Characterization and Optimization of Using *Calendula Officinalis* Extract in The Fabrication of Polycaprolactone/Gelatin Electrospun Nanofibers for Wound Dressing Applications

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ARTICLE INFO

Article history:

Received 6 May 2018
Accepted 20 October 2018
Available online 15 September 2018

Keywords:

Nanofiber
Gelatin
Polycaprolactone
Calendula officinalis
Wound dressing

ABSTRACT

Wound dressing application of nanofibers is a progressive field of research, which could be enhanced by using medicinal plant extract to bring some more advantages. Here we optimized the electrospinning method for fabrication of polycaprolactone/gelatin mixed with a medicinal plant extract, *calendula officinalis*. Characterization techniques, including fourier transform infrared spectroscopy (FTIR), scanning electron microscope (SEM), atomic force microscopy (AFM), and water contact angle analysis were performed on the electrospun nanofibers to achieve the best formulation proper for wound dressing. In results, the concentration 12% (w/v) with an applied voltage of 20 kV and 13 cm of distance between needle and collector, does pose the lowest diameters with the highest porosity among others. The hydrophilicity of the nanofibers was enhanced by adding the *calendula* extract. In addition, the analysis of mechanical strength showed that the elasticity of polycaprolactone (PCL)/gelatin (Gel)/*calendula* (*Cal*) nanofibers are still acceptable. Overall, the results of characterization tests were approved that the electrospun nanofibers of PCL/Gel/*Cal* does have appropriate characteristics to be used as wound dressing and could be suggested to clinicians.

1-Introduction

In medical sciences, wound healing is still a major challenge and scientists are making a great progress in this field, day after day. Wound healing is a complicated process which needs us to overcome many obstacles, including skin regeneration, infection, inflammation, etc. [1-3]. Application of nanomaterials is regularly increasing in the different field of medical sciences, including but not limited to drug and gene delivery, cell therapy, biosensors, and regenerative medicine [4, 5]. The latter does have a vast application field which one of the most important is wound dressing. Various nanofibers have been used in wound dressing so achieve nanofibers with enhanced capabilities

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are still a big challenge for scientists [6, 7]. Different techniques, including electrospinning, electrospraying, phase separation, sol-gel, self-assembly and 3D printing, have been used to achieve nano-dimensional fibers from polymers to be used in wound dressing [8-10].

Electrospinning is a powerful and flexible fabrication technique for producing wound dressing [11-13]. In the electrospinning process, different parameters, including polymer type, concentration, solvent, voltage, tip to collector distance should be optimized to achieve the best-fabricated fibers for wound dressing [13, 14]. In fact, the nano-dimension of the fibers and their pore size produced by electrospinning are very important in the way they interact with the skin cells for their adhesion, proliferation and even differentiation [8, 14-16]. Different types of natural and synthetic polymers, alone or in combination with other polymers, have been used to made wound dressing, for example, polycaprolactone (PCL), gelatin, chitosan, polylactic acid (PLA), poly(lactic-co-glycolic) acid (PLGA), cellulose, alginate, collagen, and many others [14, 17, 18].

Moreover, the addition of some materials, nanoparticles, drugs and medicinal plant extract into the electrospinning mixture to provide enhancement in function and application of the produced nanofibers such as enhancing the speed of wound healing, cell, and stem cell interaction and differentiation, antibacterial, antifungal effects, etc [1, 6, 19-23].

The combination of caprolactone (PCL) and gelatin have been used so far in various applications in medicine such as tissue engineering [24-31] and wound dressing [32-35]. In addition, some modification in PCL and gelatin have been made including making coaxial structures [36], mixing with other polymers and biomaterials [37, 38], adding drugs for antibacterial and anti-oxidant effect [39, 40], adding micro and nanoparticles [35, 41-44]. But, adding natural products like medicinal plant extracts are also common in this field due to their multiple advantages for the wound healing process [22, 23, 45, 46]. In this way, there are a few examples available in the literature review we have made until early 2018. Li and colleagues added a plant extract compound named eugenol for its antibacterial effects to the PCL/Gel [47]. In the same approach, Fallah et al, 2015 added curcumin to the PCL/Gel during electrospinning for antibacterial effects [48].

C. officinalis is a frequently used medicinal plant approved by many previous publications for possessing the incredible wound healing enhancement mainly because of its antibacterial, antioxidant and mostly anti-inflammation effects and even angiogenic effects [49-52].

In this research, we have applied an electrospinning method to fabricate a nanofiber from polycaprolactone-gelatin polymer mixture. We optimize the parameters in the electrospinning procedure to achieve nano-dimensional fibers with the better physical, chemical and mechanical characteristics suitable for application in a wound dressing. In addition, we have added a plant extract, *calendula*, to enhance the parameters to be used in a wound dressing application.

2-Materials and Methods

2-1- Chemicals

All reagents were of analytical grade and were used as purchased. Polycaprolactone (PCL) with the number average molecular weight of 80,000 in form of pellets was from Sigma-Aldrich Company (Cat No. 440744), while the gelatin (Cat No. 104070) and trifluoroacetic acid (TFA) (Cat No. 1082620) were purchased from Merck Company.

2-2- Fabrication of the PCL/Gel nanofibers

The electrospinning of PCL/Gel polymers in the TFA solvent was performed using a handmade machine with 6 metal needles (spinning tip). The 2 ml plastic syringes were connected to the instrument for injection of the polymer solution with the feed rate of 0.2 ml/h and 150 rpm rotation of collector. Different electrospinning solutions of the experiment were prepared separately by stirring distinct percentages of polymers (w/v) in the TFA until transparent solutions were achieved. In case of mixing solutions, the final separately prepared solution of each polymer was mixed on the stirrer for 2 h.

The concentration of each polymer was tested in 10, 12 and 14% (w/v). The applied voltage was tested in 18 and 20 kV and the distance between needle and collector was tested for 10 and 13 cm. The properties of solutions together with the

other electrospinning factors such as a tip to collector distance and voltage are shown in Table 1. As it can be seen at the end of Table1, after optimizing the best concentration of each PCL and Gel polymers, the combined mixture was prepared based on the optimum concentration percentages of each polymer with the optimum value of voltage and distance (code 19). The Resulted fibers (code 19) were carefully collected and were kept in the desiccator containing with 2.5% (v/v) glutaraldehyde vapor for 24 h to be fixed. Then *calendula* extract was added to evaluate the effect of this medicinal plant extract on the mechanical properties of the resulted nanofibers (code 20).

Table 1. Properties of electrospinning experiments (n=1).

Code	Polymer type and concentration	Voltage (kV)	Distance (cm)
1	PCL 10%	20	13
2	PCL 10%	20	10
3	PCL 10%	18	13
4	PCL 12%	20	13
5	PCL 12%	20	10
6	PCL 12%	18	13
7	PCL 14%	20	13
8	PCL 14%	20	10
9	PCL 14%	18	13
10	Gel 10%	20	13
11	Gel 10%	20	10
12	Gel 10%	18	13
13	Gel 12%	20	13
14	Gel 12%	20	10
15	Gel 12%	18	13
16	Gel 14%	20	13
17	Gel 14%	20	10
18	Gel 14%	18	13
19	PCL/Gel 12%	20	13
20	Cross-linked PCL/Gel/*Cal* 12%	20	13

2-3- Characterization of the scaffolds

The fabricated fibers were assessed for their specifications using different characterization methods. The attenuated total reflectance (ATR)-fourier transform infrared spectroscopy (FTIR) analysis was performed using the ATR-FTIR spectrometer in the spectral range of 4000–400 cm^{-1} wavenumbers (Equinox 55 from Bruker, USA).

In addition, the nanofibers were coated with a gold layer using desk sputter coater with Argon as a process gas (DSR1, Nano-structured Coatings, Iran) and analyzed by scanning electron microscope (ProX, Phenom, Netherland). The average fiber diameter and the surface area of the pores were calculated using image analysis software (ImageJ software, Version 1.46). Values were averaged and expressed as means±standard deviation (SD) by SPSS software (Version 24.0, USA).

The contact angle assessment was done by dropping colored water on the surface of electrospun fibers after 3 seconds and the picture was processed by the digimizer image analysis software (Belgium, Version 4.6.1).

Surface topography and mechanical strength were also assessed by Atomic force microscopy (AFM) instrument model easyscan 2 from Nanosurf (Switzerland) in contact mode and based on Hertz contact theory; the SPM and DP 4.2 software versions were used for image processing and analysis. In this regard, force spectroscopy tests were performed on the points of a nanofiber surface such that the applied force

direction was perpendicular to the nanofiber longitudinal axes. Force measurements were recorded at 25°C using a blunted silicon cantilever with experimentally determined spring constants of 5 N.m $^{-1}$ and a tip radius of 250 nm (Figure 7). Young's modulus (E) was calculated by converting the force-displacement curves into force-indentation curves and fitting with the Hertz model, which describes the indentation of an elastic sample using a stiff conical indenter. The half opening angle of the AFM tip was 36 ° and the Poisson ratio of the fibers was taken to be 0.5, as is typical for soft biological materials.

3-Results and Discussion

The results of electrospun nanofiber characterization, including FTIR spectroscopy, surface topography, water contact angle analysis, AFM imaging and mechanical strength test, are presented in this section.

3.1. FTIR resultsFTIR spectra of the gelatin, PCL and PCL/Gel are compared in Figure 1. In the FTIR analysis of the gelatin nanofibers, the absorption bands were observed at 1638 cm^{-1} (CO stretching vibration), 1532 cm^{-1} (NH bending vibration), 1446 cm^{-1} and 1332 cm^{-1} (CH bending and CN stretching vibration, respectively) [Figure 1]. For the gelatin at 1638 proved the amide I, while 1532 indicated to the amide II band [53].

The PCL characteristic bands were 2949 cm^{-1} (asymmetric $-CH_2$ stretching), 2868 cm^{-1} (symmetric $-CH_2$ stretching), 1726 cm^{-1} (carbonyl stretching), 1294 cm^{-1} (C–O and C–C stretching), 1239 cm^{-1} (asymmetric C–O–C stretching), and 1169 cm^{-1} (symmetric C–O–C stretching) which were similar to other researchers [54]. In Figure 1, the absorption peak of PCL/Gel with a ratio of 50/50 composite were indicated for the formation of the nanofibers which shows some different peaks which are similar to previous publications [55].

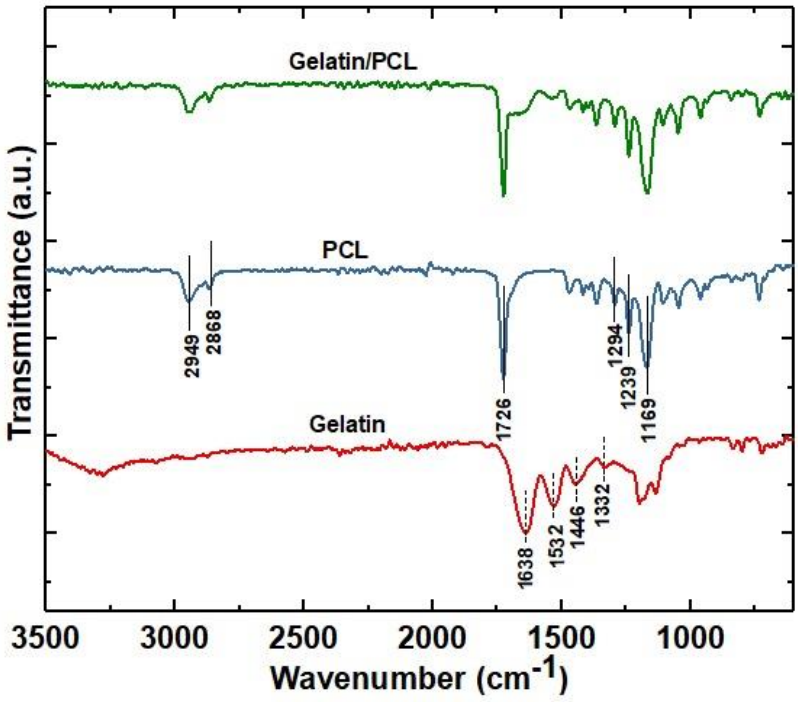

Fig 1. FTIR spectra of electrospun samples of PCL, Gelatin and PCL/Gel nanofibers.

Electrospun gelatin is often cross-linked or combined with synthetic polymers in order to maintain a fibrous structure. Several studies have demonstrated the use of different cross-linkers to reduce the limitations mentioned above, including glutaraldehyde, genipin, and glyceraldehydes [56]. Glutaraldehyde-based crosslinking structures significantly reduce

biodegradation, while protecting biological integrity, strength, and flexibility [57]. In this study, glutaraldehyde vapor was used as a cross-linking agent to improve the mechanical properties of gelatin electrospun nanofibers. In the cross-linked PCL/Gel composite scaffold [Figure 2], all the characteristic bands of PCL and gelatin were observed but the bands of gelatin have been shifted towards the lower wavenumbers. In addition, Figure 2 illustrates the comparison between cross-linked PCL/Gel nanofibers, cross-linked PCL/Gel/*Cal* nanofibers and, *calendula* extract. The *calendula* has three strong peaks on 1031 cm^{-1} suggested to terpenoid or flavonoid compounds, the band on 1398 cm^{-1} as related the germinal methyl group. Respectively, the stretching bands in 1596 and 3945 cm^{-1} are related to the ether and hydroxyl groups. Cross-linked PCL/Gel/*Cal* nanofibers also show the strong band in about 1644 cm^{-1} as related to the C=C in phenol groups of *C. officinalis* extract's flavonoids [58]. This Figure is representing the overlapping between peaks of cross-linked PCL/Gel and *calendula* extract. The results are in agreement with previous publication with the same polymers (PCL/Gel).

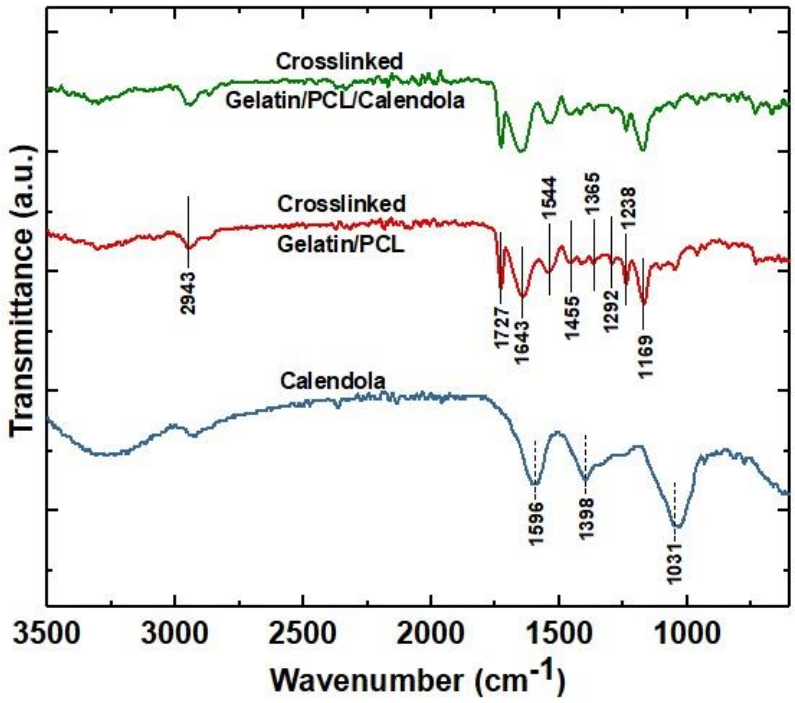

Fig 2. FTIR spectra of electrospun samples of Cross-linked PCL/Gel/*Cal*, Cross-linked PCL/Gel nanofibers and the plant extract of *calendula*.

3.2. SEM results

The important advantages of electrospinning method are the production of very thin fibers with large surface areas, ease of functionalization for various goals, good mechanical properties and easy process [59]. SEM analysis is one of the most reliable tests for assessing the morphology, size, and porosity of nanomaterials and nanofibers. The mean nanofiber diameter of SEM analysis is represented in Figure 3 for a better comparison, measuring 20 fibers per type of sample. The SEM analysis also shows that the average diameter is below 250 nm. But, SEM micrographs of different nanofibers can be seen that the fibers were formed at 12% concentration, 20 kV and 13 cm distance, is suitable composite. On the surface of these nanofibers were observed smooth without any beads. In addition, the cross-linked PCL/Gel/*Cal* nanofiber was showed an increase in nanofiber diameter; this result can be the effect of glutaraldehyde vapor. The rheological study showed a strong dependence of spinnability and fiber morphology on solution

viscosity. Solution concentrations have been found to most strongly affect fiber size, with fiber diameter increases with increasing solution concentration and the morphology of the deposition on the collector changed from spherical beads to interconnected fibrous networks [60].

The SEM pictures of the main best treatments are represented in Figure 4 for optimized samples from PCL, Gel, PCL/Gel and, cross-linked PCL/Gel/*Cal*. These figures show the fine nano-dimensional structures without any beads or other deformations. In addition, the pore diameter of these samples was calculated based on the SEM pictures. The pore size was 390 nm for the PCL sample, 529 nm for Gel, 513 nm for PCL/Gel and finally 753 nm for cross-linked PCL/Gel/*Cal* sample. Based on these results, the most suitable composition of polymers and plant extract was achieved.

3.3. Contact angle results

In addition to the chemical and morphology parameters, the hydrophilicity of the electrospun nanofibers should be assessed for those their final use is in medical applications especially wound dressing which is in contact with blood and damaged skin tissue. The results of water contact angle analysis are shown in Figure 5. Most synthetic polymers, including PCL and PLCL, are hydrophobic, whereas collagen and gelatin (a product of partially hydrolyzed collagen) are hydrophilic [61]. In order to form hydrophilic nanofibers, synthetic polymers can be blended with naturally derived polymers [62]. In this figure, it can be seen that the addition of gelatin to the PCL is a very effective way of enhancing the hydrophilicity of PCL.

Li *et al*. [63] showed that gelatin in the electrospun fibers of the PCL/Gel copolymer decreased the contact angle of the fibers. Furthermore, the addition of *calendula* extract is also enhancing the hydrophilicity of the nanofibers. This is due to increased pore size. Therefore, this medicinal plant extract does pose a suitable specification regarding the hydrophilicity to be used in a wound dressing. Hydrophilicity of the different scaffolds was characterized using three samples in each group.

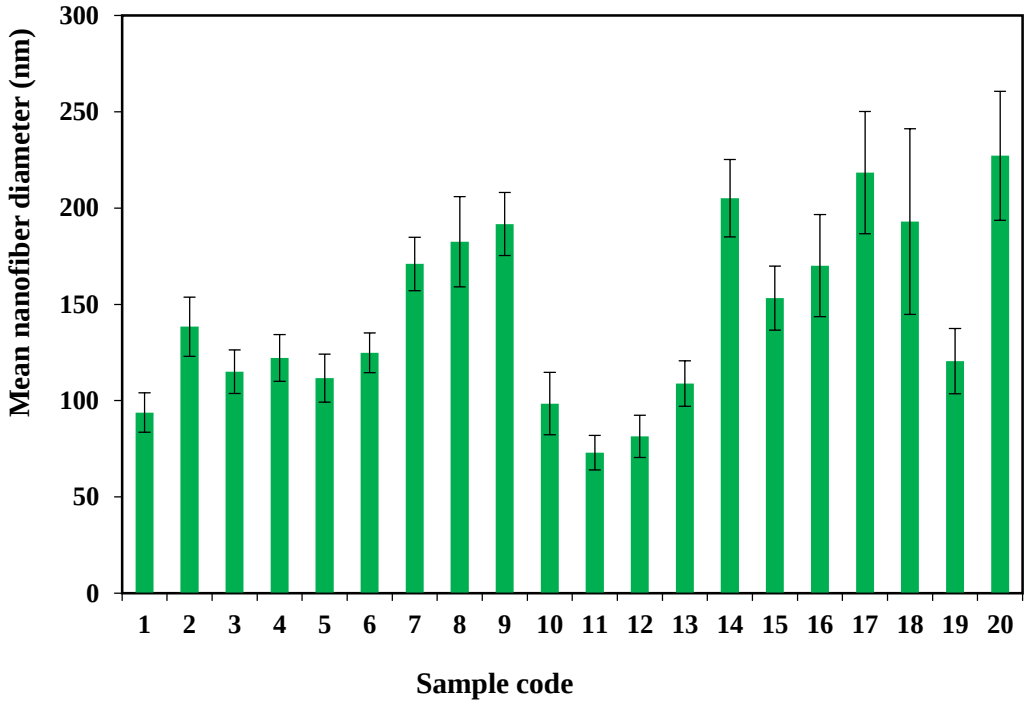

Fig 3. Mean diameters of the electrospun nanofibers evaluated by SEM analysis (n=20).

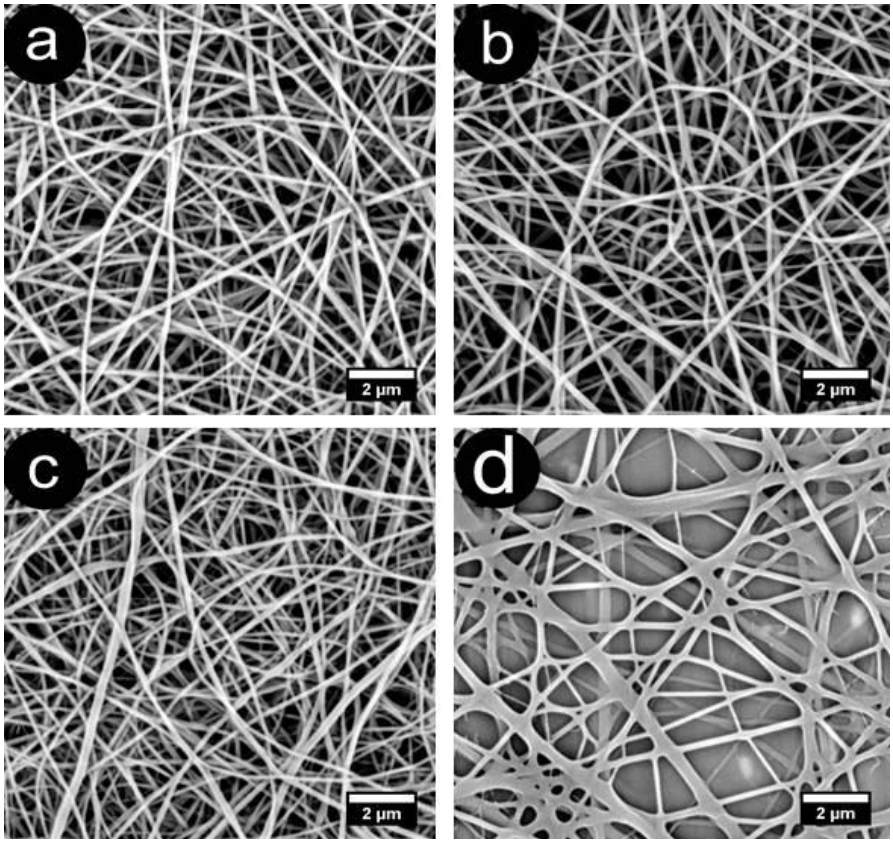

Fig 4. SEM pictures of the electrospun nanofibers from samples (a) PCL, (b) Gelatin, (c) PCL/Gel and (d) Cross-linked PCL/Gel/*Cal* (scale bar: 2 µm).

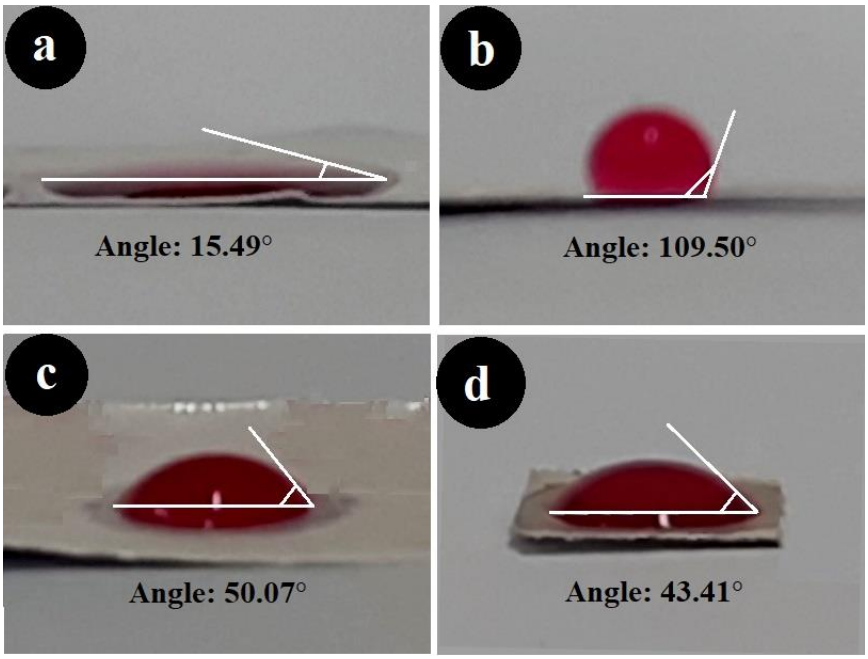

Fig 5. Water contact angle analysis of the electrospun nanofibers from samples (a) Gelatin, (b) PCL, (c) PCL/Gel and (d) Cross-linked PCL/Gel/*Cal*.

3.4. AFM and Mechanical strength results

The AFM analysis has been used for assessing the mechanical properties of different materials for many purposes, in addition to the surface imaging [64-66]. Because of higher accuracy, here, the AFM imaging was done in both 2D and 3D forms and shown in Figure 6 for PCL/Gel and PCL/Gel/*Cal* samples. As it is clear in the comparison of these two figures, the surface roughness is not different and therefore the addition of *calendula* does not affect the surface parameters. In addition, the mechanical strength of the fabricated nanofibers of PCL/Gel and PCL/Gel/*Cal* was assessed using the new technique by AFM instrument. The results are shown in the graph form which is a plot of deflection of the sample versus the force. Figure 7 is the mechanical strength graph using AFM in contact mode for sample PCL/Gel and analysis for PCL/Gel/*Cal* sample.

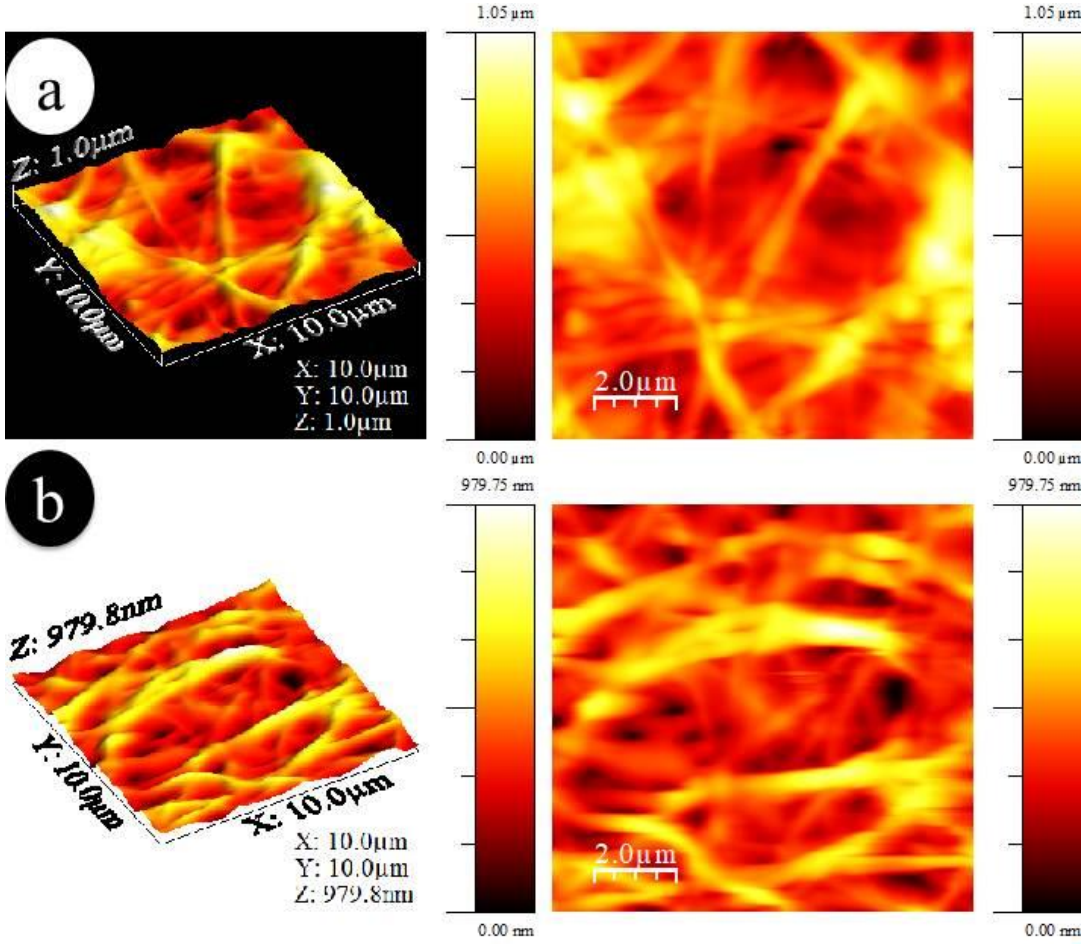

Fig 6. AFM 2D and 3D images of the electrospun nanofiber samples (a) PCL/Gel and (b) Cross-linked PCL/Gel/*Cal.*

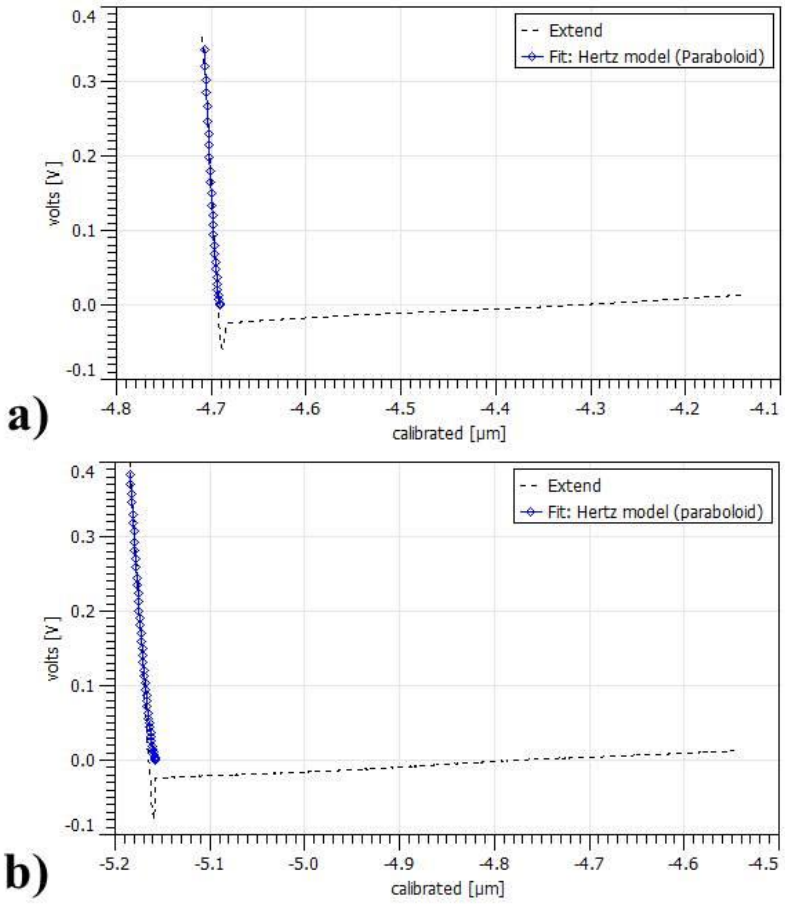

Fig 7. Mechanical strength analysis of the electrospun nanofiber samples (a) PCL/Gel and (b) Cross-linked PCL/Gel/*Cal.*

Comparison of these two graphs shows the decrease in the mechanical strength of the sample by adding the *calendula* extract, but the mechanical strength is still acceptable for future applications in a wound dressing. Application of AFM for mechanical strength assessment in polymers and composites have been evaluated in previous publications for liquid crystalline polymer (LCP) [67], PVA-Graphene composite [64], nano-hydroxyapatite/chitosan, nano-zirconia/chitosan and novel nano-calcium zirconate/chitosan composite [68], poly (p-phenylene benzobisoxazole) [65], etc. But it has not been used for PCL-Gel composite so far.

4-Conclusion

Wound dressing fabrication is still a big challenge in medicine and biomedical engineering. There are a lot of parameters that should be considered in this regard such Nano dimension and morphology of the resulted fibers, porosity, hydrophobicity, and mechanical strength of them. Is this research we presented a novel electrospun nanofiber made of a combination of PCL, Gelatin, and extract of *calendula* for application is wound dressing. The *calendula* extract does have many approved medicinal effects especially for antibacterial, antioxidant and mostly anti-inflammation effects that could be very helpful in process of wound healing. The results suggested the optimized production of PCL/Gel/*Cal* nanofibers, are acceptable in their dimension, form, porosity, surface roughness, chemical composition and hydrophobicity. Therefore, they can be used as a potential wound dressing for medical applications after being tested further by live cells *in vitro* and *in vivo* studies.

Acknowledgment

The authors are thankful to the Academic Center for Education, Culture, and Research, Yazd Branch, for their financial support of this research.

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