CHAPTER 6

EPD OF CHITOSAN WITH SILVER NANOPARTICLES ON MICRO FIBROUS PCL SUBSTRATES AND ITS BIO MEDICAL APPLICATIONS

6.1 INTRODUCTION

Wound dressings play a vital role in wound management, particularly when extensive damage is caused to the skin by burns, accidental trauma or chronic ulcerations. Recently, great interest has been shown in advanced wound care dressing products by biomedical and pharmaceutical companies (Boateng et al 2008). An ideal wound dressing, is meant to protect wounds from microorganisms (Liu et al 2013), be gas exchangeable, absorb exudates (Zahedi et al 2009), maintain a moist environment (Gu et al 2009) and painless while removing it (Zahedi et al 2009). In recent times, natural and synthetic biopolymers have gained more attention, and has been explored various forms of wound dressings such as films, foams and fibrous substrates. Micro fibrous substrates mimic the native extracellular matrix (ECM) of the skin, and have shown to be promising biomaterials for wound dressings, due to their high surface area to volume ratio, porous structure and conformability to the wound surface (Lalani & Liu 2012). Micro fibrous substrates can be prepared by melt spinning, wet spinning, electro spinning, and centrifugal spinning. The centrifugal spinning system is a recently emerging technique to produce mirco fibrous substrates. It has salient features of being voltage free,
highly productive and having a highly aligned fibrous structure compared to the electro spinning technique (Amalorpava Mary et al 2011).

Polycaprolactone (PCL) one of the biopolymers approved by the U.S. Food and Drug Administration (FDA) has been extensively used in biomedical engineering because of its inherent properties of good mechanical strength, biocompatibility and non-toxicity (Mattanavee et al 2009). PCL fibrous substrates are hydrophobic in nature and have neural charge contribution (Wan et al 2009) and these properties allow them to be an ideal low adherent material (Ng et al 2012). But they lack in hydrophilicity, which is also plays a crucial role while fabricating wound dressings. To enhance the hydrophilicity and moisture retention property, natural polymeric hydrogel can be combined with PCL fibrous substrates, because most of the natural polymers inherently possess these properties. Chitosan is a naturally derived polysaccharide polymer, which is predominantly used as a hydrogel material for wound dressings and for other biomedical applications. Extensive studies carried out on chitosan claim that it is a biocompatible, non-toxic, hydrophilic, bio degradable, non antigenic, antimicrobial, cell affinitive and bio adherent polymer (Liu et al 2008). By adhering chitosan on PCL fibrous substrates might makes it an ideal candidate for wound dressing applications. Coating is a simple approach to combine the chitosan on PCL matrices, by the cast film formation technique (Bai et al 2013) or by electrophoretic deposition (EPD) of chitosan on the substrates. However, cast film formation technique has some limitations as, they mask the microfibre substrates and submicron deposition needs some modified techniques.

EPD of chitosan on substrates has been interested in recent years to modify an implant surface (Pang et al 2009). In brief, when electrical potential is applied between the electrodes, alkaline and acidic pH gradient occur in the cathodic and anodic electrodes respectively. Chitosan is a pH
sensitive polymer and above 6.3 pKa, it turns into a sol gel state (Cheng et al 2012). A cathodic alkaline region can be effectively used as a region for chitosan deposition in metallic implants (Pishbin et al 2011). EPD also additionally offers the co-deposition of hydroxyapatite (Pang & Zhitomirsky 2005), bio glass (Pishbin et al 2011), and zinc oxide particles (Li et al 2010) with chitosan. Till date, limited efforts have been taken to deposit the chitosan on fibrous substrates.

Chitosan is mostly used in wound dressing material due to its inherent properties of biocompatibility, antimicrobial activity, hydrophilicity and biodegradability. To achieve an effective microbial resistance, bioactive compounds such as an antibiotic drug; silver nano particles, and zinc oxide nano particles are combined with chitosan and are to be released in a controlled manner. Metallic silver nano particles have attracted attention due to their broad anti microbial spectrum. Bacterial species such as *staphylococcus aureus, pseudomonas aeruginosa* are capable of colonizing on different surfaces and forming biofilms (Anghel et al 2012). These types of bacterial biofilms interrupt and delay the wound healing process (Ammons 2010). Silver nano particles (AgNPs) have been used as effective antimicrobial agent against most of the micro organisms. Nano silver loaded matrices are commonly used in wound dressing (Madhumathi et al 2010), scaffolds (Zheng et al 2010), and drug delivery applications (Hye Kim et al 2013). The AgNPs can be synthesized by different methods, but in recent years, more attention has been paid to the green synthesis of AgNPs, to avoid harmful reducing agents such as sodium borohydrate especially for biomedical applications (Wei et al 2009). Among the various green synthesis processes, electrochemical synthesis (Khaydarov et al 2008) is the recent facile and attractive technique as it avoids the toxic reducing agent, and does not require any sophisticated instruments. Additionally, hydrogel polymer such as polyethylene glycol (PEG) enhances the reduction of AgNPs particles
reduction in the synthesis. Polyethylene glycol (PEG) is one of the water
soluble hydrogel forming polymers and has been extensively used in the
preparation of AgNPs. PEG can act as a stabilizer in the synthesis of AgNPs
and additionally it can control the size of the AgNPs (Luo et al 2005). While
combining PEG with chitosan (CS)/AgNPs precursor in the EPD process, it
can reduce the AgNPs size; additionally CS/PEG blending enhances the
swelling behavior. Chitosan amine groups interact with AgNPs, and this will
restrict the water penetration for swelling (Shariatinia et al 2014) but this
property can be overcome by the addition of PEG molecules. Due to the
inherent water soluble nature of PEG, it can quickly swell and leach out from
the CS/PEG/AgNPs blend and form a porous structure in the substrates. This
porous structure additionally enhances cell adherence and gas exchangeability
of the coated substrates.

In recent years, to enhance the antimicrobial activity and wound
healing process, a low electric field was applied on wound sites. Reports
claim that low electric current directs cell migration in the wound healing
process (Kucerova et al 2011; Scarborough 2012; Zhao 2009). They also
indicate that, under the low electric current, there is a synergetic effect on the
microbes, and this effect is named as “electricidal effect” (Del pozo et al
2009). Silver nano rods coated cotton cloths and conductive composites were
introduced in a low electric field in bacterial filtration (in water), and the
results revealed that under the electric field AgNPs can be effectively inhibit
100% bacteria (Boehm & Cui 2013; Schoen et al 2010; Vecitis et al 2011).
There are no reports available on the effect of AgNPs or conductive material
loaded polymeric hydrogel on bacteria and bacterial biofilm under the electric
field.

With the detailed information on the factors mentioned, the present
work was undertaken to fabricate an ideal wound dressing material by coating
of CS/PEG with AgNPs on PCL micro fibrous substrates. First, PCL aligned micro fibrous substrates were produced by the centrifugal spinning technique. Then the PCL fibrous substrate was taken as a base material for EPD of CS/PEG/AgNPs. EPD was done by a mixture of acidic chitosan, PEG and silver nitrate solution. The Silver nitrate solution acts as a precursor for the synthesis of AgNPs. Wounds initially need vigorous treatment with antimicrobial agent, followed by sustained antimicrobial activity. The water soluble nature of PEG will lead to burst release followed by sustained release of AgNPs from the coated substrates. At the same time, the elution of PEG from the CS/PEG/AgNPs coating leaves a porous structure and gives better gas exchange and good adhesion for the cells also. Finally, the developed coated composite PCL fibrous substrate was investigated to study the surface topography, AgNPs size, silver release behavior, antimicrobial activity, swelling nature, biofilm resistance and disruption, electricidal effect on biofilm, blood compatibility and cytotoxicity and the results are discussed in detail.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Polycaprolactone (PCL) (M\textsubscript{w}-70,000-90,000), Poly ethylene glycol (PEG) (Mw 4,000 and 10,000), medium molecular weight chitosan with degree of deacetylation (≥75%) and acid citrate dextrose (ACD) were purchased from Sigma Aldrich (USA). Acetic acid, Chloroform and sodium hydroxide (AR Grade) were procured from SISCO research Laboratories Pvt Ltd (India). Regulated DC power supply unit (0-30Volt, 0-2 amps capacity with accuracy of ±0.05%) was supplied by Kpos Pvt. Ltd. Trypicase soy agar (TSA) and Trypicase soy broth (TSB) were purchased from Hangzhou Baisi Corporation of China. S. aureus and P.aeruginosa were provided by ARMAT Bio Tech., laboratory, India. Ultrapure distilled water was obtained after
purification using a Millipore Milli-Q system (USA). Cellulose acetate membrane with pore size of 0.2µ was purchased from sartorius (Germany).

6.2.2 EPD of Chitosan with AgNPs on PCL Fibrous Substrates

The chitosan solution was prepared by dissolving chitosan flakes, using dilute acetic acid as a solvent, and the pH was adjusted to 5.0 by adding NaOH drop wise into the chitosan solution. A graphite sheet was used as an electrode (both anode and cathode) for the EPD process. Centrifugally spun PCL micro fibrous substrates were prepared as mentioned in the previous chapter (pp 93). The PCL micro fibrous substrates were cut into sample sizes of 20×20mm with an average thickness of 0.11±3 mm. The sample was placed on the cathodic surface with the help of an adhesive tape. Chitosan with silver nitrate solutions were filled (50ml) in the electro deposition cell with electrode distance of 5cm. Electro deposition started by applying 5 V for 10 minutes duration. After electro deposition, the coated PCL fibrous substrates were dried at room temperature for 48 hours, to remove the excess water present in them. The dried coated composite PCL substrates were used for further characterization.

6.2.3 Characterizations

The surface topography of the coated fibrous composite substrates was characterized by the scanning electron microscope (SEM, Zeiss) operating at 10 kV in high vacuum mode after sputter-coating with a thin layer of gold metal. In addition, the surface morphology of fibrous substrates was also seen under an optical microscope (Leica, DM750) to study the macro structure of the fibrous substrates. The electro deposited AgNPs were analyzed using TEM (TECHNAI10-Philphs) at an operating voltage of 200 kV. The samples were prepared by placing carbon-coated copper grid on sol gel state chitosan/AgNPs, and the chitosan/PEG/AgNPs coating was kept for
an hour. Then the grid was taken out carefully and allowed to dry overnight in air. The shape and size distribution of the deposited AgNPs were analyzed by TEM images. To confirm the existence of the silver nanoparticles, a UV spectrophotometer (Hitachi) in absorbance mode was used, and the optical spectra were recorded. Fourier transform infrared (FTIR; Bruker Tensor 27) measurements were carried out in the range of 4000 and 400 cm\(^{-1}\) at room temperature to analyze the coated PCL fibrous substrates. The silver content in the fibrous substrates mat was determined, by immersing the weighted coated fibrous substrate samples in 25 mL of 10\% HNO\(_3\) for 1 h, and the resultant supernatant was analyzed, by inductively coupled plasma atomic emission spectroscopy (ICPOES, JY 2000). The release of silver ions from a 2\(\text{cm}^2\) coated composite substrates was evaluated in 10 mL of sterilized filtered distilled water. The tubes were incubated for 2 h, 24 h, 48 h, and 72 h at 37\(^\circ\)C. After incubation, the silver coated PCL fibrous substrates were removed aseptically, allowed to drip into the tubes for approximately 10 s, and then discarded.

6.2.4 In-vitro Antibacterial Testing

The zone of inhibition method was employed to measure the antimicrobial activity of the electro deposited fibrous substrates. It was measured by using the \textit{P.aeruginosa} and \textit{S.aureus}, bacteria as the model strains. Luria–Bertani (LB) agar nutrient medium was used for growing the bacteria. All the fibrous substrates used were sterilized by UV-light before the experiment. The coated PCL fibrous substrates were placed onto the LB agar already plated with different bacteria, and the diameter of the clear zone was measured after 24 h incubation.
6.2.5 Water Absorbance Property

The water absorption of the coated fibrous substrates was evaluated by the gravimetric method. The coated fibrous substrates were cut into 2×2 cm² pieces and their dry weights were accurately measured. The test samples were immersed in 30 ml of deionized water and incubated at room temperature. After a period of time, the swollen fibrous matrices were withdrawn from the water. The wet weight of the swollen substrates was measured after the removal of excess surface water, by gently blotting with a filter paper. The swelling behavior of the fibrous matrices was calculated using the following equation:

\[
\text{Swelling ratio (\%)} = \frac{\text{Wet weight of substrates} - \text{Dry weight of the substrates}}{\text{Dry weight of the substrates}} \times 100
\]

(6.1)

6.2.6 In-vitro BioFilm Resistant and BioFilm Disruption Testing

To examine the effectiveness of the coated composite substrates in resisting and destruction of biofilm development in wounds, an in-vitro model was designed. For biofilm resistance, sterile cellulose acetate disks (6mm) were placed on the surface of LB agar plates, and 1µL aliquots from the overnight culture (diluted into 0.1 optical densities at 600nm) were spotted on each cellulose disk. The coated composite substrates of 1cm² were placed over the bacteria seeded cellulose disks, and allowed to stand for 24 hours at 37°C. After the incubation time, the fibrous substrates were carefully removed from the cellulose disk. Then each cellulose disk and fibrous substrates were transferred into 1.5mL tube and vigorously vortexed to mechanically disrupt the biofilms to detach the bacteria from the fibrous substrates and cellulose disk (Hammond et al 2011). The suspended cells were then serially diluted (10 fold) in PBS and 10-µL aliquots of each dilution were spotted on the LB agar plates. The plates were incubated at 37°C for 16 hours, and the CFU was
calculated. Next, biofilm disruption was carried out by developing a matured biofilm on the cellulose disk by seeding the bacteria on the disk (as mentioned above), and incubated for 24 hours at 37°C. Then 1cm² of the coated composite substrates were placed on the matured biofilm containing cellulose disk and allowed to stand for 24 hours at 37°C. After the incubation time, the coated fibrous substrates were removed from cellulose disk and both were separately subjected to CFU calculation, as mentioned earlier. From the CFU calculations, the adherence of the living bacteria in the cellulose disk and fibrous substrates was identified from the examination.

6.2.7 Effect of Electric Field on Biofilm Disruption

To study the effect of the electric field on biofilm disruption, a mature biofilm was grown on the cellulose disk by seeding the overnight bacterial culture (0.1 optical density at 600 nm) and allowing it to grow for 24 hours. After the mature biofilm was grown, platinum wire (0.07mm diameter) was placed on the biofilm, and at a distance of 2cm counter platinum wire was inserted. The coated composite substrates (slightly wetted by the PBS solution) were placed over the electrode touched with biofilm and the schematic diagram of the arrangement is shown in Figure 6.1. The required amount of voltage was supplied for an hour; after that, the cellulose disk and coated composite substrates were separated and CFU was calculated as mentioned in the earlier section.

Figure 6.1 Schematic diagram of the testing arrangement
6.2.8 In-vitro Blood Compatibility Assay

Acid citrate dextrose (ACD) human blood was used to study the blood compatibility of the coated composite substrates. 1ml of ACD was mixed with 9ml of fresh human blood, and this mixture was used for further study in the blood compatibility assay. Initially, all the samples were sterilized under UV light for an hour, followed by the samples being incubated with 1 mL of sterile saline (0.9%) for about one hour. The samples were taken out after a period of time, and subsequently centrifuged at room temperature to remove excess saline present in the samples. 0.25 mL of ACD blood was added into the saline treated samples, and they were incubated for 30 minutes at 37°C. To stop hemolysis 2 mL of sterile saline was slowly added and samples again well incubated for 1 h and centrifuged at 750G for 5 min. Deionized water treated human blood served as the positive control, and saline treated blood was taken as the negative control (Zhou & Yi 1999). The supernatants absorbance was measured using Hitachi UV spectrophotometer at 545 nm. The hemolysis percentage was calculated by,

\[
\% \text{ hemolysis} = \frac{OD \text{ for the T sample} - OD \text{ for the N control}}{OD \text{ for the P control} - OD \text{ for the N control}} \times 100
\]  

\( 6.2 \)

\( \text{OD} \) - Optical density  
\( \text{T sample} \) - Test sample  
\( \text{N control} \) - Negative control  
\( \text{P control} \) - Positive control

The calculated percentages of hemolysis for all the samples were compared with ASTM standard (Chandra et al 2012),

highly hemocompatible (<5% hemolysis)  
hemocompatible (within 10% hemolysis) and  
non-hemocompatible(>20% hemolysis)
6.2.9 In-vitro Cytotoxicity

The 3T3 cells (National centre for cell science, India) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, ATCC) supplemented with 10% (v/v) of fetal bovine serum (FBS) at 37 °C in 5% CO₂ atmosphere. Before cell seeding, the coated PCL fibrous samples were sterilized under UV light for 2 hours. 3T3 cells were seeded onto the fibrous matrices in a culture medium, following which the cell viability and toxicity were assessed. The sterilized coated PCL fibrous samples (25 mg) were placed in 24well flat bottom tissue culture, and cells with density of approximately 1.2 X 10⁴ cells/well were seeded and allowed to attach for 24 hours. The proliferative potential of 3T3 cells on coated PCL fibrous samples was assessed by the MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay.

6.3 RESULTS AND DISCUSSION

This chapter is divided into two major sections. The first section focuses on finding an optimized process condition with respect to the topography of the coated substrates, and the effective antimicrobial resistance property of the substrates obtained from various conditions. The optimized parameters were used to deposit AgNPs on PCL substrates in the second section; in addition to that, different molecular weight PEGs were added, and their morphology was analyzed. Finally, the optimized CS/AgNPs coated substrate was compared with the CS/PEG/AgNPs deposited substrates with respect to the silver release behavior, biofilm resistance, biofilm disruption, blood compatibility and cytotoxicity.

6.3.1 Influence of Chitosan Concentration

The chitosan concentration used in the EPD cell was altered as 0.1, 0.3, 0.5w/v and other parameters such as concentration of the silver nitrate (1mM), deposition time (10 minutes) and voltage (5V) were kept constant.
The data for the weight add on % as a function of chitosan/silver is given in Figure 6.2. From the figure, it can be seen that the deposition of chitosan/silver complex on substrates increased with increasing the concentration of chitosan. The topography of the chitosan/silver deposition was analyzed using SEM, and it showed a different surface morphology for various polymer concentrations (Figure 6.3). Better coating of chitosan/silver composite on micro fibrous substrates was observed in 0.5 and 0.3w/v with 1mM silver nitrate combination (Figure 6.3 (c-f)). In the case of 0.1w/v concentration of chitosan, the deposition appeared as a rough coating on the surface of the substrates, as shown in the SEM images (Figure 6.3 (a, b)), and the weight add on% of chitosan/silver complex was very low, as compared to the other polymer concentrations. From these results, 0.5w/v chitosan concentrations was chosen for further studies, due to better weight add on % and better coated structure obtained on the PCL substrates.

![Figure 6.2](image.png)

**Figure 6.2** Effect of concentration, deposition time and silver nitrate concentration in weight add %
Figure 6.3  Effect of chitosan concentration with 1mM silver nitrate solution (a) (b) 0.1 w/v concentration, (c) (d) 0.3 w/v concentration (e) (f) 0.5 w/v concentration
6.3.2 Influence of Deposition Time

In order to study the net effect of chitosan/silver deposition on the PCL substrates with respect to the deposition time, the chitosan concentration was fixed at 0.5w/v and 1.0mM silver nitrate was used in the EPD cell. The time of deposition was varied from 2 minutes to 10 minutes and the pH was maintained at 5.0. The weight add on % of chitosan/silver shows significant variation, with respect to varying the deposition time, as shown in Figure 6.2. The weight add on % increased with increasing deposition time. The SEM micrograph also confirms (Figure 6.4) that at a higher deposition time (10minutes) the chitosan/silver entirely covers the PCL substrates with a smooth surface, as compared to a lower deposition time (2minutes). The surface morphology attained at a lower deposition time showed a cracked crystal like structure. It may be due to the incomplete formation of silver nano particles along with chitosan which appeared as lumps.

6.3.3 Influence of Silver Nitrate Concentration

The effect of silver nitrate concentration was altered from 0.2 mM to 1.0 mM to study its effect on weight add on % in the EPD process. The reason for choosing the maximum concentration of 1.0mM was that, above this concentration, it is unable to attain a homogeneous chitosan/silver nitrate solution at pH 5.0. The weight add on % of chitosan/silver complex with respect to variable silver nitrate concentration, is shown in Figure 6.2. The results reveal that, by increasing the silver nitrate concentrations, the weight add on % also increased. The morphology of the coated structure has not shown any significant change while comparing different silver nitrate concentrations in the EPD process (Figure 6.5 and 6.3 (e,f)). From these results it is confirmed that there is no significant change in topography with respect to the silver nitrate concentrations, but variation in the weight add on % alone is present.
From the obtained overall, it is confirmed that the deposition time plays a vital role, when compared to other parameters with respect to the weight add on % and surface topography.

Figure 6.4 Effect of voltage duration (a) (b) 2min (c) (d) 6 min (e) (f) 10 min
6.3.4 UV Spectroscopy Analysis

In the silver nitrate mixed chitosan solution, the silver ions bind with the chitosan macro molecules via electrostatic interactions. The silver nitrate added acidic chitosan turns milky white. The oxygen rich groups present in the chitosan molecules interact with the electropositive silver metal cations. The metal ions covered chitosan macro molecules, i.e., silver nitrate with acidic chitosan mixture, were kept under electric potential to be deposited on the PCL substrates. Under the cathodic reduction reaction, the electropositive cation (Ag$^+$) becomes neutral (Ag$^0$), and forms a chitosan surrounded silver nano particle in the EPD process.

In cathode, $Chitosan/Ag^+ + e^- \rightarrow Chitosan/Ag^0$ \hfill (6.1)

The reduction of silver ions depends on different parameters, such as duration of deposition, polymer concentration and silver nitrate concentration in the formation of AgNPs by the EPD process.
The successful synthesis of AgNPs was first observed by the specific color changes in the deposited chitosan structure. Light combines with metal nano particles resulting in the oscillation of metal free electrons and this effect is called surface plasmon resonance (SPR). The SPR has a strong UV–visible absorption band. Specifically, in the case of AgNPs synthesis, the silver ions change colour from colorless to yellow during the formation of silver nano particles.

Figure 6.6 UV-vis spectra of different combinations

Figure 6.6 (a) shows the UV–vis absorption spectra of AgNPs prepared using chitosan with different concentrations (0.1 to 0.5 w/v, pH of 5.0, 10 minutes of deposition). It was observed that the peak intensity of SPR increases with increasing concentration of the chitosan macro molecules. As the concentration of chitosan was increased up to 0.5 w/v a SPR appeared at 403nm ($\lambda_{\text{max}}$) which confirmed the formation of the AgNPs. At lower chitosan concentration, below 0.5 w/v decrease in the SPR peak absorbance intensity was observed with a slight shift in wavelength. The wavelength shift from 421 to 403 nm (lower wavelength) by increasing the concentration indicates that the AgNP particle’s size reduced into a smaller one, as compared to a
lower polymer concentration. An increase in chitosan concentration 0.5w/v was attempted, but the addition of silver nitrate above this concentration made the chitosan solution into a jelly-like inhomogeneous solution. From the obtained results it can be concluded, that a chitosan concentration of 0.5w/v could be an ideal concentration to synthesize AgNPs by the EPD process on PCL fibrous substrates.

Figure 6.6 (b) shows the UV–vis absorption spectra of AgNPs synthesized at different deposition times (2 to 10 minutes, pH of 5.0, 1.0 mM AgNO₃ and 0.5w/v chitosan concentration) in the EPD process. From these results it is observed that on increasing the deposition time, the peak intensity of SPR also increases, and it suggests that a higher deposition time (10 minutes) is ideal for synthesis and deposition of AgNPs on PCL substrates. With increase in deposition time to 10 minutes the formation AgNPs might increase in the EPD process but our previous studies showed that a higher deposition time leads to a lower weight add on % of chitosan. Hence, 10 minutes of deposition time was chosen for further experiments.

Figure 6.6 (c) shows the UV–vis absorption spectra of AgNPs prepared using different AgNO₃ concentrations (0.1 to 1.0 mM AgNO₃, pH of 5.0, 10 minutes deposition time and 0.5w/v chitosan concentration). The aim of the variable precursor (AgNO₃) concentration in the EPD process is to find an effective precursor concentration to deposit AgNPs with potential resistance against micro organisms. It was observed in the experimental work that addition of silver nitrate above 1.0mM concentration in the chitosan solution is unable to yield a homogeneous solution. So, the concentration of silver nitrate was varied from 0.2mM to 1.0mM. The UV spectra shows the (Figure 6.6) results of the incorporation of different concentrations of AgNO₃; on keeping the other process parameters constant, the results reveal that the peak intensity of SPR increased by increasing AgNO₃ concentration in the
EPD process. It is evident from the experiments that at all the different concentrations of AgNO₃, formation of AgNPs takes place, but an effective AgNO₃ concentration can be selected only after conducting antimicrobial tests.

### 6.3.5 Antimicrobial Activity of the CS/AgNPs Coated Substrates

The antibacterial activity of chitosan deposited PCL substrates and CS/AgNPs deposited PCL fibrous substrates (with respect to variable parameter conditions) were tested against *P. aeruginosa* and *S. aureus* by the agar diffusion method.

Figure 6.7 shows the antibacterial activity of the fibrous substrates obtained from various chitosan concentrations (0.1 to 0.5w/v). From the results it is confirmed that all the chitosan concentrations have good activity against *S. aureus* and *P. aeruginosa*. The zone of inhibition was found to be higher for higher chitosan concentration (0.5 w/v) (Table 6.1). The result suggests that different polymer concentrations with AgNPs deposited fibrous substrates deposited can stop the growth of *S. aureus* and *P. aeruginosa* bacteria.

#### Table 6.1 Effect of antimicrobial activity with respect to variable polymer concentrations

<table>
<thead>
<tr>
<th>S.No</th>
<th>Polymer concentration (w/v)</th>
<th><em>S. aureus</em> (mm)</th>
<th><em>P. aeruginosa</em> (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>0.3</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>17</td>
<td>20</td>
</tr>
</tbody>
</table>
Figure 6.7 Effect of polymer concentration on antimicrobial activity (a) \textit{S.aureus} (b) \textit{P.aeruginosa}

The antibacterial activity of chitosan deposited PCL fibrous substrates is compared with CS/AgNPs deposited at variable deposition times (2, 6, 10 minutes), and other process parameters, such as silver nitrate concentration (1.0mM), chitosan concentration(0.5w/v), pH (5.0) were kept constant in the EPD process. The antimicrobial potential of the coated bio composite fibrous substrates was tested against the same bacteria species, and the results are shown in Figure 6.8.

Figure 6.8 Effect of time duration on antimicrobial activity (a) \textit{S.aureus} (b) \textit{P.aeruginosa}
From the results it is confirmed that a lower deposition time (2 minutes) shows an insignificant zone of inhibition as compared to other deposition times. The zone of inhibition data is given in Table 6.2, and it clearly indicates that a minimum of 6 minutes of deposition time is required to attain effective antimicrobial property. The results indicate that fibrous substrates electro deposited with CS/AgNPs for 6 minutes or above will show good activity against *S. aureus* and *P. aeruginosa* bacteria.

Table 6.2 Effect of antimicrobial activity with respect to different time durations of deposition

<table>
<thead>
<tr>
<th>S.No</th>
<th>Time duration (minutes)</th>
<th><em>S. aureus</em> (mm)</th>
<th><em>P. aeruginosa</em> (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>-</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
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<td>16</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>16</td>
<td>18</td>
</tr>
</tbody>
</table>

The antimicrobial activities of chitosan deposited with varying silver nitrate concentrations (0.2, 0.6, 1.0 mM) on PCL fibrous substrates is given in Figure 6.9.

![Figure 6.9](image)

Figure 6.9 Effect of silver nitrate concentration on antimicrobial activity (a) *S. aureus* (b) *P. aeruginosa*
The results show that for all the silver nitrate concentrations taken for the study, the antimicrobial activity can be observed. The zone of inhibition is observed in Table 6.3, and it clearly indicates that a minimum of 0.2mM silver nitrate coated substrates concentration shows good antimicrobial properties on the coated PCL fibrous matrices.

### Table 6.3 Effect of antimicrobial activity with respect to different time duration of deposition

<table>
<thead>
<tr>
<th>S.No</th>
<th>Silver Nitrate (mM)</th>
<th><em>S. aureus</em> (mm)</th>
<th><em>P. aeruginosa</em> (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>0.6</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>19</td>
<td>20</td>
</tr>
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</table>

The antimicrobial activity of CS/AgNPs coated PCL fibrous substrates have a stronger activity against bacteria; because the AgNPs attach to the cell walls, and disturb the cell wall permeability and cellular respiration.

### 6.3.6 Optimum Conditions for Coating of Chitosan/AgNPs on PCL Fibrous Substrates

The optimum process conditions must be selected to obtain effective microbial resistance and biocompatibility for biomedical applications. From the previous the studies, time duration of 2 minutes for deposition was eliminated for further studies, due to low microbial activity, and poor surface topography as seen in SEM images. The other process variables, such as polymer concentration and time duration were selected at their maximum level (0.5w/v and 10 minutes), because they show better antimicrobial resistance with good morphological structure. To select optimum AgNO₃ concentrations, further testing in terms of biocompatibility
assay and silver release was conducted. A higher silver release from the substrates is toxic to the cells. A low and effective AgNO₃ concentration is to be selected based on the above studies.

Initially, a blood compatibility test was conducted for different silver nitrate concentrations (0.2, 0.6, 1.0mM) electro deposited on PCL fibrous substrates. Table 6.4 shows the hemolysis ratio of different combinations and it clearly indicates that irrespective of the silver nitrate concentration used in the electro deposition process, all the sample show below 1% hemolysis. The obtained results indicate that all samples are biocompatible in nature. From this study, a higher concentration of AgNO₃ (1.0mM) which is also biocompatible was selected for silver release study with other parameters kept constant at deposition time (10 minutes) and concentration of chitosan (0.5w/v). The results of release study are given in Table 6.5. It clearly suggests that, the amount of silver ion released from the substrates is not a burst release, and it is controlled and also within the toxic level. From these results it can be concluded that a chitosan concentration of 0.5w/v, 10 minutes of deposition time, and 1.0mM silver nitrate with 5 V were an ideal combination for biomedical applications.

Table 6.4 Effect of silver nitrate concentration on blood biocompatibility

<table>
<thead>
<tr>
<th>S.No</th>
<th>Sample</th>
<th>% Hemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PCL mat</td>
<td>0.01</td>
</tr>
<tr>
<td>2</td>
<td>CS/PCL</td>
<td>0.21</td>
</tr>
<tr>
<td>3</td>
<td>CS/Ag (0.2mM)/PCL</td>
<td>0.24</td>
</tr>
<tr>
<td>4</td>
<td>CS/Ag (0.6mM)/PCL</td>
<td>0.32</td>
</tr>
<tr>
<td>5</td>
<td>CS/Ag (1.0mM)/PCL</td>
<td>0.49</td>
</tr>
</tbody>
</table>
Table 6.5 Silver release from coated PCL fibrous matrices

<table>
<thead>
<tr>
<th>S.No</th>
<th>Time (Hours)</th>
<th>Concentrations (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>0.010</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>0.012</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>0.029</td>
</tr>
<tr>
<td>4</td>
<td>48</td>
<td>0.036</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>0.036</td>
</tr>
</tbody>
</table>

To enhance the silver nano particle activity, the particle size has to be reduced, and to increase the water uptake properties of a hydrophilic polymer polyethylene glycol (PEG) was added along with chitosan in the EPD process. The following section characterizes the morphology of silver nano particles morphology and surface using the TEM and SEM analysis with respect to the addition of different molecular weight of PEG in the EPD process. Moreover, discussion pertaining biofilm resistance and biofilm disruption activity of the developed composite fibrous substrates are given.

6.3.7 Surface Morphology Analysis

Pristine PCL fibrous substrates were successfully obtained by the centrifugal spinning technique. The chitosan/silver nano particles (CS/AgNPs), chitosan/low molecular weights PEG/silver nano particles (LMP/CS/AgNPs) and chitosan/ high molecular weight PEG/silver nano particles (HMP/CS/AgNPs) were electro-deposited on the PCL fibrous substrates. The surface topography was analyzed with the help of SEM and optical microscope images, and are shown in Figures 6.10 and 6.11. It is very clear that in all the combinations (CS/AgNPs, LMP/CS/AgNPs, and HMP/CS/AgNPs), the coating was uniform. Interestingly, embedded
polymeric spheres (Figure 6.10 (a)) and surface adhered free spheres (Figure 6.10 (c-f)) on the coated surface were also found.

Figure 6.10 (a),(b) CS/AgNPs (c),(d) CS/LMP/AgNPs (e)(f) CS/HMP/AgNPs
The sphere sizes varied from 250 nm- 1µm, and were randomly deposited on the surfaces. Still, to understand more about morphology, the coated PCL fibrous substrates were examined under optical microscope. The deep yellow background (Figure 6.11) confirmed the presence of silver nano particles by the surface plasmon resonance effect. The optical microscopic images also confirm the presence of spheres, and additionally showed the presence of spheres inside the coated structure. For the robust confirmation about the polymeric spheres, the electro deposited PCL fibrous substrates were immersed in water and viewed under the optical microscope. The free surface adhered spheres were released from the coated PCL fibrous substrates, and their hollow nature can be found from Figure 6.12. The free surface adhered micro spheres released from the coated substrates in the aqueous medium gives an advantage in wound dressing, because micro spheres can be released into the wounds on contacts with the exudates. The micro spheres can act as carriers for AgNPs to be delivered to the surrounding wound sites.

Figure 6.11 Optical microscope images of CS/HMP/AgNPs
Figure 6.12 Microspheres releasing in water medium (a), (b), (c) different magnifications

At the same time, the entrapped spheres in the matrices can be seen inside the deposited chitosan structure, and their mobility is restricted from the coated structure, as shown in Figure 6.13.

Figure 6.13 Trapped spheres inside the deposited chitosan structure on coated PCL fibrous substrates (a) lower (b) higher magnification
The schematic diagram related to AgNPs loaded polymeric microsphere formation and attachment on PCL fibrous substrates is given in Figure 6.14. When voltage is applied, at the cathode, polymeric gas bubbles are formed and these polymeric gas bubbles become a hollow micro-sphere as it gets dried. These micro spheres attached to the PCL fibrous substrates in three regions, namely, inside the structure (entrapped), embedded (partially visible), and freely adhered on the surface. An entrapped microsphere inside the coated surface occurs due to simultaneous deposition of chitosan along with microspheres on the PCL substrates. Embedded (partially viewed on the surface) spheres occurs by stopping the deposition process, when the spheres are partially covered by the deposited chitosan. Freely attached microspheres on the surface occur by already formed spheres floating in the bath. The coated PCL fibrous substrates back side was also examined under the SEM, and it reveals that most of the fibrous form is available as it is and chitosan had partially infiltrated in between the fibrous structure (Figure 6.15). The entire face side of the PCL surface was coated with hydrophilic chitosan.

Figure 6.14 Scheme I Microsphere formation (a) Embedded (b) Surface freely attached (c) Entrapped inside the deposition
6.3.8 UV Spectroscopy and TEM Analysis

In situ AgNPs were synthesized by the electro chemical reduction of AgNO$_3$, with the help of chitosan and different molecular weights of PEG. The UV–visible spectra of chitosan and chitosan/PEG loaded AgNPs are shown in Figure 6.16. On the addition of low molecular weight polyethylene glycol (LMP) and high molecular weight polyethylene glycol (HMP) in the AgNO$_3$ solution, the concentration of the silver nano particles also increases accordingly. The inset photograph in Figure 6.16 shows that the nano silver colloids are yellow in color with chitosan alone, and turn into a deep yellow color with the increasing molecular weight of PEG. From the literature it was found that, spherical AgNPs contribute to absorption approximately at around 410 nm in the UV-Vis spectra (Rivero et al 2013). In the present work also, the UV-Vis spectra show $\lambda_{\text{max}}$ approximately at 406 nm (Regiel et al 2013), indicating that the morphology of AgNPs co-deposited along with chitosan and PEG, is spherical in shape. Additionally, the TEM images also confirm the spherical shape of the silver nano particles. The incorporation of PEG in
chitosan/AgNO₃ solution reduced the silver nano particle’s size upto 15nm (Figure 6.17). It is clearly reported in the literature, that PEG acts as a stabilizing agent, and on increasing its molecular weight, the silver particle size was reduced (Vimala et al 2010). In the chitosan/PEG solution, when silver nitrate is added, it forms a metallapolymer (CS/PEG/Ag)+ complex. Under electric field, the metallapolymer complex migrates towards the cathodic surface, where the PCL fibrous substrates are attached. Due to the cathodic reduction reaction, the deposited (CS/PEG/Ag)+ complex reduced in (CS/PEG/Ag)⁰ (reduced form) and the schematic diagram explains (Figure 6.18) the formation of the silver nano particles. Interestingly, in the TEM images it is clear, that the chitosan and chitosan/PEG capped each silver nano particle well (Figure 6.19). The capped silver nano particles aid slow release of AgNPs from the matrices.

Figure 6.16 UV/Vis spectra and Photograph of the CS/AgNPs and CS/PEG/AgNPs coated PCL fibrous substrates
Figure 6.17 TEM image (a) CS/AgNPs (b) CS/LMP/AgNPs (c) CS/HMP/AgNPs deposited substrates

Figure 6.18 Schematic diagram of capped AgNPs formation
6.3.9 FTIR Analysis

The presence of chitosan/AgNPs and chitosan/PEG/AgNPs on the centrifugal spun PCL fibrous substrates surfaces was confirmed by the FTIR spectrum, and it is shown in Figure 6.20. The control sample of the centrifugal spun PCL fibrous substrates has CH₂ characteristic peaks at 2947 and 2986 cm⁻¹, and also at 1366, 1407, and 1465 cm⁻¹. The C=O stretching occurs at 1731 cm⁻¹ and -COO vibrations occur at 1184 and 1242 cm⁻¹. The pure chitosan FTIR spectrum is given in Figure 6.20 (b) for comparative purpose, and chitosan/AgNPs is shown in Figure 6.20 (c). In Figure 6.20 (c) a broad vibration occurs at 3400 cm⁻¹ due to O–H and N–H stretching. CONH₂ absorption band is observed near 1651 cm⁻¹ in pure chitosan, which is shifted from 1670 cm⁻¹ in the chitosan /AgNPs spectra. This shift indicates the binding of AgNPs to the N–H bond of chitosan. In Figure 6.20 (d) CS/PEG/AgNPs, the presence of PEG was confirmed by the peaks from 1055 to 1149 cm⁻¹ as the stretching of ether groups and also, characteristic alkyl
(R-CH$_2$) stretching mode at 2882 cm$^{-1}$, and the hydroxyl group’s contributions at wave number 3470 cm$^{-1}$ were observed. The AgNPs interact with the PEG chain molecules and are associated with the peak at 3406 cm$^{-1}$. The peak at 1652 cm$^{-1}$ indicates the C=O in amino groups, and its disappearance indicates its involvement in complexation with AgNPs. The most prominent band at 1560 cm$^{-1}$ is due to the amino group in the pure chitosan film, whose intensity lowered the wave number (1548 cm$^{-1}$) in the presence of silver nano particles. The broad spectrum is due to the presence of van der waals interactions between the hydroxyl groups of chitosan/PEG and the partial positive charge on the surface of the AgNPs (Mandal et al 2012). The result clearly identifies the involvement of primary amino groups in the interaction with the metal surface, the amino groups acting here as capping sites for the silver nano particle’s stabilization.

Figure 6.20 FTIR spectrum of coated substrates (a) PCL (b) CS film (c) CS/AgNPs coated substrates (d) CS/HMP/AgNPs coated substrates
6.3.10 Water Absorption Analysis

A moist environment is an essential requirement of a wound dressing material because it helps in the penetration of active materials that additionally support the easy removal of the wound dressing without any pain. The wound dressing also should absorb the exudates from the wound sites. These properties can be obtained by measuring the wound dressing swelling capacity. Figure 6.21 shows that the various samples attained swelling in deionized water within 2 hours.

![Figure 6.21 Water uptake % of the coated substrates: a comparison](image)

The swelling ratio of pristine PCL fibrous mat shows negligible amount swelling. Chitosan alone deposited PCL fibrous mat showed about a swelling of 143 times its dry weight, whereas chitosan/AgNPs coated mat
shows lower than chitosan alone coated mat exhibited swelling of 137%. It may be attributed to the binding of the AgNPs with the amine group present in the chitosan, and these amine groups are mainly responsible in the swelling behavior of chitosan. CS/LMP and CS/HMP show 231 and 293% of swelling compared to their own dry weight. The adding of AgNPs shows slightly reduced swelling percentage for CS/LMP/AgNPs and CS/HMP/AgNPs (212 and 274%). The results confirm that the addition of polyethylene glycol enhances the swelling behavior of the coated substrates.

6.3.11 Blood Compatibility Assay

The degree of red blood cells destroyed by a material on contact with blood is calculated in terms of the hemolysis ratio. The influence of the coated PCL fibrous substrates on the hemolysis ratio and their comparison are shown in Figure 6.22.

![Figure 6.22 Blood biocompatibility assay](image)
The results showed that both the CS/Ag NPs coated and CS/PEG/AgNPs samples did not cause any hemolysis. The PEG incorporated samples showed a slightly higher hemolysis ratio, compared to the other samples. This may be due to the quick release of the silver nano particles from the coated substrates. However, all the samples show hemolysis ratios within the acceptable limits of hemocompatibility (less than 5%) for a biomaterial (Chandra et al 2012).

6.3.12 Silver Release Study

The actual amount of silver present in the various deposited samples were found by dissolving 2cm$^2$ of the sample in 10ml of 50% nitric acid (HNO$_3$), and by digesting in mild heat for 10 minutes, followed by adjusting the volume to 100ml using millipore water. The resulting solution was used directly to determine the actual silver content by ICP-PES. The silver content was found to be 665,732, 853ppm for CS/AgNPs, CS/LMP/AgNPs and CS/HMP/AgNPs deposited substrates. The release characteristics of silver ions from the CS/AgNPs, CS/LMP/AgNPs, and CS/HMP/AgNPs were investigated for 2, 24, 48, and 72 hours by immersion method in distilled water. Initially, a 2 hours release was studied to determine the burst release from the coated substrates. It was found that initially 0.01, 0.028, 0.086ppm were released from CS/AgNPs, CS/LMP/AgNPs, CS/HMP/AgNPs coated samples. It was observed from the literature that the initial burst release of silver ions obtained was well below the toxic level (Zhao et al 2012). The amount of silver ions released from the CS/HMP/AgNPs substrates was higher than the amount released from CS/LMP/AgNPs, CS/AgNPs samples (Table 6.6). This may be attributed to the fact, that the swelling ratio was higher for high molecular weight PEG molecules. It was clear from the study that, the incorporation of PEG
molecules enhances not only swelling behavior of the coated substrates but also the release behavior of the silver ions from the coated substrates.

Table 6.6 Silver releasing from different coated substrates

<table>
<thead>
<tr>
<th>S.No</th>
<th>Release in hours</th>
<th>CS/AgNPs (ppm)</th>
<th>CS/LMP/AgNPs (ppm)</th>
<th>CS/HMP/AgNPs (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>0.010</td>
<td>0.028</td>
<td>0.086</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>0.012</td>
<td>0.064</td>
<td>0.244</td>
</tr>
<tr>
<td>3</td>
<td>48</td>
<td>0.029</td>
<td>0.272</td>
<td>0.578</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>0.036</td>
<td>0.288</td>
<td>0.608</td>
</tr>
</tbody>
</table>

6.3.13 Antimicrobial Activity

Infection is a major reason for delayed wound healing and colonies of bacteria interfere with the progress of the wound healing process. Silver and silver containing substrates have long been known to have a strong and broad spectrum of bactericidal effects, even though, the mechanisms of the bactericidal effect of silver is still not understood very clearly. In this study, the antibacterial efficacies of the samples were tested against *P. aeruginosa* and *S. aureus*. The zones of inhibition observed (Figure 6.23) for the various coated substrates are given in Table 6.7. The efficacy against *P. aeruginosa* and *S. aureus* of CS/AgNPs loaded substrates showed a lower zone of inhibition compared to different molecular PEG loaded AgNPs substrates.
Several studies has proposed that the mechanism of antimicrobial activity of AgNPs is that, (i) AgNPs attach to the bacterial cell substrates and disturb the permeability, and respiratory functions (ii) AgNPs release silver ions (iii) AgNPs penetrate inside the bacteria and cause further damage to DNA.

Table 6.7 Antimicrobial efficacy of different deposited substrates on bacteria

<table>
<thead>
<tr>
<th>S.No</th>
<th>Deposition on PCL substrates</th>
<th>S. aureus (mm)</th>
<th>P. aeruginosa (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>CS/AgNPs</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>CS/LMP/AgNPs</td>
<td>13</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>CS/HMP/AgNPs</td>
<td>15</td>
<td>16</td>
</tr>
</tbody>
</table>
6.3.14 Biofilm Resistance and Disruption Analysis

The debriment and irrigation of an infected wound removes most of the bacteria. If any bacteria remain in the wounds, they will replicate and develop a new biofilm. *P. aeruginosa* and *S. aureus* are the major cause of morbidity and mortality in wound patients. In the present study, bacteria were seeded on a porous cellulose membrane present on an agar plate. The pores presence in the cellulose disk is permeable to nutrients to the bacteria on the disk; like nutrient rich environment presents in the wound sites. This in vitro biofilm model resembles the infected wound environment. Freshly infected wounds are likely to have less number of bacteria with a nutrient rich environment. This low number of bacteria can multiply and develop a mature biofilm within 24 hours. First an in vitro biofilm prevention test was conducted for the CS/AgNPs, CS/LMP/AgNPs, and CS/HMP/AgNPs deposited samples. To mimic the fresh wound situation, initially 0.1 OD (optical density) value of 12 hours bacterial culture (0.1µl) was applied on cellulose disk. The deposited samples were slightly wetted with water to adhere well on the bacteria seeded cellulose disk. After that, it was allowed to stand for 24 hours at 37°C, and a quantitative analysis was carried out. From the experimental results, it was determined that all AgNPs deposited along with CS and PEG (LMP,HMP), showed complete inhibition or prevented (Figure 6.27) the development of a bacterial biofilm. But the chitosan alone coated substrates were unable to control the complete growth of the bacterial biofilm, as compared to the AgNPs coated deposited samples.
Figure 6.24 Colonies developed by (a) control (b) CS (c) CS/AgNPs (d) CS/LMP/AgNPs (e) CS/HMP/AgNPs deposited PCL substrates by \textit{P.aeruginosa} bacteria
Figure 6.25 Colonies developed by (a) control (b) CS (c) CS/AgNPs (d) CS/LMP/AgNPs (e) CS/HMP/AgNPs deposited PCL substrates by *S. aureus* bacteria
Figure 6.26 CFU of (a) *P. aeruginosa* and (b) *S. aureus* bacteria

![Figure 6.26 CFU of (a) P. aeruginosa and (b) S. aureus bacteria](image)

Figure 6.27 Colonies developed by (a) control (b) CS/AgNPs coated substrates (c) CS/AgNPs substrates treated disk on *P. aeruginosa* bacteria

![Figure 6.27 Colonies developed by (a) control (b) CS/AgNPs coated substrates (c) CS/AgNPs substrates treated disk on P. aeruginosa bacteria](image)
Chitosan alone deposited substrates partially retard the growth of bacteria and showed CFU (log \(_{10}\) 5.8, 5.1 in disk for \(P.\) aeruginosa and \(S.\) aureus (Figure 6.26). Additionally it was found that there is no bacterial colonies adhered on the all AgNPs deposited substrates respectively.

Chitosan deposited substrates (placed on cellulose disk) showed adhered bacteria CFU of log \(_{10}\) 1.3 and 1.7 for \(P.\) aeruginosa and \(S.\) aureus. The qualitative analysis by SEM showed (Figure 6.27) that the controlled sample (\(P.\) aeruginosa) produced a well developed mature biofilm on the disk, CS/AgNPs coated substrates, and the placed disk showed no biofilm growth on it. Finally, it was concluded from the studies that the chitosan alone coated substrates are unable to prevent complete development of biofilm formation but it partially retards the growth. On the other hand, all AgNPs loaded along with CS, CS/LMP and CS/HMP showed complete inhibition or prevention against both gram positive and gram negative wound pathogen bacteria.

The second goal of the experiment is to examine the effectiveness of various coated substrates, against the already formed matured biofilm bacteria. The matured biofilm was formed by seeding over night bacterial culture on the cellulose disk, and allowing it to stand for 24 hrs. After formation of a matured biofilm, the samples were placed on the cellulose disk. After a period of 24 hours, the samples were separated from the disk and involved in a quantitative colony counting test for both the cellulose disk and the coated substrates.

The colony counting results of the cellulose disk and coated substrates are shown in Figure 6.28. With exceptions of control biofilm, and chitosan alone coated substrates, the other coated substrates such as CS/AgNPs, CS/LMP/AgNPs, and CS/HMP/AgNPs showed reduced biofilm
produced by both the bacteria (Figure 6.29 and 6.30). In contrast, bacterial presences in the coated substrates were high as compared to the cellulose disk. This might be because chitosan and chitosan/PEG coated substrates are hydrophilic in nature and the bacterial colonies adhere well on them. Even the AgNPs loaded substrates were unable to inhibit the complete biofilm presence in the cellulose disk. However, all the AgNPs coated substrates showed a reduction in bacterial biofilm growth in the following order of CS/AgNPs < CS/LMP/AgNPs < CS/HMP/AgNPs. The burst release and high surface area of the silver nano particles present in high molecular weight PEG/chitosan in the PCL substrates are the major reasons for better reduction found in this combination. The SEM visualization of the biofilm produced by *P. aeruginosa* is shown in Figure 6.31. From the SEM images it can be confirmed that the presence of bacteria in the coated substrates (CS/AgNPs) was higher compared to the cellulose disk. Additionally, the tested agar plate is shown in Figure 6.32 for *P. aeruginosa* and *S. aureus*. Visualization of agar plates show retarding effect in bacteria growth in case of AgNPs coated samples. Even though all the silver nano particles loaded substrates are unable to disrupt the entire matured biofilm, they effectively retard the growth of the bacterial biofilm. In addition to that, the greater biofilm adherent nature of the coated substrates prevent the adhered bacteria spreading further from the substrates.
Figure 6.28 CFU of (a) *P. aeruginosa* (b) *S. aureus*
Figure 6.29 Colonies developed by (a) CS (b) CS/AgNPs (c) CS/LMP/AgNPs (d) CS/HMP/AgNPs deposited PCL substrates by *P. aeruginosa* bacteria and the subscript (1) indicates the colonies developed from cellulose disk and (2) indicates the colonies developed from coated substrates.
Figure 6.30 Colonies developed by (a) CS (b) CS/AgNPs (c) CS/LMP/AgNPs (d) CS/HMP/AgNPs deposited PCL substrates by S.aureus bacteria and the subscript (1) indicates the colonies developed from cellulose disk and (2) indicates the colonies developed from coated substrates.
Figure 6.31 Colonies developed by (a) control (b) CS/AgNPs coated substrates (c) CS/AgNPs substrates treated disk on *P. aeruginosa* bacteria

Figure 6.32 In-vitro biofilm disruption tested agar plate after samples removal from (a) *S. aureus* (b) *P. aeruginosa* bacteria; (1) blank (2) CS alone (3) CS/AgNPs (4) CS/LMP/AgNPs (5) CS/HMP/AgNPs coated substrates
From the biofilm prevention model, all AgNPs deposited substrates completely inhibit bacteria present in the disk, and there are no bacteria found on the substrates also. The mechanisms of the inhibition of bacteria are similar to the planktonic bacteria explained in the agar diffusion study. In the case of the biofilm disruption model, the same AgNPs deposited substrates are unable to completely inhibit or disrupt the entire biofilm. It is universally accepted that, to disrupt the mature biofilm, a higher concentration of antimicrobial agent required, compared to the concentration required to kill susceptible planktonic forms. Unfortunately, it is not advisable to increase the concentration of AgNPs, because it also creates problems to the normal cells. To enhance the existence of AgNPs present in the coated substrates, they were electrically activated by low electric voltage. Additionally, recent research reports suggest, that a low electric stimulus enhances cell proliferation in the wound healing process.

6.3.15 Bioelectric Field Effect of Biofilm Disruption

To enhance the antimicrobial property of the AgNPs loaded substrates, three different voltages were applied on the (1.5, 3.5 and 5.5 V) substrates in the duration of one hour. The surviving bacterial presence in the disk and coated substrates were determined, with respect to the variable voltage, by the quantitative colony counting method. The results are shown in Figure 6.33 and 6.34. There is a very high decrease in the surviving bacteria in both the disk and the coated substrates. From the results, it was found that 1.5 V reduced the bacteria CFU of $1.3\log_{10}$ for CS/AgNPs deposited substrate, which indicates that electric stimulus drastically reduced the bacteria within one hour. But, the other voltages 3.5 and 5.5 V showed complete inhibition of biofilm bacteria. The control sample without any substrates and chitosan alone coated substrates also showed slight reduction in
the bacterial biofilm. From these results it can be confirmed that not only are
the AgNPs involved in the bacterial inhibition, but the additionally applied
current also played a vital role in the bacterial reduction. To visually view the
results, the bacterial turbidity with respect to 3.5V is shown in Figure 6.35.
The CS/HMP/AgNPs deposited substrates show a clear solution as compared
to the control sample. As explained in an earlier section, the drastic release of
silver with the high surface of AgNPs, and the applied voltage completely
inhibit the biofilm bacteria. The mechanism for the biofilm disruption of
AgNPs coated PCL fibrous substrates can be either one or the combination of
(i) the release of silver ions, (ii) liberation of reactive oxygen species (ROS)
or by (iii) contact killing in an acidic pH environment of the coated substrates.
The release of silver ions alone is unable to control the bacterial growth and it
should be a combined effect on the destruction of biofilm bacteria in both
gram positive and gram negative organism. It was found that in the
experiment that the pH on the substrates reaches around 5.0, 3.5 and 1.0 for
1.5, 3.5 and 5.5 V respectively. All the previous reports suggest (Choi & Hu
2008; Chaloupka et al 2010 2009) that liberation of ROS is the major reason
for the inhibition of the bacteria. It can be concluded from the experiment that
3.5 V applied for an hour is enough to completely inhibit the biofilm.
Figure 6.33 Colonies developed 3.5 V applied to (a) blank (b) CS (c) CS/AgNPs (d) CS/HMP/AgNPs deposited PCL substrates by *P. aeruginosa* bacteria and the subscript (1) indicates the colonies developed from cellulose disk and (2) indicates the colonies developed from the coated substrates.
Figure 6.34 Effect of Voltage on deposited substrates

Figure 6.35 Effect of 3.5 V on (a) cellulose disk alone; (b) CS coated (c) CS/AgNPs (d) CS/HMP/AgNPs coated substrates
6.3.16 MTT Assay

The toxicity of the coated PCL fibrous substrates was evaluated by the MTT assay, which quantitatively measures metabolically viable cells. All combinations of the electro deposited substrates were subjected to cell culture studies for 24 hours. The results of the MTT assay are given in Figure 6.36. A slight decline was observed in the optical density (OD) value with respect to the addition of AgNPs and HMP/AgNPs with chitosan. It demonstrated that the viability of cells was slightly retarded by the incorporation of silver nanoparticles. But all the coated substrates had shown more than 87% viability of cells.

![Figure 6.36 MTT assay of different deposition on PCL substrates](image)

The CS/HMP/AgNPs substrates showed the least OD value as compared to the CS/AgNPs substrates, which indicates the higher cytotoxicity due to burst release of silver ions from the substrates. In general, CS/AgNPs substrates showed better cell viability as compared to other combinations of the deposited substrates due to controlled slow release of silver ions.
6.4 CONCLUSIONS

The AgNPs was successfully synthesized and co-deposited on the PCL substrates. The presence of AgNPs was confirmed by UV spectrophotometer and TEM image analysis. It was found that, at 0.5%w/v concentration of chitosan along with 1.0mM concentration of silver nitrate, with a deposition time of 10minutes showed a potential microbial resistance against gram positive and gram negative bacteria. In was found that, the addition of PEG molecules effectively reduce the AgNPs size and swelling property of the coated substrates. Further, it was found that, microspheres were formed and attached in places of the coated substrates. The silver ion release study confirmed that released silver ions were within the toxic levels and the samples are of biocompatible in nature for blood and cytotoxic studies. All AgNPs loaded showed effective resistance against biofilm formation but it failed in biofilm disruption model. The AgNPs loaded substrates activity against bacteria can be enhanced by application of low electric voltage. The entire bacterial biofilm growth was destroyed at 3V. From the obtained results, it was confirmed that the developed coated substrates are promising materials for wound dressing applications.