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A Novel Graphene Oxide-Para Amino Benzoic Acid Nanosheet as Effective Drug Delivery System to Treat Drug Resistant Bacteria

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ABSTRACT
A novel graphene oxide-para amino benzoic acid nanosheet was synthesized by modified chemical exfoliation of expandable graphite using $K_2Cr_2O_7$. Physico-chemical characteristics of graphene oxide-para-amino benzoic acid nanosheet were determined by field emission scanning electron microscopy, fourier transform spectroscopy, fluorescence spectroscopy and biocompatibility assay. Experimental results showed that the average size and zeta potential of graphene oxide-para amino benzoic acid nanosheet sheets 100 nm and -34.9 (±7.03) mV, respectively. Afterwards, tetracycline drug loading efficiency and drug releasing efficiency were also monitored. Drug loading and drug releasing efficiency were found to be 64.075 (±2.74) and 38.35 (±0.07) %, respectively. Finally, antimicrobial activity of tetracycline loaded this nanosheet was determined in terms of minimal inhibitory concentration and putative mode of action on tetracycline resistant bacteria *Escherichia coli* XL-1. The minimal inhibitory concentration of this nanosheet was found to be 110 µg/ml. Hence, it has been clearly shown that graphene oxide-para amino benzoic acid nanosheet has very unique features as drug delivery system to treat drug resistant bacteria.

Keywords: graphene oxide, chemical exfoliation, minimal inhibitory concentration, drug delivery system, drug resistant bacteria.

INTRODUCTION

Graphene and graphene based materials are currently have an outstanding significance due to their fantabulous electronic [1] and mechanical properties [2] including especially high surface area, useful non-covalent interactions with aromatic drugs, higher aqueous solubility and comparatively gamier stability in physiological solution like human serum. As a result, graphene based nanocomposites are now unique materials for formulations of micro electrical devices [3], nanosensors [4], biomedicines [5-6], mechanic resonators [7], and ultra capacitors. [8] It has been previously reported that graphene oxides are synthesized by chemical exfoliation of expandable graphite using various oxidizing agents such as $KMnO_4$ [9-10], $KClO_3$ [11], and m-CPBA. [12] But these chemical means are still suffering from various hindrances. These major limitations include removal of MnO_2 during $KMnO_4$ oxidation, generation of copious fumes of chlorine dioxide on $KClO_3$ and m-CPBA treatments. Even production of graphene oxide through dichromate oxidation incorporates some additional problems including larger size and comparatively lower presences of carboxylic groups on the surface.

On the other hand, rapid emergence of drug resistance or multi drug resistant infection by pathogenic bacteria has become another burning issue, especially for tetracycline resistance in the field of medicinal chemistry. Prior to mid 1950s tetracycline is the most widely used antibiotic in human medicine and animal farms. Because this drug has some special features like inexpensiveness, easy oral administration, and fewer side effects. [13] But massive usage of this antibiotic for several years has emerged a burning problem of tetracycline resistance in 1980s. Genetic and biochemical mechanism of tetracycline resistance is mainly associated with the efflux gene transfer by R-plasmid, transposons and ribosomal protection. Efflux gene encoded proteins block the uptake of tetracycline. Even ribosomal protection is associated with some mutated cytoplasmic proteins that protect the ribosome from tetracycline binding in resistant bacteria. [14]

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Emergence of this tetracycline resistance has adversely affected the two major fields. These two major fields are therapeutics and environment. Excess use of tetracycline in human has developed potent tetracycline resistance in various pathogenic bacteria and increased the difficulties in disease treatments. [15] Even a toxic compound anhydrotetracycline has been formed during a high-temperature treatment of animal-derived feed contaminated with excess tetracycline. [16] On the other hand, various veterinary farms have been still using tetracycline in non therapeutic level or excess since last 50 years for animal medications. [17] Moreover, effluents of these farms and public hospitals have been entered in to the water streams [18], soil [19] through municipal sewage system. As a result, it increases the risk of further spread of tetracycline resistance in the ecosystem.

Recent developments in medicinal chemistry have incorporated various novel remedies to resolve this drug resistance problem in human and animals. These novel developments include use of chemically modified drug [20], combinatorial therapy [21], use of helper drug or efflux pump inhibitors [22], phage application [23], and plasmid curing technology. [24] Still these mentioned technologies are not potentially effective. Sometimes it produces drastic side effects like human apoptotic pathway induction [25] and depression of skin collagen synthesis. [26] Even more recently some research group has prepared the tetracycline encapsulated microspheres of chitosan [27] to solve these undesirable side effects. Moreover, hyaluronic acid, alginic acid were also used for microencapsulation of this drug. Even several research groups have been working with chitosan based biopolymer to prepare the nanoparticles because chitosan is biocompatible, non-toxic, biodegradable, and cheap chemical compound. [28] But major problems have been still associated with its comparatively larger size, high molecular weight which indirectly causes inefficient transport of drug and induces immunogenic clearance. [29]

Contextually in the present study, graphene oxide-para amino benzoic acid (GO-PABA) nanosheet has been synthesized following dichromate oxidation. Diazonium salts of PABA has been reacted with GO to increase the carboxylic acid groups. It has automatically increased the water dispersability of the final product GO-PABA nanosheet. Moreover, these carboxylic acid groups have also been served as a better anchor for drug tetracycline through the formation of amide linkage. Afterwards, physico-chemical characterization of GO-PABA and tetracycline loaded GO-PABA (GO-PABA-tet) nanosheet has been done. Tetracycline loading and release efficiency have also been monitored. Finally, GO-PABA-tet nanosheet has been applied on tetracycline resistant bacteria *Escherichia coli* XL-1 to determine its minimal inhibitory concentration (MIC). Even the putative molecular effect of GO-PABA-tet nanosheet on *Escherichia coli* XL-1 has been established.

MATERIALS AND METHODS

Materials

Graphite (Loba Chemie), $K_2Cr_2O_7$ (Sigma, Aldrich), H_2SO_4, $KMnO_4$, HCl, $NaNO_2$, NaOH (Merck, India), $NaNO_3$ (Sigma, Aldrich), para-aminobenzoic acid (SRL, India), tetracycline (Merck, India), nutrient broth (CDH, India) and *Escherichia cell* XL-1 stock (Stratagene, India)

Synthesis of GO-PABA nanosheet

Graphene oxide (GO) was synthesized by modified chemical exfoliation of expandable graphite using $K_2Cr_2O_7$ (Sigma, Aldrich) as an efficient oxidant. [30] Briefly, 5g graphite and 3.75 g $NaNO_3$ (Sigma, Aldrich) were mixed in a conical flask and kept in an ice bath. 375 ml conc. H_2SO_4 (Merck) was poured into this mixture with constant stirring. Subsequently 37.6 g $K_2Cr_2O_7$ were slowly added over about 2 h with constant stirring. Stirring was again continued for 2 h in ice bath. After that mixture was continuously stirred for 5 days at room temperature. The initial color of the mixture was appeared as dark yellow, which has turned into dark green color after 4 days. After 5 days of stirring, 750 ml of 5 % aqueous H_2SO_4 was slowly added to the above mixture over about 1 h and during mixing the temperature of the whole system was kept at 98^oC. After complete addition of deionized water (to make the final volume of the mixture 1000 ml) it was again kept at 98^oC for 2 h at constant stirring condition. Then the mixture was cooled down to room temp and stirred the entire mixture vigorously for another 2 h. The solid mass was separated from the reaction mixture using centrifuge to remove the water soluble oxidant and other inorganic salts. The collected solid mass was washed 5 times with 3 % (v/v) HCl. Each time of washing, solid mass was suspended by ultra-sonication using a probe-type sonicator (Sigma Ultrasonic Processor, Sonix-600, having power output 12W) for 2 min and was collected by centrifugation. The resultant graphite oxide was then readily exfoliated to completely water dispersed graphene oxide (GO) by ultrasonication. It was dried and collected. For the preparation of GO-PABA nanosheet at first 50 ml of ~2 mg/ml water dispersed graphene oxide was produced by the sonication of the solid GO in deionized water. Higher amount of NaOH was added to the solution to make it highly alkaline. After that diazotization was done by using the diazotized salt of PABA with the above alkaline solution of GO. The resultant Solution was highly acidic in nature, which was neutralized by NaOH (2M), to adjust the pH of solution at 7.0.

Tetracycline drug loading on GO-PABA nanosheet

The GO-PABA nanosheet was dispersed in deionized distilled water and tetracycline (Merck, India) (15 mg/ml) was dissolved in 50 % (w/v) ethanol. Then the GO-PABA nanosheet dispersed solution and tetracycline solution were mixed together in equal volumetric ratio (1:1) and incubated for overnight (18 h) at 37^oC temperature in orbital shaker (250 rpm). Then the mixture was subjected to ice cold 100% ethanol and kept at 4^oC to facilitate precipitation for overnight (18 h). Then the visible precipitate was subjected to centrifugation at 14,000 rpm for 30 min and pellet was vacuum dried. The dried pellet was dispersed in 5 ml of deionized distilled water by sonication and finally subjected to lyophilisation. Finally Lyophilized pellet was kept at 4^oC for physico-chemical characterization and minimal inhibitory concentration (MIC) assay at different concentrations in deionized distilled water.

Physico-chemical characterization

Fourier transform spectroscopy (FTIR) spectra (4000-500 cm^{-1}) were recorded from a Perkin- Elmer spectrum RX-1 IR spectrophotometer using 5 mg of dry GO, GO-PABA and GO-PABA-tet nanosheet samples and KBr discs under dry air at room temperature. Each FTIR spectrum represents 16 scan with 4 cm^{-1} spectral resolution. Fluorescence spectral analysis (at 380 nm excitation) was performed by using

fluorescence spectrophotomer Hitachi (F-7000) equipped with Xenon lamp in the visible range. The concentration of free tetracycline and GO-PABA-tet nanosheet for florescence analysis was same (100µg/ml). Fluorescence spectra were taken separately for both free tetracycline and GO-PABA-tet nanosheet to determine the interaction between tetracycline and GO-PABA nanosheet.

Zetapotential of GO, GO-PABA and GO-PABA-tet nanosheet was measured by using zeta potentiometer (Zetasizer 4, Malvern Instruments UK) to confirm the polydispersity. GO-PABA and GO-PABA-tet nanosheet in deionized water were used to assess the zetapotential using DTS1060C type clear possible zeta cell at 25^{o}C temperature. The Smoluchowsky approximation was applied in the calculation of the zetapotential. The field emission scanning electron microscopy (FE-SEM) provides useful analysis for average nanosheet size. The GO-PABA and GO-PABA-tet nanosheet samples were spread on a double-sided conducting adhesive tape pasted on a metallic stub, were coated with gold (100µg) in a sputter coating unit for 5 min and observed under FE-SEM machine (Olympus) at 20 kV.

Biocompatibility assay

Serum assay was performed to determine the stability of GO and GO-PABA nanosheet both. The stability was determined in the media include deionized water, phosphate buffer saline (pH 7.4), cell nutrient medium (nutrient broth), human serum. As generalized protocol, firstly we mixed 500 µl of each medium with 500 µl sample (GO or GO-PABA nanosheet) containing 0.1 gm.ml^{-1}. The reaction mixture were incubated for 1 h at 37^{o}C and followed by centrifugation at 10,000 rpm for 5 min and allowed to serum assay for biocompatibility.

Physical tetracycline drug loading efficiency

Physical loading efficiency (PLE %) was calculated based on the weight ratio of the amount of incorporated tetracycline to that of total tetracycline used for GO-PABA-tet nanosheet formation. Briefly, the GO-PABA-tet nanosheet was precipitated by absolute ethanol (ice cold) to extract the tetracycline in supernatant (dilution factor was taken into account for each set). The supernatant was centrifuged at 14,000 rpm for 30 min and finally was filtered through 0.22 µm membrane filter. Free tetracycline in supernatant suspension was the drug amount not loaded into GO-PABA nanosheet; its concentration was assayed by spectrophotometry (Shimatzu, Japan) at 423 nm. [31] The PLE % was calculated from the equation [1]:

$$PLE\ \% = [(W_{total} - W_{free}) / W_{total}] \times 100\ \% \qquad [1]$$

Where; W_{free} is the free tetracycline amount in supernatant and W_{total} is the total tetracycline amount used for GO-PABA-tet nanosheet formation.

Studies on in vitro tetracycline drug release efficiency

In vitro release of tetracycline from GO-PABA-tet and unloaded GO-PABA was determined using shaking water test at 37^{o}C. The shaking speed was placed set at 100 rpm. About 100 mg of GO-PABA-tet nanosheet sample was placed in a dialysis bag and 5 ml of preheated phosphate buffer saline (PBS) of pH 7.4 was subsequently added. The dialysis bag was then sealed and put into a glass bottle. Another 95 ml of PBS solution was added in the clean grease free glass bottle. At each time point, 2 ml of PBS was taken out for analysis and another 2 ml of fresh PBS was added to keep total volume constant throughout experimental assay. The amount of released tetracycline was assayed spectrophotometrically at 423 nm.

Antibacterial effect of GO-PABA-tet nanosheet on drug resistant bacteria

Antimicrobial effect of the GO-PABA-tetracycline was evaluated by minimal inhibitory concentration (MIC) assay. MIC was ultimately defined as the lowest concentration of the drug or drug loaded nanoparticle required to inhibit bacterial growth that of control uninoculated growth medium (nutrient broth) after 48 h. In brief, Bacteria (*Escherichia coli* XL-1) were incubated at 37°C, shaken at 250 rpm aerobically. At the exponential phase, bacteria were harvested by centrifuge at 6000 rpm for 10 min at 4°C, then washed twice with10 mM phosphate buffer saline (PBS, pH7.4). The bacteria were suspended in PBS and adjusted to ~1 × 10^{7} CFU per ml for further use. Then the GO-PABA-tet nanosheet sample was gradually diluted for final reaction concentration range of 30-130 µg/ml in nutrient broth medium. Bacteria were inoculated to achieve a bacterial concentration of 1-2 × 10^{5} colony forming unit (CFU) per ml. The MIC was read after 48 hours of incubation at 37°C, equivalent to the concentration of the tube without visible growth. In this study, MIC of GO-PABA-tet nanosheet sample was determined to inhibit the growth of tetracycline resistant (*tet*^{R}) bacteria *Escherichia coli* XL-1. Dilution of nutrient broth containing the bacteria and test sample (at MIC) was allowed for FE-SEM after 12 h incubation to confirm the morphological changes by lytic effect, wherein complete disappearance of the cells was observed after 48 h.

Statistical Analysis

Mean values and standard deviations were calculated from the data of tests performed three times per sample. Results were compared by least significant difference test and multiple regressions (R^2) analysis using minitab Version 15 statistical software.

RESULTS

Physico-chemical characterization

The FTIR spectrum [Fig. 1A] of the graphite oxide (GO) illustrates the presence of C=C, C=O, O-H, C−O−C and C−O bonds from the peaks at 1584, 1706, 3416, 1205 and 1059 cm^{-1} respectively. Again the IR spectra of GO-PABA [Fig. 1B] reveals the presence of O-H (3475 cm^{-1}) and C=O (1640 cm^{-1}) group of carboxylic acid whereas that of GO-PABA-tet nanosheet [Fig. 1C] demonstrates the presence of amide (1639, 1589, 1539 cm^{-1}) and O-H (3437 cm^{-1}) groups. The ultra-small size of the GO-PABA nanosheet was caused by the presence of more carboxylic acid group in GO-PABA nanosheet than in GO, which was confirmed by the FTIR spectra. Fluorescence measurements [Hitachi (F-7000)/ Xenon lamp] in the visible range revealed that the free tetracycline and GO-PABA-tet nanosheet emissions peaked at ~595 nm and 580 nm respectively at 380 nm excitation. The fluorescence spectra of free tetracycline and GO-PB-tet nanosheet at the same concentration showed drastic fluorescence quenching of free tetracycline in the case of GO-PABA-tet nanosheet, suggesting close proximity of free tetracycline to GO-PABA-tet nanosheet [Fig. 1D].

Zetapotential of GO, GO-PABA and GO-PABA-tet nanosheet were found to be -8.1 (±2.34), -34.9 (±7.03) and -56.4 (±7.81) mVs, respectively. It has clearly shown that the GO-PABA and GO-PABA-tet nanosheet have higher solubility in water in normal physiological condition. From

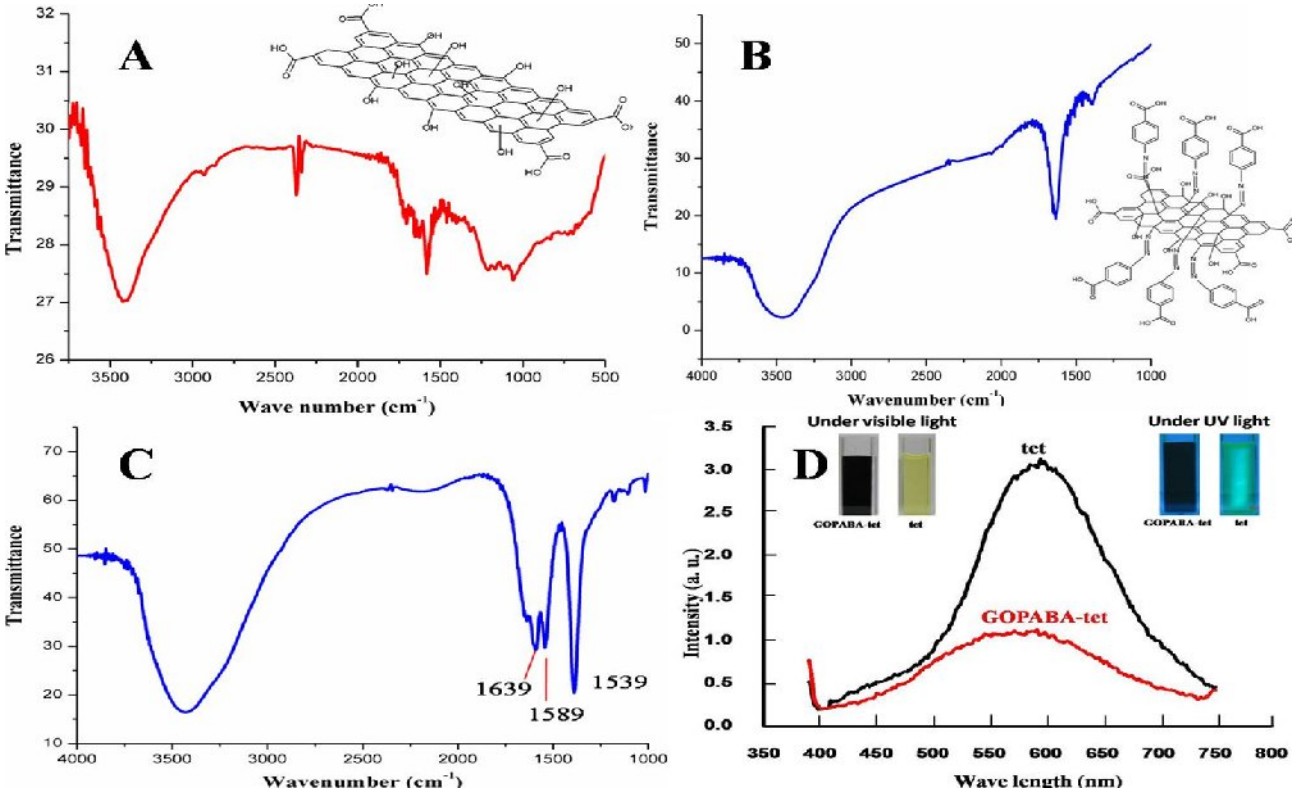

Fig. 1: Physico-chemical characterization. (A) FTIR profile for GO; (B) FTIR profile for GO-PABA; (C) FTIR profile for GO-PABA-tet; (D) fluorescence spectra for both GO-PABA-tet and free tetracycline drug (tet)

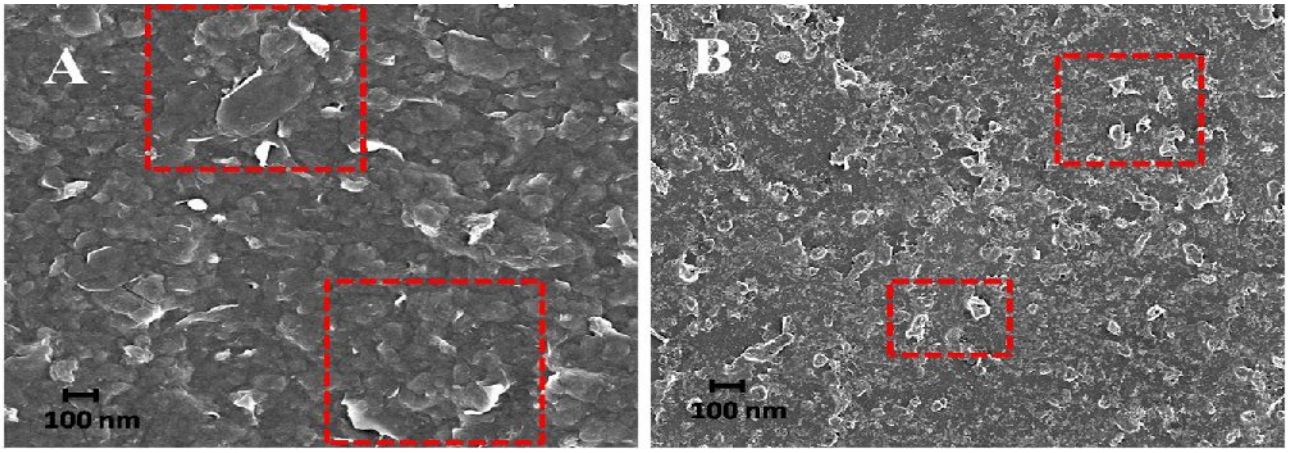

Fig. 2: FE-SEM image analysis for GO and GO-PABA. (A) GO and (B) GO-PABA under 10,000X magnification

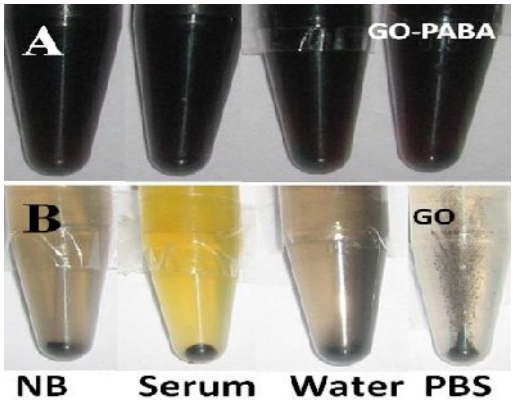

Fig. 3: Biocompatibility assay for GO and GO-PABA. (A) GO-PABA nanosheet sample; (B) GO sample on various media including nutrient broth (NB), serum, water, and phosphate buffer saline (PBS)

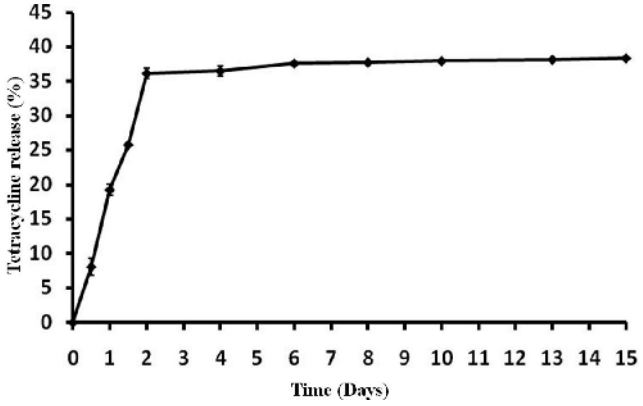

Fig. 4: *In vitro* release profile of tetracycline from GO-PABA-tet nanosheet (Each data point is the mean of three sample and error bars are standard deviation).

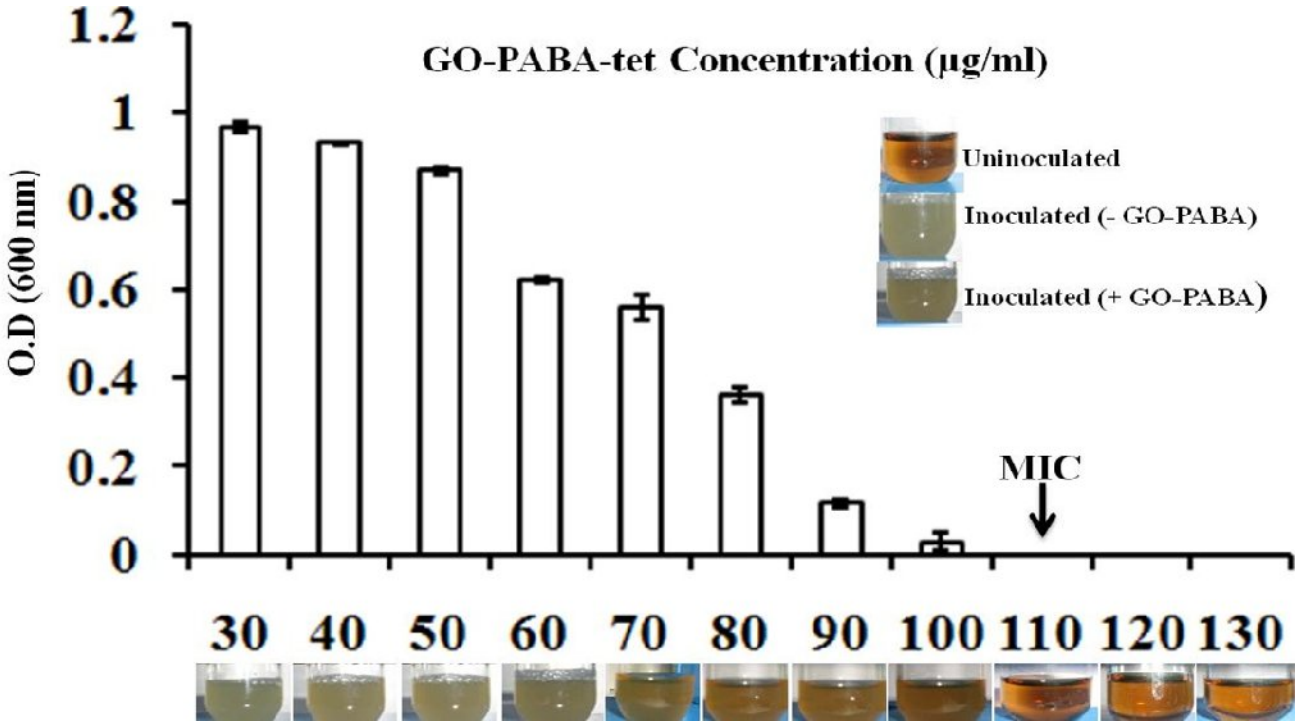

Fig. 5: Evaluation of MIC of GO-PABA-tet nanosheet on tetracycline resistant bacteria *Escherichia coli* XL-1. "Uninoculated" represents the only autoclaved nutrient broth (negative control); "Inoculated (–GO-PABA)" represents the autoclaved nutrient with tetracycline resistant bacteria *Escherichia coli* XL-1 without GO-PABA nanosheet; "Inoculated +GO-PABA" designates inoculated medium with GO-PABA nanosheet but without tetracycline loading (positive control).

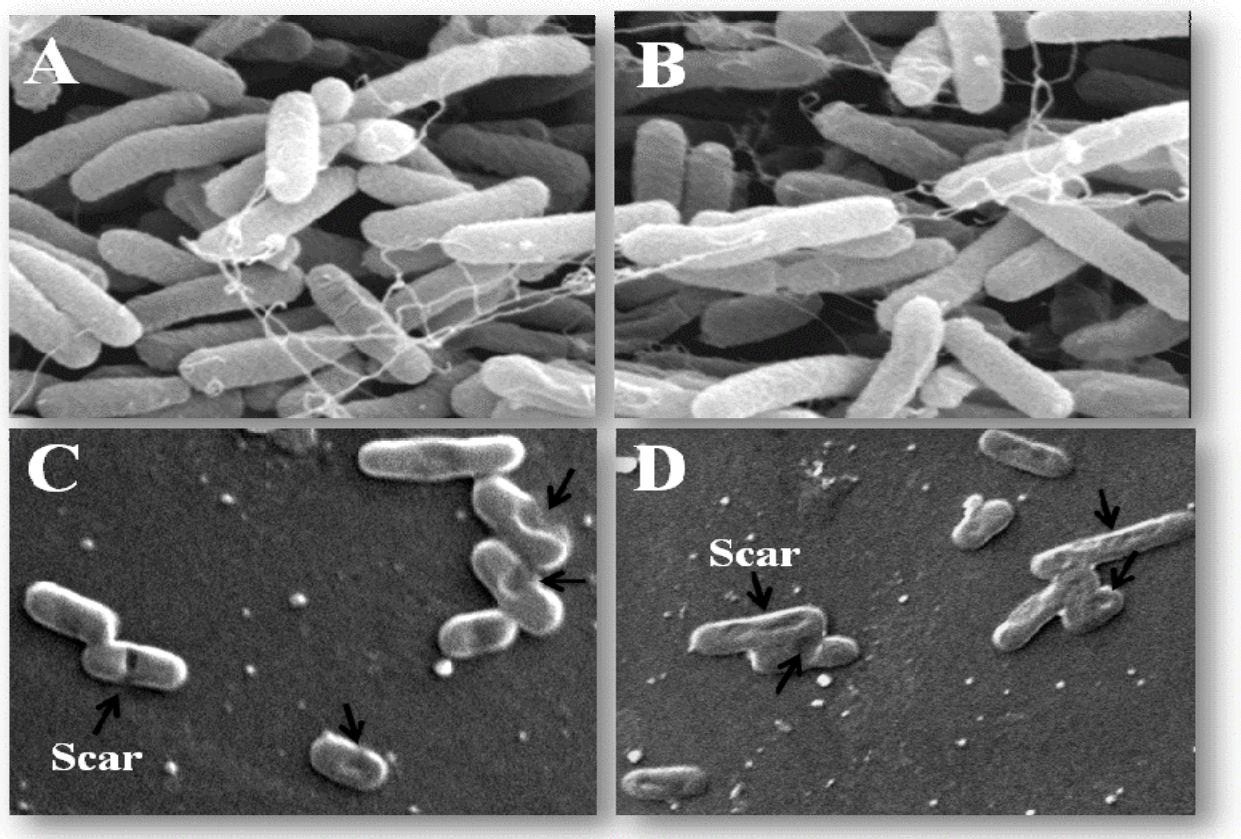

Fig. 6: Evaluation of a putative mode of action of GO-PABA-tet nanosheet on tetracycline resistant bacteria *Escherichia coli* XL-1. (A) Untreated *E. coli* XL-1; (B) treated *E. coli* XL-1; (C) treated after 12 hrs; (D) treated after 24 hours under 20,000 X magnification. In (C) scar was appeared in middle region of bacteria; in (D) the scar become bigger and spreading toward the Polar Regions (Considering the same dilution in each FE-SEM analysis of GO-PABA-tet treated bacterial sample preparations before gold coating).

the FE-SEM images, it was observed that the average size of GO and GO-PABA nanosheet sheets are 500 nm and 100 nm respectively [Fig. 2].

Biocompatibility assay

Biocompatibility of GO and GO-PABA nanosheet was evaluated by a simple serum assay. The resulting GO (single layered and few-layered) was soluble in deionized water but aggregated in solutions rich in salts or proteins such as nutrient broth and serum [Fig. 3]. This is likely due to screening of the electrostatic charges and nonspecific binding of proteins on the GO. [32] In contrary, GO-PABA nanosheet exhibited excellent stability in nutrient broth and serum.

Determination of physical tetracycline drug loading efficiency

Drug can be loaded into a nanosheet system in two ways: during the preparation of the particles or following their formation. In such systems, the drug molecules are embedded physically in to the matrix or absorbed onto the surface. Drug loading can be maximized by incorporating the drug during the formation of the nanosheet system. In the present study the tetracycline PLE % in GO-PABA nanosheet system was 64.075 (±2.74) %.

Evaluation of tetracycline drug loading efficiency

Due to the mutation in the tetracycline drug transporter protein in tetracycline resistant bacteria, effective release of tetracycline from GO-PABA-tet nanosheet is desired. Tetracycline *in vitro* release is shown in [Fig. 4]. The profile shows two stages. First stage belongs to fast release till first two days. The second stage belongs to after third day to 15 days with much more slower and gradual release. Within first two days, 36.15 (±0.78) % of tetracycline was released and after 15 days a total of 38.35 (±0.07) % of tetracycline was released. The initial burst of tetracycline may be attributed to tetracycline existing at or near the surface of GO-PABA nanosheet.

However, the gradual release of tetracycline at second stage was probably caused by slow diffusion of tetracycline from the highly ordered layer structure of GO-PABA nanosheet. In addition even after 15 days, the release rate of tetracycline is still constant and there is no tail off or burst. This indicated that this GO-PABA-tet nanosheet is stable at least for 15 days.

Antibacterial activity of GO-PABA-tet nanosheet with special emphasis of putative molecular mode of action on drug resistant bacteria

The antibacterial effect of GO-PABA and GO-PABA-tet nanosheet against tetracycline resistant bacteria *Escherichia coli* XL-1 was determined by using MIC assay. The minimal inhibitory concentration of GO-PABA-tet nanosheet was found to be 110 µg/ml [Fig. 5] for *Escherichia coli* XL-1. A further FE-SEM study was performed to find out the putative mechanism of antibacterial activity of GO-PABA-tet nanosheet on drug resistant bacteria. Antibacterial activity may be occurred due to following reasons. Block of nutrient transport across the bacterial cell membrane is one major reason. Blockage of the transport system may be feasible if the GO-PABA-tet nanosheet may form visible turbidity or bacterial aggregation after addition to the test cultures in the nutrient broth. While GO-PABA-tet nanosheet may show a similar bactericidal action, owing to their smaller size and being higher water dispersion, they can transverse the bacterial membrane, carry the drug and release them in drug resistant bacteria.

In the present study, there was no turbidity appeared after the GO-PABA-tet nanosheet addition into the test culture media. It clearly pointed out that the aggregation did not occur due to comparatively smaller molecular size, higher solubility of GO-PABA-tet nanosheet. Further, FE-SEM studies indicated scar formation on the bacterial cell surface especially in the bacterial polar ends after 24 h treatment with GO-PABA-tet nanosheet [Fig. 6]. Scar formation might be happened due to erroneous proteins synthesis and membrane assembly after tetracycline entry inside the bacterial cell through GO-PABA nanosheet.

DISCUSSION

In discussion, this study showed that GO-PABA-tet nanosheet is very efficient system for drug delivery in drug resistant bacteria. On the other hand, release of tetracycline which was clearly indicated by the unique electron microscopy based antibacterial experiments. Thus, the novel GO-PABA nanosheet, combined with multi-functionalities including biocompatibility, efficient physical drug loading efficiency and delivery in drug resistant bacteria, suggest promising applications of graphene materials in biological and therapeutic fields.

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