RESEARCH PAPER

Preparation and characterization of electrospun apigenin-loaded polycaprolactone nanofibers for wound dressing applications

Nayereh Niazi [1, 2], Masoumeh Zahmatkeshan [1, 2], Fariba Esmaeili [3], Farzaneh Soltani-Jazi [2], Seyed Mahdi Rezayat [3, 4], Maryam Zahmatkeshan [5], Moein Adel [1, 6]*

[1]Cellular and Molecular Research Center, Iran University of Medical Sciences, Tehran, Iran
[2]Department of Medical Nanotechnology, Faculty of Advanced Technologies in Medicine, Iran University of Medical Sciences, Tehran, Iran
[3]Department of Medical Nanotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran.
[4]Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[5]Department of Nursing and Midwifery, Estahban branch, Islamic Azad university, Estahban, Iran
[6]Department of Medical Nanotechnology, Tabriz University of Medical Sciences, Tabriz, Iran

ABSTRACT

Objective(s): This study uses the blend-electrospinning method to explore the development of apigenin) APG(-loaded PCL nanofibers as a promising wound dressing material.

Materials and Methods: The approach combines APG's anti-inflammatory and antioxidant properties with the advantages of nanofibers for wound healing. The research investigates the electrospinning process for optimal parameters and characterizes the resulting nanofibers using FE-SEM, FTIR, and contact angle measurements.

Results: The findings demonstrate successful APG incorporation into PCL nanofibers at concentrations up to 0.5 wt%. The APG release profile indicates a sustained release over 48 hours. Biocompatibility and cytotoxicity assessments using the Alamar Blue assay reveal excellent biocompatibility of APG-loaded PCL nanofibers (over 90% viability). Additionally, the nanofibers exhibit a porous, bead-free structure with improved hydrophilicity due to APG incorporation.

Conclusion: Overall, this study highlights the development of APG/PCL nanofibers with promising characteristics for wound dressing applications. The combination of APG's therapeutic properties, sustained release profile, and biocompatible nanofiber structure suggests their potential for effective wound healing.

Keywords: *Apigenin, Electrospun, Nanofibers, Polycaprolactone, Wound dressing*

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INTRODUCTION

Nanofibers are a type of nanomaterial that has been widely used in biomedical and other applications due to their inherent properties [1]. They can be synthesized using various polymers or compounds, including natural and synthetic polymers. Electrospinning is the most widely used method for producing nanofibers and has proven to be both cost-effective and highly effective at establishing these materials with molecules ranging from nm to micrometers in size [2].

Nanofibers can serve as a drug delivery system for both hydrophobic and hydrophilic drugs and can be used for topical or systemic treatments. They can efficiently deliver drugs with polymer-encapsulated drug molecules [3-6].

Wounds can be healed using various biomedical textile materials created using different production methods. An ideal wound dressing should have biocompatibility, non-toxicity, protection against infection, absorbency, permeability, and quick drug delivery [7-10]. Electrospun nanofibrous mats are a cutting-edge alternative to traditional

* *Corresponding author: Email: masoumeh.education@gmail.com; zahmatkeshan.m@iums.ac.ir*

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wound dressings because they have numerous special advantages over others. These advantages include morphological similarities to the ECM, a high surface area to volume ratio, greater porosity, oxygen permeability, continuous and flexible nanostructured fibers, and drug delivery capabilities. These structural characteristics permit tissue regeneration, transfer wound fluid, and ensure breathability for cellular expansion and proliferation [11, 12]. Antibacterial, anti-inflammatory, and healing agents can be easily incorporated into electrospinning to create multifunctional nanofibrous membranes. All types of wounds can be treated with bioactive electrospun dressings that also have sufficient physicochemical and mechanical properties to protect the wound, promote healing progression, and eliminate bacteria from the wound [9, 12-17].

Apigenin (APG), also known as 4',5,7-trihydroxyflavone, is a flavonoid that occurs naturally in various vegetables, fruits, beans, and tea leaves. Celery has the highest concentration of APG. APG has C2C3 double bonds and 4' hydroxyl groups at positions 5 and 7, which together contribute significantly to its special physicochemical properties [18-20]. In comparison to other flavonoids like quercetin and kaempferia brass, APG demonstrates a superior safety profile [21].

By preventing the production of tumor necrosis factor (TNF), interleukin-6 (IL-6), and interleukin-1 (IL-1), APG has anti-inflammatory effects. APG increases the expression of vascular endothelial growth factor (VEGF) to encourage vascular regeneration after ischemia [18, 19, 22]. Because of its anti-inflammatory, antioxidant, and pro-angiogenic properties, APG treatment has been investigated as a method to improve the survival rate of random skin flaps [21, 23-25]. For example, following surgery, the topical application of PLX gel improved the degree of reepithelialization and inflammation and promoted neo-vascularization of the wounds at two and seven days [26]. Moreover, Shuklaa et al. demonstrated that APG-loaded gellan gum–chitosan hydrogel (GGCH-HGs) promotes wound contraction and enhances collagen content in both diabetic and normal wound tissues. This was supported by increased hydroxyproline content and protein levels. These findings suggest that APG could be beneficial for managing diabetic wound healing through its free radical scavenging effects [27]. Rajab et al. also investigated the healing effects of a topical cream containing 2% APG on rabbit skin. They concluded that APG promotes wound healing in rabbits due to its promising properties, including antibacterial, antiviral, anti-inflammatory, antioxidant, and free radical scavenging activities [28]. However, a significant drawback of APG is its poor water solubility and limited bioavailability [20]. Therefore, it is necessary to develop new drug delivery systems or formulations to enhance the bioavailability of APG.

Poly (ε-caprolactone) (PCL) is one of the polymers that has been widely investigated in the field of tissue regeneration and wound healing applications due to its biocompatibility and biodegradability. The similarity of electrospun nanofiber matrices to the diameter of collagen fibers in the natural extracellular matrix has recently attracted considerable attention for a variety of biomedical applications [29]. Additionally, PCL is an ideal material for tissue engineering and wound dressing due to its non-toxic nature and flexible mechanical properties [30, 31].

Hydrogel- and polymeric electrospun nanofiber-based wound dressings have been extensively studied for wound healing. However, these structures can promote bacterial growth in wounds by trapping exudates, which is a major drawback of drug-free, nonabsorbent wound dressings [32, 33].

In this regard, the production of polymeric electrospun nanofibers for the manufacture of drug-loaded wound dressings with anti-inflammatory and anti-infective properties is a crucial requirement. An ideal wound dressing should possess unique qualities like the capacity to exchange gases, the ability to deliver cells into wounds, and a particular surface area for angiogenesis. Thus, the best structure for regulating the wound environment is provided by drug-loaded electrospun nanofibers, which have a porous structure and a high surface-to-volume ratio.

APG-loaded PCL in the form of electrospun nanofibers is proposed as a novel template for wound dressing scaffolds due to APG's antibacterial activity, which has not been previously reported. Therefore, the goal of the current study is to produce electrospun PCL nanofibers that contain APG for use in wound dressing applications.

MATERIALS AND METHODS

Materials

PCL (Mn = 70,000–90,000) was purchased from Sigma-Aldrich (U.S.A).APG powder was kindly supplied by Prof. Farnaz Nikbakht from the Department of Physiology at the University

of IUMS in Tehran, Iran. Dichloromethane (DCM), dimethylformamide (DMF), and methanol were obtained from Merck Chemical Co. All chemicals and solvents were used as received.

Fabrication of electrospun PCL and APG-loaded PCL nanofibers

A weighed amount of PCL (15, 16, 14.5, 14.75, 15.5, and 15.75 wt%) was separately dissolved in a mixture of DCM: DMF: methanol (at a 60:30:10 weight ratio) by agitating the mixture for 2 hours at room temperature (25 °C) with a magnetic stirrer. To fabricate PCL nanofibers loaded with APG, the PCL solution that contained 14.75, 15.75, 14.5, and 15.5 wt% was first mixed with the appropriate amounts of APG (0.25 and 0.5 wt%), then stirred for one hour to produce PCL 15 and 16 wt%. Each prepared solution was placed into a standard 5 mL plastic syringe with a blunt 22-gauge stainless steel hypodermic needle and connected to a high-voltage power source (Chungpa EMI, Korea). To control the flow rate of the solution, a syringe pump (Model KDS200, KD Scientific, USA) was used. A rotating collector with an aluminum sheet covering it was used to collect electrospun nanofiber mats. All electrospinning procedures were carried out at 20 kV with a 21 cm distance between the needle tip and the collector and a solution flow rate of 0.5 mL/h. The mats were dried in a vacuum dryer for 12 hours at room temperature to make sure that DMF did not remain in the nanofiber mats.

Characterization of nanofibers

To characterize the morphology and diameter of the electrospun nanofibers, field emission scanning electron microscopy (FE-SEM) was used (HITACHI S-4700, Japan). Image J software was used to measure the average fiber diameters of 100 fibers randomly selected from each electrospun mat (National Institute of Health, USA).

Fourier Transform Infrared Spectroscopy was applied to characterize the chemical structures of nanofibers and APG powder (Thermo Nicolet FTIR, Nexus 670).

The surface hydrophobicity of the manufactured nanofibers was assessed using measurements of contact angle. Contact angles were assessed with a video contact angle device quickly after deionized water was permitted to fall freely onto the surfaces of flat non-woven mats (Samsung FA-CED camera, South Korea). The final contact angle value was determined by calculating the average of five measurements made at various points on the surface of a non-woven mat.

APG release profile from nanofibers

APG-loaded PCL nanofiber matrices (1cm^2) were incubated for 48 hours at 37°C in 5 mL of PBS/ethanol 50%. At set intervals, 200 µl of the extraction media were taken out and replaced with the same volume of fresh media. Using a UV-visible spectroscopy fluorometer (excitation 430, emission 535 nm), the amount of APG released was measured, and the outcomes were compared to an APG standard curve in 50% ethanol.

Cell seeding on APG-loaded PCL nanofibers

Cell viability assay

To reduce bacterial contamination, PCL, and APG-loaded PCL nanofibers (1 cm^2) were treated with 10% ethanol solution. HUVEC cells were seeded and grown in DMEM containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin until becoming confluent in tissue culture flasks (75 cm^2). In a tissue culture incubator with 5% CO2 and 37°C, the media was replaced every other day. Cells were trypsinized and seeded onto the nanofiber matrices when they had reached 80% confluency. The Alamar Blue assay was used to assess the viability of cells adhered to the surface of nanofibers. At the same time, relevant blank samples (PCL and APG-loaded PCL nanofibers without cells) were collected.

The Alamar Blue method measures the biocompatibility and cytotoxicity of nanofibers using a calorimetric approach. A decrease in cellular metabolism is detected using the oxidation-reduction of the Resazurin molecule (REDOX), which changes color in response to reduction or oxidation. A reduced form of the compound is pink and highly fluorescent, and the intensity of the fluorescence varies with the number of living cells. As a direct indicator of viability and cytotoxicity during respiration, Alamar Blue detects levels of oxidation during respiration. The Alamar Blue solution (10% v) was applied to each well. For several months, the Alamar blue solution can be kept at 4 °C and in the dark. To perform the Alamar blue test, a predetermined volume of medium containing roughly 20,000 cells was poured into each well of a 48-well plate. This process was repeated four times for each sample. Following that, the plates were incubated for 48 hours. After 4 hours of treatment with Alamar blue solution, the contents of each well were transferred to another plate, and an ELISA reader was used to measure each well's absorption at 570 and 630 nm.

Statistical analysis

The data was statistically analyzed using one-way analysis of variance (ANOVA), and $p \leq 0.05$ was considered statistically significant. Results are presented as mean ± standard deviation, so the error bars show the standard deviation. The software program Origin Pro 7.5 was used to analyze the data. One-way ANOVA with a Tukey test (p 0.05) was employed to statistically compare PCL nanofibers with and without APG loading.

RESULTS AND DISCUSSION

The morphology and properties of polymer nanofibers are affected by the experimental conditions for electrospinning. Therefore, the results will be discussed while considering the optimization of electrospinning parameters and the characterization of samples obtained under optimized conditions.

Characterization of nanofibers

Morphology of electrospun nanofibers

The fiber morphology was analyzed using FESEM imaging. Fig. 1A, B displays the images of PCL electrospun fibers from 15% and 16% PCL solutions. The study found that beads started to form along the fibers when the PCL solution concentration was below 14% (w/w). Electrospinning of 15% (w/v) PCL in DCM/DMF/methanol gave rise to bead-free nanofibrous matrices with a median diameter in the range of 300-400 nm. Under the optimal electrospinning conditions mentioned above, Fig. 1C, D displays the morphology of APG-loaded (0.25% and 0.5% w/w) PCL nanofibers. PCL nanofibers with APG loaded (0.25% and 0.5% w/w) displayed bead-free morphology, which was like that of the PCL nanofibers. The distribution of fiber diameter, however, was significantly altered by the loading of APG. After APG was added, fibers with a wide range of diameters (200-600 nm) were produced (Table 1). The highest concentration of APG that could be loaded into PCL nanofibers under optimal conditions was found to be 0.5% (w/w).

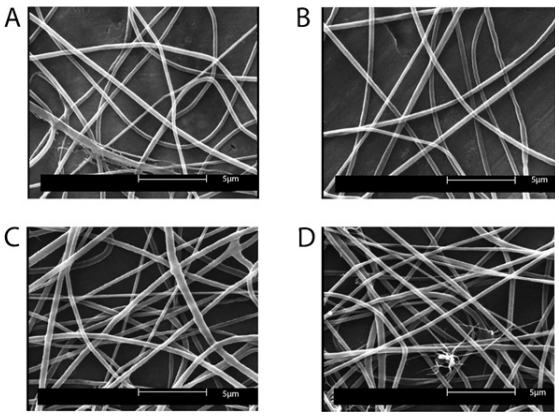


Fig. 1. SEM images of the electrospun nanofibers (A) PCL15, (B) PCL16, (C) PCL15.5APG 0.5, (D) PCL15.75 APG 0.25

With a further increase in APG concentration, APG precipitated from the polymer solution and formed aggregates on the surface of the fibers.

Chemical structure studies

FTIR was used to investigate the interactions between functional groups of PCL and APG. The FTIR spectra of PCL, APG, and APG-loaded PCL nanofibers are illustrated in Fig. 2. The characteristic peaks of PCL include 2,949 cm^{-1} (asymmetric tensile band CH_2), 2,865 cm-1 (asymmetric tensile band CH_2), 1,727 cm^{-1} (carbonyl tensile band), 1,293 cm^{-1} (C-O and C-C tensile bands) and 1,240 cm^{-1} (COC asymmetric tensile bands) [34, 35].

In the APG spectrum, some tensile bands in 2710-2580 (2612) are related to the O-H band. The broad peak at 3261 is most likely related to the O-H tensile vibration of the OH phenol groups, and bands in 1600-1400 and 800-250 can be related to C = O tension and C-H curvatures related to the aromatic group [36-38]. The peak observed in the spectrum of the APG sample in 1649 is related to the C = O tensile band of the C = O group of the central heterocyclic ring [36, 39] while the C-O vibration occurs in the range of 1110.

The results of FTIR analysis of the APG-loaded

Table 1. the mean diameters of nanofibers produced with 20 kV and 21 cm; Values are expressed as mean ± S.D

No.	Sample	Concentration Weight/Volume	Average diameter of nanofibers (nm)
1	PCl %16	16%	368 ± 69.92
2	PCL %15	15%	374 ± 74.8
3	PCL %15.5 + APG %0.5	16%	353 ± 77.6
4	PCL %15.75 + APG %0.25	16%	398 ± 79.6
5	PCL %14.5 + APG %0.5	15%	358 ± 75.18
6	PCL %14.75 + APG %0.25	15%	400 ± 80

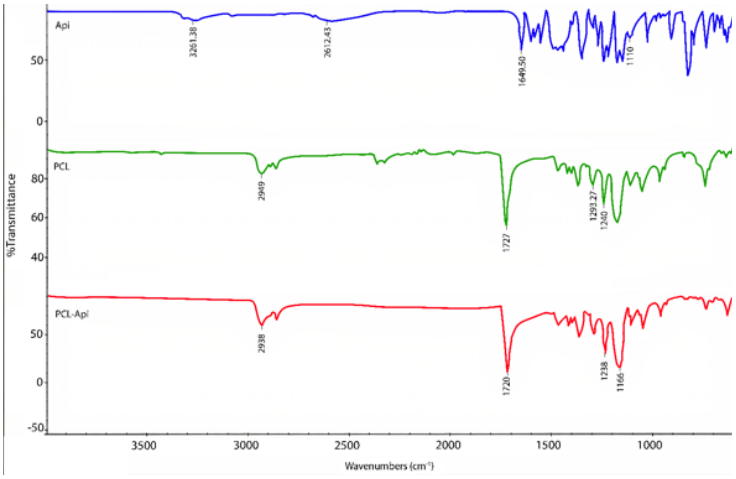


Fig. 2. FTIR spectrum of PCL nanofibers, APG powder, and PCL/ APG nanofibers.

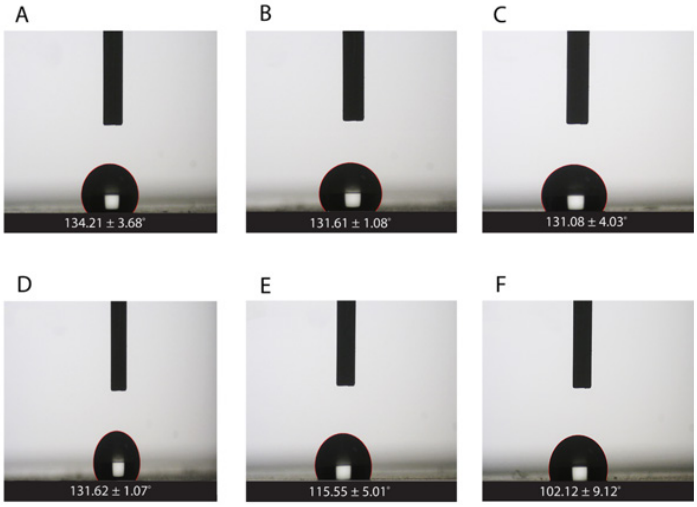
PCL nanofiber (APG-PCL) have also been shown in Fig. 2. The APG / PCL spectrum shows characteristic peaks for both PCL and APG. In summary, all characteristic peaks of both APG and PCL have been transferred to the polycaprolactone composite nanofiber containing APG, indicating that APG and PCL are bonded together to form a more stable structure and homogeneous nanofibers.

Contact angle measurements of the nanofibers

PCL is a hydrophobic polymer. As expected, PCL has a high contact angle, which indicates that it is highly hydrophobic. The addition of APG changes the contact angle and decreases it. The results of investigating two different concentrations of APG (0.25 and 0.5 w% in PCL solution) were shown in Fig. 3. APG generally does not dissolve in water, but the oxygen in APG can bond with water as a proton donor. Therefore, with the addition of APG, the contact angle was decreased. In other words, nanofibers became more hydrophilic.

In vitro APG release from nanofibers

Fig. 4 shows the *in vitro* release of APG into the media over 2 days. As can be seen, loaded nanofibers exhibited a burst release of 15% in the first 2 hr, followed by a slow and sustained release of 52% over 2 days. Overall, the cumulative release



	Sample	Contact angle
A	PCl %16	134.21 ± 3.68°
B	PCL %15	131.61 ± 1.08°
C	PCL %15.75 + APG %0.25	131.08 ± 4.03
D	PCL %14.75 + APG %0.25	131.62 ± 1.07
E	PCL %15.5 + APG %0.5	115.55 ± 5.01
F	PCL %14.5 + APG %0.5	102.12 ± 9.12

Fig. 3. Contact angle images and measurement of nanofibrous samples. Values are expressed as mean ± S.D (n=3).

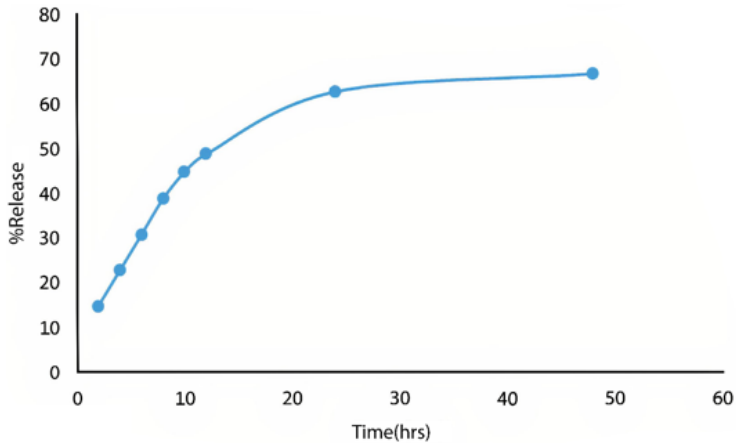


Fig.4. The release diagram of APG 0.5% from PCL nanofibers in 2 days

of APG was 67%.

In vitro cytotoxic assay

Fig. 5. shows the viability of HUVEC cells cultured directly on APG-loaded PCL nanofibers and PCL nanofibers. After 48 hr of culture on 0.5 and 0.25% APG-loaded PCL fibers, about 90% of cells were alive while there was no significant difference with control PCL nanofibers, indicating biocompatibility and non-cytotoxicity of PCL nanofibers containing APG.

DISCUSSION

According to previous studies, ES has successfully fabricated PCL nanofibers using a variety of solvents [40]. We chose DCM/DMF/methanol as the solvent in our research because DCM is known to be a superior solvent for PCL and APG. Additionally, the inclusion of DMF and methanol can enhance the electrospinability of the solution [41]. A fixed electric field of 20 kV/21 cm was used for the ES of these solutions. The results showed that the PCL nanofibers had uniform, bead-free structures, and smooth surfaces. PCL nanofibers demonstrated good characteristics in terms of diameter, uniformity, and non-beaded area, as shown in Fig. 1. Moreover, Fig. 1 and Table 1 show the morphology and average diameter of PCL nanofiber mats that were electrospun and loaded with APG.

Different concentrations of PCL and APG-PCL solutions (15 wt%, and 16 wt% containing 0.25 wt% and 0.5 wt% APG) were used in the study. The concentration of the polymer solution plays an important role in the formation of fibers during the electrospinning process. When the concentration is very low, polymer nanoparticles are obtained. In this case, due to the low viscosity and high surface tension of the solution, electrospraying takes place instead of electrospinning. When

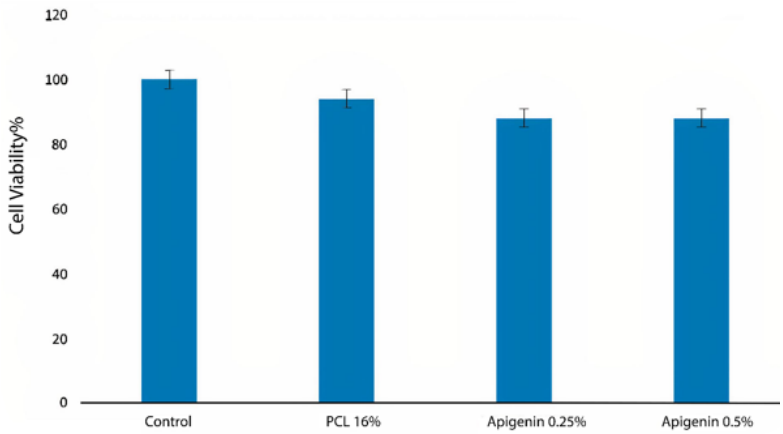


Fig. 5. Cell viability assay of HUVEC cells on PCL, and APG-loaded PCL nanofibers after 48 hr. Values are expressed as mean ± S.D (n=3)

the concentration is slightly higher, a mixture of beads and fibers is formed. Nanofibers are obtained when the concentration is suitable. If the concentration is increased a lot, spiral bands are obtained at the micro level. Actually, in a suitable concentration of the solution, the fiber diameter increases with increasing concentration [42, 43].

The study found that when the PCL concentration was below 15% and the APG concentration was above 0.5%, the polymer jet was unstable and resulted in beaded fibers. In APG-PCL nanofibers, the beaded structure indicates that a stable Taylor cone could not be formed. The presence of 0.25 and 0.5 wt% of APG eliminated bead formation.

The APG-loaded PCL nanofibers had a three-dimensional interconnected porous structure that was randomly oriented and like that of the pure PCL fiber. According to the results, adding APG to PCL fibers reduced the average diameter of the electrospun nanofibers. The average diameter of PCL nanofibers was 372 nm, while that of PCL nanofibers loaded with APG was 353 nm. This is most likely due to a change in the viscosity of the PCL solution, which is consistent with Fallah et al.'s findings [44]. Furthermore, the results obtained in this study are in agreement with those reported by Zeng et al., who observed that the incorporation of lipophilic drugs such as rifampin and paclitaxel could decrease the diameter and distribution of hydrophobic electrospun fibers made from PLLA [45, 46].

Additionally, considering that APG is a hydrophobic substance, it has been observed that the incorporation of hydrophobic medications into the polymer solution leads to the disruption of its components, reduces surface tension, and ultimately increases the bending instability while electrospinning [46, 47].

The interaction between lipophilic drugs and hydrophobic polymers occurs through hydrophobic binding, as observed with the lipophilic APG, which is highly soluble in a PCL/DCM/DMF solution. As the solution jet is rapidly stretched out and the solvent evaporates quickly, APG continues to attach to PCL and functions as a trap between the polymer chains [45]. An electrospun fiber mat with a porous interconnected structure can mimic the natural extracellular matrix, which promotes cell growth and attachment.

Assessing cell viability is crucial in determining the cytotoxicity of nanofiber mats for potential use as wound dressings. In this study, the HUVEC cells were cultured on PCL, and APG-loaded PCL nanofiber mats for 48 hours. Alamar blue assay was performed to evaluate cell viability, as shown in Fig.5.

The cells cultured on polystyrene well plates as a control gradually proliferated during 48 hours of cell incubation. However, when seeded on the PCL nanofiber mat, their viability was lower than that of cells cultured on the control well ($P > 0.05$). This might be attributed to the hydrophobicity of the PCL nanofibers. For all the nanofiber mats, there was a negligible difference in cell viability after 2 days of culture. After 2 days of culture, more than 80% of the cells were viable on both the APG-loaded PCL and PCL nanofibers, indicating low cytotoxicity of APG-loaded PCL nanofibers and therefore their biocompatibility.

The drug release process can be divided into three stages: a) burst drug release, b) linear increase in drug release, and c) stationary or unchanged stage [48, 49]. In this study, the APG release profile demonstrated a burst release equal to 14% in the first 2 hours of incubation. Then, the APG release increased linearly up to 16 hours, which is equivalent to 45% of the APG. Finally, the amount of APG released was decreased over time. Only 6% of APG was released from 16 to 48 hours.

It is accepted that APG concentration in cell culture media is extremely low. The results suggest that the contact between cells and APG on the surface of nanofibers affects cell viability more than the concentration of released APG. Consistent with these results, Merrell et al. showed that on 3% (w/w) Cur-PCL, HFF-1 cell viability was more than 70% after 48 hours of culture. Nguyen et al. reported that including 0.125 wt% Cur in PLA, nanofibers could increase cell proliferation by contact between cells and Cur on the surface of nanofibers rather than by their morphology. Findings by Fallah et al. also confirmed these results.

Ultimately, our findings indicate that the newly developed APG/PCL nanofibers show potential for wound healing applications. However, further studies, including antibacterial tests and *in vivo* studies, are necessary to fully evaluate their potential.

CONCLUSION

In this study, the electrospinning process successfully fabricated continuous nanofibers of PCL and blends of the polymer with APG. For

the first time, the combination of therapeutic APG with antioxidant and anti-inflammatory properties with PCL nanofibers exhibited a useful and convenient method for wound dressing application. The addition of the APG reduced the diameter size of the electrospun fibers and maintained their interconnected porous structure. Contact angle measurements showed that adding APG decreased contact angle significantly and created a hydrophilic surface. The fabricated APG/PCL nanofibers possess a porous, nanoscale, and beadless structure with optimum biocompatibility. FTIR results confirm the APG loading. Moreover, APG-PCL nanofibers had a long-release profile and showed good cell biocompatibility compared to drug-free nanofibers. Overall, the study's findings demonstrate that the newly developed APG/PCL nanofibers have high biocompatibility, and low cytotoxicity, which can be a promising wound dressing platform for wound-healing applications.

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