Preparation of PVA/Chitosan samples by electrospinning and film casting methods and evaluating the effect of surface morphology on their antibacterial behavior

Samira Nokhasteh¹, Amir Mahdi Molavi¹,², Mohammad Khorsand-Ghayeni¹,³ and Alireza Sadeghi-Avalshahr¹,⁵,⁶

¹ Materials Research Group, Iranian Academic Center for Education, Culture and Research (ACECR), Mashhad Branch, Iran
² Materials Engineering Department, Tarbiat Modares University, Tehran, Iran
³ Department of Biomaterials, Iran Polymer and Petrochemical Institute (IPPI), Tehran, Iran
⁴ Research Department, Researchassist Company, Mashhad, Iran
⁵ Department of Biomaterials, Amirkabir University of Technology, Tehran, Iran
⁶ Author to whom any correspondence should be addressed. E-mail: sadeghi_av@ymail.com

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Abstract
Chitosan due to outstanding properties including biocompatibility, biodegradability, nontoxicity and antibacterial activity has received considerable attention in different fields of biomedical engineering. To study the effect of morphology and topology on antibacterial activity of chitosan, two samples of PVA/Chitosan blend with the same concentration and volume ratio were prepared using electrospinning and film casting methods. To improve the electrospinability of chitosan, it was hydrolyzed by 50% V NaOH solution (95 °C for 48 h.) and PVA was used as an auxiliary polymer for electrospinning. The best electrospinning parameters for producing beadless structure were determined at a voltage of 21 kV and distance of 15 cm. Different physical and chemical characterizations of produced samples were performed using scanning electron microscopy (SEM), Fourier Transform Infrared Spectroscopy (FTIR), viscosimetry, Atomic Force Microscopy (AFM) and antibacterial assay by two different bacterium strains including Escherichia coli (E.coli) as the gram-negative and Staphylococcus aureus (S.aureus) as the gram-positive bacteria. Antibacterial assays revealed higher sensitivity of E.coli in comparison with S.aureus in the two samples. Also, it revealed that nanotopography of surface wielded influence on the antibacterial activity of samples.

1. Introduction
Chitin is the second most abundant polysaccharide after cellulose in nature [1]. It is a primary component in exoskeletons of crustaceans such as crabs, lobsters and shrimps. Alkaline deacetylation of chitin when deacetylation is > 50% yields chitosan. Chitosan is a copolymer with cationic nature that makes it distinctive among the other biopolymers for biomedical and environmental applications [2]. The inherent properties of chitosan such as biocompatibility, biodegradability, nontoxicity and antibacterial activity are the main reasons for its extensive application in biomedical fields including scaffolds of wound dressing and drug delivery [3–5]. Among these properties, growing attention has been paid to the antibacterial ability of chitosan to inhibit a broad diversity of fungi, yeasts, bacteria and viruses. Three models have been proposed for mechanism of antibacterial activity of chitosan [6, 7], the most notable of which is based on the electrostatic forces between NH₃⁺ groups in chitosan and negative charge of microbial cell membranes. According to the second mechanism, chitosan binds with microbial DNA and inhibits the mRNA and protein synthesis by invading the cell nucleus. The third mechanism suggests the chelation of metals, suppression of spore elements and binding to essential nutrients for microbial growth. However, the antibacterial effect of chitosan on gram-positive or gram-negative bacteria is a controversial issue. Some authors have claimed that it is more effective in inhibiting the activity of
gram-positive bacteria while others support its greater effect on gram-negative bacteria [6]. One of the most appealing features of chitosan is its analgesic effects, which induces remarkable pain relief, especially when was applied as a topical agent to open wounds such as burns, skin graft and skin ulcers [3]. It has been shown that alleviated inflammation results in decreased pain, which is due to the absorption of proton ions released in the site of inflammation. Moreover, it has been claimed that chitosan suppresses the expression of proinflammatory cytokines [3, 8, 9], while increasing the expression of the anti-inflammatory cytokine, interleukin-10 [8].

Some authors have suggested that the analgesic effect of chitin is mostly due to the absorption of bradykinin, which is one of the main pain-related substances [8]. Due to its hydrophilicity and structural similarity to glycosaminoglycan, chitosan is extensively used as a wound healing agent and chitosan-based dressings have been developed for biomedical applications [10]. N-acetyl glucosamine is the monomeric unit of chitosan, which is involved in the initiation of fibroblast proliferation, improves the deposition of ordered collagen and increases synthesis of hyaluronic acid at the wound site. NH$_3^+$ groups in chitosan interact electrostatically with negatively charged molecules such as proteoglycans and glycosaminoglycans which exist in the membrane of cells [11, 12]. Many animal studies have investigated the wound healing abilities of chitosan-based products, which have revealed the promising results such as histocompatibility for burn wounds in rats [13], satisfactory tissue regeneration for burn injuries in dogs [14], and fast wound closure and re-epithelialization in rat burns have been reported [15, 16]. It has been shown [17] that the behavior of major cell types of skin including keratinocytes and fibroblasts (e.g. adhesion, growth and differentiation) are improved by fibrous scaffolds of chitosan in comparison with other forms like films, sponges or gels. Large specific surface area, high absorbance capacity, high porosity, and nano-level roughness are some unique features of fibrous structures [18].

Electrospinning is a versatile method for producing nanofibers from various synthetic and natural polymers, but the electrospinning of chitosan is hard to achieve due to the complicated behavior of its solutions. Auxiliary polymers such as PEO [19, 20] or PVA [5, 21] have been used to improve electrospinnability of chitosan. However, to the best of our knowledge, there is no report on evaluating the effect of production method on morphology of produced samples and their antibacterial effects, which is the subject of this research. This paper aims to investigate fiber formation by high molecular weight chitosan via electrospinning using PVA as the auxiliary polymer for application in wound healing. The antibacterial activity of chitosan has been investigated with respect to different material properties such as molecular weight, degree of deacetylation, solvent type and concentration and under varying test conditions involving physiological state of bacteria strains, culture medium, pH, temperature, ionic strength, etc.

2. Material and methods (experimental)

2.1. Materials
Chitosan (with high molecular weight and degree of deacetylation $= 85\%$) and polyvinyl alcohol ($M_w = 89–98$ KDa, degree of hydrolysis $= 99\%$) were obtained from Sigma-Aldrich Chemical Company. Acetic acid (99.9\%) was purchased from Carlo Erba Company (Italy) and sodium acetate (NaAc) with HPLC grade was obtained from Merck Company. All other reagents were of analytical grade and used as received.

2.2. Electrospinning
2.2.1. Hydrolysis
The molecular weight of crude chitosan powder was decreased by hydrolysis in NaOH (50\% V/V) (aq) as follows. Firstly, the chitosan powder was mixed in NaOH solution with gentle stirring at 95 $^\circ\text{C}$ for 48 h. Then, it was filtered and rinsed with distilled water several times and finally kept in an oven at 60 $^\circ\text{C}$ for 24 h to dry.

2.2.2. Solution preparation
Chitosan powders (both crude and hydrolyzed) were dissolved in aqueous acetic acid (90\% v/v) at room temperature (concentrations of 2 and 4\% w/v) and at 60 $^\circ\text{C}$ (concentrations of 4 and 6\% w/v) with gentle stirring for 3 h. In addition, PVA solution was prepared by dissolving PVA in deionized water (10\% w/v) at 90 $^\circ\text{C}$ under magnetic stirring for 5 h, and it was then cooled to room temperature. Subsequently, these two solutions were mixed for an additional 2 h with volume ratios of 90/10, 70/30, 50/50, 30/70 and 10/90 (PVA/Chitosan).

2.2.3. Fiber formation via electrospinning
The final solutions were loaded into four plastic syringes with needle gauge 22. Electrospinning was carried out at room temperature with a relative humidity of around 30\%. Collector speed ($S$) and flow rate ($I$) were set to 800 rpm and 0.5 ml h$^{-1}$, respectively. The voltage ($V$) applied to the nozzle and the nozzle tip to collector distance ($D$) were considered as variable parameters. After choosing the best volume ratio for PVA/Chitosan...
according to SEM results, the machine parameters were changed to select the best condition for electrospinning of final samples.

2.3. Preparation of PVA/chitosan film
The solution of hydrolyzed chitosan in acetic acid (4% w/v) was prepared at 60 °C. Then, the PVA solution (10% w/v) with a PVA: Chitosan ratio of 30:70 (v/v) was prepared. After 2 h mixing at room temperature, the solution was cast into a Teflon mold. After being dried at room temperature, the PVA/Chitosan film was removed from the mold.

2.4. Characterization
2.4.1. Viscosity and molecular weight determination
Intrinsic viscosities of both crude powder and hydrolyzed chitosan were determined by measuring dilute solution viscosity in aqueous solution of acetic acid and sodium acetate (0.25 M HAc/0.25 M NaAc) using a cannon–Ubbelohde viscometer (Cannon Instrument USA, capillary number 150). On brief, the solution was prepared as follows: chitosan (0.13% w/v) dissolved in acetic acid and sodium acetate solvent system was stirred for approximately 22 h and then filtered by a 0.45 μm cellulose acetate filter. The viscosimetric parameters were measured at 25 ± 0.1 °C and the intrinsic viscosity was determined by Tang and Rao’s plots. The following Mark–Houwink–Sakurada equation (equation (1)) was used to determine the average molecular weight for both crude and hydrolyzed chitosan with 1.57 and 0.79 for constants K and α respectively [22].

\[ \eta = k M^a = 1.57 \times 10^{-4}M_w^{0.79} = 1.49 \times 10^{-4}M_w^{0.79} \]  
(1)

where η is the intrinsic viscosity and \( M_v \) and \( M_w \) are viscosity and weight average molecular weight, respectively. In addition, the degree of deacetylation was determined using the following equations [23]:

\[ DA\% = 31.92 \frac{A_{1320}}{A_{1420}} - 12.20 \]  
(2)

\[ DD\% = 100 - DA\% \]  
(3)

where DA is the degree of acetylation and DD is the degree of deacetylation.

2.4.2. Morphological and topological observations
Field emission scanning electron microscopy (FE-SEM) (Mira3tescan-XMU, Czech Republic) was used to evaluate the morphological microstructure of the samples. Fiber diameter measurements were carried out by image processing using ImageJ software (1.47 v, NIH, USA). At least 50 fiber diameters were measured and the average was reported. Also, for topographical evaluations, the atomic force microscopy (AFM) (Ara Pajoohesh, Iran) was used and experiments were carried out at a scanning area of 10 μm × 10 μm in the tapping mode.

2.4.3. FTIR analysis
FTIR spectroscopy of both untreated and hydrolyzed chitosan were performed using the KBr pellet method in a Shimadzu (8400 S, Japan) spectrometer.

2.4.4. Antibacterial assay
Two bacterial strains, which were at their logarithmic phase of exponential growth, were used. Staphylococcus aureus (ATCC 6538, PTCC 1112) as the gram positive and Escherichia coli (ATCC 25922, PTCC 1399) as the gram-negative bacteria. The antibacterial activity was evaluated according to the method proposed by Ko et al [24]. In brief, 15 μl of bacterial suspension (1.5 × 10^6 CFU/ml) was added to each prepared circular sample (22 mm in diameter) and then samples were incubated at 37 °C for 1 h. Afterward, samples were placed into sterile plastic tubes, diluted with PBS and then homogenized by centrifuge at 10^4 rpm for 1 min. The solution was diluted by PBS, cultured on plate count agar and incubated at 37 °C for 1, 8 and 24 h, respectively. The number of cells was determined and compared to blank controls (bacterium suspension alone).

3. Results and discussion
3.1. Fiber formation through electrospinning
3.1.1. Electrospinning of pure chitosan
Solution viscosity of chitosan is being affected considerably by molecular weight, concentration and solvent characteristics. Solution viscosity in turn effects on fiber diameter and morphological structure. On the other hand, solvents play a key role in the electrospinning process through influence on surface tension of the solution [21]. In this work, the \( M_w \) of chitosan crude powder, based on viscosimetry results and equation (1), was about
860 KDa, which is a relatively high molecular weight for chitosan. Acetic acid was used as the solvent for its benign and non-hazardous properties. A concentration of 90% v/v was chosen as the optimum acetic acid concentration for electrospinning [18]. It has been shown that acetic acid concentration is the main electrospinning parameter, which decreases the surface tension of chitosan solution and increases the charge density of jet without remarkably affecting the viscosity [25]. The maximum concentration for dissolving the above-mentioned chitosan powders in the acetic acid solution 90% at room temperature was 4% w/v, which was insufficient to form a stable jet for electrospinning, and shown to be non-electrospinnable. By increasing the mixing temperature to 60 °C, the chitosan solubility rose to 6% w/v, but it still lacked electrospinning ability. In chitosan high molecular weight, it seems that long polymer chains, which forge strong entanglement force between each other and repulsive forces between ionic groups prevent the electrospinning and formation of continuous fibers [23, 26].

3.1.2. Electrospinning of hydrolyzed chitosan

The $M_w$ of hydrolyzed chitosan was determined as explained in section 3.1 based on viscosimetry results (557 KDa). Also, the degree of deacetylation was estimated at 87.4% for the treated chitosan and 83.5% for the crude powder using equations (2)–(4). Due to sensitivity of $A_{1320}/A_{1420}$ to chemical composition, it was selected for these calculations [27]. 48 h hydrolysis in 50% aq NaOH was chosen due to its best satisfactory results for electrospinning [5, 28]. Some researchers have reported that the solubility is related inversely to the molecular weight and directly to the degree of deacetylation. The less molecular weight and higher degrees of deacetylation the better solubility will be obtained [2].

In this work, it seems that electrospinnability of solution was improved due to elevated deacetylation and reduced molecular weight [5], figure 1, shows FTIR results for treated and untreated chitosan. As can be seen, resonance bands at 1157, 1087 and 895 cm on FTIR spectrum reveal the presence of saccharide group in the treated chitosan. Other bands at 1598, 1646 and 3433 cm indicate the bending of N–H in primary amino groups, stretching of the carbonyl group and N–H stretching of the primary amino groups, respectively. Small shifts in the location of resonance bands such as N–H stretching bands in crude and hydrolyzed chitosan can be attributed to variations in secondary bonds like hydrogen bonds [23, 28]. Therefore, it can be inferred that the chemical structure of chitosan remained unchanged after 48 h of hydrolysis treatment.

Figure 2, shows the FESEM results for the electrospinning of treated chitosan with varying concentrations at ambient and high (60 °C) temperatures. The maximum possible concentration of chitosan in the solution was 2% w/v and 4% w/v for ambient temperature (2a, 2b) and 4% w/v and 6% w/v for high temperatures. As can be seen, hydrolysis enabled the electrospinning but there were still some beads in the microstructure. Increased temperature to 60 °C, probably improved solving and mixing of solution, which in turn reduced beads in the microstructure compared with that of the same concentrations at ambient temperature (2b and 2c). Meanwhile, increasing concentration to 6% w/v at high temperature resulted in expansion of beads in the microstructure (figure 2(d)). According to deacetylation percent and molecular weight calculations, it can be observed that hydrolysis lowered molecular weight by about 35% but deacetylation percent rose by about 4%, indicating the greater effect of molecular weight on improving the electrospinnability of the solutions. It has been shown that the surface energy of a cylindrical jet of fluid is higher than that of its divided droplets in the same volume. Thus, it seems that beads are formed by a kind of instability driven by surface tension [29]. Also, at 2% w/v chitosan solution, as shown in figure 2(a), it seems that lack of proper chain entanglements and repulsive forces between polycations produced a bead-only structure rather than fiber formation [5]. In addition, some smaller fibers are visible in figures 2(e)–(h), which can be ascribed to the branching of jet during electrospinning process. Instable shape of the jet and formation of secondary jets during the process can be attributed to the imbalance of electrical forces and surface tension arising from the elongation of jet, variation of charge density and evaporation of solvent [30, 31].

3.1.3. Electrospinning of PVA/hydrolyzed chitosan

PVA was used as an additive polymer due to its nontoxicity, biodegradability, biocompatibility, solubility in acetic acid and possibility of making hydrogen bonds with chitosan [5, 21, 32–35]. Electrospinning of PVA/Chitosan solutions was performed at different ratios and machine parameters as described in section 2.2.3 ($V = 15$ kV, $D = 15$ cm and $Q = 0.5$ ml h$^{-1}$). Chitosan chains interact with PVA molecules through amino or hydroxyl groups to form hydrogen bonding, which improves mixing, formation of stable jet and distribution of PVA and chitosan nanofibers in the electrospun microstructures [5, 21, 23, 36].

According to the SEM results shown in figure 3, it seems that there is an optimum amount of PVA that facilitate the electrospinning of chitosan with good microstructure. Hence, the best beadless microstructure was selected at a ratio of 30/10 (PVA/Chitosan) for electrospinning. In the next step, machine parameters were changed to create optimized conditions for achieving a beadless microstructure. According to the results of different experiments that were conducted on a trial-and-error basis, it was found that for evaluating the
Figure 1. FTIR spectroscopy for (a) crude; (b) treated chitosan.

Figure 2. SEM images for electrospun samples of treated chitosan: (a), (e) 2% and (b), (f) 4% at room temperature; (c), (g) 4% at 60 °C, and (d), (h) 6% at 60 °C.
possibility of a successful electrospinning, considering the cationic nature of chitosan, voltage and distance (V & D), especially voltage, are the most effective machine parameters whereas flow rate (Q) and collector speed (S) have the lowest effect.

Nozzle tip to collector distance (D) affects jet flight time and electric field strength. The high voltage provides the necessary charge distribution on the solution for initiating the electrospinning process by dominating the electrostatic force over the surface tension of the solution but using of too high electric field can cause repulsion between ionic groups of polymer backbone, which could disturb the formation of continuous fibers [10]. Thus, these two parameters were changed (D: 10, 15 and 20 cm and voltage varied in the range of 15 ~ 30 KV with 3 KV step) and the flow rate and collector speed were held constant during the experiments. (Q = 0.5 ml h$^{-1}$ and S = 800 rpm). All of the experiments were carried out at room temperature (figure 3). As depicted in figure 3, microstructure in figure 3(f) is beadles. The following parameters were chosen to produce final electrospun samples (figure 4) for the evaluation of antibacterial activity in the next step. (V = 21 kV, D = 15 cm, S = 800 rpm and Q = 0.5 ml h$^{-1}$). The corresponding distribution of fiber diameters are shown in figure 4(c). The average diameter is 56.9 nm.

Figure 3. SEM images for electrospun samples with different PVA/Chitosan volume ratios: (a) 10/90; (b) 30/70; (c) 50/50; (d) 70/30; (e) 90/10; (f) (magnified image of b).
the surface topography plays an important role in bacterial attachment and hence biofilm formation. It has been shown that the surface topography plays an important role in bacterial attachment and hence biofilm formation [37–39]. In this paper, using an identical composition for film-casting sample (A) and electrospun sample (B), we attempted to evaluate the effect of morphology on antibacterial activity of samples. As shown in table 1, due to the antibacterial characteristics of both sample surfaces (chitosan blend), the higher antibacterial activity of sample B in comparison with sample A for both bacterium strains reflects the better adhesion of bacteria suspension to the sample B surface. The surface roughness has been shown to influence the amount and nature of bacterial cell attachment. Bacterial cells can sense an average roughness change of about one nm [40]. It seems that, due to different roughness (figure 5) of film-casting and electrospun samples, the antibacterial activity is significantly higher in both gram-positive and gram-negative strains for sample B. There appears to be a minimum roughness requirement for attachment of bacteria onto the sample surface. Considering the micron size of two bacterium strains, it seems that more surface roughness in sample B has a positive effect on the attachment of bacteria onto its surface. (Sample A with \( R_a = 1.43 \) nm and sample B with \( R_a = 25.20 \) nm, respectively). In addition, as depicted in figure 4(c), the average diameter of fibers in sample B is 56.9 nm, which is significantly smaller than the length of E.coli. It has been known that for rod-shaped bacteria such as E.coli, the attachment of bacteria onto the nanofiber structure in diameters smaller than the bacterial length leads to the conformational changes of bacteria, as they wrap around each fiber [41]. This in turn could increases the antibacterial effect of the surface. Hence, the highest antibacterial activity against E.coli can be observed in sample B (table 1).

On the other hand, as table 1 suggests, the tested samples have varying antibacterial effect on gram-positive (S.aureus) and gram- negative (E.coli) bacteria. There are divergent views about this issue. Some of authors believe that chitosan has a greater effect on gram-negative bacteria while others espouse the opposite view [6]. This is probably due to variations in experimental conditions (materials, methods, medium pH, etc) [42, 43]. According to the results of this study, the antibacterial effect of samples against E.coli are stronger than that of S. aureus for all contact times (1, 8 and 24 h). It has been shown that the hydrophilicity of gram-negative bacteria is significantly higher than gram- positive bacteria, which makes them highly susceptible to chitosan. It appears

### Table 1. Results of antibacterial assay against two bacterium strains for samples A and B in 3 iterations (Condition of Blank control for all samples was 1.5 × 10⁵ CFU/ml).

| Microorganism | Culture time (h) | Sample | (CFU/ml) | Antibacterial activity (%) |
|---------------|-----------------|--------|----------|---------------------------|
| **E.coli (ATCC25922)** | | | | |
| 1 | A | \(8.9 \times 10^3\) | 40.0 |
| | B | \(6.0 \times 10^3\) | 60.0 |
| 8 | A | \(8.5 \times 10^3\) | 43.0 |
| | B | \(5.5 \times 10^3\) | 63.0 |
| 24 | A | \(7.5 \times 10^3\) | 50.0 |
| | B | \(4.8 \times 10^3\) | 68.0 |
| **S.aureus (ATCC6538)** | | | | |
| 1 | A | \(9.7 \times 10^3\) | 35.0 |
| | B | \(6.4 \times 10^3\) | 57.0 |
| 8 | A | \(9.0 \times 10^3\) | 40.0 |
| | B | \(5.8 \times 10^3\) | 61.0 |
| 24 | A | \(8.6 \times 10^3\) | 42.0 |
| | B | \(5.0 \times 10^3\) | 66.0 |

### 3.2. Antibacterial assay

Adhesion of bacteria to surfaces is the first stage of colonization and biofilm formation. It has been shown that the surface topography plays an important role in bacterial attachment and hence biofilm formation [37–39]. In this paper, using an identical composition for film-casting sample (A) and electrospun sample (B), we attempted to evaluate the effect of morphology on antibacterial activity of samples. As shown in table 1, due to the antibacterial characteristics of both sample surfaces (chitosan blend), the higher antibacterial activity of sample B in comparison with sample A for both bacterium strains reflect the better adhesion of bacteria suspension to the sample B surface. The surface roughness has been shown to influence the amount and nature of bacterial cell attachment. Bacterial cells can sense an average roughness change of about one nm [40]. It seems that, due to different roughness (figure 5) of film-casting and electrospun samples, the antibacterial activity is significantly higher in both gram-positive and gram-negative strains for sample B. There appears to be a minimum roughness requirement for attachment of bacteria onto the sample surface.

**Figure 4.** SEM images for (a), (b) electrospun sample under optimized conditions: 30/70 PVA/Chitosan volume ratio (\(V = 21\) kV, \(D = 15\) cm, \(S = 800\) rpm and \(Q = 1\) ml h⁻¹); (c) Normal distribution curve of fiber diameters.
that chitosan damages the outer membrane of gram-negative bacteria, changes its permeability and hence disrupts its function by inducing leakage the drainage of cellular proteins [6, 44–46].

4. Conclusion

Two different samples of PVA/Chitosan blends with an identical composition (concentration, degree of deacetylation, and volume ratio) were fabricated using casting and electrospinning methods, and then effect of morphology on their antibacterial activity was evaluated. Optimized conditions for bead-less electrospinning of chitosan was 70/30 volume ratio of PVA/Chitosan and the machine parameters were estimated at $V = 21$ kV, $D = 15$ cm, $S = 800$ rpm, and $Q = 0.5$ ml h$^{-1}$. The results of antibacterial assay revealed that nanofibrous structure and greater roughness of the electrospun sample have a positive effect on its antibacterial activity. Also *E.coli*, as a gram-negative bacterium, is more sensitive to the antibacterial effect of chitosan blend in comparison with *S.aureus*, which is a gram-positive bacterium.

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ORCID iDs

Mohammad Khorsand-Ghayeni  
https://orcid.org/0000-0001-8996-6693

Alireza Sadeghi-Avalshahr  
https://orcid.org/0000-0001-6681-1840

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