ANTIMICROBIAL NANOCOMPOSITE OF SILVER AND GELATIN NANOFIBERS FOR MEDICAL APPLICATIONS

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Abstract: ***Bactericidal property of silver nanoparticles was exploited for the preparation of gelatin nanofibers with antimicrobial properties. Common textile fibers lack antimicrobial properties. Therefore, antimicrobial nanocomposite of silver nanoparticles and gelatin polymer nanofibers was made for medical applications such as antimicrobial filters, surgical dressing, water disinfection and protective clothing. Gelatin is the cheap biopolymer with good biocompatibility. Gelatin polymer is only soluble in high polarity organic solvents due to which it can be used alone or as a blend component to prepare nanofibrous membranes for tissue scaffolds, wound healing and health caring devices. Antimicrobial nanocomposite of silver and gelatin nanofibers was prepared by electrospinning using 16% gelatin in acetic acid and 34% silver nitrate aqueous solution. The electrospun nanofibers were weak and found soluble in water, hence inter-crosslinking was carried out with 0.5% glutaraldehyde solution to increase the stability of polymer. Antimicrobial silver nanoparticles (AgNPs) were prepared in situ by reduction of silver nitrate with sodium borohydride. Scanning electron micrograph image showed 200 nm diameters of gelatin nanofibers. Due to the presence of AgNPs, nanofibers exhibited high antibacterial activity against pathogenic bacteria Staphylococcus aureus.***

Keywords: ***Antimicrobial Nanofibers, Gelatin Nanofibers, Nanocomposite, Silver Nanoparticle, Staphylococcus Aureus.***

I. INTRODUCTION

Nanotechnology is a rapidly growing science which provides the ability to engineer the properties of materials by controlling their size, and this has driven research towards a multitude of potential uses for nanomaterials. Within the connotation of nanotechnology and nanostructured materials, a nanofiber generally refers to a fiber having a diameter less than 100 nm. A number of processing techniques such as melt-blown, drawing [1] template synthesis [2] phase separation, self-assembly [3] electrospinning [4,5] etc have been used to prepare polymer nanofibers in recent years.

Amongst all fabrication methods, electrospinning is the most popular and preferred technique to use. It is simple, cost-effective and able to produce continuous nanofibers of various materials from polymers to ceramics. Until now a wide variety of synthetic polymers have been electrospun into ultrathin fibers and these include biodegradable synthetic polymer such as PLA, PGA, PLGA, PCL, PAN, PVP, PVA, PVC, Polypropylene, chitosan fiber, poly(amic acid) and cellulose acetate. An important characteristic of electrospinning is the ability to make fibers with diameters in the range of nanometers to a few microns. Consequently these fibers have a large surface area per unit mass so that nanowoven fabrics of these nanofibers collected on a screen can be used for filtration of submicron particles in separation industries and biomedical applications, such as wound dressing in medical industry, tissue engineering scaffolds and artificial blood vessels. The use of electrospun fibers at critical places in advanced composites to improve crack resistance is also promising. Electrospinning seems to be the only method which can be further developed for large scale production of continuous nanofibers for industrial applications. Natural biopolymers are more suitable for human body due to biocompatibility. Gelatin is a natural biopolymer, prepared by partial hydrolysis of collagens, the most abundant structural proteins found in skin, tendon, cartilage, bones and connective tissues of animals such as bovines, porcines and pisces. There are two types of gelatin depending on the method by which collagens are pre-treated. The gelatin obtained from acid-treated collagens is called type-A gelatin, while that obtained from alkali-treated ones is called type-B gelatin. Gelatin has biological origin with non-immunogenicity, biodegradability, biocompatibility, commercial availability at relatively low cost and biopolymer with strong polarity. Gelatin is commonly used in food, photographical, cosmetic, pharmaceutical and medical applications. It can be fabricated into many forms, e.g. films, micro or nanoparticles, and dense or porous hydrogels. By some post-treatment or mixed with another (synthetic) biodegradable polymer, gelatin can be used alone or as a blend component to prepare nanofibrous membranes for tissue scaffolds, wound healing, health caring devices and other biomedical applications. Gelatin is a biopolymer with strong polarity. There are very few high polarity organic solvents available for dissolving this biopolymer. Gelatin nanofibers have been fabricated by electrospinning technique either by gelatin alone or mixed with other polymers [6,7,8, and 9]. Solvents for preparing an electrospinnable gelatin solution are: 2,2,2-trifluoroethanol (TFE) [10], 98% formic acid [11], 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) [12,13] and Glacial acetic acid [14]. As electrospun nanofibrous structure of gelatin is water soluble and mechanically weak. This can limit its applications. For a long-term biomedical application, an electrospun gelatin nanofibrous membrane must be crosslinked. Hexamethylene diisocyanate, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide,

Genipin Glutaraldehyde vapor, Formaldehyde, Dextran dialdehyde and Siloxane. Glutaraldehyde (GTA) based crosslinking agents enhanced the thermal stability and mechanical performance of gelatin nanofibers [15]. Crosslinking treatment would be able to improve both water-resistant ability and thermo mechanical performance of the resulting nanofibrous membranes. Several physical and chemical methods have been reported for crosslinking collagenous materials. Nanocomposite of metal nanoparticles dispersed in polymer nanofiber combine the unique properties of metal nanoparticles (e.g., the high ratio of surface atoms to inner sphere atoms and quantum size effect etc.) with the outstanding characteristics of polymer nanofibers (e.g., the high specific surface area and high interpenetrating capacity in other materials etc.).

Among the metal nanoparticles, silver nanoparticles have been drawing much interest because they have been found to exhibit remarkable catalytic activity, surface-enhanced Raman scattering activity, high electrical conductivity and antimicrobial activity etc. Silver nanoparticles have been doped in polymer film by a variety of chemical and physical methods in which conventionally the formation of silver nanoparticles are performed firstly, and then the nanoparticles are doped in polymer solution to prepare silver/polymer composite film. It is extremely difficult to disperse silver nanoparticles homogeneously into the polymer matrix by these methods because of the easy agglomeration of nanoparticles and the high viscosity of polymer solution. In recent years, more attention has been paid to the in situ synthesis of metal nanoparticles embedded in polymer film. This method is based on the reduction of metal ions that are dispersed in polymer matrices. Methods for synthesis of silver nanoparticles include chemical reduction (chemical reduction of silver ions in aqueous solutions or non-aqueous solutions), template method, electrochemical or ultrasonic-assisted reduction, photoinduced or photocatalytic reduction, microwave-assisted synthesis, irradiation reduction, microemulsion method and biochemical reduction.

Amongst all the above methods synthesis of silver nanoparticles by chemical reduction is the best. Formaldehyde, Sodium borohydride, Polyethylene, Glycol, Dimethyl Formamide, Trisodium citrate, Ascorbic Acid and Glucose are used as reducing agents [16,17]. Silver ions and silver compounds have been extensively studied in various fields like antimicrobial filters, wound dressing material, water disinfection, sensors, chemical and gas filtration, protective cloth and air filtration, etc. Positively charged silver nanoparticles interact electrostatically with the negatively charged bacterial cells and thus act as bactericidal material. The silver cation, AgNP being a potent antimicrobial agent binds to and damages the bacterial cells at multiple sites. The mechanism of action for AgNP is to strongly bind to electron donor groups containing sulphur, oxygen, or nitrogen and bring about structural and functional changes in the cell eg. AgNP binds to proteins in the cell wall, the wall ruptures and the internal cell content leaks out, resulting in the death of the bacterial cell. Consequently, this prevents from performing functions, like carrying out processes necessary for respiration or process nutrients leading to subsequent death of the bacterial cell. Antimicrobial polymers are used in several areas like medical devices, health care, hygienic application, water purification systems, hospital, dental surgery equipment, textiles, food packaging, and storage.

II. MATERIALS AND METHODS

1) Materials and Instruments

Gelatin powder (type A, porcine skin, bloom strength - 175) was purchased from Sigma Aldrich. Acetic Acid and Distilled water was used as solvent. Silver nitrate used as antimicrobial agent. Glutaraldehyde (25%) used as crosslinking agent. Reducing Agents used Sodium borohydride, Ethylene glycol, Tri-sodium citrate, and Glucose. Micro organism Staphylococcus aureus used as received without any purification. Electrospinning set up consists of : Syringe pump (kdScientific Hooliston, MA, model no : KDS-100-CE frequency 50/60 Hz) and High voltage DC supply (Gamma High voltage Research Model No : D-ES40PN-10W).

2) Preparation of Dope Solution

Dope solution is used so as to get beadless and fine gelatin fiber. Two types of Dope Solution are prepared :

a) **For control sample**

Compostion : Gelatin 16%
Acetic acid 50%
Distilled water 34%

b) **For test sample**

Compostion : Gelatin 16%
Acetic acid 50%
10mM Silver Nitrate 34%

As gelatin forms gel with water instantaneously, dope solution was heated at 50° C and stirred at 1100 rpm over hot plate for 1 hour. This completely mix dope solution was kept for 2 hours at room temperature.

3) Stability of Dope Solution with time

Stability was evaluated by measuring viscosity with the help of Brookfield rheometer at 10rpm, 20rpm, and 50rpm. Viscosity was measured just after preparation of dope solution, after 2 hours, 24 hours and 48 hours at the time of electrospinning.

3) Electrospinning

Dope solution was taken in syringe (0.6mm internal diameter) and placed in electrospinning setup. Electrospinning was done at a flow rate of 1ml/h needle voltage -25 kv collector voltage -25kv and the distance between needle and collector plate was 22 cm (Fig. 1). Fiber was collected on filter paper for 2-30 min. Sample was dried in vacuum oven (760mmHg vacuum) at 80° C for 1 hour and kept in dessicator for further use.

a) **Characterization of gelatin nanofibers**

Electrospun and crosslinked fibers obtained were characterized by two methods :

- Leica polarizing microscope at 500 X magnification.

- Scanning electron microscope (Zeiss EVO 50 model) at X 12.0 k and X 3.0 k magnification and 20.0 kV voltages.

b) **Crosslinking of gelatin nanofibers**
As the electrospun gelatin nanofibers were weak and dissolve in water so crosslinking was done to increase mechanical strength. For this purpose glutaraldehyde was used as crosslinker. 50 % aqueous glutaraldehyde solution was heated in a beaker and vapors were given to gelatin nanofibers for different timings (1sec. to 15 min). All work performed in fume hood. Crosslinked fibers kept in vacuum oven for 5 hours at room temperature to remove excess GTA.

c) **Selection of reducing agent for AgNO3**
For AgNO3 reduction, a variety of reducing agents were screened and tested such as Formaldehyde, Sodium borohydride, Trisodium citrate, Glucose, Ethylene Glycol in the following combinations :

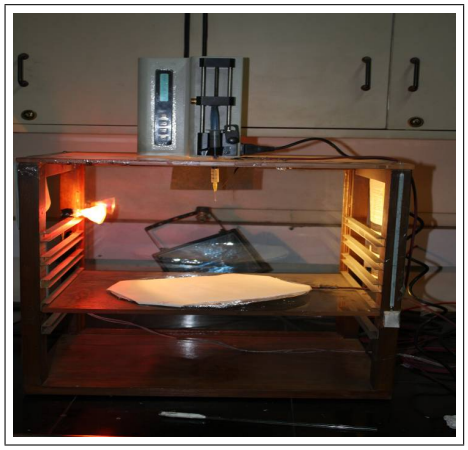


Figure 1: Electrospinning Setup

Dope solution + heat at 80° C for 1 hoour.
Dope solution + 10mM AgNO3 +heat at 80° C for 1 hour.
Dope solution + 10mM AgNO3 + ethylene glycol (0.025M) + heat at 100° C for 1 hour.
Dope solution + 10mM AgNO3 + glucose (0.05M) + heat at 100° C for 1 hour.
Dope solution + 10mM AgNO3 + trisodium citrate (20 mM) + heat.
Dope solution + 10mM AgNO3 + sodium borohydride (0.025M).

d) **Reduction of AgNO3**
When 10 mM AgNO3 was mixed with sodium borohydride it gave black color immediately. Thus, Sodium borohydride selected as best reducing agent. Gelatin nanofibers kept in sodium borohydride solution till black color appeared to confirm that reduction has occurred. Gelatin nanofibers were then taken out for further use.

5) Antibacterial Test
Luria broth media (0.2gm in 9ml water) was prepared and sterilized. Gelatin nanofibers test and control samples were placed over the mouth of flask containing LB media and 1ml of S. aureus bacterial culture passed over it. This assembly was then placed in shaker at 200 rpm and at 37° C for 24 h. Agar-agar and Luria agar solution was sterilized, put in petriplate and allowed to cool. 1ml of sample without AgNO3 was diluted up to 10^6 and sample with AgNO3 diluted up to 10^4. 1μl of solution from 6th test tube was spread on petriplates containing Luria agar + agar agar media. The petriplates were then kept in shaker at 37° C for overnight. Bacterial colonies were counted to calculate the activity. The activity was then measured with AATCC 100 method.

Control sample = Gelatin nanofibers without silver nitrate
Test sample = Gelatin nanofibers with silver nitrate
R (%) = (B-A)/B*100
Where,
R= % of bacterial reduction
B= number of surviving bacterial colonies from the control sample
A= number of surviving colonies from test samples

III. RESULT AND DISCUSSION

a) **Stability of the dope solution with time**
Stability was evaluated by measuring viscosity change with Brookfield rheometer. Viscosity was lower at the time of formation of dope solution because solution hot and viscosity decreases with temperature. After 2 h it increases firstly but decreases with time and sample degrades with time. Sample was electrospinnable for up to 48 h after that solution did not remain electrospinnable (Table 1).

b) **Electrospun gelatin nanofibers with or without AgNO3**
Voltage was applied to the dope solution which spins into nanofibers. A self supporting web was collected on whatman filter paper (Fig. 2). Fiber was collected on filter paper (for 2-30 min). Sample was dried in vacuum.

c) **Concentration optimization of gelatin**
Various conc. of gelatin were used from 5% to 18% with acetic acid and water. No fiber formed at 5% gelatin concentration. Beaded fibers formed between 8% to 15%. And at 18% large diameter fibers were formed. Beadless and fine fibers were obtained with 16% gelatin (Table 2).

d) **Characterization of gelatin nanofibers**
Gelatin nanofibers were characterized by Leica Polarizing Microscope and Scanning Electron Microscope (fig. 3). The average diameter of gelatin nanofibers was found to be 200 nm.

TIME	RPM	Viscosity (in centipoise)	EFFECT ON ELECTROSPINNABILITY
0 h (at the time of formation of dope solution)	10	88.0	Solution was hot but electrospinnable
	20	89.0	
	50	93.6	
After 2 h	10	134.0	Solution was electrospinnable
	20	140.0	
	50	146.0	
After 24 h	10	132.0	Solution was electrospinnable
	20	139.0	
	50	141.8	
After 48 h	10	128.0	Solution did not remain electrosppinable
	20	136.5	
	50	139.0	

Table 1: Viscosity evaluation of dope solution with time.

Concentration of Gelatin(%)	Concentration of Acetic Acid(%)	H2O (%)	Temperature(0C)	Humidity	Observe (through Leica at 500X)
5	90	0	23.0	40	Fiber not formed
8	87	5	21.0	32	Highly beaded fiber
8	82	10	22.0	35	Beaded fiber
10	90	0	22.0	36	Beaded fiber
10	80	10	22.6	38	Beaded fiber
11	89	0	23.3	41	Beaded fiber
12	88	0	23.4	42	Beaded fiber
15	50	35	22.0	36	Few beads fiber
16	50	34	23.6	42	Beadless and fine fiber
18	50	32	22.7	39	Large diameter fiber

Table 2: Concentration optimization of gelatin for electrospinning.

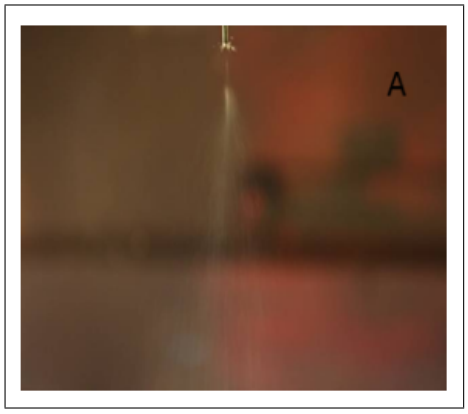
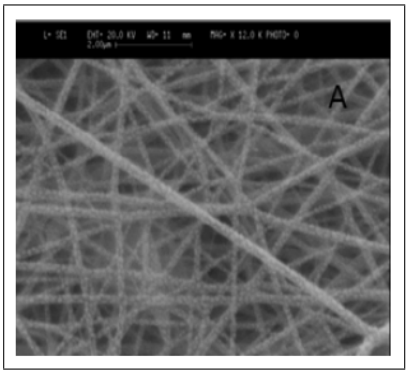


Figure 2: Fiber formation during electrospinning

e) **Crosslinking of gelatin nanofibers**
In order to increase the mechanical strength of electrospun gelatin nanofibers, 50% glutaraldehyde aqueous solution was used for crosslinking of gelatin nanofibers. Fibers were crosslinked for different timings from 2 second to 30 minutes. When the vapors were given to gelatin nanofibers, they shrinked and got yellowish. Gelatin nanofibers crosslinked due to the formation of aldimino linkage between aldehyde group of glutaraldehyde and amino group of gelatin. Due to the nanoscale size of gelatin fibers; the co-existence of water moisture with GTA vapor during crosslinking treatment has affected the fiber morphology to some extent.

f) **Effects of various reducing agents on silver nitrate**



EXPERIMENT	RESULT
Dope solution + heat at 800C	No color change
Dope solution + AgNO3 + heat at 80° C for 1 h	No color change
Dope solution + AgNO3 + Formaldehyde +Ammonia + heat	No color change gel formed
Dope solution + AgNO3 + glucose + heat at 40° C for 30 min	No color change
Dope solution + AgNO3 + trisodium citrate + heat	No color change
Dope solution + AgNO3 + sodium borohydride	Black color observed

Table 3: Reduction of AgNO3 with different reducing agent.

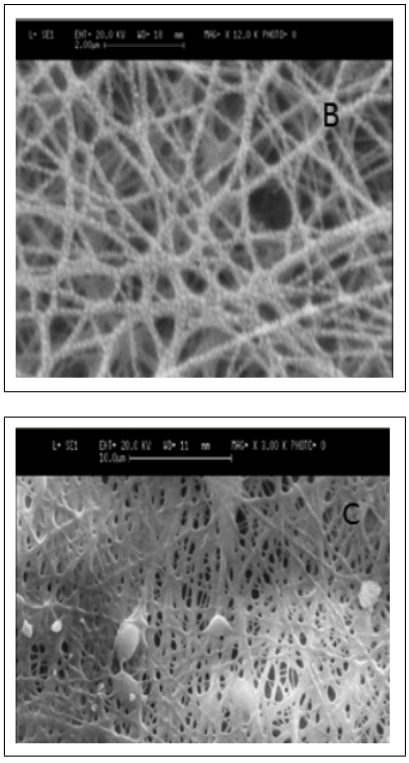


Figure 3: (A) Gelatin nanofiber without AgNo3 (B) Gelatin nanofibers with AgNo3 (C) Crosslinked gelatin nanofibers

g) **Evaluation of bacterial activity of silver nanoparticles on gelatin nanofibers**
1ml of sample without AgNO3 which was diluted up to 10^6 and sample with AgNO3 diluted up to 10^4. 1μl of solution from 6th test tube of solution without AgNO3 and

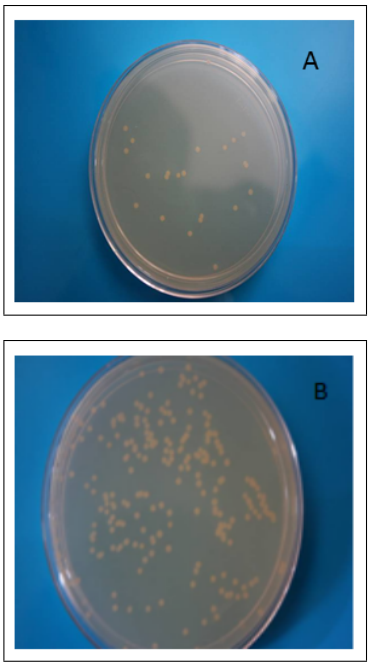


Figure 4: (A) Test sample (B) Control sample for antibacterial testing
4th test tube of solution with AgNO3 was spread on petriplates containing Luria agar. The petriplates were then kept in shaker at 37° C for overnight. Bacterial colonies were counted to calculate the activity. The 99.9% activity was measured with AATCC 100 method (Fig.4). These result showed that only spun fibers with silver nanoparticles exhibited good antibacterial activity. Gelatin/Ag nanocomposite fibers with silver nanoparticles reduced S. aureus by 99.9%.

IV. CONCLUSION

Silver nanoparticles on gelatin nanofibers and their antibacterial activity have demonstrated the successful application of nanofibers in water disinfection, surgical dressing antimicrobial filter, protective clothing and air filter.

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