Characterization of Graphene Oxide reduced by Bacillus clausii and its activity against MDR uropathogenic isolates

Muktad Fadel Almamad¹, Nawfal Hussein Aldujaili²
¹Department of Microbiology, Faculty of Science, University of Kufa, Iraq
²Department of Biology, Faculty of Science, University of Kufa, Iraq
Email.nawfal.aldujaili@uokufa.edu.iq

Abstract

Due to the emergence of high virulence pathogenic strains of bacteria that are resistant to most antibiotics, this study was conducted to find alternative materials for antibiotics or work with antibiotics against bacterial strains that are resistant to antibiotics. In this study, we used bacterially reduced graphene oxide (BrGO) for this purpose and through the experiment showed that Graphene oxide (GO) nanosheets can biologically reduce by Bacillus clausii by reaction GO with cell free supernatant, where the reaction mixture incubates for 72hrs at 37°C in a shaking incubator. The general properties of reduced Graphene Oxide (rGO) nanosheets by B. clausii were determined through Ultraviolet–visible (UV-vis) Spectroscopy, Scanning Electron Microscope (SEM), Energy Dispersive X-Ray Spectroscopy (EDS), Fourier Transform Infrared Spectroscopy (FTIR), Atomic Force Microscopy (AFM), and X-Ray Diffraction (XRD) analysis. The results showed the successful bacterial synthesis of rGO nanosheets via removal of water molecules and oxygen functional groups from interlayer of GO. So, the reduced Graphene oxide by Bacillus clausii considered excellent and eco-friendly. BrGO nanosheets exhibited potent noticeable antibacterial activity at different concentrations (0.1, 0.5, 1, 5, 10 mg/ml) against both Gram (-ve) and Gram (+ve) tested MDR uropathogenic isolates when used alone or at combining with other antibiotics. Also the results showed that potent growth inhibition zones was increased with increasing concentrations of BrGO.

Keywords: Bacillus clausii, Reduced Graphene Oxide, Bacterially reduced Graphene Oxide, Uropathogenic isolates, antibacterial activity.

Introduction

Urinary tract infection is a term which involve the infection that occurs in any part of the urinary tract, whether the upper side (kidneys and ureters) or the lower side (bladder and urethra).¹

Nanoparticles are manufactured or natural materials have unbound, aggregated or agglomerated particles, and possess one or more external dimensions with size range between 1-100 nm, according to the European Commission (EU Commission).²

The NMs synthesized by several methods include biological, physical and chemical methods, but the biological methods are considered the best methods of NMs synthesis; inexpensive and harmless to the environmental and human health.³
Biological reduction of GO became the focus of researchers’ working on bionanotechnology due to this strategy is efficient and eco-friendly in nature, and subsequently decreasing the high cost and risks of toxic chemicals involved in the conventional chemical routes. Moreover, most of biological reduction routes act at the moderate conditions such as room temperature and atmospheric pressure making them effective, easy to handle, and affordable (4).

The effect of NMs on microorganisms are various due to difference in their physical, chemical, and biological properties which in turn highly affect their behaviors, activities, and interactions with microorganisms. Graphene is a modern NMs that has revolutionized the field of nanobiotechnology and emerged as another new tool for a various modern application according to its physical, chemical, and biological unique properties as in drugs and genes delivery, biosensing, bioimaging, tissue engineering, and as antimicrobial agents (5).

The best alternative materials to antibiotics are nanomaterials (NMs) that have proven to inhibit the growth of pathogenically bacteria resistant to several groups of antibiotics, therefore some NMs used as antimicrobial agents. The antimicrobial activity of NMs is varies according to their different types and the physical properties of NMs itself (6).

Experiment part:

2. Materials

Graphene oxide powder were bought from Alfa Aesar (Karlsruhe, Germany), the rest of experiment materials involves; Methyl Red, Gram stain Kit, Glucose (C₆H₁₂O₆), Glycerol (C₃H₈O₃), Kovac’s Reagent, Oxidase Reagent, N,N,N,N-tetramethyl paraphenylen diamine dihydrochloride, Absolute Ethanol, Ionized water, Hydrogen peroxide (H₂O₂), α-naphthol (C₁₀H₈O), Distilled water, Agarose gel, 6X Loading dye, and MacConkey agar, Brain heart infusion (BHI) broth, Brain heart infusion (BHI) agar, Muller-Hinton agar, Peptone water broth, Nutrient agar, Nutrient broth, Agar -Agar, were supplied by (Hemedia / India), Blood agar Base, Trypticase soy broth, were supplied by (Oxoid / England). All these chemicals were used as received without further purification.

2.1 Isolation and Diagnoses of MDR Uropathogens

A total of 350 urine samples were collected from patients suffering from UTIs and six bacterial isolates (E. coli, K. pneumonia, P. aeruginosa, E. cloacae, S. saprophyticus and MRSA) were selected for this study from among the 50 MDR isolates were diagnosed through morphological properties, biochemical tests and VITEK2 system.

2.3 Selection and Identification of M12 (B. clausii) for Reduction of Graphene Oxide

Different species of Bacillus from different sources was testing their ability to reduction of GO nanosheets to select the efficient isolate for extracellular production, the bacterial isolate Bacillus clausii namely (M12) selected from among 46 different samples depending on the morphological properties, biochemical tests and VITEK2 system.

2.4 Antibiotics sensitive tests of uropathogenic isolates

The antibiotics sensitive tests of uropathogenic isolates were carried out depending to the VITEK2 system.

3- Results and discussion:

3.1 Biosynthesis of rGO Nanosheets Using B. clausii
**Bacillus clausii** (*M12* isolate), showed an ability for extracellular biosynthesis of rGO nanosheets through adding the GO solution at (0.5mg/ml concentration) to cell free supernatant in a ratio 1:1 (volume ratio), after the incubation of mixture for 72 hrs at 37°C in a shaking incubator, the Changing in the color of reaction mixture from a clear, brown to black with precipitate considered as indicator for successful biosynthesis of rGO. (7). The reduction of GO by *M12* isolate was confirmed by changing the color as shown in the Figure (3-1) (A: before a reduction reaction) and rGO (B: after a reduction reaction).

![Figure (3-1): Bacterial reduction of GO obtained by *M12* isolate.](image)

A: Supernatant and GO dispersion, B: BrGO.

The reduction of GO is doesn’t necessarily only removal of oxygen functionalities and other atomic-scale lattice defects, but also involves the replay arrangement or repair of the conjugated graphitic network figure (3-2) (8,9).
3.2. Characterization of BrGO

The general properties of reduced Graphene Oxide (rGO) nanosheets by *B. clausii* were determined through Ultraviolet-visible (UV-vis) Spectroscopy, SEM, EDS, AFM, FTIR, and XRD analysis. Where the results showed the successful bacterial synthesis of rGO nanosheets via removal of water molecules and oxygen functional groups from interlayer of GO. So, the reduced Graphene oxide by *Bacillus clausii* considered excellent and eco-friendly. And as shown in the following analyses:

3.2.1 Ultraviolet-visible (UV-vis) Spectroscopy:

The absorption spectrum demonstrated that the peak of the BrGO suspension was about 268 nm as shown in Figure (3-3). This indicates that the process of reducing GO was successful. The big shift of BrGO can be attributed to the increase in aromatic rings, opposite to the decrease in oxygen functional groups, causing electrons to be easily excited even at lower energy state. So, the ultraviolet spectrum analysis reaffirms the restoration of electronic conjugation after the reduction. Therefore, the peak of rGO or the max value observed of rGO was represented in efficiency of the reducing agent used to reduction of GO. For instance, in the present study, the BrGO produced through using *B. clausii* cell free supernatant showed an absorption peak at 268 nm, which is similar to the rGO that ready by hydrazine derivatives (270nm).
3.2.2. Scanning Electron Microscopy (SEM) analysis of BrGO

The scanning electron microscope (SEM) was used to study the morphological properties such as surface shape, structure and size of (BrGO) samples reduced by *B. clausii*, where the SEM pictures clearly showed rGO composed from very thin, extended sheets closely associated with each other and with wrinkles morphology and lateral dimensions ranging among a few micrometers in length with thickness about (40 nm), Figure (3-4). The presence of the attachment of oxygen functional groups in GO makes its surface furry and coarse, while the folded and flaky morphologies in GO are, according to the presence of water molecules, trapped between the layers of GO. The presence of the attachment of oxygen functional groups in GO sheets dislocates the original conjugation (14).

In previous studies, (15) asserted that the rGO exhibited a typical wrinkled structure through SEM analysis. (16) other examinations of GO and BrGO by SEM were images of GO in the form flakes,
hexagonal shape. The images uncovered that the rGO substance consists of individual sheets closely associated with each other.

3.2.3 Energy Dispersive X - Ray Spectroscopy (EDS) Analysis of BrGO

The chemical elements present in the BrGO sample were determined by using energy dispersive x-ray spectroscopy (EDX) analysis. The amounts of each of elemental carbon and oxygen on the surface of the sample were quantified via observing the optical absorption peaks of carbon and oxygen elements in the sample. The reduction of GO in the reaction mixture into rGO was discovered through the weight percentages of carbon and oxygen.

The record of EDS spectrum was in the spot-profile mode, where optical absorption peak was observed in rGO, in which the strongest signal was noticed from the (C) atoms while the medium signal came from (O₂) atoms and the weaker signal was from (S) atoms. The weight percentages of elemental components in the BrGO biosynthesized by M12 isolate were 79.96% Carbon, 19.71% Oxygen. This points to the partial removal of oxygen containing functional groups as shown in the EDS analysis (Figure 3-5).

In a previous study, [17] used hydrazine solvent as a reducing agent through chemical reduction. They reported that a high carbon peak was found from the rGO sample (88.10%), while the oxygen peak was decreased up to (11.90%). This result was due to the strong reduction reaction by the hydrazined agent, which led to a significant increase in carbon content, and also due to the well bonding of carbon-carbon atoms with each other.

| Element  | Series | Atom (wt. %) |
|----------|--------|--------------|
| Carbon   | K-series | 79.96        |
| Oxygen   | K-series | 19.71        |

Figure (3-5): EDS analysis of BrGO nanosheets biosynthesized by M12 isolate. The weight percentages of Carbon (79.96%) and Oxygen (19.71%).

3.2.4 Fourier Transform Infrared Spectroscopy (FTIR) Analysis of BrGO

FTIR is technique used to identify the bonding formation of different kinds of oxygen. The FTIR spectroscopy analysis of BrGO biosynthesized by M12 isolate in the wave length ranging between 400 to 3800 cm⁻¹ showed that the distinctive peaks associated with oxygen functional groups significantly decreased while the hydroxyl and alkoxy peaks completely vanished, Figure (3-6). The absorption peaks were noticed at 2918.30 cm⁻¹ (for C–H stretching vibrations), 1627.92 cm⁻¹ (for C=O stretching vibrations).
vibrations from the carbonyl groups), 1469.76 cm\(^{-1}\) (for C=C configurable vibrations from the aromatics rings), and 1053.13 cm\(^{-1}\) (for C–O vibrations from the epoxy groups). These results can indicate the removal of most oxygen functional groups from GO nanosheets (18). Thus, the results clearly show that \(B. clausii\) successfully reduced GO to rGO, Figure (3-6).

Figure (3-6): FTIR spectrum of rGO biosynthesized by \(M12\) isolate, was recorded in the 400 to 3800 cm\(^{-1}\) range.

3.2.5 Atomic Force Microscopy (AFM) Analysis of rGO Synthesized by \(B. clausii\)

Through the AFM analysis of rGO nanosheets synthesized by \(B. clausii\), information emerged about the external morphology, roughness, and the average diameter of BrGO nanosheets. Thus, the present density and engraving time may be used in the control of shape and size of the final structures. The average diameter of the GO nanosheets synthesized by \(B. clausii\) was 45.26nm. Figure (3-7) exhibited three-dimension pictures and granularity accumulation distribution charts of rGO nanosheets.

Figure (3-7) Atomic force microscopic analysis of rGO nanosheets synthesized by \(B. clausii\). A- Three dimension of rGO nanosheets images. B- Granularity cumulation distribution chart of rGO nanosheets.
3.2.6 X-Ray Diffraction (XRD) Analysis of rGO synthesized by B. clausii

To study the crystalline structure and interlayer spacing of (BrGO), XRD analysis was used and it confirmed the reduction of GO to the rGO by B. clausii. Thus, the results showed that the broad diffraction peak of rGO synthesized by M12 isolate was centered at $2\theta = 24.5^\circ$, matching to the interlayer distances of 0.3 nm (Figure 3-8). The diffraction peak at $2\theta = 24.5^\circ$ indicates the organized crystalline structure of (BrGO). So, the interlayer distances proved the removal of most oxygen functional groups and water molecules present the interlayer through the reduction of GO nanosheets (16,19).

Figure (3-8): X-Ray Diffraction pattern of rGO biosynthesized by M12 isolate.

A previous research reported that the d-spacing value of Graphene oxide was within the range 0.8-0.9nm in the various styles of oxidation such as: Hummer method, modified Hummer method, and optimized biosynthesis. Also, when the biological reduction agents are to be used for reducing GO, the interlayer distances will decrease because of the removal of most oxygen functional groups and the opening of epoxy rings in the GO nanosheets (20).

Earlier studies discovered that the interlayer spaces of rGO decreased to a great extent after diverse reduction reactions. (21) stated that the optimized broad diffraction peak was near $2\theta =11^\circ$ (d= 0.81 nm) of GO, which was vanished after the reduction reaction by Azotobacter chroococcum, while the range of a broad peak of rGO emerged at 17-24$^\circ$ due to the parallel stacking of the reduced GO nanosheets.

3.2.7 Antibacterial activity of BrGO nanosheets:

The agar well diffusion method on Muller-Hinton agar dishes was used for detecting the antibacterial activity of BrGO nanosheets by add equal volume (100μl) from BrGO solution in each well and incubated at 37°C for 24 hrs. The results showed that the antibacterial activity of different concentrations (0.1, 0.5, 1, 5, 10) mg/ml of BrGO nanosheet biosynthesized by B. clausii was potent effective against almost all the tested MDR uropathogens. A potent growth inhibition zones was increased with increasing the concentrations of BrGO, as shown in the Figure (3-9) and the table (3-1).

Table (3-1): Growth Inhibition Zones (mm) of Different Concentrations of BrGO Against (MDR) Uropathogens Isolates.
Figure (3-9) Antibacterial activity of different concentrations from BrGO against MDR uropathogenic bacteria.

One of the defining qualities of graphene materials is that it has potent broad-spectrum antibacterial activities against both Gram-positive and Gram-negative bacteria and biofilm forming microorganisms. There are many beliefs about the mechanism graphene nanosheets action, it may cause physical malfunctioning or causes structural damage to the bacterial cell walls and membranes, or by isolate bacteria from the microenvironment, and generate reactive oxygen species (ROS). (22)

The largest growth inhibition zone by BrGO nanosheets was showed in Gram (-ve) in contrast to Gram (+ve) bacteria. This difference was possibly due to the difference of the peptidoglycan layer in the bacterial cell between Gram (+ve) and Gram (-ve) bacteria. In the Gram-positive, the cell wall is composed of lipoteichoic acid, thick peptidoglycan layer and cell membrane, while the Gram-negative cell wall is composed of outer membrane, thin peptidoglycan layer, and cell membrane. Thus, the GONPs biosynthesized using B. clausii was considered an effective agent against microorganisms (23). Also, the high concentrations of biosynthesized rGO lead to an increase in its antimicrobial effectiveness which causes the rupture of cell membrane (24).
The presence of oxygen on the GO surface, and other unique bactericidal and disinfecting properties of GO, make it a desirable material to the products composition that accelerate the wound healing process. The complex built of silver and GO acting on the destroy of Gram (+ve) bacteria cells by about 87% and Gram (-ve) bacteria by as much as 100% (25).

3.2.8 The Effect of BrGO when Combinated with Antibiotics

Four bacterial isolates (S. aureus, K. pneumoniae, P. aeruginosa, and E. coli) and six types of antibiotics (AMC, CFM, CRO, CTX, CN, and IMP) were selected for testing the effect of combination antibiotics with BrGO. The method used in this test was Kirby- Bauer disk diffusion method. These bacterial isolates were selected because they were more resistant than other isolates. Also, these antibiotics were chosen because the selected bacterial isolates were totally or partially resistant to these antibiotics. The selected bacterial isolates were cultured on Muller Hinton agar media. The antibiotics discs were spreading on bacterial culture, then 30µl of BrGO solution (1mg/ml concentrations) were added to each of the antibiotic discs separately, and incubated for 24 hours at 37 °C. After incubation, the growth inhibition zone (by millimeter) was measured by a transparent ruler. By comparing the results of the growth inhibition zone of antibiotics alone with the results of the growth inhibition zone after combination with BrGO, it was shown that the inhibition zones of the combination of antibiotic and BrGO is larger than those without BrGO, as shown in Figures (3-10) and (3-11). In addition, the results showed that BrGO enhanced the antibacterial activity of antibiotic against all the selected bacterial isolates (Gram-negative and Gram-positive bacteria), as shown in Table (3-2).

Table (3-2): the effectiveness of antibiotics alone and when combinated with BrGO (0.5mg/ ml) against MDR uropathogens.

| Antibiotics with / without BrGO | MDR bacterially isolates | E. coli | K. pneumoniae | P. aeruginosa | S. aureus |
|--------------------------------|-------------------------|--------|---------------|---------------|-----------|
|                                 |                         | inhibition zone (millimeter) |        |               |           |           |
| AMC                            | 0                       | 0      | 0             | 0             | 0         |
| AMC + BrGO                     | 12                      | 11     | 11            | 12            |           |
| CRO                            | 0                       | 0      | 10            | 9             |           |
| CRO + BrGO                     | 13                      | 11     | 12            | 16            |           |
| CTX                            | 0                       | 0      | 0             | 0             |           |
| CTX + BrGO                     | 13                      | 12     | 13            | 12            |           |
| CFM                            | 0                       | 0      | 0             | 0             |           |
| CFM + BrGO                     | 12                      | 9      | 9             | 0             |           |
| CN                             | 17                      | 15     | 16            | 14            |           |
| CN + BrGO                      | 25                      | 20     | 19            | 20            |           |
| IMP                            | 33                      | 22     | 31            | 33            |           |
| IMP + BrGO                     | 36                      | 29     | 34            | 35            |           |

AMC: Amoxicillin/clavulanic acid; CRO: Ceftriaxone; CFM: Cefixim; CN: Gentamicin; CTX: Cefotaxime; and IMP: Imipenem; BrGO: Bacterially reduced Graphen Oxide.
In a previous study, \cite{26} stated that antibacterial activity of antibiotics increased when combined with BrGO nanosheets against both gram-positive and gram-negative uropathogenic bacteria. Also, \cite{27} stated that the growth inhibition capacity of the combined BrGO with antibiotics can be due to "carrier effect".

Obviously, the effectiveness of antibiotics increases dramatically when mixed with BrGO synthesized by \textit{B. clausii} as shown in figures (3-10) and (3-11).

Figure (3-10): Growth inhibition zone of MDR \textit{K. pneumoniae} uropathogenic isolate. (A): Antibiotic alone, (B): combination Antibiotics with BrGO nanosheet. BrGO: Bacterially reduced Graphene Oxide; 1: Ceftriaxone; 2: Amoxicillin /clavulanic acid; 3: Cefixim; 4: Gentamicin; 5: Cefotaxime; 6: Imipenem.

Figure (3-11): Growth inhibition zone of MDR \textit{S. aureus} uropathogenic isolate. (A): Antibiotic alone, (B): combination Antibiotics with BrGO nanosheet. BrGO: Bacterially reduced Graphene Oxide; 1: Ceftriaxone; 2: Amoxicillin /clavulanic acid; 3: Cefixim; 4: Gentamicin; 5: Cefotaxime; 6: Imipenem.

It is believed that the physical interaction between bacterial cell membrane and the acute edges of Graphene nanosheet leads to an action disorder of the cell membrane and facilitates the passage of antibiotics particles across the cell membrane \cite{28}.
This study observed that (Imipenem and Gentamicin), being alone, exerted great effectiveness against uropathogenic isolates. This effectiveness increased in the presence of BrGO that was mixed with them. This increase in the antibacterial activity of (Imipenem and Gentamicin) can be seen through increasing the inhibition zones of growth, as shown in Table (3-2). This antibacterial enhancement may be attributed to the "carrier effect".

The increase in the antibacterial activity of the combination of antibiotics with BrGO could be as a result of several factors including the unique physicochemical characteristics of reduced GO, the antibiotics bioavailability, and sensitivity of bacteria to antibiotics, as well as the strain of the selected bacterium (29).

5- Conclusions

1- Bacillus clausii was capable of reducing of GO nanosheets by the reaction of GO with supernatant. It can be used as ecofriendly synthetic protocol for reducing Graphene Oxide.

2- Through (UV-vis, EDS, XRD, FTIR, AFM, and SEM) analyses of BrGO nanosheets synthesized by Bacillus clausii, the successful bacterial synthesis of rGO nanosheets was discovered via the removal of water molecules and oxygen functional groups from interlayer of GO.

3- The rGO nanosheets synthesized by Bacillus clausii exhibited a potent noticeable antibacterial activity at different concentrations (0.1, 0.5, 1, 5, 10 mg/ml) against both Gram (-ve) and Gram (+ve) tested MDR uropathogenic isolates such as (E. coli, Ps. aeruginosa, K. pneumoniae, S. saprophyticus, and S. aureus).

4- The antibacterial activity of BrGO nanosheets against the tested uropathogenic isolates was increased at the combination of BrGO with broad-spectrum antibiotics, compared to the effects of antibiotics alone.

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