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Ag/silk fibroin nanofibers: Effect of fibroin morphology on Ag+ release and antibacterial activity

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Abstract

Silk based nanofibers are potential carrier systems for the controlled delivery of antibiotics and other antimicrobial agents. For silk based biomaterials, silk morphology; random coil (Silk I) or β sheets (Silk II) is an important factor affecting their mechanical, thermal and hydrolytic stability and also release of drug/biocides. In this study, Ag/fibroin composite nanofibers were produced in the form of both random coil (Silk I) and β sheet (Silk II) morphologies via glutaraldehyde vapor and methanol post-treatments, respectively and effect of fibroin morphology on Ag ion release and concomitant antibacterial activity was investigated. Ag nanoparticles were synthesized in fibroin nanofibers with diameter of 200–600 nm, by reduction of Ag ions to Ag0. It was proven that random coil (Silk I) and β sheet (Silk II) morphologies cause significant differences on Ag+ release profiles, thermal properties and hydrolytic stability of nanofibers. According to the kinetics data, Ag+ was released by the first order kinetics for both random coil (Silk I) and β sheet (Silk II) morphologies cause significant differences on Ag+ release profiles, thermal properties and hydrolytic stability of nanofibers. According to the disc diffusion test results for both nanofiber structures, containing 1% (w/v) of AgNO3, clear zones of inhibition were observed against Staphylococcus aureus, Staphylococcus epidermidis and Pseudomonas aeruginosa.

1. Introduction

Efficient delivery of antibacterial drugs from pharmaceutical dosage forms are noticeable fields in biotechnological and pharmaceutical industries. During the last decade many pharmaceutical dosage forms like nanocapsules, liposomes, injectable hydrogels and nanofibers have been fabricated in order to achieve controlled drug release. In this regard, antibacterial drug loaded electrospun nanofibers have influential advantages like high drug loading capacity, high encapsulation efficiency, simultaneous delivery of multiple drugs [1]. Hence, particular attention has been devoted to the design of the electrospun nanofibers as promising antibacterial drug carrier systems. Also, in recent years centrifugal spinning technology has provided low cost possibilities to produce nanofibers in mass industrial scale [2,3].

Different antibacterials e.g., antibiotics [4], biocides [5], quaternary ammonium salts [6] and antibacterial polymer...
networks [7] have been incorporated into nanofibers by various techniques that exhibit strong antibacterial activity against the wide range of microorganisms. Also, metallic nanoparticles such as Ag, Au, and Cu are known to have antimicrobial activity and have been widely used in medical field since 1990s. Among these metallic nanoparticles, silver nanoparticles have many advantages, such as low cost, unique antimicrobial properties, and versatility of applications in many commercial products such as wound healing materials, antimicrobial coatings, fillers and medical biotextiles [8].

Like other metallic nanoparticles, colloidal silver nanoparticles have a tendency to form aggregates to minimize their surface energy. The aggregation of silver nanoparticles also decreases their antimicrobial activity [9]. In order to overcome this aggregation, silver nanoparticles have been incorporated into polymer solutions and electrospun as nanofibers [10,11]. Also, in situ synthesis of silver nanoparticles in nanofibers by reduction of silver nitrate is an alternative method.

Silver nanoparticle loaded nanofibers are proposing main advantages like high nanoparticle loading capacity and tunable release mechanisms. Compared with conventional materials, a higher Ag⁺ release is achieved due to the high surface area and the short diffusion length of electrospun nanofibers. Also, controlled release mechanisms can reduce dose related systemic toxicity of silver ions and increase the local antibacterial activity. The release mechanism of silver ions from polymeric nanofibers take place in multiple ways; diffusion due to swelling of the polymer matrix (swelling-controlled system), degradation of the polymer, or a combination of both. Controlled release performance can be considered an important factor for long term antibacterial activity. Previous studies have shown that silver nanoparticles containing electrospun polymers such as gelatin [12], cellulose [13] chitosan [14] and poly(methyl methacrylate) have strong antibacterial activity against broad spectrum of bacteria.

Alternatively, silk based nanofibers are potential carrier systems for the delivery of silver and other antimicrobial agents [15]. As a biomaterial, silk fibroin offer unique properties including biocompatibility, permeability, biodegradability, minimal inflammatory reaction and mechanical properties. Silk fibroin can be processed into many forms and morphologies suitable for variety of biomedical applications. The primary structure of silk fibroin mainly consists of [Gly-Ala-Gly-Ala-Gly-Ser] repeating sequence. Secondary structure of silk fibroin can exist in two general crystalline states: random coil (Silk I) and β-sheet (Silk II) [16]. The physical properties of silk fibroin such as degradability, mechanical and thermal properties depends on the secondary structure and the type of processing [17].

Silk nanofibers which are produced by the electrospinning method have lower β-sheet contents. The transition of silk fibroin molecules from the random coil to β-sheet crystalline state can be induced by treatment with an organic solvent [18], water vapor annealing [19] and stretching process [20]. Organic solvents such as methanol and ethanol have been applied in order to increase the stability of silk nanofibers [21,22]. Also, glutaraldehyde has been used as the chemical crosslinker in order to increase the stability of silk based nanofibers. On the other hand random coil (Silk I) or β sheets (Silk II) morphologies affect the release mechanism of drug/biocides from nanofibers. To date, a few studies regarding silk fibroin nanofibers containing silver nanoparticles have been reported. However, there is no report about release kinetics of silver ions, the effect of fibroin morphology on Ag⁺ release kinetics and concomitant antibacterial activity. In this study, silver nanoparticles were produced in situ in electrospun fibroin nanofibers by UV reduction of silver nitrate and composite nanofibers were treated with glutaraldehyde vapor and methanol. The effect of silk morphology; random coil (Silk I) or β sheets (Silk II) on Ag⁺ release kinetics, antibacterial activity, thermal and hydrolytic stability and cytotoxicity were discussed in detail.

2. Materials and methods

2.1. Materials

Bombyx mori cocoons were obtained from Institute for Silkworm Research (Bursa, Turkey). Silver nitrate 99.8% (Sigma–Aldrich), Formic acid (Sigma–Aldrich 98%), Dialysis membrane (12 kDa, Sigma), NaHCO₃ (Sigma–Aldrich), CaCl₂ (Sigma–Aldrich), Glutaraldehyde; GA (Sigma–Aldrich 25% in water), Methanol (Sigma–Aldrich 99.9%), L929 Fibroblast cells were purchased from Ministry of Food, Agriculture and Livestock (Turkey). Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum (FBS), L-Glutamine, trypsin-EDTA, penicillin-streptomycin solution were purchased from BIOCHROM Berlin. MTT; (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma–Aldrich), Staphylococcus aureus (ATCC 6538), Staphylococcus epidermidis (ATCC 35984), Pseudomonas aeruginosa (ATCC 15442), Tryptic Soy Agar; TSA (Acumedia Manufactures Inc. Lansing, Michigan).

2.2. Preparation of regenerated silk fibroin solution

Native Bombyx mori silk fibers were degummed with 0.5% (w/w) NaHCO₃ solution at 100 °C for 30 min and then rinsed with warm distilled water to extract the glue-like sercin proteins. The degummed silk fibroin was dissolved in the ternary solvent system of CaCl₂/CH₃OH/H₂O (1:2:8 in molar ratio) at 70 °C for 3 h. This solution was dialyzed against distilled water using a dialysis membrane (12 kDa, Sigma) at room temperature for 3 days to remove the salts. After dialyzing for 3 days, the silk fibroin solution was filtered and lyophilized for electrospinning.

2.3. Processing of Ag/Fibroin composite nanofibers by electrospinning

Lyophilized fibroin was dissolved in formic acid (98%) to prepare 13% (w/v) electrospinning solution. Then, AgNO₃ solutions with different ratios (0.1%, 0.5% and 1%, w/v) were added to silk fibroin solution. Each solution was placed in a glass syringe (10 mL) bearing a metal capillary (0.8 mm i.d.) which was connected with a high voltage power supply (Gamma High Voltage Research, Model...
ES40P-20 W). A constant volume rate of 0.100 mL/min was maintained using a syringe pump. The polymer solutions were electrospun at 17 kV, a tip-to-collector distance of 13 cm. The nanofibers were collected on a collection plate covered with aluminum foil (50 cm²). Then nanofibers were irradiated with UV light having wavelength of 254 nm for 30 min in order to reduce Ag⁺ to metallic silver.

Ag/Fibroin nanofibers, which was denoted as Ag(0.1)/Fib, Ag(0.5)/Fib and Ag(1)/Fib for the sample with the mass ratio of AgNO₃ to fibroin at 0.1%, 0.5% and 0.1%, respectively. The composition of Ag/Fibroin composite nanofibers are given in Table 1.

2.4. Treatment of electrospun nanofibers

In order to increase the stability of nanofibers, some nanofibers were treated by methanol and others were exposed to glutaraldehyde (GA) vapor. Briefly, for methanol treatment, electrospun silk fibroin nanofibers were immersed in 90% (v/v) methanol solution for 30 min at room temperature and dried for 24 h. On the other hand, the cross-linking procedure was carried out with GA vapor in a desiccator. Samples were treated with vapor of 20 mL 25% glutaraldehyde aqueous solution in a desiccator at room temperature for 3 h. Then nanofibers were rinsed with ultra pure water.

2.5. Characterization of nanofibers

Morphological characterization of nanofibers was investigated by Scanning Electron Microscope (SEM) (Nova NanoSEM), with the accelerating voltage of 10 kV. Average diameters of nanofibers were measured by using Image J software (n = 20). Energy Dispersive X-ray Spectroscopy (EDX) analysis were carried out to determine the existence of silver in composite nanofibers. Also, silver nanoparticles were examined by transmission Electron Microscope (TEM) (FEI TECNAI G2 S-TWIN).

In order to observe the conformational changes of fibroin nanofibers via glutaraldehyde vapor and methanol post-treatments, chemical characterizations were carried by Attenuated Total Reflectance-Fourier Transform Infrared Spectrometer (ATR-FTIR) (Perkin Elmer Spectrum BX FT-IR system). The spectra were given as the mean of 32 scans taken in the range of 1400–1800 cm⁻¹ and 600–4000 cm⁻¹ with resolution of 4 cm⁻¹.

Thermal properties of nanofibers were investigated by Differential Scanning Calorimeter (DSC) (Perkin Elmer Diamond DSC) and Thermogravimetric Analyzer (TGA) (Setaram Labsys TGA). DSC analysis were performed under N₂ atmosphere between 0 and 300 °C and at a heating rate of 10 °C/min. The thermal degradation profiles of nanofibers up to 800 °C were obtained by TGA under nitrogen atmosphere at heating rate of 10 °C/min.

2.6. Ag ion release kinetics from composite nanofibers

Silver content in composite nanofibers were determined before the post-treatment. Briefly, each sample was cut into 1 x 1 cm, weighed (5 mg) and dissolved in PBS (0.1 M, pH 7.4). Then, Ag content of each solution was analyzed by Atomic Absorption Spectrometer (AAS) (Perkin Elmer AAnalyst 800).

The Ag⁺ release studies were performed with all compositions of Ag/Fib-M and Ag/Fib-G nanofibers. For this purpose samples were cut into 1 x 1 cm, weighed (5 mg) and immersed in 5 mL of PBS (0.1 M, pH 7.4) at 37 °C in a shaking incubator. At each predetermined time interval, the release solution was collected and the amount of Ag⁺ released was analyzed by AAS. All experiments were performed in triplicate for statistical relevance.

2.7. Degradation of composite nanofibers

In order to examine the hydrolytic degradation, nanofiber mats were cut into squares (1 x 1 cm²) and weighed. Then each sample was placed in 10 mL of PBS and incubated for 14 days at 37 °C. After 14 days period, the samples were weighed again. According to the following equation, the weight loss of the samples were calculated.

\[
WL\% = \left(\frac{W_i - W_f}{W_i}\right)\times 100
\]

WL is weight loss, Wi is the initial weight of sample, whereas Wf is dry weight of the sample after 14 days. Also, the morphological changes of samples were observed by SEM.

### Table 1

Composition and properties of the nanofibers.

<table>
<thead>
<tr>
<th>Sample code</th>
<th>% AgNO₃ in electrospinning solution (w/v)</th>
<th>Average diameter (nm)</th>
<th>Ag⁺ content (ppm/cm² fiber)</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fib</td>
<td>–</td>
<td>337 ± 63</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ag(0.1)/Fib</td>
<td>0.1</td>
<td>293 ± 45</td>
<td>38 ± 5.1</td>
<td>–</td>
</tr>
<tr>
<td>Ag(0.5)/Fib</td>
<td>0.5</td>
<td>226 ± 85</td>
<td>45 ± 4.5</td>
<td>–</td>
</tr>
<tr>
<td>Ag(1)/Fib</td>
<td>1</td>
<td>202 ± 37</td>
<td>56 ± 4</td>
<td>–</td>
</tr>
<tr>
<td>Fib-G</td>
<td>–</td>
<td>406 ± 72</td>
<td>–</td>
<td>Glutaraldehyde vapor</td>
</tr>
<tr>
<td>Ag(0.1)/Fib-G</td>
<td>0.1</td>
<td>626 ± 88</td>
<td>–</td>
<td>Glutaraldehyde vapor</td>
</tr>
<tr>
<td>Ag(0.5)/Fib-G</td>
<td>0.5</td>
<td>504 ± 32</td>
<td>–</td>
<td>Glutaraldehyde vapor</td>
</tr>
<tr>
<td>Ag(1)/Fib-G</td>
<td>1</td>
<td>385 ± 42</td>
<td>–</td>
<td>Glutaraldehyde vapor</td>
</tr>
<tr>
<td>Fib-M</td>
<td>–</td>
<td>342 ± 85</td>
<td>–</td>
<td>Methanol</td>
</tr>
<tr>
<td>Ag(0.1)/Fib-M</td>
<td>0.1</td>
<td>295 ± 44</td>
<td>–</td>
<td>Methanol</td>
</tr>
<tr>
<td>Ag(0.5)/Fib-M</td>
<td>0.5</td>
<td>227 ± 84</td>
<td>–</td>
<td>Methanol</td>
</tr>
<tr>
<td>Ag(1)/Fib-M</td>
<td>1</td>
<td>210 ± 35</td>
<td>–</td>
<td>Methanol</td>
</tr>
</tbody>
</table>
2.8. Antibacterial activity of Ag/Fibroin composite nanofibers

Antibacterial activity of the composite nanofibers was tested against gram-positive S. aureus (ATCC 6538), S. epidermidis (ATCC 35984) and gram-negative P. aeruginosa (ATCC 15442) based on the Clinical Laboratory Standards Institute (CLSI) document M2-A9:26:1 Disc Diffusion Test Method. Mueller-Hinton agar were used as bacterial medium. The agar surface was inoculated by using a swab dipped in a cell suspension adjusted to the turbidity of a 0.5 McFarland standard [23]. Ag/Fibroin composite nanofibers were cut into circular discs (5 mm in diameter). All compositions of Ag/Fib-M, Ag/Fib-G nanofibers and control discs were placed on each S. aureus, S. epidermidis and P. aeruginosa growth Mueller-Hinton agar plates and incubated overnight at 37 °C. The inhibition zone diameters are measured to the nearest millimeter with a ruler after incubation at 37 °C for 24 h.

2.9. Cytotoxicity of Ag/Fibroin composite nanofibers

The indirect cytotoxicity evaluation of Ag/Fibroin nanofibers were conducted according to ISO10993-5 standard test method. For this purposes, nanofiber mats were cut into squares (1 × 1 cm², n = 6) and the specimens were sterilized by UV radiation for 1 h and then were placed in wells of TCPS. Then, extraction media (DMEM, supplemented by 10% fetal bovine serum, 1% L-glutamine and 1% antibiotics (Penicillin/Streptomycin)) were added to each sample containing well and incubated for 3 days. On the other hand, L929 mouse fibroblast were cultured in wells of TCPS at 1 × 10⁴ cells/well in DMEM, supplemented by 10% fetal bovine serum, 1% L-glutamine and 1% antibiotics (Penicillin/Streptomycin) for 1 day at 37, in 5% CO₂ to allow cell attachment. After that, the medium was replaced with an extraction medium and cells were reincubated further 1 day. Finally the extraction medium was removed from wells of TCPS and 100 µL fresh DMEM and 50 µL MTT were added to each well of TCPS. After incubation for 4 h the medium was removed from each well and 100 µL HCL-isopropanol solution was added. The TCPS was kept in a dark media for 30 min and the solutions were transferred in a 96-well TCPS. The viability of the cells cultured by extraction medium was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, with the viability of the cells cultured by fresh DMEM being used as control. MTT, a yellow dye, is taken up by viable cells and converted to formazan. The absorbance was measured at 570 nm by Microplate Reader.

2.10. Statistical analysis

All the nanofiber diameter are expressed as mean ± standard deviation and Kruskal-Wallis one way ANOVA was performed using SPSS (IBM SPSS Inc. version 20) for statistical analysis of cell culture studies.

3. Results and discussion

3.1. Ag/silk fibroin nanofibers

In the present work, randomly oriented Ag/Fibroin composite nanofibers were produced with a smooth and uniform morphology under controlled parameters (Fig. 1).
Polymer concentration is a critical parameter to control morphology of nanofibers. When fibroin solutions were prepared by low concentrations (<8% (w/v)) bead formation was observed. To overcome this problem, fibroin concentration were gradually increased and optimized at 13% (w/v) while other parameters were held constant (voltage: 20 kV, distance: 20 cm, flow rate: 0.1 ml/min). Fibroin nanofibers had an average diameter of 337 ± 63 nm (Fig. 1a). Addition of AgNO$_3$ to spinning solution caused a decrease in fiber diameters without any bead formation. Ag(0.1)/Fib nanofibers had an average fiber diameter of 293 ± 45 nm and showed a porous structure with smooth fiber orientation (Fig. 1b). At 0.5% (w/v) AgNO$_3$ weight ratio, the average fiber diameter decreased to 226 ± 85 nm (Fig. 1c). The increase of AgNO$_3$ concentration to 1% (w/v) caused a further decrease in fiber diameter to 202 ± 37 nm (Fig. 1d). It can be suggested that the addition of Ag$^+$ into fibroin solution increase the cationic charge density in fibroin solution and result in thinner fibers compared to fibroin nanofibers. High concentration of silver nitrate (more than 2% w/v) resulted in instabilities in the electrospinning jet and the fiber formation was not observed. Optimum silver nitrate concentration was chosen between 0.1 and 1% (w/v). The elemental silver signal in EDX confirmed the presence of Ag nanoparticles in fibroin nanofibers (Fig. 1e-g) and the amount of silver content was found as 38 ± 5.1, 45 ± 4.5 ppm/cm$^2$ for Ag(0.1)/Fib, Ag(0.5)/Fib and Ag(1)/Fib, respectively (Table 1).

Strong signals of C, N and O were also detected mainly from amorphous silk I structure (Fig. 4b). This result proved that nanofibers have silk II structure. For Ag(1)/Fib and Ag(1)/Fib-G nanofibers amide I showed a strong band at 1653 cm$^{-1}$ that are characteristic bands of amorphous silk I structure (Fig. 4c). Additionally, there was no difference in the ATR-FTIR spectra between Fib and Fib/G nanofibers; amide I showed a strong band at 1626 and 1518 cm$^{-1}$ whereas the absorption at 1648–1654 and 1535–1542 cm$^{-1}$ belong to silk I conformation [24]. As seen in Fig. 4b, for the Fib and Fib-G nanofibers; amide I showed a strong band at 1651 cm$^{-1}$ and amide II showed its corresponding band at 1534 cm$^{-1}$ related to silk I structure. The β-sheet structure was observed for all methanol stabilized nanofibers (Fib-M) with bands at 1626 and 1518 cm$^{-1}$ (Fig. 4b). This result proved that nanofibers have silk II structure. For Ag(1)/Fib and Ag(1)/Fib-G nanofibers amide I showed a strong band at 1653 cm$^{-1}$ and amide II had its corresponding peak at 1535 cm$^{-1}$ that are characteristic bands of amorphous silk I structure (Fig. 4c). Additionally, there was no difference in the ATR-FTIR spectra between Fib and Ag/Fib nanofibers. Consequently, ATR-FTIR spectroscopic studies prove the transition of fibroin structure from random coil (Silk I) to β sheets (Silk II) by methanol treatment.

3.3. Thermal properties of composite nanofibers

DSC thermograms of composite nanofibers are given in Fig. 5. In all DSC thermograms endothermic peaks around 70–80°C can be referred to water loss due to humidity of samples. Glass transition temperature of untreated nanofibers was observed around 190°C and degradation started at 270°C (Fig. 5a and d). Thermal degradation temperatures of GA treated samples were shifted to 5°C higher than degradation temperature of untreated samples. It
was an expected result since GA vapor treatment did not change the morphology of silk nanofibers but stabilized the structure by crosslinking (Fig. 5b and e). Because of amorphous morphology of GA treated samples (Fib-G and Ag(1)/Fib-G), Tm was not observed and Tg values were around 190°C. Corresponding degradation temperatures ($T_{\text{deg}}$) were detected as 278°C and 281°C for Fib-G and Ag(1)/Fib-G nanofibers, respectively.

In methanol treated samples (Fib-M, Fig. 5c), Tg was observed around 190°C due to the amorphous content of crystalline morphology. Crystallization temperature ($T_c$) and melting temperature (Tm) were also observed around 225°C and 275°C, respectively. This results confirmed the β-sheet structure of methanol treated samples and were consistent with IR results. Also, thermal degradation of methanol treated samples were observed around 288°C. Introduction of silver into nanofibers (Fig. 5f) did not alter the thermal properties of silk β-sheet structure and similar Tm and Tg values were observed.

TGA results of composite nanofibers are given in Fig. 6. For all samples thermal degradation profiles can be divided to two main regions. Loss of water was recorded at 100°C in region I and the second weight loss occurred between 270 and 370°C in region II, due to fibroin degradation which is associated with the breakdown of peptide bonds. The amorphous samples of silk fibroin (Fib, Fib-G, Ag(1)/Fib and Ag(1)/Fib-G) showed lower thermal stability compared to crystalline samples (Fib-M and Ag(1)/Fib-M) (Fig. 6a and b). 50% degradation was observed for Fib and Fib-G samples at 395°C whereas, for Fib-M it was around
Fig. 4. ATR-FTIR spectra of Ag/Fib composite nanofibers prepared after post treatment: (a) Fib and Ag/Fib nanofibers, (b) Fib, Fib-M and Fib-G nanofibers, (c) Ag(1)/Fib, Ag(1)/Fib-M and Ag(1)/Fib-G nanofibers.

Fig. 5. DSC thermograms of the nanofibers; (a) Fib, (b) Fib-G, (c) Fib-M, (d) Ag(1)/Fib, (e) Ag(1)/Fib-G and (f) Ag(1)/Fib-M. DSC measurements were performed at a heating rate of 10 °C/min. Temperature range from 0 to 300 °C.
530 °C. So, it can be concluded that crystalline β sheet structure is more stable than amorphous random coil against thermal degradation.

3.4. Ag ion release kinetics and hydrolytic degradation

The effect of glutaraldehyde vapor and methanol treatment on Ag ion release kinetics for composite nanofibers with different silver loading are given in Figs. 7 and 8. For both glutaraldehyde vapor and methanol treated fibers, typical two-step release was observed so that the initial rapid release was due to the diffusion, while the second step was due to both diffusion and degradation of the matrix. All nanofibers released silver ion more than 10% in 24 h and obtained data correlated with the literature. Within 8 h, amount of Ag ion release increased exponentially for all fibers and followed a first order kinetic (Fig. 10) [25,26]. As seen in Fig. 8, silver ion reached to a plateau after 72 h. At the end of 72 h, about 30% of loaded silver was released from the Ag(1)/Fib-G composite nanofibers which increased to 63% within 336 h. Both glutaraldehyde vapor and methanol treated fibers showed almost similar release profiles. However, due to silk II structure (β sheet) the cumulative release of silver ions from Ag/Fib-M was lower than glutaraldehyde treated fibers (Ag/Fib-G). In the first 72 h, only 20% of silver released from the methanol treated composite nanofibers. Slow release from methanol treated fibers is a result of crystalline structure which reduces chain mobility, decreases diffusivity and dissolution of matrix. Thus, structure of the polymer matrix play an important role in the silver ion release process.

According to the literature, degradation of silk fibroin is related to the type of processing and the corresponding content of β-sheet crystallinity. Fig. 11 indicate that due to the amorphous structure of glutaraldehyde treated nanofibers (Silk I) their degradation rate was higher than methanol treated nanofibers. At the end of 14 days, % weight loss was 61 for Ag(1)/Fib-G and 41 for Ag(1)/Fib-G samples (Fig. 11). As a result, Ag ion release was slower and in a controlled fashion for methanol treated samples as compared to glutaraldehyde treated samples. At the end of the release study, morphological characteristics of nanofibers were given in Fig. 9.

On the other hand, amount of released silver ion is a crucial parameter in order to get a substantial antibacterial effect and should be above minimum inhibitory concentration (MIC). In this regard MIC of silver nanoparticles for different bacteria have been studied by several researchers. Cho et al. reported that 5 ppm silver nanoparticle concentration in colloidal solution had excellent antibacterial effect against gram positive S. aureus [27]. Kvitik et al.
have found that 1.69 ppm colloidal silver nanoparticles in solution was enough to inhibit gram positive *S. epidermidis* [28]. Also, Nowroozi et al. have reported the MIC of silver nanoparticles against gram-negative *P. aeruginosa* as...
3.5 ppm [29]. According to our kinetics data, in the first hour, released silver ion was 8.56 ppm and 5.62 ppm from Ag(1)/Fib-G and Ag(1)/Fib-M, respectively. Thus, silver ion release from both random coil (Silk I) and β sheets (Silk II) morphologies was higher than the MIC. Therefore, it can be concluded that amount of released silver ion from Ag(1)/Fib-G and Ag(1)/Fib-M composite nanofibers was enough to show antibacterial activity against S. aureus, S. epidermidis and gram negative P. aeruginosa within 1 h.

3.5. Antibacterial activity of Ag/Fibroin nanofibers

The antibacterial activity of composite nanofibers against gram-positive S. aureus, S. epidermidis and gram negative P. aeruginosa was tested and results are shown in Fig. 12 and the diameters of the inhibition zones are given in Table 2. According to the results of disc diffusion test, post-treated Ag/Fibroin nanofibers prepared with 0.1 and 0.5% (w/v) silver concentration had no antibacterial activity against S. aureus and S. epidermidis strains (Table 2). At higher silver concentration (1%, w/v), all composite nanofibers possessed antibacterial property against S. aureus, S. epidermidis and P. aeruginosa strains (Fig. 2a–d and g, h). In contrast, Ag/Fib composite nanofibers also showed antibacterial activity against P. aeruginosa even at low silver contents (0.1, 0.5%, w/v) (Fig. 2e and f). The diameters of the inhibition zones were found as 8.3, 7.3 and 9.3 mm for the Ag(0.1)/Fib-G, Ag(0.5)/Fib-G, Ag(1)/Fib-G samples, respectively. Whereas, they were 8.0, 8.3 and 8.6 mm for Ag(0.1)/Fib-M, Ag(0.5)/Fib-M, Ag(1)/Fib-M samples, respectively (disc diameter of samples was 5 mm, Table 2). Also, the inhibition zone was found to be
slightly higher for *P. aeruginosa* in comparison to *S. aureus* and *S. epidermidis*. Consequently, our results show that *P. aeruginosa* is more susceptible to silver ions than gram positive bacteria (*S. aureus* and *S. epidermidis*). The main reason for this difference between the results for *P. aeruginosa* and *S. aureus*, *S. epidermidis* is because of their gram positive and gram negative variance. Similarly, Eid and Araby [30] reported that silver nanoparticles show promising activity against *P. aeruginosa* and slightly active against *E. coli*, methicillin-resistant *S. aureus*, and *K. pneumonia*. The exact mechanism of action for silver nanoparticles and silver ions on gram negative and gram positive bacteria is still unclear. The general consensus is that metallic silver, silver ions and silver nanoparticles causes morphological and structural changes in the bacterial cells [31]. Furthermore, Feng et al. have explained the mechanism as: silver ion causes the inactivation of cellular proteins by annihilating the DNA replication ability [32]. We pondered whether due to the electrostatic interaction between negatively charged bacterial membrane and positively charged silver ions, they can easily enter into the bacterial cells and penetrate through the cell which may result in protein denaturation in *P. aeruginosa*. Also, some reports in the literature confirm the idea that electrostatic attraction between negatively charged bacterial cells and positively charged nanoparticles is crucial for the activity of nanoparticles as bactericidal materials [33]. In this regard, literature shows that metallic antimicrobial agents are more effective in comparison to antibiotics for *Pseudomonas* species [34].

### 3.6. Cytotoxicity

The cytotoxicity of the Ag/Fibroin composite nanofibers was evaluated based on the ISO10993-5 standard test method. Fig. 13 shows the cell viability of composite nanofibers, tested on L929 fibroblast cell line, after 72 h.

![Fig. 10. Kinetic results of Ag⁺ release from composite nanofibers for the first 8 h; (a) Ag(0.1)/Fib-G, (b) Ag(0.5)/Fib-G, (c) Ag(1)/Fib-G, (d) Ag(0.1)/Fib-M, (e) Ag(0.5)/Fib-M. All the kinetic results showed first order Ag⁺ release.](image-url)
results showed that cell viability of fibroin nanofibers was almost the same as the control group and were non-toxic. Since fibroin consists of [Gly-Ala-Gly-Ala-Gly-Ser] repeating amino acid sequences and therefore, degradation products of fibroin is nontoxic for cells. On the other hand cell viability of crystalline Ag(1)/Fib-M composite nanofibers treated with methanol were decreased but with no significant differences in comparison to control group. The slight decrease in cell viability was related to released silver ions. For the Ag(1)/Fib-G samples% cell viability was around 50 and it was considered as toxic. According to the results of release experiment in Section 3.4, 63% of silver ion was released in cell culture medium within 72. In addition to this, GA treatment may also caused some toxicity. Several researches have been reported for silver ions to

<table>
<thead>
<tr>
<th>Type of samples</th>
<th>Inhibition zone diameter (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol treated nanofibers</td>
<td></td>
</tr>
<tr>
<td>Fib-M</td>
<td>None</td>
</tr>
<tr>
<td>Ag(0.1)/Fib-M</td>
<td>None</td>
</tr>
<tr>
<td>Ag(0.5)/Fib-M</td>
<td>None</td>
</tr>
<tr>
<td>Ag(1)/Fib-M</td>
<td>None</td>
</tr>
<tr>
<td>Glutaraldehyde vapor treated nanofibers</td>
<td></td>
</tr>
<tr>
<td>Fib-G</td>
<td>None</td>
</tr>
<tr>
<td>Ag(0.1)/Fib-G</td>
<td>None</td>
</tr>
<tr>
<td>Ag(0.5)/Fib-G</td>
<td>None</td>
</tr>
<tr>
<td>Ag(1)/Fib-G</td>
<td>None</td>
</tr>
</tbody>
</table>

* The diameter of disc was 5 mm.

Fig. 11. Percent weight loss of composite nanofibers after release studies.

Fig. 12. Images of the inhibition zones for samples: (a) Ag(1)/Fib-M against S. aureus, (b) Ag(1)/Fib-G against S. aureus, (c) Ag(1)/Fib-M against S. epidermidis, (d) Ag(1)/Fib-G against S. epidermidis, (e) Ag(0.1)/Fib-M and Ag(0.1)/Fib-G against P. aeruginosa, (f) Ag(0.5)/Fib-M and Ag(0.5)/Fib-G against P. aeruginosa, (g) Ag(1)/Fib-M against P. aeruginosa and (h) Ag(1)/Fib-M against P. aeruginosa.

Fig. 13. The cytotoxicity of the composite nanofibers was evaluated based on a procedure ISO10993-5 standard test method. * Significant differences between groups ($p < 0.01$). Data points are the average of $n = 6$ and error bars represent $\pm$ SD.
be cytotoxic in many cell lines, but the molecular mechanism of its cytotoxicity has not been defined yet.

Different mechanisms induce the toxicity of silver nanoparticles. Generally, it is believed that silver nanoparticles penetrate through cell membrane and cause induction of reactive oxygen species (ROS) and in vitro studies demonstrated that silver ions can cause induction of DNA strand breaks and chromosomal aberrations.

4. Conclusion

In this study, Ag/fibroin composite nanofibers with ultrafine texture were fabricated in both random coil (Silk I) and β sheet (Silk II) structures via glutaraldehyde vapor and methanol post-treatments, respectively. It was confirmed that composite nanofibers which contain β sheet structure result in higher hydrolytic and thermal stability (up to 530 °C) than random coil structure. Silver ion release from both nanofiber structures followed first order release kinetics within first 8 h. Also, due to the amorphous random coil structure, cumulative amount of silver ions released from Ag/Fib-G (random coil) was significantly higher than the amount released from Ag/Fib-M (β sheet). This study shows that silver ion release can be controlled by fibroin morphology. Ag/Fibroin nanofibers showed promising antibacterial activity against P. aeruginosa even at low Ag content (0.1% Ag, w/v) and slightly active against S. epidermidis and S. aureus at 1% Ag (w/v) content with minimal cytotoxicity to L929 cells.

In summary, the antimicrobial property of Ag/fibroin composite nanofibers is related to the amount of silver in nano fiber structure, morphology of nanofibers and the composite nanofibers is related to the amount of silver nanoparticles. Langmuir 2008;24(5):2051–6.

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