Chitosan/Nanosilver Nanofiber Composites with Enhanced Morphology and Microbiological Properties

Hosnie Baheri and Seyed Hajir Bahrami

Abstract— **In recent years natural polymers have been widely used in biomedical applications. Application of natural and biocompatible polymers in wound dressing, medical sutures and tissue engineering are extensively growing. Additional properties are provided when metal nanoparticles such as silver and gold are incorporated in to the fibers. However, nowadays nanofibers due to their inherent properties such as higher surface to area with these nanoparticles are used for biomedical application. In this study chitosan has been converted into nanofibers and the effect of silver nanoparticle on the antibacterial properties of the nanocomposite fibers has been investigated. Chitosan (Cs)/poly (vinyl alcohol) (PVA) solutions in 2% (v/v) aqueous acetic acid were electrospun. The effects of different total concentrations of polymer solutions and mass ratios of Cs and PVA on the fibers formation and its morphology have been investigated by SEM. Effect of spinning parameters on the nanofiber diameter have been investigated. Fine nanofibers, without bead were obtained from 8% total concentration of polymer in aqueous acetic acid solution and 40/60 mass ratio of Cs/PVA. To improve the antibacterial properties of nanofibers silver was incorporated in to the electrospinning solution by two different ways ie., i) addition of silver nanoparticles into the electrospinning solution and ii) addition of silver nitrate salt and then reducing it to silver. Antibacterial activity of nanofibers against St.aureus as gram-positive and P.aeruginosa as gram-negative bacteria shows that nanofibers containing silver nanoparticles have stronger antibacterial activity than nanofibers without silver. Moreover, cell culture test shows that cells can grow easily on these nanofibrous webs.**

Keywords: Chitosan, poly (vinyl alcohol), electrospinning, nanosilver, nanofiber.

I. INTRODUCTION

Electrospun nanofibrous materials of several polymers have been characterized with respect to their applications as biocompatible or bioresorbable materials. Because of its abundant production in nature, excellent biocompatibility, biodegradability and nontoxicity, the cationic polysaccharide chitosan (Cs) is a very promising polymer for this purpose [1]. Chitosan, poly [(1,4)-2 amino-2-deoxy-D-glucose], is an environmental friendly, nontoxic polymer derived by partial deacetylation of chitin. Chitin is the principal structural polysaccharide of the arthropods (for example, crabs and insects) and the second most abundant polysaccharide next to cellulose. Depending on the chitin source and the methods of hydrolysis, chitosan varies greatly in its molecular weight (MW) and degree of deacetylation (DDA). The MW of chitosan can vary from 30 kDa to well above 1000 kDa. The typical DDA of chitosan is over 70%, making it soluble in aqueous acidic solutions [1, 2]. It is an interesting polymer because of its physicochemical properties, including its solid state structure and the dissolving state conformation. In the solid state, relatively rigid crystallites form due to the regularly arranged hydroxyl and amino groups at the equatorial positions in the β(1,4)-linked D-glucosamine repeating units, while in solution, hydrogen bonding drives the formation of microfibrils, depending on the chitosan concentration. Recent attention has been on making chitosan fibrous membranes by electrospinning for tissue engineering applications [3-9].

An additional property is provided when metal nanoparticles such as silver and gold are incorporated in to the fibers. Nowadays nanofibers with these nanoparticles are used for biomedical application. Chitosan can be used as a suitable matrix for antibacterial nanoparticles.

It has long been known that silver nanoparticles have bacteriocidial and fungicidal activity. The silver nanoparticles are very effective antimicrobial and antifungal at low concentration. The antibacterial activity of silver is higher than other metals, such as mercury, copper, lead, chromium and tin [10]. The possible use of silver nanoparticles as antibacterial agent has therefore been investigated as a means of arresting increasing bacterial resistance to conventional bactericides and

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antibiotics. The proposed mode of action of silver nanoparticles is that they attach to the bacterial cell wall via thiol-containing proteins. They may bind to DNA after penetrating cell membranes with compromised permeability [11]. There are, only few reports on the antimicrobial activity of a nanosilver- chitosan nanocomposite [12].

In the recent years, much attention has been paid to electrospinning process and it has received a dramatic revival of interests because of its potential to produce ultra fine fibers with diameter in the range of nanometer or sub-micrometer. In this technique, a high voltage is applied to overcome the surface tension of polymer solution or polymer melt, and a charged jet is ejected. The jet extends in a straight line for a certain distance and then bends and follows a looping and spiraling path. These jets get dried to form nanofibers which are collected on a target (an electrically grounded metal sheet or winder) as nanofibers. The electrospinning phenomenon itself involves basic and significant issues in polymer science for solution dynamics, in which viscoelastic parameters, surface free energy (surface tension) and electro conductivity are critical factors for the successful spinning of nanofibers. These nanofibers are of considerable interest for various applications such as wound dressing, tissue engineering, filtration, etc due to their specific surface area and porous structure [2].

Fiber formation from chitosan has however, been met with challenges because of its high viscosity. For example blending with PEO [13], PVA [14] or using UHMWPEO as the fiber forming facilitating additive [15], can improve electrospinning ability of chitosan with high molecular weight.

In the present work, nanofibrous mats from Cs/PVA blend were produced by electrospinning method. The effect of different total concentrations of polymer solution and mass ratio of Cs and PVA on the fibers formation and its morphology were investigated by SEM technique. To improve the antibacterial properties of nanofibers two methods were utilized: First addition of silver nanoparticles into electrospinning solution and second, addition of silver nitrate salt to the solution and reducingt it to silver, where chitosan plays role in reducing and acts as stabilizing agent [16]. Nanofibers containing silver nanoparticles were produced by electrospinning method.

II. Experimental

A. Materials

Chitosan (Mw=500 KDa, 85%DDA), poly (vinyl alcohol) 98% hydrolyzed (Mw=94-120 KDa) and acetic acid were purchased from MERC, Co. Colloidal silver nanoparticles (800 ppm) was obtained from American Nanogroup Co.

B. Electrospinning Apparatus

The apparatus for the electrospinning experiments was assembled based on previous studies. The electrospinning experiments were performed at room temperature. The polymer solution of known concentration was placed into a 20 ml syringe with a capillary tip having an inner diameter of 0.7 mm. A high voltage power supply (10-25 KV) was used to generate the electric field. The positive side was connected to the needle tip and the negative side was connected to the collector.

C. Electrospinning Procedures

Because of high viscosity of chitosan solution, it could not be electrospun. The repulsive interaction among the polycations along the chitosan chains was thought to prevent sufficient chain entanglement which is necessary for fiber formation. Poly(vinyl alcohol) (PVA) was used to moderate the repelling interaction between polycationic chitosan molecules and enhance the molecular entanglement.

In order to obtain the optimal polymer blend solution concentration proper ratios of Cs and PVA were mixed and the blend solutions were prepared in 2% (V/V) aqueous acetic acid by heating and stirring. To investigate the effect of total polymer concentration on the fiber morphology, polymer solutions containing 25/75 mass ratio of Cs/PVA, with total concentration 0f 4%, 6%, 8% and 10% were prepared and electrospun.

Polymer blend solutions with different amount of chitosan and poly (vinyl alcohol) ie., 25/75, 30/70, 35/65, 40/60, 50/50, 60/40 and 70/30 were prepared and electrospun under constant electrospinning conditions. The applied voltage was 15 KV and the electrospinning distance (tip-to-collector distance) and the federate were fixed at 15 cm and 1.5 ml/hr, respectively.

D. Applying Silver Nanoparticles

To improve the antibacterial properties of nanofibers, nanosilver was added to the polymer solution by two methods. First addition of silver nanoparticles into electrospinning solution, directly, and second addition of silver nitrate salt to the polymer solution and then reducing it to silver.

i) Silver Nanoparticles:

The colloidal silver nanoparticles (800 ppm) were mixed with electrospinning solution. Proper amount of colloidal nanoparticles were added to the solvent (acetic acid 2%) and mixed in ultrasonic bath. Then both of the polymers (Cs and PVA) were added to solvent and dissolved by heating and stirring. Three polymer solutions containing different nanosilver concentrations (50, 100 and 500 ppm

bases on the weight of polymer) were prepared and electrospun.

ii) Silver Nitrate Salt:

Three polymer solutions with different concentrations of silver nitrate salt, including 0.3, 0.6 and 1% silver salt based on the weight of polymer, were prepared. The solutions were stored at the room temperature for 10 days and then kept in ultrasonic bath for proper mixing before electrospun.

E. Cell Culture

Cell culture is a sufficient method for investigating the cell viability and biological compatibility of nanofibers. Cell culture was performed on nanofibrous mats of Cs/PVA and Cs/PVA/Ag blends, using rat mesenchymal stem cells (MSCs). This cell culture test method is suitable for adoption in specifications and standards for materials for use in the construction of medical devices that are intended to be implanted in the human body or placed in contact with tissue, tissue fluids, or blood on a long term basis. However, care should be taken when testing materials that are resorb able to make sure if the method is applicable. Briefly the procedure was: (1) Cells grown to confluent monolayer, (2) biomaterial sample placed on top, (3) incubated for 24 hours, (4) evaluated cells underneath or beside material.

The cell culture medium was containing DMEM (Dulbecco's Modified Eagle's Medium) and FBS (Fetal Bovine Serum). The number of cells was 1×10^4 that was cultured in the medium. The cells were exposed to Cs/PVA and Cs/PVA/Ag nanofibers for 7 days in 5% CO_2 incubator, 99% RH and at 37^{o} C.

F. Antimicrobial Experimentation

The agar plate method was used for investigating the antimicrobial effect of Cs/PVA and Cs/PVA/Ag nanofibrous mats. We used two microorganisms: staphylococcus aureus (ATCC25923) as gram-positive bacteria and pseudomonas aeruginose (ATCC27853) as gram-negative bacteria.

A loop of each bacterium was inoculated on 5 ml of nutrient broth and incubated at 37 ^{0}C for 24 hours. They were then, cultured on the nutrient agar plate (Muller Hinton Broth and Muller Hinton Agar). The 1 X 1 cm^2 samples (Cs/PVA/Ag mats with different Ag concentration) and control (Cs/PVA mat) were sterilized by using UV irradiation. They were soaked in ethanol 75% and placed in each plate. After that the plates were incubated at 37±0.5 ^{0}C for 24 hours. The antibacterial behavior of the mats was investigated by measuring of the zone of inhibition. According to the defined standard the zone of inhibitory shows three responses for bacteria behavior against the samples: Sensitive (S), Semi sensitive (SS) or Resistant (R). Sensitive is suitable result for our samples whereas semi sensitive and resistance are not suitable [17].

III. RESULTS AND DISCUSSION

A. Effect of Total Concentration of Polymer Solution

PVA was added to the chitosan solution prepared in 2% V/V aqueous acetic acid to moderate the repelling interaction between polycationic chitosan molecules and to enhance the molecular entanglement. Nanofiber formation could be obtained from electrospinning of mixture with constant mass ratio 25/75, at increasing total concentration from 4% to 10%. Figure 1 shows micrographs of Cs/PVA nanofibers with different concentrations under the same processing condition. From Figure 1, at low concentration (4%), the nanofibers diameter were as low as 80±12 nm and interspersed with spindle-like sections called beads. SEM pictures shows that increasing solution concentrations decreases number of beads in nanofibers structure and at 8% concentration nanofibers surface were smooth without any beads but with higher diameter (190±21 nm). At concentration higher than 8%, the electrospinning did not produce continuous fiber because of high viscosity.

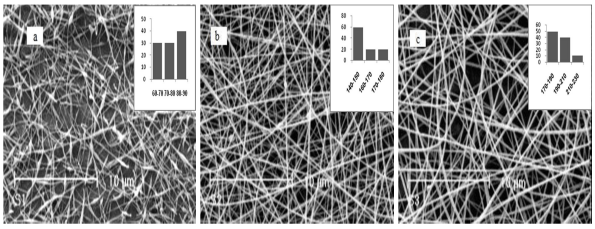

Fig. 1. SEM photographs (2000X) of nanofibrous Cs/PVA mats with constant mass ratio 25/75 at different total concentrations: (a) 4%,(b) 6% and (c) 8%.

Similar to increasing the molecular weight, an increase in the concentration will result in greater polymer chain entanglements within the solution which is necessary to maintain the continuity of the jet during electrospinning. Many experiments have shown that a minimum viscosity for each polymer solution is required to yield fibers without beads. At a low viscosity, it is common to find beads along the fibers deposited on the collection plate. When the viscosity increases, there is a gradual change in the shape of the beads from spherical to spindle-like until a smooth fiber is obtained. At a lower viscosity, the higher amount of solvent molecules and fewer chain entanglements, surface tension will have a dominant influence along the electrospinning jet causing beads to form along the fiber. When the viscosity is increased which means there will be a higher amount of polymer

chains entanglement in the solution, the charges on the electrospinning jet will be able to fully stretch the solution with the solvent molecules distributed among the polymer chains. With increasing the viscosity, the diameter of the fiber also increases. This is probably due to the greater resistance of the solution to be stretched by the charges on the jet [18]. However, at higher polymer concentration (10%) the surface tension and the molecular entanglement will exceed the dragging force of the electrical field and prevents the jet to flow at a straight line and interrupts this flow resulting in electrospraying rather than electrospinning.

B. Effect of Blend Ratio

Chitosan is a cationic polysaccharide with amino groups at the C2 position, which are ionizable under acidic or neutral pH conditions. Therefore, the morphology and diameter of electrospun nanofibers will be seriously influenced by the weight ratio of Cs/PVA. Figure 2 shows SEM images of Cs/PVA blend fibers with different mass ratio of chitosan to PVA under the same processing condition.

It shows that finer nanofibers were produced with increasing chitosan content in the blend solution due to higher chitosan polarity than poly (vinyl alcohol). Because the number of amino groups which can be protonated in acidic media increases, therefore, the density of electrical charges on the surface of the jet increases and the jet gets affected more by the electrical field. Thus the jet can be drawn more and fibers with finer diameter are obtained. So the nanofibers diameter was decreased from 190±21 nm in 25/75 mass ratio of Cs/PVA, to 140±17 nm in 40/60. However, after that ratio with increasing chitosan content the viscosity of solution increased due to higher molecular weight of chitosan and nanofibers diameter varied. Figure 3 shows the average diameter of the electrospun fiber as a function of the mass ratio.

On the other hand, chitosan is a fragile polymer and PVA play plasticizer role in the blend, so by increasing chitosan content in the blend, PVA ratio decreases, and the nanofibers became more fragile and as it is clear from the SEM images cracks are formed along the nanofibers. It seems that 40/60 mass ratio of Cs/PVA is the best ratio under these condition.

C. Cs/PVA/Ag Nanofibers (Nanoparticle)

Three polymer solutions containing different nanosilver concentrations (50, 100 and 500 ppm with respect to the weight of polymer) were prepared with 8% total polymer concentration and 40/60 mass ratio of Cs/PVA and electrospun under the said condition (V=20 KV, d=15 cm, r=1.5 ml/h).

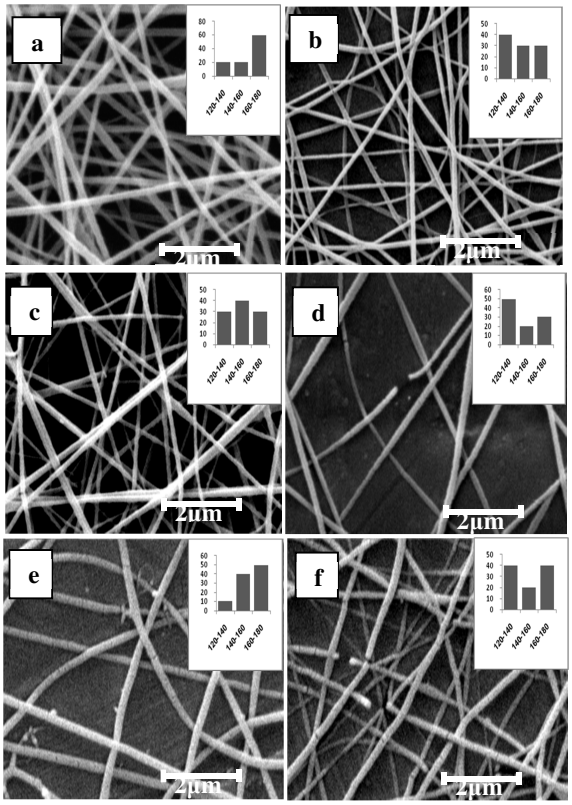

Fig. 2. SEM photographs (7500X) of nanofibers electrospun from Cs/PVA solution at 8% total concentration with mass ratio a) 30/70, b) 35/65, c) 40/60, d) 50/50, e) 60/40 and f)70/30; V=15 KV, d= 15 cm, r= 1.5 ml/hr.

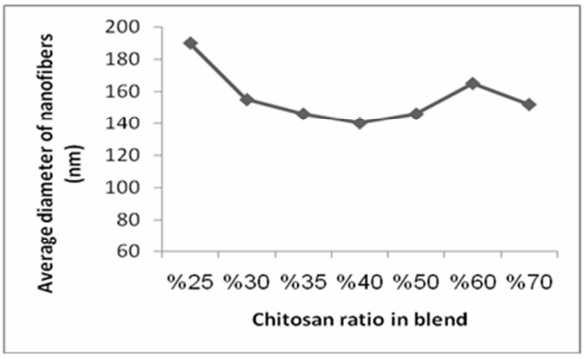

Fig. 3. Average diameter of the electrospun fibers (nm) as a function of the mass ratio.

The SEM photographs of produced nanofibers (Figure 4.) show that presence of nanosilver in electrospinning solution, affected the nanofibers diameter. From Figure 4 it is clear that increasing nanosilver concentration increases the diameter of electrospun nanofibers as the average diameter of nanofibers were 140±12, 165±19 and 260±37 nm, for 50, 100 and 500 ppm nanosilver respectively.

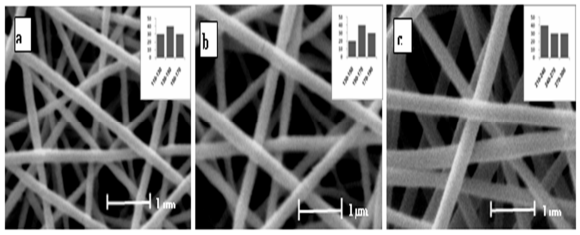

Fig. 4. SEM photographs (15000X) of nanofibrous Cs/PVA mat with different nanosilver concentrations: a)50 ppm, b)100 ppm and c)500 ppm; V=15 KV, r=1.5 ml/hr, d=15 cm.

D. Cs/PVA/Ag Nanofibers (Silver Salt)

Three polymer solutions containing different silver nitrate concentrations (0.3, 0.6 and 1% with respect to the weight of polymer) were prepared with 8% total polymer concentration and 40/60 mass ratio of Cs/PVA. These solutions were stored at room temperature for ten days. By ageing the color of solution was changed from bright yellow to brown.

In the presence of chitosan solution, Ag^+ can be capped well. To change the Ag^+ into free or metallic Ag, an electron supplier or a reducing agent must be used. Twu et al., in their study reported that low molecular weight chitosan degradation products may supply electrons and function as a reducing agent [19]. Moreover, organic macromolecules, such as poly (vinyl pyrrolidone) and poly (vinyl alcohol), are usually used as stabilizers for metal nanoparticles [20]. It has been reported that chitosan can be used as stabilizer for silver and gold nanoparticles [21] in the chemical reduction preparation method. So chitosan acts as a reducing agent in the absence of other chemicals, and as a stabilizer of nanoparticles.

After 10 days, the solutions were exposed to ultrasonic bath and then electrospun using previous conditions. Figure 5 shows SEM photographs of the solution (1% $AgNO_3$) and nanofiber surface prepared from its electrospinning at high magnification. In these images, produced silver nanoparticles are visible.

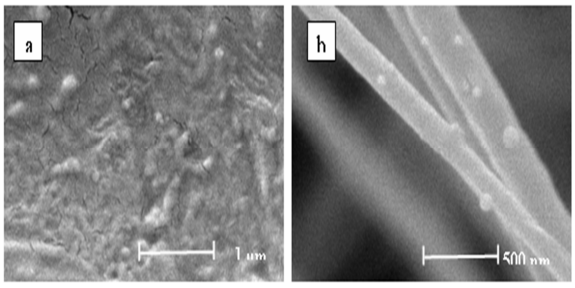

Fig. 5. (a) SEM photograph(15000X) of Cs/PVA solution containing 1% $AgNO_3$, after 10 days storing at the room temperature, (b) SEM photograph (30000X) of the nanofibers produced from electrospinning of this solution.

Figure 6 shows the SEM images of the nanofibers produced from solutions containing nanosilver. It is clear that with increasing silver salt concentration finer fibers are obtained. The average diameters of nanofibers with 0.3%, 0.6% and 1% are 142±12, 126±13 and 103±8 nm, respectively. When a small amount of salt or polyelectrolyte is added to the solution, the increased charges carried by the solution will increase the stretching of the solution. As a result, smooth fibers are formed. The increase in the stretching of the solution also will tend to yield fibers of smaller diameter. However, there is a limit to the reduction in the fiber diameter. As the solution is being stretched, there will be a greater viscoelastic force acting against the columbic forces of the charges [18].

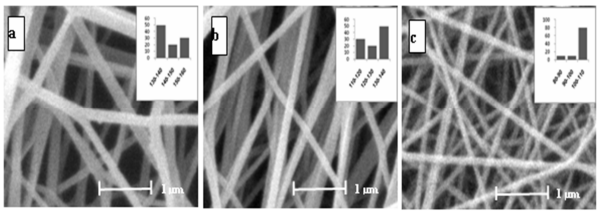

Fig. 6. SEM photographs (15000X) of nanofibrous Cs/PVA mat with different $AgNO_3$ concentrations: (a)0.3%, (b)0.6% and (c)1%; V=15 KV, r=1.5 ml/hr, d=15 cm.

E. Cell culture results

Cell culture is a sufficient method for investigating the cell viability and biological compatibility of nanofibers. We selected two nanofibrous mats for cell culture studies ie., Cs/PVA/Ag blend with 500 ppm nanosilver concentration and Cs/PVA mat without nanosilver. The cell culture was carried out using msenchymal stem cells (MSC) of rat.

Figure 7 shows SEM photographs of these mats when the cells were exposed to them for 7 days in CO_2 incubator, 99% RH and 37^0 C. As it is clear, the cells were grown and covered overall surface of the mats. So they have very good compatibility with the Cs/PVA and Cs/PVA/Ag nanofibrous samples and these nanofibrous mats are able to let the cells grow and can be use as scaffold in tissue engineering applications.

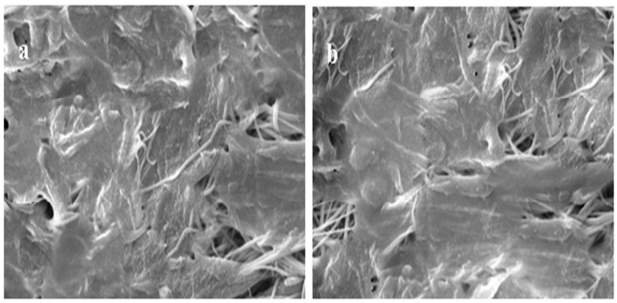

Fig. 7. SEM photographs (2000X) of cell cultured mats a) Cs/PVA nanofibers without nanosilver b) Cs/PVA nanofibers containing 500 ppm silver nanoparticles

F. Antibacterial Properties

Table I shows the results of antibacterial test of nanofiber webs produced from solution containing nanosilver particles. Two microorganisms were used staphylococcus aureus (ATCC25923) as gram-positive bacteria and pseudomonas aeruginose (ATCC27853) as gram-negative bacteria.

In Table I we see the effect of different minimum inhibitory concentration (MIC) (50, 100 and 500 ppm) for several bacteria concentration ie., minimum bactericidal concentration (MBC) for each sample. According to the standards of gram-positive and gram-negative bacteria [17], the zone of inhibitory in every state explains one of the three results for bacteria behavior against the samples: sensitive(S), semi sensitive (SS) or resistant (R). Samples containing silver particles are sensitive whereas Cs/PVA sample is semi sensitive for gram negative and sensitive for gram positive with zone of inhibition lesser then the samples containing nanoparticles. The PVA sample is resistant.

Several researches show that chitosan can inhibit the growth of some bacteria. The cationic amino groups of chitosan probably bind to these microorganisms resulting in growth inhibition. However there is limitation for this act, because of specific microorganism structure. For example, gram-negative bacteria have a difficult structure with three surface layers as wall, so destruction of wall and penetration into its body are not easily.

It has long been known that silver ions can be bacteriostatic as well as bactericidal. The possible use of silver nanoparticles as antibacterial agent has therefore been investigated as means of arresting increasing bacterial resistance to conventional bactericides and antibiotics. The proposed mode of action of silver nanoparticles is that they attach to the bacteria cell wall via thiol-containing proteins. They may bind to DNA after penetrating cell membranes with compromised permeability [11].

The effect of the nanofibrous mats on the growth of the bacteria was investigated by measuring zone of inhibitory. According to the Table I, the samples containing more silver concentration (MIC), inhibited the growth of higher bacteria concentration. The results show increasing nanosilver in the nanofibers effected on the antibacterial behavior of samples.

Table II shows the result of antibacterial test of the nanofiber samples prepared from solutions containing different concentrations of $AgNO_3$. The samples containing more silver concentration (MIC), inhibited the growth of higher concentration of bacteria. It seems the results are more or less similar. The better results may be obtained by controlling of nanoparticles size. Obviously, smaller silver nanoparticles are more effective in penetration into microorganism body and stopping its activity [19, 20].

TABLE I
ANTIBACTERIAL TEST

P.aeruginosa (ATCC 27853) Gram negative			St.aureus (ATCC 25923) Gram positive			Silver contant (MIC)
Result	Zone (mm)	MBC (µg/ml)	Result	Zone (mm)	MBC (µg/ml)	
R	-	4	R	-	4	PVA
S.S	16	4	S	21	4	Cs/PVA
S	25	16.3	S	32	16.3	50 ppm
S	27	16.3	S	35	16.3	100 ppm
S	25	33	S	30	33	500 ppm
Gram Negative Bactera Result: Sensitive (S) ≥ 20mm Semi Sensitive(SS) = 15-19 Resistant(R) ≤ 14mm			Gram Positive Bactera Result: Sensitive (S) ≥ 21mm Semi Sensitive(SS) = 18-22 Resistant(R) ≤ 17mm			Zone standard [10]

TABLE II
ANTIBACTERIAL TEST

P.aeruginosa (ATCC 27853) Gram negative			St.aureus (ATCC 25923) Gram positive			Silver Contant (MIC)
Result	Zone (mm)	MBC (µg/ml)	Result	Zone (mm)	MBC (µg/ml)	
R	-	4	R	-	4	PVA
S.S	16	4	S	21	4	Cs/PVA
S	20	16.3	S	23	16.3	0. 3%
S	23	16.3	S	34	16.3	0.6%
S	21	33	S	35	33	1%
Gram -ve Bactera Result: Sensitive (S) ≥ 20mm Semi Sensitive(SS) = 15-19 Resistant(R) ≤ 14mm			Gram +veBactera Result: Sensitive (S) ≥ 21mm Semi Sensitive(SS) = 18-22 Resistant(R) ≤ 17mm			Zone standard [10]

IV. CONCLUSIONS

Chitosan is one of the most important natural polymers used for many applications. In this study its solution with PVA in acetic acid was prepared and silver in the form of nanoparticles as well as silver salt ($AgNO_3$) was added to this solution. The solutions were electrospun using electrospinning technique. The effect of spinning parameters on the diameter of the nanofibers was examined. It was found that with increasing the polymer concentration and flow rate the diameter of the nanofibers produced increases. Presence of nanoparicles also affects the diameter of the nanofibers. msenchymal stem cells (MSC) of rat was cultured on the nanofiber webs. The results showed good cell culture behavior. staphylococcus aureus (ATCC25923) as gram-positive bacteria and pseudomonas aeruginose (ATCC27853) as gram-negative bacteria were used for investigating antibacterial properties. The nanofibrous mates produced showed good antimicrobial properties towards both bacteria. Presence of silver in the nanofibers in the form of nanoparticles or reduced form its salt did not show any significant change in the antibacterial properties. On the other hand the

concentration of the silver particles had an important effect on the antibacterial properties of the composite webs.

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