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Synthesis of Graphene Oxide Using Simplified Hummer's Method for Antibacterial Application

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Abstract. In order to promote the biological applications of graphene based materials, great exploration for nano-scale preparation of graphene oxide (GO) has been made with a novel facile processing method and low cost. For these reasons this method has been carried out in the following study. The GO nanosheets have been prepared depending on Hummer's method, which represents an easy and efficient technique since 1958. Alternations have been done to simplify this method in order to prepare the GO with a novel way without a need to add $NaNO_3$. Also this preparation has been achieved at room temperature without a need to water bath at temperatures of 35 °C and 98 °C. Structural and morphological properties and contents of the synthesis nanosheets were characterized by using XRD, SEM, and EDS techniques, respectively. The purpose of this work is to evaluate the antibacterial activity of GO nansheets against Gram-negative and Gram-positive bacteria of *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*), respectively. Bacteriological test were achieved by colony forming units (CFUs) assay. CFUs assay has ensured potential of GO as an anti-infective agent for controlling the growth of two spices of bacteria. The results have showed that Gram-positive were more effective by nanosheets than Gram-negative.

Keywords: Graphene Oxide; Simplified Hummer's Method; XRD; FE-SEM; EDS; CFU.

1. Introduction

Nanomaterials are very small in size and have a large surface area per unit volume. These novel physical characteristics of nanomaterials can be produced drastically various chemical and biological properties with respect to the properties of the same material in bulk form [1]. Researches into graphene and graphene based materials have expanded into medicine and life sciences focusing on imaging, biosensors, drug delivery, and pathogen control [2]. Graphene is the thinnest and strongest known material in the world, where a lot of attention has been received by researchers to study this material, due to its attracting properties in chemistry, physics and spacious range of potential applications involving transparent conductors, nano–electronic devices and disease diagnosis [3]. Graphene is a single atomic plane of graphite that was first obtained from the micromechanical exfoliation of graphite. From the chemistry point of view, GO is a graphene sheet with carboxylic groups at its edges and hydroxyl and epoxide groups on its basal plane [4]. Usually the GO nanosheets were prepared by Hummer's method, however in the current work this nanomaterial have been prepared by simplified Hummer's method without adding $NaNO_3$ and without using water bath [5]. Until now, biomedical applications of graphene based materials especially GO have been relatively

finite. However, recently considerable interests have been directed toward biological applications of GO [6]. It is well known that GO is a biocompatible nanomaterial and possess antimicrobial properties, which have been used as anti-bacterial agents. They have tremendous applications in catalysis, composite materials, solar cells, biosensors and biomedical applications [7, 8].

The application of the prepared GO nanosheets has been studied for the antibacterial activity by using well diffusion method and the morphological changes depending on the SEM technique for the two type's pathogens *E. coli* and *S. aureus* [5]. However, this work is deal with the antibacterial application for the above species by using CFUs assay. The *E. coli* and *S. aureus* are the common bacterial forms and the major cause of infectious diseases in humans and animals [9]. Therefore, it is important to look for new antibacterial materials as alternative of antibiotics for therapy from these bacterial strains.

2. Experimental Detailas

2.1. Preparation of Graphene Oxide

The GO was prepared by a novel method from graphite powder (212 μm mesh) by using a simplified Hummer's method. Briefly, 1 g of graphite powder (Sigma Aldrich Company (Germany)) was first oxidized by reacting them with 100 ml of concentrated sulfuric acid (95-97% H_2SO_4, Schwefelsäure Company (Netherlands)) under constant stirring. Actually, the reaction was continuing for about 3 days to fully oxidize graphite to graphite oxide after adding 3 g of potassium permanganate (99% $KMnO_4$, GCC Company (England)) gradually to the above solution while the solution temperature has been kept less than 20 ℃ in order to prevent over heat and explosion. In order to terminate the oxidation process and hence destroying the excess $KMnO_4$, solution of hydrogen peroxide (37% H_2O_2 (5% v/v), United Horizon Company (USA)) has been added. For purification, the mixture was washed by rinsing and centrifuged by Gemmy Industrial Corporation – Taiwan, with speed 8000 rpm several times with 1 M of hydrochloric acid (HCl, Romil company (UK)) solution to remove the metal ions. Then, it was washed repeatedly with deionized water to eliminate the acid in the mixture. Ultrasonic apparatus model (1740QT) supplied by VGT Company – China, has been used for 20 min to convert graphite oxide to GO [10]. A vacuum oven at 80 ℃ has been used to dry the sample by using (VO-27) oven supplied by Hysc Company - Korea, in order to obtain GO as a powder.

2.2. Characterizations:

The XRD measurements of drop of GO, dried on glass slides have been investigated by using Shimadzu - Japan X-Ray Diffraction (XRD 6000), a CuKα tube with radiation of wavelength λ=1.5406 Å operated at 40 kV and 30 mA was used to generate the x-rays, with scanning speed 8 deg/min, scan mode: continuous. The FE-SEM morphology, microstructural features, and cross section images of dried GO drops on glass substrates have been investigated by using type (Hitachi FE-SEM model S-416, Japan). A typical accelerating voltage of 15 kV was used for secondary electron imaging (SEI) of 1500 nm thickness Au coated samples. Finally, the EDS analysis was performed by using a spot size of 0.5 nm, the Bruker, Germany (EDS) analyzer software has been used to obtain and identify the elemental spectra of various points of interest. The thin film of prepared GO was deposited on a glass slide by dropping few drops of the GO on the slide and dried at 80 °C under vacuum oven.

3. Antibacterial Activity Test

3.1. Microorganisms and Required Materials:

An *E. coli* isolates has been supplied by the (medical microbiology laboratory, branch of biotechnology, department of Applied Science, University of Technology, Baghdad – IRAQ) and *S. aureus* has been provided by Nanotechnology Center (Microbiological Laboratory, University of Technology, Baghdad – IRAQ). The used materials for antibacterial activity of GO were nutrient broth has been supplied by Himedia, India.

3.2. Preparation of Inoculums:

In this method, 1.3 g of nutrient broth has been dissolved in 100 ml of distilled water (D.W.) in two universal tubes and sterilized. In one universal tube clinically isolated strain of *E. coli*, has been inoculated and in the other universal tube clinically isolated strain of *S. aureus* has been added. The bacterial cultures inoculated nutrient broth has been kept on incubated at 37 °C for 24 h.

3.3. Inoculation of Test Plate:

Nutrient agar is prepared (2.8 g dissolved in 100 ml of D.W.) and sterilized. The agar suspension is poured into sterile petri–plates and allowed to solidify. Then the dilutions of two pathogenic strains *E.coli* and *S. aureus* with and without GO nanosheets were spreaded evenly over the entire surface of the plate by spreader technique.

3.4. Experimental Techniques:

The GO nanosheets have been tested as antibacterial activity by CFUs assay. The standard plate count assay consists of diluting the sample with sterile saline or phosphate buffer saline (PBS) until the bacteria are diluted enough to accurately be counted. The plate in the series which includes 30 to 300 colonies is taken into account. The plates that contain fewer than 30 colonies are not agreeable for statistical reasons (too few to count may be not representative of the sample), while plate with more than 300 colonies are likely to produce colonies too nearby to each other (too numerous colonies to be counted), and hence difficult to be distinguished as distinct CFUs [11].

There are four groups of specimens were tested, for *E. coli* and *S. aureus* cells before and after GO nanosheets treatment. The used samples for CFUs assay have been prepared in four universal tubes. The bacterial cultures in an inoculated nutrient broth, for untreating (control) and treating groups, have been kept on incubating in shaking incubator with 150 rpm at 37 °C for 24 h.

Over a period of time (overnight), the four groups suspensions was serially diluted 10 fold in normal saline (0.1 ml of each dilution of each group spread out on a solid nutrient agar medium) by using spreader technique. The plates were incubated overnight at 37 °C, and then the number of viable organisms in the plates per 1 ml was determined by the number of colonies divided by the dilution used [11] and compared with control which is represented bacterial suspension without GO nanosheets for *E. coli* and *S. aureus*. All of these tests have been achieved in triplicate.

$$CFU/ml = \frac{Number\ of\ colonies}{Volume\ of\ culcture\ plate\ (ml) \times dilution\ used} \qquad \text{………..……... (1)}$$

The percentage reductions have been calculated on the basis of the initial count according to the following equation. Also, the percentage reductions of bacteria have been estimated by using bactericidal rate *K* [12]:

$$K = \frac{(A-B)}{A} \times 100\% \qquad \text{……………... (2)}$$

where

A and *B* are the numbers of bacteria colonies corresponding to the control samples (without GO nanosheets) and samples with GO nanosheets, respectively.

4. Results & Discussions

4.1. The X-Ray Diffraction:

Figure 1 illustrates the XRD pattern of the prepared GO nanosheets. As shown in the XRD pattern, a broad peak near 11.88º was observed. This peak corresponds to the interlayer spacing of 7.44 Å which represents the diffraction from [001] plane; this indicates that GO has been exfoliated successfully. This result is in a good agreement with that has been reported by *Alam et al.* [13] who have also prepared GO using modified Hummer's method where a broad peak around 11.95º has been observed in the XRD pattern. Additionally, the number of layers of GO (2θ=11.88º) are about 5 layers, while the crystallite sizes (D) of prepared nano GO has determined using Debye-Scherrer's equation [14], and it is found to be 3.7 nm.

$$D = \frac{0.89\lambda}{\beta \cos\theta}$$ …………………… (3)

And the number of layers is given by;

$$Number\ of\ layers = {}^{D}\!/_{d}$$ …………………… (4)

where

λ is the wavelength of incident X-ray (nm), β is the full width at half maximum, and θ is the diffraction angle. The variable d is the distance between atomic layers in a crystal.
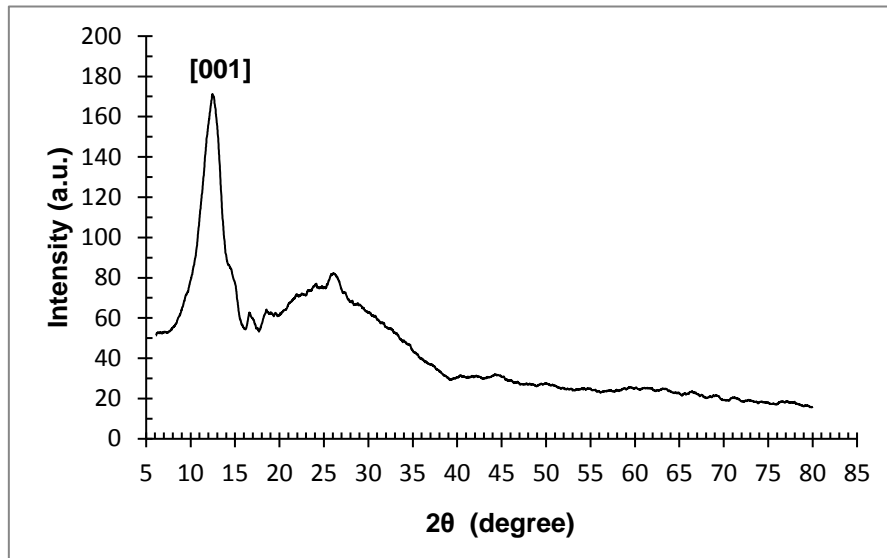

Figure 1. XRD pattern of synthesized GO nanosheets.

4.2. The Field Emission - Scanning Electron Microscope and the Energy Dispersive Spectroscope Analysis:

Figure (2 a) shows the morphology of GO which represents an ultrathin and homogeneous of the GO film with a corrugated surface. However, from figure (2 b), it can be seen that the cross section photograph of GO has a layer structure with tightly packed.
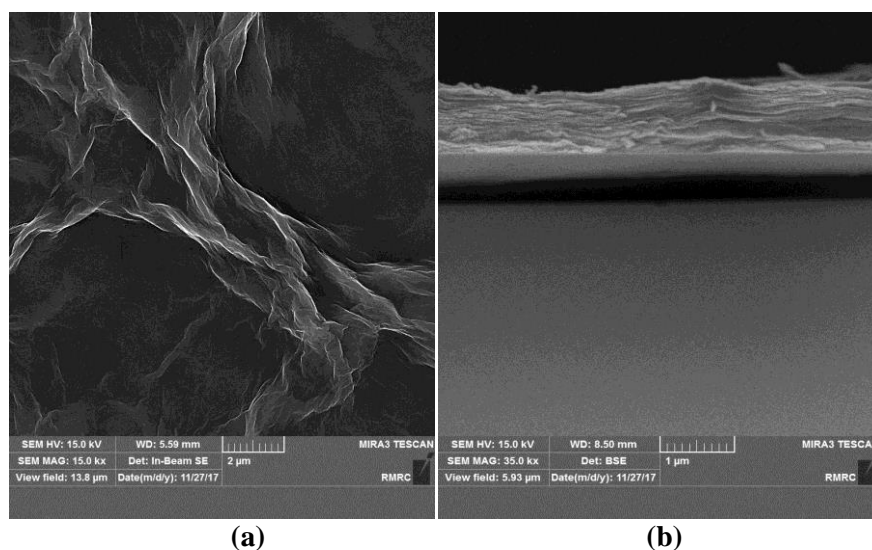

| (a) | (b) |

Figure 2. FE-SEM of the synthesized GO nanosheets.
(a) The morphology image.
(b) The cross section image.

The energy dispersive spectra (EDS) of the samples obtained from the SEM-EDS analysis has confirmed the presence of Carbon (35.08 wt%) and Oxygen (47.45 wt%) elements of the weight in the analyzed sample, as shown in figure 3. Also there are other peaks with very small percent due to glass substrate impurities, while the presence of sulfur (S) (3 wt%) is due to the chemical reaction of the used H_2SO_4 compound.
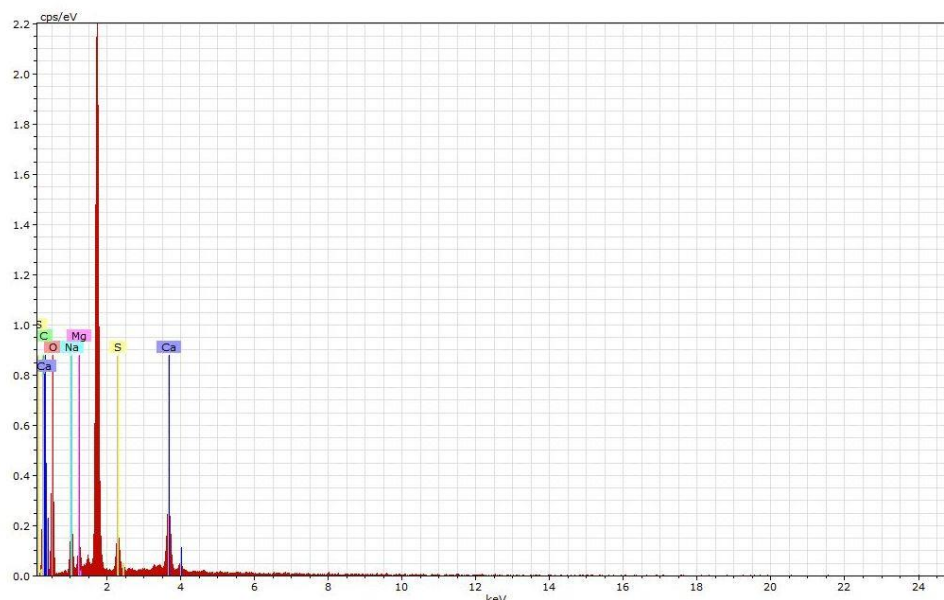

Figure 3. EDS of GO nanosheets.

4.3. Colony Forming Units Assay:

The degree of antibacterial activity of GO nanosheets against two species bacteria are determined by measuring the CFU values by using serial dilutions according to viable count assay. The CFUs methodis an in vitro cell survival method based on the capability of a single cell to grow into acolony. The antibacterial activity of GO nanosheets for Gram-negative and Gram-positive bacteria cells have been evaluated as comparison with control samples; the numbers of bacteria colonies have been decreased after treated with GO nanosheets for two species. The reduction of colony formation may lead to the fact that the bacterial cells have been inhibited within the 24 hrs of treatment. The percentage of inhibition of *E.coli* and *S. aurues* are 24% and 40%, respectively. The GO nanosheets exhibited minor efficiency on the colony formation against *S. aurues* more than *E. coli* bacterial cells as shown in figure 4.

Structural variations in the bacterial cell wall led to the classification of bacteria into Gram-negative and Gram-positive. Where, the last bacterial cell wall involves a thick peptidoglycan layer and another coat of polysaccharide usually created from teichoics and teichuronic acids. However, the cell wall of Gram-negative bacteria has more complexity, include thin layers of peptidoglycans, lipoproteins, lipopolysaccharides and an outer membrane. This structure explains, why Gram-positive would be more influenced by nanoparticles than Gram-negative bacteria [15].

Generally, there are several mechanisms proposed for the antibacterial activity of graphene materials such as direct contact mechanism, oxidative stress, and also due to the trapping of microorganism within the aggregated graphene nanosheets. Membrane damage may be caused by the atomically sharp edges of graphene, which could penetrate the cell membrane and physically disrupt its integrity. Reactive oxygen species (ROS) are probably familiar in biology due to their ability to cause oxidative stress. The antibacterial activity of the GO nanosheets relates on the production of hydroxyl radicals which will attack the carbonyl groups present in the peptide links of the bacterial cell wall and thus leading to the destruction of the bacteria [16, 17].
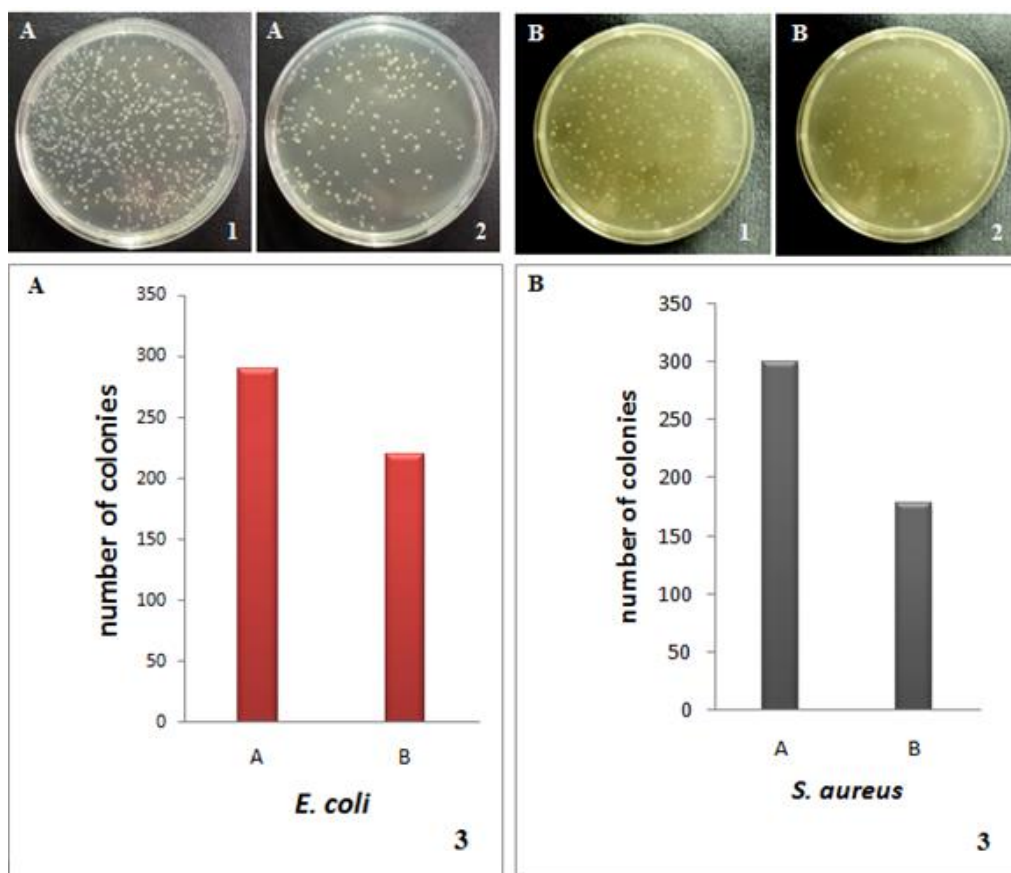
Figure 4. shows the antibacterial effects on (A) *E.coli* and (B) *S. aureus* bacterial cells by using CFUs assay, (1) before treatment, (2) after treatment, and (3) statistical accounts.

5. Conclusions

This study demonstrates that the GO nanosheets have been prepared successfully by using novel simplified Hummer's method and characterization results confirm the GO formation.

The results of XRD have confirmed the existence of a broad nano peak of GO with the grain size and the number of layers of the prepared GO nanosheets measured from XRD. The SEM has provided the morphology of nanomaterial, and the 2D sheets were very clear from the cross section view of the sample. The EDS has confirmed the existence of carbon and oxygen.

In this study, the antibacterial activity has been studied of the prepared GO nanosheets toward two prokaryotic bacterial species. The GO nanomaterial exhibits excellent antibacterial properties, and the obtained data allowed to formulate the conclusion that nano-GO may inhibit bacterial growth.

TheiCFUs assay of *E.coli* and *S. aureus* cells with GO sheets, has indicated that this nano-scale material was reduced the number of colonies for two species bacterial cells. From these results, one can predict that GO nanosheets will ensure its importance as a potential antibacterial agent in the development of future nanomedicine.

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Conflict Of Interest

The corresponding author states that there is no conflict of interest, financial or otherwise.