Effect of chitosan coated chemogenic silver nanoparticles coated syringes against biofilm of clinical isolate of *Staphylococcus aureus*

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**ABSTRACT**

Biofilm represents the most prevalent type of virulent factor of most of the pathogenic microorganism and involved in crucial development of clinical infection and exhibit resistance to antimicrobial agents. Now the biofilm is considered as major target for the pharmacological development of drugs. A biofilm serves to promote bacterial persistence by resisting antibiotic treatment and host immune responses. Antibiotics are rendered ineffective when biofilms form due to their relative impermeability, the variable physiological status of microorganisms, subpopulations of persistent strains, and variations of phenotypes present. Metal nanotechnology chemistry has the potential to prevent the formation of these life-threatening biofilms on life supporting devices. In the present study, anti biofilm effect of silver nanoparticles coated syringes against clinical isolate of *Staphylococcus aureus* was studied. Chitosan stabilized silver nanoparticles synthesized by chemical reduction method and the synthesized particles were coated on the surface by ultrasonication. Coated syringes were characterized by scanning electron microscopy (SEM) which reveals complete dispersion of the nanoparticles on the fibre surface and the size, shape of the particles shows uniform spherical particles with the size of 60-70 nm. Distinct effect of biofilm inhibition was recorded in the nanoparticles coated syringes and maximum inhibition was observed during 72 hour of incubation. Biochemical composition of biofilm matrix mainly total carbohydrates and total protein was highly reduced. The present study would suggests the development of anti microbial coated medical devices against pathogenic microorganism.

**Keywords.** Biofilm, *Staphylococcus aureus*, Silver nanoparticles, Chitosan

1.**INTRODUCTION**

Nanobiotechnology, the convergence of nanotechnology and biotechnology and in particular its applications in the medical sector are considered as one of the most promising and most advanced areas of nano technology[1]. The application of nanotechnology in the field of healthcare has come under great attention in recent times. There are many treatments today that take a lot of time and are also very expensive. Using nanotechnology, quicker and much cheaper treatments can be developed. By performing further research on this technology, cures can be found for diseases that have no cure today. The application of such a technology can be used for the inhibition of biofilm formation on the surgical and medical devices which are of higher threat in the process of treatments. Bacteria are able to grow adhered to almost any surface, forming architecturally complex communities termed biofilms [2,3]. Microbial biofilms develop when microorganisms irreversibly adhere to a submerged surface and produce extracellular polymers that facilitate adhesion and provide a structural matrix. This surface may be inert, nonliving material or living tissue. Biofilm-associated microorganisms behave...
differently from freely suspended organisms with respect to growth rates and ability to resist antimicrobial treatments and therefore pose a public health problem [4, 5]. Due to increasing tolerance of the biofilm community to antibiotics, biocides and mechanical stress, it has become just as difficult to completely eradicate mature biofilms as it is to completely avoid the presence of planktonic cells, the origin of the biofilm in the water. Common treatments to prevent or remove biofouling include using disinfection, minimizing nutrients in the feed or altering surface materials to prevent bacterial attachment, or clean-in-place (CIP) to remove mature biofilm by chemical or mechanical shear. Several studies have examined the effect of various types of antimicrobial treatment in controlling biofilm formation on medical devices[6, 7, 8]. The vast majority of the chemical agents currently available for biofilm control are broad-spectrum non-specific micro biocide agents [9]. Chlorhexidine, triclosan, and essential oils (e.g., Listerine) are the most commonly used and clinically tested antimicrobials [10]. Biofilm-control strategies based on disruption of EPS formation on the surface could be an effective alternative (or adjunctive) approach [11]. In order to control biofilm formation on medical devices and all costs associated, a large number of new strategies and approaches have been developed in the last few years, including: antimicrobial locks (in the case of catheters) [12]; surface modification of biomaterials with antimicrobial coatings [13]; the use of quorum sensing (QS) inhibitors [14], antimicrobial peptides as a new class of antibiotics [15]; enzymes that dissolve biofilms [16], nitric oxide [17], electrical [18] or ultrasound [19] enhancement of antimicrobial activity, or even the application of light activated antimicrobial agents [20]. Nevertheless, nanoscale materials have recently appeared as one of the most promising strategies to control biofilm infections related to indwelling medical devices, especially due to their high surface area to volume ratio and unique chemical and physical properties [21]. A nanomaterial has a diameter ranging from 1 and 100 nm, and they can be made from different materials, like copper, zinc, titanium, magnesium, gold, alginate and silver. The use of silver nanoparticles (NPs) is now considered as one of the most promising strategies to combat biofilm infections related to indwelling medical devices [22]. Drug delivery nano carriers systems, such as liposomes [23] and polymer-based [24] carriers have also arisen as appealing methods with a great potential in the treatment of biofilm infections, due to several factors especially good biocompatibility and ample range and extent of drugs that they can carry. Another important factor is the protection provided by the encapsulation of the drug in the biological milieu, decreasing toxicity and allowing the drug to reach the specific site [25]. Chitosan is another natural polymer has been reported as a polymer-based protective agent to stabilize the metal nanoparticles[26].Because of the biocompatibility,biodegradability, nontoxicity and adsorption properties of chitosan, it was used as a stabilizing agent to prepare Ag, Au and Pt nanoparticles. These chitosan-protected nanoparticles can be easily integrated into systems relevant for pharmaceutical, biomedical, and biosensor applications. Therefore, it has attracted considerable interest due to its medicinal properties, such as antifungal, antibacterial, antiprotzoal, anticancer, antiplaque, antitartar, hemostatic, wound healing and potentiates anti-inflammatory response, inhibits
the growth of cariogenic bacteria, immunopotentiating, antihypertensive, serum cholesterol lowering, immune enhancer, increases salivary secretion (anti-xerostomial) and helps in the formation of bone substitute materials[27]. The present study is aimed to evaluate anti biofilm effect of biocompatible polymer stabilized metallic nanoparticles coated syringes against clinical isolate of Staaph. aureus under in vitro condition.

2. MATERIALS AND METHODS

Coating of metallic nanoparticles on syringes
Syringes were obtained from Solaguard (Chennai, Tamil Nadu, India). The outer transparent portion of syringes were cut into 4 pieces and transferred to beaker containing 0.1 molar AgNO₃ and 0.1 molar tri sodium citrate placed in ultrasonicator. Freshly prepared 0.1 molar sodium borohydride was added drop by drop till reaction mixture turned into brown. The preparation was left in ultrasonicator for 2 hours to facilitate complete dispersion of nanoparticle on surface. The coating of nanoparticles on syringe was primarily confirmed by colour change of cut pieces of syringe into brown colour. The pieces were dried at 40°C overnight to remove excess moisture. The dried pieces were kept in sterile petriplate for further study. Chitosan coated nanoparticle was also coated by chemical reduction method of respective metal precursor with reducing agent and 0.1 molar chitosan as a stabilizer agent. Chitosan was obtained from SRL laboratory and deacetylation process was done and degree of deacetylation was determined using Viscometric method. The pre-treated chitosan as described earlier was dissolved in 1% w/v acetic acid (1 mL of acetic acid in 100 mL of distilled water) and suspension was transferred to a beaker containing respective metal precursor and a reducer. The homogenous slurry thus obtained was coated with cut pieces. Before coating, the suspension was characterized by scanning electron microscopy (SEM equipped with energy dispersive x ray atomic spectroscopy (EDAX), the mixtureThe coated cut pieces thus obtained were dried at 40°C as described earlier.

Biofilm inhibition assay
The metallic nanoparticles coated syringes kept in sterile Petri plates were inoculated with 5 mL of S. aureus culture. The plates were allowed for incubation at 37°C for 72 hours. After incubation period the treated syringes were stained with 0.1% w/v of crystal violet solution for 15 minutes at room temperature. After staining the syringe pieces were washed with phosphate buffered saline (PBS) solution to remove free planktonic cell. Further washing was carried out with 95% of ethanol for 3 times at room temperature and the washed solution was collected and absorbance was measured spectrophotometrically at 540nm.

The percentage of biofilm inhibition was calculated by following formula:

\[
\text{Biofilm inhibition (\%) = \frac{OD \text{ in control} - OD \text{ in treatment}}{OD \text{ in control}} \times 100}
\]
Biofilm Kinetics

Biofilm kinetics was done to study the inhibition percentage of nanoparticles coated syringes against biofilm of Staphylococcus aureus with respect to time. Fresh syringe was taken and dispersed in metallic nanoparticles - AgNp, AgNp-CS. The syringe was kept in beaker containing AgNp and kept for sonication in water bath sonicator for 3 hours likewise performed for AgNp-CS and kept them in water bath sonicator for 3 hours. Once coating was done, the Petri plates were kept in dry air oven at 46°C for 2 hours. After drying, inoculation was done by spraying 5 mL of Staphylococcus aureus on different nanoparticle coated syringes and kept for incubation for different time interval. Fresh syringe was taken as control in another petri plate. After 12 hours of incubation, Ag coated syringe was cut into 1st part with surgical blade and the remaining part was kept for further incubation. Similarly AgNp-CS coated syringe was cut and the remaining portion was kept for incubation. Incubated coated syringes and control syringes were dipped in 2 mL of 0.1% w/v of crystal violet in sterile boiling test tubes each, shaken properly and kept for incubation at room temperature for 15 minutes. After the incubation period crystal violet was removed with sterile micro tip then the syringes were washed with 2 mL of sterile phosphate buffered solution twice. It was aspirated and PBS was discarded, 5 mL of ethanol was added to each tube and was kept on ultrasonicator for 15 minutes. Elutants were measured at 540nm spectrophotometrically and reading was kept for tabulation.

Evaluation of effect of nanoparticles on biochemical composition of biofilm matrix

Biochemical composition of biofilm matrix mainly total carbohydrate and total protein was carried out. The control syringe pieces and respective nanoparticle coated syringe pieces (3 in each treatment) was transferred to test tube each containing 5 mL of culture containing S.aureus prepared overnight. Test tubes containing pieces and culture were kept for incubation at 37°C for 3 days for allowing formation of biofilm on the syringes.

After incubation period, the inoculated pieces were transferred to screw cap vials containing 5 mL of 0.9% NaCl. The bottles were sonicated for 10 minute in an ultrasonicator water bath and vortexed vigorously for 1 minute to disturb biofilm. Cell suspensions were then folded and centrifuged at 10000 rpm at 4°C for 10 minutes. The collected suspension was used as source for studying biochemical composition in terms of total protein determined by Lowry et al and total carbohydrate by Anthrone method.

3.RESULT AND DISCUSSION

Chitosan stabilized silver nanoparticles were synthesized by chemical reduction of metal salt precursor with nontoxic and biocompatible polymer chitosan which primarily confirmed by FTIR, SEM and EDAX. When the FTIR spectrum of free and stabilized nanoparticles were compared, it was found that almost all the absorbed peaks were modified upon coating with chitosan. FTIR spectra of chitosan coated silver nanoparticles are presented in (Figure 1 a,b). The IR spectra of the chitosan capped Nano silver shows prominent peaks at * ~ 3788 cm\(^{-1}\), 3427.4005 cm\(^{-1}\)
corresponding to O – H stretching, strong polymerization, at 2928.1733 cm\(^{-1}\) for aliphatic C – H stretching. Peaks at approximately 2369.0387 cm\(^{-1}\), 2345.3187 cm\(^{-1}\) represent N – H stretching vibration. Peaks at 1637.5845 cm\(^{-1}\) and 1389.4649 cm\(^{-1}\) represent N –H bending and a peak at 1026.6957 represents C – N vibration in aliphatic compounds. A is also observed at 617.7611 cm\(^{-1}\) showing the presence of inorganic metal ions (silver ions). SEM analyzer built in with and EDAX analyzer allows a quantitative deduction on localization of elements in the nano specimens. Scanning electron microscopy (SEM) study of chitosan stabilized silver nanoparticles reveals the uniform spherical smooth morphology, within the size range of 101.78 nanometers and electron dense thin chitosan coating shell of diameter 3-5 nanometers(Figure 2 a) Such size distribution analysis primarily confirms that the particles are well dispersed and less aggregated. The EDAX images illustrated the presence of large amounts of C, O, N (Figure 2 b).

Coating of respective nanoparticles on syringes by ultrasonicator was primarily confirmed by fine dispersion of particles on the surface which can be easily visualized. Surface topography with SEM clearly reveals uniform spherical particles with nano range embedded on the syringe surface (Fig. 3.) SEM Micrograph reveals complete disturbance of biofilm, less aggregates, weakened cell mass was observed (Figure 3a). Biofilm inhibition study clearly reveals all the nanoparticles (both free and coated) inhibited biofilm in significant manner (P.0.05). Maximum inhibition of 80.4% was recorded in AgNp-CS treatment. followed by 72.8% was reported from free Ag and (Table 1).

**Biofilm kinetics**

Biofilm kinetics study clearly reveals all the tested nanoparticles inhibited biofilm with respect to different time interval ranging from 12, 24, 36, 48, 60, 72 hours, but distinct effect was observed in chitosan coated nanoparticle and linear increase in inhibitory effect was inferred during late inhibition period.

In the case of free AgNps the biofilm inhibition at respective time period was found to be 9.8, 11.7, 15.9, 21.05, 34.3, 41.6 % (Table .2.). Improved inhibitory activity was reported from CS coated AgNp as 11.2, 14.6, 19.3, 26.1, 40.7, 49.8 % during 12, 24, 36, 48, 60, 72 hours respectively (Table 3.)

**Effect of Nanoparticles on biochemical composition of biofilm matrix of S.aureus**

All the tested nanoparticles (both free and coated) reduced biochemical composition mainly total protein and total carbohydrate of biofilm matrix (Table 4). Maximum reduction of protein was recorded in chitosan coated syringes (12 mg/mL) followed by AgNp-CS (15 mg/mL). free Ag nanoparticle recorded was (17 mg/mL).Similar effects on carbohydrate content was also reported from CS and AgNp-CS nanoparticles which recorded least total carbohydrate content (14 mg/mL) each followed by AgNp (16 mg/mL).

The developments of nanoparticles with antimicrobial properties have recently received growing interest from both academic and industrial sectors due to the increasing resistance of pathogenic microorganisms to the diverse conventional chemotherapeutics.
present study demonstrated that silver nanoparticles synthesized by chemical reduction method and stabilized with biocompatible polymer chitosan coated on the syringes showed distinct anti biofilm effect against *Staphylococcus aureus* can be used in to prevent or to minimize bacterial infections and will lead to new generation of development of antimicrobial agents to prevent pathogens infection

REFERENCES


[26]. H. Huang, Q. Yuan, X. Yang, Colloid Surf. B., 39, 31–37, 2004

### Table 1: Biofilm inhibition (%) of *S. aureus* with metallic nanoparticles

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Treatment</th>
<th>Biofilm inhibition (%)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>AgNp</td>
<td>72.8</td>
</tr>
<tr>
<td>2</td>
<td>AgNp-CS</td>
<td>80.4</td>
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</table>

### Table 2: Biofilm inhibition (%) of *S. aureus* with Silver Nanoparticles (AgNps)

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Time-Interval (hrs.)</th>
<th>Biofilm inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>9.8</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>11.7</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>15.9</td>
</tr>
<tr>
<td>4</td>
<td>48</td>
<td>21.05</td>
</tr>
<tr>
<td>5</td>
<td>60</td>
<td>34.3</td>
</tr>
<tr>
<td>6</td>
<td>72</td>
<td>41.6</td>
</tr>
</tbody>
</table>

### Table 3: Biofilm inhibition (%) of *S. aureus* with Chitosan coated Silver Nanoparticles (AgNp-CS)

<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Time-Interval (hrs.)</th>
<th>Biofilm inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>11.2</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>14.6</td>
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</table>
Table 4. Effect of Free and Chitosan coated metallic nanoparticles coated syringes against biofilm matrix biochemical composition of S.aureus.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total Carbohydrate (mg/mL)</th>
<th>Total Protein (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgNp</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>AgNp-CS</td>
<td>14</td>
<td>15</td>
</tr>
</tbody>
</table>

*Figure 3. SEM image of nanoparticles coated syringe*

*Figure 3 a. Biofilm of Staph.aureus on un coated syringe*
Figure 3b. SEM image of nanoparticles coated syringe showed disturbed biofilm