Improved Bactericidal Activity of Polyethylenimine Grafted Graphene Oxide Nanocomposite against Staphylococcus aureus and Escherichia coli

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Abbreviations: 2-D: 2-Dimensional; GO: Graphene Oxide; PEG: Polyethylene Glycol; ROS: Reactive Oxygen Species; PEI: Polyethyleneimine; QPEI: Quaternate Polyethyleneimine; NHS: N-hydroxy-Sulfosuccinimide Sodium Salt; UPW: Ultrapure Water; EDC: Ethyl Dimethylamino Carbodiimide; TEM: Transmission Electron Microscopy; FTIR: Fourier-Transform Infrared Spectrophotometer; LB: Luria Bertani

ABSTRACT

Global emergence of pathogenic bacteria is becoming a major concern to human's health because of the resistance of bacteria towards antibiotics overuse. Nanotechnology has gained intense attention to overcome these challenges with the development of antibacterial nanomaterial with distinctive physicochemical properties. In this work, we have prepared antibacterial Polyethylenimine grafted graphene oxide (PEI-GO) by functionalization of GO with PEI through the formation of C―N bond with amine groups of PEI and carboxyl and epoxy groups from GO. The characterization of PEI-GO was done by FTIR, XRD, TEM, Raman spectroscopy, and UV spectrophotometry for the confirmation of the as-prepared nanocomposite. PEI-GO showed high antibacterial activity as compared to GO and PEI against Gram-positive Staphylococcus aureus (S. aureus) and Gram-negative Escherichia coli (E. coli), killing almost 100 % S. aureus and 90.5 % E. coli bacteria with 20 μg/mL of PEI-GO. Moreover, time dynamic bactericidal activity reveals that antibacterial toxicity of PEI-GO is inversely proportional to the concentration and contact time of bacteria.

The high antibacterial action of PEI-GO was assigned to the synergistic effect of both material, the positive charge of PEI-GO interact with the negatively charged membrane of bacteria disturbing the membrane while sharp edges of GO penetrate the membrane resulting in the disruption of bacterial cell structure and ultimately cell death. The higher susceptibility of S. aureus was associated with lacking outer membrane, whereas E. coli with an outer membrane indicated slight resistance compared to S. aureus. Our result shows that PEI-GO could be an attractive antibacterial agent for its use to combat the development of bacterial resistance.

Keywords: Graphene Oxide; Antibacterial; Nanocomposite; Polyethylenimine

Introduction

Recently the worldwide emergence of bacterial resistance towards antibiotics and drugs is a serious threat to public health, resulting in the prevalence of infectious disease and increase morbidity rate around the world. Furthermore, various effective antibiotics, that were developed to fight bacterial infections are no longer potent due to the emergence of bacterial resistance [1-3]. To control the evolution and environmental adaptation of numerous microbial strains is one of the prime challenges for the research community [4]. Therefore, the development of new approaches needs to be immediately explored to combat drug-resistant bacteria. In the past decade, the massive advancement in nanotechnology has attracted intensive attention [5], leading to the development of non-antibiotic, biocompatible, antimicrobial nanomaterial with...
distinctive physical and chemical properties [6]. Graphene, since after its discovery has acquired colossal attention of scientists, because of its scale-up production, its synergistic effect with other material, significant physicochemical [7,8], electronic properties [9], and large surface area [10], allowing its application in the field of drug delivery [11], tissue engineering [12], sensors and biosensors development [13,14], cell imaging [15], photothermal therapy [16] as well as in bacterial growth inhibition [17].

Graphene is the 1st 2-dimensional (2D), single atomic sheet of graphite, hexagonally arranged lattice structure of sp² hybridized carbon atoms [7,18,19]. Likewise, Graphene oxide (GO) is the derivatives of graphene with sp³ hybridization of carbon atoms, highly oxidative, obtained by chemical oxidation and subsequently exfoliation [20], hydrophilic in nature, possessing a large number of oxygen-bearing functional groups including epoxy and hydroxyl on the basal plane, and carboxyl groups on the edges [21,22]. These oxygen-containing functional groups assist the chemical functionalization of GO sheet with other molecules [22]. Innumerable studies of GO functionalization with polyethylene glycol (PEG), Polyethylenimine, chitosan, cystamine, for their application in drug delivery, tissue engineering, bioimaging, gene delivery and bactericidal activity [12,23-26] has been reported. Various studies on the antibacterial effect of GO has reported its bactericidal property through somatic damage and oxidative stress [17,27,28]. Along with physical damage [27], oxidative mechanism also plays its part in deactivating bacterial cell by reactive oxygen species (ROS) production in GO [26,29].

Liu et al. have studied the antibacterial effect of four different graphene-based material (GO, Gt, Gr, rGO) and found GO with higher bactericidal property comparing to others [30]. In another study done by Barbolina et al. observed no antibacterial effect of pure GO against Gram-positive \textit{S. aureus} and Gram-negative \textit{E. coli} [31]. Furthermore, other studies on the functionalization of GO with various nanoparticles and polymers has been reported to improve its antibacterial property and stabilization [32,33]. Polyethylenimine (PEI) is polycationic synthetic polymer prepared from aziridine by polymerization, hydrophilic, branched or unbranched, containing primary, secondary and tertiary amino group (―NH₂) in its structure, that can be protonated [34,35]. Because of being highly positive charge, high membrane permeability and exceptional transferability, PEI is extensively used in gene transfer and drug delivery [34,36]. PEI itself is considered as microbicidal agent, and it is also used as an enhancer in the field of microbiology to strength the antibacterial property of both hydrophilic and hydrophobic antibiotic [37].

Gao et al. prepared quaternate Polyethylenimine (QPEI), that exhibited excellent antibacterial activity, associated with the degree of cationic charge on its backbone chain [38]. A study by Azevedo et al. used nanoPEI to inhibit bacterial growth and concluded that the antibacterial property of PEI mainly depends on high concentration as well as species specificity [39]. PEI can be linked to GO via nucleophilic addition reaction between the PEI amine group and carboxyl and epoxy group of graphene oxide [40,41]. Here in, keeping in view the antibacterial property of both the materials, we have prepared PEI grafted graphene oxide (PEI-GO) nanocomposite through the functionalization of PEI on the graphene sheet via C―N bond. The schematic illustration is presented in Figure 1. The nanocomposite was observed through various characterization techniques. The antibacterial activity of as produced PEI-GO was conducted against two bacterial strains (\textit{S. aureus} and \textit{E. coli} as the representative bacteria), besides that time dynamic bactericidal activity was also studied. It is expected that our nanocomposite will have excellent biocidal activity because of the synergy effect of PEI and GO.

![Figure 1: Schematic illustration of PEI-GO nanocomposite.](image-url)
**Materials and Methods**

**Reagents**

Graphene Oxide (GO) was obtained from Nanjing XFNANO Materials Tech Co., Ltd. (Nanjing, China). Polyethylenimine (PEI) was acquired from Aladdin Chemistry Co., Ltd. (China). Ethyl-3-(3'-dimethylamino-propyl) carbodiimide (EDC) and N-hydroxysulfosuccinimide sodium salt (NHS) were bought from Sigma Aldrich co., (Shanghai, China). Ultrapure water (UPW) was used for preparing all the aqueous solution.

**Preparation of PEI-GO Nanocomposite**

Graphene oxide was grafted with PEI according to the previously reported process with little adjustment [42]. 20 mg of graphene oxide was ultrasonicated in 10 mL of ultrapure water for 2 hours to get a homogeneous dispersion. Then 100 mg PEI was added and sonicated for an additional 30 minutes. After that, 60 mg EDC and 30 mg NHS were added gradually in the dispersion and sonicated for a further 15 minutes. Next, the material in the flask was shifted on the magnetic stirrer for one day, and speed of the stirrer was adjusted to 850 rpm. The whole procedure was performed at ambient temperature. The composite was obtained by centrifugation followed by three washing with ultrapure water and subsequently dried in the oven at 50 °C.

**Characterization**

Transmission electron microscopy (TEM) was performed at an accelerating voltage of 200 kV on a JEOl model 2100 HR instrument (TEM, JEOl, Ltd., Japan). Shimadzu UV-1800 spectrophotometer was used to carry out the UV–vis absorption spectra in the range of 200 to 800 for nanocomposite (Shimadzu, Japan). Fourier-transform infrared spectrophotometer (FTIR) was used to obtain FTIR spectra on Nicolet Nexus 470 FTIR Spectrophotometer (Thermo Electron co., USA) at a wavelength of 500 to 4000 cm⁻¹. The X-ray diffraction patterns were taken on a Bruker D8 advance X-ray diffractometer with Cu-Kα X-ray radiation (λ=1.5406 Å) with 40 kV voltage at the 2°/minute scanning rate (Bruker AXS Ltd., Germany). Raman spectra were measured using LABRAM HR 8000 microscopy confocal Raman spectrometer using 532 nm excitation wavelength with x50 objective (HORIBA Corporation, Japan). Nanocomposite zeta potential was measured using zeta potential instrument by diluting the sample in water (Malvern zeta-sizer nano ZS).

**Bacterial Culture**

First two bacterial strains Gram-positive *Staphylococcus aureus* (*S. aureus*) ATCC 29213 and Gram-negative *Escherichia coli* (*E. coli*) ATCC 25922 were grown overnight on Luria Bertani (LB) agar plates at temperature 37 °C in an incubator. Then a single colony of bacterial strains from above plates were cultivated overnight in liquid LB media at 37 °C in an incubator shaker. Afterwards, the bacteria were harvested by centrifugation (8000) at the mid-exponential growth phase and washed using sterilized saline solution (0.85% NaCl) to remove extra residual macromolecules [17]. Optical density (OD₆₀₀ₐₚ) was measured by spectrophotometer after resuspending the pellets in saline solution and diluted to 10⁶ CFU/mL.

**Antibacterial Test**

For antibacterial assay, 10 μL of bacterial suspensions were mixed in 900 μL of saline solution comprising a different concentration of GO, PEI, PEI-GO. Tubes containing the above suspensions were transferred into incubator shaker for 5 hours at 37 °C with shaking speed 200 rpm. Afterwards, 100 μL of suspension from the above samples were spread evenly on the LB agar plates, and all the plates were incubated overnight at 37 °C [43]. The same procedure was adopted for the control sample without any material treatment. Bacterial colonies were counted on LB agar plates and survival percentage was calculated by comparing control group. All experiments were repeated three times.

**Minimum Inhibitory Concentration (MIC)**

Minimum inhibitory concentration of GO, PEI, PEI-GO were ascertained by the previously reported microdilution technique [44] with little modification. In brief, log phase *S. aureus* and *E. coli* (10⁶ CFU/mL) were incubated in 96-well microtiter plates with different concentrations (2, 4, 8, 16, 32, 64, 128 μg/mL) of material at 37 °C for 18 hours. MIC value was devoted to the least minimum material concentration that observes no bacterial colony.

**Time Dynamic Bactericidal Activity**

Time dynamic antibacterial assay was conducted to evaluate the efficiency of PEI-GO nanocomposite over a period of time along with nanocomposite concentration. *S. aureus* and *E. coli* (10⁶ CFU/mL) were incubated with a different concentration (10, 20, 40 μg/mL) of PEI-GO nanocomposite. A blank sample as control was also incubated without nanocomposite. Then 100 μL sample from above suspension was taken every 30 minutes on time scale zero to 4 hour, diluted in gradient, spread on the LB agar plates and place in incubator for 12 hours at 37 °C. The bacterial colony were counted, and survival % was calculated in comparison with control samples.

**Results and discussion**

PEI-GO nanocomposite synthesis was done by chemical functionalization of GO with PEI by using EDC and NHS as a facilitator. Figure 1 illustrates formation of PEI-GO nanocomposite through the reaction of amine group of PEIs with functional (epoxy and carboxyl) group of GO resulting in the formation of C–N bond. The surface morphology of PEI-GO was investigated by TEM (Figures 2a & 2b). The Figure 2a express the thin layer of GO morphology can be attributed to the formation of PEI-GO. UV-vis absorption spectrum was carried out for GO, PEI and PEI-GO nanocomposite. As shown in Figure 3a, the spectrum of bare GO indicates two representative peaks, the sharp peak is observed at 270 nm correspond to electronic π−π* transition of C−C aromatic.
bonds, and the shoulder peak is noticed at 300 nm link to the n−π* transitions of C=O bonds [45]. For PEI, a sharp absorption peak is observed at 200 nm. On the other hand, the spectrum of PEI-GO is observed same as GO with a fresh absorption peak at 200 nm, witness the conjugation of PEI with GO.

![TEM image of GO and PEI-GO](image)

**Figure 2:** TEM image of (a) GO and (b) PEI-GO

![UV-vis. absorption spectra, FTIR spectra, XRD patterns, and Zeta-potential graphs](image)

**Figure 3:** (a) UV-vis. absorption spectra of PEI-GO, GO, and PEI.  
(b) FTIR spectra of PEI-GO, GO and PEI.  
(c) XRD patterns of PEI-GO and GO.  
(d) Zeta-potential of PEI-GO, PEI and GO.

The FTIR spectrophotometer was used to confirm the formation of PEI-GO complex (Figure 3b). In the bare GO spectrum, the main absorption peak at 3424 cm⁻¹ is ascribed to −OH stretching vibration. The peak at 1714 cm⁻¹ shows the Stretching vibration of carboxyl functional group of GO. Whereas the band 1624 cm⁻¹ is considered as C=C aromatic Stretching, that at 1224 cm⁻¹ and 1063 cm⁻¹ are assigned to epoxy C−O stretching vibration [46]. The Infrared spectrum of PEI indicates absorption peak at 1573 cm⁻¹ and 1111 cm⁻¹ are linked to the bend vibration of N−H bond of primary and secondary amine groups, respectively [38]. After
PEI and GO reaction, downshift in a peak at 3418 cm\(^{-1}\) is observed assign to hydroxyl stretching, and two other peaks are gained at 2916 and 2855 cm\(^{-1}\), ascribed to the symmetric and asymmetric stretching mode of PEI methylene groups [47]. The peak at 1714 cm\(^{-1}\) is hardly noticeable, and a new strong peak is detected at 1608 cm\(^{-1}\) indicate the stretching mode of amide bond because of the reaction of polyethylenimine –NH\(_2\) groups with graphene oxide – COOH groups. Furthermore, disappearance of peak at 1224 cm\(^{-1}\), and the prominent peak at 1067 cm\(^{-1}\) also indicate the formation of C–N bond via epoxy groups of GO and amine groups of PEIs.

XRD measurements were performed to determine the changes in the crystalline structure of GO before, and after functionalization with PEI. As presented in Figure 3c, the XRD pattern of bare GO expresses one characteristic peak at \(2\theta = 11.2^\circ\) corresponding to the (001) crystalline plane having an interlayer-spacing 0.793 nm. This interlayer-spacing is because of the oxygen-bearing functional groups of GO present in the layers [12]. After PEI conjugation with GO, two peaks are observed, the broad peak at \(2\theta = 20.2^\circ\) might be assigned to the non-crystalline diffraction of PEI, and the peak at \(2\theta = 12^\circ\) relate to the (001) plane with an interlayer spacing 0.741 nm compared to GO. This right-shift and reduction in interlayer spacing are probably due to the presence of PEI in the PEI-GO nanocomposite. The zeta potential (mV) measurement of GO, PEI, and PEI-GO is shown in Figure 3d. GO exhibit negative zeta potential of -34.2 mV owing to its functional groups (hydroxyl, epoxy and carboxyl) on its basal and edges plain, whereas PEI is highly cationic polymer having zeta potential of -34.2 mV owing to its functional groups (hydroxyl, epoxy and carboxyl) on its basal and edges plain, whereas PEI is highly cationic polymer having zeta potential of 45.6 mV. After functionalization of GO, our nanocomposite indicate positive zeta potential of 39.4 mV, confirms the successful functionalization of GO with PEI.

Figure 4: Raman spectroscopy spectra of bare graphene oxide and PEI-GO nanocomposite.

Figure 5: Survival rate (in %) of *S. aureus* and *E. coli* after 5 hours of treatment with 10, 20, and 40 μg/mL, concentration of GO, PEI, PEI-GO. Error bars indicate a standard error in three experiments.

Figure 4 presents the Raman spectra for bare graphene oxide and PEI-GO nanocomposite. Raman spectra of both bare GO as well as PEI-GO shows two main noticeable peaks denoted as D band and G band. The D band demonstrate the breathing mode of \(\kappa\)-point phonons of \(A_{1g}\) symmetry and structural imperfection in graphene sheet; however, the G band arise from the first order scattering of \(E_{2g}\) phonons of \(sp^2\) carbon atom [48,49]. The Raman spectra of GO is found at 1358 and 1593 cm\(^{-1}\), for D band and G band, respectively, whereas for PEI functionalized graphene oxide the D and G band obtained are located at 1361 and 1596 cm\(^{-1}\), respectively. Additionally, the degree of disorder in GO structure can be estimated by calculating the \(I_D/I_G\) ratio. From our results,
the Raman shift in D band and G band are clearly observed after functionalisation of GO with PEI. Moreover, the increase in I_D/I_G ratio in PEI-GO as compared to GO from 1.86 to 2.17 clarifies the increase in structural disorders in PEI-GO.

Table 1: MIC values of different material against S. aureus and E. coli. “-” represent the MIC value were undetectable even at concentration 128 μg/mL.

| Material | MIC μg/mL |
|----------|-----------|
|          | S. aureus | E. coli |
| GO       | -         | -       |
| PEI      | 64        | 128     |
| PEI-GO   | 8         | 16      |

Antibacterial Activity

The antibacterial activity of GO, PEI, PEI-GO were tested against S. aureus and E. coli by calculating the survival cells after 5 hours incubation with require concentration of well-dispersed each nanocomposite followed by growth on agar plate. For comparison, control samples were incubated in 0.85% saline solution without any nanocomposite. Figures 5a & 5b describe the survival % of bacterial colonies after treatment with nanocomposite. As compared to the control sample, all three materials indicate concentration dependent reduction in the bacterial viabilities. It can be seen in Figure 5, at concentration of 40 μg/mL of PEI-GO, 100 % reduction of S. aureus and E. coli are achieved, whereas in case of GO and PEI, the same above concentration of 40 μg/mL could only reduce the S. aureus viabilities to 46.9%, 22.5% and E. coli viabilities to 66.1%, 34.2%. In Figure 5, at concentration of 40 μg/mL of PEI-GO, 100 % reduction of S. aureus was inhibited entirely at concentration 128 μg/mL for both S. aureus and E. coli showing very week antibacterial activity and highest MIC value. However, MIC of PEI is obtained to be 64 μg/mL for S. aureus and 128 μg/mL for E. coli. In addition, with PEI-GO the growth of S. aureus was invisible at concentration 8 μg/mL or above, and growth of E. coli was inhibited entirely at concentration 16 μg/mL or above making their MIC values 8 μg/mL and 16 μg/mL respectively.

Lowest MIC value of PEI-GO is due to the synergistic effect of both constituents of nanocomposite. Antibacterial analysis reveals that PEI show superior inhibition activity towards S. aureus than E. coli [35]. The time dynamic bactericidal activity of PEI-GO was assessed against S. aureus and E. coli. The bacteria were exposed to different concentration of PEI-GO nanocomposite, and their survival rate was calculated in correlation with blank control over a half-hour (h) time interval from 0 to 4 h. we kept the concentration of PEI-GO nanocomposite same as we used in the antibacterial activity assay to understand the change in survival rate with time and concentration. Figure 6 shows the time dynamic curves of S. aureus and E. coli treated with PEI-GO. Bacterial survival rate inversely correlates with the concentration and exposure time of PEI-GO, with high concentration of nanocomposite the bactericidal time was shortened, and survival rate of bacteria decreased. As in Figure 6a, the survival rate of S. aureus at concentration 40 μg/mL after 2.5 h treatment was 1.9%, later half more hour (after 3 h) that was found 0, which means that S. aureus was inhibited entirely in-between 2.5 to 3 h duration. In case of E. coli (Figure 6b) after 2.5 h with 40 μg/mL nanocomposite, the survival rate was 7.3%, and complete reduction was achieved near 3.5 h.
Furthermore, *S. aureus* survival rate after 2.5 h, with concentration 10 and 20 μg/mL were 41.5% and 17.3% respectively, while those of *E. coli* after 2.5 h exposure to 10 and 20 μg/mL, the survival rate were 54.9% and 29.3% respectively. Comparison of *S. aureus* and *E. coli* revealed that the decrease in survival rate was observed quicker in *S. aureus* than *E. coli* with PEI-GO nanocomposite. In addition, with 40 μg/mL PEI-GO about 70% and 60% of the reduction was achieved within first-hour exposure of *S. aureus* and *E. coli*, then moved gradually to the complete reduction about/after 3 and 3.5 h respectively. With 10 and 20 μg/mL concentration, the reduction in survival rate was bit quicker in the beginning than in later stage, after 4 h the survival rate of *S. aureus* and *E. coli* was 1.9% and 12.4% with 20 μg/mL nanocomposite. The difference in reduction of survival rate between *S. aureus* and *E. coli* is because of bacterial structural difference, which has been discussed above and enhanced destructive capability of PEI against Gram-positive bacteria [53]. From the graph, it is revealed that with increasing concentration, rapid reduction in survival rate can be obtained. However, less concentration requires more time to attain high bactericidal activity. The difference in reduction rate in the beginning and later stage could be attributed to the reduction in live bacterial cells and covering of nanocomposite with bacterial residues.

### Possible Bactericidal Phenomena

The bactericidal property is attributed to the damage caused by the PEI-GO by direct contact to the bacterial membrane. The positive charge PEI has ability to bind negatively charged cell surface and increase the cell permeability causing destruction of bacterial membrane integrity. In addition, PEI can bind to phospholipid and various cellular material disturbing the cell functionality [53]. The sharp edges of GO can pierce the bacterial membrane and produce membrane stress in the cell that degrades the bacterial morphology and outflow of protoplasm, cause the destruction of bacteria leading to osmotic difference and ultimately bacterial death [27,28,54]. The even distribution of PEI on the GO surface increase the binding affinity to the bacterial cell membrane. The amine group of PEI attach to the bacterial membrane inducing structural damage whereas the sharp edges of GO penetrate and disrupt the membrane, resulting in the release of protoplasmic fluid and consequently cell lysis. The enhanced bactericidal activity of PEI-GO is because of the synergistic effect of both materials.

### Conclusion

In the present study, we have synthesized Polyethylenimine grafted graphene oxide nanocomposite (PEI-GO) via C–N bond by utilizing EDC and NHS as a facilitator agent, which was confirmed by characterization. The antibacterial property of prepared material was investigated against Gram-positive and Gram-negative bacteria. MIC value of *S. aureus* was found to be lower (8 μg/mL) than that of *E. coli* (16 μg/mL), which might be because of *E. coli* resistance to the direct contact interaction with material owing to the presence of an outer membrane and the high susceptibility of *S. aureus* to PEI, besides the lack of outer membrane in its structure can be a possible reason. Moreover, results from time dynamic bactericidal curves show that the survival rate is inversely proportional to the concentration of material as well as to contact time. The antibacterial test shows the significant bactericidal property of PEI-GO nanocomposite at low dosage compared to PEI and GO alone, which was attributed to the better charge transfer of PEI onto the negative charge membrane and sharp edges of GO causing the ultimate cell destruction. Our prepared nanocomposite can be a promising antibacterial agent to fight against pathogens from attaining resistance.

### Competing Interest

The authors declare no competing interest.
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