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RESEARCH ARTICLE

MIC and MBC of grapheme oxide

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Graphene and graphene oxide (GO) have been extensively explored as some of the most promising biomaterials for antibacterial applications due to their unique properties: two-dimensional planar structure, large surface area, chemical and mechanical stability, superb conductivity and appropriate biocompatibility. The antibacterial applications of graphene-based materials have grown rapidly in the past few years. This property results in promising bio-applications for the design of advanced drug delivery systems by focusing on infectious and antibacterial disease of a broad range of therapeutics.

In this study, we have reported the antibacterial activity of GO nano sheets (NSs) against general and more pathogenic bacteria, which is named Escherichia coli (E.coli). GO NSs with different dispersion concentrations were prepared. Then, the samples were putted intoculture plates *and were inoculated* with E.coli. We reported the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The results of the GO NSs MIC and MBC tests againtsE.coli were 10 and 100 mg/ml respectively.

INTRODUCTION

Graphene oxide (GO) is an atomic sheet of graphite decorated by several oxygenated functional groups on its fundamental planes and at its edges, resulting in a hybrid structure comprising a mixture of sp^2 and sp^3 hybridized carbon atoms[1-2]. GO has attracted great attention because of the easy availability of bulk quantities, ready functionalize by chemical reaction, good water-dispersion, and high cell compatibility. It is promising for potential applications in electronics, conductive thin films, biochemical sensing, drug delivery, cancer therapies [3-10]. Recently, there are some reports about interactions of Go with the human body, our environment and microorganisms and evidenced the strong antibacterial activity of GO [11-17]. The antibacterial activity of GO is found to relate to oxidative stress [18], membrane puncture by sharp edges of graphene NSs [19], leading to the loss of bacterial membrane integrity, the leakage of RNA[18] and maybe consecutively leads to DNA fragmentation[20]. E. coliO157: H7 is an importantglobal cause of diarrhea, hemorrhagic colitis and hemo-lytic-uremic syndrome. The antibacterial properties of different shape of GO was investigated but the mechanistic action is still undetermined. Well-dispersed GO NSs have been demonstrated the strongest antibacterial activity among several graphene-family nano materials [21].

In this work, well-dispersed GO NSs were prepared by sonication and were added to bacteria suspension which was Escherichia coli (E.coli). Then loose of E.coli viability after 24 and 48 h were investigated. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were evaluated.

Experimental details

Synthesis of GO:

GO was prepared using the modified Hummers method [1]. At first step, 9:1 mixture of concentrated H_2SO_4/H_3PO_4 (180:20 mL) was added to a mixture of 3.0 g graphite flakes (Sigma-Aldrich, cat #332461, 150 µm lateral dimensions) and 9.0 g $KMnO_4$. Although temperature of the exothermic reaction was 40–45◦C, the mixture was heated to 55◦C and stirred for 12 h. The reaction was cooled and poured onto ice (400 mL) followed by addition of H_2O_2 (3ml) until the color changed to brilliant yellow. The solution was centrifuged at 6000 rpm for 6 h to eliminate the supernatants and washed with 200 ml of 30% HCl , 200 ml of 70% ethanol aqueous solution and 200 ml de-ionized water (2x). Each washing process was completed by centrifuging at 6000 rpm for 30 min, and removing the supernatants. The obtained materials were exfoliated under sonication for about 3h then the sonicated aqueous suspensions were centrifuged at 6000 rpm for 4 h and the supernatants were removed. The obtained materials were coagulated with 100 mL de-ethyl ether and filtered by using a poly tetrafluoroethylene membrane (0.45 µm pore size).

Purification of bacterial culture and preparation of inoculums:

The bacteria E. Coli were used as organisms test. Lyophilized bacteria were cultured in Brain Heart Infusion (BHI) broth for 24 h at 35°C. Stock cultures were prepared on TSB slant agar and maintained at 4°C. The inoculums were prepared by transferring bacteria from stock cultures to tubes of Mueller Hinton broth (MHB) followed by incubation at 35°C for 18 h. Second subcultures were prepared and overnight cultures were adjusted to the McFarland 0.5 turbidity (optical density (OD) of 0.1 at 600 nm). The number of cells in the suspensions was verified by duplicate surface plating from tenfold serial dilution on BHI agar and counting the colonies after 24 h incubation at 35 °C. Final suspensions obtained contain between 107 and 108 cfu/ml.

Determination of Minimum Inhibitory Concentration (MIC)

MIC was determined using the broth micro-dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI). Stock solution of the tested GO NSs in sterile Mueller Hinton Broth (MHB) in saline phosphate buffer (ph 7.2) was prepared. Serial dilutions of the stock solutions in broth medium were prepared (200 µl per well) in a 96 well micro-titer plate. Then all wells were inoculated by bacterial suspension (final bacterial concentration was 5×10^5cfu/ml), and incubated at 37 °C for 24 hours. The MIC was defined as the lowest concentration of the GO NSs at which the microorganism does not demonstrate visible growth. A positive control containing the bacterial culture and DMSO without the essential oil and a negative control containing only the medium were performed as well. The first well without turbidity reported as MIC in mg/ml.

Minimal bactericidal concentration MBC

To determine MBC, samples were taken from each tube showing no growth and were inoculated into Mueller Hinton agar containing Petri plate by 0.1 sterile micropipette and separate 0.1 ml sterile tips in drop method and incubated for 24 h at 37 ˚C. The MBC is defined as the lowest concentration of the GO NSs at which inoculated bacteria were completely killed.

Cell viability assay (Time to kill assay)

GO concentrations of MIC, 2MIC and 4MIC were inoculated with E. coli cells (10^6 Cfu/ml) in isotonic saline solutions and incubated under 200 rpm shaking speed for 4 h. Bacterial count was performed at 30min, 1, 2, 3 and 4h of incubation by surface plating 100 µl of different tenfold serial dilutions made from the treatments. The plates were incubated at 37^o C for 24h and bacterial cell numbers were calculated by counting visible colonies. Isotonic saline solutions without GO were used as control.

Result and discussion

The Raman spectrum was acquired to study the carbon structure of the GO . The main features in the Raman spectra of carbons are the so-called G and D peaks, which usually are at 1560 and 1360 cm^{-1} respectively[22].However, some variations in the relative intensities were observed.The Raman spectrum of thesample, shows a peak at 1594 and 1310 cm^{-1} correspond to G and D band (Fig. 1). The evolution of the G band as a function of the number of layers has reported [23]. It indicated monolayer graphene has a much broader and

upshifted G band with respect to graphene. This band is also quite different from bulk graphite. Compare to other reports the G band has a shift about 34 cm^{-1} to higher wave number correspond to monolayer graphene. The G band of graphite is characteristic of all sp^2 sites, including alkenic C= C sites and not just those in aromatic rings [24]. The uncongugatedalkenic C=C bonds are shorter than aromatic bonds, so they have higher vibration frequencies. Thus, during gradual transformation from sp^2a bonded conjugated hexagonal ring to sp2 bonded chains; a shift in the G band to higher wave numbers is expected. On the other hand, the D band intensity is proportional to the carbons only in the aromatic rings in clusters with small sizes, whereas the D band broadening is proportional to the distribution of clusters containing hexagonal aromatic rings with different orders and dimensions. In other words, the D band intensity is proportional to the number of hexagonal aromatic rings in the cluster whereas carbons in non-aromatic bonds do not contribute to the intensity of the D band [24].For diamond or samples containing a significant fraction of diamond phase, the diamond sp^3 peak at 1332 cm^{-1} is seen [25]. Fig. 1 shows a shift about 50 cm^{-1} to lower wave number for D peak which means the structure of graphene is more like diamond . Intensity of the D band in the Raman spectra is important and has been interpreted as in-plane crystallite sizeson an atomic scale [26]. Increasing of the D intensity may be regarded as an increase of the graphite edges because of the smaller size of the crystallites.The ratio of the integrated intensities of the G band to D band is proportional to the average in-plane crystallite size L_a by the equation [26];

$L_a(nm)=(2.4\times10^{10})\,\lambda^4_{laser}(I_D/I_G)^{-1}$

Where λ_{laser} is the frequency of the exciting laser in nm [27]. This indicates the number of laterally broken graphene sheets and therefore the amount of boundary regions along the fracture lines. The result of calculation was L_a=58.44 nm.

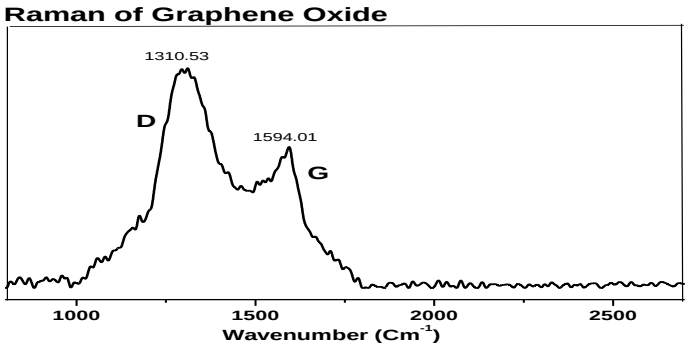

Fig. 1 Raman spectra of graphene

MIC, defined as the lowest concentration of an anti-microbial agent that inhibits the growth of microorganisms after overnight incubation, determined by monitoring the growth of bacteria. MBC, the lowest concentration of nanoparticles thatkills ≥ 99.9% of the bacteria, was also determined (Fig. 2).

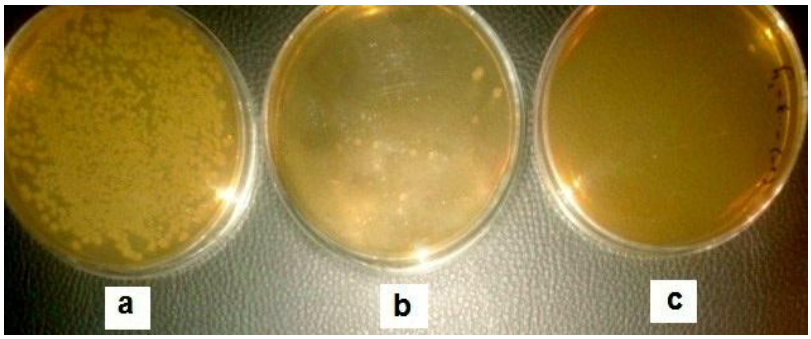

Fig. 2 The images of viability of E. coli cells after incubation with 40 mg/ml GO sheets for 24 h at 37˚C. a) time =0, b)after 30 min and c)after 60 min.

The results showed that value of the MIC and MBC of GO NSs were 10 and 100 mg/ml respectively. Then time dependent MIC efficiency of GO NSs evaluated. GO NSs concentration of MIC, 2MIC and 4MIC were inoculated with E. coli cells (10^6Cfu/ml) in isotonic saline solutions and incubated under 200 rpm shaking speed for 4 h. Bacterial counting was performed at 30min, 1, 2, 3 and 4h after incubation by surface plating 100 µl of different tenfold serial dilutions. The plates were incubated at 37˚ C for 24 h and bacterial cell numbers were calculated by

counting visible colonies. The results for different concentration of GO NSs are shown in Table 1. Isotonic saline solutions without GO were used as a controler. As shown in Fig. 3, for 10, 20 and 40 mg/ml of GO NSs concentration all bacteria were killed after 240, 120 and 60 minutes, respectively. It is clear that for GO NSs, a large fraction of cell death occurs in the first hour of incubation[5].

Table 1:The results for different concentration of GO NSs.

	E.coli count (log cfu/ml)					
Time(min) / GOmg/ml	0	30	60	120	180	240
0(Controler)	6.05	6.02	6.08	6.01	5.89	5.94
10	6.05	4.75	4.08	3.3	2.34	0
20	6.05	3	2.34	0	0	0
40	6.05	1.6	0	0	0	0

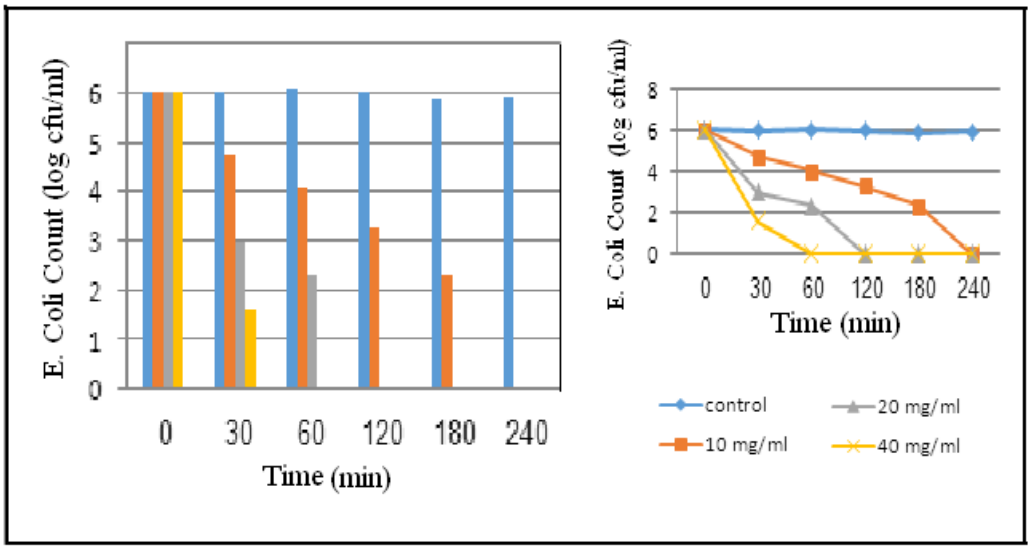

Fig. 3 For 10, 20 and 40 mg/ml of GO NSs concentration all bacteria were killed after 240, 120 and 60 minutes, respectively

Conclusion

In this study, we reported the antibacterial activity of GO NSs against E.coli bacteria. GO was prepared using the modified Hummers method. Raman spectroscopy proved that GO NSs were monolayer and had diamond like structure. The average in-plane crystallite size L_a was found about 58.44 nm. GO NSs were putted intoculture plates and were inoculated with E.coli. Then time dependent MIC efficiency of different dispersion concentrations of GO NSs like MIC, 2MIC and 4MIC evaluated. We reported the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The results of the GO NSs MIC and MBC tests against E.coli were 10 and 100 mg/ml respectively.

Acknowledgements

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