Development and characterization of the electrospun melittin-loaded chitosan nanofibers for treatment of acne vulgaris in animal model

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Abstract
Acne is an inflammatory disease of the sebaceous glands. Melittin (Mel) is one of the principal toxic components of bee venom that can cause antibacterial and anti-inflammatory effects. Chitosan is a biodegradable polysaccharide has anti-inflammatory, antimicrobial, and regenerative properties. In this study, chitosan (Ch)/Mel 0.001 and 0.003% nanofibers were fabricated for topical treatment of acne vulgaris.

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Physicochemical properties of Ch/Mel were evaluated using FTIR and FESEM. Furthermore, encapsulation efficiency, tensile strength, water absorption capacity, enzymatic activity, drug release, antimicrobial, and cell cytotoxicity were assayed. Animal models were exerted to study the impact of Ch/Mel nanofibers on the treatment of Propionibacterium acnes. The FESEM results showed that nanofibers were successfully prepared in nano-scale fiber diameter. The FTIR confirmed the presence of Mel in structures. The addition of melittin to the polymer solution reduced electrical conductivity, induced viscosity and tensile strength of the fibers, and decreased the percentage of the swelling. The Mel loading into Ch/Mel 0.003% was reported at 86.74±1%. This Mel releasing process (89.65%) of Ch/Mel 0.003% was slowly completed during 72 h. The Mel retained its hemolytic activity after being loaded into the polymer structure. The drug toxicity study displayed the lack of any significant toxicity from Ch/Mel nanofibers on normal Human Dermal Fibroblasts (HDF). The Ch/Mel 0.003% achieved the highest growth inhibition in P. acnes in vitro and animal studies, this group showed the greatest reduction in inflammation and redness. The Ch/Mel 0.003% structure is proposed as a suitable topical drug delivery system for treating acne vulgaris.

Keywords
Acne vulgaris, melittin, chitosan, nanofibers, electrospinning, P. acnes

Introduction
Acne is an inflammatory disease of the sebaceous glands and a common skin condition that causes inflammation on the skin, neck, chest, and back. The signs of this disorder are majorly observed in young people due to various factors such as hormonal imbalance, bacterial infection, stress, food, and cosmetics consumption.1 Propionibacterium acnes (P. acnes), an anaerobic Gram-positive bacterium, was discovered to be the main reason of acne.2 Acne vulgaris is the chronic inflammatory for sebaceous glands, is induced by the high growth of P. acnes3 and is recognized as the most common skin disease.

The current treatments for acne vulgaris include using compounds with anti-inflammatory or bactericide properties, including benzoyl peroxide, azelaic acid, retinoids, tetracycline, erythromycin, macrolides, and clindamycin.4 However, these therapeutic agents cause numerous and even dangerous side effects. Therefore, recent researchers attempted to focus on introducing novel therapeutic methods for acne that would be more efficient with fewer side effects.5,6

Treating of acne requires compounds with both anti-inflammatory and antimicrobial qualities since the primary goal of these medicaments is to eliminate inflammation, prevent the formation of subsequent comedones, and inhibit disease progression.7,8 Purified bee venom (PBV) stands as a novel suggestion and promising candidate for the treating acne vulgaris.9,10 Relative studies indicated the ability of PBV to significantly inhibit P. acnes in a concentration-dependent manner.10
Melittin is the principal toxic component of bee venom and is recognized as a cationic hemolytic peptide with antibacterial, antiviral, and anti-inflammatory properties. Discoveries claimed the ability of melittin to inhibit cell cycle and cell growth, as well as induce apoptosis in various tumor cells. According to the work of Kim et al., *P. acnes* could increase the number of apoptotic cells, the secretion of pro-inflammatory cytokines, and the breakdown of caspases three and eight and poly (ADP-ribose) polymerase (PARP) in THP-1 (human monocyte cells) and HaCaT (human keratinocytes). However, the application of Melittin can decrease the effects of *P. acnes* through the regulation of NF-κB and MAPK pathways. Based on these findings, researchers suggest melittin’s potential for the prevention and treatment of inflammatory skin diseases.

Chitosan is a natural, hydrophilic, biocompatible, and biodegradable polymer. Chitosan has various applications in the clinic due to its different biological properties, including wound healing, tissue engineering, and drug delivery in the form of hydrogels, films, nanofibers, microparticles, and nanoparticles. Chitosan is a polymer that does not show any toxic effects and does not cause damage or inflammation. On the other hand, due to its bioadhesion properties, increasing permeability, and having suitable physicochemical properties, it is used as a carrier for skin delivery.

Electrospinning is one of the most widely used methods in the production of pharmaceutical systems. The advantage of the electrospinning method compared to other methods is ease and speed of work, high encapsulation efficiency, simultaneous transfer of various therapeutic compounds, and cost-effectiveness. The better features of this technique make it attractive for use in medications for wound healing. There are some limiting factors for the electrospinnability of neat chitosan, like chain stiffness and positive charges on the chitosan molecule. It was shown in various studies that the blending of chitosan and polyethylene oxide (PEO) solutions makes their blends electrospinnable by strong hydrogen bonding between chitosan and PEO chains.

In this study, we synthesized a novel anti-acne formulation based on chitosan-nanofiber containing melittin using the electrospinning method. The loading of melittin in nanofibers resulted in increased their antimicrobial effects. Furthermore, we investigated the characteristics of this drug delivery system, which included the diameter and morphology of nanofibers, porosity, hydrophilicity, rate of loading, drug release profile, antimicrobial effects on *P. acnes*, and cytotoxicity on HDF cells. In addition, animal models were exerted to study the impacts of this product on inflammation and acne manifestations.

**Materials and methods**

**Materials**

Low molecular weight Ch powder (50,000–190,000 Da), Polyethylene Oxide (PEO average Mv 200,000), Melittin (C₁₃₁H₂₂₉N₃₉O₃₁), and Triton X100 were purchased from Sigma-Aldrich (Germany). Acetic acid glacial, methanol, sodium hydroxide (NaOH), sodium phosphate monobasic (NaH₂PO₄), potassium dihydrogen phosphate (KH₂PO₄), Sodium chloride (NaCl), Potassium chloride (KCl), and formaldehyde were procured.
from Merck (Germany). Dulbecco’s modified Eagle’s medium (DMEM) and Fetal bovine serum (FBS) were purchased from Gibco (USA). Xylazine/Ketamine, Clindamycin Topical Gel, and Alamar blue were obtained from Trittau (Germany), Kiahs-Mediparm (Iran), and Bio-Rad (Poland), respectively. *P. acnes* was purchased from the Institute of Pasture (Iran), while normal Human Dermal Fibroblasts (HDFs) were isolated from the skin samples of our previous study.\(^2\) The animals (mice) were achieved from the Institute of Mashhad Medical Sciences (Iran). All animal experiments were performed under the approval (IR.MUMS.PHARMACY.REC.1397.028) of the Institutional Research Ethics Committee of Mashhad University of Medical Sciences (Mashhad, Iran).

**Materials and methods**

**Polymer solution preparation for nanofiber synthesize**

The polymer solution was prepared by adding 0.001 and 0.003% w/v of melittin (Figure 1) to the mixture of chitosan (2.5% w/v) and polyethylene oxide (2.5% w/v). Briefly, a stock solution of melittin (1% w/v) was prepared by dissolving 1 mg of melittin powder in 100 μL of PBS buffer.\(^1\)\(^,\)\(^2\)\(^,\)\(^5\) Then, five or 15 μL of this melittin (1% w/v) solution was added to 5 mL of acetic acid (80%) solution containing 125 mg chitosan and 125 mg...
polyethylene oxide and stirred overnight, which resulted in the preparation of 0.001 and 0.003% w/v of Ch/Mel, respectively.

**Measurement of viscosity and conductivity of polymer solution**

The viscosity of chitosan and Ch/Mel 0.001% and 0.003% solutions were measured applying a rotational rheometer (Brookfield Co, Middleboro, MA) at room temperature using a 25 mL model of the spindle at the shear rate of 0–200 s\(^{-1}\). The electrolytic conductivity of every polymer solution was measured through an RS232 conductivity meter (Goldpoint Co Ltd, Taiwan) at room temperature.\(^6,\ 21\)

**Preparation of nanofibers structures by the electrospinning method**

A two-axis electrospinning device ((Fanavaran Nano-Meghyas, FNM Co, Tehran, Iran)) and a 5 mL syringe with a stainless steel needle of 0.9 mm in inner diameter (G 18) were exerted to prepare the nanofibers structures. The optimum conditions of the electrospinning method were settled as the following: 200 mm of the distance between needle and collector, a voltage of 9 kV, a flow rate of 0.5 mL/h, a drum diameter of 8 cm, a drum length of 20 cm, a drum rotation speed of 200–240 rpm, the temperature of 28°C, and 30% of relative humidity. Electrospun nanofiber sheets (20 × 25 cm with a thickness of about 1 mm) were collected on a piece of aluminum foil.\(^6,\ 21,\ 22\) Melittin and chitosan were dissolved in sterile solvents. An attempt was made to perform electrospinning under aseptic conditions. Nanofibers were sterilized under UV light.

**Characterization of Electrospun Nanofibers**

**Scanning Electron Microscope Imaging (FE-SEM).** Electrospun nanofibers were coated with gold and platinum, and their diameter distribution, and surface morphology were evaluated by imaging with field emission scanning electron microscopy (FESEM, MIRA3, Tescan Co, Czech Republic). The images were analyzed through the usage of Image-J software (1.46), and the diameter distribution of nanofibers was assessed by measuring at least 50 fiber diameters for each structure.\(^6,\ 21,\ 22\)

Infrared Fourier Transfer Analysis (FTIR) of nanofibers structures

Infrared spectroscopy was performed to identify the functional groups and determine the type of existing bonds between chitosan, melittin, and polyethylene oxide throughout the electrospun Ch/PEO/Mel nanofibers. All of the experiments were performed by Thermo Nicolet spectrometer AVATAR 370 FTIR (Waltham, MA) within the wavelength range of 400–4000 cm\(^{-1}\).\(^6,\ 21,\ 22\)

**Mechanical properties**

H50KS universal testing machine (Hounsfield, UK) was utilized to examine the mechanical properties of pure and drug-loaded electrospun nanofibers. For this purpose, nanofibers structures were cut into strips (1 cm×4 cm) and placed into the grips of the
device. Then, the strips were stretched up to the point of breakage with a strain rate of 5 mm/min to report the young’s modulus afterward.6,21,22

In Vitro Studies

Calculation of melittin encapsulation efficiency in nanofibers. The encapsulation efficiency of melittin in Ch/Mel 0.001 and 0.003% w/v nanofibers was measured by dissolving a certain amount of each nanofiber structure in acetic acid at room temperature. The amount of melittin in these polymer solutions was determined by applying a BCA kit (Pars Toos). In addition, a standard calibration curve was used for the case of melittin, while the encapsulation efficiency was calculated by applying the following formula:

\[
\text{Melittin encapsulation efficiency } \% = \left( \frac{\text{Total amount of encapsulated melittin in nanofibers}}{\text{Theoretical amount of melittin}} \right) \times 100.\]

Drug release assay

To evaluate the profile of melittin release from Ch/Mel 0.001 and 0.003% w/v nanofiber structures, the nanofibers pieces weighed approximately 2 mg were floated in 10 mL of phosphate buffer saline (0.1 M, pH 7.5) and incubated in a shaker incubator at 37°C for 1, 2, 3, 4, 24, 48, and 72 h. At each time interval, one ml of the sample was transferred to the test tube to be replaced with one ml of fresh phosphate buffer solution. Then, the amount of released melittin was measured using a BCA kit (Pars Toos). Finally, the concentration of melittin at each time point was calculated through the standard calibration curve and expressed as the percent of cumulative drug released.6,27,28

Study of the swelling ratio of nanofibers

The swelling ratio of nanofiber structures was evaluated by their water uptake capacity. In brief, pre-weighed dry nanofiber structures were immersed in a PBS buffer at 37° C and incubated within a wet incubator for 1 and 24 h. The water uptake capacity (WC) was calculated by applying the following equation: WC = ((Weight of swollen material - Initial weight of dry sample)/Initial weight of dry sample).29

Toxicity test using the Alamar Blue

The exertion of the Alamar Blue assay was considered to evaluate the cytotoxicity of chitosan and Ch/Mel 0.001 and 0.003% w/v nanofiber structures against HDF cells.6,21,28 The structures were punched and placed in a 96-well plate. Then, HDF cells with a concentration of 5 × 10³ cells/100 µL of DMEM culture medium containing 10% of FBS were poured into each well and incubated at 37°C and 5% CO₂. The culture medium containing cells without any nanofiber structure or melittin was considered the control. After 72 h, 10 microliters of Alamar Blue (10% v/v) were added to each well, and the plates were incubated for 3 h at 37°C and 5% CO₂. The amount of Alamar blue absorption

was read at 605 nm by an ELISA reader (BioTek, Bad Friedrichshall, Germany). Lastly, the percentage of cell growth was calculated through the following formula:

\[
\% \text{The growth rate compared to the control sample} = \frac{\text{(Test sample absorbance-Blank absorbance)}}{\text{(Control sample absorbance (cell alone) - Blank absorbance)}} \times 100
\]

Blank absorption: The amount of light absorbed by Alamar Blue

**Evaluation of the hemolytic activity of melittin**

To confirm the maintenance of melittin activity after being loaded in Ch/Mel 0.001 and 0.003% w/v nanofiber structures, the hemolytic activity of melittin was assayed on fresh human red blood cells (hRBCs) by the exertion of a standard procedure. In brief, 3 mL of the blood of a healthy volunteer was collected in the presence of an anticoagulant (3.2% sodium citrate) and washed three times by PBS. Then, the suspension of red blood cells in PBS (6% v/v) was mixed with the samples and incubated at 37°C for 35 min. The samples were consisted of free melittin 0.001 and 0.003% w/v in PBS, Ch/Mel 0.001 and 0.003% w/v nanofiber structures, and the drug release medium of Ch/Mel 0.001% and 0.003% w/v. The samples were then centrifuged at 2000 rpm for 10 min. The amount of released hemoglobin in the supernatant was evaluated by measuring the absorbance of supernatants at 540 nm by an ELISA reader (BioTek, Bad Friedrichshall, Germany). Meanwhile, we exerted hRBCs and Triton X100 with a final concentration of 0.2% as the negative and positive controls, respectively. The percentage of hemolysis was calculated by following the equation below:

\[
\% \text{Percentage of hemolysis} = \frac{\text{(Test sample absorbance - Blank absorbance)}}{\text{(Triton uptake absorbance - Blank absorbance) \times 100}}
\]

**Evaluation of the antimicrobial effects**

*P. acnes* was cultured in Brain Heart Infusion (BHI) solid culture medium at 37°C under anaerobic conditions for 2 days and collected in the mid-log phase. Bacteria were washed three times with 10 mM of PBS, and then the number of bacteria was counted. The tested samples, which included free melittin 0.001 and 0.003% w/v in PBS, Ch/Mel 0.001 and 0.003% w/v nanofiber structures, drug-released medium of Ch/Mel 0.001 and 0.003% w/v nanofibers, and the positive control (Isotretinoin 0.1% w/w), was dissolved in 1 mL of BHI medium to be incubated with *P. acnes* (1 × 10^5 CFU) in a final volume of 100 μL in each well. Once the plates were incubated at 37°C for 48 h, 10 microliters of Alamar Blue (10% v/v) were added to each well, and the plates were incubated for 3 h at 37°C. Subsequently, the amount of Alamar blue absorption was read at 605 nm by an ELISA reader, and the inhibition percentage of *P. acnes* growth was calculated as the following:

\[
\% \text{Inhibition of } P. \text{ acnes growth compared to the positive control} = \frac{\text{(Test sample absorbance - Blank absorbance)}}{\text{(Positive control absorbance (P. acnes alone) - Blank absorbance) \times 100}}
\]
Blank absorption: The amount of light absorbed by Alamar Blue

The effect of melittin on inflammation caused by *P. acnes* growth in a mouse model

Eight-week-old BALB/c mice with a weighing range of 20–25 g were procured and randomly divided into five groups (four mice per group), including positive control (treated with 1% clindamycin gel), Ch/Mel 0.001 and 0.003% w/v nanofiber structures, and free melittin 0.001 and 0.003% w/v in vaseline. The experimental mice were anesthetized by exerting 50 μL of xylazine 2% and ketamine 50 mg/mL mixture in a ratio of 1:1. Then, 20 μL of *P. acnes* suspension that contained 10⁷ CFU was subcutaneously injected into each ear of every mouse. After 4 h, the entire right ear surface of mice was covered with free melittin 0.001% and 0.003% w/v in vaseline (groups A and B), Ch/Mel 0.001% and 0.003% w/v nanofiber structures (groups C and D), and 1% clindamycin gel as a positive control (group E). Nanofibers were fixed on the ear using dressing adhesive. Meanwhile, the left ears of all groups remained untreated as the negative control. Subsequent to 24 h, the thickness of the ear at three different points in each ear was measured by a caliper. The mice have sacrificed afterward, and the ears were placed in a formalin solution 10% v/v for preventing tissue damage. The formalin solution was removed after 24 h, and the ear sections were moved into paraffin to be stained with hematoxylin/eosin for histological examination.¹,³²,³³

Statistical calculations

All tests were repeated at least three times, and the results were stated in mean± SD (standard deviation). The data were analyzed by the utilization of statistical software GraphPad Prism 9.0.0 was used to analyzing the data. ANOVA followed by Tuckey’s Post Hoc test to compare the outcomes of different groups. In this study, *p*-value < 0.05 was considered significant.

Results

Characterizations of polymer solution

The results of viscosity measurements for three groups, including chitosan solution, Ch/Mel 0.001%, and 0.003% w/v (Table 1), indicated that the lowest value was obtained in drug-free chitosan solution. Furthermore, the addition of melittin increased the viscosity while directly dependent on the loaded concentration of melittin. The viscosity of Ch/Mel 0.003% with chitosan solution and Ch/Mel 0.001% melittin was statistically significant (*p* < 0.0001). According to the electrical conductivity results, the highest electrical conductivity was observed in the case of chitosan solution, which was followed by Ch/Mel 0.001% w/v in the second case. Lastly, the lowest value was reported from Ch/Mel 0.003% (Table 1). A significant difference was found between (*p* < 0.0001) chitosan and Ch/Mel 0.003%, as well as (*p* < 0.0001) between the two mixtures that contained melittin.
Table 1. The average viscosity and electrical conductivity of polymer solutions and the average fiber diameter of structures.

| Sample            | Viscosity (shear rate 100 S⁻¹) (mPa.s) | Conductivity (µS/cm) | Fiber diameter (nm) |
|-------------------|--------------------------------------|----------------------|--------------------|
| Chitosan          | 270 ± 70                              | 838 ± 19.3           | 355 ± 164.5        |
| Ch/Mel 0.001% w/v | 284 ± 64                              | 758 ± 21.5           | 560 ± 149.2***     |
| Ch/Mel 0.003% w/v | 409 ± 11***                           | 572 ± 26.2***        | 620 ± 156.08***    |

*p-value was defined as ***p < 0.001 compared to chitosan as the control sample.

Morphological studies of electrospun nanofibers using FE-SEM images

Figure 1 exhibits the scanning electron microscopy (FE-SEM) images of electrospun samples of chitosan and Ch/Mel 0.001% and 0.003% w/v nanofibers. Drug-free chitosan nanofibers resulted in a more uniform structure (Figure 2(a)) when compared to that of electrospun nanofibers Ch/Mel 0.001 and 0.003% w/v (Figures 2(b) and (c)). Moreover, a higher rate of non-uniformity was observed in the case of Ch/Mel 0.001% than Ch/Mel 0.003%. The diameter of nanofibers in all of the three groups was normally distributed (Figure 2(d) and Table 1). A significant difference was observed between the average diameter of chitosan nanofibers and Ch/Mel 0.001% and 0.003% w/v nanofibers (p < 0.001).

Fourier transform infrared (FTIR) spectroscopy

Figure 3 displays the FTIR spectra of chitosan, Ch/Mel 0.001%, and Ch/Mel 0.003% in 400–4000 cm⁻¹. Regarding pure Ch nanofibers, the characteristic broadband of hydroxyl group (O-H) tensile vibrations were observed at 3358 cm⁻¹ while overlapping with the vibrations of NH₂ bands. The symmetric vibrations peaks were recorded at 2883 cm⁻¹ (CH₂ in CH₂-OH), 1526 cm⁻¹ (for carbonyl groups (C = O) attached to secondary amine (NH₂)) for the stretching band, and 1643 cm⁻¹ (amine I stretching band at C=O-NH) for Ch nanofibers. The N–H aromatic peak (designated the amide II (N–H) bending vibration and the absorption of amide III) were observed at 1539 cm⁻¹ and 1324 cm⁻¹, respectively.

The FTIR spectra of aromatic group (C = C groups), aliphatic group of C–H stretching, and C = O (carbonyl ketone stretching) were also observed throughout 1508 cm⁻¹ and 2833 cm⁻¹, respectively. The spectrum of Ch nanofibers reflected the bending of C-H₂ by displaying peaks at 868 cm⁻¹, whereas the detected peaks at the points of 1148 cm⁻¹ and 1110 cm⁻¹ traced back to the stretching of carbonyl (C-O-C) bands.

Every related peak to the case of Ch nanofibers was observed throughout the structure of Ch/Mel. However, in the case of Ch/Mel nanofibers, the observed peaks of hydroxyl groups OH and amide O = C-NH₂ were less intense and seemed to be shifted towards lower regions. The observed peak at 3250–3450 cm⁻¹ indicates the free vibrations of N–H stretching of Mel in Ch/Mel. The FTIR spectrum of Mel confirmed the characteristic amide bands, i.e. amide I (1651 cm⁻¹), amide II (1532 cm⁻¹), and the bands at 1112 cm⁻¹.
Figure 2. FE-SEM images of electrospun nanofibers. (a): Chitosan nanofibers, (b): Ch/Mel 0.001% w/v nanofibers, and (d): Ch/Mel 0.003% w/v nanofibers, (d): The average diameter of 50 nanofibers is reported as mean ± SD (**** means p < 0.0001).
and 1040 cm$^{-1}$ that indicate unsystematic coil conformation. The identified peak at 1058 cm$^{-1}$ in Ch/Mel nanofibers can be attributed to the symmetric vibrations of O-C-O, while the vibrations of C = O-H were associated with the protein in melittin. In addition, the asymmetric vibrations of carbonyl groups (O-C-O) in the polysaccharide structure of melittin were observed at 750 cm$^{-1}$.

Also, the FTIR spectrum of Ch/Mel demonstrated that the Mel absorbance peak for N–H stretching at 3318 cm$^{-1}$ was reduced and shifted to 3421 cm$^{-1}$ (Figure 3) and the amide I indicated band in the Mel at 1651 cm$^{-1}$ was also shifted to a higher wavenumber (1654 cm$^{-1}$) in Ch/Mel.

**Evaluation of Mechanical properties of electrospun nanofibers**

The Young’s modulus of chitosan nanofibers (before and after loading of melittin 0.001% and 0.003% w/v) are presented in Figure 4. In comparison to the case of Ch nanofibers as the control sample, Young’s modulus was observed increase by 11% and 65% as a result of increasing the melittin concentration from 0.001% to 0.003% w/v in chitosan solution. According to the obtained results, the highest and the lowest tensile strength was reported from Ch/Mel 0.003% nanofibers (58.06 ± 3.7 MPa) and chitosan nanofibers (35.35 ± 1.8 MPa), respectively, while displaying a significant difference ($p < 0.001$).

**The evaluation of drug encapsulation and drug release profile**

In conformity to the evaluation outcomes of melittin encapsulation efficiency in nanofiber structures, the maximum encapsulation percentage for nanofibers containing Ch/Mel 0.001 and 0.003% w/v were 74.61± 2.5% and 86.74±1%, respectively. Indicated by the
drug release assay, the release rate of melittin from nanofibers 0.001 and 0.003% w/v was almost equal in the early hours without displaying any significant differences (Figure 5). However, over time from 4 h, the release rate of nanofibers faced a significant increase of 0.001%. Ch/Mel 0.001% reached a releasing rate of 100% after 48 h, while Ch/Mel 0.003% was released at a slower rate, and 89.65±2% of melittin was released after 72 h.

**Water absorption**

The swelling ratio of nanofiber structures was evaluated by their water uptake capacity. The results indicated proper water uptake capacity for all nanofibers, which increases with increasing exposure time to the aqueous environment. The highest water uptake occurred in chitosan nanofibers, followed by Ch/Mel 0.001% and 0.003%, respectively (Table 2). There was a lack of any significant differences between chitosan and Ch/Mel 0.001% (p < 0.05), while the water uptake of Ch/Mel 0.003% was significantly lower than that of chitosan (p < 0.001). Moreover, the comparison between Ch/Mel 0.001% and 0.003% revealed a significant difference (p < 0.01) in their water absorption.
The hemolytic activity of melittin

The obtained percentage of hemolysis is presented in (Figure 6). The lowest percentage of hemolysis was reported from Ch/Mel 0.001% in the release medium after 24 h (8.39% ± 3.44), which is significantly different from the results of free melittin 0.001% in PBS ($p < 0.01$). On the other hand, the percentage of hemolysis by melittin increased in the release medium over time while being proportional to the rate of drug release. There was no significant difference between the hemolysis percentage of Ch/Mel 0.001 and 0.003% w/v with their release medium after 72 h ($p < 0.05$). These findings were in line with the results of drug release, which indicated that almost all of the melittin was released after 72 h.

Table 2. Water uptake capacity (WC) of Ch/Mel nanofiber structures.

| Sample              | After 1 h   | After 24 h  |
|---------------------|-------------|-------------|
| Chitosan            | 7 ± 0.2     | 10.25 ± 0.15|
| Ch/Mel 0.001% w/v   | 6.94 ± 0.14 | 9.16 ± 0.21 |
| Ch/Mel 0.003% w/v   | 4.63 ± 0.1***| 7 ± 0.11*** |

$p$-value was defined as ***$p < 0.001$ in comparison with Chitosan as control sample.

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Figure 5. Melittin release profile from Ch/Mel nanofiber structures.

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$p$-value was defined as ***$p < 0.001$ in comparison with Chitosan as control sample.
Cytotoxicity assay

The cytotoxicity of chitosan nano fi bers without drug and after loading of melittin 0.001% and 0.003% w/v was investigated on normal Human Dermal Fibroblasts (HDF) (Figure 7). Next to the lack of observing any significant toxicity in the experimental groups, only the cells adjacent to free melittin 0.003% displayed a reduced growth in comparison to the control group (74 ± 16.73% after 24 h and 67.26 ± 26.71% after 72 h).

The antimicrobial effects of Ch/Mel nano fi bers

The results of Ch/Mel nano fi bers antimicrobial properties against \(P.\) acnes are provided in Figure 8. Compared to the positive control group, the highest inhibition percentage of bacterial growth occurred in Ch/Mel 0.003% after 72 h (98.02 ± 3.53), while the second stance belonged to the case of free melittin 0.003% in PBS (97.86% ± 1.84). The lowest
percentage of bacterial growth inhibition was reported from Ch/Mel 0.001% in the release medium after 24 h (53.9 ± 0.54), with a significant difference with the percentage of growth inhibition of free melittin 0.001% in PBS ($p < 0.001$). On the other hand, the percentage of bacterial growth inhibition by melittin in the release medium increased over time while being proportional to the results of drug release. There was no significant difference between the inhibition percentage of bacterial growth by Ch/Mel 0.001 and 0.003% w/v with their release medium after 72 h ($p < 0.05$).

Evaluation of the effect of melittin on inflammation caused by $P.\ acnes$ growth in a mouse model Figures 9 (a) and (b) presents the results of a qualitative and quantitative study performed on an animal model subsequent to dressing the right ear of every experimental mouse with Ch/Mel 0.001 and 0.003% w/v nanofiber structures, free melittin 0.001 and 0.003% w/v in Vaseline, and 1% clindamycin gel as a positive control during 24 h. In comparison to the negative control sample (left ear), the inflammation (the thickness of the ear) of all treated groups faced a significant reduction, except for the case of free melittin with a concentration of 0.001% w/v. There was no statistically significant difference between the effectiveness of the positive control group and the groups treated
with free melittin 0.003%, Ch/Mel 0.001%, and Ch/Mel 0.003%. However, the highest reduction in inflammation was reported in the group treated with Ch/Mel 0.003%. Meanwhile, observations were indicative of a more significant reduction in ear thickness throughout the groups treated with Ch/Mel nanofibers than that of the groups treated with free melittin (Figure 9(b)).

**Discussion**

As an inflammatory disease, acne is mainly caused by *Propionibacterium acnes* (*P. acnes*). The most commonly applied antibiotics for the treating acne vulgaris can inhibit inflammation or annihilate bacteria⁴; however they also lead to the
Figure 9. Evaluation of the effect of melittin on inflammation caused by *P. acnes* in a mouse model. A: Qualitative and B: Quantitative results of the average thickness of different parts of the ear in the animal model. The results of three replications and three measurements each time are shown as mean ± SD (so that ** means $p < 0.01$ and *** means $p < 0.001$).
inducement of numerous and even dangerous side effects. Therefore, it is valuable to
discover new therapeutic agents with higher antibacterial activity and lower significant
side effects. Melittin, a cationic amphiphilic linear peptide (NH₂-GIGAVLKVLTTGLPALISWIKRKRQQ-CONH₂), is the principal toxic component of bee
venom. According to available data, the high concentrations of this substance can
induce inflammation and pain while displaying anti-nociceptive and anti-inflammatory
properties at lower concentrations (up to ∼35 mM). Furthermore, melittin proved to
contain antibacterial features against different Gram-positive and Gram-negative bacteria
as well. However, its clinical application is limited due to its threatening properties such
as rapid degradation, high cytotoxicity, non-specific cellular lytic activity, low perme-
ability across the stratum corneum, and strong binding to the lipid bilayers of the skin.
Although several strategies were established to improve the efficiency and stability of
melittin, this study developed a chitosan nanofiber structure loaded with melittin and
had it evaluated for the treatment of acne vulgaris by exerting an animal model.

The antimicrobial and anti-inflammatory properties of chitosan were previously re-
ported by various researchers, which proposed this natural polymer as a potential agent
against acne. Chitosan can display antimicrobial properties against P. acnes. Ji-
Hoon Kim et al. reported the dramatic impact of chitosan-containing phytochemicals such
as Chitosan-Caffeic-Acid in a combined treatment for acne vulgaris with mild side ef-
effects. In addition, In vitro and in vivo studies explained the inhibitory effects of melittin
on P. acnes growth, which occurs by suppressing the expression of inflammatory cy-
tokines in keratinocytes.

In our study, the lowest amount of viscosity was observed in the case of chitosan
solution. The addition of melittin resulted in increased viscosity in a concentration-
dependent manner, and accordingly, Ch/Mel 0.003% displayed the highest rate of vis-
cosity. On the other hand, the assessment results of electrical conductivity related the
lowest electrical conductivity to Ch/Mel 0.003%, was followed by Ch/Mel 0.001% and
chitosan, respectively. These findings were consistent with some reports indicating an
inverse relationship between electrical conductivity and viscosity. Moreover, normal
fiber diameter distribution suggested the achievement of favorable electrospinning
conditions. Meanwhile, the minor diameter was obtained in the case of Chitosan
nanofibers. Melittin caused an increase in the average diameter of nanofibers, which was
consistent with the electrical conductivity results. According to previous studies, per-
forming drug loading in chitosan-PEO nanofibers results in increasing the diameter of
nanofibers while decreasing the electrical conductivity. Therefore, it can be assumed
that Ch/Mel nanofibers follow the general rule of the direct relationship between viscosity
and fiber diameter, as well as the inverse relationship of viscosity and fiber diameter with
electrical conductivity.

In conformity to the FTIR results, the successful loading of melittin in the fabricated
chitosan nanofibers structure was confirmed by the percentage of symmetric vibrations of
O-C-O and C = O-H peaks in Ch/Mel nanofibers. Related research indicated many
parameters that can influence the mechanical properties of electrospun chitosan nano-
fibers (with and without drug), such as polymer solution parameters and electrospinning
conditions. Results also indicated that the average fiber diameters of drug-loaded
nanofibers could be increased by adding melittin into the chitosan polymer solution. In agreement with the reported outcomes by Liu et al., the achievement of the highest average fiber diameter and Young modulus by Ch/Mel 0.003% nanofibers, among the other nanofibers, was confirmed by the performed examination of mechanical properties.

Moreover, the highest water uptake was observed in the case of non-melittin nanofibers, and the lowest value was reported from Ch/Mel nanofibers 0.003%. The formation of a more robust network through the addition of melittin to chitosan, as well as the occurrence of possible reactions, may cause a reduction in water uptake capacity. Chitosan contains more hydrophilic properties that can be decreased due to being combined with melittin. Also, other studies reported the observance of a decrease in the water uptake of chitosan nanofibers after the drug loading procedure.

The evaluated amounts of drug encapsulation indicated a very high rate of melittin encapsulation in chitosan nanofibers, which is a notable advantage for these formulations. Ch/Mel 0.001% and 0.003% displayed an almost similar release rate in the early hours. However, the release rate of Ch/Mel 0.001% faced a significant increase over time, in which Ch/Mel 0.001% released about 100% of melittin after 48 h, while Ch/Mel 0.001% released up to 80% of melittin in the course of 72 h. A higher concentration of melittin and a tighter network may be the reason behind these observations. This specific property of 0.003% nanofibers can be applied in the preparation of slow-release formulations.

We evaluated the enzymatic activity of melittin and realized that the loading of this product into chitosan nanofibers did not affect the activity of this enzymatic mixture. On the other hand, the percentage of hemolysis by melittin in the release medium was observed to be increased over time, with a significant difference between the hemolysis percentages of Ch/Mel nanofibers 0.001% and 0.003% after 72 h. This finding was equivalent to the results of the drug release rate and also indicated that almost the entire amount of melittin was released by the appointed time.

The cytotoxicity evaluation showed that the toxicity of Ch/Mel nanofibers was very low when compared to that of free melittin and control groups, suggesting that the encapsulation of melittin in chitosan nanofiber could reduce its toxicity. A study conducted by Zhang et al. labeled melittin as an antimicrobial peptide with very low toxic effects on human cells. On the other hand, the toxicity of drug-free chitosan nanofibers was not significantly different from the control, which indicates the lack of natural polymer cytotoxicity. These results are consistent with other studies that confirmed the biocompatibility of chitosan nanofibers.

According to our obtained results of the antimicrobial assay, Ch/Mel nanofibers 0.003% in the release medium displayed the highest inhibition percentage of bacterial growth compared to the positive control after 72 h. Meanwhile, the inhibition percentage of bacterial growth by melittin in the release medium was increased over time with the lack of any significant differences between the inhibition percentages of Ch/Mel 0.001 and 0.003% w/v with their release medium after 72 h. These findings were in line with the results of drug release, which claimed that almost all of the melittin was released after 72 h.

Consistent with the microbial testing results, the most significant reduction in inflammation and redness of lesions among the mouse model was observed in the group
treated with Ch/Mel nanofibers 0.003%. On the other hand, there was a more significant reduction in the ear thickness of groups treated with Ch/Mel nanofiber structures than those treated with the corresponding concentration of free melittin. Furthermore, other reports also indicate a significant increase in the anti-inflammatory and antimicrobial properties of chitosan as a result of being combined with other drugs.\textsuperscript{51,52} Many studies proved the anti-inflammatory properties of melittin. This substance was used to treat periodontitis and displayed satisfying anti-inflammatory effects.\textsuperscript{53} Another study reported the ability of melittin-containing PBV to significantly reduce the redness and inflammation of the skin as the main component.\textsuperscript{54,55} The anti-inflammatory effects of melittin on acute liver failure were also confirmed by observing the inhibition of inflammatory cytokines.\textsuperscript{56} Lee et al. tried to recognize the anti-inflammatory mechanism and the anti-apoptotic effect of melittin in \textit{P. acnes} infected cells. They showed that \textit{P. acnes} increased the expression of Toll-like receptors and pro-inflammatory cytokines. At the same time, melittin treatment could significantly suppress these expressions through regulation of the MAPK, and NF-\kappa B signaling pathways.\textsuperscript{1} They also showed that \textit{P. acnes} increased the cleavage of caspase-3, -8 and PARP, while melittin treatment could inhibit these effects.\textsuperscript{25}

**Conclusion**

In this study, chitosan nanofibers loaded with melittin were prepared by applying an electrospinning device. The addition of melittin to chitosan solution increased the viscosity while decreasing the electrical conductivity of the mixture. Furthermore, this combination caused an increase in the diameter of electrospun Ch/Mel compared to that of chitosan nanofibers, which is a potential reason behind the induced increase in mechanical strength and slower release of melittin from Ch/Mel nanofibers 0.003% w/v. However, the loading of melittin into chitosan nanofibers did not affect its enzymatic activity. Notably, the nanofibers containing melittin displayed much less toxicity on human dermal fibroblasts than free melittin. The highest inhibition of \textit{P. acnes} in vitro growth was reported from the case of Ch/Mel 0.003% w/v, which exhibited a higher significant reduction in inflammation and redness throughout animal studies. Considering these results, Ch/Mel 0.003% w/v nanofiber structure can stand as an applicable topical drug delivery system for treating acne vulgaris to provide adequate concentrations while avoiding the side effects of systemic drugs.

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**Appendix**

**List of Abbreviations**

- BHI: Brain Heart Infusion
- Ch: Chitosan
- DMEM: Dulbecco’s modified Eagle’s medium
- FBS: Fetal bovine serum
- FE-SEM: Scanning Electron Microscope Imaging
- FTIR: Infrared Fourier Transfer Analysis
- HaCaT: Human keratinocytes
- HDF: Human Dermal Fibroblasts
- hRBCs: Human red blood cells
- Mel: Melittin
- PEO: Polyethylene oxide
- P. acnes: Propionibacterium acnes
- PBV: Purified bee venom
- PARP: Poly(ADP-ribose) polymerase
- THP-1: Human monocyte cells