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Production and characterization of bactericidal wound dressing material based on gelatin nanofiber

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ABSTRACT

Gelatin is a biocompatible and biodegradable natural polymer obtained by collagen. Gelatin nanofibers meet all the necessary requirements when used as wound dressing material. However, their lack of antimicrobial properties limits their use. The purpose of this study is to expand the field of use of gelatin by providing it with antimicrobial properties. For this purpose, poly([2-(methacryloyloxy)ethyl] trimethylammonium chloride) (PMETAC), was used. In this study, the polymers were dissolved in formic acid-acetic acid and nanofibers were synthesized by electrospinning. The obtained nanofibers were characterized with SEM, FTIR, and TGA. The antibacterial effect, degradation tests, and cell viability, adhesion and proliferation were investigated. The SEM studies show that the nanofibers are homogeneous and smooth. At the end of 14 days, all nanofibers lost >90% of their mass. The nanofibers containing PMETAC showed good bactericidal activity against *Staphylococcus aureus*, *Escherichia coli*, *methicillin-resistant Staphylococcus aureus* and *Acinetobacter baumannii*. MTT test demonstrated that low doses of the nanofibers were biocompatible. The cell adhesion study has been shown that many cells attachment and proliferate on the surface of nanofibers. It has been found that the obtained nanofibers can be used safely and effectively as antimicrobial wound dressing material.

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1. Introduction

Wound dressing materials play an important role in the treatment of certain types of open wounds. An ideal wound dressing material should allow gas to be exchanged, provide a moist environment at the wound interface, be easily removed without trauma, create a barrier against microorganisms and leak out excess fluids. It must also be made of a biomaterial that is antimicrobial, non-toxic, biocompatible, biodegradable [1,2]. Traditionally used bandages are ideal places for the growth of microorganisms on the wounded area. It is important to develop new wound dressing materials to shorten the healing process and make it less painful, while at the same time contributing to the regeneration of the structure and function of the skin in a short period of time [3,4]. In order to develop a wound dressing material that can be used effectively and can meet all the requirements sought, this material must be structurally adaptable and its properties controllable. For this purpose, the use of nanofibers obtained by electrospinning has increased lately [5,6]. Electrospinning technique is a process which is used in obtaining micro or nano size fibers. It is a simple, cheap, fast processing that process parameters can be changed easily and that can be used in a wide variety of polymers and which is suitable for commercial production. In this process, polymer solution is withdrawn from one plate to another between two plates loaded with high voltage and fibers are thus formed [7]. An important characteristic of the nanofibers obtained by this method are very similar to the natural extracellular matrix (ECM) with a very large surface area per unit mass, highly porous microstructure, high gas permeability and small pore size between fibrils. The ability of electrospun porous nanofibers to mimic natural ECM increase cell behaviors such as adhesion and proliferation desired for wound healing [7–10]. This ensures that the expected wound healing effect is achieved more quickly and effectively.

Open wounds provide appropriate environments for bacterial colonization and infection. For these reasons it is very important to prevent microbial infection in order to accelerate wound healing in emergency care of skin wounds. Therefore, an ideal wound dressing material must have antimicrobial property. Quaternary ammonium compounds are among the most commonly used antibacterial agents due to their good antibacterial properties, low toxicity, low skin irritation, low corrosion properties, good environmental stability and excellent cell penetration properties. However, since the low molecular weight active substances used in designing the dressing material are slowly released into the environment, their usage times are very limited. Besides, the release of antibacterial agents may cause toxic properties and bacterial resistance in humans. It is expected that the use of polymeric materials instead of low molecular weight materials as antimicrobial agents will

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enhance the ability to kill in contact with harmful microorganisms by increasing the hygienic surface area. [4,11–13]. The main part of our study is based on the use of an antibacterial polymer in nanofibers for the first time. For this purpose, PMETAC was used as antimicrobial polymer in our study.

Gelatin, which has nearly the same composition and biological properties as collagen, is a natural biopolymer obtained by collagen hydrolysis. The gelatin obtained from collagen is also known to increase the adhesion and proliferation of cells due to its biological origin. Given these important properties and examining clinical outcomes, it has been determined that gelatin accelerates wound healing and tissue regeneration [9,14]. In addition to the above-mentioned properties, its non-toxic, biocompatible and biodegradable properties suggest that the gelatin nanofibers obtained by electrospinning will have a wide application area as wound dressings, controlled release systems, and tissue engineering scaffold [15–21]. However, their low mechanical properties and water solubility narrow the fields of use of gelatin [10]. Therefore, it is necessary to cross-link gelatin nanofibers in order to increase their stability in aqueous media [22,23]. Gelatin nanofibers meet all the necessary requirements when used as wound dressing material but their lack of antimicrobial properties limits their use.

The aim of this study is to improve the low mechanical properties of gelatin and give it antibacterial properties. For this purpose, poly([2-(methacryloyloxy) ethyl] trimethylammonium chloride) (PMETAC), a quaternary ammonium polymer, will be used. This substance, as a quaternary ammonium polymer, has important properties to be used on the body, such as good antibacterial effect, low toxicity and low skin irritation. In addition, since the polymer to be used as antimicrobial agent is an acrylate polymer, it is thought that the addition of this in the nanofibers structure will highly increase the mechanical properties as well as the other properties of the gelatin.

The nanofibers were synthesized by the electrospinning by dissolving PMETAC and gelatin in a formic acid-acetic acid mixture. The obtained fibers were cross-linked with glutaraldehyde vapor in order to increase their resistance to water and mechanical properties. The obtained and cross-linked fibers was characterized with the scanning electron microscopy (SEM), the Fourier transform infrared spectroscopy (FTIR), the thermogravimetric analysis (TGA) methods and degradation tests. At the last stage, the antimicrobial effect of gram–positive *Staphylococcus aureus* and *methicillin-resistant Staphylococcus aureus* and gram–negative *Escherichia coli* and *Acinetobacter baumannii* cultures was investigated in order to determine the usability of the fibers as antimicrobial wound dressing materials. Finally, its biocompatibility with the body was evaluated by cytotoxicity, cell adhesion and proliferation studies using L929 fibroblast cells.

2. Materials and methods

2.1. Materials

Gelatin (from porcine skin type A) and [2-(Methacryloyloxy) ethyl]-trimethylammonium chloride (75% w/w aqueous solution), sodium chloride, acetic acid, acetone, and formic acid were purchased from Sigma (America). *Staphylococcus aureus*, *methicillin-resistant Staphylococcus aureus* and *Escherichia coli* were supplied from Kırıkkale University Center Research Laboratory (Turkey). *Acinetobacter baumannii* were supplied from Ankara University Hospital, microbiology laboratory. L929 fibroblast cell were obtained from Şap Institute (Turkey). Ammonium peroxy disulphate, potassium chloride, disodium hydrogen phosphate, potassium hydrogen phosphate, basic alumina, glutaraldehyde were purchased from Merck (Almanya). Aluminum foil (thin is 12 μm) were purchased from, Horeca (England). Nutrient broth was purchased from Scharlau (Spain). Dulbecco's modified eagle's medium, Fetal bovine serum, penicillin-streptomycin, trypsin, trypan blue, L-glutamine were purchased from Biological Industries. Ribonuclease-A, Hoecht, propidium iodide, flasks and the other plastic materials were purchased from Serva (Israel).

2.2. Synthesis and characterization of the PMETAC

First of all, the METAK monomer was removed from its stopper by passing through a glass column filled with basic alümina. In order to polymerization, the required amount of monomer was taken into a 3-necked flask and diluted with water. Nitrogen gas was passed through this mixture for 30 min. The METAK was then added as ammonium peroxidisulfate initiator and polymerized under reflux for 4 h at 70 °C [24,25]. This mixture was then removed by precipitation in cold acetone and dried at 35 °C in a vacuum oven. The molecular weight of the polymer was determined using the Dynamic Light Scattering method [26,27].

2.3. Synthesis and characterization of nanofibers

Gelatin and gelatin/PMETAC nanofibers were produced in this study. At first, the gelatin polymer was stirred with a magnetic stirrer in a 3/1 (v/v) mixture of formic acid/acetic acid until a homogeneous solution was obtained. PMETAC was added in the range of 20–80% (w gelatin/w PMETAC) in the gelatin solution to prepare the gelatin/PMETAC nanofibers. Then, the solution was sonicated in the sonicator for 5 min in case there were still undissolved parts. After the solutions were obtained, the mixture was taken up in a 10 mL glass syringe. The aluminum sheet size to collect nanofibers was kept constant at 10 × 15 (length × width) cm. The effects of the distance between the needle and the collector, the applied voltage and the flow rate on the formation of nanofibers were investigated (the parameters of the experimental studies are listed in Supplementary Data 1).

The resulting nanofibers were cross-linked by exposing them to glutaraldehyde vapor for 24 h in a desiccator in order to expand their field of use. These nanofibers were then washed with 0.1 M glycine solution to remove glutaraldehyde residues [20]. SEM images were taken in order to determine the surface morphology and diameter of the prepared nanofibers. Fourier transform infrared spectroscopy (FTIR), elemental analysis and thermogravimetric analysis (TGA) studies were carried out to elucidate the structure of the nanofibers.

2.4. In vitro degradation test

Degradation tests were made to test the biodegradability of each nanofiber. Approximately 30 mg was taken from each sample and put into a 5 mL pH 7.4 phosphate buffer (0.8 g NaCl, 0.2 g KCl, 2.9 g Na_2HPO_4, and 0.2 g KH_2PO_4 in 1 L distilled water) [28]. The initial weights of the nanofibers kept in a 37 °C water bath for 24 h were taken. The excess liquid on the samples was removed with the help of filter paper every other day. The samples were then weighed and put again in 5 mL of fresh phosphate buffer. The degradation percentage was determined using the following equation. Moreover, SEM images of the nanofibers were taken to confirm the degradation.

$$\text{Degradation Percentage} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}} \times 100 \qquad (2.1.)$$

2.5. The antimicrobial effect test

The *Staphylococcus aureus* (SA) and *methicillin-resistant Staphylococcus aureus* (MRSA) as gram positive and *Escherichia coli* (EC) and *Acinetobacter baumannii* (AB) bacteria as gram negative were selected for the microbial effect test. The antimicrobial effect experiments were carried out based on a slightly modified version of the broth microdilution procedure described by Wiegend et al. [29]. For our study, a 12-well plate was used in place of a 96-well. Each well was inoculated with a cell of 5×10^5 cfu/mL. The biggest difference in the

procedure is that the cell count was done by optical density measurement. First of all, the bacteria were incubated in liquid culture medium for 24 h. The cross-linked fibers were taken into a 12-well plate and 5 $\times 10^5$ cfu/mL of prepared bacteria were added to each well into 2 mL nutrient broth culture medium (The all experiments have been run with 3 replicates). The bacteria were incubated for 24 h and cell densities were determined using a UV-VIS spectrophotometer at a wavelength of 600 nm. The percent of inhibition of bacterial growth was determined following equation;

$$\text{Inhibition } (\%) = \frac{A_c - A_s}{A_c} \times 100 \qquad (2.2.)$$

where A_c is an average of optical density values of the control groups, and A_s is an average of optical density values of the samples including nanofiber.

MIC90 was calculated as the nanofiber concentration that inhibited 90% of the visible growth of bacteria. For MIC determination, 2 mL nutrient broth medium was first added to each well in 12-well plates. Cross-linked nanofibers were added to these culture media at concentrations ranging from 0.05 mg/mL to 30.0 mg/mL. Each experiment was run with 3 replicates. 5×10^5 cfu/mL of prepared bacteria (SA and EC) were added to each well and plates were incubated at 37 °C for 24 h. Culture medium that was cultivated only bacteria was used as a control. Bacterial growth was determined by recording changes in optical density at 600 nm.

2.6. Determination of cell viability, adhesion and proliferation

The MTT assay is based on the calculation of cell viability by metabolic activity. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolyumbromide), which is a yellow water-soluble

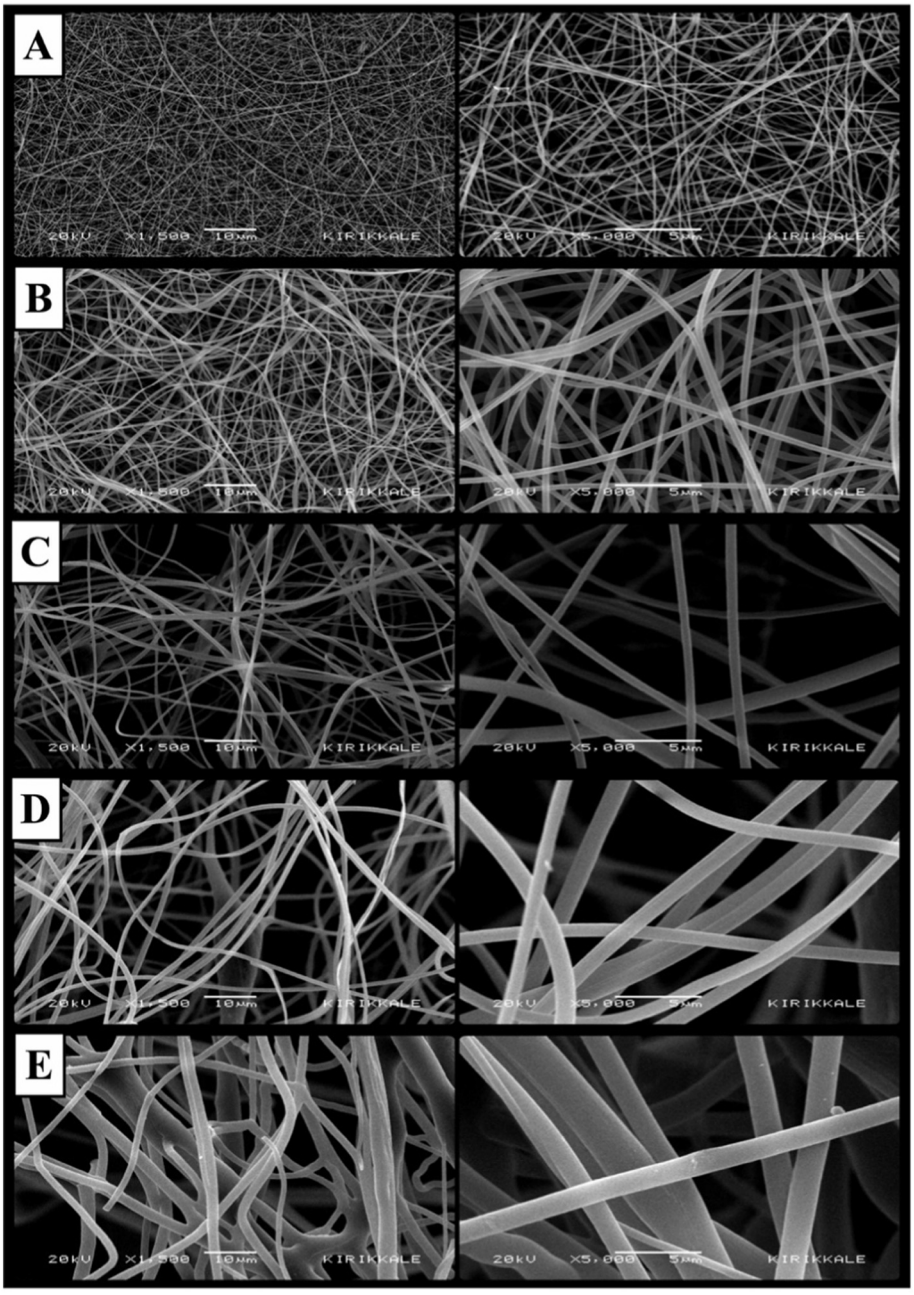

Fig. 1. SEM images of nanofibers (magnifications of ×1500 and ×5000) A: Gelatin nanofibers (G0), B: Gelatin nanofibers containing 20% PMETAC (G20), C: Gelatin containing 40% PMETAC (G40), D: Gelatin nanofibers containing 60% PMETAC (G60), E: Gelatin nanofibers containing 80% PMETAC (G80).

structure, is metabolically reduced to blue-violet colored and water-insoluble formazan in living cells. The number of viable cells correlates with the color intensity of formazan determined by photometric measurements after solving in alcohol. L929 fibroblast cells in the frozen stock were passaged to flasks, then the cells were removed from culture flasks by using trypsin EDTA and the cell suspension was centrifuged at 200*g* for 3 min. Cells were then resuspended in culture medium (Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum and 1% penicillin–streptomycin) and seeded in a 96-well plate at a cell density of 1×10^5 cells/mL per well. The cells were incubated for 24 h (5% CO_2, 37 °C, >90% humidity) until they formed a semi-confluent layer. This incubation period allowed the cells to recover, adhere and develop into the exponential growth phase. Cells were examined under a microscope to check that the cells were spread uniformly in a 96-well culture flask. This check was performed to determine experimental errors. After 24 h incubation, the culture medium in the wells was removed. The per well from various concentrations of extracted nanofibers (0.2 g/mL) in culture medium were added (1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 (the ratio of the volume of nanofiber extract to the volume of culture medium)). 100 μL of medium was added to the control group. Cells are incubated for 24 h at 5% CO_2, 37 °C, >90% humidity. After 24 h incubation, each well on the plate was examined under a microscope in order to determine the cell growth errors and the proliferation of control and treated cells. After examination of the cells in the flasks under the microscope, culture medium in each well was removed. 50 μL of MTT (at 1 mg/mL concentration) was added to each well and the culture flasks were incubated for 2 h at 37 °C. The MTT solution was then withdrawn and 100 μL isopropanol was added to each well to solve the formazan. The absorbance values were analyzed by a microplate reader at 570 nm. The decrease in the number of viable cells also leads to a reduction in the metabolic activity of the sample. This reduction is directly related to the number of formazan crystals formed in blue-purple, read as optical density at 570 nm.

The decrease in viability compared to the control value is calculated as follows;

$$\text{Cell viability } (\%) = \frac{OD570_s}{OD570_c} \times 100 \quad (2.3.)$$

$OD570_s$ = the value of optical density of the sample.

$OD570_c$ = optical density of the control group.

Nanofibers were cultured with L929 fibroblast cells to evaluate the adhesion and proliferation properties of the cells. The samples were crosslinked and washed with 0.1 M glycine solution, then sterilized under UV radiation for 1 h and then placed in 24-well microplates. After the cells were passaged into flasks, the cells were removed from the culture flasks by trypsin EDTA and the cell suspension was centrifuged at 200*g* for 3 min. Cells were then resuspended in culture medium (Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum and 1% penicillin–streptomycin) and seeded in each well at a cell density of 1×10^5 cells/mL per well. The microplates were incubated at 37 °C and 5% CO_2 at various times. After the cells were adsorbed onto the nanofibers, non-adherent cells were removed. Cells adhering and proliferating onto the fibers were stained with 4′,6-diamidino-2-phenylindole (DAPI) and photographed by fluorescence microscopy. DAPI dye is used for staining living cells and especially for qualitative determination of cells.

3. Results and discussion

3.1. Molecular weight analysis results for PMETAC

The molecular weight of the obtained PMETAC was determined at the METU Center Research Laboratory. The molecular weight of the polymer was determined as 2.643×10^5 g/mol.

3.2. Characterization of the nanofibers

3.2.1. SEM analysis

SEM analyzes were carried out to examine the structure, shape and diameters of the obtained and cross-linked fibers (with glutaraldehyde). The results are shown in Fig. 1.

When the SEM images in Fig. 1 are examined, it is observed that the nanofibers have a smooth, beadless and homogeneous size distribution. Besides, it is observed that the parameters modified during the production affected the nanofibers diameters. The results are presented in Table 1. The size distributions of nanofibers range from 201.16 to 2410.12 nm. The most important parameters in the production of nanofibers from polymers by the electrospinning method are: the applied voltage, the distance between the needle and the collector, and the flow rate of the polymer.

Nanofibers were prepared from the polymer mixture containing 20% by mass of PMETAC in order to examine the effects of the modified parameters on the nanofiber diameters. In order to examine the effect of the applied voltage value, the distance and the flow rate were kept at 10 cm and 30 μL/min. The average diameters with the voltage increased by 15, 20 and 25 kV were respectively 429.93 ± 19.50; 449.10 ± 41.65; 485.56 ± 42.58 nm. Similar results have been shown in other studies [30,31]. Tiwari et al. [31] studied polycaprolactone/human serum albumin nanofibers. It has been determined in this study, by increasing the value of the voltage from 12 to 20 kV, the diameter of the nanofibers went from 356 nm to 1280 nm.

In order to examine the effect of the distance between the needle and the collector, the applied voltage and the flow rate were kept at 25 kV and 30 μL/min. The average diameter of the obtained nanofibers with the distance between the needle and the collector increased by 8, 10, 12, 14 and 18 cm were respectively 475.60 ± 38.61, 485.56 ± 42.58, 464.51 ± 31.16, 480.80 ± 48.37, 452.08 ± 41.05 nm. When examining the values, it was found that increasing the distance hardly affects the diameter of the nanofibers. When the distance between the needle tip and the collector was increased to 18 cm, the average nanofiber diameter decreased [31–33]. Increasing the distance between the syringe and the collector ensures complete evaporation of solvent from the structure. This reduces the diameters of nanofibers.

Tiwari ve et al. [31] synthesized polycaprolactone/human serum albumin electrospun nanofibers. It has been determined in this study, by increasing the value of the distance between the needle tip from 6 to 15 cm, the diameter of the nanofibers decrease from 832 nm to 354 nm.

In order to examine the flow rate of the polymer, the applied voltage and the distance were maintained at 25 kV and 10 cm. The average diameter of the nanofibers with the flow rate of the polymer increased by 30,

Table 1
Variation of the average diameters of nanofibers obtained due to the changing parameters in the electrospinning process.

Percent of PMETAC (w/w%)	Flow rate of polymer (μL/min)	Distance (cm)	Voltage (kV)	Average diameters of nanofibers (nm)
0	120	10	25	201.16 ± 29.95
20	30	10	15	429.93 ± 19.50
20	30	10	20	449.10 ± 41.65
20	30	10	25	485.56 ± 42.58
20	30	8	25	475.60 ± 38.61
20	30	12	25	464.51 ± 31.16
20	30	14	25	480.80 ± 48.37
20	30	18	25	452.08 ± 41.05
20	40	10	25	477.91 ± 33.11
20	60	10	25	431.30 ± 34.45
20	80	10	25	434.72 ± 32.81
20	120	10	25	421.61 ± 32.63
40	120	10	25	622.02 ± 59.41
60	120	10	25	1151.82 ± 164.41
80	120	10	25	2410.12 ± 258.65

40, 60, 80 and 120 μL/min were respectively 485.56 ± 42.58; 477.91 ± 33.11; 431.30 ± 34.45; 434.72 ± 32.81; and 421.61 ± 32.63 nm. In this study, increasing the flow resulted in a decrease in the average diameter of nanofibers. Similar results were demonstrated by Moutsatsou et al. [34]. In their study, polyaniline-doped sulfonic acid/polyethylene oxide conductive nanofibers were prepared with a voltage of 25 kV, a collector distance of 10 cm, and a flow rate of 120 μL/min.

In our study, it was determined that the percentage of PMETAC in the nanofibers significantly affected the diameters of nanofibers. Only the nanofibers obtained using gelatin were determined to have a diameter distribution of 201.16 ± 29.95 nm. When 20, 40, 60 and 80% of PMETAC (weight by gelatin weight) were respectively added to the gelatin solution, it has been observed that the diameters of the nanofibers were 421.61 ± 32.63; 622.02 ± 59.41; 1151.82 ± 164.41; 2410.12 ± 258.65 nm. It has been found that the addition of PMETAC into the gelatin increases the concentration of the polymer solution and accordingly increases its viscosity. Several studies have shown that nanofibers increase their diameters because of the increased viscosity of the polymer solution [30,35,36].

Similar results are observed in the study of Tarus et al. [35] on cellulose acetate and poly (vinyl chloride) nanofibers. Increasing the cellulose acetate concentration from 10% to 16% in acetone/*N*,*N*-dimethylacetamide increased the viscosity and the nanofiber diameters from 60 nm to 122 nm. It was determined that increasing the concentration of poly (vinyl chloride) dissolved in *N*,*N*-dimethylformamide/tetrahydrofuran mixture from 12% to 16% and thus increasing the viscosity also increased the nanofiber diameters from 121 nm to 275 nm.

Only gelatin nanofibers, and gelatin nanofibers containing PMETAC in the range of 20–80% (w/w) have been named G0, G20, G40, G60, and G80, respectively.

Protecting their distinctive biomimetic morphology and pores during the use of nanofibers is important for medical applications requiring a large surface area and high porosity. Therefore, the gelatin fibers must be crosslinked before use. Besides, it was found that non-crosslinked

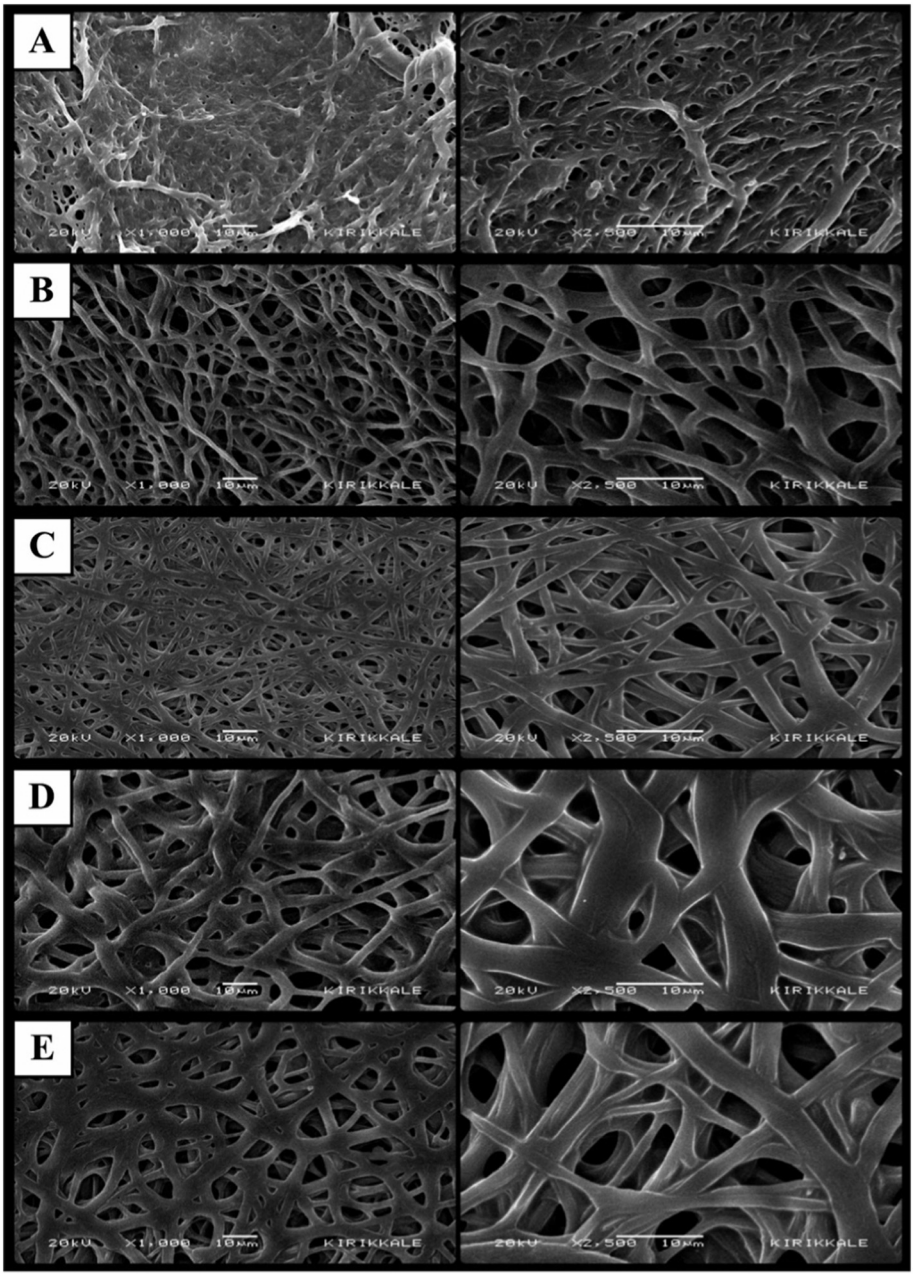

Fig. 2. SEM images of nanofibers exposed to glutaraldehyde vapor (magnifications of ×1000 and ×2500) A: G0, B: G20, C: G40, D: G60; E: G80.

gelatin-based nanofibers were rapidly dissolved in water. When these nanofibers are thrown into the water, the fibers begin to fuse with each other and the porous openings on them are destroyed. This is an undesirable property for use.

The obtained nanofibers were cross-linked by exposure to glutaraldehyde vapor in order to increase their water resistance and mechanical properties. SEM images of the cross-linked nanofibers with glutaraldehyde are shown in Fig. 2.

It was found that the cross-linked nanofibers were stable and their morphology had hardly changed, as it was the case after the electrospinning. However, after crosslinking, the nanofibers have become considerably smaller; the nanofibers strongly entrained at the intersection points and have been strongly bonded to each other. It is observed that nanofibers form a strong and hard network structure. Various studies with gelatin nanofibers have found similar results [22,37]. Lu et al. [22] crosslinked the nanofibers obtained by the spiral electroplating method in liquid and vapor phase glutaraldehyde and characterized the resulting nanofibers. Both methods were found to cross-link nanofibers successfully and the liquid-phase crosslinked nanofibers formed a stronger network. However, they found that even

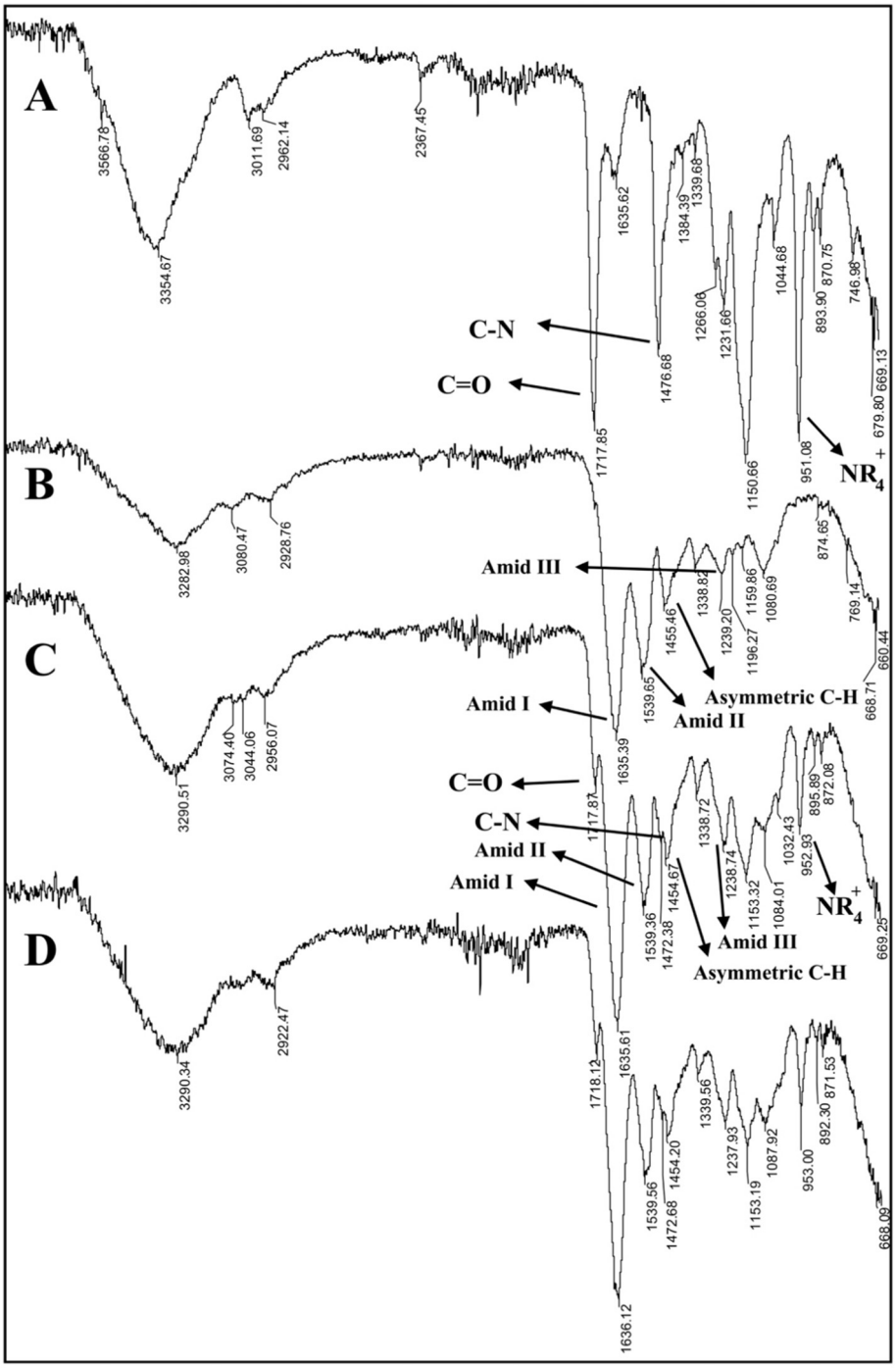

Fig. 3. FTIR spectra A: PMETAC, B: G0, C: G60, D: cross-linking nanofiber of G60 with glutaraldehyde vapor.

in the vapor phase cross-linked nanofibers, the tensile strength increased by about 2-time.

3.2.2. FTIR analysis results

To confirm the synthesis of PMETAC and to elucidate the chemical structure of the produced nanofibers, the nanofibers' structures were investigated by FTIR studies and the results are presented in Fig. 3.

When the Fig. 3.A is examined, the peaks at 1717, 1476, and 951 cm^{-1} were respectively attributed to the (C=O) stretching vibration of the ester group, the (C—N) stretching of amine groups, and the vibration of the quaternary ammonium group in PMETAC [38,39].

When the Fig. 3.B is examined, the pic at 1635 cm^{-1} is considered to correspond to (C=O) stretching vibration of the amide I, at 1539 cm^{-1} to (N—H) stretching of amid II, at 1239 cm^{-1} to the (C—N) stretching vibration for amid III and at 1455 cm^{-1} to the asymmetric (C—H) stretching vibration for the ($-CH_2$) groups [40].

When the Fig. 3.C is examined, the peaks at 1717, 1472 and 952 cm^{-1} are considered to belong to the PMETAC, and the peaks at 1635, 1539, 1454 and 1238 cm^{-1} are thought to be gelatine bands. According to the results, it was determined that gelatin and PMETAC can be successfully used together in nanofiber production.

Fig. 3.D shows the FTIR spectra of G60 nanofibers cross-linked by exposure to glutaraldehyde vapor in the desiccator. The results obtained have shown that some peak intensities and wave density are relatively reduced due to the cross-linking compared to the Fig. 3.C [22]. However, no change in the wavelength of the peaks was observed. This result showed that the cross-linking did not affect the functional groups on the obtained nanofibers.

3.2.3. TGA test results

The TGA analysis was performed at 0–900 °C in order to determine the thermal stability of the produced nanofibers and the results are presented in Fig. 4.

It is observed in the TGA thermogram of G0 in Fig. 4.A that the degradation occurred in two steps. The first step of the degradation occurred between 40 and 175 °C. It is thought that the nanofibers lost their 8.285% of their mass and that this loss was caused by the loss of water from the structure. It is observed that the maximum degradation temperature is 325 °C and that the nanofiber lost about 60% of its mass. The mass loss in this step is thought to be due to the thermal degradation of the polymer chains of the gelatin [20].

It is observed in the TGA thermogram of the PMETAC in Fig. 4.B that the degradation occurred in three steps [41]. The first step of the degradation occurred between 30 and 175 °C and the nanofibers lost their 15% of their mass. It is thought that this loss was caused by the loss of water from the structure. The degradation occurred in the second step between 210 and 275 °C and it is determinate that the maximum degradation temperature is 250 °C. It is found that this mass loss was due to the degradation of the quaternary ammonium groups and breakage of the polymer chains and that about 52% of the mass loss occurs mainly at this stage. The final step of the degradation occurred between 375 and 450 °C, and the maximum temperature of degradation was found to be 430 °C. Due to the breakage of the polymer chains in this step, a loss of about 30% of the polymer mass is observed [42,43].

Fig. 4.C shows TGA thermogram of G60 nanofibers by mass. It is observed in the thermogram that the degradation occurred in four basic steps. The first step is attributed to the 8.5% of mass loss and to the loss of water from hydrophilic nanofibers. The degradation in the second and fourth steps, which occurred between 210 and 275 °C (24.5% of mass loss) and 375–450 °C (18.2% of mass loss), is thought to be due to thermal degradation of polymer chains of PMETAC in the structure. The maximum degradation temperatures at this stage were found to be respectively 250 and 410 °C. This result coincides again very well with the maximum degradation temperatures of the PMETAC. The mass loss that occurs between 275 and 375 °C in the third step (35.5% loss in mass) is thought to be due to the thermal degradation of the gelatin. Moreover, these results correspond very well to the maximum decomposition temperature (325 °C) of gelatin nanofibers.

The TGA thermogram of G60 nanofiber cross-linked by exposure to glutaraldehyde vapor nanofibers is presented in Fig. 4.D. When the TGA thermogram is observed, it can be seen that that the degradation occurred once again in four basic steps. The first step is attributed to

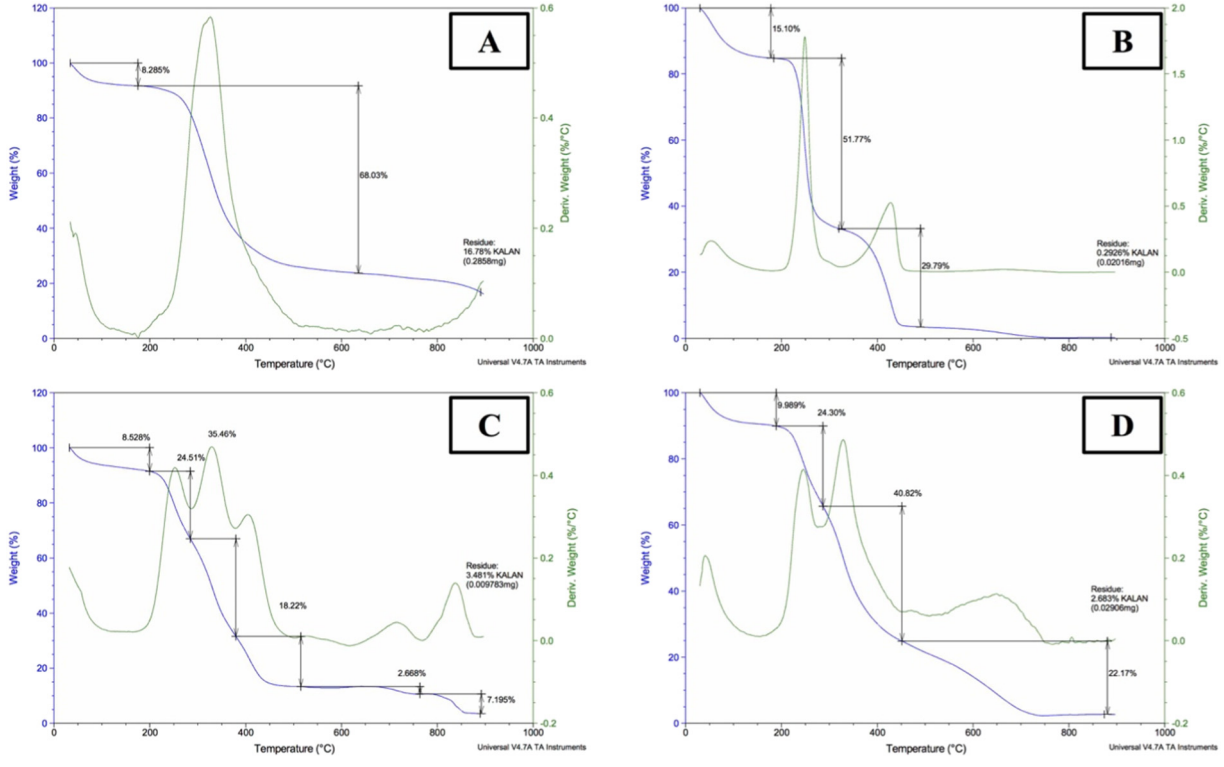

Fig. 4. TGA thermogram A: G0, B: PMETAK, C: G60, D: cross-linking nanofiber of G60 with glutaraldehyde vapor.

Table 2
% PMETAK quantities that should be and calculated for nanofibers prepared with different PMETAC contents.

Sample code	PMETAC% should be in nanofibers	PMETAC% calculated in nanofibers
G20	16.67	26.22
G40	28.57	33.68
G60	37.50	43.32
G80	44.40	57.93

about 10% of mass loss and to the loss of water from hydrophilic nanofibers. The second step remained unchanged at temperatures between 210 and 280 °C with a mass loss of 24.5% and a maximum degradation temperature of 250 °C. The initial and final temperatures and the amount of lost mass in the first two steps of the degradation remained mainly the same. The effect of the cross-linking began to emerge in the third step. The degradation occurred in the third step between 280 and 450 °C with 40.8% of mass loss and it is determinate that the maximum degradation temperature is 325 °C. Once the thermograms were examined, it was found that the non-cross-linked fibers suffered a total loss of 53% in a range of 210–450 °C and that approximately 40% of the nanofibers were degraded after cross-linking with glutaraldehyde. It is observed that the nanofibers cross-linked at 450 °C suffer approximately 12% less mass loss. This remaining amount of fibers has undergone thermal degradation in the range of 450–720 °C. These results showed that the thermal stability of the cross-linked nanofibers increased and this is an expected result [20].

3.2.4. Elemental analysis

The elemental analysis was carried out to prove the rate at which the PMETAC was incorporated into the structure. During the preparation of nanofibers, the amount of gelatin has never been changed (25% by mass) and PMETAC was added in the range of 20–80% (w/w) to the gelatin mass. The contents of PMETAC, that should be into the nanofiber and calculated from elemental analysis, are presented in Table 2 (the results of the elemental analysis are presented in Supplementary Data 2).

The calculated PMETAC content of nanofibers has been found to be higher than expected. This result shows us that the PMETAC having a (+) net ionic charge is better spinned by electrospinning than the gelatin.

3.2.5. Experiment results of the degradation test

A biotechnological material used as a dressing material or implant placed in a damaged organ or tissue is expected to self-degrade without any treatment in the region during a set period of time. For this reason, we have tested the degradation of the wound dressing material in an environment similar to the skin layer of the body. Degradation tests were performed every two days for 14 days using mass measurements of glutaraldehyde cross-linked nanofiber samples taken in a phosphate degradation solution prepared in the manner of Gong et al. [28]. The degradation results are presented in Fig. 5.

The mass loss was fairly rapid and occurred at a high rate in the first week. A decrease in the rate of degradation in the gelatin composed nanofiber sample is observed in the second week. When the obtained values are examined, it is seen that after seven days, gelatin and G20 lost approximately 80% of their masses. However the other nanofibers lost >50% of their masses. It was determined that the gelatin and G20 nanofibers were degraded at a higher rate especially in the first 7 days compared to other nanofibers. The G20 nanofibers showed a degradation close to the gelatin nanofibers within the first 7 days. However, it was determined that the degradation of other nanofibers (G40, G60, and G80) was low. These results show that the addition of PMETAC to the structure increased the resistance of nanofibers during the first 7 days. The graph shows clearly that at the end of 14 days, all the nanofibers lost >90% of their masses.

At the end of the 7th day, samples were taken from the highly degraded nanofibers. Then, SEM images of those samples were taken and the results are given in Fig. 6. The SEM images show clearly that the morphology of the cross-linked nanofibers and the pores between the cross-linked structures in the degradation process evidently expand and become a large hole [44].

3.3. Experiment results of the antimicrobial effect test

Open wounds provide appropriate environments for bacterial colonization and infection. Traditionally used wound dressing materials in the treatment of open wounds can cause infection in this area and are ideal environments for the growth of microorganisms on the wounded area by providing a moist, warm and nourishing environment. These infections can prolong the treatment and the healing process of the wound. For these reasons, it is very important to prevent microbial infection in order to accelerate wound healing. Therefore, an ideal wound dressing material should have an antimicrobial property [4,11–13].

Although gelatin meets all the requirements sought for a wound dressing material, one of its major deficiencies is that it is not antimicrobial. Giving antimicrobial properties to gelatin is the basis of this work.

Two control groups were formed in order to investigate the antimicrobial effect of cross-linked nanofibers. At first, a bacterial culture

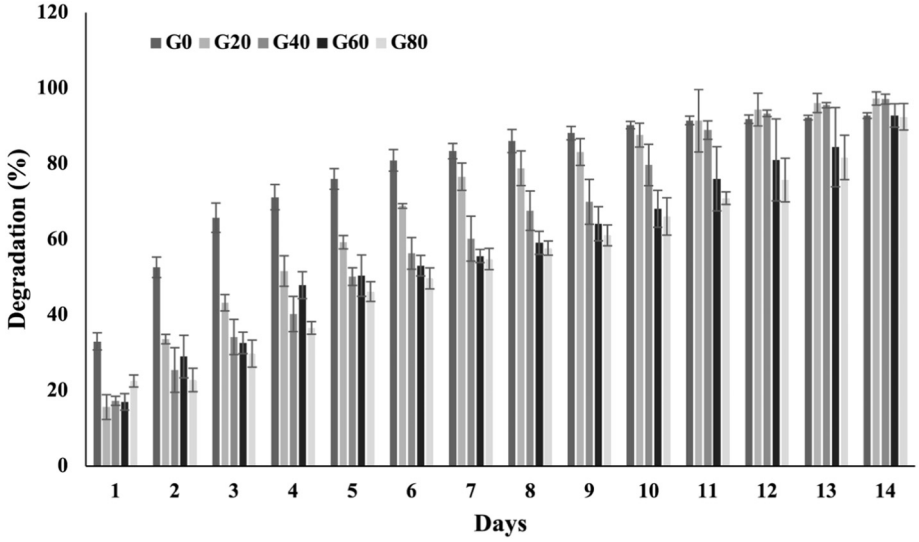

Fig. 5. Day-to-day mass losses of the samples in the phosphate buffer (n = 3).

medium with no fiber in it and then a bacterial culture medium with gelatin nanofibers added in it were used. The nanofibers were taken up in 24-hour pre-grown bacterial cultures. Bacterial growths were measured as optical density at 600 nm using the UV-VIS spectrophotometer and the results were given for both bacteria in Fig. 7.

Culture media with only bacteria and G0/bacteria added were used as a control group. It was determined that bacterial cultures and G0 added bacterial cultures used as control group had almost the same optical density. Antibacterial test results were showed that all PMETAC containing nanofibers exhibit >90% inhibition for EC, SA and AB bacteria. However, the inhibition results for MRSA remained around 75%. These results exhibited that nanofibers showed very high inhibition against bacteria even when the lowest rate of PMETAC is used. These results show us that gelatin nanofibers prepared by mixing METAC polymer can be used effectively as an antimicrobial wound dressing material. Similar results have been obtained in other studies on nanofibers as wound dressings using various antibacterial agents.

Similar results have been obtained with nanofiber studies in literature used as dressings containing various antibacterial agents. Sarhan et al. [45] have created honey/poly (vinyl alcohol)/chitosan nanofibers

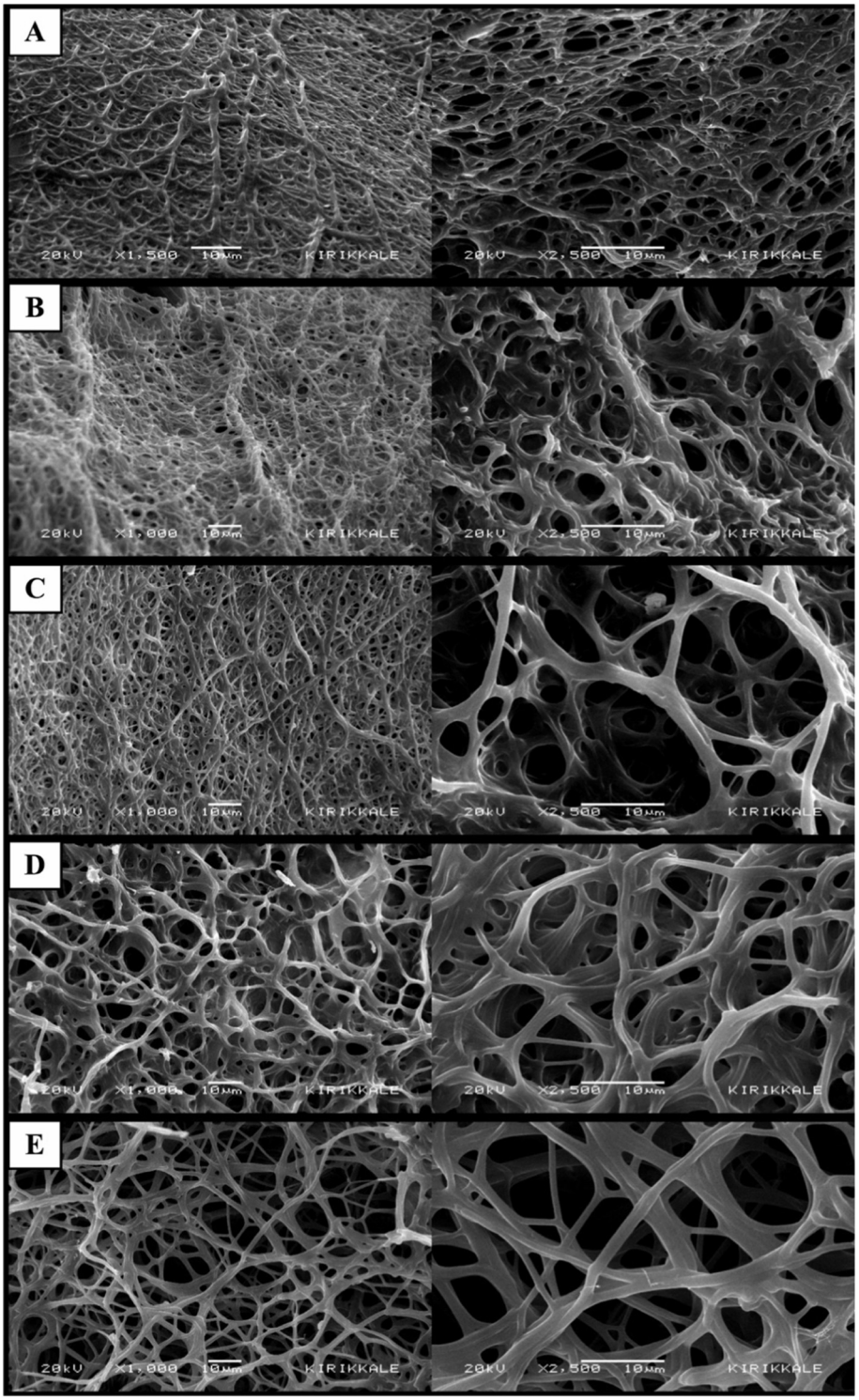

Fig. 6. SEM images of the nanofiber samples taken after 7 days in phosphate buffer A: G0, B: G20, C: G40, D: G60; E: G80.

containing aqueous extracts of Cleome droserifolia and *Allium sativum*. They investigated the antibacterial effects of these nanofibers against SA, EC, MRSA and multidrug resistant *Pseudomonas aeruginosa* bacteria. They determined that the nanofibers obtained had an antibacterial effect against SA and MRSA, but not against EC and multidrug resistant *Pseudomonas aeruginosa*.

Ramalingam et al. [46] examined the antibacterial effect of polycaprolactone/gelatin fibers containing *Gymnema sylvestre* extract against gram positive SA and *Staphylococcus epidermidis*, and gram negative *Pseudomonas aeruginosa* and EC bacteria. In liquid culture studies, the researchers determined that 40% inhibition for gram positive bacteria and 30% inhibition for gram negative bacteria.

Li et al. [47] have synthesized polycaprolactone/gelatin fibers containing short-chain polycaprolactone modified with antibacterial eugenol and a peptide called REDV for vascular applications. They have found that fibers containing 30% eugenol inhibited 71% of EC bacteria and 78% of SA.

Shi et al. [48] have synthesized polycaprolactone/gelatin hybrid fibers containing trimethoxysilylpropyl octadecyldimethyl ammonium chloride (QAS), a cationic antimicrobial agent in various ratios (5–20%). They investigated the antibacterial effect of these fibers against SA and *Pseudomonas aeruginosa* bacteria. They found that fibers containing 15% and 20% QAS inhibited approximately 100% of SA and *Pseudomonas aeruginosa* bacteria in respectively 6 h and 12 h.

Kwak et al. [49] have synthesized gelatin fibers containing silver nanoparticles for biomedical applications with the wet spin method. The researchers synthesized Silver nanoparticles in gelatin solution under UV light. The fibers were then formed by wet spinning and crosslinked with fructose. Fibers synthesized with 10 ppm of silver inhibited EC bacteria by 42% and SA by 50%. Fibers prepared with 50 and 100 ppm silver inhibited 100% of these bacteria.

Kwak et al. [50] synthesized gelatin nanofibers containing 0.5 and 1.0% *Phaeodactylum tricornutum* extract. They examined the antibacterial effect of these nanofibers against EC and MRSA bacteria. They determined that the fibers synthesized with both extracts inhibited approximatively 100% of the bacteria.

Nanofiber studies that have been conducted using many different antibacterial agents are given above. Once the results examined, it was determined that all the substances used provided 100% inhibition in a similar way. However, as already mentioned, although all materials have the same bacterial effect, their duration of use is very limited and can lead to toxic effects and bacterial resistance in humans because these low molecular weight materials are slowly released into the environment. The PMETAC, which we use in our study, is supposed to eliminate the unwanted side effects of these materials.

The MIC90 results were given for bacteria in Table 3. Before the MIC 90 values were calculated, the antibacterial effect assays were carried out to contain 15 mg/mL nanofiber. However, when MIC90 values were calculated, they were found to be antibacterial at much lower concentrations. Especially, it was determined to be lower values for SA bacteria. It has been found that even at very low concentrations, nanofiber provides the desired antibacterial effect in both bacteria. The extract concentration used for cytotoxicity of our nanofibers was taken as 0.2 g/mL according to ISO standards. Therefore, the cytotoxicity of nanofibers was found to be high. When cytotoxicity results and MIC 90 values were compared with each other, the desired antibacterial effect can be easily achieved with a small amount of the concentration used for cytotoxicity. Considering the MIC90 values, the amount of PMETAC used in the nanofibers is much less usable and the cytotoxic effect is deliberated to be much lower under these conditions. Besides, in order to support this idea, cytotoxicity studies using 1 mg/mL extract were found to have no toxic effects, especially G20 and G40 fibers.

3.4. The cell viability, adhesion and proliferation tests results

The toxic effect of the obtained gelatin and gelatin nanofibers containing PMETAC at different ratios against fibroblast cells was determined by MTT test. The obtained results are given in Fig. 8.A. When the viability values of obtained from the MTT test were examined, it was found that G20, G40, G60, and G80 nanofibers were found toxic due to their cell viability being below 60% at 1:1, 1:2, 1:4, 1:8 concentrations, but they were determined nontoxic at 1:16 and 1: 32 concentrations in L929 fibroblast cells. However, it was calculated that only 1: 1 concentration showed a toxic effect in L929 fibroblast cells where G0 was applied.

Sarhan et al. [45] produced honey/poly(vinyl alcohol)/chitosan nanofibers enriched with *Allium sativum* and Cleome droserifolia extracts for use as wound dressings. The cytotoxicity of obtained wound dressings was compared with Aquacel®Ag that is a currently available commercial wound-dressing on the market in this study. In addition, fibroblast cells were also cultured at different concentrations of nanofiber extract solutions (100%, 75, %, 50%, and 25%) and cytotoxicity was determined by cell density. In the cytotoxicity study of the nanofibers with 100% extract, the cell viability was found to be 68%. However, Aquacel®-Ag was found not to exceed 20% even at the lowest concentration of cell viability.

Cytotoxicity and antibacterial activity studies were carried out by Yunoki et al. on 5 different silver containing commercial wound dressings [51]. Cytotoxicity tests were performed according to ISO 10993-5 standards. In this study, the cell viability of Mepilex®Ag, Algisite™Ag, PolyMem®Ag, and Aquacel®Ag were found to be below 50%.

Amirabad et al. [52] coated banknotes with cellulose nanocrystals and chitosan nanofibers. They investigated the antifungal activity of the coated papers. They found that cytotoxicity improved with the

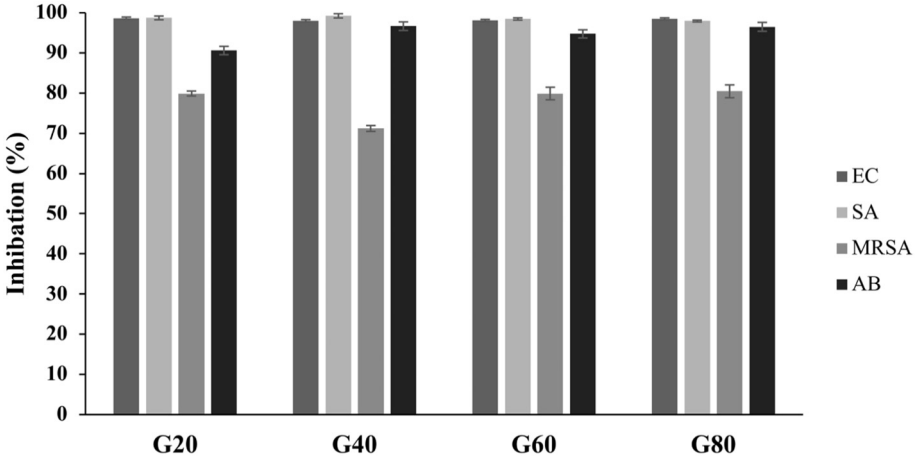

Fig. 7. Change of antimicrobial inhibition of nanofibers for EC, SA, MRSA, and AB bacteria (n = 3).

Table 3
MIC 90 values (μg/mL) of nanofibers for EC and SA bacteria.

Sample code	EC	SA
G20	922.57 ± 32.95	376.76 ± 10.56
G40	687.81 ± 28.83	274.65 ± 14.08
G60	340.96 ± 27.95	158.45 ± 10.56
G80	193.57 ± 20.59	109.15 ± 10.76

increase in the amount of chitosan nanofiber used in the coating. Cell viability was found below 65% with papers coated at 6 and 8 mg/mL chitosan nanofibers. It was determined that although both coating materials that were used in this study were natural materials, they could show a cytotoxic effect.

According to the results of all these studies, it was found that even commercially available products or natural materials on the market now may cause high cytotoxic effects. When the results were compared with our study, the cytotoxic effect of nanofibers at lower concentrations (1:16 and 1:32) was found to be much better than that of, especially commercial products. It is thought that the cytotoxic effect of nanofibers produced by us is not caused by PMETAC that was added to the fibers as an antibacterial. Because quaternary ammonium compounds have low toxicity, low skin irritation, and excellent cell penetration properties compared to other antibacterial agents. The reason for the cytotoxic effect is believed to be due to glutaraldehyde used as a crosslinker. In a study related to this, it was found that cellulose-collagen nanofibers had no cytotoxic effect in the non-crosslinked form, but showed high cytotoxicity after crosslinking with glutaraldehyde [53]. Increasing the washing time and number of nanofibers with glycine solution after crosslinking is thought to reduce cytotoxicity.

The cell adhesion and proliferation of nanofibers were studied and the obtained results are given in Fig. 8.B. According to the images

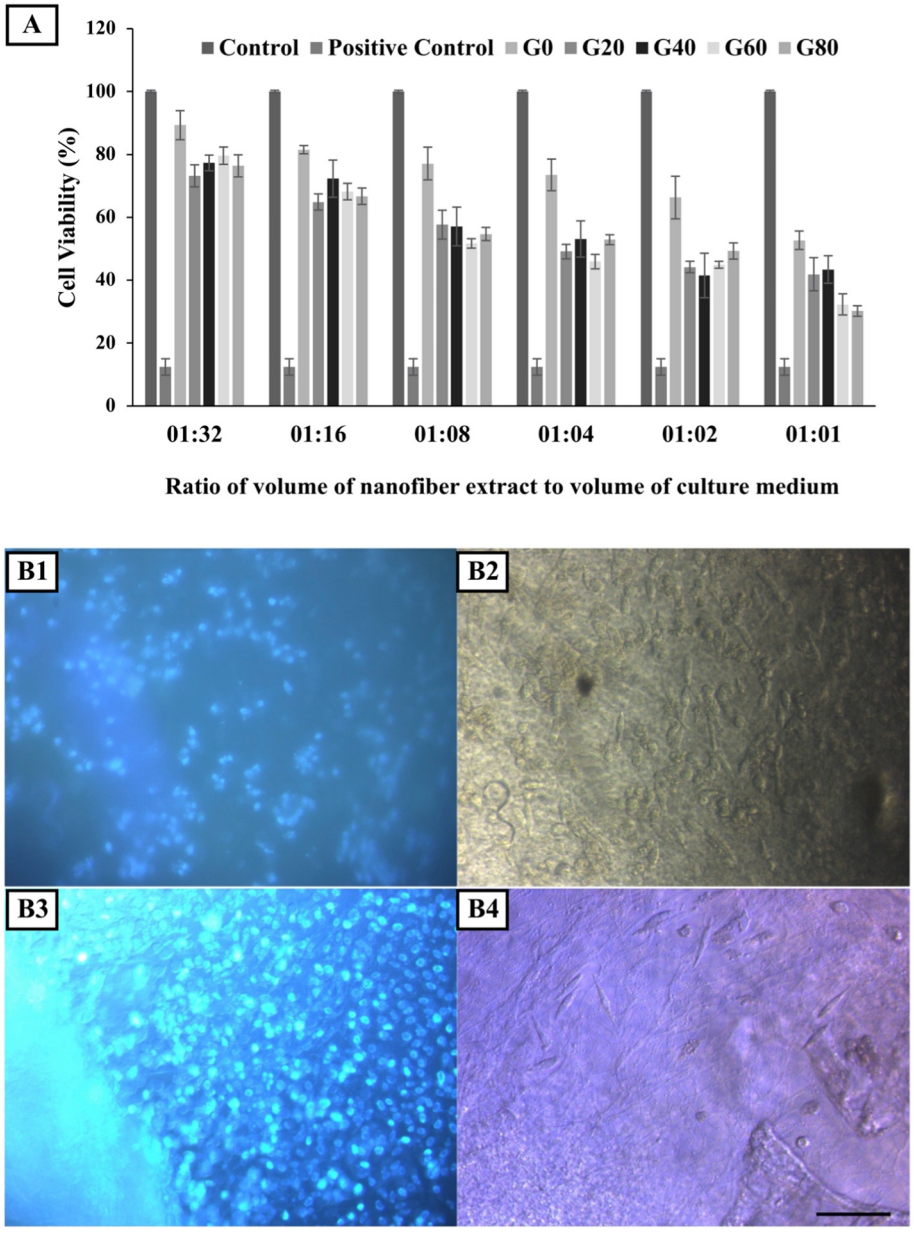

Fig. 8. A: The cell viability results obtained from MTT studies B: Fluorescence microscope and light microscopy images showing the adherence of cells on G20 and G40 samples. 1) Showing of L929 fibroblast cells applied on G20 by DAPI fluorescent staining. 2) Display of L929 fibroblast cells on G20 sample under light microscope. 3) Showing of L929 fibroblast cells applied on G40 by DAPI fluorescent staining. 4) Display of L929 fibroblast cells on G40 sample under light microscope. Photographs were taken on a Leica DMI 6000B inverted microscope. The scale bar shows 100 μm (n = 3).

taken from the inverted microscopy, it is clear that the cells grow significantly by acquiring fibroblast shapes on the surface of the G20 and G40 nanofibers interacting with the cells for 72 h. In addition, the fluorescence microscopy images of the stained nanofibers could be said that the cells proliferate on the nanofibers and the number of G40 nanofibers especially is quite high. As a result, PMETAC-containing gelatin nanofibers were found to be biocompatible. Similar results have been shown in several studies [54–56]. Atyabi et al. [54] investigated the adhesion and viability of poly (ε-caprolactone) nanofibers modified with cold plasma on L929 fibroblast cells. Compared with unmodified nanofibers, modified nanofibers have been shown to significantly increase cell adhesion and proliferation.

4. Conclusions

Gelatin and gelatin-PMETAC nanofibers were successfully produced by the electrospinning method. The SEM images show that the nanofibers have homogeneous size distribution and have smooth and beadless structures. It was found that the parameters such as the applied voltage, the distance between the needle and the collector and the flow rate of the polymer in the electrospinning system did not really change the fiber diameters, but the percentage of PMETAC in the nanofiber greatly influenced the diameters of the nanofibers. According to the results of FTIR, TGA and Elemental Analysis, it has been observed that the gelatin and the PMETAC successfully enter the nanofiber structure and can be effectively used together. Again, according to the TGA results, it was found that the thermal stability of the nanofibers increased significantly after crosslinking with glutaraldehyde. According to the degradation tests, it was determined that all nanofibers could be biodegradable. These results also show that the addition of PMETAC to the structure increases the resistance of nanofibers. The nanofibers showed very good antibacterial properties against SA, MRSA, EC, and AB bacteria by introduction of PMETAC in the gelatin structures. When cytotoxicity results were examined, especially low doses of the nanofibers were biocompatible. In cell adhesion experiments, it is determined that the cells attach to the surface of nanofibers and they proliferate significantly. The results have shown that the nanofiber recommended as antimicrobial wound dressing material has a great using potential especially in health field because of its superior properties such as its easy to apply, its low cost and its production in a short time, its biodegradability, its very good antibacterial properties and its very low cytotoxic effect.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2019.06.119.

Declaration of Competing Interest

There are no conflicts to declare.

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