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Controlled Release of Gentamicin from Electrospun Poly(Vinyl Alcohol)/Gelatin Nanofibers: The Effect of Crosslinking Time Using Glutaraldehyde Vapor

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In this study, polyvinyl alcohol (PVA), gelatin (GEL) and gentamicin (GEN) were used and 13PVA/0.5GEL/GEN nanofibers were fabricated with the electrospinning method. These nanofibers were crosslinked chemically with glutaraldehyde (GA) at different time intervals (2, 3, 4, 5, 5.5 and 6 h) to observe the crosslinking effect on the properties of the nanofibers. Morphological analysis reported that as the crosslinking time increased, the nanofiber diameters also increased from 369.26 nm (non-crosslinked) to 447.72 nm (6 h/crosslinking), respectively. The thermal characterization results demonstrated that crosslinking with different times noticeably shifted the thermal points.The tensile testing results proved that application of crosslinking enhanced the mechanical strength of the nanofibers from 3.31 MPa (non-crosslinked) to 5.8 MPa (5.5 h/crosslinking), respectively. The GEN release profiles from the nanofibers showed similar behaviors under crosslinking and indicated that the crosslinking time did not have a significant effect on the amount of the GEN released.

Introduction

Drug release is the process where the drug is let off from the polymer matrix to the release medium.[1] Biopolymeric materials have the feature of being the best choice for drug release carriers.[2] To upgrade bioefficacy, enable clinical applicability, naturally derived and synthetic materials are generally used in controlled drug release.[1] According to the literature, PVA is a semicrystalline polymer produced by partial or complete hydrolysis of acetate group from polyvinyl acetate.[3] PVA has high processability, biocompatibility, superior mechanical strength, excellent biodegradability, and chemical resistance. These properties have led to its extensive use in drug delivery systems.[4,5] Polyvinyl alcohol (PVA) has remarkable properties such as high surface stabilization, chelation properties and low protein adsorption resulting in poor cell adhesion compared to other hydrogels for use as a drug delivery system.[6,7] Besides, it shares structural similarities with tissues like skin, cartilage, the cornea, and others.

Gelatin (GEL) is one of the most frequently used FDA-approved natural biopolymers because of its biocompatibility and biodegradability.[8,9] Thanks to its innate protein structure and a large number of distinct functional groups, GEL presents modification opportunities to bind the crosslinker, which is useful in developing of drug delivery and release systems.[2] GEL has been successfully studied in controlled release.[9] GEL is used in the literature as drug delivery system, especially for inflammatory drugs, antineoplastic compounds, antibacterial agents, nucleic acids, and hydrophobic materials.[10]

Nanofibers produced by electrospinning technique from polymer solution have attracted a lot of attention due to their widespread applications.[11,12] Electrospinning is a technology that continues to evolve day by day. Some of these are as follows: It is a suitable system for large-scale fiber production.[13] It allows the production of fibers in many different morphologies, thanks to its various modifications from a single-needle system[14] to a multi-needle system such as coaxial,[15] triaxial,[16,17] side by side biocomponents[18] and other complex processes.[19] The third method, in which the choice of crosslinker and chemical reaction parameters is an important factor, is the combination of traditional physical and chemical methods.[20]

After production process, the obtained structure must be processed to encourage its longtime shape protection and to prevent constructional failure.[21] A common strategy to receive stable structure is crosslinking process. Crosslinking of nanofibers provides a complex three-dimensional linkage between polymeric chains, which increases the stability of nanofibers.[22] In addition crosslinking process enhances the mechanical and physicochemical characteristics of the tissue-engineered polymeric constructs. Depending on the chemical structure of the polymer constructs can be crosslinked using chemical, physical

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or enzymatic methods.[23] Chemical crosslinking takes place on the molecules by covalent bonding.[24] Formaldehyde, Glutaraldehyde, Genipin, Carbodiimide are the crosslinkers that are used in biomedical applications.

Glutaraldehyde (GA) is widely used as a chemical crosslinker for polymeric scaffolds and hydrogel constructs. Glutaraldehyde is often used to crosslink with high efficiency, short reaction time, and low cost hydroxyl groups-containing polymers such as PVA or GEL. Compared to other crosslinking agents, glutaraldehyde possess lower cytotoxicity.[25] GA reacts with amine or hydroxyl functional groups of polymers by connecting polymer chains. GA shows a reaction with the amine or hydroxyl functional groups of polymers by connecting polymeric chains.[26] GA is more effective than other aldehydes in forming chemically stable crosslinks.[27] Yang et al. reported that PVA/CNC/LNP films crosslinked by glutaraldehyde possess better mechanical properties, modified thermal stability and swelling behavior.[28] Martinez et al. investigated the effect of crosslinking on polymeric films and reported that vapor phase reaction of GA increased the mechanical stiffness, cell adhesion and proliferation, and extended the drug release time.[29] According to the report by Mi et al., higher crosslinking rate of chitosan hydrogel results in a decreased drug-release rate.[30] In another study performed by Teixeira et al. the authors fabricated nanofibrous dressings using poly(vinyl alcohol) (PVA) and cellulose acetate (CA) and crosslinked them with GA for 7 h. They investigated the mechanical resilince and thermal stability after crosslinking, and found that after GA-based crosslinking process, the mechanical resilince and thermal stability of the fiber mats were enhanced.[31] There are many studies in the literature examining the effect of crosslinking on the properties of fibers and fibrous structures. However, for the best of authors' knowledge, no study on the effect of crosslinking time on the morphologies, degradability, chemical, thermal and mechanical properties of the drug-loaded PVA/GEL-based nanofibers, and its effect on the release behavior of the antibiotic has been conducted. Thus, this study aims to develop GEN-loaded 13PVA/0.5GEL nanofibers and to evaluate the crosslinking effect on the properties of the nanofibers and gentamicin release behavior.

Materials and Methods

Materials

Polyvinyl alcohol (PVA, $M_W = 89{,}000–98{,}000$), gelatin (GEL, from bovine skin, type B), phosphate buffer saline (PBS, pH = 7.4), glutaraldehyde solution (25 wt.% in H_2O, Mw ~ 100.12) and gentamicin solution were purchased Sigma Aldrich (Saint Louis, MO, USA). Tween 80 was obtained from Merck (Merck KGaA, 64271, Germany).

Preparation of the Solutions

Firstly, 13% PVA (w/v) was added to 20 mL of distilled water and mixed on a magnetic stirrer at 120 °C. To diminish the surface tension, 3% Tween 80 (v/v) was added to the solution after dissolution of PVA. Then, 0.5% Gelatin (w/v) was added to the hydrogel solution and mixed at 60 °C, followed by addition of 400 μL of GEN and further stirring for an hour.

Electrospinning Process

The electrospinning system consisted of two syringe pumps (NE-300, New Era Pump Inc., Toledo, OH, USA) and a commercial laboratory-scale electrospinning machine (Inovenso, Istanbul, Turkey). In iorder to produce fibers, first,10 mL solutions were loaded into two syringes and placed on the syringe pumps and attached to two brass needles. The electrospinning parameters were: 120 mm – working distance, 22 kV – high voltage, and 0.2 mL/h-flow rate, respectively.

Crosslinking Process

Crosslinking process of produced nanofibers was performed using glutaraldehyde vapor. 25% glutaraldehyde water solution was poured into a 35 × 10 mm glass plate and placed on the bottom of the desiccator. The nanofibers were placed on the top of the glass chamber and the chamber was inserted inside an incubator at 60 °C. Nanofiber was divided into pieces and exposed to glutaraldehyde vapor for specific time intervals (2, 3, 4, 5, 5.5, 6 h).

Scanning Electron Microscopy (SEM)

SEM (EVO LS 10, ZEISS) was utilized to obtain nanofiber's morphology and diameters. Surfaces of the samples were coated with gold-palladium for 120 s with a Quorum SC7620 sputter-coated before the analyzys. Each type of nanofibers was evaluated to obtain morphological properties and diameter differences. The average fiber diameter was calculated by taking the average of 100 fibers' diameter using Image software (Olympus AnalySIS, USA).

Fourier Transform Infrared Spectroscopy (FT-IR)

To observe the functional groups on the furface of the fibers, nanofibers were analyzed using Jasco FT/IR4700 model machine at room temperature over the range of 4000–400 cm^{-1} in the transmission mode with 4 cm^{-1} resolution.

Tensile Testing

To determine the mechanical properties, a tensile testing device was used (Shimadzu Corporation, EZ-LX, Kyoto, Japan). For this, nanofibrous patcheswere cut on a rectangular dimension of 10 × 50 mm. Before starting the test, the thickness of the nanofibers was measured using a digital micrometer (Mitutoyo MTI Corp., USA). The test speed was adjusted to 5 mm/min, and a 5 kN load cell was applied during the test. The measurement was repeated 3 times for each group.

Differential Scanning Calorimetry (DSC)

Thermal properties of the produced nanofibers were determined with DSC (Shimadzu, Japan) with a temperature range of 25–300 °C. The nanofibers were weighed (~1 mg) and put into an aluminum pan. The constant 10 °C/min heating rate was used during the tests.

Degradation Behaviors of Nanofibers

To evaluate degradation rates relative to crosslinking time, nanofibers were incubated at 37 °C in phosphate-buffered saline solution (PBS; pH 7.4) for 10 days. For the degradation test, initial weights of small pieces of nanofibers were measured (Wo). Then, each piece was kept in 1 mL PBS (pH 7.4) in a thermal shaker (BIOSAN TS-100) at 37 °C for 24 h. At specific time intervals (1–10 days), nanofibers were taken from PBS medium and dried in a dryer at 37 °C for 24 h. The dry weights of nanofibers were measured (Wt), and the degradation index (D) was calculated by using the equation given below:[32]

$$D = \frac{Wo - Wt}{Wo}.100 \quad (1)$$

Drug release test

The *in vitro* release profiles of the 400 μL GEN from the 13PVA/0.5GEL nanofibers were carried out in a thermal shaker. The first step of the drug release testwas to get the linear calibration curve of the GEN. Five different drug amounts were used and absorbance values were detected at 190–300 nm using UV spectroscopy (Shimadzu UV-3600, Kyoto, Japan). To start the drug release test, ~5 mg GEN loaded into 13PVA/0.5GEL nanofibers were weighed and placed into eppendorf tubes with 1 mL PBS (pH 7.4). The measurements were taken at different time intervals. The fresh PBS was used during the test, and absorbance values were detected at 196 nm.

Statistical analysis

The statistical analysis was determined by using a single factor ANOVA Turkey analysis program. Measurements of the pore size were made successfully by using SPSS 17.0 analysis program. The level of significance was taken $p < 0.05$, and data were labelled with (*) for $p < 0.05$, (**) for $p < 0.01$, (***) for $p < 0.001$.

Results and Discussion

Morphological Analysis of the Nanofibers

Crosslinking mechanism occurs by forming both intramolecular and intermolecular acetal bridges between the aldehyde ends of glutaraldehyde and the hydroxyl groups of PVA nanofibers.[34] The surface morphologies and fiber diameters of the non-crosslinked nanofibers and nanofibers crosslinked for different times were examined using SEM (Figure 1). Diameters were calculated by taking an average of 100 random readings for each sample and a diameter distribution was generated. The mean diameter of the as-spun fibers not exposed to crosslinking agent was measured as 369 ± 58 nm (Figure 1(a)). The mean diameters of the crosslinked nanofibers were calculated as 382 ± 31, 406 ± 39, 406 ± 53, 427 ± 48, 428 ± 56, 447 ± 41 nm for the nanofibers exposed to glutaraldehyde vapor for 2, 3, 4, 5, 5.5, and 6 h, respectively. According to the results obtained, it can be noticed that the fiber diameter slightly increases as the exposure time to glutaraldehyde vapor increases. During crosslinking with glutaraldehyde vapor, the water vapor softens and swells the surface of the nanofibers, thus making them thicker.[34] Moreno-Cortez et al., in their study, found that PVA nanofibers had a flawless morphology and an average diameter of 116 nm before crosslinking. It was observed that the average diameter of the nanofibers increased up to 174 nm after exposure to glutaraldehyde vapor, but apart from that, the other morphological features has been preserved. They suggested, that the increase of the diameter may be related to the absorption of GA vapor in the nanofiber structure.[35] Vashisth et al. study reported that the fiber diameters of gellan/PVA nanofibers, which were chemically crosslinked using methanol and glutaraldehyde, increased compared to the the non-crosslinked fibers, and the surface area decreased.

Fourier Transform Infrared Spectroscopy (FT-IR)

FTIR analysis was performed to examine the structural change of gentamicin-loaded PVA nanofibers before and after GA crosslinking. For pure GEL (Figure 2(a)), characteristic absorption bands were observed N–H stretching of secondary amide at 3277 cm^{-1}, C–H stretching at 2933 cm^{-1}, C–O stretching at 1626 cm^{-1}, and N–H bending at 1525 cm^{-1}.[36] For pure PVA (Figure 2(b)), sharp bands were observed for O–H stretching at 3268 cm^{-1}, C–H stretching of the alkyl groups at 2910 cm^{-1}, C=O and C–O stretching of the acetate groups at 1646 cm^{-1}, and C–H_2 stretching at 1417 cm^{-1}.[37] In Figure 2(c), the characteristic infrared bands of gentamicin were observed equally to the amide NH bending vibrations of primary aromatic amines at 1619 cm^{-1} and 1525 cm^{-1}. In addition, the S–O bending vibration peak at 1031 cm^{-1} and the S–O stretching peak at 605 cm^{-1} are observed.[38] FTIR spectrum of non-croslinked PVA included sample, showing the peaks at 916 and 831 cm^{-1} thus confirming the presence of skeletal vibration of PVA.[39] The magnitude of the peak at 916 cm^{-1} decreases correspondingly with the crosslinking time. At the same time, the position of the peak at 831 cm^{-1} shifted to 836 cm^{-1}. The low amount of gelatin also caused the effect of crosslinking on amide peaks to be unclear. These confirmations shows that the perceivable effects of cross-linking is especially on PVA.

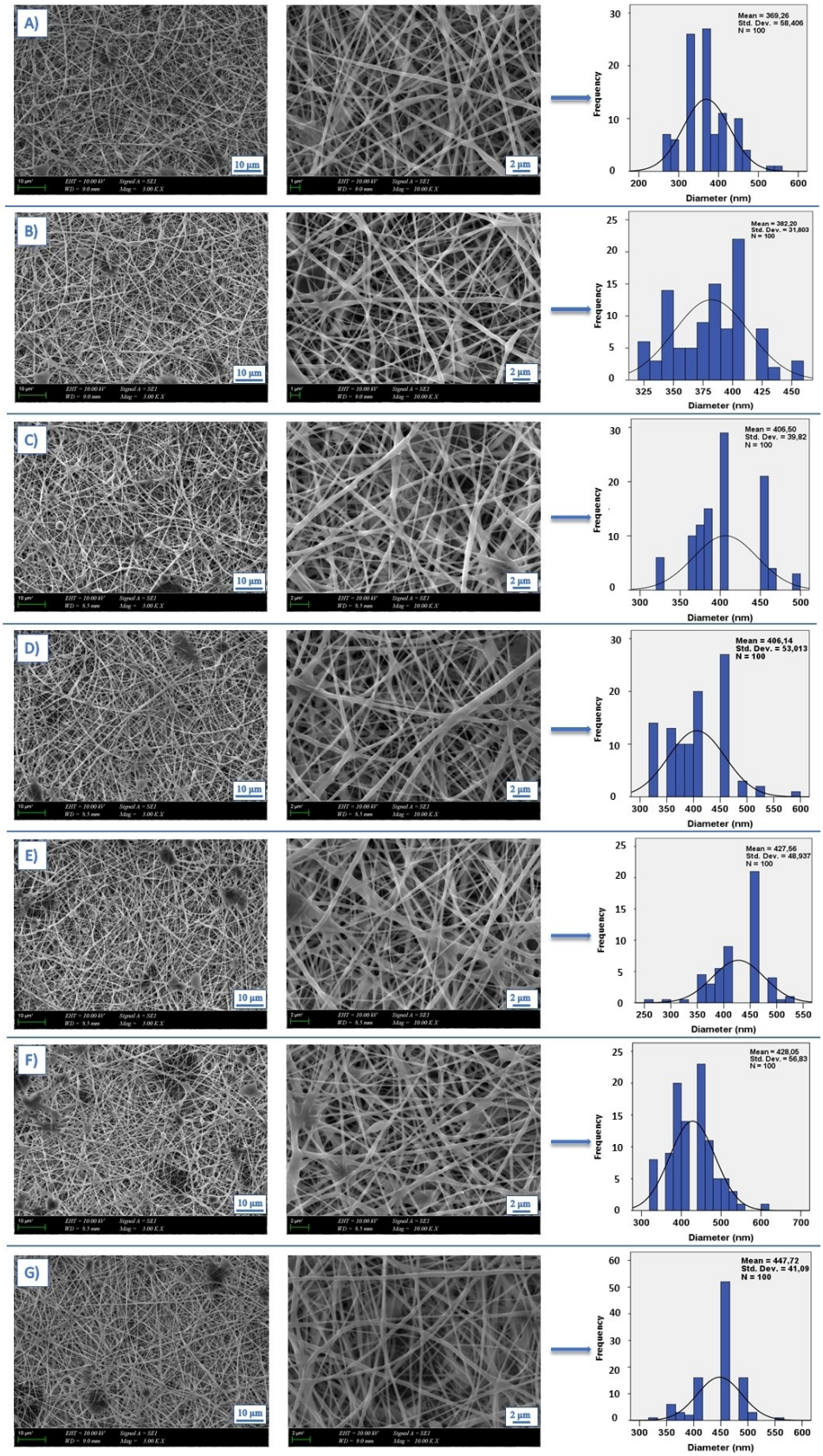

Figure 1. SEM images and fiber diameter distribution of electrospun nanofibers non-crosslinked (a) and after crosslinking for: 2 h/ (b), 3 h (c), 4 h (d), 5 h (e), 5.5 h (f) and 6 h (g). Calculated by averaging the diameter of 100 fibers.

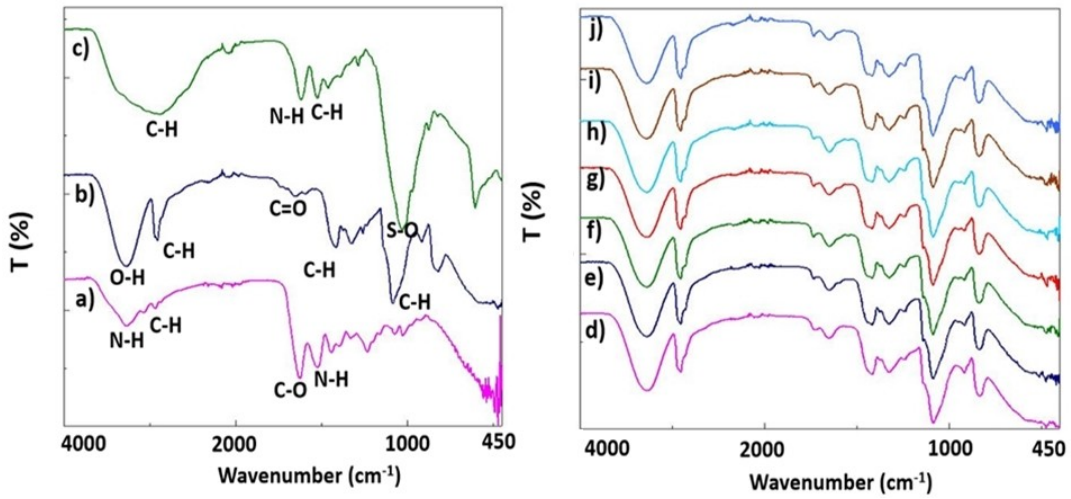

Figure 2. FTIR spectra of pure GEL (a), pure PVA (b), Gentamicin (c), non-crosslinked fibers (d), and fibers after crosslinking for: 2 h (e), 3 h (f), 4 h (g), 5 h (h), 5.5 h (i), and 6 h (j).

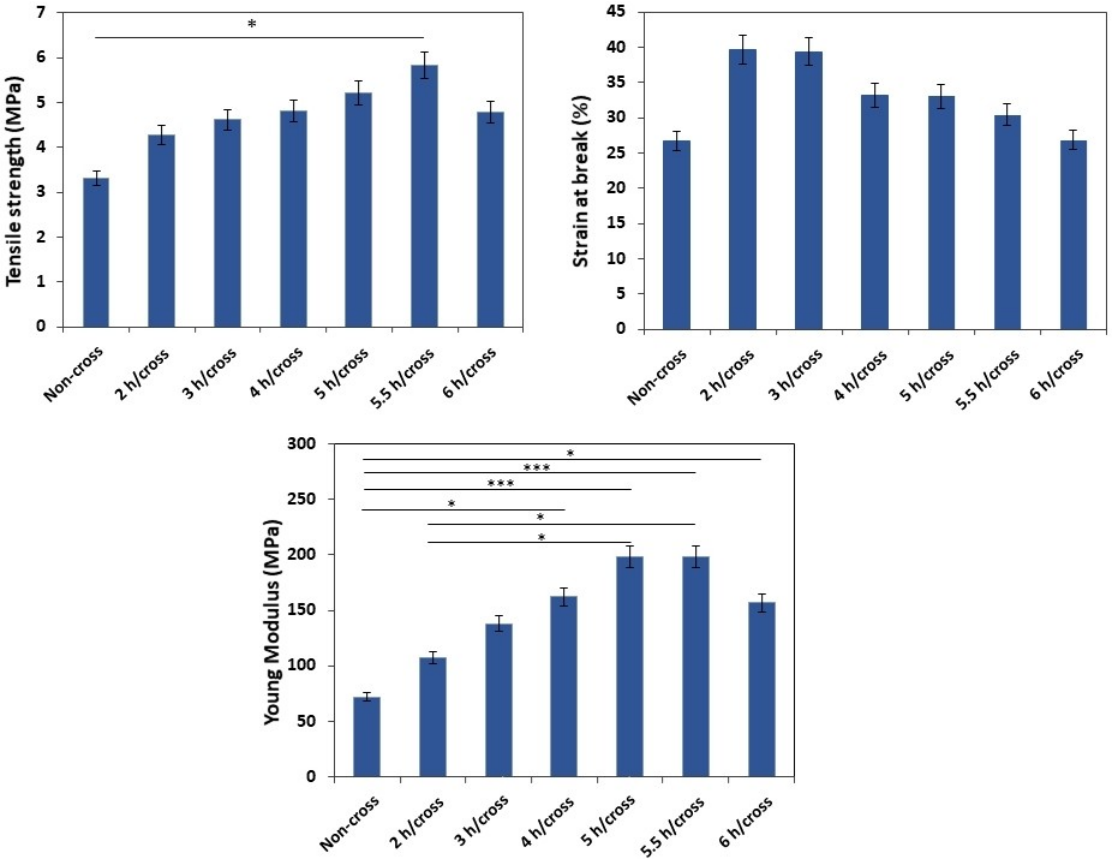

Figure 3. Mechanical properties of PVA fibers before and after crosslinking with GA for different time periods (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). The measurement was repeated 3 times for each group. Error bars represent the range of results obtained and data presented are mean ± SD, n = 3.

Mechanical Properties of the Nanofibers

The effect on the mechanical stability of gentamicin-loaded PVA nanofibers crosslinked with GA is shown in Figure 3. Tensile strengths strain at break, and young modulus were evaluated. The results show that the non-crosslinked 13PVA/0.5GEL/GEN nanofibers have minimum tensile strength of (3.31 MPa) and minimum strain at break of 26.73 % compared

to the croslinked fibers. The overall tensile strength and elongation at break increased when PVA nanofibers were crosslinked with glutaraldehyde at different time ranges.[40,41] While the tensile strength and elongation at break increased continuously to up to 5.5 h, there was a slight decrease after 6th h. According to the literature, although the cross-linking time increased up to a certain hour initially increases the mechanical properties, exposure to GA vapor after 5 h mostly turns the material into a more brittle structure.[42] In this study, it is observed that the young module of nanofibers increases as the cross-linking time increases up to 5.5 h. This increase can be attributed to the formation of both inter- and intramolecular covalent bonds during cross-linking.[43] As a result, it was observed that the mechanical properties of 13PVA/0.5GEL/GEN nanofibers were changed by crosslinking treatment. Crosslinking improved the mechanical performance of the fibrous structure.[44]

Thermal behaviours of the nanofibers after crosslinking process

DSC analysis was carried out to observe the thermal behaviors of the pure GEN, non-crosslinked and crosslinked nanofibers. In Figure 4 in thermogram b obtained for non-crosslinked 13PVA/0.5GEL/GEN, the peak detected at 228 °C represents the melting point of the PVA. Another peak detected at 58 °C is associated with relaxation in the amorphous region of 13PVA/0.5GEL.[45] The decomposition stage of the PVA started at 270 °C which is related to the backbone decomposition.[46] Thermogram (c) represents 13PVA/0.5GEL/GEN after 2 h of crosslinking and results showed no difference betwenn this sample and non-crosslinked material. After 3 h of crosslinking, the melting point of the PVA was shifted from 228 °C to 220 °C (Thermogram (d)). The reason for this decrease can be due to the morphological effects caused by the reaction between PVA and GA.[47] In Thermogram (e), the peak observed at 58 °C for non-crosslinked material shifted to 60 °C after 4 h of crosslinking, and the new peak was observed at nearly 240 °C. Thermogram (f) belongs to the fibers crosslinked for 5 h, in this curve, the melting temperature of the PVA decreased to 220 °C and the peak observed at 58 °C for non-crosslinked fibers also shifted to 60 °C. In addition, the broad new curve was observed at 200 °C. In Thermogram (g), the peak of the melting point at 220 °C disappeared and another peak was detected at 240 °C for fibers crosslinked for 5.5 h. Thermogram (h) representing the 6 h crosslinked fiber patches exhibited many additional small peaks and the peak at 240 °C was observed as more narrow and sharp compared to peaks obtained for other crosslinked fiber patches. The peaks that occur between 200 °C and 240 °C in crosslinked fibers can be explained by degradation induced by crosslinking.[46]

Degradation Behaviors of the Nanofibers

Nanofibers fabricated for tissue engineering must have certain degradation properties to provide sufficient space to allow cell in growth and tissue formation.[48] The static conditions degradation test is a method that evaluates the decrease in mass of structures exposed to PBS over time.[49] The rate and mechanism of degradation of polymeric component affect the mechanical properties of the fibrous structure.[50] As shown in Figure 5, the non-crosslinked PVA-based nanofibers lost approximately 75 % of its initial mass at the end of the 10th day. Keeping all the other parameters constant, the degradation rates of nanofibers crosslinked with GA vapor for 2, 3, 4, 5, 5.5, and 6 h were calculated as 59 %, 57 %, 51 %, 45 %, 40 %, 37 %, respectively. The results clearly showed that degradation

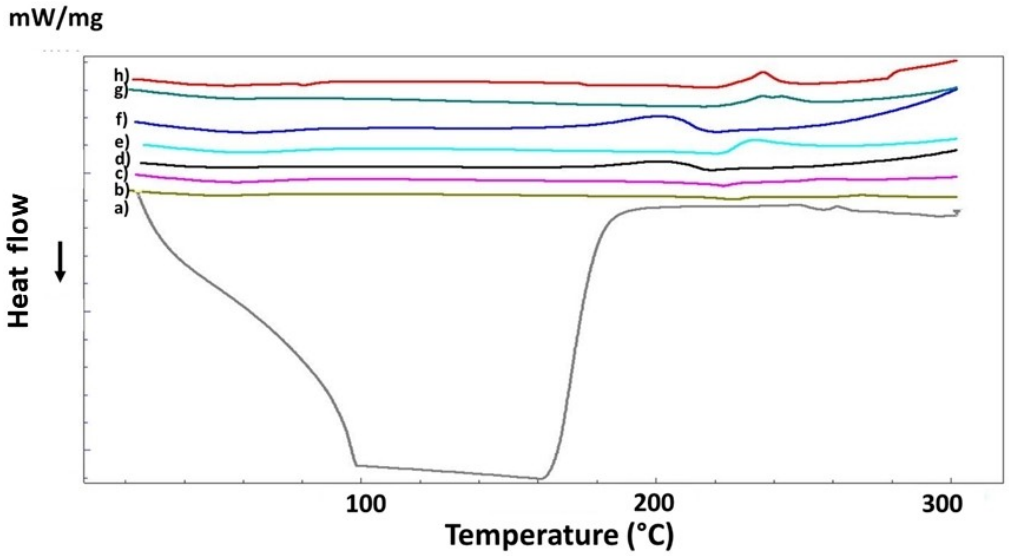

Figure 4. DSC thermograms of GEN (a), the non-cross (b) and crosslinked GEN-loaded 13PVA/0.5GEL nanofibers, during the 2 h (c), 3 h (d), 4 h (e), 5 h (f), 5.5 h (g), and 6 h (h).

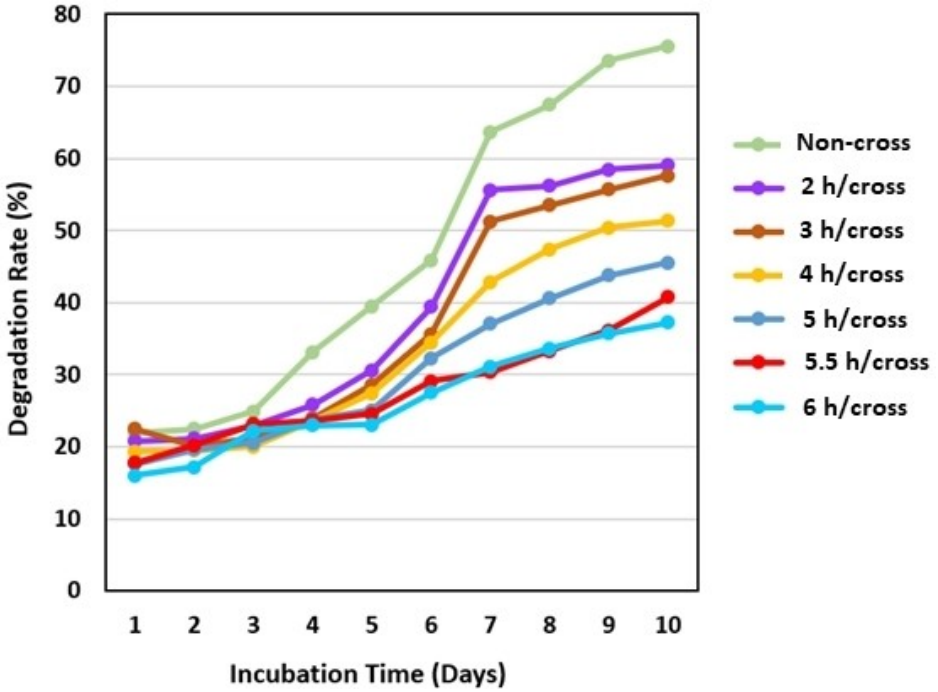

Figure 5. Degradation graphics of non-crosslinked fibers and fibers after crosslinking for, 2 h, 3 h, 4 h, 5 h, 5.5 h, 6 h. The measurement was repeated 3 times for each group.

decreased as the exposure time of the fibers to crosslinking agent increased. The presence of a large number of intermolecular hydroxyl groups in PVA/Gel nanofibers results in a strong hydrogen bond, which affects the solubility of the nanofibers.[51] When PVA nanofibers are crosslinked with GA vapor, intermolecular acetyl bridges (CH–(CH_2)–CH)are formed between the aldehyde ends of GA and the hydroxyl groups of nanofibers.[52] When the hydrophilic hydroxyl groups in the PVA macromolecules fully react with the GA vapor and the PVA is completely crosslinked, fibers becomes insoluble in water. However, in partially crosslinked PVA nanofibers, the non-crosslinked fragments dissolve in water over time. From this, it is possible to say that the low degree of crosslinking increases the amount of water solubility.[53] In this study, the decrease in mass loss with increasing crosslink time can be attributed to this reason.

In Vitro Drug Release Profiles

The release of GEN from the nanofibers was examined in a PBS at a thermal shaker. The calibration curve of the GEN was determined with five differently concentrated solutions (0.25–1 μg/mL) and the graph was given in Figure 6 (A). Figure 6 (B) represented the absorption graph of the GEN obtained at 196 nm. Figure 6 (C) showed the cumulative release of GEN from the different formulations. According to Figure 6 (C), the non-crosslinked nanofiber exhibited a rapid release profile and released approximately 80% of the drug in the first 6 h. When the literature is examined, it is expected that the drug release of nanofibers cross-linked with GA vapor will be longer.[54] It can be attributed to this that the non-crosslinked nanofiber showed a burst release compared to the cross-linked samples. In adition, completion of drug release from the non-crosslinked nanofiber was achieved at the end of the 2th day. It was observed that the release of the cross-linked nanofiber at 2 and 3 h was completed to 100% on the 4th day. The release of cross-linked nanofibers at 4, 5.5, 6 h reached 100% on the 5th day. When Figure 6(C) is examined, the increased cross-linking time from the 3rd h to the 6th h did not further prolong the GEN release. As a result, on the 5th day of release, the GEN was fully released from all nanofibers. According to the results, there was no significant difference between the cross-linking time extending from 4 h to 6 h and the total amount of GEN released. It can be concluded that the crosslinking application can extend the GEN release through decreased hydrophilicity of the samples, and their stability in the water environment. Up to a certain time higher crosslinking caused the lower drug diffusion ratio out the fiber matrix.[54] It is known that crosslink application makes the structures denser than non-crosslinked structures. Therefore, the release of a drug from the crosslinked network is more difficult than non-crosslinked network.[55] In addition, it can be noticed that the hydrophilic nature and poor cell penetration of the GEN causes better integration within the PVA/GEL nanofibers and prolongs the release by the effect of crosslinking.[56,57] Acetyl bridges are formed between PVA nanofibers exposed to GA steam, Hydroxyl groups of PVA nanofibers and aldehyde ends of GA. These acetyl bridges reduce the water solubility of the material. In this study, PVA nanofibers cross-linked at different times allow controlled release of the drug.[58]

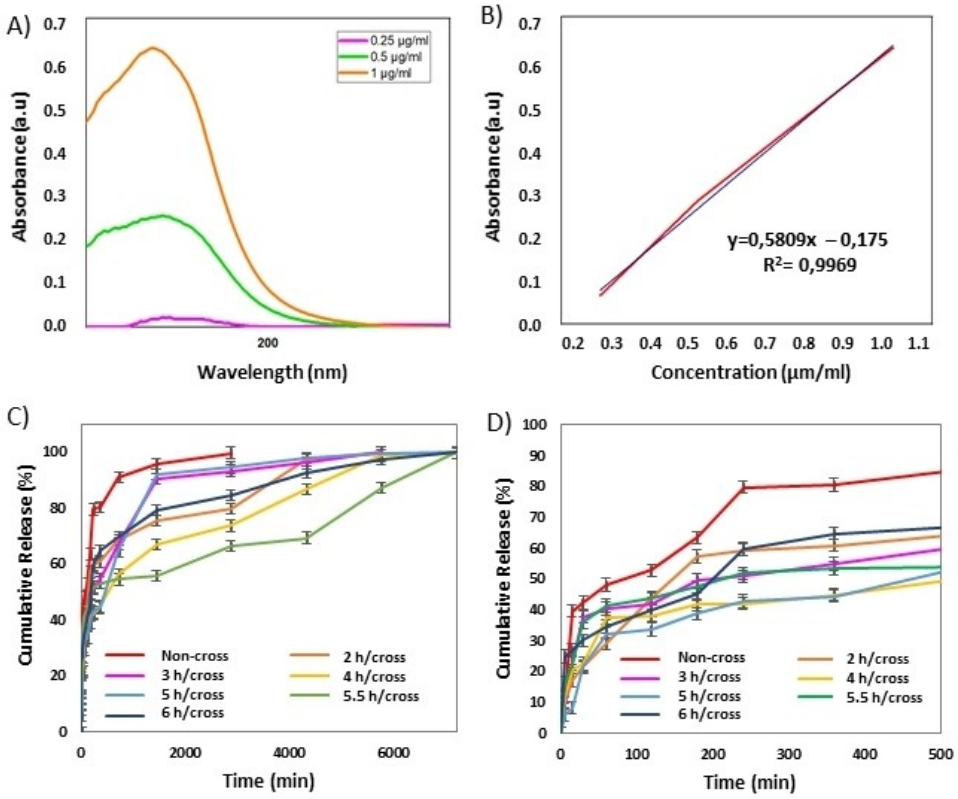

Figure 6. Absorbance spectra of the GEN acquired from the different amounts (A), the standard calibration curve of the GEN (B) and the cumulative release graphs of the non-crosslinked, 2 h/cross, 3 h/cross, 4 h/cross, 5 h/cross, 5.5 h/cross, and 6 h/cross (C), Cumulative release graph of nanofibers between 0–500 minutes (D). The measurement was repeated 3 times for each group. Error bars represent the range of results obtained and data presented are mean ± SD, n = 3.

Conclusion

In this study, GEN-loaded PVA/GEL nanofibers were successfully produced by the electrospinning method. As spun nanofibers were chemically crosslinked by exposition to GA vapors for different time intervals (2, 3, 4, 5, 5.5, and 6 h) and the effect of crosslinking on the fibers was investigated. It was observed that as the crosslinking time increased, the nanofibers became thicker by absorbing more steam and their diameters increased from 369 nm to 447 nm under SEM. The thermal analysis results showed that the time of crosslinking changed the degradation temperature and melting temperature. Moreover, the crosslinking time to up to 5.5 h increased the mechanical strength and elongation at breakon the other hand crosslinking time of 6 h made the material brittle and reduced its mechanical strength. In addition, it can be concluded that the duration of the crosslinking has a significant effect on the degradation behavior of the nanofibers. It is also observed that GEN release occurs faster from non-crosslinked nanofibers, while the time of release for crosslinked fiber is slightly longer. According to all obtained results, differences are observed in the physicochemical, morphological, mechanical and thermal properties, along with fibers degradation and drug release behavior of 13PVA/0.5GEL/GEN nanofibers dependly to crosslinking times.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Research data are not shared.

Keywords: Crosslinking · electrospinning · gelatin · nanofiber · polyvinyl-alcohol

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