka *et al.,* 2015). Therefore, blending a natural polymer such as gelatin (Zhao *et al.,* 2007) and collagen (Jin *et al.,* 2009) with PCL is expected to modulate its bioactivity, degradation rate, and hydrophilicity (Kucinska-Lipka *et al.,* 2015).

The combination of PCL and gelatin has been studied in the previous studies (Chong *et al.,* 2007; Zhang *et al.,* 2005; Zhao *et al.,* 2007). In most of these studies, a solvent was used to dissolve gelatin and PCL. For example, hexafluoro-2-propanol was employed as a common solvent for PCL and gelatin. However, this solvent is toxic and unavailable. Due to the fact that there is no common, non-toxic, and well-known solvent for both PCL and gelatin, researchers have sought to find a suitable solvent. For example, (Gautam *et al.,* 2013) used acetic acid for gelatin and chloroform/methanol for PCL. They claimed that with this solvent system, it is possible to try these two polymers in one syringe. Regarding the literature review, in this study, two syringes were used to electrospin two solutions separately.

In the present study, we first investigated the appropriate composition for fibrous scaffolds and hydrogels separately based on heart tissue features. PCL and gelatin were used for fibers fabrication. For hydrogel composition, the best material is ECM derived from the heart due to its similarity to the native cardiac tissue. In order to improve the mechanical properties of the ECM-derived hydrogel, chitosan was applied. Therefore, a new biomaterial composition was developed from PCL/gelatin fiber embedded within the hydrogel of chitosan and ECM to use in cardiac tissue engineering.

Experimental

Materials and methods

PCL, gelatin, sulfuric acid (H_2SO_4), Phosphoric acid (H_3PO_4), hexafluoro-2 propanol, sodium dodecyl sulfate (SDS) were bought from Sigma-Aldrich. To crosslink the samples, glutaraldehyde was obtained from Merck.

Decellularization

Decellularization of the bovine heart was performed using enzymatic detergent based on Seif-Naraghi's protocol (Singelyn *et al.,* 2009). Briefly, the bovine heart was kept on ice during transportation to the laboratory to avoid matrix damage. Then the heart was cut to the thin sheets with 2 mm thickness and then washed with PBS solution for 2 h. Next, the samples were immersed in SDS solution (0.1% w/v) in the presence of protease inhibitors and placed in the shaker-incubator for 24 h three times. The tissues were finally washed with a Triton X-100 solution (1% v/v), and then with sterile PBS for 24 h with vigorous stirring, frozen overnight at -70 °C and lyophilized for 48 h. The decellularized powder was dissolved in pepsin solution (1 mg/mL).

Hydrogel fabrication

Hydrogel samples were fabricated according to a certain protocol, which was optimized for cardiac tissue engineering applications and reported by Esmaeili et al (Esmaeili Pourfarhangi *et al.,* 2018). Briefly, a chitosan solution (3.5% wt.) was prepared in acetic acid (2% v/v) under agitation condition at 60 °C for 24 h. To make ECM solution, initially 0.1 M HCL solution was made and 1 mg of pepsin was dissolved in 1 Ml of acid solution (pepsin solution). Then, 10 mg of decellulaized ECM was dissolved in pepsin solution for about 65h stirring. After that, by adding 1 M NaOH and PBS 10X, the solution was reached to pH 7.4. ECM and chitosan solutions were then mixed with the same volume ratio and then stirred for 2 h to obtain a homogenous solution.

Electrospinning

Here, two-nozzle electrospinning method was applied to produce nanofibers from PCL and gelatin. A syringe containing PCL solution (in chloroform/dimethylformamide solvent with volumetric ratio of 4 to 1) and another syringe of gelatin solution was used. Gelatin solution in acetic acid (25% wt.) was prepared under agitation for 24 h. Then, PCL and gelatin solutions were poured in two separate syringes located at 16 and 24 cm and two voltages of 17 and 11 kV were applied to the needle of each syringes, respectively. By changing the flow rate of both solutions, samples with different properties can be obtained. Three groups of the fibrous samples were fabricated with different gelatin to PCL mass ratio of 1, 2, and 3, which termed as GP1, GP2, and GP3. For production of GP1, GP2, and GP3, flow rate ratios of PCL to gelatin were 0.2/0.1, 0.5/0.5, and 0.3/0.5, respectively. Finally, glutaraldehyde steam was used to crosslink the polymeric fibers.

Fiber/hydrogel composite fabrication

Fibrous scaffolds of PCL/gelatin were embedded within the hydrogel of chitosan and ECM. To create the constructs from hydrogel and fibers, the fibrous scaffolds were cut in 3 × 2 cm and then embedded in 10 mL hydrogel solution for 5 min. In the next step, 10 µl glutaraldehyde (2% wt.) was added. The thickness of the samples was about 2 mm. The electrospun scaffolds were placed in a plate containing certain amount of the non-cross linked solution of the hydrogel. The solution of the hydrogel was allowed to penetrate into porous structure of the electrospun scaffolds. After 5 minutes, glutaraldehyde was added to crosslink the hydrogel. Based on the constant composition of the hydrogel, here we fabricated three groups of the fiber/hydrogel

composite; Gel/GP1, Gel/GP2, and Gel/GP3. Fiber/hydrogel composites showed a thickness of about 2 mm.

DNA quantification

DNA content of the decellularized samples was analyzed using proteinase K (Rajabi-Zeleti *et al.,* 2014). Briefly, the samples were incubated in digestion buffer solution containing 1% proteinase at 55 C overnight. Then, a phenol/chloroform extraction was performed using centrifuge for 15 minutes at 13700 rpm and the over layer was pick up. In the following step, ethanol 100% was added and DNA was deposited and separated from the aqueous solution and then washed with ethanol 70% and then dissolved in RNase-free water. Finally, spectrophotometer (ELISA reader) was applied to determine DNA concentration at 260 nm. We repeated all of the experiments three times for both native and decellularized tissue and reported the average amount of DNA as mg/mg dry weight of samples (Rajabi-Zeleti *et al.,* 2014).

Scanning electron microscopy (SEM)

To study microstructure of the nano fibers and hydrogels, SEM test was employed. Toward this goal, the dried specimens were coated with gold. Then, different parts of the samples were observed to measure fibers diameter and choose the optimum composition based on the absence of granolas.

Contact angle

To analyze the degree of hydrophobicity of the fibrous scaffold, contact angel test was applied. A drop of water with a specific volume was pipetted and on the surface of fibrous scaffold at room temperature. After 10 s, the angel between the drop and surface was observed using a camera (Dino camera). By analyzing these images with ImageJ, the angle of contact can be measured.

Fourier transform infrared spectroscopy (FTIR)

Chemical interaction between functional groups of chitosan and ECM in the presence of glutaraldehyde was studied using FTIR test (ABB Bomem-100).

Mechanical properties

To study mechanical properties of the samples, uniaxial mechanical test was used. Using this method, elastic modulus and maximum shear stress can be obtained. A load (20 N) with a velocity of 0.5 mL/min was used for each sample.

Statistical analysis

In this study, each experiment was performed at least three replicates. We present the obtained data as mean ± standard error (SE). Statistical analysis was performed by using One-way analysis of variance (ANOVA) and *P*-values less than 0.05 were considered significant.

Results and discussion

In recent years, combination of synthetic materials and natural polymers to develop electrospun scaffolds has been widely used (Zhao *et al.,* 2007). PCL is a well-known

synthetic biomaterial which possess great properties including biocompatibility and mechanical strength. However, its drawbacks such as hydrophobicity and lack of active positions for cell attachment have limited its application in tissue engineering (Cruz *et al.,* 2019). To overcome this challenge, gelatin with high level of cell-recognition domains can be used in combination with PCL (Binulal *et al.,* 2014). In this study, all of the processing parameters related to electrospinning was considered constant as mentioned above.

DNA content

In the previous studies (Nasiri and Mashayekhan, 2017; Sivandzade and Mashayekhan, 2018; Esmaeili Pourfarhangi *et al.,* 2018), which the same protocol was used to extract ECM from the native tissue, the success of decellularization process was confirmed using Hematoxylin & Eosin staining test. In this study DNA content of ECM was determined. Figure 1 shows the result of this test for both native tissue (control) and decellularized construct. A significant difference between the samples (4800 ng/mL in the heart tissue and 100 ng/mL in ECM) can be seen which shows that decellularization was performed successfully.

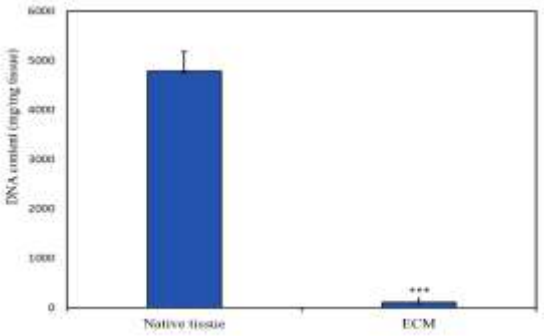

Figure 1. DNA content of the heart tissue and decellularized scaffold (***: *P*-value<0.002)

FTIR

Figure 2 shows the results of FTIR test for a combination of PCL and gelatin with mass ratio of 2 (GP2). Based on this figure, peaks at 1639 cm^{-1}, 1542 cm^{-1}, 1240 cm^{-1} and, 3300 cm^{-1} indicates C=O, N_H (Amide I), N_H (Amide II), and Amide A bonds, respectively. These peaks are related to gelatin content of the scaffolds. Moreover, two peaks at 2850 cm^{-1} and 1640 cm^{-1} are related to C_H and C=O bonds, showing PCL. In crosslinking reaction, aldehyde groups (-CHO) of glutaraldehyde reacts with the amino groups of protein lysine. An index of gelatin crosslinking is a graduate color change in the samples from white to yellow. This phenomenon occurs due to the formation CH=N bond, which its peak appears in the range 1640-1690 cm^{-1}. It is worth mentioning that cross-linking reaction does not involve any change in the molecular structure of PCL. The similar results have been reported in the previous studies (Pereira *et al.,* 2014; Safaeijavan *et al.,* 2013). We also studied chemical interaction between pure chitosan and ECM using FTIR test (Figure 2b). The main peaks of chitosan can be easily seen at 3439 (N-H and O-H bond), 2925 (CH3 stretch), 1661 (C=O bond), 1438 (C-N), 1155 (C-O-C), and 1073 (C-OH bond) cm^{-1}. FTIR spectra of ECM shows peaks at 1290, 1550, and 1680 cm^{-1} relating to Amid I, Amid II, and Amid III, respectively. The effect of cross-linking reactions on FTIR spectra of the hybrid

hydrogel is detectable from the peaks at 1640-1690 cm^{-1} , a slight increase in amid bond at 1103 cm^{-1} , and a significant reduction of peak at 3423 cm^{-1} corresponding to free amine groups.

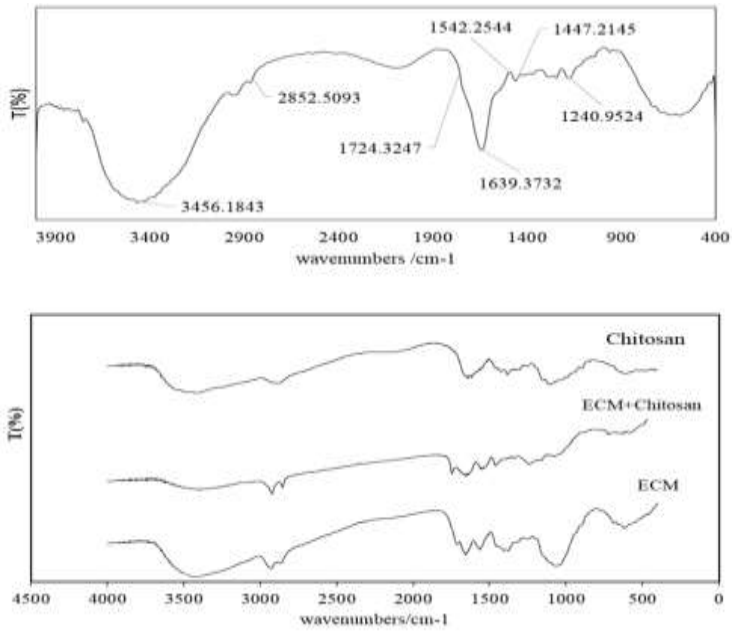

Figure 2. FTIR spectrum of A) PCL/gelatin scaffold and B) pure chitosan, ECM, and hybrid hydrogel

Morphological characterization of the fibrous scaffolds

The effect of gelatin to PCL ratio on the fiber diameter was studied using SEM. Figure 3 presents the results of SEM test for 3 groups of the fibers. As can be seen, the samples containing the least amount of gelatin showed the lower average diameter (507.21±24 μm) in comparison with other groups (Figure 3a). Moreover, based on Figure 3b-c, the average fiber diameter for 1:2 and 1:3 hybrid mats increased to 580.08±41 and 654.99±49 μm, respectively.

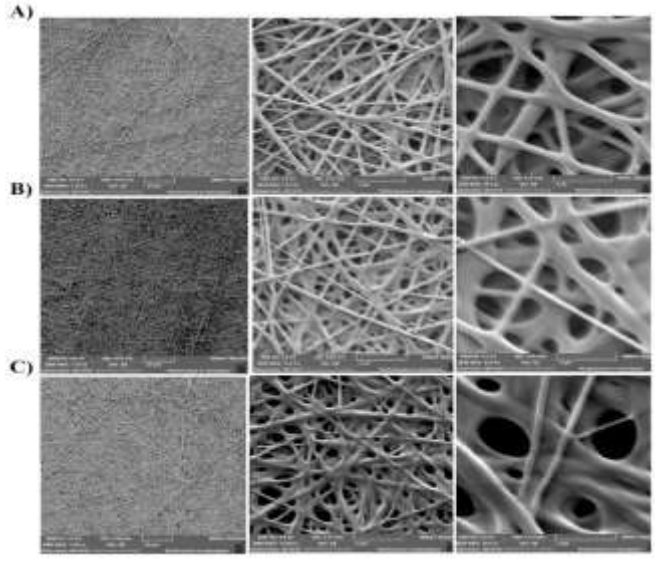

Figure 3. SEM images from the fibrous scaffolds containing different weight ratio of gelatin to PCL of A) GP1, B) GP2, and C) GP3

Using ImageJ software, the average diameter of three different groups of the electrospun scaffold was estimated. The results of the fiber diameter measurement were shown in Figure 4. From Figure 4, it can be concluded that fiber diameter increased with enhancement of gelatin content in gelatin/ PCL hybrid scaffolds. Given that all processing parameters in spinning were considered constant, the reason of changing in fiber diameter is related to viscoelastic properties of gelatin (Jose *et al.*, 2009). It is well known that viscosity of the polymeric solution is a key parameter that has a significant effect on the fiber diameter because a highly viscoelastic solution requires a high level of tensile force and as a consequence a thick jet and finally a thick fiber is generated (Meechaisue *et al.,* 2006). Furthermore, higher molecular weight of gelatin solution leads a higher viscoelasticity behavior and increases fiber diameter (Aghdam *et al.,* 2014). (Chong *et al.,* 2007) fabricated an electrospun scaffold of PCL and gelatin and observed the same changes in the fibers diameter. They reported that the samples showed an interconnected pores and smooth micro-structure.

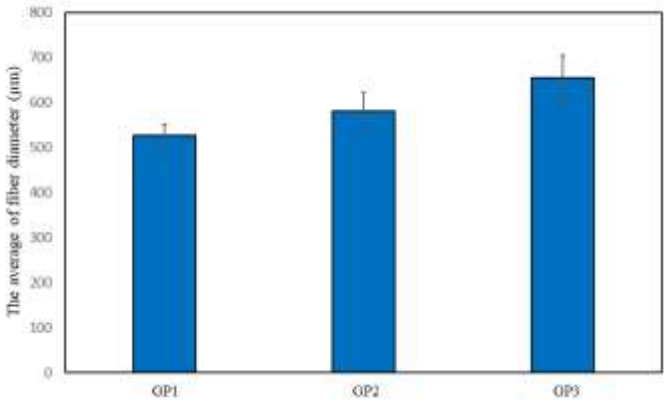

Figure 4. The average diameter of three groups of the produced fibers

Contact angle

The hydrophilicity and hydrophobicity features of an implantable construct is an influential factor in tissue engineering strategies due to its important effect on different cell behaviors such as attachment and migration (Aghdam *et al.,* 2014). To analyze the influence of gelatin content in the hybrid PCL/gelatin electrospun scaffolds on their hydrophilicity features, contact angle of water drop test was performed. Figure 5 displays the results of contact angle measurements for PCL, PCL/gelatin fibrous samples with various gelatin content.

Based on Figure 5, it can be seen that the contact angel of pure PCL scaffolds is 84°, which indicates that the polymer is hydrophobic and does not absorb water well. By enhancement of gelatin content in the fibrous scaffold, the drop water spread gradually and so, the contact angle was reduced, showing the improved hydrophilicity feature of PCL/gelatin composites. The reason for this is the abundant presence of hydrophilic groups such as hydroxyl in gelatin molecular structure. Therefore, it is reasonable that the increase of gelatin in the hybrid scaffolds would leads to a significant reduction in the contact angle, showing more hydrophilicity (Aghdam *et al.,* 2014). (Zhang *et al.,* 2005) developed electrospun scaffolds of PCL and gelatin and studied the hydrophilicity

of two components and its composition. They obtained similar results for gelatin and PCL scaffolds and reported that an improved wettability was observed for gelatin/PCL hybrid scaffolds in comparison with gelatin or PCL alone.

It is shown the combination of PCL and gelatin provides a material with better wettability compared to both pure components. Moreover, because the purpose of this study is to embed these fibers in the hydrogel, the improved hydrophilicity facilitates better interaction between the constituents of the hydrogel.

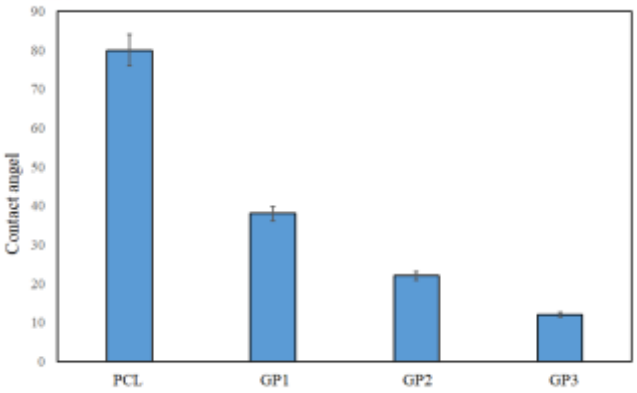

Figure 5. Contact angel of PCL fiber (gelatin to PCL ratio of 0) and GP1, GP2, GP3

Mechanical properties

Mechanical strength is an important feature of the engineered scaffolds in tissue engineering applications. Because scaffolds should tolerate different forces when are implanted in the body. Moreover, mechanical properties also can direct cell behaviors, which influence tissue regeneration. To control cardiac cell behaviors, it is necessary to develop a scaffold with similar mechanical properties of the heart tissue. Thus, an engineered cardiac tissue should have appropriate Young modulus and sufficient mechanical tensile to resist contracting force of the heart (Vunjak-Novakovic *et al.,* 2010; Dunn *et al.,* 2014).

Figure 6 presents the results of tensile mechanical test of the native heart tissue and different groups of the samples. As shown in this Figure, the nature of PCL scaffold displays a very high stretch, and strong material. As can be seen, gelatin Young's modulus is greater than all of the groups Young's modulus. It can be seen that as gelatin content in the constructs increases the Young's modulus enhances. The similar results have been reported in the previous studies (Zhao *et al.,* 2007; Denis *et al.,* 2015). For example. (Zhang *et al.,* 2005) also reported that the blend of PCL and gelatin produced a higher elongation, energy and Young at break tension point which shows that hybrid scaffolds have appropriate flexibility.

Moreover, it is clear that Young modulus of all samples is much more than that of the native tissue (31.25±1.5). As mentioned above, an ideal scaffold for cardiac tissue engineering must show a similar mechanical feature. Therefore, it is necessary to embed these electrospun scaffolds in the hydrogels with lower Young modulus. Figure 6 shows the results of tensile test of the fiber/hydrogel composites.

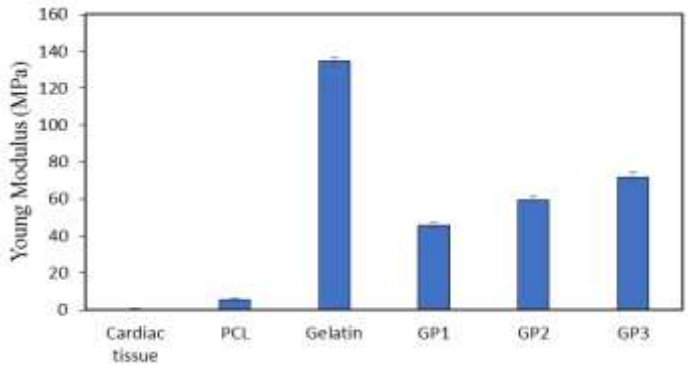

Figure 6. Young's modulus of the native heart tissue, PCL, gelatin, three different groups of the hybrid fibers

According to Figure 7, GP2 embedded in the hydrogel provides Young's modulus of 32.8±1.3 kPa, which is the most similar amount to the native tissue (31.25±1.5 kPa).

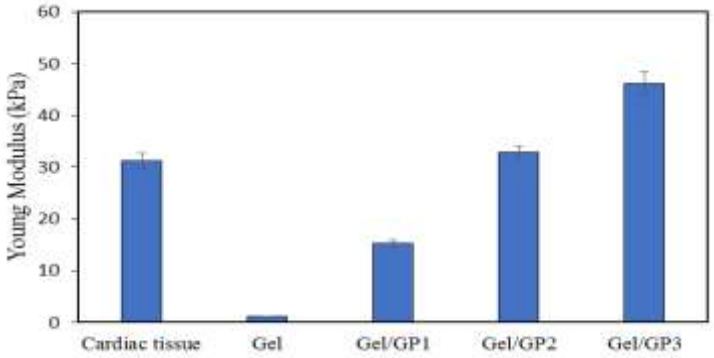

Figure 7. Young's modulus of the native heart tissue, hydrogel of chitosan and ECM (Gel), and fiber/hydrogel composites

Conclusion

In this study, a promising biomaterial of PCL/gelatin fibers embedded in the hybrid hydrogels of chitosan and ECM was successfully developed for cardiac tissue engineering applications. Microstructure, chemical interactions, contact angel, mechanical properties of the samples were investigated. Young's modulus of GP2 fibrous scaffolds embedded in the hydrogel from chitosan and ECM (mass ratio of 1) was reported to be 31.25 kPa, which is very close to the heart tissue. Therefore, this biomaterial can be considered as a potential substrate for cardiac cell culture. However, more *in vitro* experiments such as MTT assay and *in vivo* studies are needed to optimize its performance.

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