Activity of Biosynthesized Reduced Graphene Oxide against Multidrug resistant Uropathogenic Bacteria

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Abstract

The effect of Bacterially Reduced Graphene Oxide (BRGO) as inhibitory agent alone and in combination with antibiotics against some multidrug resistant (MDR) uropathogenic bacterial isolates as well as the antibiofilm activity was investigated. BRGO nanosheets were synthesized biologically by *Escherichia coli* strain E-NO.7 (accession no. MK685205). Different concentrations 0.1, 0.5, 1 and 10 mg/ml of BRGO nanosheets showed potent inhibitory effects of all concentrations against tested MDR uropathogenic *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and Methicillin-Resistant *S. aureus* (MRSA) isolates. Results showed that the growth inhibition zones increased with increasing in BRGO concentration. The combinations of BRGO (0.5 mg/ml) and different broad spectrum antibiotics exhibited enhanced antibacterial activity against all the studied isolates in comparison with the effects of antibiotics alone. It has been observed that BRGO effectively restricted biofilm formation, and the antibiofilm effect was dose- dependent, since the biofilm inhibition gradually increased with increasing in BRGO concentration.

Key words: *Uropathogenic bacteria, Bacterially Reduced Graphene Oxide, Antibacterial activity, Antibiofilm effect.*

Introduction

Urinary tract infections (UTIs) are among the most common types of bacterial infections occurring in both the community and hospital settings [(1)] Gram-negative bacteria, specifically *Enterobacteriaceae*, are common causes of both community and hospital associated UTIs. The most common pathogenic organism is uropathogenic *Escherichia coli* (UPEC), responsible for 80% or more of the cases, while other Gram- negative rods and Gram- positive cocci, such as *Staphylococcus saprophyticus* and enterococci, are responsible for the remaining cases [(2)].

In spite of the availability and use of the antimicrobial drugs, UTIs caused by bacteria have been showing increasing trends. Much of this increase has been related to the emergence of antibiotic resistance among urinary tract pathogens. The widespread use of broad-spectrum antibiotics has led to the appearance of MDR isolates [(3)]. Biofilms are considered an important virulence factor that causes persistent chronic and recurrent infections. They are highly resistant to antibiotics and host immune defenses [(4)]. Bacteria protected within biofilms are up to 1,000 fold more resistant to antibiotics than their planktonic counterparts, which generates serious consequences for therapy and complicates treatment options [(5)] Unfortunately, antimicrobial resistance is still a major cause of morbidity and mortality. Each year more than 700,000 people die due to antimicrobial resistant infections worldwide. Antibiotic resistance is estimated to cause around 10 million deaths by 2050 [(6)].

Nanomaterials (NMs) have emerged as a novel alternative to defeat MDR bacteria because of their microbicidal nature and the development of bacterial resistance to NMs are less likely when compared to antibiotics [(7)]. The physical structure of the NM itself may have inherent antibacterial properties due to its membrane damaging abrasiveness, as seen in graphene nanosheets [(8)]. Graphene materials have potent broad-spectrum antibacterial activities against both Gram-

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positive and Gram- negative bacteria and biofilm forming microorganisms. The unique properties of graphene provide surfaces with anti-adhesive properties and it is particularly effective to inhibit bacterial attachment and the biofilm formation [9].

Experimental part

Bacterial isolates

Four uropathogenic isolates *E. coli*, *K. pneumonia*, *P. aeruginosa*, and MRSA were identified according to morphological, biochemical and VITEK2 System as MDR after screening their antibiotic sensitivity against 12 selected antimicrobials of different classes using disc diffusion method. MDR uropathogens were also identified as biofilm producers by Congo Red Agar (CRA) according to the method described by [10] and by Tissue culture plate (TCP) method according to [11, 12]. The interpretation of biofilm production was done according to [13].

Antibacterial activity of BRGO

The antibacterial activity of BRGO nanosheets was tested against the MDR uropathogens by agar well diffusion method [14].

Combination of antibiotics and BRGO

The combination between BRGO nanosheets and antibiotics against uropathogens was done using the Kirby- Bauer disk diffusion method. Each standard antibiotic disc was impregnated with 30µl of BRGO solution (0.5 mg/ml) and used against indicated bacterial isolates to assay their antibacterial activity. The plates were incubated at 37°C for 24 hr. After incubation, the plates were checked for the zones of growth inhibition. The combination effect of antibiotics and BRGO was evaluated by calculating the increase in the zones of inhibition [15].

Antibiofilm activity of BRGO

Tissue culture plate method (TCP) was also used with some modifications to detect the antibiofilm activity of BRGO solution against biofilm producers *in vitro*. Three concentrations 1, 0.5, and 0.1 mg/ml of BRGO solution were prepared. Bacterial suspensions were prepared for each isolate as, 150µl of the bacterial suspensions were added to each polystyrene 96-well microtiter plate wells. Then an amount of 50µl of each 4X concentration was added to the corresponding wells to get the final concentrations. An amount of 200µl of autoclaved distilled water was added in peripheral wells to decrease loss of water. Microtiter plates were incubated in 37ºC for overnight. After incubation, contents of each well of microtiter plates were treated and the OD values were measured.

Results

Antibacterial activity of BRGO

Results showed that BRGO had potent inhibitory effects against all the tested MDR uropathogenic isolates with higher activity against MRSA. The growth inhibition zones clearly revealed that BRGO inhibited the growth of tested isolates where the highest concentration of BRGO has the strongest antibacterial activity (Figure 1, Table 1). Treatment with different concentrations of BRGO nanosheets showed higher antibacterial activity against *E. coli* compared to other Gram-negative (*K. pneumonia* and *P. aeruginosa*), the growth inhibition zones increased in a concentration dependent manner.

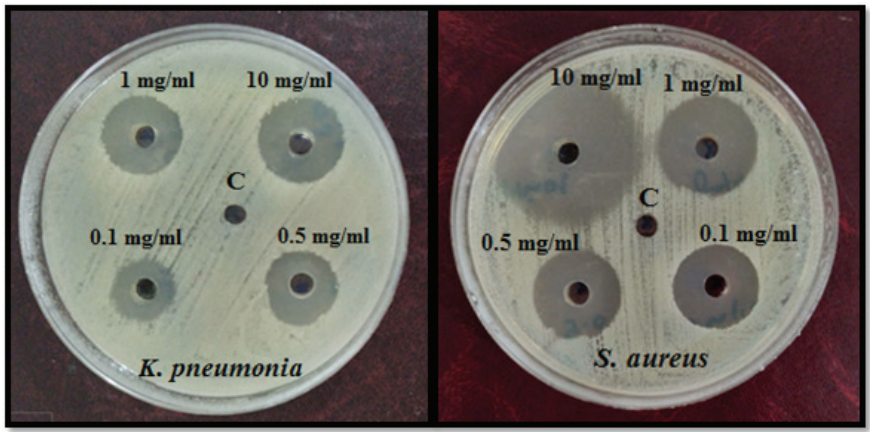

Fig. 1- Antibacterial activity of different concentrations of BRGO against MDR uropathogenic isolates. C, represents control (D. W.)

Table 1. Growth inhibition zones of different concentrations of BRGO nanosheets against MDR uropathogens.

BRGO MDR Bacteria	0.1 mg/ml	0.5 mg/ml	1 mg/ml	10 mg/ml
	Zone of inhibition (mm)			
E. coli	16	22	23	25
K. pneumonia	15	18	19	21
P. aeruginosa	10	12	18	20
MRSA	21	22	24	35

BRGO, Bacterially Reduced Graphene Oxide; MDR, Multi Drug Resistance

Combination effect of antibiotics and BRGO

The selected antibiotics were divided into two groups, the first group included four different β-lactam antibiotics (cefepime, ceftriaxon, cefazolin, and cefotaxim) that were not effective against the studied isolates, while the second group included imipenem and gentamicin that were highly active against these isolates. Results revealed that antibiotic-BRGO combinations exhibited enhanced antibacterial activities against all the studied isolates as compared to the effect of antibiotics alone with higher activity against MRSA (Table 2 Figure 2 and Figure 3).

Table 2. Individual and combined activity of antibiotics and BRGO nanosheets (0.5 mg/ ml) against MDR uropathogenic isolates.

	Bacterial isolates			
	E. coli	***K. pneumonia***	***P. aeruginosa***	**MRSA**
	Zone of inhibition (mm)			
FEP	0	0	8	0
FEP + BRGO	10	9	14	17
CTX	0	0	0	0
CTX +BRGO	9	10	10	15
CRO	0	0	0	6
CRO +BRGO	11	10	15	15
CZ	0	0	0	0
CZ + BRGO	10	11	9	16
IPM	32	28	30	36
IPM + BRGO	34	30	33	38
CN	18	18	15	17
CN + BRGO	25	20	18	24

FEP, Cefepime; CTX, Cefotaxime; CRO, Ceftriaxone; CZ, Cefazolin; IPM, Imipenem; CN, Gentamicin; BRGO, Bacterially Reduced Graphen Oxide.

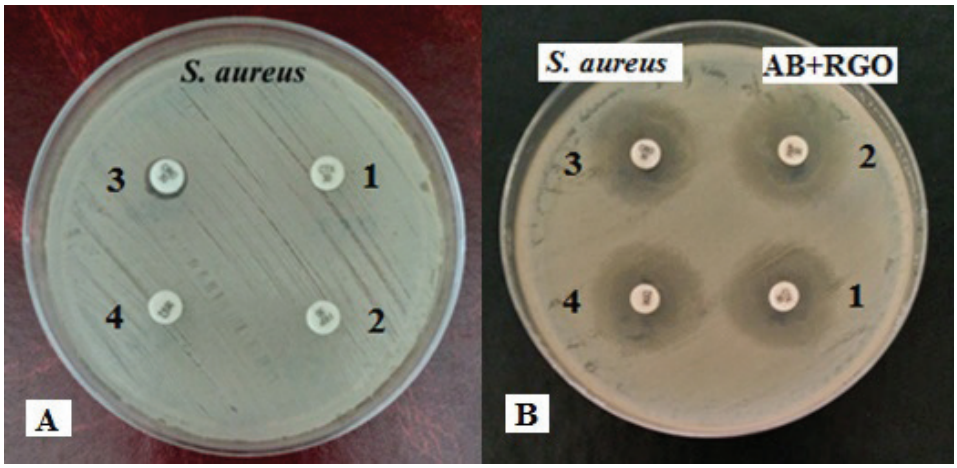

Fig. 2- Zone of growth inhibition of MDR MRSA isolate with antibiotic alone (A) and in combination with BRGO nanosheets (B). AB, Antibiotic; RGO, Reduced Graphene Oxide; 1, Cefotaxime; 2, Cefepime; 3, Ceftriaxone; 4, Cefazolin.

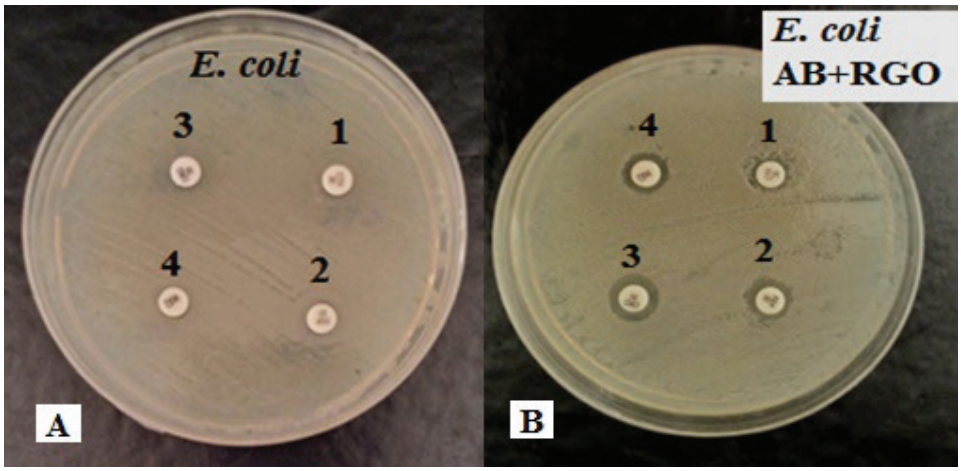

Fig. 3- Zone of growth inhibition of MDR *E. coli* isolate with antibiotic alone (A) and in combination with BRGO nanosheets (B). AB, Antibiotic; RGO, Reduced Gpaphene Oxide; 1, Cefotaxime; 2, Cefepime; 3, Ceftriaxone; 4, Cefazolin.

Biofilm formation by uropathogens

Multidrug resistant uropathogens (*E. coli*, *K. pneumoniae*, *P. aeruginosa*, and MRSA) were identified as biofilm producers by CRA method, it was observed that all isolates produced black colonies which is indication for the production of biofilm. The experiment of TCP was performed in triplicate. The OD values of stained adherent biofilm was determined with an ELISA reader at wavelength of 590 nm. The average of OD values obtained for individual isolate were considered as an index of bacterial adherence to surface and biofilm formation. Results revealed that all the studied uropathogens were strong biofilm producers by TCP method [Table 3]. The average OD value (2.034) for *P. aeruginosa* indicated the strongest biofilm production, followed by 1.930, 1.575, and 1.537 for *K. pneumoniae*, MRSA, and *E. coli*, respectively.

Table 3. Antibiofilm activity of different concentrations of BRGO against MDR uropathogenic isolates.

Bacterial isolate	Cont.	Absorbance (at 590 nm) after treatment BRGO		
		0.1 mg/ml	0.5 mg/ml	1 mg/ml
E. coli	1.537	1.220	0.620	0.153
K. pneumonia	1.930	1.238	0.621	0.191
P. aeruginosa	2.034	1.304	0.720	0.215
MRSA	1.572	1.215	0.619	0.124

Evaluation of antibiofilm activity of BRGO

The OD values showed that BRGO effectively restricted biofilm formation of the studied uropathogens. It has been observed that the antibiofilm effect was dose- dependent, since the biofilm inhibition gradually increased with increasing in BRGO concentration (Table 3). Three different concentrations (1, 0.5, and 0.1 mg/ml) of BRGO nanosheets exhibited higher antibiofilm effect against MRSA than Gram- negative isolates. The effect of concentrations corresponding to 0.5mg/ml and 1mg/ml of BRGO caused notable reduction of the biofilm formation of MRSA from approximately 1.572 to levels of 0.619 and 0.124, respectively. The OD values also revealed that concentrations of BRGO caused notable reduction in the biofilm formation of *E. coli* and *K. pneumoniae* and the best biofilm inhibition activity was observed in higher concentration. The effect of concentrations corresponding to 1, 0.5 and 1mg/ml of BRGO caused reduction of the biofilm formation of *E. coli* from approximately 1.537 to levels of 1.220, 0.620 and 0.153, respectively. Similarly, these concentrations showed reduction in biofilm formation of *K. pneumoniae* from approximately 1.930 levels of 1.238, 0.621, and 0.191, respectively. Against *P. aeruginosa*, treatment with BRGO showed relatively lower antibiofilm effects compared to the other isolates. The biofilm formation reduced from 2.034 to levels of 1.304, 0.720, and 0.215 corresponding to 1, 0.5 and 1mg/ml concentrations of BRGO respectively.

Discussion

Graphene nanosheets can cover on the external surface of cells, which might lead to indirect toxicity by biologically isolating them from growth medium, and consequently the bacterial cells can neither proliferate nor consume the nutrients [16]. In addition, the direct contact between the sharp edges of RGO nanosheets with cells can physically damage cell membrane, resulting in leakage of intracellular material and negatively affecting cell metabolism [17]. Many researchers have investigated the potential toxicity of GNMs against several bacterial species [18, 19]. The increase in graphene concentration led to a continuous increase in its antibacterial activity [20]. The antimicrobial effectiveness of biosynthesized RGO is enhanced at higher concentrations due to the rupture of cell membrane [21].

The inhibition ability of antibiotics combined with BRGO may be attributed to "carrier effect" [22]. Namely, the coating of BRGO on bacteria can cause the cell membrane damage and will facilitate the release of the deposited antibiotic on BRGO surface in high quantity. The cell entrapment property of graphene ensures high local concentrations of antibiotic molecules in the immediate proximity of the cell membrane. It is also possible that RGO contributed to increased permeation of antibiotic into the bacterial cells [23].

Bacterial biofilms play an important role in UTIs, being responsible for both acute and persistent infections in which biofilm can become a serious problem [24]. Many

studies documented a higher proportion of antibiotic resistance in biofilm producers in comparison to non-producers [25, 26]. The EPS matrix confers antibiotic resistance through expression of chromosomally encoded resistant genes, restricted penetration of antibiotics, and decreased growth rate [27, 28]. On the other hand, it is possible that the biofilm can facilitate the accumulation of antibiotic degrading enzymes such as β-lactamases [29].

The penetration and deposition of NMs within the biofilms are key components for the design of biofilm therapeutics. The bioavailability of NMs allows them to penetrate a mature biofilm and target bacterial cells not only at the surface but also within the deep layers of biofilm [30]. The antimicrobial and antibiofilm effect of graphene can be attributed to different mechanisms originated by the direct interaction between graphene and bacteria cells [31]. Graphene nanosheets possess biocidal properties based on their ability to act as nano-knives, penetrating and disrupting the cell membrane [32]. Additionally, their basal planes which are free of functional groups possesses anti-adhesion properties against biofilm and it is particularly effective in preventing biofilm formation [9].

t The BRGO nanosheets exhibited remarkable antibacterial and antibiofilm activities against the tested MDR uropathogenic *E. coli*, *K. pneumonia*, *P. aeruginosa*, and MRSA isolates. The activity increased with increasing in BRGO concentration. The combination of BRGO and broad- spectrum antibiotics showed enhanced antibacterial activity against uropathogens as compared to effects of antibiotics alone.

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Ethical Clearance: Authors are in accordance with the ethical standards of the responsible committee on human experimentation (institution and national) and the Helsinki Declaration of 1975.

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