Original Article

Palestinian Medical and Pharmaceutical Journal (PMPJ). 2016; 1(2): 65-72

Non-Covalent Functionalization of Graphene Sheets with Surfactants and their Antibacterial Activity

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Received: (12/11/2016), Accepted: (1/12/2016)

ABSTRACT

Graphene is a monolayer of tightly packed carbon atoms that attracted tremendous research interest in recent years, owing to its interesting physical, chemical, electrical, mechanical and optical properties and has numerous exciting applications. However, it suffers from low water solubility which limits its biological application. Therefore, the aim of this work is to functionalize the graphene sheets non covalently with various charged surfactants (cationic- Cetrimide, nonanionic-Tween 80, anionic-Sodium dodecyl sulfate) to get a highly stable conjugate soluble in water and to improve the antibacterial activity of this nano-derivative. The morphology and the size of the functionalized graphene were determined by atomic force microscopy. Moreover, the functionalized graphene was quantified by thermogravimetric analysis obtaining the maximum functionalization in the case of citrimide functionalized graphene. The antibacterial activity against Staphylococcus aureus and Pseudomonas aeruginosa has been studied. The results indicate that only the functionalized graphene with citrimide has significant antibacterial activity against the two strains of bacteria *S. aureus* and *P. aeruginosa* with MIC 0.98, 7.81 mg/L respectively.

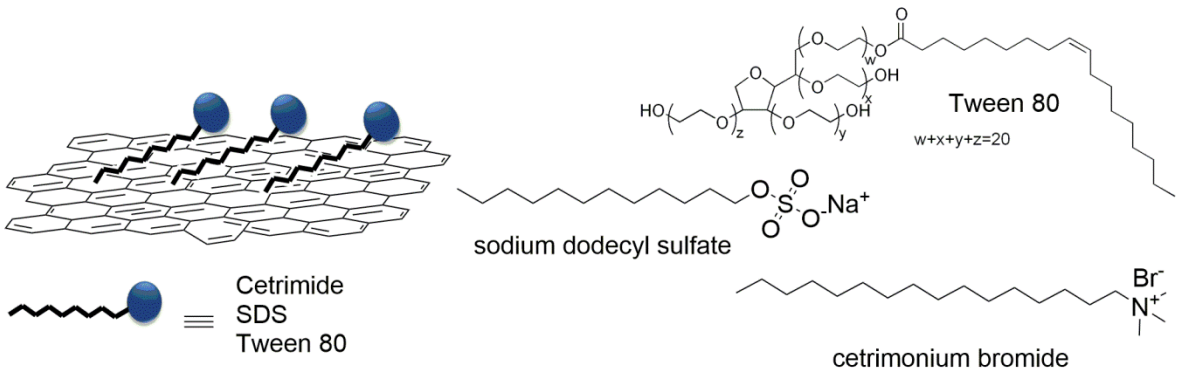
Keywords: Graphene, Surfactants, Antibacterial Activity.

INTRODUCTION

Graphene is a two-dimensional, single-layer sheet of sp^2 hybridized carbon atom arranged in six-membered rings and has a high specific surface area of 2630 m^2 g^{-1}. Graphene was firstly isolated from graphite by Andre Geim and Kostya Novoselov at Manchester University in 2004 using micromechanical exfoliation, where in this technique the graphene flakes become visible under an ordinary optical microscope when substrate has been prepared with an ultrathin layer of silicon dioxide on top (1). This material has shown amazing thermal, mechanical, optical and electrical properties. Despite it acts as semimetal, the behavior of graphene as charge carrier has a zero gap with a little overlap between valence and conductance bands. Consequently, the charge carriers move as relativistic massless at a constant speed of 10^6 m/s under ambient conditions (2, 3). Moreover, graphene is a nearly transparent material as it absorbs about 2.3% of the intensity of the white light that reaches its surface (4, 5). Regarding its thermal conductivity, graphene has a value of conductivity at room temperature of 3000-5000 W m^{-1} K^{-1}, which is considered 2 to 50 times greater than those of copper and silicon, respectively, which makes it ideal candidate as composite materials of high thermal conductivity (6). Due to these interesting properties, graphene is considered as a promising material with tremendous applications in the field of electronic, optics and energy storage (7-9). Regarding its application in medicine and biology, it is necessary to improve its solubility in the biological fluid and this can be achieved through the surface functionalization of graphene sheets covalently or non-covalently (10-12). The covalent functionlization includes amidation reaction with the carboxyl

groups of graphene oxide (13), free radical addition reaction (14), nucleophilic substitution reaction (15) and cycloaddition reaction (16, 17). This approach has the advantage of great stability of the obtained functionalized graphene; however its main drawback is the destruction of the pi system of the graphene sheet which affects the electrical properties of the graphene. On the other hand, the non-covalent approach doesn't interrupt the electronic properties of graphene sheets and can be occurred through *pi-pi* stacking between the aromatic surface of the graphene and aromatic compounds or through Van Der Waals interaction with polymers and biomolecules (18-20). Therefore, the functionalized graphene has shown numerous biomedical applications in the field of drug and gene delivery (21, 22), photothermal therapy of cancer (23) and tissue engineering (24). Various studies have shown the antibacterial activity of graphene and they explain its possible antibacterial activity against sensitive and resistant bacteria (25-27).

Herein, we aim to functionalize the graphene sheets with various surfactants in non-covalent manner and study the enhancement of the water solubility and the antimicrobial activity as shown in scheme 1. The studied surfactants are cationic (cetrimide), anionic (sodium dodecyl sulfate - SDS) and non-ionic (Tween 80) in order to evaluate the effect of the charge on the antibacterial activity and solubility of the graphene sheets.



Scheme (1): Describes the non-covalent functionalization of graphene sheets with different surfactants (Cetrimide, SDS, Tween 80) through van der waals interactions.

MATERIALS AND METHODS

Reagents and instrumentation

All materials used in this study were of analytical grade and they were used without further purification. Graphene nanopowder (thickness: 0.55-3.7 nm), sodium dodecyl sulfate (SDS), tween 80, citrimide were purchased from (Sigma-Aldrich Company, USA). BBLTM Muller Hinton II Broth (catalog # 212322) and DifcoTM Muller Hinton agar (catalog # 225250) were purchased from (BD Company, USA). A weighing balance (Adventurer®, OHAUS Corporation, USA) was used. Rotary Evaporator (VV2000 OB2000, Heidolph, Germany) was used for solvents drying. Centrifuge (UNIVERSAL 320, Hettich Zentrifugen, Germany) and water bath sonicator (Elmasonic S 70 H, Elma®, Germany) were used in preparation and dispersion of functionalized graphene. Thermogravometric analysis spectra were recorded by (STA 409 PC Luxx®, NETZSCH) in range of 0-800℃, flow 20℃ under nitrogen (100cc/min). Atomic Force Microscopy (AFM) using a tapping mode-AFM system (Alquds University, Abu Dees) with WSxM software designed by Nanotec Electronica (Madrid, Spain) was used for image analysis. Rectangular commercial Si_3N_4 cantilevers (NSG 10, NT MDT Co., Ltd.) with spring constants of 5.5–22.5 Nm^{-1} and resonance frequencies in the range 190 to 325 kHz were used. Plate-reader used for antibacterial test obtained from Stat Fax® 2100-Microplate reader, Awareness Technology INC, FL, USA.

Non-covalent functionalization of graphene sheets with surfactants

1% w/v of the surfactant (citrimide, SDS or tween 80) was added to 1 mg of the graphene sheets and sonicated for 30 mins in order to obtain a homogeneous suspension of graphene sheets. After that, a centrifugation process was done at 15000 rpm for 10 mins in order to remove the excess and the un-functionalized graphene. Figure 1 summarizes the method of the non-covalent functionalization of graphene sheets.

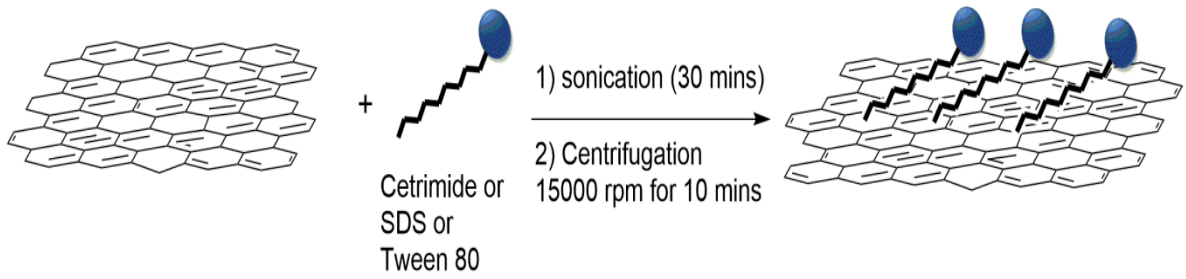


Figure (1): The method of non-covalent functionalization of graphene sheets.

Preparation of AFM Sample

The sample has been deposited on a substrate (5 × 5 mm^2) of mica and let to dry at room temperature. The AFM images were treated with WSxM 5.0 Develop 6.5 software (28).

Thermogravimetric analysis

Thermogravometric analysis spectra were recorded by (STA 409 PC Luxx®, NETZSCH) in range 0-800℃, flow 20℃ under nitrogen (100cc/min).

Antibacterial activity

Bacterial strains

The antibacterial activity of functionalized graphene was studied against *S. aureus* (ATCC 25923) and *P.aeruginosa* (ATCC 27853) strains.

McFarland preparation

Bacterial culture preparation was adjusted using the turbidity of bacterial

suspensions according to the 0.5 McFarland standard solution that represent 1.5×10^8 colony forming unit (CFU)/ml. This solution was prepared using 0.50 ml of 1.175% (w/v) $BaCl_2.2H_2O$ and 99.5 ml of 1% (v/v) H_2SO_4. Then, the optical density was measured on spectrophotometer at wavelength λ 620 nm, distilled water was used as the standard blank, to obtain turbidity within 0.08 – 0.1 that reflect bacterial concentration of about 1.5×10^8 CFU/ml. The McFarland solution was closed tightly to prevent evaporation and foiled with aluminum foil to protect it from light. The final standard bacterial

concentration was adjusted to 5×10^5 CFU/ml in each well.

Mueller Hinton broth preparation

Mueller Hinton broth was used to prepare the serial dilutions in order to reveal the antibacterial activity. According to the manufacturer, it was prepared by adding 22.0 g of Mueller Hinton broth powder to 1.0 L of distilled water and dissolved by heating on Bunsen burner with shaking. This mixture was boiled for 1 min. Mueller Hinton broth was sterilized at 121℃ for 15 min. Sterilization was confirmed by the control blanks and the usage of the sterilization indicator tapes. Sterilized Mueller Hinton broth was used as required for making the dilutions and preparing the bacterial cultures.

Mueller Hinton agar preparation

Mueller Hinton agar was used to culture the bacteria to detect the antibacterial activity. According to the manufacturer, it was prepared by adding 38.0 g of Mueller Hinton broth powder to 1.0 L distilled water and dissolved by heating on Bunsen burner with shaking. This mixture was boiled for 1 min. Mueller Hinton agar was sterilized at 121℃ for 15 min. Sterilization was confirmed by the control blanks and the usage of the sterilization indicator tapes.

Broth microdilution method

This is the basic technique in this project to obtain the minimum inhibitory concentration (MIC) for each compound.

Briefly, the functionalized graphene solutions were serially diluted (2-fold) 12 times (12 wells) with BBL Muller Hinton II broth (BD, USA). In order to detect the presence of antibacterial activity for surfactants (citrimide, tween 80, SDS) in broth microdilution method conditions, citrimide, tween 80, SDS respectively was serially diluted (2-fold) with Muller Hinton broth to achieve concentrations form 0.024% to 50%. Then, overnight grown bacterial isolates were applied to all wells to obtain final bacterial concentration of 5×10^5 CFU/ml in each well. After inoculation of bacteria, the plates were incubated for 24 h at 37℃. Broth microdilution method was performed in duplicate for each isolate. Minimal inhibitory concentration (MIC) was considered to be lowest concentration that did not show any visible growth in the test media. MIC can be read visually, or absorbance of each plate was taken at λmax 620 nm for all strains by (Stat Fax® 2100-Microplate reader, Awareness Technology INC, FL, USA), using Muller Hinton broth as the blank.

RESULTS

Herein, we aim to functionalize the graphene sheets non-covalently with three types of surfactants (cationic, non-ionic and anionic) as considered amphiphilic structure, in which its polar head exhibits a strong affinity for polar solvents, particularly water, and the hydrophobic or lipophilic tail binds to the graphene sheet through van der waals interaction to disperse it sufficiently in water. The used three surfactants are Cetrimide (cationic surfactant), Tween 80 (non-ionic surfactant) and sodium dodecyl sulfate -SDS- (anionic surfactant). Firstly, the graphene sheets were sonicated for 30 minutes with 1% solution of cetrimide getting a homogenous dispersion that exhibits positive charge on its surface as shown in figure 2a. Another solution was prepared through the sonication of graphene with 1% of Tween 80 obtaining a soluble dispersion of the graphene as shown in figure 2b. The third dispersion was the solubilization of graphene sheets with 1% of SDS to obtain homogenous dispersion as can be observed in figure 2c. As graphene sheets are insoluble in water, once they functionalized with the surfactants they became totally dispersed as shown in the three different eppendorfs in figure 2.

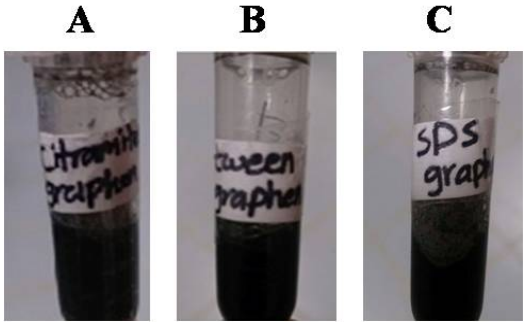


Figure (2): The formed dispersion of the non covalent functionalization of graphene sheets with a) Cetirmide; b) Tween 80; c) SDS.

The all three dispersions have shown great stability for more than three months without the formation of any precipitate. The solubility of the functionalized graphene was 1 mg in 1 ml of the surfactant. In order to determine the morphology and the diameter of the solubilized graphene, the obtained samples were examined under atomic force microscopy (AFM) in the nanotechnology lab at Alquds University-Palestine. As can be observed in figure 3, single graphene sheets were obtained in three samples with a totally dispersed sheets which confirmed the successful functionalization and dispersion of the graphene sheets. The diameter range in all cases range from 400-800 nm with a thickness of the sheets range from 0.5-1.5 nm. According to the literature, the thickness of the unfunctionalized graphene is approximately 0.25 nm (29). The increase in the thickness indicates the successful functionalization with the different types of surfactants.

Once the solubility and the morphology of the functionalized graphene were determined, the amount of the functionalization has measured by conducting a thermogravimetric analysis (TGA).

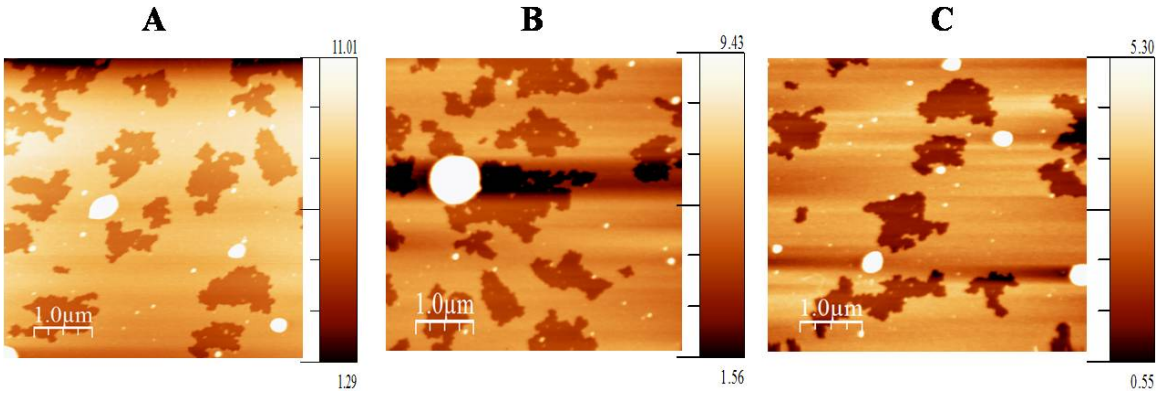


Figure (3): AFM images of the non-covalent functionalization of graphene sheets with the three different surfactants: A) Cetrimide; B) Tween 80; C) SDS.

Figure 4 demonstrates the TGA spectra of the three functionalized graphene sheets with the various surfactants. As graphene is stable for heating and will not be degraded, so the heating process will only degrade the attached surfactant. Accordingly, the percentage of the mass loss will be related to the percentage of the functionalization. From the observed results, the percentage of the functionalization was the maximum with the case of the cetrimide surfactant with an amount of functionalization of 45%, which is high in the case of the non covalent functionalization followed by the case of tween 80 with a percentage of 30% and the least amount was in the case of the SDS. Therefore, the optimum functionalization was in the case of the cetrimide in its potency in the functionalization and solubilization of the graphene sheets.

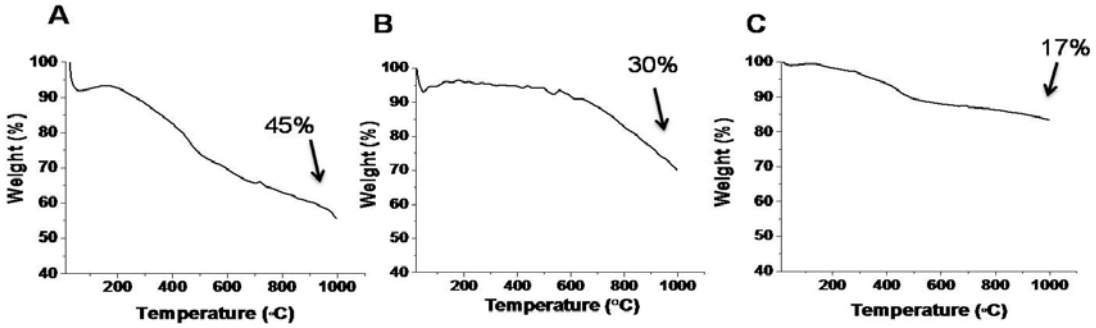


Figure (4): TGA spectra of the functionalized graphene with the three different surfactants: A) Cetrimide; B) Tween 80; C) SDS.

After that, we have measured the antibacterial activity of the three functionalized graphene sheets using the broth microdilution method in order to determine the minimum inhibitory concentration (MIC). The antibacterial activity was measured on two types of bacteria the gram positive bacteria (*Staphylococcus aureus*) and gram negative bacteria (*Pseudomonas aeruginosa*). The antibacterial activity of the functionalized graphene with SDS and tween 80 has shown negligible antibacterial activity in comparison to the graphene sheets functionalized with citrimide. This result could be due to the positive charge of the citrimide which enhances the penetration of the *f*-graphene to the bacteria by the interaction with the phosphate groups of the cell wall in comparison to the tween 80 that doesn't have a charge or SDS that owns a negative charge that repelled with the cell wall. Therefore, we have conducted the antibacterial activity of the graphene sheets functionalized with citrimide. Table 1 demonstrates the antibacterial activity of the citrimide alone and the functionalized graphene with citrimide. According to the literature, the MIC of the citrimide against *S. aureus* was

16 mg/L and against *P. aeruginosa* was 512 mg/L (30). The functionalized graphene with citrimide has shown improvement of the antibacterial activity in comparison to citrimide alone by 16 fold against S. aureus and 65 fold against P. aeruginosa. So, the antibacterial activity of the graphene was improved significantly due to its functionalization with citrimide that enhances the solubility and the penetration to the cell wall of the bacteria.

Table (1): MIC of citrimide alone and *f*- graphene sheets with citrimide against S. aureus and P. aeruginosa.

Species	MIC of citrimide (mg/L)	MIC of f-graphene citrimide (mg/L)	Folded
S. aureus	16	0.98	16
P. aeruginosa	512	7.81	65

DISCUSSIONS

Graphene sheets are the two dimensional allotrope of carbon with a unique structure and properties that can be viewed as a macromolecule with molecular weights of more than 10^6–10^7 g/mol (31). Graphene sheets are constructed by sp^2-hybridized carbon atoms arranged in a honeycomb lattice (32). These sheets are found as a stick layers due to the π-π stacking interactions. Therefore, it is difficult to disperse graphene homogeneously in solution due to its tendency to form aggregates with tightly bounded packages (33). The application of graphene in the biological field implies the necessity to improve its solubility, especially in the water as it is the biological medium. One approximation that can separate and disperse individual graphene sheets is through the non-covalent interaction of the graphene sheets with different species, such as polymers, aromatic compounds and biomolecules (34, 35). This type of functionalization is very attractive due to the ability to solubilize the graphene without affecting its electronic structure (10). Non-covalent interactions are based in most cases on van der Waals forces or π-π stacking, hydrogen bonds and electrostatic interactions (36). Herein, we successfully functionalized the graphene sheets with three various surfactants. The functionalized graphene sheets have characterized by AFM and the morphology of the graphene sheets confirmed the functionalization. The functionalized graphene has shown great stability in water and the optimum functionalization was in the case of citrimide surfactant as confirmed with TGA.

The functionalized graphene has shown great biological applications in various fields. One of the interesting and promising application is its usage as healing agent and the antibacterial activity in wide range of bacteria. Liu et al. have concluded that the reduced graphene oxide sheets have antibacterial activity due to the oxidative stress. The proposed mechanism of action includes: cell deposition on graphene sheets, membrane stress caused by its direct contact and the formation of the superoxide anion-independent oxidation (26). On another work, the same results were obtained confirming the mechanism of the antibacterial activity of graphene sheets through the damage of the cell wall and membrane of the bacteria (25). A study conducted by Krishnamoorthy et al. describes the effective antibacterial activity of graphene sheets in comparison to the antibiotic kanamycin on different types of bacteria such as Escherichia coli, Salmonella typhimurium, Enterococcus faecalis, and Bacillus subtilis. They proposed that the antibacterial activity of the graphene sheets is due to the involvement of reactive oxygen species (37).

In our case, the functionalized graphene with citrimide has shown a potent antibacterial activity against gram positive and gram negative bacteria due to the synergistic effect of the graphene sheets with the citrimide surfactant. Moreover, the positive charge on the surface of graphene sheets improve its penetration and consequently the antibacterial activity.

CONCLUSION

The graphene sheets were successfully functionalized non-covalently with the three

different surfactants (citrimide, Tween 80 and SDS) obtaining a highly soluble nano-conjugates. The morphology and size confirmed by atomic force microscopy. The level of functionalization was determined by TGA obtaining the maximum functionalization with citrimde with 45% of functionalization. The anitbacterial activity of *f*-graphene with citrimide has improved against the citrimide alone by 16 fold against *S.aureus* and 65 fold against *P.aeruginosa*. This improvement can be explained due to the positive charge of citrimide which interacts with the cell wall of bacteria and the direct contact by the graphene sheets. These results will be in huge interest in the field of tissue engineering and its utilization as healing agent.

CONFLICT OF INTERESTS

The authors report no conflicts of interest in this manuscript.

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