Synthesis of Biocompatible carboxylic acid functionalized Graphene Oxide as a stimulator of bacterial growth

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ABSTRACT

Researchers have shown great interest towards Graphene and its potential applications in various fields such as electronics, energy, materials and biomedical areas. The effects of Graphene, graphene oxide (GO) and its derivatives on bacteria activities is still controversial. Thus, how graphene and its derivatives interact with microorganisms and the mechanisms of their interactions are important issues for nanotechnology which need proper exploration. In the present investigation, graphene oxide (GO) has been synthesized and functionalized by the chemical method. The GO and COOH-functionalized GO have been characterized by Fourier transforms infrared spectroscopy; Raman spectroscopy and SEM analysis. Further, haemocompatibility study has been performed to check the biocompatibility of functionalized graphene oxide. The effect of GO and COOH-GO on the bacterial growth has been observed. The FTIR, Raman and SEM data confirm the successfully functionalization of GO with carboxyl (-COOH) group. The haemolysis test shows that GO and GO-COOH are highly hemocompatible. Interestingly, functionalized graphene oxide, can significantly stimulate bacterial growth for gram positive and gram negative bacteria, whereas as-made GO shows no effect. It can be concluded that carboxylic acid functionalized GO may act as a new, positive regulator for the growth of bacterial cells.

Keywords:
Graphene oxide; FTIR; SEM; Raman; Biocompatibility; Bacterial culture
1. Introduction

Recently, graphene and its derivatives have shown their prompt significance in the field of nanobiotechnology having their potential applications in widespread fields. Graphene is simply, a single or few layered two dimensional sp$^2$ bonded carbon sheet which is the basic unit of other carbon allotropes. Among the graphene derivatives, graphene oxide (GO) has been considered as a potential material for nanomedicine as it is a water-soluble derivative containing abundant functional groups and the large surface area. The abundant functional groups (epoxide, hydroxyl and carboxyl groups) in GO increases its solubility in water and forms a stable colloidal suspension. These groups also provide various active sites for the process of functionalization and hybridization with other materials like metals, genes, drugs and proteins through electrostatic, coordinate and other approaches (Wang et al., 2014).

A large number of literature reports related to biomedical research, demonstrate the applications of graphene and its derivatives in various fields such as bio-imaging (Pal, 2015; Urbanova et al., 2015; Zhang et al., 2012), biosensing (Liu et al., 2014), cancer therapy [9 - 11], drug and gene delivery [12-14], protein modulation [15], antibacterial activities [16,17], as well as tissue engineering [18,19]. Numerous studies conclude that Graphene or graphene oxide (GO) usually impose adverse toxic effects on the cells or animals such as genotoxicity, immunotoxicity, dose-dependent or size-dependent toxicities [20,21]. However, when these materials are modified or functionalized by proper methods/modifications, their toxic effects could be ameliorated significantly.

Recently, the scientists have shown substantial interests for the effects of graphene derivatives on the microorganisms. Various reports revealed that GO possess antibacterial activity, thus it can be used to inactivate bacteria. The antibacterial activity of GO may be due to ROS generation, interaction between sharp edges of GO sheets and bacterial cell wall to disrupt cell integrity, and wrapping bacteria with GO sheets to reduce their activities [22]. On the other side, a few reports have demonstrated that GO may enhance bacterial activities. [23]. The effects of Graphene oxide alone on the bacterial cells have been explored [24,25]. The controversies in the reported results may be due to the differences in the experimental protocols (e.g. preparation of samples, samples size variations and their chemical states, as well as the culture procedure of bacteria cells). As the effects of GO on the bacteria cells have been demonstrated, the studies related to the interactions of biocompatible, carboxylic acid functionalized GO with the microorganisms along with the underlying mechanisms have not been explored. Such interactions and effects are very important for nanotechnology as well as microbiology, and therefore need great attention.

Thus, in the present investigation, graphene oxide (GO) has been synthesized and functionalized chemically to form carboxylic acid (-COOH) functionalized graphene oxide. The GO and functionalized GO have been characterized by Fourier transforms infrared spectroscopy; Raman spectroscopy and SEM analysis. Further, haemocompatability study has been performed and the interaction of GO and functionalized GO with gram positive bacteria (B. subtilis, S.aureus) as well as gram negative bacteria (E. coli, P. aeruginosa) has been analysed to explore its potential applications in microbiology and nanotechnology.

2. Experimental

2.1. Materials

Graphite and all other reagents have been purchased from Sigma Aldrich. All the reagents are freshly prepared with ultrapure water. The microorganisms Pseudomonas aeruginosa, Bacillus subtilis, Escherichia.coli and S.aureus have been used for the study.

2.2. Preparation of Graphene oxide and Carboxylic Acid Functionalized Graphene oxide.
Graphene oxide (GO) is produced from graphite using Hummers method with slight modifications [26]. Synthesized Graphene oxide (GO) is suspended in water in a concentration of 2 mg/ml to form GO aqueous suspension, and then the bath is sonicated for 1 h. The resultant 2 mg/ml GO suspension (100 ml) has been mixed with NaOH (1.2 g, 30 mmol) and chloroacetic acid (Cl-CH-COOH) (1.0 g, 10.6 mmol), and then the bath is sonicated for 2 h. The -OH groups on the GO are being converted to -COOH moieties. The resulting GO-COOH solution has been neutralized with hydrochloric acid and purified by repeated rinsing and filtrations until the product is well dispersed in water.

2.3. Characterization of Graphene oxide and Carboxylic Acid Functionalized Graphene oxide

The synthesized GO and COOH-GO have been characterized using Raman spectroscopy (in Via Reflex Raman Spectrometer, Renishaw, U.K.), Fourier transform infrared (FTIR) spectroscopy (SHIMADZU IR prestige-21, Japan), and scanning electron microscopy (SEM) (JEOL JSM 6390LV, USA). FTIR spectra are recorded in Attenuated Total Reflectance mode at ambient temperature and pressure. SEM measurements are carried out at 10 kV.

2.4. Biocompatibility study

The haemolysis test has been carried out to investigate the biocompatibility of the synthesized COOH-GO. Different concentrations of COOH-GO (20, 40, 60 and 100 µg/ml) are taken in test tubes containing N-saline (0.9% NaCl) and incubated at 37 °C for 30 min for providing temperature stabilization. The anticoagulated and diluted blood (N-saline : blood; 8 : 10) (0.2 ml) is then added to the test tube, incubated for 60 min after mixing properly. The optical density (OD) of the incubated solution is measured in an UV spectrometer at 545 nm. N-saline has been used as a negative control and Sodium carbonate (1%) as a positive control. The haemolysis percentage is calculated using the formula [27,28]

$$\text{Haemolysis(\%) = } \frac{\text{OD(Uncd sample)} - \text{OD(negative)}}{\text{OD(positive)} - \text{OD(negative)}} \times 100.$$ 

The accepted norm is that if the haemolysis percentage is less than 10 the test sample is considered to be haemocompatible and if it is less than 5 the sample may be highly haemocompatible.

2.5. Bacterial Culture

The microorganisms Pseudomonas aeruginosa, Bacillus subtilis, Escherichia.coli and S.aureus have been used for the study. A fresh subculture is prepared for each microorganism individually. One loopful of individual microorganism is transferred to each autoclaved test tube containing media and incubated in a shaking incubator for 24 hours at 37°C. Further, the culture is again inoculated (1:100) into fresh medium and grown in incubator at 37°C for 2-3 h till an optical density of 0.5 at 600 nm is obtained.

2.6. Bacterial viability assay

The required amount of prepared media is transferred into each sterilized test tube. The different concentrations of graphene oxide and Functionalized graphene oxide (20, 40, 60 and 100 µg/ml) are added into the media. When graphene oxide solution is added it forms a distinct lower layer which is solubilised by shaking the test tube 1-2 minutes. After that, one loopful of microorganisms are added into the all test tubes and incubated for 24hours at 37°C. Finally, all test tubes were checked for turbidity. The cultures are further diluted and seeded in the 96-well plates. The medium containing the same amount of nanomaterials but no bacterial cell is considered as the blank for each culture. The growth as well as the metabolic activities of bacteria have been analyzed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay [25]. All the readings have been measured in triplicate.

The another method used to analyze the numbers of viable bacterial cells is Colony
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forming units (CFU) counting method. The, gradient dilutions of each culture are plated on agar plates in triplicate. The plates are further incubated at 37°C for 24 h. The number of bacterial colonies formed have been counted.

2.7 Cell viability assay of mammalian cells

Lymphocytes are collected from albino mice and the single cell suspension is prepared under sterile conditions. The suspension is filtered through 100 micron filter and centrifuged at 3000 rpm for 10 min at 4°C. Lymphocytes are re-suspended in RPMI-1640 medium [29]. The cells at a density of 1.0×10^4 cells per well are plated in a 96-well plate along with different concentrations of COOH-GO (20-200 µg/ml) and incubated at 37°C in a CO2 incubator for 72 h.

Ten microlitres (µl) of 3(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 5mg/ml in Phosphate buffered saline is further added to each well, 4 h before the completion of incubation. The plate is centrifuged, supernatant is removed and 100 µl of dimethyl sulfoxide (DMSO) is further added to dissolve the formazan formed. The absorbance is read at 530 nm in a microplate reader [30].

3. Results and Discussion

3.1. Preparation and characterization of functionalized GO

Graphene oxide has been prepared from graphite by following Hummer's method with few changes and further, GO has been functionalized chemically to form carboxylic acid functionalized graphene oxide. The GO and functionalized GO have been characterized by Fourier transform infrared spectroscopy; Raman spectroscopy and SEM analysis. FTIR is the widely used technique to characterize the bonding and chemical bonding present in the material. In the FTIR spectra of GO (Fig. 1a), a broad absorption peak has been observed at 3012 cm-1. This absorption peak is due to the –OH stretching vibration of GO. The peak at 1,706 cm-1 correspond to carbonyl group (C=O stretching vibration), the peak at about 1,600 cm-1 is due to the skeletal vibrations of sp² carbon atoms. Stretching peaks at about 1,218 cm-1 and 939 cm-1 are due to the vibrations of COOH and C–O–C groups, respectively. From the FTIR spectra of GO-COOH it has been observed that the –OH stretching peak (3101 cm-1) became sharper than the native GO (Fig. 1b). This peak sharpening may be due to the introduction of carboxylic acid functional group and the nitrogen containing surface group (-NH). In addition, the characteristic bands at 1708, 1511 and 1088 cm-1 correspond to C=O stretching vibration of carboxyl group, the O-H vibration in carboxyl acid and C-O vibration in epoxy group, respectively.

The another most reliable tool to confirm the formation of GO-COOH is Raman spectroscopy. Graphite material has dominant Raman features at 1,620 cm-1 (G band) and 1,375 cm-1 (D band) as shown in figure 2. The G mode, present in most graphite-like materials, is related to the planar vibrations of carbon atoms. The D mode, present in all graphite-like carbon materials, corresponds to the structural defects. The increase in the intensity of D mode (probably becomes higher than the G mode) may be due to the structural disorder in sp2 pattern induced by oxygen containing groups on the carbon basal plane or at the edges. The changes between Graphene Oxide and COOH- functionalized Graphene oxide can be observed by analyzing the plot which shows that the shifting of the intensity at 1,620 cm-1 and 1,375 cm-1 is more sharp and wide in functionalized Graphene oxide rather than Graphene oxide. Hence, it proves that the structural changes occurred and the functionalization is done successfully.

The SEM images of GO and modified GO (1000x and 5000x) are presented in figure 3. From the SEM images it can be observed that GO is having more or less smooth arranged, brick by brick structure. It is also lacking any type of surface growth and fluffiness. However, in modified GO the arranged structure is totally disrupted. Moreover, some petals like surface growth is appeared as observed in higher magnification. These changes in surface property and topology may be an indication, that
Table 1. Biocompatibility study of COOH-GO

<table>
<thead>
<tr>
<th>S. no</th>
<th>Samples</th>
<th>Absorbance</th>
<th>% Haemolysis</th>
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</thead>
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<tr>
<td>1</td>
<td>NaCl (-ve control)</td>
<td>0.234</td>
<td>0%</td>
</tr>
<tr>
<td>2</td>
<td>Na$_2$CO$_3$ (+ve control)</td>
<td>0.714</td>
<td>100%</td>
</tr>
<tr>
<td>3</td>
<td>20 µg/ml</td>
<td>0.238</td>
<td>0.83%</td>
</tr>
<tr>
<td>4</td>
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<td>0.242</td>
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</tr>
<tr>
<td>5</td>
<td>60 µg/ml</td>
<td>0.246</td>
<td>2.5%</td>
</tr>
<tr>
<td>6</td>
<td>100 µg/ml</td>
<td>0.257</td>
<td>4.8%</td>
</tr>
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Figure 1. FTIR plot of (a) Graphene oxide and (b) –COOH functionalized Graphene oxide.
Figure 2. Raman spectra of Graphene oxide and (-COOH) functionalized Graphene oxide.

Figure 3. The SEM micrographs of (e)GO (f) Functionalized GO at 1000x, 10µm (g)GO (h) Functionalized GO at 5000x, 5µm.
Figure 4. Effects of COOH-GO on bacterial viability (a) E. coli, (b) P. aeruginosa, (c) B. subtilis, (d) S. aureus.

Figure 5. Effects of COOH-GO on CFU counting of viable bacteria
GO is successfully modified to its carboxylic form.

3.2 Biocompatibility of synthesized COOH-GO

The biocompatibility is the most important requirement for all the materials used in biotechnology. The biomaterial should also not cause any effect on the proteins adsorbed on the surface during the growth of microorganisms as well as should not percolate any substance that can induce toxic effects to the organisms. The biocompatibility of synthesized COOH-GO has been observed by the haemolysis test. The different concentrations of COOH-GO (20, 40, 60 and 100 µg/ml) have been used for the test. The result of the haemolysis test (Table 1) implies that carboxylic acid functionalized GO is biocompatible.

3.3 Effect of functionalized GO on Bacterial growth

The effects of as-made GO and COOH-GO on the viability of gram positive bacteria (B. subtilis, and S.aureus) as well as gram negative bacteria (E. coli and P. aeruginosa) have been analyzed using MTT assay. The results demonstrate that without functionalization, GO alone has not shown any sort of effect on the cell viability of any bacteria (Figure 4). This result is almost consistent with the literature reports. The functionalized COOH-GO at the concentration of 20 µg/ml, has also shown no effect on the viability of all the bacterial cells. Interestingly, COOH-GO has strongly increased the E. coli viability to around 165% at the concentration of 40 µg /ml. The higher concentration (60 µg /ml) has also shown significant increase in the cell viability but in some less extent. In, P. aeruginosa and B. subtilis, both the concentrations of COOH-GO (20 and 40 µg /ml) have shown significant cell growth. The highest concentration (100 µg /ml) could not affect the viability of cell in all the bacterial species. In S.aureus, only 60 µg /ml concentration has enhanced the cell viability in a significant manner.

The results of the CFU counting method also confirm the growth stimulating effect of COOH-GO (Figure 5). The growth of all the bacterial species has been increased by COOH-GO treatment. This result is similar with the results of MTT assay. Such stimulation effect on bacterial growth, may be due to surface chemistry modification, is fascinating. The interaction between bacterial cells and COOH-GO might be different from the interaction of cells with GO.
Therefore, it is possible that COOH-GO with more carboxylic groups may provide a different biocompatible interface between the bacteria and the nanomaterial, and thus affect the bacterial growth. Its mechanism needs to be explore further.

### 3.4 Effect of functionalized GO on murine lymphocyte viability

Since carboxylic functionalized GO can stimulate the activity and growth of bacterial cells, its effect on the growth of eukaryotic cells has been analyzed further. The murine lymphocytes are being used in the study and the similar concentrations of COOH-GO are chosen those are used in the bacterial study. As shown in Figure 6, murine lymphocytes, after being treated with COOH-GO (20-200 µg/ml) for 24 hrs, only about 15 % increase in cell growth could be obtained with the lowest concentration i.e 20 µg/ml. Further increase in the concentration of COOH-GO (up to 200 µg/ml) could not enhance the cell growth, but exhibited the cytotoxic effects towards lymphocytes. Higher concentrations (100 and 200 µg/ml) have shown even more than 50 % cell death.

### 4. Conclusion

In summary, GO and COOH-GO have been synthesized chemically and characterized using Raman spectroscopy, FTIR and SEM. The biocompatibility study of synthesized samples is carried out and their effects on Pseudomonas aeruginosa, Bacillus subtilis, Escherichia.coli and S.aureus have been carefully investigated. The GO alone has not shown any effect on the growth and viabilities of bacteria, whereas COOH-GO has shown a rather significant stimulating effect on the growth of bacterial cells (both Gram positive and Gram negative). The further investigation of the mechanism underlying the stimulation of the growth of bacteria and any type of toxicity towards bacteria is required in future. Since, the present work reveals that the interactions between nanomaterials (GO) and microorganisms (Bacterial cells) could be closely related with the surface properties of nanomaterials. In the present investigation, specific carboxylation on the GO surface could generate a different surface chemistry for interaction with bacteria. Thus, it can be concluded that carboxylic acid functionalized GO may act as a new, positive regulator for the growth of bacterial cells. This indicates the role of carboxylic acid functionalized GO as a promising nanomaterial in microbial engineering.

**Conflict of interest** - The authors declare that they have no conflict of interest

### References


