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HEMOLYSIS ACTIVITY AND CELL VIABILITY AGAINST *E. COLI* OF FEW LAYERS AND MULTILAYERS GRAPHENE OXIDE (GO) NANOSHEETS

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Abstract

In the last years, the biological applications of graphene oxide (GO) have been gained more attention. Many prominent characteristics and features of GO make it a candidate material for biological applications. A few layers and multilayers of graphene oxide nanosheets (GO) were prepared using the electrochemical exfoliation method were used to study the biocompatibility of each of them and to study the effect of the number of layers on hemolysis activity in addition to their effect as an antibacterial activity against the *Escherichia coli* (*E. coli*) bacteria. The antibacterial activity of GO was tested by cell viability assay. In this work, contrasting results were shown regarding hemolysis and antibacterial activity against the *E.coli* bacteria caused by GO nanosheets for the few and multi layers. The morphological, structure and optical properties were investigated by using Field emission scanning electron microscope (FE-SEM), X-ray diffraction(XRD) , Fourier Transform infrared spectroscopy(FTIR). FE-SEM was used to confirm existence few and multilayers GO nanosheets. The number of GO layers and crystalline size were calculated using XRD analysis. The functional groups of GO were determined by FTIR analysis. Moreover, the stability of GO was determined by Zeta potential analysis.

Keywords: Graphene Oxide, Nanosheet, Electrochemical Exfoliation, Hemolysis, cell viability, *Escherichia coli*.

Introduction

The biological effect of nanomaterials have attracted great interest both *in vitro* and *in vivo* because of its distinctive properties such as high surface area, small size, and chemical composition(Liu *et al.*, 2014, Kaminskas *et al.*, 2011, Reddy *et al.*, 2012). The unique characteristics of carbon based nanomaterials, especially graphene oxide, which make them promising materials for use in biomedical applications (Maiti *et al.*, 2019, Mohajeri *et al.*, 2019).

GO is a 2d nanomaterial (Perrozzi *et al.*, 2015). Its characteristics are similar to graphene, but it is distinguished by the presence of functional groups (Stankovich *et al.*, 2007). The presence of functional groups makes it the GO hydrophilic material thus easy to disperse in aqueous solution (Stankovich *et al.*, 2007), while graphene is observed to be hydrophobic (Xu *et al.*, 2014). Graphene oxide has gained more interest due to its unique properties such as the specific surface area volume ratio is large and production cost is low. GO became a promising nanomaterial for biological application due to these properties (Gupta *et al.*, 2015).

The bad employ of antibiotics has led to the development of bacterial strains that are resistant to many known drugs. This status has resulted to search for new treatment (Soria-Mercado *et al.*, 2012; Shukla, 2015). One of these treatment alternatives is the application of nanomaterials as an antibacterial activity.

E. coli bacteria is a gram-negative, anaerobic bacterium, of the genus Escherichia, which is usually found in human intestine and warm-blooded animals (Katouli, 2010; Mogna *et al.*, 2012). Some strains of *E. coli* are pathogenic and cause diseases such as bloody diarrhea, watery diarrhea, meningitis, urinary tract infections and poisoning (Nataro and Kaper, 1998; Gyles, 2007).

Also, it is necessary to study the extent of blood compatibility of nanomaterials that are used to be an alternative to antibiotics. Hemolysis is the breakdown of red blood cells. A material that leads hemolysis is a hemolysin (Wei, 2015).

Experimental (Material and Methods)

Synthesis of few layers and multilayers GO nanosheets

The electrochemical exfoliation method has been adopted for the purpose of preparing GO using graphite rods. High purity graphite rods were used as cathode and anode. Three centimeters was the distance between them. These electrodes were fixed in a beaker filled with five types of acids. PH was standardized for each acid at PH = 4.The acids used in exfoliation are (H_2SO_4, CH_3COOH, HNO_3, HCl, H_2O_2,). After the acid solution was prepared, the electrochemical exfoliation of the graphite rods was carried out by connecting them to a power supply. The voltage applied across the graphite rods was 10 volts.

After 24 hours, bubbles were observed around the electrodes, which indicate the exfoliation process began and the color of the solution seemed to change to a bright yellow color. The peeled graphite layers begin to expand, separate and then spread to the solution (Parvez, 2014).

Following that, the solution was sonicated for 3hrs to vibrate and dismantle the molecules of the solution. Thereafter, the solution was centrifuged 3times with distilled water at 4000rpm for 20min. After centrifugation, the suspended solution was separated from the precipitate. The precipitate was sonicated for half an hour for in order to decouple the accumulation of precipitate **which** was represent multilayer GO nanosheet. The suspension that represent few layers GO nanosheet.

Finally, both suspension and precipitate were dried in the vacuum oven for 2 hours.

Cell viability assay:

One ml of *E. coli* cells (10^8 Cell /ml) were incubated with 1 ml of each concentration of a few layers and multilayers GO nanosheets dispersions in isotonic saline solutions at 37 ℃ under 250 rpm shaking speed for 3 hrs. The percentage of loss of cell viability was estimated by colony counting method.

Fifty microliters of different concentrations (**25**, 50, 75 and 100 µg /ml) with *E. coli* cells were spread onto and Macconkey agar plates and left to grow overnight at 37 ºC. Colonies were calculated and compared with control. The *E. coli* bacteria in isotonic saline without a few layers and multilayers GO nanosheets were used as control (Liu *et al.*, 2011).

Hemolysis assay

Hemolytic toxicity of few layers and multilayers GO nanosheets was done by mixing 200 µL of the separated blood with 1600 µL normal saline. Different concentrations of few layers and multilayers GO nanosheets (25, 50, 75 and 100 µg/ml) were completed with 1 mL distilled water and then 200 µL from each concentration was added to diluted blood . Distilled water was used as positive control (100% lysis) and normal saline was applied as negative control (0% lysis). The controls and concentrations were incubated for 1 h at 37°C and centrifuged at 4000 rpm for 10 min. The Absorbance was measured at 541 nm spectrophotometry and the hemolysis percentage was calculated using the formula(1) in following (Joshy *et al.*, 2016).

$$Hemolysis(\%) = \left(\frac{Abs.sample - Abs.negative\ control}{Abs.positive\ control - Abs.negative\ control}\right) \times 100\%$$

...formula(1)

Characterization Techniques

(1) FE-SEM

Figure 1A shows the micrograph of few layers GO nanosheets with sharp edges, which it has the structure of layers like papers. While Figure 1B shows the micrograph of multilayers GO nanosheets with smooth edges, which it has structure of folded layers.

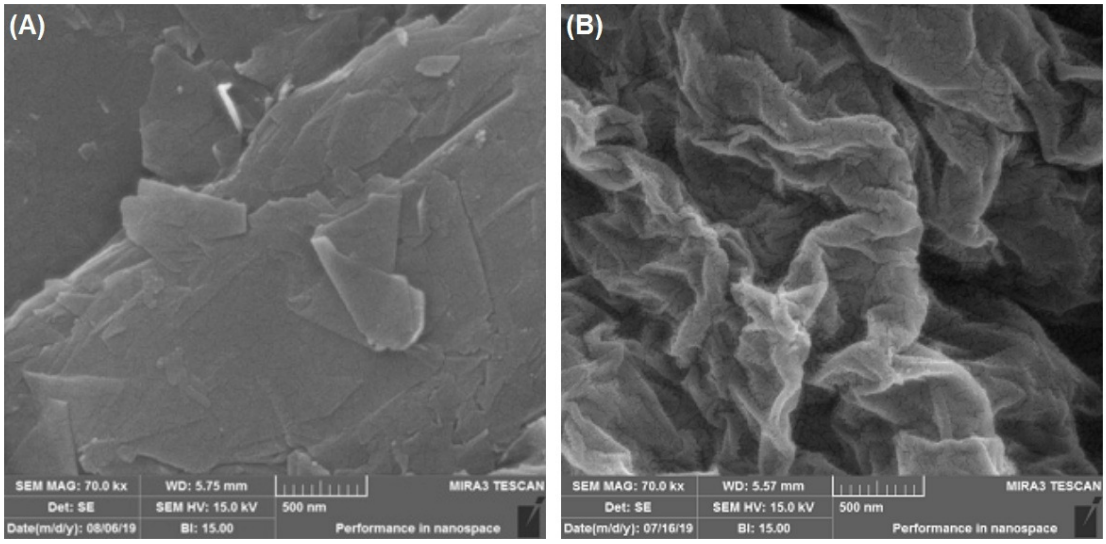
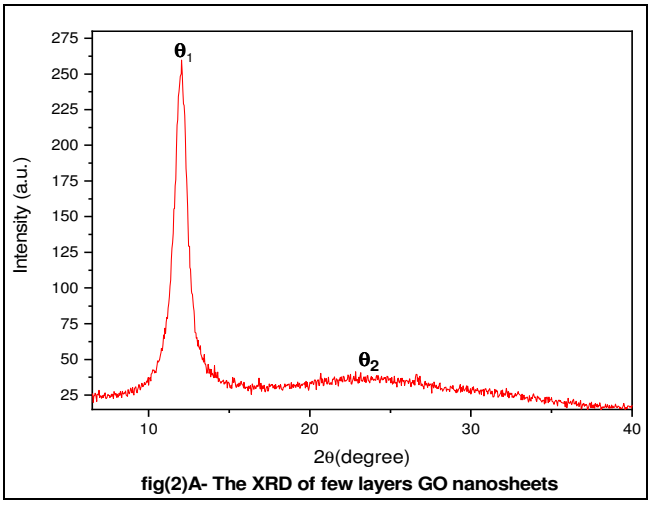


Fig. 1 : FESEM of GO nanosheets (A) few layers (B) multi layers

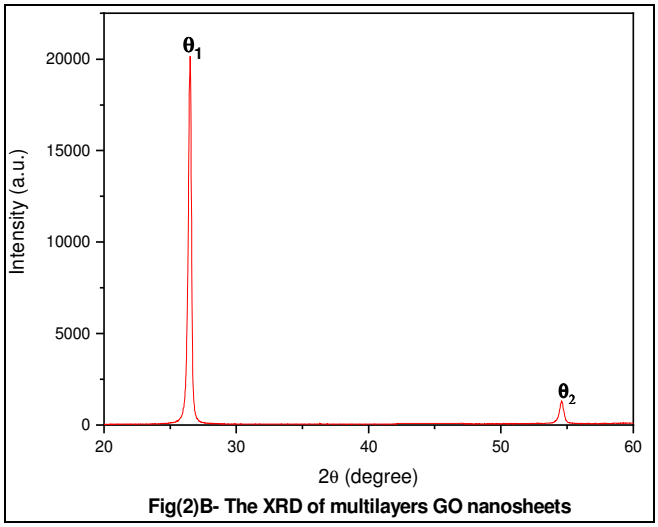
(2) XRD

By analyzing XRD, the few layers and multilayers of GO nanosheets which were prepared via electrochemical exfoliation method. The crystal structure was compared between both of them. Where the major peak of the few layers of GO nanoseets was at 2θ = 12.04 ° and a crystalline size was 6.8 nm number of layers were 9 (Figure 2A).



fig(2)A- The XRD of few layers GO nanosheets

While the major peak of the multilayer GO nanosheets was 2θ = 26.52°. Additionally, crystalline size and number of layers were found to be 15.1nm and 48, respectively.

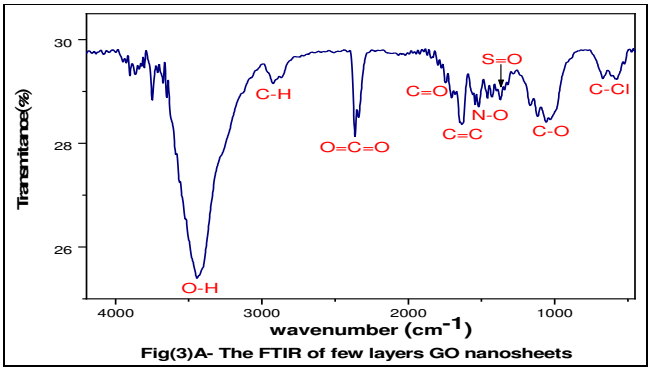


Fig(2)B- The XRD of multilayers GO nanosheets

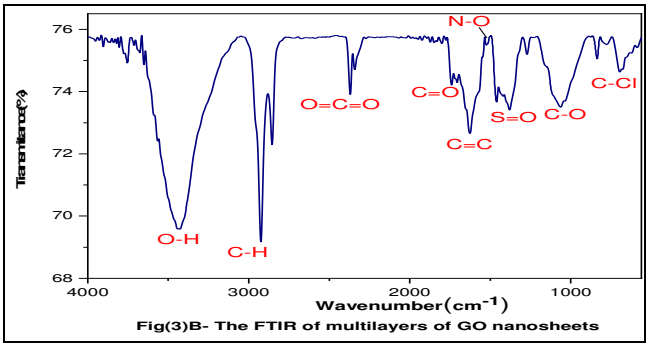
(3) FTIR

The FTIR spectrum of a few layers and multilayers of GO nanosheets presents the following functional groups: (O-H), (C-H), (CO_2), (C=O), (C=C), (N-O), (S=O), (C-O), (C-

Cl). The presence of these functional groups indicates the success of graphene oxidation.



Fig(3)A- The FTIR of few layers GO nanosheets



Fig(3)B- The FTIR of multilayers of GO nanosheets

Results and Discussion

1 - Bcterial cell Viability with GO:

The antibacterial activity of the few layers and multilayers of GO nanosheets against *E. coli* were investigated by colony counting method as shown in Figure 5(a and b), respectively. The loss of cell viability in liquid medium was estimated by using the number of colonies forming onmacconkey agar plates which incubated at 37 ºC for 24 hrs .The colony counting method showed that few layers GO nanosheets inhibited the growth of *E.coli* depending on a concentrations. The four concentrations were measured against *E. coli* are 25, 50, 75 and 100 µg/ml, respectively. The colony counting method confirmed the anti-bacterial properties of GO, where it inhibited the growth of *E. coli* bacterium and killed them. The highest of loss of cell viability at 100 µg/ml while the lowest of loss of cell viability was 25 µg/ml for both few layers and multilayers of GO nanosheets. The figure 4 (a,b and c) represent control, *E. coli* with few layers GO nanosheets and *E. coli* with multilayers GO nanosheets, respectively. It should be noted that the loss of cell viability with few layers GO nanosheets is much higher than the multilayers GO nanosheets. Finally, it is important to mention that the toxicity of nanomaterials towards bacterial kinds depend on the bacterial structure and the enzymatic activity and other factors (Oh *et al.*, 2018).

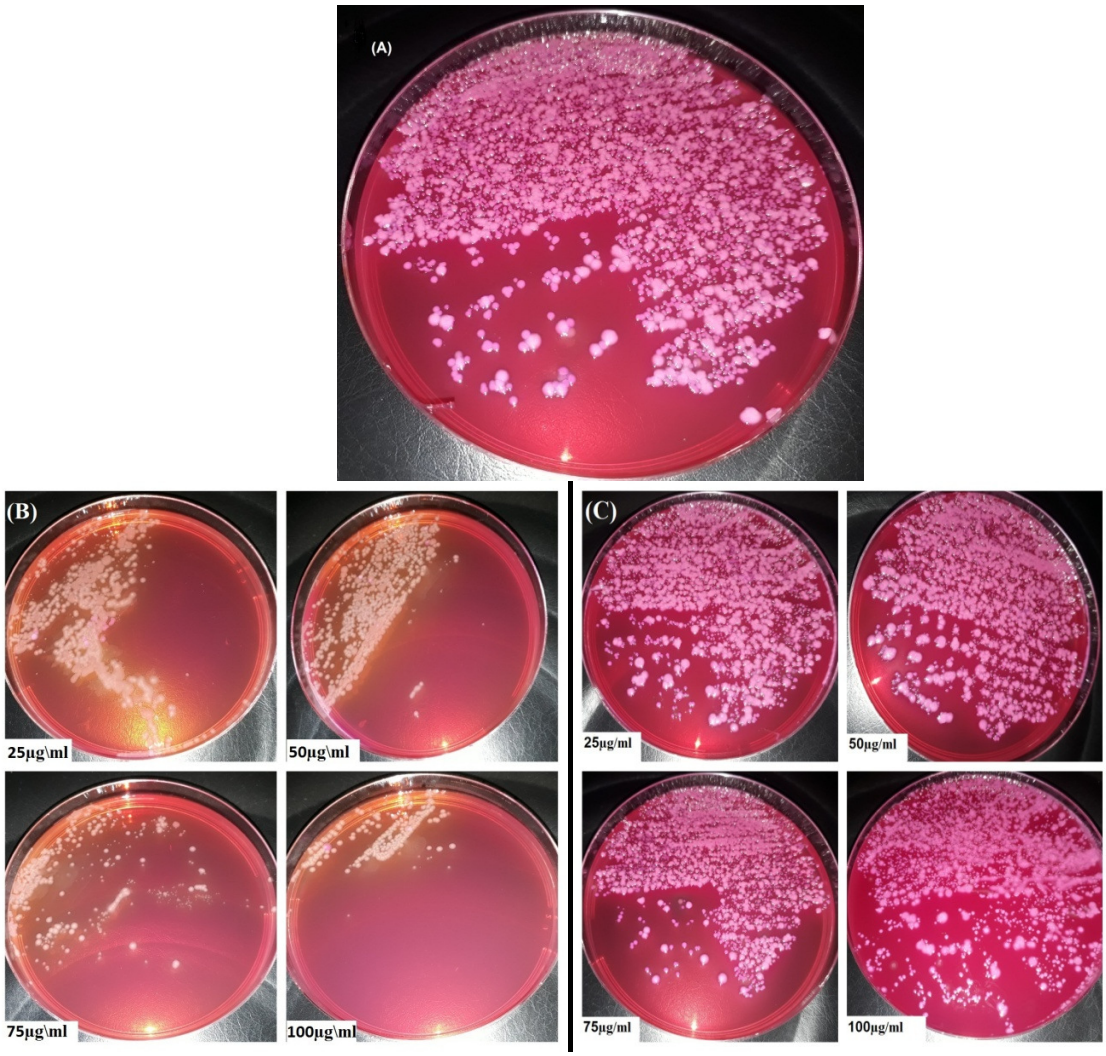


Fig. 4 : A- the control, B- *E.coli* colonies in the presence of few layers GO Nanosheets on macconkey agar plate., C- *E.coli* colonies in the presence of few layers GO Nanosheets on macconkey agar plate.

The figure (5)A and B show the relationship between loss of cell viability of few layers and multilayers of GO nanosheets with different concentrations.

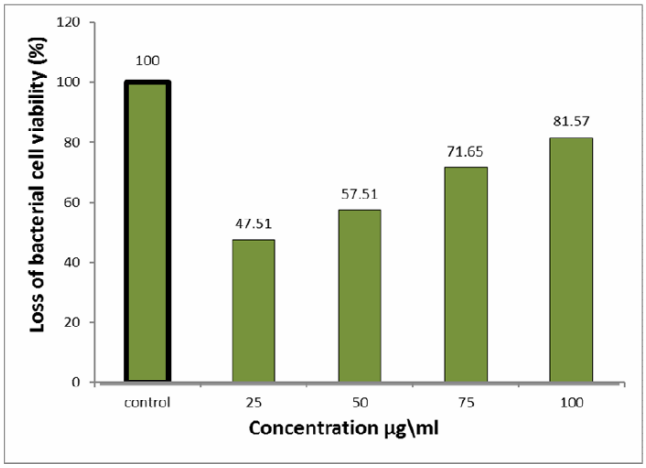


Fig. 5A : Determination of antibacterial activities of few layers GO Nanosheets

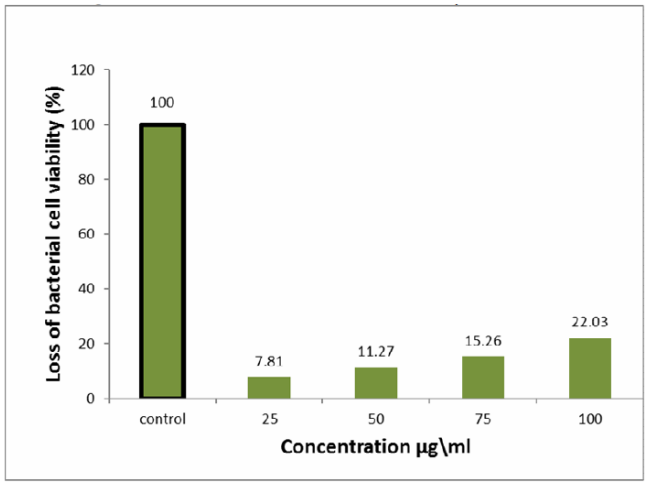


Fig. 5B : Determination of antibacterial activities of multilayers GO nanosheets.

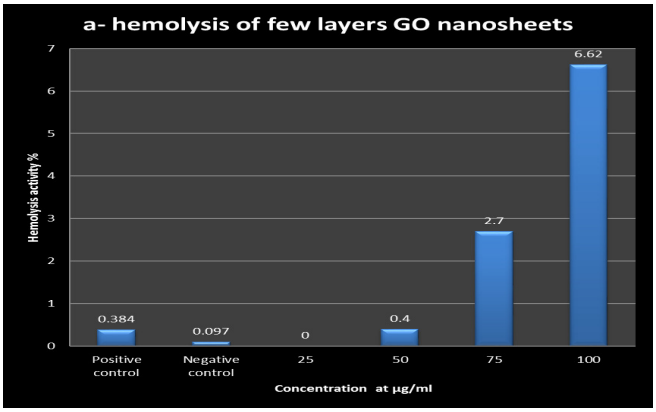
Hemolysis activity of GO

The hemolysis of RBCs when incubated with 25, 50, 75 and 100 µg/ml after exposure to four concentrations of both few layers and multilayers GO nanosheets and incubated for 1 h. Also it shows the values of hemolysis for each concentration. It was apparent that hemolysis percentage of few layers GO nanosheets was less than that multilayers GO nanosheets, as shown in figure 6 (a and b).

No hemolysis activity of few layers GO nanosheets was observed when 25 µg/ml was applied, while hemolysis value at the concentration of 100 µg/ml was 6%. On the other hand, the hemolysis activity of multilayers GO nanosheets were 11 and 22 % when 25 and 100 µg/ml were used, respectively.

Briefly, the few layers GO nanosheets did not aggregate and behaved in the same manner as that of normal saline. While the aggregation of RBC with multi layers GO nanosheets was revealed. The multilayers GO nanosheets is reported to have toxicity and behaved like positive control. Where the positive controls in blood studies are materials that induce aggregation of the blood cells.

Blood compatibility is an important measure that must be studied to evaluate responses between particles whether nanomaterials (few layers and multi layers) or micromaterials with the blood, thus it determines the effectiveness of these material within the human body (Joshy *et al.*, 2016).



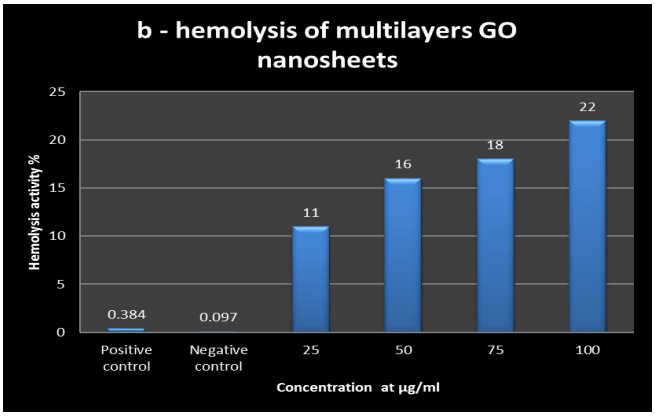


Fig. 6 : Determination of hemolysis activity of (a)few layers GO, (b) multilayers GO nanosheets at different concentrations.

Conclusions

Few layers and multilayers GO nanosheets have been successfully synthesized by simple and inexpensive electrochemical exfoliation method. The results of bacterial cell viability assay showed that GO possesses anti-bacterial activity against *E. coli* bacteria. The increased inhibition and killing of *E. coli* bacteria is related to the number of layers of GO nanosheets. It was found that the antibacterial activity of few layer GO nanosheets against *E.coli* bacteria is higher than the multilayers GO nanosheets. Moreover, few layer GO nanosheets have relatively acceptable proportions of hemolysis activity, while the multilayers GO nanosheets causes hemolysis with proportions higher than the few layer GO nanosheets.

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