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In Situ Synthesis of Silk Fibroin Mediated Silver Nanoparticles in Chitosan-Peo Film and Studies on Release Kinetics for Wound Dressing Application

Saraswathy Nachimuthu*, Balaji Sadhasivam, Veerabhuvaneshwari Veerichetty and Ramalingam Ponnusamy, P. Muthukumaran, Sankavi T, Divyapriya
Department of Biotechnology, Kumaraguru College of Technology, Coimbatore-641049, Tamil Nadu, India
***Corresponding author: E-Mail: saraswathy.n.bt@kct.ac.in, Telephone: 0422 2661100**

ABSTRACT

Silver Nanoparticles (AgNPs) are gaining importance in the place of traditionally used silver nitrate for wound dressings with antimicrobial properties. AgNPs were synthesised *in situ* in the chitosan-polyethylene oxide (PEO)- silk fibroin (SF) film. The characterization of chitosan-PEO-SF film with AgNPs using FTIR, SEM-EDAX techniques showed the presence of AgNPs and its distribution in the film. Tensile strength and % elongation of chitosan-PEO-SF film with AgNPs showed good mechanical property. Release kinetics of silver nanoparticles from chitosan blend film was studied using various mathematical models such as zero-order kinetics, first order kinetics, Higuchi model and Power law model. From all the above models, based on coefficient of correlation (R^2) value (0.92), it was confirmed that the release of AgNPs from chitosan-PEO-SF film followed power law model. The release of silver nanoparticles from chitosan film depends on dissolution of the AgNPs. Antimicrobial efficacy of the chitosan- PEO- SF film with AgNPs was comparatively studied against two wound colonizing pathogens such as *B.subtilis* and *P.aerugenosa* and it showed significant reduction in cell density. Therefore, the film developed in present study can be used to prepare wound dressings in the clinical set up.

KEY WORDS: Silk fibroin, AgNPs, Chitosan, PEO, Antimicrobial, and Release kinetics.

1. INTRODUCTION

Nanoparticles are gaining importance in many fields due to their unique optical, electrical and physicochemical properties. Because of their high surface area to volume ratio their effective interaction with surrounding molecules is increased a lot. Therefore, nano forms of materials are preferred over their bulkier counterparts (Raveendran, 2003). Silver is used in many biological applications including water purification, wound dressing material preparation etc., for centuries. Silver shows high toxicity to microbes and less toxic to animals (Kaba and Egorova, 2015). Silver based ointments and wound dressings as broad spectrum antimicrobial agent has been used for decades due to its efficacy and non-toxicity to human being (Boateng, 2008; Peppas, 2006). In recent years natural product mediated silver nanoparticle synthesis is explored. This is called as green synthesis of silver nanoparticles as the process does not involve harmful or harsh chemicals. Many reports are available on green synthesis of nanoparticles using environment friendly green sources like bacteria (Parikh, 2008), fungi (Bhanisa and D'souza, 2006) plant extracts (Harekrishna, 2009) and natural products (Vigneshwaran, 2008).

About 15% of the diabetic people are affected by the diabetic foot ulcer (DFU). Diabetic wounds are shown to be heavily exudating and act as good substratum for microbial growth (Boateng, 2008). The wound dressing material for diabetic wound-care must absorb excess exudates; thereby it reduces microbial infection and further colonization. Another important aspect of diabetic wound healing is preventing microbial infection by antimicrobial agents. The diabetic foot ulcer must be suitably dressed all the time for proper and faster healing. There are new types of wound dressing designed for diabetic wound healing such as hydrogels loaded with bioactive compounds (Moura, 2013).

Hydrogels are water insoluble cross-linked polymers used for biomedical applications for more than four decades due to their soft nature, ability to absorb moisture and their minimal interactions with surrounding tissues etc. (Peppas, 2006). The hydrogels have pores in them and can hold drugs and release the drugs in a slow manner at the wound site. Hydrogels can be prepared using synthetic chemicals, natural proteins (collagen) and polysaccharides like alginate, chitosan, gellan gum, dextran, etc. (Tomme and Hennink, 2007). The hydrogels prepared using natural materials are preferred due to their biocompatibility, biodegradability, non-toxic and natural substances which are able to interact with body cells and promote wound healing process. Biocompatible, biodegradable biopolymers including chitosan were tested for their potential in developing wound dressing material and used as slow releasing base materials for drugs (Kashyap, 2005). Silk fibroin (SF) is a biocompatible and a biodegradable natural protein from silk worm. It is being used in medical applications for decade (Zhang, 2012). When the SF is included with other polymeric materials it helps in maintaining good tensile strength and extension (Gosline, 1999). Silk fibroin has been combined with many other polymers to prepare composite materials with enhanced performance (Luangbudnark, 2012). Blending of Polyethylene Oxide (PEO) with other polymers increases the functionality of the films over the films formed by individual components. PEO is hydrophilic and readily forms hydrogen bonds; thereby it increases bio adhesiveness nature of the chitosan film. This will help in self-adhesive nature of the film

for wound dressing applications. PEO is used in pharma industries to prepare capsules. Blending of PEO with chitosan makes the chitosan more flexible otherwise chitosan film is brittle (Zivanovic, 2007).

2. MATERIALS AND METHODS

Chitosan (High Molecular weight with >75% deacetylation) was purchased from Sigma-Aldrich (St. Louis, USA), Silver nitrate, acetic acid, mono basic sodium phosphate, cellulose dialysis tube, dibasic sodium phosphate, glycine, sodium hydroxide, bovine serum albumin, lithium bromide and sodium carbonate were purchased from Himedia, Mumbai, India. All the chemicals used for the study were of analytical grade. Silk cocoons were purchased from Silk Board Association, Coimbatore, Tamilnadu.

Preparation of silk fibroin: Silk fibroin was prepared by following Sah (2016), method. The cocoons were cut open; worms were removed and washed several times with distilled water. Dried silk cocoons were cut into small pieces and shade dried. The pre-weighed cocoon pieces were boiled in 0.02 M sodium carbonate for 60 min and filtered through coarse filter paper. The degummed raw silk fibres thus obtained were washed several times with milli-Q water and dried under shade to remove moisture. Silk fibroin solution (6% w/v) was prepared in 9.3 M lithium bromide at 60 for 4 h. The silk fibroin solution was dialysed for three days using 12kDa cut-off membrane to remove lithium bromide. After dialysis, the undissolved silk fibroin and other impurities were removed by centrifugation (9000 rpm for 20 min). The homogenous solution was then lyophilized and stored at 4˚C until further use. The silk fibroin concentration was determined using Lowry et al method (Lowry, 1951).

Preparation of chitosan solution: Chitosan powder (2% w/v) was added to 1% (v/v) acetic acid solution and stirred for 2-3 h at room temperature to completely solubilize the chitosan. The homogenized solution was filtered to remove any insoluble matter. The pH of chitosan solution was adjusted to 7.0 before using it for composite preparation.

***In situ* synthesis of silver nanoparticles in chitosan-PEO-SF films:** To the 2% (w/v) chitosan solution, 1% (w/v) PEO was added and stirred till clear solution was obtained and then 5 % (v/v) propylene glycol was added as a plasticizer. The mixture was continuously stirred for another hour to get homogenous solution. To the chitosan-PEO solution silver nitrate (final concentration 10 mM) and silk fibroin (1% (v/v) were added and the solution was kept in sun light for 15 min and poured in the Teflon coated glass plates and allowed to dry for 48 h at 37˚C. The film thus formed was peeled off and stored at room temperature in a sealed cover for further study.

Characterization of chitosan-PEO-SF film AgNPs: Mechanical properties such as thickness, tensile strength and elongation were measured using Universal Testing Machine (Instron, USA). The chitosan-PEO-SF films with AgNPs were subjected to FTIR and SEM-EDAX analysis.

Antimicrobial Activity of chitosan-PEO-SF film with AGNPs: Antimicrobial activity of the chitosan-PEO-SF film with AgNPs was tested against *B.subtilis* as Gram positive test bacteria and *P.aerugenosa* as Gram negative test bacteria (Wei et al., 2009). Overnight culture of each bacterial culture was used to prepare as culture to test the antibacterial efficacy. The chitosan-PEO-SF film with AgNPs was cut into 2 cm x 2 cm size and added into the 10 ml of the nutrient broth inoculated with respective bacterial culture and incubated at 37°C for 24 h. Flask without chitosan-PEO-SF film alone inoculated with respective bacterial culture and treated as control. Cell density was measured using UV-visible spectrophotometer at 660 nm.

AgNPs release kinetics study: Chitosan-PEO-SF film with AgNPs was subjected to *in vitro* release test using phosphate buffered saline (pH 7.4). AgNPs release was worked out from the standard calibration curve by measuring the absorbance of free AgNPs at 440 nm. AgNPs release kinetic was calculated by plotting the cumulative AgNPs release data verses time. Then the AgNPs release kinetics and mechanism was analysed by fitting the data to the following four mathematical equations;

- Zero order : $Mt/M\infty=K_0t$
- First order : $Mt/M_\infty= 1-\exp(-k_1t)$
- Higuchi model : $Mt/M_\infty= K_Ht^{1/2}$
- Korsmeyer-Peppas model (power law model): $Mt/M_\infty=Kt^n$

Where $Mt/M\infty$ is the fraction of drug released at time t and K_0, k_1, k_H and k represent zero order release constant, first order release constant, Higuchi constant respectively (Vimala, 2011).

3. RESULTS AND DISCUSSION

***in situ* synthesis of AgNps in chitosan-PEO-SF film:** AgNPs were synthesized *in situ* in the chitosan-PEO-SF film using silk fibroin as reducing agent under the sun light at room temperature. Chitosan-PEO-SF blended film with AgNPs was prepared using solution casting method. The chitosan-PEO-SF film was transparent whereas Chitosan-PEO-SF with AgNPs film was dark brown in colour due to synthesis of AgNps mediated by chitosan as well as silk fibroin (Fig.1). Silk fibroin is a natural protein with alternative hydrophobic and hydrophilic blocks. The reducing property of the silk fibroin is attributed to the tyrosine residues in the silk fibroin which acts as reducing sink (Fei, 2013).

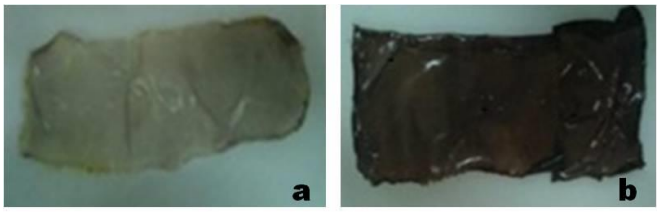

Figure.1. Chitosan-PEO blend films A) chitosan-PEO-SF film; B) chitosan-PEO-SF film with AgNPs

This film is termed as chitosan-PEO-SF film with AgNPs. Chitosan is a biodegradable polysaccharide used in biomedical field for various applications like preparation of wound dressing materials, drug carriers, and scaffolds for tissue engineering and also it has good antimicrobial properties (Fei, 2013). PEO incorporated in the chitosan film enhances the physical properties of chitosan film which elevate the elasticity and water absorption capacity of the film. Further, PEO also contributes transparency to the film. SF acts as reducing agent, capping agent and stabilizing agent in the film (Shengjie, 2013).

Characterization of film (thickness, tensile strength and elongation): Mean thickness values of the chitosan-PEO-SF film with AgNPs showed no change in the thickness of the film compared to the control film. Tensile strength and elongation of the film was also tested for all the films. It was found that chitosan-PEO-SF with AgNPs film had good physical properties when compared with chitosan-PEO-SF films (Table.1).

Table.1. Tensile Strength, elongation and Thickness, for chitosan-PEO-SF and chitosan-PEO with AgNPs film

Film type	Tensile stress at Maximum Load (MPa)	Tensile strain at Maximum Load (%)	Extension at Break (Standard) (mm)	Tensile stress at Break (Standard) (MPa)	Tensile strain at Break (Standard) (%)	Thickness (mm) Mean± S.D
chitosan-PEO-SF film (control)	0.52	28.92	25.24	0.01	37.11	24.20± 0.98
chitosan-PEO-SF film with AgNPs (test)	0.85	21.94	20.68	0.02	30.41	24.92± 0.79

FTIR analysis of chitosan-PEO-SF with AgNPs film: The Fig.2, shows the FTIR spectrum of chitosan-PEO-SF film with AgNP which has shown broad peak at 3500-3100 cm^{-1} is attributed to NH and OH-H vibrations and hydrogen bonding. Peak at 1661.07 cm^{-1} is due to amide bond, amine $–NH_2$ absorption and C=O stretching. Similar result has been described with chitosan- PEO blend film (Ling-hao, 2009). The peak at 1530.62 cm^{-1} is random coil and ß-sheet of silk fibroin.

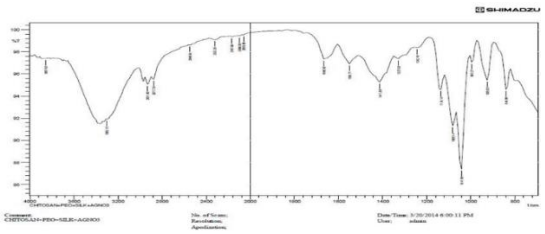

Figure.2. FTIR spectrum of Chitosan-PEO-SF film with AgNPs

SEM with EDAX analysis of the chitosan-PEO-SF with AgNPs film: Scanning electron microscopic analysis was carried out in order to study the surface morphology of the chitosan-PEO-SF blend film. Morphology of the chitosan-PEO-SF film was examined from the scanning electron microscopic image. The microstructures of the chitosan-PEO-SF film prepared using solution casting technique showed the uniform distribution of AgNPs in the chitosan-PEO-SF film (Fig.3). EDAX analysis gave the elemental composition of the chitosan-PEO-SF film and it was observed that silver nanoparticles are present in the film (Fig.4).

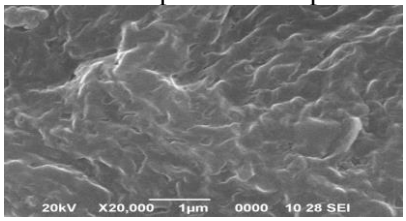

Figure.3. Scanning Electron Microscopic(SEM) image of Chitosan-PEO-SF film with AgNPs

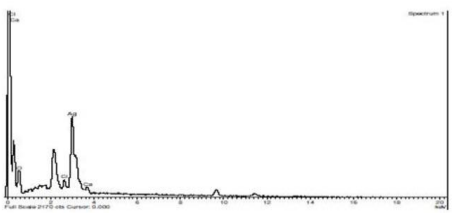

Figure.4. EDAX profile of Chitosan-PEO-SF film with AgNPs

Release studies of AgNPs from chitosan-PEO-SF films: The release of silver nanoparticles from the chitosan-PEO-SF films was studied using four different model such as the zero order kinetics model, first order kinetics, Higuchi model and power law. It was observed from the results that the release of silver nanoparticles from chitosan-PEO-SF films followed Hixson-Crowell order kinetic model which is dependent on both surface area and the thickness of film and contributes to controlled release of silver nanoparticles from the film. From the entire above model with respect to R^2 value (Table.2), it was confirmed that the release of silver nanoparticles follows power law model. Hence, there was controlled release of silver nanoparticles from the chitosan-PEO-AgNP film. This model follows a diffusion pattern for the release of silver nanoparticles from chitosan-PEO-SF silver nanoparticle film. It is exponentially related to time.

Table.2. Comparison of AgNP release fitted in different models (Based on R^2 values)

S.NO	Model	R^2
1	Zero order kinetics model	0.8871
2	First order kinetics model	0.4961
3	Higuchi model	0.6432
4	Power law model	0.9274

Antimicrobial studies of chitosan-PEO-SF film with AgNPs against *B.subtilis* and *P.aerugenosa:* AgNPs are preferred over commonly used silver nitrate based ointments for wound dressing applications due to their high surface area to volume ratio and high efficacy. Preparation of ready to use wound dressing materials need to have good mechanical properties such as tensile strength, greater flexibility and able to release the bioactive molecule (Thomas et al. 2009). The antimicrobial study of the Chitosan-PEO-SF film with AgNPs was carried out against two wound colonizing pathogens. Inoculated flasks with chitosan-PEO-SF with AgNPs film (test) and the remaining flasks without films (control) were incubated at 37^0C for about 24 h. The Fig.5, shows the results for the antimicrobial properties of the chitosan-PEO-SF film with AgNPs and control flasks. Growth of bacterial culture is significantly reduced in the test flask due to the release of AgNPs. It is evident that the flask with chitosan-PEO-SF film with AgNPs showed the reduction in cell density as it appeared clear compared to the respective control flasks. Percentage reduction in cell density for the two test pathogens is represented in the Table.3.

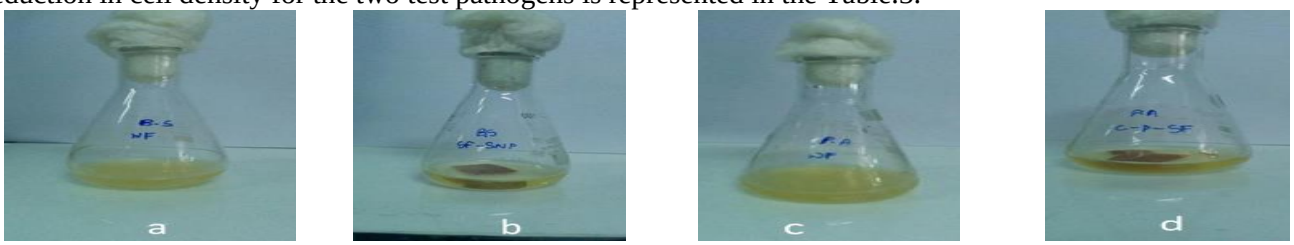

Figure.5. Antimicrobial activity of Chitosan-PEO films against *B.subtilis* and *P.aerugenosa*
***a. B.subtilis* (without film) b. *B.subtilis* (with chitosan-PEO-SF with AgNps film)**
c. *P.aerugenosa* (without film)d. *P.aerugenosa* (with chitosan-PEO-SF with AgNPs film)

Table.3. Antimicrobial activity of chitosan –PEO-SF film with AgNPs against *B.subtilis* and *P.aerugenosa*

Wound colonizing pathogen	Cell density measurement –OD at 600nm		Percent reduction in cell density
	Control (without chitosan-PEO-SF Film)	Test (Inoculated with Chitosan-PEO-SF film)	
B. subtilis	0.949	0.007	99.56
P. aerugenosa	0.885	0.041	97.38

4. CONCLUSION

Chitosan-PEO-SF with AgNPs film was prepared by solution casting method using direct sunlight as source. Chitosan as well as silk fibroin mediates the synthesis of AgNPs. Antimicrobial efficacy of the chitosan-PEO-SF with AgNPs film against *B.subtilis* and *P.aerugenosa* showed up to 99% reduction in cell density. Release of AgNPs from chitosan-PEO-SF film into the PBS solution was recorded and was fitted in various mathematical models and is concluded that release of AgNPs from the film follows power law model and dissolution of the AgNPs. Since chitosan, PEO and SF are used in preparing wound dressing materials, the film prepared in the present study could be used to develop wound dressing material with improved performance.

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