5.1 Introduction

Bacterial biofilm are posing a global health concern as they are highly resistant to antibiotics and are cause of several chronic infections [Costerton et al. 1999; de la Fuente-Núñez et al. 2013]. Biofilms are a well-organized association of bacteria embedded in the pool of self-produced polymeric matrix [Høiby et al. 2010]. They have tendency to grow on surfaces of medical implants such as sutures, catheters, dental implants and cause infections which can only be treated by their removal. Thus, not only increasing the cost of treatment, but also imposing mental stress to patients [Costerton et al. 2005; Høiby et al. 2011]. Alternate strategies for inhibition of biofilm and its control are urgently needed. Nanoparticle based antimicrobials have been widely studied in recent years [Hernández-Sierra et al. 2008; Eshed et al. 2012]. In particular, silver nanoparticles (Ag-NPs) and their composites have gained major attentions as they possess exceptional antibacterial properties, broad antimicrobial spectrum and negligible tendency to induce bacterial resistance [Radziga et al. 2013; Gupta et al. 2014; Rai et al. 2014]. The suggested mechanisms behind the antibacterial activity of Ag-NPs are slow and sustained release of Ag\(^+\) ions, direct damage to cell wall and production of reactive oxygen species (ROS) [Prabhu & Poulose 2012].

The major drawback of nanoparticles is colloidal instability and tendency to aggregate which reduce their antibacterial efficacy. Loading of nanoparticle on the supporting matrix is one of the strategies to avoid this problem [Zhou et al. 2013]. Graphene oxide (GO) has emerged as an excellent supporting material for nanoparticles. GO contain a single atomic sp\(^2\) hybridized carbon layer with various functional groups like hydroxyl, epoxy, carbonyl, carboxyl on both accessible sides which are reducible [Zhang et al. 2012]. Furthermore, it is highly hydrophilic and readily forms stable colloidal dispersions. Hence, it acts as a good matrix for growing and stabilizing nanoparticles [Ocsoy et al. 2013; Kulshrestha et al. 2014]. However, widely used reducing agents like Sodium borohydride (NaBH\(_4\)), hydrazine are highly toxic and poisonous. They have both biological and environmental hazard [Wang et al. 2011]. Moreover, these processes are time consuming and cost ineffective as handling of hazardous waste generated may significantly increase the production cost on an industrial scale. Green synthesis of nanoparticles is an eco-friendly method and has potentials to replace chemical and physical methods [Mohanpuria et al. 2008]. Plant extracts offer a superior platform for
the formation of nanoparticle because along with being non-toxic they also act as natural capping agents. They are cheap and easily available. Furthermore, they contain large quantities of secondary metabolite which can be used as reducing and stabilizing agent [Kharissova et al. 2013; Philip et al. 2011; Patra et al. 2015]. *Lagerstroemia speciosa* (L.) Pers. (LS) plant belongs to the family Lythraceae and has been known to possess medicinal properties [Chan et al. 2014; Park et al. 2014]. Its leaf extract has been widely studied for its therapeutic properties and have recently been used in formation of silver nanoparticles [Sundararajan et al. 2014]. Although antioxidant and antibacterial properties of floral extract of LS have been reported, but there are no studies on its use in nano-material formation.

It is the first report where we have bio-fabricated silver nanoparticle onto the surface of graphene oxide using a floral extract of *Lagerstroemia speciosa* (L.) plant and have obtained a highly dispersed and stable graphene oxide-silver nanocomposite (GO-Ag). The method is simple and have no environmental and biological hazards. Recently, many stable silver nanocomposites have been developed by researchers with GO sheets utilizing both chemical and biological synthesis [Upadhyay et al. 2014; Tang et al. 2013; Shao et al. 2015]. But there are scarce reports on the antibiofilm efficacy of these composites. In view of this fact, our study highlight the potentials of GO-Ag as an antibiofilm agent on both gram-negative (*Enterobacter cloacae*) and gram-positive (*Streptococcus mutans*) bacteria and provide an in-depth analysis on its mode of action in both the bacteria.

### 5.2 Experimental Overview

The synthesis of GO and green synthesis of graphene oxide silver nanocomposite was performed using methodology described in section 2.2.3.1 and section 2.2.3.4 respectively. The characterization of GO and GO-Ag was done by UV-visible spectroscopy (2.2.4.1), TEM (2.2.4.2), XRD (2.2.4.5) and EDX (2.2.4.6). The sub-MIC concentrations of GO and GO-Ag against *S. mutans* and *E. cloacae* were estimated by method outlined in section 2.2.5. The effect of sub-MIC concentration of GO and GO-AG on biofilm forming abilities of both the strains were evaluated by crystal violet assay described in section 2.2.7. Growth curve pattern of *S. mutans* and *E. cloacae* was investigated in presence of sub-MIC concentrations of GO and GO-Ag by method
provided in section 2.2.11. Furthermore, effect of sub inhibitory concentration of GO-Ag on cell membrane integrity of both the strains was evaluated (Section 2.2.15). Amount of ROS produced in the presence of sub-MIC concentration of GO-Ag in both the strains was also estimated (section 2.2.16). SEM and CLSM analysis of GO-Ag treated biofilms was performed using methodology outlined in section 2.2.17. Moreover, the effect of GO-Ag on expression of gene involved in S. mutans virulence pathway was studied by quantitative RT-PCR as outline in section 2.2.18. Cytotoxicity assay was also performed on HEK-293 cell line (section 2.2.21).

### 5.3 Results

#### 5.3.1 Characterization of GO-Ag

![Figure 5.1 Characterization of GO-Ag](image)

The TEM micrograph of GO (Figure 5.1a) displayed a single layer of graphene oxide sheet. While image of GO-Ag (Figure 5.1b) revealed well dispersed silver nanoparticle embellished on the surface of GO nanosheets. The average size of silver nanoparticle was in range of 60-100 nm (Figure 5.1c). Formation silver nanoparticle on the surface of GO
was monitored using UV-visible spectroscopy (Figure 5.1d). Figure 5.2a depicts the X-Ray diffraction pattern of GO. The sharp diffraction peak of GO was observed at 9.8°. While in XRD pattern of GO-Ag (Figure 5.2b) along with peak at 9.8° a broad peak appeared at 24.26°. Energy-dispersive X-ray spectroscopy (EDX) was used to analyse the chemical composition of GO and GO-Ag. Peaks corresponding to C and O were observed in spectrum of GO (Figure 5.3a) while spectrum of GO-Ag shows peaks corresponding to C, O and Ag (Figure 5.3b). The inset tables give the weight percent and atomic percent of elements present in both the compound.

![Figure 5.2 XRD pattern of (a) GO and (b) GO-Ag.](image)

### 5.3.2 Antibacterial concentrations of GO-Ag nanocomposite

The MIC values of GO-Ag were much lower in *E. cloacae* as compared to *S. mutans* although the MIC value of GO was same for both the strains (Table 5.1). The MBC of GO-Ag for *E. cloacae* and *S. mutans* were 94 µg ml⁻¹ and 188 µg ml⁻¹ respectively (Figure 5.4). As the antibacterial concentrations were different for both the categories of bacteria so we used different sub inhibitory concentrations of GO-Ag in *E. cloacae* (24 µg ml⁻¹ and 12 µg ml⁻¹) and *S. mutans* (47 µg ml⁻¹ and 24 µg ml⁻¹) for further experiments.
Chapter 5

Figure 5.3 EDX spectra of (a) GO and (b) GO-Ag.

Table 5.1 MIC values of GO and GO-Ag

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Nanoparticle</th>
<th>S. mutans</th>
<th>E. cloacae</th>
</tr>
</thead>
<tbody>
<tr>
<td>1)</td>
<td>Graphene oxide</td>
<td>&gt;1500 µg/ml</td>
<td>&gt;1500 µg/ml</td>
</tr>
<tr>
<td>2)</td>
<td>Graphene oxide - silver nanocomposite</td>
<td>94 µg/ml</td>
<td>47 µg/ml</td>
</tr>
</tbody>
</table>

Figure 5.4 MBC of GO and GO-Ag against S. mutans and E. cloacae
5.3.3 Inhibitory effect on biofilm forming abilities *Streptococcus mutans* and *Enterobacter cloacae*

There were 90% and 49% reduction in the presence of 24 µg ml\(^{-1}\) and 12 µg ml\(^{-1}\) of GO-Ag respectively in *E. cloacae*, while, at the same concentrations GO reduced the biofilm to 35% and 20% (Figure 5.5b). Similarly, 89% and 34% reduction was observed in *S. mutans* biofilm when treated with 47 µg ml\(^{-1}\) and 24 µg ml\(^{-1}\) of GO-Ag respectively, however, in the presence of same concentrations of GO there was only 18% and 15% reduction in *S. mutans* biofilm (Figure 5.5a).

![Figure 5.5](image)

**Figure 5.5** (a) Effect of sub inhibitory concentrations of GO and GO-Ag on *S. mutans* biofilm formation, where T1 is 47 µg ml\(^{-1}\) and T2 is 24 µg ml\(^{-1}\) (b) Effect of sub inhibitory concentrations of GO and GO-Ag on *E. cloacae* biofilm formation, where T1 is 24 µg ml\(^{-1}\) and T2 is 12 µg ml\(^{-1}\) (c) Effect of GO and GO-Ag on growth curve pattern of *S. mutans* (d) Effect of GO and GO-Ag on growth curve pattern of *E. cloacae* (* means p value <0.05).
5.3.4 Effect on growth curve

Growth curve assay was performed in the presence of sub inhibitory concentrations of GO and GO-Ag. There was no change in pattern of growth curve in *S. mutans* (Figure 5.5c), while in *E. cloacae* the growth pattern of GO-Ag treated bacteria was altered considerably, a delay in exponential phase was observed (Figure 5.5d).

5.3.5 Protein leakage assay

Further, the effect of GO-Ag on cell membrane integrity was evaluated by protein leakage assay. Figure 5.6a revealed that in *E. cloacae* after 4h of treatment amount of protein released was 0.13 mg ml$^{-1}$ while in *S. mutans* it was only 0.03 mg ml$^{-1}$ which is negligible.

5.3.6 Reactive oxygen species production

Reactive oxygen species detection assay revealed the amount of reactive oxygen species (ROS) generated in *E. cloacae* was much higher than *S. mutans* after 12 h of incubation (Figure 5.6b).

![Figure 5.6](image)

**Figure 5.6** (a) Effect of GO-Ag on cell membrane integrity of *S. mutans* and *E. cloacae* (b) Amount of reactive oxygen species generation by GO-Ag in *S. mutans* and *E. cloacae* (Data is mean ± Standard deviation, * represent p value < 0.05).

5.3.7 Microscopic analysis of biofilms

The scanning electron micrographs of *S. mutans* and *E. cloacae* are shown in Figure 5.7. Upper panel represents the effect of GO and GO-Ag (47 µg ml$^{-1}$) on *S. mutans* biofilm (Figure 5.7a, b) while the lower panel displays the effect of GO and GO-Ag (24 µg ml$^{-1}$)
on *E. cloacae* biofilm (Figure 5.7e, f). The results depict a substantial decrease in biofilm architecture on treatment of GO-Ag in both the cases. Furthermore, the magnified view of cells of biofilm show no changes in cell morphology of *S. mutans* in the presence of GO-Ag (Figure 5.7c, d). However, GO-Ag treated *E. cloacae* displayed a damage in cell wall and decreased intracellular density (Figure 5.7g, h). Confocal microscopy analysis was performed on both the bacterial biofilms by using SYTO 9 (green fluorescence, live) and PI (Red fluorescence, dead). Images of SYTO9/PI stained biofilms of and EC15 in shown in Figure 5.8.

**Figure 5.7** Scanning electron microscopy images of biofilm treated with sub inhibitory concentration of GO-Ag: (a, b) inhibition of *S. mutans* biofilm, (e, f) inhibition of *E. cloacae* biofilm, (c) magnified view of *S. mutans* in control biofilm, (d) magnified view of *S. mutans* in treated biofilm showing no change in cell wall integrity, (g) magnified view of *E. cloacae* in control biofilm, (h) magnified view of *E. cloacae* in treated biofilm, red arrow depicting loss of intracellular component.
Figure 5.8 Confocal laser scanning microscopy images stained with SYTO9 (green, live) and PI (red, dead): (a, c) control biofilm of S. mutans, (b, d) treated biofilms of S. mutans, (e, g) control biofilms of E. cloacae, (f, h) treated biofilms of E. cloacae. Scale bar = 5μm.

5.3.8 Quantitative RT-PCR analysis

Figure 5.9 shows the gene expression profile of three important genes of S. mutans (com DE, spa P and vic R) which play major role in process of biofilm formation. There was downregulation in gene expression of all these genes.
Figure 5.9 Gene expression profile of specific genes involved in the formation of *S. mutans* biofilm. Quantitative RT-PCR was carried out in triplicate. Data presented were generated from at least four independent sets of experiments (Data is mean ± Standard deviation, * represent p value < 0.05).

### 5.3.9 Cytotoxicity on HEK-293 cell line

MTT assay was performed to assess the effect of GO-Ag on HEK-293 cell line (Figure 5.10). The IC$_{50}$ value of GO-Ag was 750µg ml$^{-1}$ while it was 1500µg ml$^{-1}$ for GO. This value was much higher than the concentrations used in present study suggesting that these nanoparticle are non-toxic to human normal cell line at antibacterial concentrations of these nanoparticles (94 µg ml$^{-1}$ in *S. mutans* and 47 µg ml$^{-1}$ in *E. cloacae*).

Figure 5.10 Effect of GO and GO-Ag on viability of HEK-293 cell line.
5.4 Discussion

Plant extracts have been known to possess large amount of secondary metabolites which can act as reducing and capping agents for nanoparticles [Kharissova et al. 2013; Philip et al. 2011; Patra et al. 2015]. The green synthesis of nanoparticle is environment friendly mode and is less hazardous [Mohanpuria et al. 2008]. In the present study graphene oxide-silver nanocomposite (GO-Ag) was synthesized by simultaneous reduction and stabilization of silver nanoparticle on to the surface of graphene oxide using Lagerstroemia speciosa floral extract. The schematic representation of the process of GO-Ag preparation is shown in Figure 5.11.

![Figure 5.11](image)

**Figure 5.11** Schematic representation of green synthesis of GO-Ag: (a) Graphene oxide was prepared by Hummers method, (b) plant extract was used to reduce GO to RGO, (c) silver nanoparticle was reduced and stabilized onto the surface of GO with help of plant extract.

Morphological aspects of GO and GO-Ag were analysed using Transmission electron microscopy (TEM). The average size of silver nanoparticle was in range of 60-100 nm. The results validate that graphene oxide is acting as a supporting agent for silver nanoparticle and also reducing the agglomeration of nanoparticles [Zhou et al. 2013]. Formation silver nanoparticle on the surface of GO was monitored using UV-visible spectroscopy. A peak was observed at 230 nm in UV-visible spectrum of GO which is characteristic peak of GO due to π−π* transitions of the aromatic C−C bonds [Shao et al. 2015]. The UV-visible spectrum of GO-Ag showed an additional peak at 420 nm along with peak at 230 nm. The peak at 420 nm corresponds to surface plasmon resonance peak.
of silver nanoparticle [Chevirona et al. 2014], suggesting the formation of silver nanoparticles onto the surface of graphene oxide. Broadening and shifting of the XRD peak of GO to around 24º suggest the formation of reduced graphene oxide [Seema et al. 2012]. Furthermore, sharp peaks at 38.1º, 44.3º, 64.4º and 77.4º in XRD pattern of GO-Ag can be assigned to the (111), (200), (220), and (311) diffraction cubic crystal planes of Ag (JCPDS No. 04-0783), demonstrating the formation of metallic silver nanoparticles [Ma et al. 2011]. The strong signal of silver in the EDX analysis of GO-Ag along with carbon and oxygen confirms the formation of silver nanoparticle on graphene oxide sheets.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) were estimated for gram-positive and gram-negative bacterial species by taking *Streptococcus mutans* and *Enterobacter cloacae* as model organisms. It has been demonstrated that enhanced antibacterial activity of graphene nanocomposites are due to nonspecific binding abilities of GO sheets to microbes [Park et al. 2010]. Thus, stabilization of silver nanoparticle onto the GO sheets provide better platform for interaction of nanoparticle with microbes [Xu et al. 2011]. Silver nanoparticles are well known for their antibacterial properties although exact mechanism has not yet been reported. It has been suggested that production of reactive oxygen species (ROS) and release of silver ions are the main antibacterial properties of silver nanoparticles which may lead to oxidative stress and cellular damage [Shao et al. 2015]. Furthermore, the difference in antibacterial concentrations of GO-Ag for gram-negative and gram-positive bacteria may be attributed to the difference in cell wall composition with gram-positive bacteria having multi-layered peptidoglycan on their surface [Tang et al. 2013]. Thus, higher concentration of GO-Ag was required to kill *S. mutans* as compared to *E. cloacae*.

The best way of to deal with the biofilm related infections is the prophylaxis treatment of biofilm [Lynch et al. 2008; Anghel et al. 2012]. Keeping this in view the biofilm forming abilities of both the bacteria were assessed in the presence of GO-Ag. It is apparent from the results that GO is acting as an inimitable nano-interface for interaction of microbe with silver nanoparticles and thus, considerably inhibiting the biofilm formation [Kulshrestha et al. 2014; Tang et al. 2013].

It is important to investigate whether the effect on biofilm is due to inhibition of biofilm forming pathway or because of the killing of planktonic cells. From the growth curve data
it may be elucidated that the reduction in biofilm in *E. cloacae* is due to killing of its planktonic cells. These results of cell membrane integrity assay were in accordance with the growth curve assay suggesting that GO-Ag is affecting the planktonic cells of *E. cloacae* by cell membrane disruption which may be the reason of decreased biofilm formation. On contrary, *S. mutans* possess multilayer peptidoglycan which renders GO-Ag unable to damage its, thus, the inhibition in its biofilm formation is due to some other factors.

Reactive oxygen species (ROS) production is one of the primary mechanisms by virtue of which nanoparticle effects the bacterial cell functioning [Premanathan *et al.* 2011]. When the ROS production by nanoparticles exceeds the capacity of cellular antioxidant defence system, it can cause oxidative stress which can further initiate lipid per oxidation, thus damaging the cell membrane and eventually leading to cell death [Lovrić *et al.* 2005; Khan *et al.* 2012]. Hence, it can be elucidated from present results that amount of ROS produced by GO-Ag in *S. mutans* is not exceeding its antioxidant system subsequently not affecting its membrane integrity but in *E. cloacae* there is higher amount of ROS which is causing oxidative stress and cell death. It is apparent from SEM results that *E. cloacae* cells are killed by pit formation in their cell wall while *S. mutans* are not directly damaged by GO-Ag and there is only reduction in biofilm. A considerable reduction of biofilm architecture was visualized by CLSM in *S. mutans* but there was no effect on viability of cells as indicated by green fluorescence of Syto-9. In *E. cloacae* there was a decrease in biofilm but the cell viability was affected as yellow fluorescence was visible due to entry of both the dyes. The microscopic analysis results were in accordance with the previous results validating that inhibition of biofilm in *E. cloacae* in presence of GO-Ag is because of loss of viability of planktonic cells while in *S. mutans* the cells are viable after treatment with sub inhibitory concentrations of GO-Ag and the biofilm inhibition may be due the release of silver ions which can affect the biofilm cascade on genetic level.

In the presence of GO-Ag there was downregulation of expression of important genes associated with process of biofilm formation of *S. mutans*. Gene *spaP* (Ag I/II) helps in sucrose independent initial adherence of *S. mutans* while *vicR* is a two component regulatory system and have been reported to regulate genes which are necessary for sucrose dependent adherence of *S. mutans* [Hasan *et al.* 2012; Khan *et al.* 2010]. Thus,
down regulation of these genes will affect the adherence of *S. mutans* to the surface which is first step of biofilm formation. *comDE* plays a crucial role in quorum sensing cascade of *S. mutans* [Hasan *et al.* 2014; Li *et al.* 2002]. Its downregulation will suppress the regulation of genetic tolerance, acid production and biofilm formation. From the results it became apparent that in *S. mutans* the inhibition in biofilm in presence of GO-Ag is due to effect on biofilm associated genes cascade.

In conclusion, we demonstrate a simple and environment friendly approach for synthesis of well dispersed silver nanoparticle onto the surface of graphene oxide by using flower extract of *Lagerstroemia speciosa* (L.) Pers. The sub inhibitory concentrations of nanocomposite were found to inhibit biofilm formation of both gram-negative and gram-positive bacteria but the mechanism of inhibition of biofilm is different in both the microbes. Moreover, nanocomposite was found to be non-toxic against HEK-293 cell line at used concentrations. Hence, GO-Ag may be assigned as potential prophylaxis for biofilm based infections although further research is required to elucidate specific biofilm inhibition pathway by proteomics and metabolomics approaches.