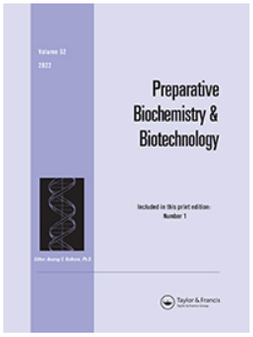

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Fabrication and *in vitro* evaluation of thermally cross-linked gelatin nanofibers for drug delivery applications

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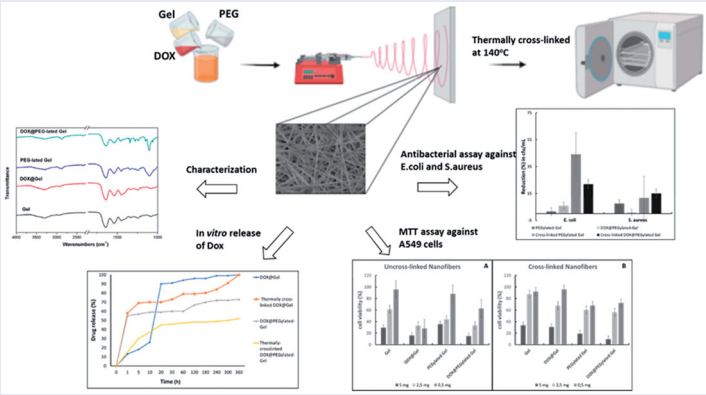
ABSTRACT

In this study, four different nanofibers consisting of gelatin (Gel), doxorubicin (DOX) with gel (DOX@Gel), a composite of gel with poly(ethylene glycol) (PEGylated-gel), and DOX@PEGylated-gel were fabricated. Subsequently, the nanofibers were thermally cross-linked in order to offer a stable and biocompatible alternative for the biological applications of nanofibers such as drug delivery and tissue engineering. Nanofibers were characterized by scanning electron microscopy, Fourier Transform-Infrared Spectroscopy (FT-IR), and confocal microscopy. The formation of smooth, continuous, and uniform nanofibers was observed and the addition of PEG resulted in an increase whereas the incorporation of DOX into nanofibers had no significant change in the diameter of nanofibers. Crosslinking also enlarged the diameter of all nanofibers and the most dramatic increase was observed 53% by DOX@PEGylated-gel. Afterward, the biological performance of the nanofibers was investigated by drug release profile, cytotoxicity on A549 cell line as well as antimicrobial activity with *E. coli* and *S. aureus*. The results indicate an enhanced drug release profile, moderate antimicrobial activity, and reasonable cytotoxic efficiency for thermally cross-linked nanofibers compared to uncross-linked nanofibers.

KEYWORDS

Electrospinning; drug delivery; gelatin; nanofiber; stability; thermal crosslinking

GRAPHICAL ABSTRACT



Introduction

Drug delivery systems (DDSs) have emerged as a way of improving drug efficiency. Most chemotherapeutics have their own drawbacks, simply because drugs cannot stay long enough in the body fluid as well as they cannot reach to the targeted section of the body with the desired drug concentration.[1] Therefore, it is highly important to develop DDSs which will promote the desired drug release profile while minimizing the undesired consequences. Up to date, there have been diverse nanocarrier studies for DDSs such as nanoparticles,[2,3] liposomes,[4,5] polymeric micelles,[6,7], and nanofibers.[8–11] Particularly, electrospun nanofibers are an attractive platform for the drug delivery applications because of high and interconnected porosity with small pore size, a large surface/volume ratio, as well as high encapsulation efficiency.[12] Owing to these properties, nanofibers have become an attention-grabbing topic of research recently in drug delivery applications.[9,13–16]

Gelatin, a hydrophilic natural biopolymer derived from partial hydrolysis of collagen,[17] is a promising biomaterial for many diverse applications[18–20] including drug delivery[21–23] owing to its biocompatible and biodegradable nature. However, gelatin displays poor fiber formation and lacks stability in water,[24,25] therefore, it is generally applied as a composite in the process of nanofiber production.[26] Furthermore, in order to improve the water stability of

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gelatin fibers, it can be cross-linked using genipin[24] and glutaraldehyde.[27] Yet these cross-linking agents have their own limitations in drug delivery applications because of their toxicity.[28] In the related cases, thermal cross-linking is an alternative choice over the use of these agents. Although the thermal cross-linking of gelatin is already known in the literature,[29] to our best knowledge, there is no study merging and investigating the applicability of thermal cross-linking with Gel and Gel with PEG as a composite nanofiber in drug delivery applications.

In this regard, gel and composite of PEGylated-gel nanofibers were fabricated firstly. Subsequent to that, DOX was performed as a model drug and DOX@Gel and DOX@PE Gylated-Gel nanofibers were also produced. Later on, the nanofibers were thermally cross-linked and the performance of these nanofibers as a drug delivery agent was investigated by drug release behavior, cytotoxicity effect on A549 cell line. All fabricated nanofibers were well characterized via physically using SEM, UV-Vis light, and confocal microscopy; chemically using FTIR. The biological importance of fabricated nanofibers was elucidated by cell viability and antimicrobial activity in order to be utilized for possible applications such as drug delivery and wound dressing in the future.

Materials and methods

Electrospinning of gel/PEG solution

Gelatin and PEG were dissolved in acetic acid (25 wt%) with various concentrations, separately and then PEG added into the gelatin solution with Gel/PEG ratio of 5/0 and 4/1. DOX (1 mg) was added to these solutions. The mixtures were sonicated for an hour and stirred overnight to obtain homogenous solutions at room temperature. The solutions were transferred to a 20 mL plastic syringe fitted with a needle (diameter = 0.47 mm) and set up in the electrospinning apparatus. A piece of aluminum foil, used as a collector and grounded, was located 20 cm apart from the capillary tip. The electric potential was controlled at 18–22 kV and the electrospinning was performed 0,8 mL/h at room temperature.[30]

Morphological and structural characterizations

The chemical structures of Gel, DOX@Gel, PEGylated-Gel, and DOX@PEGylated-Gel nanofibers were measured by Fourier transform infrared spectroscopy (Perkin Elmer FT-IR System Spectrum BX) in the range of 400–4000 cm^{-1}. A scanning electron microscope (SEM, JEOL-5600LV, Japan) was used to observe the surface morphology and size distribution of the electrospun fibers. Before SEM observation, all of the samples were sputter-coated with gold. Its accelerating voltage was 5 kV. The average diameter and diameter distribution of nanofibers were obtained from the SEM micrographs of 100 individual fibers by using Fiji ImageJ software. Compositional analysis was performed with confocal microscopy (Andor Revolution).

In vitro drug release study

In-vitro release studies of DOX from Gel and PEGylated-Gel nanofibers in the buffer solution were carried out by a UV–Vis spectrophotometer at the wavelengths of 234, 254, and 471 nm. The drug-loaded fiber sample (20 mg) was incubated at 37 °C and 100 rpm in 15 mL of phosphate-buffered saline (PBS, pH 7.4). Samples of 2.0 mL released solutions were taken from the dissolution medium at predetermined intervals, while an equal amount of fresh buffer solutions were added back to the incubation media. At the required incubation time, the sample was transferred to 2 mL of fresh buffer solution, and the released DOX in the buffer solution was determined. All the drug release experiments were repeated for three times.

Thermal cross-linking of gelatin nanofibers

The gelatin nanofibers were cross-linked thermally using a vacuum oven at 140 °C for 12, 24, 48, or 72 h[29]

Water solubility of gelatin nanofibers

The percentage (w/w) of cross-linking of Gel, DOX@Gel, PEGylated-Gel, DOX@PEGylated-Gel nanofibers was determined using water solubility of the Gelatin nanofibers. For this purpose, 10 mg piece of gelatin nanofibers (Thermally cross-linked and uncross-linked Gel, DOX@Gel, PEGylated-Gel, and DOX@PEGylated-Gel) were incubated in distilled water at 37 °C for 24, 48, and 72 h. The undissolved nanofibers were subsequently taken, dried in the vacuum oven at 80 °C for 2 h and weighted. The percentage of remaining undissolved piece of nanofibers (Ws) was calculated according to the following equation: Ws (%) = 100(Wu/Wi), where Wi is the initial weight of the sample and Wu is the weight of the undissolved desiccated sample[29]

Cell proliferation effect of gelatin nanofibers

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) proliferation assay was performed to evaluate the cytotoxicity of the gelatin nanofibers, reflecting the cell viability of A549 (human lung carcinoma) cell lines in the presence of the nanofibers. Cells were seeded at a density of 5×10^4 cells/cm^2 and incubated at 37 °C in % 95 air, %5 CO_2 environments for 24 h. The obtained nanofibers (0.5, 1, 2.5, 5 mg) were weighted and sterilized under UV light for 40 min. The sterilized nanofibers were placed on 24-well cell culture plate and then incubated for 24 h. After incubation, the medium was removed and replaced with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) -containing medium. Plates were incubated for an additional 4 h at 37 °C. MTT medium was removed and 100 µl of DMSO was added to dissolve the formazan crystals. The absorbance was determined using a plate reader at a wavelength of 540 nm.

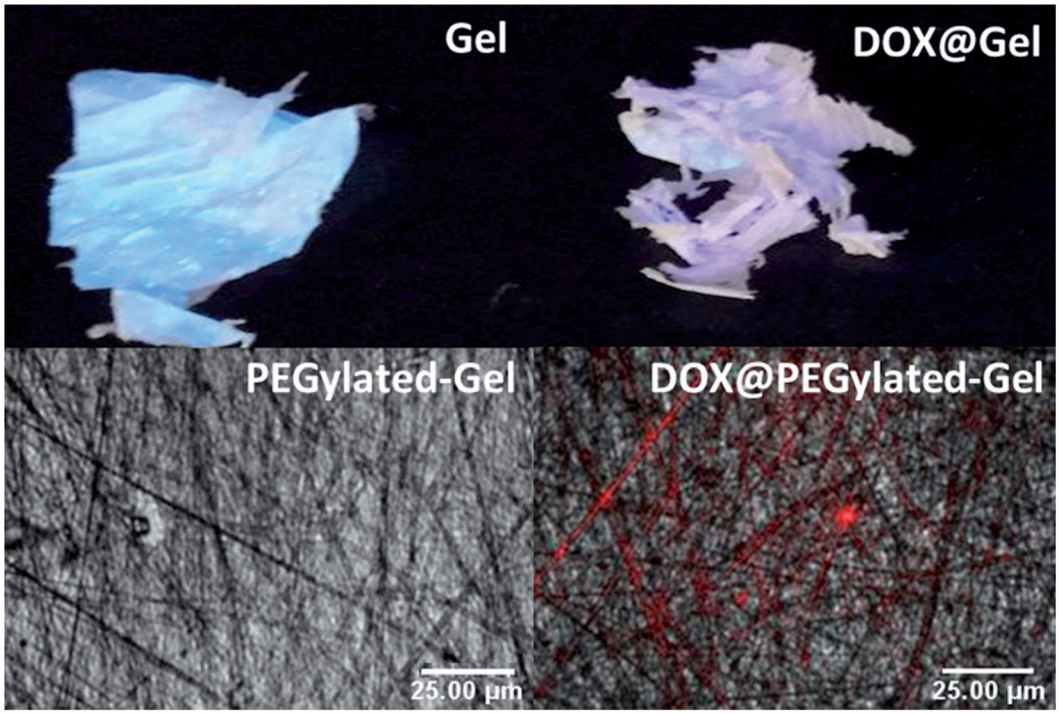

Figure 1. Photographic images of electrospun nanofibers; Gel, DOX@Gel under UV light and PEGylated-Gel, DOX@PEGylated-Gel under confocal microscopy.

Antibacterial effect of gelatin nanofibers

The antibacterial activity of the electrospun fibers was tested against *E. coli* (Gram-148 negative) and *S. aureus* (Gram-positive) as model microbes. Initially, the concentration of the bacterial cultures was set to 0.5 McFarland standard, then using a cotton swab, the final bacterial suspensions were adjusted to $\sim 1.25 \times 10^7$CFU/mL for *E. coli* and 1.7×10^7CFU/mL for *S. aureus*. PEGylated-Gel and DOX@PEGylated-Gel electrospun nanofibers for uncross-linked and cross-linked (33.0 mg) were placed separately in 24 well plates, incubated at 37 °C under shaking condition at 150 rpm for 24 h. After incubation, one hundred microliter of the culture inoculum was retrieved from each well, serially diluted (10^{-3} to 10^{-6}) and plated on a Mueller Hilton agar at 37 °C. The number of viable bacteria persisted in the plates were enumerated using colony counter after 24 h incubation. The experiment was conducted in duplicate.

Statistical analysis

The results represented the mean ± standard deviation from at least three independent experiments. Statistical errors of the data were determined by OriginLab 2016 version (OriginLab Corporation, Northampton, MA) (https://www.originlab.com/2016).

Results and discussion

In this study, four different nanofibers namely Gel, DOX@Gel, PEGylated-Gel, DOX@ PEGylated-Gel were fabricated and thermally cross-linked with the hope of improving stability, water resistance property and controlled drug release behavior. Then, the nanofibers were characterized by various techniques and their biological activities were investigated.

First of all, the photographic images of Gel and DOX@Gel under UV-Vis light were applied and confocal microscopy for PEGylated-Gel and DOX@PEGylated-Gel was performed in order to prove the presence of DOX molecules within the nanofibers. It is necessary here to mention that PEGylated-Gel and DOX@PEGylated-Gel nanofibers displayed almost no obvious difference with naked-eye under the UV-Vis light, therefore, the presence of DOX molecules in these nanofibers was examined by confocal microscopy. According to the images (Figure 1), it is possible to conclude the equal distribution of DOX molecules within all electrospun fibers since they exhibited red and uniform fluorescence. Similar to Dox-loaded PEG-PLA fibers, the surface of gelatin fibers was smooth and no drug crystals were detected, indicating that DOX was incorporated into the nanofibers.[31]

In literature, it is possible to see some examples of chemically cross-linked nanofibers of gelatin.[32–36] However, the use of these methods for the nanofibers is limited due to the toxicity of solvents in the biological application. In contrast to chemical cross-linking, thermal cross-linking is a green and user-friendly alternative when it comes to biomaterials production. In this regard, Gel, PEGylated-Gel, DOX@Gel, DOX@PEGylated-Gel nanofibers were cross-linked at 140^0C. Subsequently, the degree of cross-linking is evaluated as a function of time (Table 1). According to the results, it can be stated that there is an increasing trend in the cross-linking degree of all nanofibers. Therefore, for further structural characterization and biological applications, 72 h of cross-linking time was used.

In order to observe the fiber morphology, SEM images were taken before cross-linking (Figure 2). The formation of

Table 1. The percentage (w/w) of cross-linking of Gel, DOX@Gel, PEGylated-Gel, and DOX@PEGylated-Gel nanofibers.

Duration for crosslinking	Gel nanofiber	DOX@Gel nanofiber	PEGylated-Gel nanofiber	DOX@PEGylated-Gel nanofiber
24 h	57	49.1	44.6	63.1
48 h	59.9	70.1	62.3	74.4
72 h	62.3	74.8	62.4	84.5

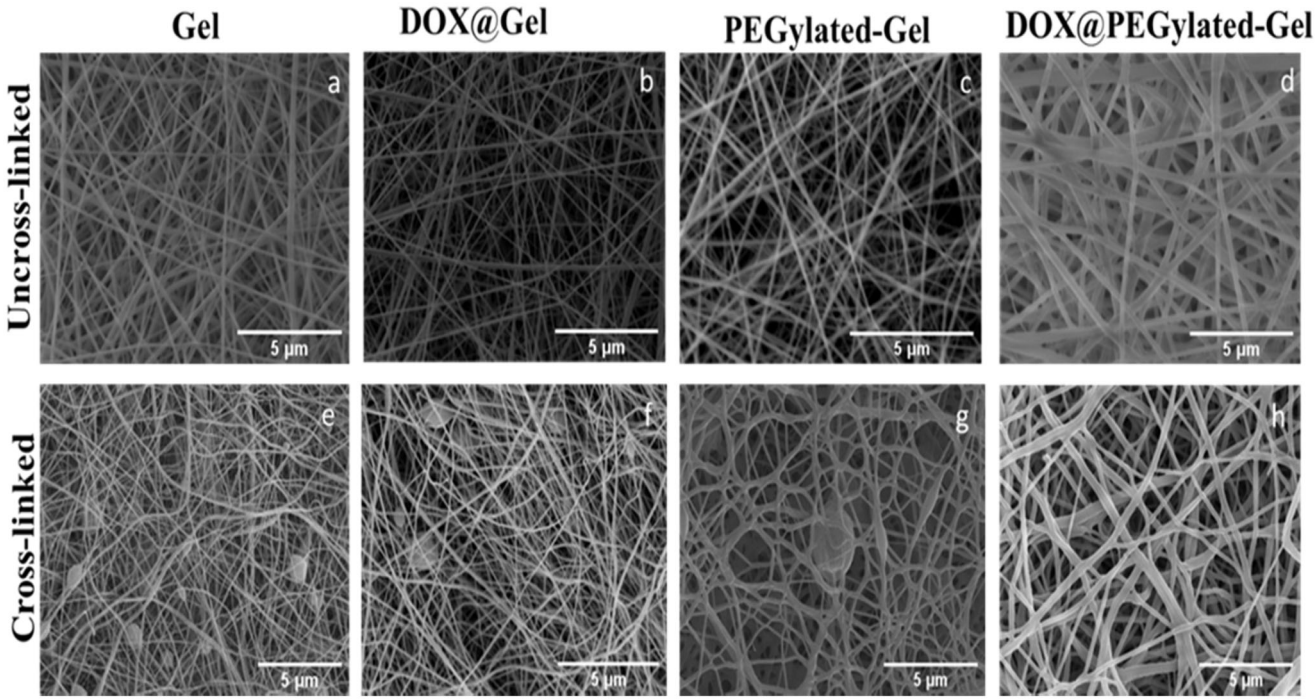

Figure 2. SEM micrographs of (a) Gel, (b) DOX@Gel, (c) PEGylated-Gel, (d) DOX@PEGylated-Gel, (e) cross-linked gel, (f) cross-linked DOX@Gel, (g) cross-linked PEGylated-Gel, and (h) cross-linked DOX@PEGylated-Gel.

Table 2. Diameter of Gel, DOX@Gel, PEGylated-Gel, DOX@PEGylated-Gel nanofibers according to SEM measurement.

	Diameter (nm)	
Nanofiber	Uncross-linked	Cross-linked
Gel	112.88 ± 24.48	159.08 ± 26.75
DOX@Gel	123.02 ± 14.81	212.80 ± 40.50
PEGylated-Gel	219.80 ± 34.62	268.75 ± 44.90
DOX@PEGylated-Gel	195.35 ± 32.93	298.78 ± 44.90

smooth, continuous and uniform Gel, DOX@Gel, PEGylated-Gel, and DOX@PEGylated-Gel nanofibers were observed. In general, the addition of PEG resulted in an increase in the diameter of Gel nanofibers from 112.88 ± 24.48 nm to 219.80 ± 34.62 nm whereas the incorporation of DOX into nanofibers had no significant change in the diameter of nanofibers (Table 2). The seen phenomena can be linked to the decrease in electrostatic repulsion forces due to the drop in conductivity and surface charge density of the solution.[37] Furthermore, cross-linking also enlarged the diameter of all nanofibers (Table 2) for Gel and PEGylated-Gel nanofibers whereas the change was almost two times in the size of DOX@Gel and DOX@PEGylated-Gel. This result points out the importance of thermal cross-linking in this study.

Furthermore, FT-IR analysis of all nanofibers was performed (Figure 3). According to the results, there is no significant difference between Gel and DOX@Gel as well as PEGylated-Gel and DOX@PEGylated-Gel, which indicates the successful incorporation of drugs into the nanofibers. Additionally, it is possible to see the characteristic protein bands of gelatin, amide I and II, at 1650 cm^{-1}, 1540 cm^{-1}, which can be attributed to both stretching of C = O and C-N as well as bending of N-H of gelatin.[38] Noteworthy to mention that the presence of amide I is related with related both with coil and and α-helix conformation.[39] Also, PEGylated-Gel and DOX@PEGylated-Gel display additional bands at 3409, 1099, 2875 cm^{-1} attributed to OH stretching, C–O–C stretching, and also C-H stretching bands, respectively. Asides from those, the effect of thermal cross-linking were investigated by FT-IR, which suggest no significant structural change in the nanofibers.

The drug, DOX, release profiles from Gel, PEGylated-Gel and also thermally cross-linked Gel, PEGylated-Gel nanofibers at 37 °C in the phosphate buffer solutions (pH = 7.4) are depicted in Figure 4. DOX is freed from Gel and PEGylated-Gel fibers with an initial burst release of 89% and 59% during the first 20 min, followed by further gradual release over time. After immersion for 360 min, the cumulative release reached approximately 99% for Gel and 73% for PEGylated-Gel fibers. DOX is a hydrophilic drug and is easier to diffuse into the water, leading to a fast release phenemona. The pores of gelatin could be responsible for the release of drugs easily from nanofibers because the addition of PEG slowed down DOX penetration. Thermally cross-linked Gel and PEGylated-Gel nanofibers exhibited a better drug release behavior compared to uncross-linked fibers

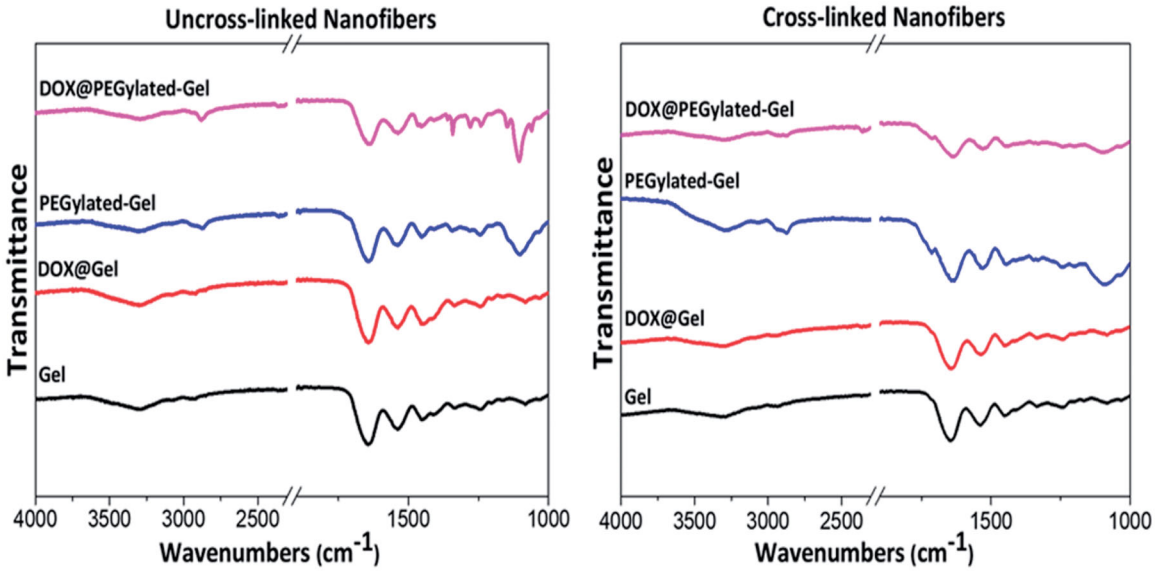

Figure 3. FT-IR spectra of uncross-linked and cross-linked nanofibers of Gel, DOX@Gel, PEGylated-Gel, and DOX@PEGylated-Gel.

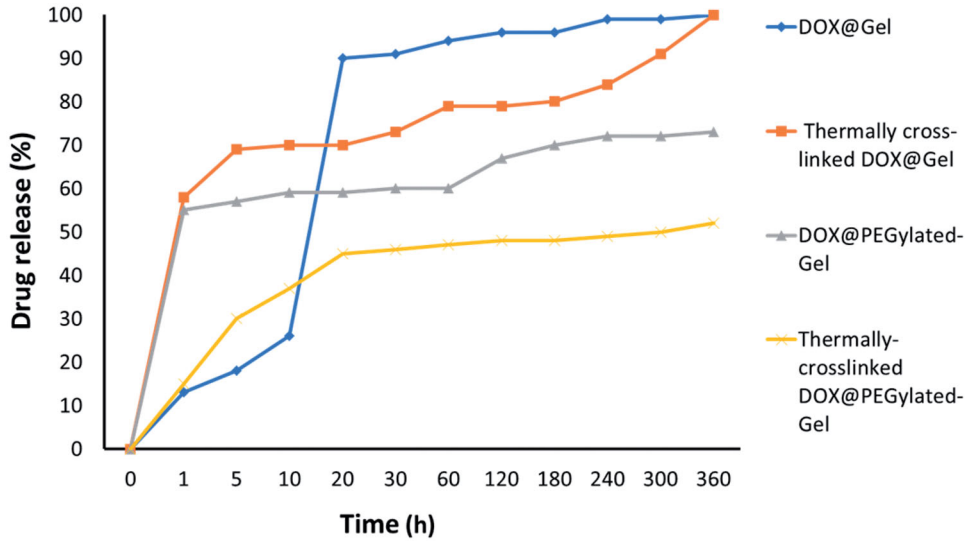

Figure 4. *In vitro* drug release profile of DOX from (a) DOX@Gel, (b) DOX@PEGylated-Gel, (c) cross-linked DOX@Gel, (d) cross-linked DOX@PEGylated-Gel nanofibers through 360 min.

(Figure 4). The initial bursts of release observed with thermally cross-linked Gel and PEGylated-Gel nanofibers were 70 and 45%, respectively, for the first 20 min. The gradual release ensues for about 360 min up to 99 and 52% for gel and PEGylated-gel fibers, respectively. The results clearly show that 90% DOX release with gel nanofibers was achieved at 20 min, while the same results were achieved at 300 min with thermally cross-linked gel nanofibers. Drug release behavior from these types of systems might be controlled by diffusion, not fiber erosion because the nanofibers preserved their fiber structure during immersion for 360 min.[40]

Cell cytotoxicity of gel, DOX@Gel, PEGylated–gel, and DOX@PEGylated–gel fibers for A549 cells were investigated as shown in Figure 5A. Cell viability was concentration dependent as the amount of nanofiber material was increased, the cell viabilities were decreased. Although 0.5 mg Gel nanofiber has shown no toxicity on A549 cells, DOX@Gel nanofiber increased cell cytotoxicity approximately 60% as expected.[41] No significant difference was observed in the survival rate of A549 cells due to the addition of PEG to Gel nanofiber. Cytotoxicity of thermally cross-linked nanofibers on A549 cells was also investigated and compared to uncross-linked nanofibers in Figure 5B. A slight change in cell viability of A549 cells was observed when cross-linked Gel nanofibers applied because of slower DOX release.

The antibacterial activity test was carried out for two model bacterial strains; *E. coli* (Gram-negative) and *S. aureus* (Gram-positive). The results of microbial inhibitory expressed as the number of viable bacteria persisted in the plates enumerated using colony counter after 24 h incubation in the presence of cross-linked and uncross-linked, PEGylated-Gel and DOX@PEGylated-Gel nanofibers (Table 3). The cross-linked nanofibers exhibited superior killing effect against both bacterial strains. The best

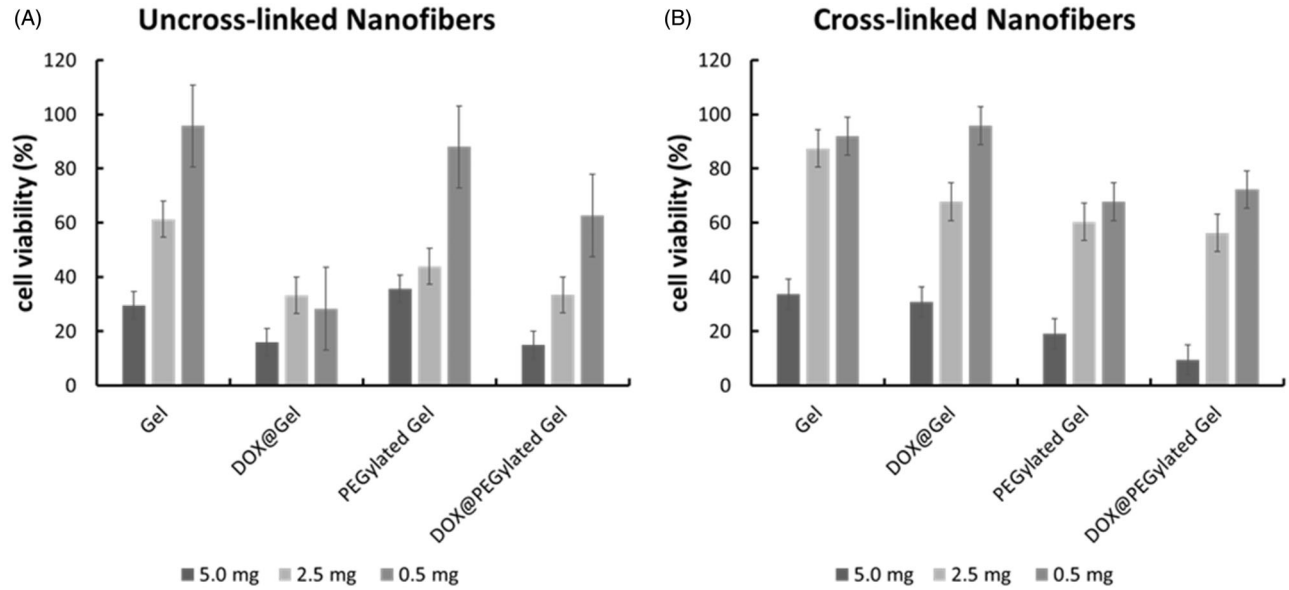

Figure 5. *In vitro* cell viability (%) of A549 cell line in the presence of 0.5, 2.5, and 5.0 mg of (A) uncross-linked and (B) cross-linked nanofibers.

Table 3. Microbial inhibitory effect expressed as the number of viable E. coli and S. aureus in the presence of cross-linked and uncross-linked nanofibers after 24 h incubation.

Nanofibers	*E. coli* (cfu/ml)	*S.aureus* (cfu/ml)
Control	8.80×10^8	1.90×10^8
Uncross-linked		
PEGylated-Gel	6.40×10^8	1.90×10^8
DOX@PEGylated-Gel	2.6×10^8	7.3×10^8
Cross-linked		
PEGylated-Gel	1.10×10^5	8.00×10^7
DOX@PEGylated-Gel	9.6×10^6	4.00×10^7

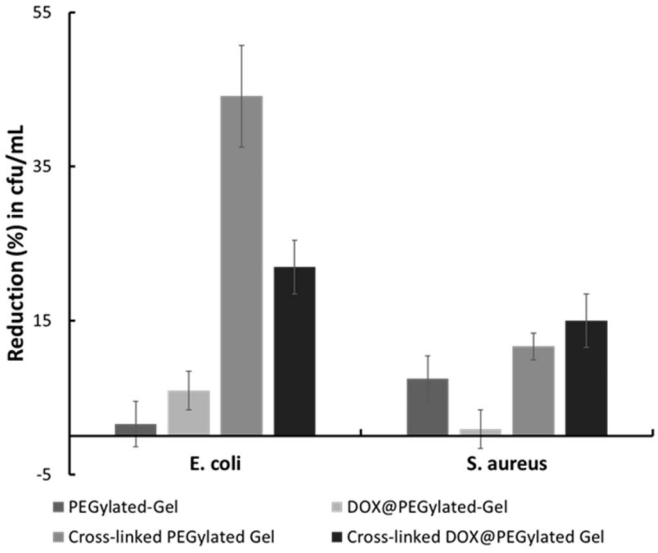

Figure 6. The antibacterial activity of the uncross-linked and cross-linked PEGylated-Gel and DOX@PEGylated-Gel nanofibers against *E. coli* and *S. aureus*.

antibacterial effect was observed using cross-linked PEGylated-Gel nanofibers calculated to be 44% of reduction in cfu/ml after incubation for 24 h against *E. coli* strain (Figure 6). *E. coli*. strain demonstrated better antibacterial activity compared to *S. aureus* in all nanofiber conditions. Similar to literature studies, PEGylated-Gel and DOX@PEGylated Gel nanofibers showed antimicrobial activity against *E. coli.*[40] It is noteworthy to mention here that electrospun nanofibers are widely studied in wound dressing application areas[42,43] and it is known that PEG can display antimicrobial activity.[44,45] This was one of the underlying reasons why PEG was chosen as a composite material in this study. It is thus clear that cross-linked PEGylated-Gel has a potent antibacterial property among all nanofiber conditions.

Conclusion

In the case of polymer-based nanofibers, one of the biggest struggles is the burst release behavior and low delivery capacity, which still remains a problem to be solved. In this research, novel gelatin nanofiber composites were successfully synthesized by electrospinning and cross-linked through thermal treatment. Incorporated and molecularly distributed DOX into the nanofibers was illustrated by confocal microscopy and not only morphology but also the size of the fabricated Gel nanofibers was determined via SEM images. Chemical characterization of nanofibers confirmed that Gel nanofibers were obtained well and the effect of thermal cross-linking created no significant structural change in the nanofibers investigated by FT-IR. Better and slower DOX release behavior was achieved from thermally cross-linked gel and PEGylated-gel nanofibers compared to uncross-linked fibers. *In vitro* investigations of gel nanofibers on A549 cell line showed no significant difference in the survival rate with the addition of PEG while the incorporation of DOX to nanofiber increased cell toxicity. Additionally, a slight change in cell viability of A549 cells were observed when cross-linked Gel nanofibers applied because of slower DOX release. The antibacterial activity was investigated and *E. coli.* demonstrated better antibacterial activity compared to *S. aureus* in all nanofiber conditions. The cross-linked compared to uncross-linked nanofibers inhibited the growth of both bacterial strains.

Taken together, newly fabricated and well-characterized gel nanofibers here with thermal cross-linking resulted in a potential material for drug delivery and wound dressing applications.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Informed Consent

All authors are consent to publish the manuscript.

Authors' contributions

D.M.: research, methodology, investigation, data collection, analysis, writing - original draft. G.G.: data collection, analysis. G.Ş.M.: research conceptualization, supervision, project administration, writing-review, and editing.

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