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Faculty of Graduate Studies

Functionalization of graphene sheets and their antibacterial activity

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Dedication

To greatest and affectionate father and mother in the world, who supporting and believe me and raised me to be what I am today and stay beside me in every step of all the way.

To my grandmother spirits

To my brothers, sister, my aunts and her lovely families who have supported me from the first step to the end

To my lovely friends

I dedicate this work

Acknowledgement

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Last but not least, a big thanks to my lovely family, who stood with me throughout my study and provided me with psychological support and encouragement.

Deema Alsouqi

الإقرار

انا الموقعة أدناه مقدِّمة الرسالة التي تحمل عنوان:

تفعيل صفائح الجرافين و تاثيرها المضاد للبكتيريا

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Declaration

The work provided in thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

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Table of contents

List of tables

List of figures

List of schemes

List of abbreviations

Symbol	Abbreviation
0D	Zero Dimensional
1D	One Dimensional
2D	Two Dimensional
3D	Three Dimensional
Å	Angestrom
AFM	Atomic Force Microscopy
anhydrous $CuSO_4$	Anhydrous copper sulfate
Boc	Di-tert-butyl-dicarbonate
^{o}C	Celsius
C_2H_3N	Acetonitrile
C_3H_8O	Isopropyl alcohol
$(C_2H_5)_2O$	Diethyl ether
CEG	Chemically-assisted Exfoliated Graphene
CFU	Colony Forming Unit
$CHCl_3$	Chloroform
CLSI	Clinical and Laboratory Standards Institute
$Cm^2/V.s$	Centimeters squared per volt-second
CNTs	Carbon Nanotubes
CTAB	Cetyl-Trimethyl Ammonium Bromide
CVD	Chemical Vapor Deposition
DCM	Dichloromethane
DEG	Diethylene-glycol
DIPEA	Di-isopropylethyl amine
DMAc	N,N′-dimethylacetamide
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethyl Sulfoxide
DNTB	Ellman's reagent, 5,50-dithio-bis-(2-nitrobenzoic acid)
DOC	Sodium Deoxycholate
E.coli	*Escherichia coli*
EDC	Ethylcarbodiimide hydrochloride
Et_3N	Triethylamine
EtOAc	Ethylacetate
GO	Graphene oxide
GSH	Glutathione
Gt	Graphite

GtO	Graphite Oxide
h	Hour
H_2SO_4	Sulfuric acid
Hex	Hexane
HOPG	Highly Oriented Pyrolytic Graphite
$HRBC_S$	Human Red Blood Cells
HRP	Horse Radish Peroxidase
ITO	Indium-Tin-Oxide
LB	Lennox Broth
LCST	Lower Critical Solution Temperature
LDS	Lithium Dodecyl Sulfate
MeOH	Methanol
MIC	Minimal Inhibitory Concentration
Na_2SO_4	Sodium Sulfate
$NaBH_4$	Sodium Borohydride
NaCl	Sodium Chloride
NaN_3	Sodium azide
Ni	Nickel
NMP	1-methyl-2-pyrrolidinone
NMR	Nuclear Magnetic Resonance
O_2 $\bullet^-$	Superoxide anion
ODA	Octadecyl Amine
ODCB	1,2-dichlorobenzene
PB^-	1-pyrenebutyrate
PBS	Phosphate buffer saline
p-CCG	polydisperse Chemically-Converted Graphene
PNIPAAm	Poly(N-isopropylacrylamide)
PSS	Poly (Sodium 4-Styrenesulfonate)
RAFT	Reversible Addition Fragmentation chain Transfer
RBF	Round bottom flask
rGO	reduced Graphene Oxide
ROS	Reactive oxygen stress
Rpm	Round per minute
S.aureus	*Staphylococcus aureus*
SC	Sodium Cholate
SDBS	Sodium Dodecyl Benzene Sulfonate
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy
SiC	Silicon Carbide
$SOCl_2$	Thionyl Chloride
TBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate

TDOC	Sodium Taurodeoxycholate
TEG	Tetraethylene glycol
TEM	Transmission electron microscope
TFA	Trifluoroacetic acid
TGA	Thermogravimetric analysis
THF	Tetrahydrofuran
TLC	Thin layer chromatography
Tris-HCl	Trizma Hydrochloride
TsCl	Tosyl chloride
TTAB	Tetradecyltrimethyl Ammonium Bromide
UV-Vis	Ultraviolet-Visible
$Wm^{-1}K^{-1}$	Watts per meter-kelvin
λ_{max}	Lambda max

Functionalization of graphene sheets and their antibacterial activity

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Abstract

Graphene is a thin flat monolayer of carbon atoms tightly packed into a two-dimensional (2D) honeycomb lattice. It is one of the most studied materials nowadays because it has a high surface area and unique properties. However, graphene is considered a hydrophobic material (has a low solubility and dispersability in physiological solution), which leads to aggregation and precipitation in cell tissue and causing toxicity for different types of cells. For this purpose, functionalization of graphene has its purpose to improve its dispersability in water and its biocompatibility for various biomedical applications.

The aim of this work is to covalently functionalize the graphene sheets with various charged groups (positive, negative and neutral) to improve its water dispersability, study its antibacterial activity and to determine the effect of charge on the activity.

The graphene sheets had successfully functionalized covalently with three different groups (COOH, amine and tetraethylene glycol). The functionalization was confirmed by infrared spectroscopy and transmission electron microscope images. The functionalization demonstrates good dispersability in water and the degree of functionalization was quantified

using the thermogravimetric analysis obtaining 32% of the functionalization in the case of graphene-TEG, 33% of graphene-amine and 47% of graphene-COOH.

The antibacterial activity was determined primarily by agar diffusion disk- and well-variant method. Agar diffusion well-variant demonstrated the presence of the antibacterial activity for all graphene derivatives. In additional step, the antibacterial activity was detected and quantified through determining the MIC of the graphene derivatives by broth microdilution method. MIC was 250µg/ml for graphene-amine and graphene-TEG and 125µg/ml in the case of graphene-COOH. Moreover, the reduction of bacterial concentration after exposure to graphene derivatives was detected by the plate count method, the result shows that the bacterial reduction was increased in functionalized graphene nanomaterials with the complete growth inhibition in the case of graphene-COOH on both bacteria (*E. coli* and *S. aureus*). Graphene toxicity mechanism was investigated by *in vitro* graphene-mediated oxidation of glutathione, the loss of glutathione activity was the maximum for graphene-COOH with a reduction of 83%. Finally, graphene-COOH and graphene-TEG showed good hemo-compatibility, which supports their further *in vivo* studies.

Chapter one
Introduction

1.1. Graphene

Graphene, one of the carbon nanomaterial allotropes, is a thin flat monolayer (single atom thick) of carbon atoms tightly packed into a two-dimensional (2D) honeycomb lattice, with a carbon-carbon distance of 0.142 nm and thickness of 3.35 Å. It is formed by a mesh of hexagonal structures of sp^2 hybridized carbon and has a high specific surface area of 2630 m^2 g^{-1} and density 0.77 mg/m^2.These characteristics confer its exceptional thermal, mechanical, optical and electrical properties [1].

Graphene is a basic building block of all graphitic materials (Figure 1). It can be wrapped up into 0D fullerenes (bucky balls), rolled into 1D carbon nanotubes or stacked into 3D graphite [1-3].

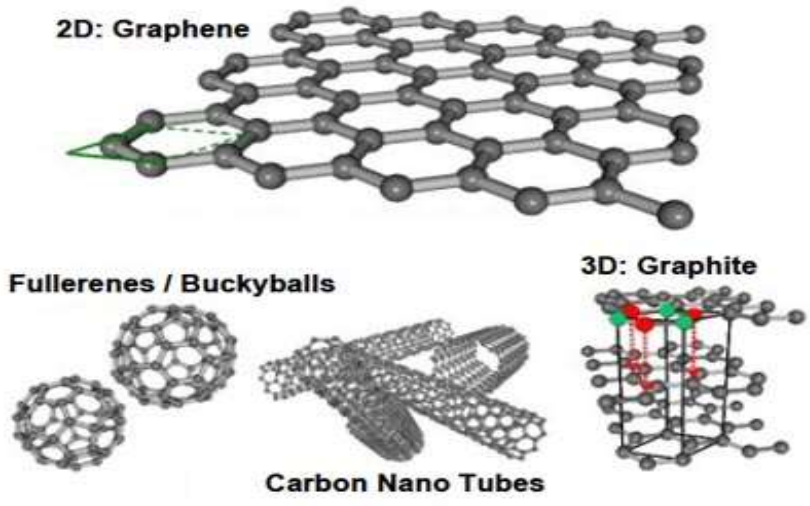


Figure 1.1: Mother of all graphitic forms**.** Graphene is a 2D building material for carbon materials of all other dimensionalities. It can be wrapped up into 0D buck balls, rolled into 1D nanotubes or stacked into 3D graphite.

1.2. Discovery of Graphene

Andre Geim and Konstantin Novoselov from the University of Manchester (UK), and the Institute for Microelectronics Technology in Chernogolovka (Russia) have succeeded for the first time in producing, isolating, identifying and characterizing Graphene and they published their results in October of 2004 in Science and honored with Nobel Prize in Physics 2010 [1, 3]. They used a simple but effective mechanical exfoliation method for extracting thin layers of graphite from a graphite crystal with Scotch tape and then transferred these layers to a silicon substrate. This method was first suggested and tried by R. Ruoff's group, who were, however, not able to identify any monolayers. The Manchester group succeeded by using an optical method with which they were able to identify fragments made up of only a few layers. An AFM picture of one such sample is shown in Figure 1.2 [3].

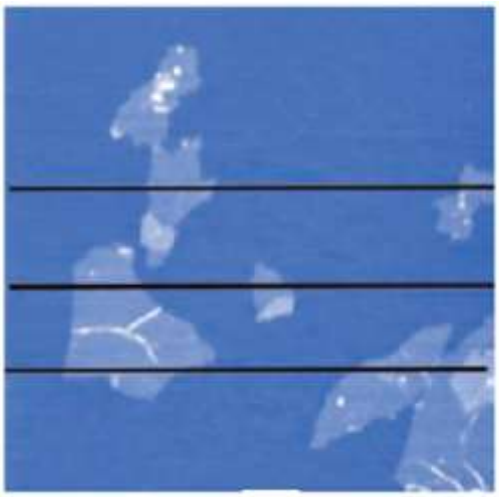


Figure 1.2: AFM image and line profile of graphene sheets with ~1 nm thickness [4].

1.3. Properties of Graphene

Graphene and carbon nanotubes (CNTs) are two of the most studied materials these days but the two-dimensional Graphene has a special attention because it has a high surface area [2, 5] and a unique electrical properties such as very high carrier mobility. Electrons in graphene, obeying a linear dispersion relation, behave like massless relativistic particles or quantum billiard balls. This results in the observation of a number of very peculiar electronic properties – from an anomalous quantum Hall effect to the absence of localization – in this, the first two-dimensional material. It also provides a bridge between condensed matter physics and quantum electrodynamics, and opens new perspectives for carbon-based electronics [6, 7]. Electrons in bilayer Graphene possess an unusual property: they are chiral quasi-particles characterized by Berry phase 2π. Researchers at the University of Manchester observed that electrons move more easily in graphene than all other materials, including gold, silicon, gallium arsenide and carbon nanotubes and have singled graphene out as the "best possible" material for electronic applications. With a high electronic quality - measured at around 200,000 $cm^2/V.s$ and more than 100 times higher than for silicon – these researchers believe graphene has the potential to improve upon the capabilities of current semiconductors and open up exciting new possibilities, using the layer thickness we get a bulk conductivity of 0.96x106 $\Omega^{-1}cm^{-1}$ for graphene. This is somewhat higher than the conductivity of copper which is 0.60x106 $\Omega^{-1}cm^{-1}$ [8].

Regarding the optical properties, graphene is almost transparent, it absorbs only 2.3% of the light intensity, independent of the wavelength in the optical domain [9, 10].

Moreover, graphene is considered the strongest material ever measured, and show that atomically perfect nano-scale materials can be mechanically tested to deformations well beyond the linear [11].

The thermal conductivity of Graphene is dominated by phonons and has been measured to be approximately 5000 $Wm^{-1}K^{-1}$. Copper at room temperature has a thermal conductivity of 401 $Wm^{-1}K^{-1}$. Thus graphene conducts heat 10 times better than copper [12].

1.4. Synthesis of graphene

Synthesis of Graphene refers to any process for fabricating or extracting graphene, depending on the desired size, purity and efflorescence of the specific product.

As mentioned earlier, Graphene was firstly obtained by micromechanical exfoliation of highly oriented pyrolytic graphite (HOPG) using adhesive tape and subsequently deposited on a silicon substrate, subsequently analyzed by atomic force microscopy (AFM) and transmission electron microscopy (TEM) as shown in figure1.3 [1].

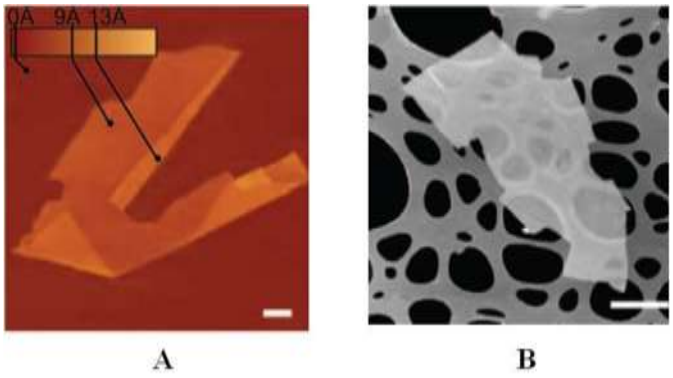


Figure 1.3: A) Graphene visualized by AFM. The folded region exhibits a relative height of ≈ 4 Å indicating that it is a single sheet. B) Single sheet of Graphene observed by TEM. Reproduced with permission from ref. 14. Copyright 2005, PNAS.

Although this methodology has allowed obtaining individual graphene sheets of highest quality and with dimensions of the order of 10 microns, the main problem lies in the extremely low yield. Therefore, alternative methods are developed for the preparation of graphene with low production costs while increasing the amount of the material obtained. Therefore, the methods that are currently most promising are:

1.4.1. Chemical vapor deposition (CVD) [13-15] : CVD processes generally use the transition metal surface for the growth of graphene using hydrocarbon gases as precursors at a deposition temperature of approximately 1000 °C. For example, the group of Kong [16] , presented a scalable and low cost technique using ambient pressure CVD on polycrystalline Ni films, for which they prepared films with an elevated area of one or a few sheets of graphene and these prepared layers can be transferred to various nonspecific substrates.

1.4.2. Epitaxial growth of graphene on silicon carbide (SiC) [17-19]: It is a very promising method for the synthesis of graphene sheets with a uniform size in which a crystalline SiC substrate is heated in vacuum at high temperatures in the range of 1200-1600 °C. Since the sublimation rate of silicon is larger than that of carbon, excess carbon remains on the surface, being rearranged to form the layer of graphene. The necessity to work on ultra-high vacuum and the required high temperature to produce the sublimation of silicon greatly limits their widespread application on large scale.

1.4.3. Chemical reduction of Graphene oxide (GO): the oxidation and exfoliation of natural graphite by thermal technique or oxidation followed by the chemical reduction has been appreciating one of the most efficient methods for the production of large scale graphene and low cost. However, the major drawback of this method is the low structural quality of the sheets. With this method, strong oxidation of the graphite generates mixture (epoxy, hydroxyl, carbonyl and carboxyl ...) in both the edges and in the plane of the sheet which interrupting the electronic conjugation of the plane, figure1.4 [20].

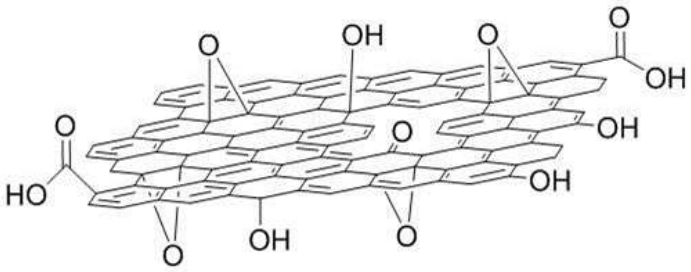


Figure 1.4: Structure of Graphene oxide layers.

These functional groups interrupt numerous van der Waals interactions between the layers of the graphene oxide giving them a hydrophilic character. However, due to its strong hydrophobicity, graphene obtained from the reduction of Graphene oxide usually suffers from low solubility and irreversible agglomeration, thus limiting further processing and application. So far, many types of reduction methods have been developed to obtain a reduced graphene oxide sheets such as chemical reducing agents such as Hydrazine and $NaBH_4$ [5, 21] thermal reduction [22], photochemical reduction [23], photo-thermal reduction [24], electrochemical reduction [25], and microwave-assisted reduction [26]. Lee *et al.* have recently reviewed the effective techniques in the reduction of graphene oxide [27]. In this regards, it should be mentioned that the reducing reagents such as hydrazine and $NaBH_4$ are highly toxic. Since hydrazine is toxic and unstable, it is desirable to explore a new route within the green chemistry to reduce the graphene oxide. The group of Zhang has reported an environment friendly methodology using ascorbic acid as a reducing agent and an amino acid as a stabilizer [28]. Meanwhile, Loh's group reported a simple and clean hydrothermal dehydration route to convert graphene oxide to graphene using supercritical water (SC) which

could act as a reducing agent under hydrothermal conditions and promote recovery of the π conjugation after dehydration [29]. More interestingly, the Tour's group demonstrated the use of environmental bacteria (*Shewanellaoneidensis*) as an electron donor to reduce graphene oxide. They have found that the conductivity and physical characteristics of graphene converted by the bacteria were comparable to other forms of chemically converted graphene [30]. These results are important in this regard not only of the use of environmental bacteria for the bioremediation but also in a more specific form in materials synthesis using green chemistry conditions.

1.5. Application of Graphene

Graphene has a number of properties which makes it interesting for several different applications. It is an ultimately thin, mechanically very strong, transparent and flexible conductor. Its conductivity can be modified over a large range either by chemical doping or by an electric field. The mobility of graphene is very high [31] which makes the material very interesting for electronic high frequency applications [32]. Recently it has become possible to fabricate large sheets of graphene. Using near-industrial methods, sheets with a width of 70 cm have been produced [33, 34]. Since graphene is a transparent conductor can be used in applications such as touch screens, light panels and solar cells, where it can replace the rather fragile and expensive Indium-Tin-Oxide (ITO). Flexible electronics and gas sensors [35] are other potential applications. The quantum Hall effect in graphene could also possibly contribute to an even more accurate

resistance standard in metrology [36]. New types of composite materials based on Graphene with great strength and low weight could also become interesting for use in satellites and aircraft [37, 38]. However, the biological applications of graphene sheets have been hampered due to its low water solubility in water and low dispersability. Therefore, there is a need to increase its water solubility through functionalization.

1.6. Functionalization of Graphene

Graphene is substantially insoluble material, or it disperses with difficulty, in any type of solvent. Therefore, the potential application of Graphene in the biological field implies the necessity to improve its solubility, especially in the water as it is the biological medium. For this purpose, several methodologies have been developed for its functionalization that can be classified into two main groups: covalent manner and non-covalent routes.

1.6.1. Covalent functionalization of graphene

Covalent bonds can be established in two different ways, either by the use of the carboxyl groups of graphene oxide through amide or ester formation, or by direct reaction with the graphene sheet. The main advantage of this strategy is the great stability of the nanomaterial obtained after the functionalization; however its main drawback is the destruction of the π system of the graphene sheet which affects the electrical properties of the graphene.

1.6.1.1. Amidation and esterification reactions of graphene oxide

As have seen earlier, the production techniques of graphene generate different sizes of graphene sheets whose structure is furnished with oxygenated functional groups, mainly carbonyl and carboxylic groups which have been utilized for further functionalization using acid chloride as intermediates or directly using activating agents such as carbodiimide, figure 1.5 [39].

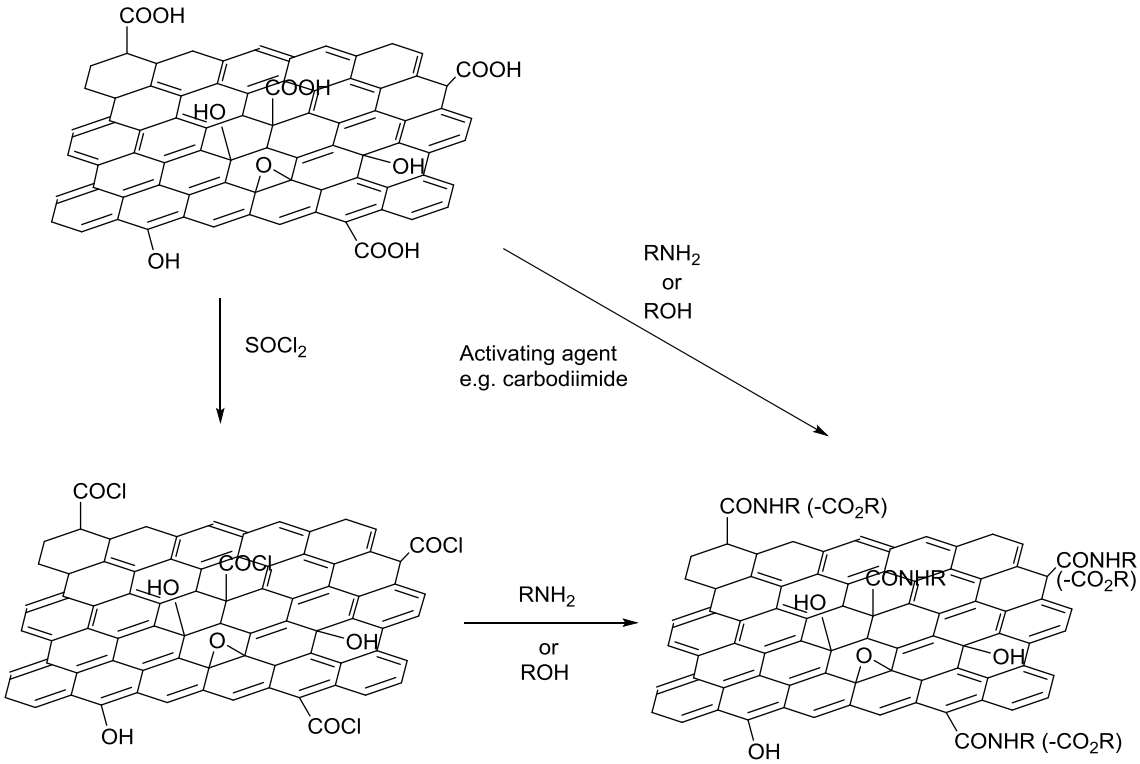


Figure 1.5: Acylation reactions through the carboxylic acid groups appeared on the Graphene oxide sheet.

The group of Haddon has reported for the first time the grafting of octadecylamine (ODA) on the surface of graphene oxide after the activation of the carboxyl groups by thionyl chloride ($SOCl_2$). The functionalized graphene has a solubility of 0.5 mg/mL in THF and is also soluble in CCl_4 and 1,2-dichloroethane [39].

1.6.1.2. Direct formation of covalent bond with graphene sheet.

This section will mainly cover the following chemical reactions: free radical addition, nucleophilic substitution and cycloaddition reactions.

1.6.1.3. Free radical addition reaction.

One of the most investigated lines within this type is the utilization of diazonium salts as functionalizing agents, figure 1.6. This methodology was developed in the group of Tour by electrophilic substitution of aryl diazonium salt on the surface of surfactant-wrapped graphene [40]. They demonstrated that the functionalization of the graphene oxide wrapped with the surfactant sodium dodecyl benzene sulfonate (SDBS) with the diazonium salt derivative can be achieved firstly by reducing the oxy groups with hydrazine monohydrate; followed by grafting of the aryl diazonium salt on the surface of SDBS wrapped graphene at room temperature. The degree of functionalization was estimated by TGA up to 1 functional group per 55 carbon atoms and the functionalized graphene is highly dispersible in N,N′-dimethylformamide (DMF), N,N′-dimethylacetamide (DMAc), and 1-methyl-2-pyrrolidinone (NMP).

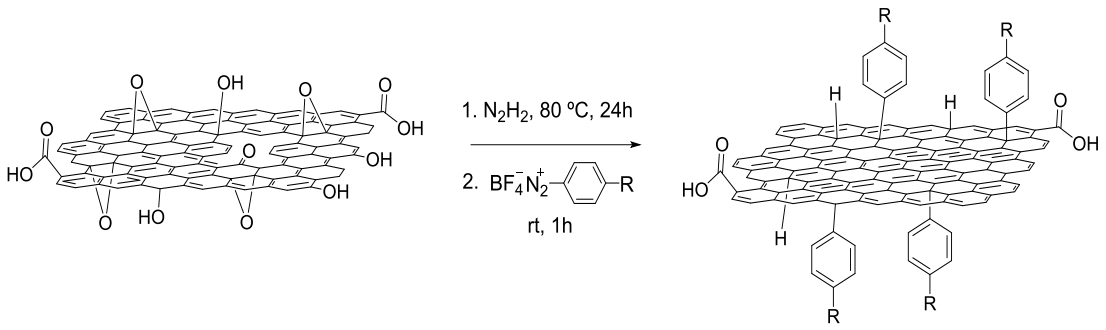


Figure 1.6: Reduction of the GO and functionalization with different derivatives of diazonium salts.

The same group has extended the functionalization to expanded graphite by using the diazonium salt of 4-bromo analine [41]. The obtained chemically-assisted exfoliated graphene (CEG) sheets are more soluble than pristine graphene in DMF after the ultra-sonication and without the addition of surfactant and the microscopic data has shown that more than 70% of the CEG flakes have less than 5 layers.

1.6.1.4. Nucleophilic substitution reaction.

The epoxy groups of Graphene oxide are the main active sites for the nucleophilic substitution. The amine group in the organic compounds has lone pair of electrons that can attack the epoxy groups of the graphene oxide. Niu and co-workers have reported the functionalization of graphene oxide using amine- terminated ionic liquid (IL-NH_2) [42]. The IL-NH_2acts as a nucleophile and opens the ring of the epoxy group of the GO. The obtained functionalized graphene is totally dispersed in water, DMF and DMSO without the addition of any surfactant as stabilizer, figure 1.7.

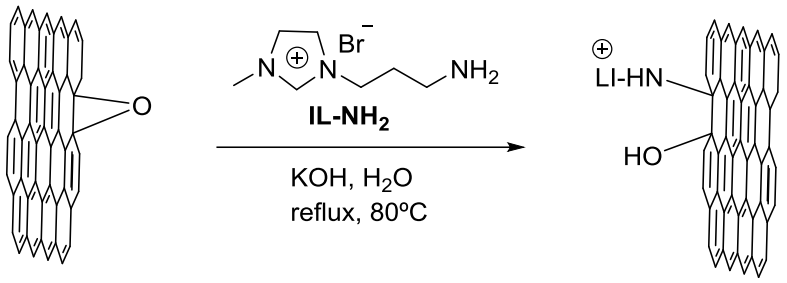


Figure 1.7: Preparation of poly disperse chemically-converted Graphene (p-CCG) sheets.

1.6.1.5. Cycloaddition reaction.

In this section three major cycloaddition reactions will be mentioned, the [2 + 1] cycloaddition is one of the earliest methods that have been used to functionalize the graphene. One of the important examples of [2 + 1] cycloaddition is the introduction of aziridine adduct onto the graphene sheet by the formation of reactive intermediate nitrene, which can be achieved by thermal or photo decomposition of azide group [43]. Various research groups have used this strategy to introduce different organic compounds onto graphene. Yan and co-workers have successfully functionalized exfoliated graphene by a variety of derivatives of perfluoro-phenylazide containing alkyl, ethylene oxide and perfluoroalkyl chains though their thermal and photochemical activation [43], figure 1.8. The functionalized graphene with alkyl and perfluoroalkyl chains were soluble in *o*-dichlorobenzene whereas the graphene functionalized with ethylene oxide chains was soluble in water.

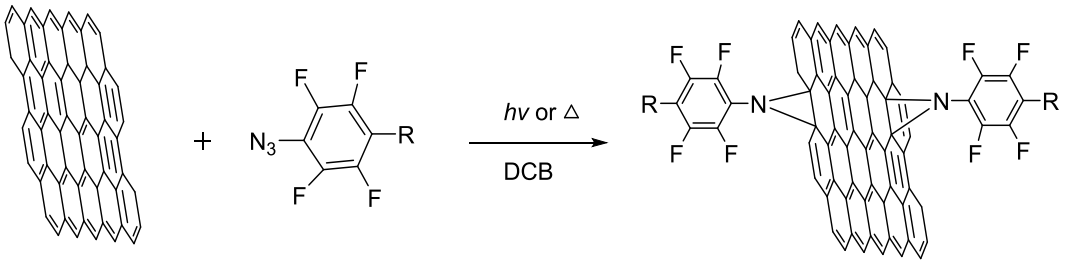


Figure 1.8: Functionalization of Pristine Graphene with azide derivatives.

The other type of cycloaddition that has been applied on the chemistry of Graphene is [2 + 2] cycloaddition. The reaction results in the formation of four-membered ring on the Graphene sheet. Zhong *et al.* have demonstrated the addition of three derivatives of benzene moiety which have been attached covalently to the graphene sheet [44], figure 1.9. Based on the TGA data, the degree of functionalization was estimated to be 1 functional group per 17 carbon atoms and the functionalized graphene demonstrated good solubility in various solvents such as DMF, ethanol, chloroform, 1,2-dichlorobenzene and water.

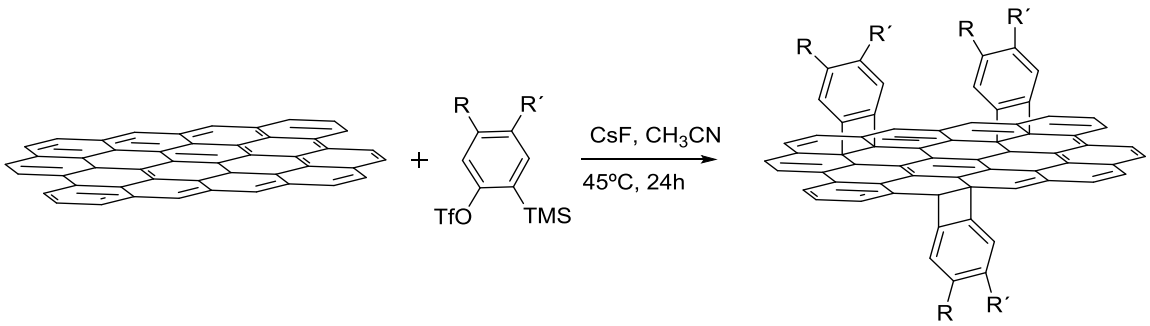


Figure 1.9: Illustration of the chemical modification of Graphene sheets via arynecycloaddition.

The third type is 1,3-dipolar or [3 + 2] cyloaddition reaction consists in the addition of azomethineylides, generated *in situ* by thermal condensation between an α-amino acid and an aldehyde with a double bond of the graphene sheet resulting in pyrrolidone ring fused to the surface.

Georgakilas *et al.* were the first who utilized the 1,3-dipolar cycloaddition to functionalize graphene π carbon network with high degree of functionalization reached 1 functional group per 40 carbon atoms as highlighted by TGA data [45]. The product demonstrated good solubility in polar organic solvents and water with high stability, figure 1.10.

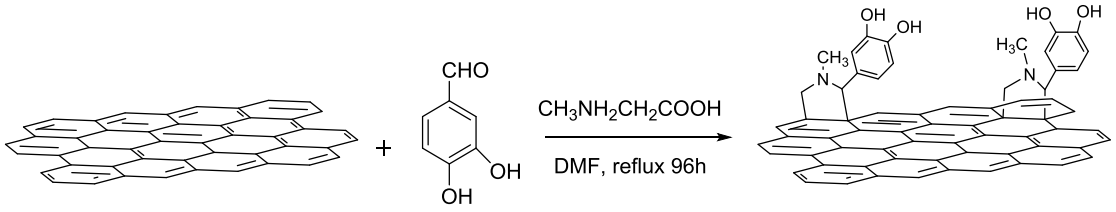


Figure 1.10: Schematic representation of the 1,3 dipolar cycloaddition of azomethineylide on grapheme.

1.6.2. Non-covalent functionalization of graphene

The covalent functionalization of graphene has the drawback to modify the surface of the sheet and thus alter its physical and chemical properties, due to the conversion of the sp^2 carbons to sp^3 ones which decreases the mobility of the carrier, so the non-covalent approach, that does not alter the graphene sheet, is of greater interest.

Graphene sheets are difficult to disperse homogeneously in solution due to their tendency to form aggregates with tightly bounded packages. One approach that has been used to separate and obtain individual graphene sheets is to establish non-covalent interactions with the surface of the sheet with different species, such as polymers, aromatic compounds, detergents and biomolecules.

This type of functionalization is very attractive because it offers the ability to anchor molecules without affecting the electronic structure of the graphene. Non-covalent interactions are based in most cases on van der Waals forces or π-π stacking, hydrogen bonds and electrostatic interactions.

The first example of the non-covalent functionalization of graphene using poly(sodium 4-styrenesulfonate) (PSS) has been reported by Stankovich *et al.* in which the functionalized graphene nano platelets have been obtained by the exfoliation and the reduction in situ of GO in the presence of PSS. These nano platelets have shown high dispersability in water and other organic solvents [46].

Among the examples of non-covalent functionalization through π-π stacking interactions, pyrene derivatives have been mainly used. Thereby, Xu *et al.* have successfully functionalized a reduced graphene oxide non-covalently utilizing 1-pyrenebutyrate (PB^-), as a stabilizer since the pyrene moiety has strong affinity with the basal plane of graphite via π-stacking. The obtained graphene is highly dispersed in aqueous media and has showed high magnitude (7 orders) of conductivity in comparison to the GO precursor [47]. Likewise, Liu *et al.* have synthesized pyrene-terminated poly(N-isopropylacrylamide) (PNIPAAm) using reversible addition fragmentation chain transfer (RAFT) polymerization which is capable of the non-covalent functionalization of Graphene sheet via π-π stacking between the pyrene moiety and π network of the Graphene, figure 1.11 [48]. The obtained Graphene-PNIPAAm composite is soluble and thermo-

sensitive in water and has a reversible lower critical solution temperature (LCST)-induced dispersability at 24 ºC.

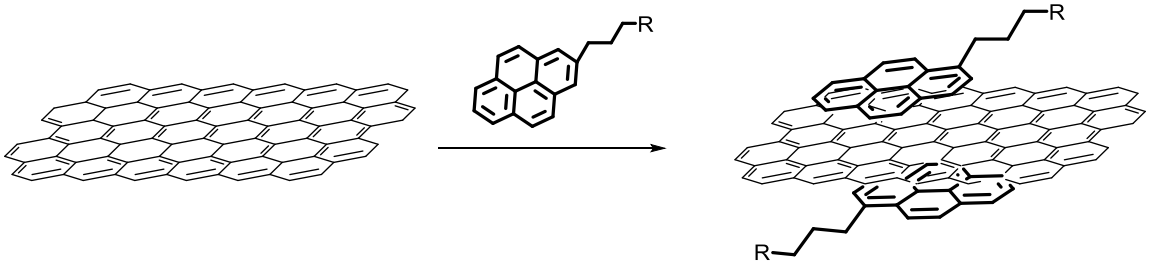


Figure 1.11: Interaction between derivatives of pyrene and Graphene.

Other type of non-covalent functionalization of graphene could be occurred by coating the surface of graphene with proteins, surfactants and polymers through van der Waals forces. The Suslick's group has utilized the sono-chemical method to functionalize graphite starting from a reactive monomer of styrene obtaining polystyrene functionalized graphene. The ultrasonic irradiation of graphite in the presence of styrene exfoliates the graphite flakes to mono or few layers of Graphene sheets. In addition, a radical polymerization of styrene has been initiated sono-chemically forming polystyrene on the surface of the graphene sheets. The obtained graphene has showed high solubility in various organic solvents such as DMF, THF, toluene and chloroform [49]. Regarding the surfactants that have been used in the functionalization of graphene, Zeng *et al.* have reported the self-assembly of sodium dodecyl benzene sulfonate (SDBS) on the surface of the graphene which has been further attached with horseradish peroxidase (HRP) by electrostatic attraction between the negative charges of SDBS and the positive charge of the HRP [50].

The group of Coleman has functionalized the graphene with various ionic and non-ionic surfactants and has studied the effect of these surfactants on the dispersion of the graphene in water [51]. They have studied 8 ionic surfactants: SDBS, sodium dodecyl sulfate (SDS), lithium dodecyl sulfate (LDS), cetyltrimethyl ammonium bromide (CTAB), tetradecyltrimethyl ammonium bromide (TTAB), sodium cholate (SC), sodium deoxycholate (DOC) and sodium taurodeoxycholate (TDOC), and 4 non-ionic surfactants which are IGEPAL CO-890, Triton X-100, Tween 20 and Tween 80. In all cases, the concentration of the surfactant was 0.1 mg/ml and the concentration of the graphite was 5 mg/ml, the dispersion was obtained by the ultra-sonication method in aqueous solution. They have found that the degree of exfoliation, as characterized by flake length and thickness, was independent of the surfactant type; however the stability was dependent on the solvent type. The dispersed graphene was characterized to have 750 nm long and four layers thick on average.

1.7. Biomedical application of Graphene

Graphene expanding its territory beyond electronic and chemical applications toward biomedical such as precise bio sensing through graphene-quenched fluorescence and graphene-enhanced cell differentiation and growth, and Graphene-assisted laser desorption/ionization for mass spectrometry as shown in figure 1.12 [4].

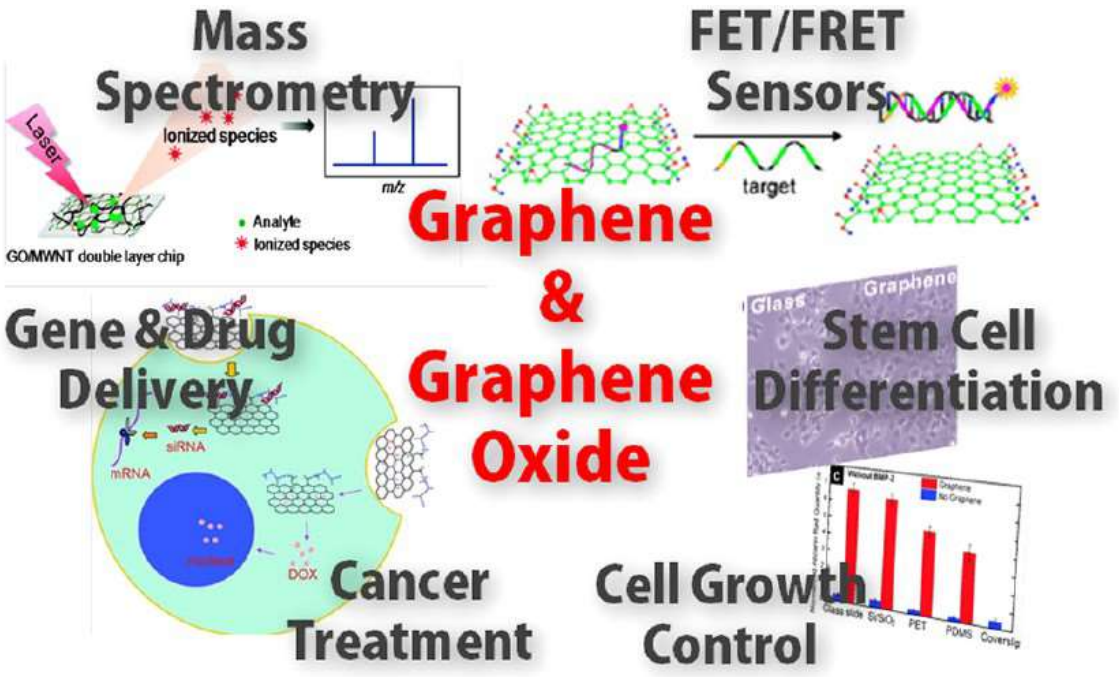


Figure 1.12: Graphene application

Due to Graphene unique properties – large surface area and high conductivity- a group of researchers from Harvard University and MIT have demonstrated that graphene sheets could make a big improvement over the currently used membranes for nano-pore DNA sequencing- a technique that promises to accelerate and simplify the sequencing of long DNA chains [52], there has been increasing interest in using graphene and its derivatives for drug delivery system [53], because the planar structure and ultra-high surface area (2600 m^2/g) of graphene facilitate molecular loading and bio-conjugation with a high ratio [54].Graphene and its derivatives, have been evaluated as novel nano-carriers for a variety of therapeutic applications, including the delivery of conventional drugs, because their use may alleviate problems due to multi drug resistance and nonspecific targeting [55, 56].

Some in vitro and in vivo studies confirmed that Graphene is a highly efficient in the targeted delivery of the anticancer drugs, doxorubicin and SN38 (a camptothecin analogue), and it is a promising platform for cancer therapy involving insoluble drugs [57, 58].

Some reports indicated that graphene and some derivatives noticed a measurable cytotoxicity in both in vitro and in vivo studies in various types of bacteria, human cells, and animal models [59]. Recently, it was reported that graphene nanoparticles exhibit strong antibacterial activity. The antibacterial activity of graphene has been attributed to membrane stress caused by sharp edges of graphene nano-sheets, which may result in physical damages on cell membranes, leading to the loss of bacterial membrane integrity and the leakage of RNA and intracellular electrolytes, uptake of membrane impermeable dyes, and changes in the trans-membrane potential [60-62]. Graphene has shown an antibacterial activity in both Gram-positive *Staphylococcus aureus* (*S. aureus*) and Gram-negative *Escherichia coli* (*E. coli*) to investigate the antibacterial actions of large-area monolayer graphene film. The results show that the graphene films inhibit the growth of both bacteria [63].

1.8. Literature review

Many attempts were carried out in order to synthesize a graphene derivatives like graphene oxide and reduced graphene and to study their antibacterial activities against different bacterial species. In 2011, the researchers at School of Chemical and Biomedical Engineering, Nanyang

Technological University, Singapore, had studied the antibacterial activity of graphite, graphite oxide, graphene oxide, and reduced graphene oxide. They found that graphene has strong cytotoxicity toward bacteria, by comparing the antibacterial activity of four types of graphene-based materials (graphite (Gt), graphite oxide (GtO), graphene oxide (GO), and reduced graphene oxide (rGO)) toward a bacterial model of *E.coli*. Under similar concentration and incubation conditions, GO dispersion shows the highest antibacterial activity, sequentially followed by rGO, Gt, and GtO. No superoxide anion (O_2 $\bullet^-$) induced reactive oxygen species (ROS) production is detected and the four types of materials can oxidize glutathione, which serves as redox state mediator in bacteria. Conductive rGO and Gt have higher oxidation capacities than insulating GO and GtO. The results obtained from this study confirmed that the antimicrobial actions of the graphene are contributed by both membrane and oxidation stress [64]. In 2014, the researchers from Huazhong Agricultural University, China, studied the antimicrobial activity against bacterial phyto-pathogens and fungal Candida and found that GO interacts with the pathogens by mechanically wrapping and locally damaging the cell membrane and finally causing cell lysis [65]. In 2015, Perreault and his collaborators studied the relationship between size of graphene oxide nano-sheets and its antimicrobial properties. They found that the antimicrobial activity of graphene oxide increased four-times when graphene oxide sheet area decreased from 0.65 to 0.01 μm^2. The higher antimicrobial effect of smaller GO sheets is attributed to oxidative mechanisms associated with the higher defect density of smaller sheets [66].

1.9. Objectives of the Research

It is obvious from the literature review that there is no previous report that study the effect of different charges on the antibacterial of the graphene or interaction with the membrane of the bacteria.

Therefore, in this project we aim to:

1. Functionalization of Graphene with different charges including positive, negative and neutral.
2. Characterization of the resulted functionalized Graphene with a transmission electron microscopy (TEM) and thermo-gravimetric analysis (TGA).
3. Study the antibacterial activity of the developed nano-sheets on different types of bacteria (both gram positive such as *Staphylococcus aureus* and gram negative such as *E. coli*).
4. Determine the possible mechanism of interaction of the functionalized graphene with the various types of bacteria.

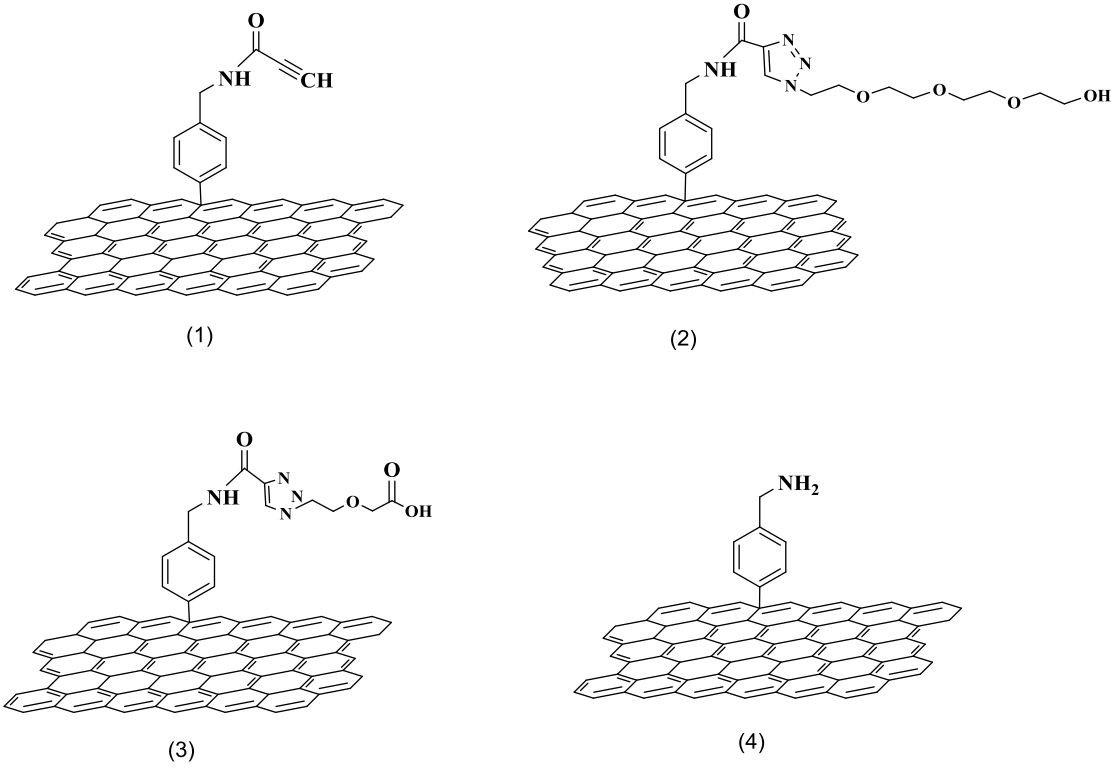
1.10. Significance of the research

1. Synthesis for the first time the graphene sheets with different charges (positive, negative and neutral).
2. This study will determine if the charge has effect on the antibacterial activity of graphene or not.

3. The project will be the first in its kind to determine the method of interaction and the possible mechanism of antibacterial activity of graphene.

1.11. General approach of the synthesis and functionalization of graphene derivatives

The aim of our study is to develop new compounds based on graphene sheets through covalent functionalization with different groups which have positive, negative and neutral charges. Alkyne, TEG, carboxyl and amine groups were attached as shown in the following scheme1. The antibacterial activity was determined against *E.coli* and *S.aureus* bacteria.



Scheme 1: covalent functionalization of graphene, (1) graphene-alkyne, (2) graphene-TEG, (3) graphene-COOH, (4) graphene-amine.

Chapter two
Experimental

Materials and Methods

2.1. Materials

All materials that used in the experiments were of analytical grade. 1,2-ichlorobenzene (*o*-DCB) (catalogue # 65152), 1-(3-Dimethylaminopropyl) -3- ethylcarbodiimide hydrochloride (EDC) (catalog # A10807) 4-(dimethylamino)pyridine (DMAP) (catalog # 1122583) 4-nitrobenzylamine hydrochloride (catalog # 191434) 4-[N-Boc)aminomethyl]aniline (catalog # 1001918209), acetic anhydride (catalog # 320102), acetonitrile (catalogue # 5550070) solvents were purchased from (Frutarom Company, Israel). Anhydrous copper sulfate (catalog # 451657), copper (catalog # 266086), diethylene glycol (catalog #A14728). Ethyl acetate (EtOAc) (catalogue # 2355516100024). Graphene nano-powder (stock #2191YJ) was purchased from (nanostructured and amorphous materials, USA), isoamyl nitrite (catalog # 110463), L-ascorbic acid sodium salt (catalog # A17759), propiolic acid (catalog# STBB7815V), Sodium azide (catalog # 0E30428), toluene-4sulfonyl chloride (catalog# 89732), TBTU (catalog # B23597) and tetraethylene glycol (catalog # B23990) were purchased from (Alfa Aesar Company, England). Trifluoro-acetic acid (TFA) (catalog # A12198), TRIZMA base (Tris-HCl) (catalog # 6066-T) and *N*,*N*-diisopropylethylamine (DIPEA) (catalog # 496219) were purchased from (Sigma-Aldrich Company, USA). Methanol (MeOH), dichloromethane (DCM), isopropyl alcohol, acetone

and ethanol were purchased from (C.S. Company, Israel), n-hexane (Hex) (catalogue # 2355544800024).

All reactions were stirred under ambient conditions. Column chromatography using silica gel (pore size 60 Å) purchased from Sigma Aldrich Company was used to purify the products. TLC (DC-Fertigfolien ALUGERAM®SIL G/UV_{254}, MACHEREY NAGEL Company, Germany) was used to monitor the reactions. Centrifuge (UNIVERSAL 320, Hettich Zentrifugen, Germany) and water path sonicator (MRC DC-200H Digital Ultrasonic Cleaner) were used in preparation and dispersion of functionalized graphene. Rotary evaporator (MRC, ROVA-100, laboratory equipment manufacturer) was used for solvents drying.

For biological test, 5,5'-Dithiobis(2-nitrobenzoic acid) (catalog #D8130), phosphate buffered saline (REF # 02-023-1A), and L-Glutathione reduced (catalog # G4251) were purchased from (Sigma-Aldrich Company), Difco™ LB Broth, Lennox and Difco™ Nutrient Agar (Catalog #213000).

2.2. Techniques and equipment

- **NMR spectra** were recorded with Bruker 500 MHz −Avance III, Switzerland. Chemical shifts were reported in ppm, and coupling constants were reported in Hz.

- **Thermogravimetric analysis spectra** were recorded by (STA 409 PC Luxx®, NETZSCH) in range 0-600 ºC, flow 20 ºC under nitrogen (100 cc/min).

- **TEM images:** were taken by using Morgagni 286 transmission microscope (FEI Company, Eindhoven, Netherlands) at 60 kV.

- **Infrared spectroscopy (IR):** Nicolet iS5, Thermo Fisher Scientific Company, USA.

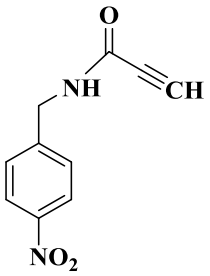
- **Ultraviolet-Visible (UV-Vis) spectr**a: were recorded on with (7315 Spectrophotometer, Jenway, UK), using quartz cuvettes.

- **Analytical balance:** 5 digits balance.

2.3. Synthesis and characterization of the products:

All the synthetic procedures and antibacterial activity were prepared at An-Najah University labs. Except for NMR measurements, TEM and TGA measurements, which were conducted at the University of Jordan.

2.3.1. Synthesis of *N*-(4-nitrobenzyl) propiolamide (1):



P-Nitrobenzyl amine (200mg, 1.06mmol), propiolic acid (72µl, 1.17mmol) and TBTU (374.4mg, 1.17mmol) were combined in a 250 ml flask and stirred under vacuum. DIPEA (555µl, 3.18mmol) and DCM (12ml) were added to the flask. The reaction mixture was stirred over night at room temperature. The product was extracted with DCM (60ml) and washed with

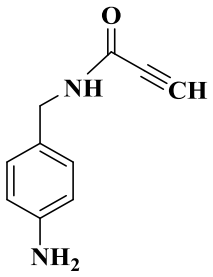
HCl 1M (30ml) and dried over Na_2SO_4. The solid was then purified using column chromatography with a mobile phase of Hex: EtOAc (1:2) to yield product (**1**) (175 mg, 81%) as yellowish powder.

R_f: 0.5 (Hex: EtOAc) (1:2)

^{1}H NMR (500 MHz, $CDCl_3$): δ 8.20 (d, 2H, *J* = 4.9 Hz, Ph), 7.44 (d, 2H, *J* = 4.8 Hz, Ph), 6.42 (bs, 1H, NH), 4.57 (d, 2H, *J* = 5.9 Hz, CH_2NH), 2.84 (s, 1H, C≡CH).

^{13}C NMR (125.7 MHz, $CDCl_3$):δ159.0, 152.3, 147.6, 128.4, 124.1, 75.4, 74.4, 43.1.

2.3.2. Reduction for *N*-(4-nitrobenzyl) propiolamide (2):



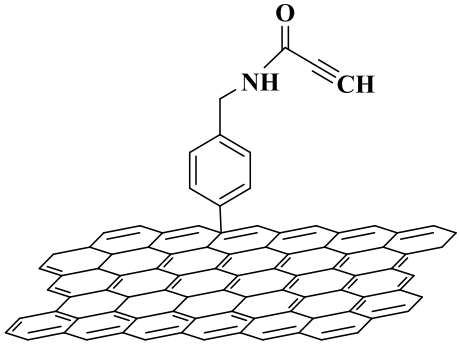
Compound1(175 mg, 0.86 mmol) was dissolved in a mixture of methanol:H_2O (2:1), and then zinc powder (504 mg, 7.7 mmol) and ammonium chloride (100mg, 1.88mmol) were added and stirred under reflux for 4 h. Then the reaction mixture was filtered using silica, washed with ethyl acetate and evaporated to yield yellowish product with yield of 95% (142mg).

R_f: 0.36 (Hex/EtOAc 1:1)

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.28 (t, 1H, *J* = 7.8 Hz, NHCO), 7.08 (d, 2H, *J* = 8.8 Hz, Ph), 6.64 (d, 2H, *J* = 8.5 Hz, Ph), 5.63 (s, 2H, NH_2), 4.38 (d, 2H, *J* = 5.6 Hz, C$\underline{H_2}$NH), 3.72 (s, 1H, C≡CH).

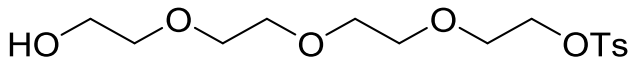
^{13}C NMR (125.7 MHz, $CDCl_3$): δ 148.4, 145.2, 130.9, 129.3, 129.0, 115.3, 73.2, 72.2, 43.4.

2.3.3. Graphene-alkyne (3)



Compound 2 (170 mg, 0.98mmol) was added to graphene powder (40 mg) and dried under vacuum. 1,2-dichlorobenzene (ODCB) (13ml) and acetonitrile (7ml) were added. The reaction was sonicated for 10 minutes under argon. Iso-amyl nitrite (786µl, 5.86mmol) was added drop wise manner and stirred over night at 60 ºC. After that, a centrifugation was done at 15,000 rpm for 10 min and washed with MeOH (2 x 30 ml), DCM (2 x 30 ml) and Et_2O (2 x 30 ml). The product was collected with diethyl ether and dried under reduced pressure. Dried black powder (90mg) was obtained.

2.3.4. Synthesis of OH-TEG-OTs (4)



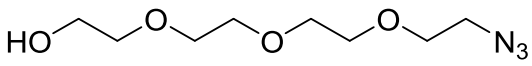
A solution of tetraethylene glycol (3 g, 15.45 mmol), tri-ethylamine (2.6 ml, 18.54 mmol) and THF (50 ml) were stirred for 5 min in a 250 ml flask and cooled at 0 ºC in ice bath, tosyl chloride (2.9 g, 15.45 mmol) was added drop wise over a period of 30 min and stirred vigorously over night at R.T. The product was extracted with DCM (100 mL) and saturated NaCl (50 mL). The aqueous layer washed again with (50ml) DCM, and washed with HCl 1M (30ml), NaCl (30 ml) and dried over Na_2SO_4, filtered and evaporated and was purified by column chromatography with a mobile phase DCM: MeOH (20:1) to obtain an oily product (**4**) of yield (1.3g, 26%).

R_f: 0.52 ((DCM: MeOH) (20:1).

^{1}H NMR: (500 MHz, $CDCl_3$): δ 7.76 (d, 2H, *J* = 8.5 Hz, Ts), 7.35 (d, 2H, *J* = 7.9 Hz, Ts), 4.12 (t, 2H, *J* = 5.4 Hz, CH_2OTs), 3.68-3.55 (m, 14H, $6CH_2O$, $C\underline{H_2}OH$), 2.40 (s, 3H, CH_3).

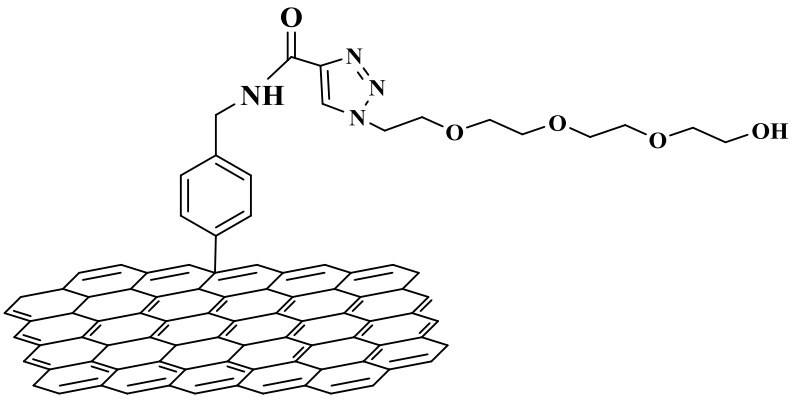
^{13}C NMR (125.7 MHz, $CDCl_3$):δ144.8, 133.0, 129.8, 128.0, 72.5, 70.7, 70.6, 70.4, 70.3, 69.3, 68.7, 61.7, 21.6.

2.3.5. Synthesis of OH-TEG-N_3 (5):



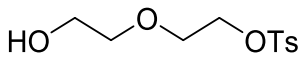
The product was synthesized and characterized according to the literature [67].

2.3.6. Graphene-TEG (6)



Ascorbic acid (20 mg, 0.1 mmol), anhydrous copper sulfate (50mg, 0.31 mmol) were dissolved in distilled H_2O (3ml), then the solution was added to a sonicated solution of compound **5** (150 mg, 0.68 mmol) and graphene-alkyne **3** (50 mg) dissolved in DCM (3 ml) and was left at R.T. for 24 h. MeOH (20 ml) was added and a centrifugation was done for 10 min. at 15,000 rpm, the supernatant was discarded followed by washing steps with MeOH (2 x 20 ml) and Et_2O (2 x 20 ml). The product was collected with Et_2O and dried to obtain black powder (55 mg) as product (**6)**.

2.3.7. Synthesis of OH-DEG-OTs (7)



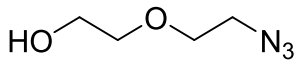
In a 250 RBF a mixture of diethylene glycol (DEG) (1 g, 9.4mmol) and triethylamine (1.3ml, 9.4mmol) in DCM (10 ml) and stirred for 5 min, then cooled to0 ºC in ice bath. Tosyl-chloride (1.8g, 9.4mmol) was added drop wise for 30 min. The reaction was stirred overnight at R.T. The product was extracted with DCM (100ml), saturated NaCl (50 ml) and HCl 1M (50ml). The product was dried over Na_2SO_4. The obtained crude was purified by silica column chromatography with a mobile phase of DCM: MeOH (20:1) to obtain a yield of 25% (600 mg) of an oily product.

R_f: 0.4 (DCM: MeOH 20:1).

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.76 (d, 2H, *J* = 8.6 Hz, Ts), 7.31 (d, 2H, *J* = 8.1 Hz, Ts), 4.14 (t, 2H, *J* = 3.4 Hz, CH_2OTs), 3.64 (t, 4H, *J* = 4.1 Hz, $2CH_2O$), 3.49 (t, 2H, *J* = 2.1 Hz, $C\underline{H_2}OH$), 2.40 (s, 3H, CH_3).

^{13}C NMR (125.7 MHz, $CDCl_3$): δ 145.0, 142.0, 129.2, 127.9, 72.3, 69.2, 68.6, 61.6, 21.6.

2.3.8. Synthesis of OH-DEG-N_3 (8):



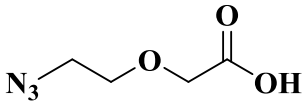
Sodium azide (120mg, 1.85 mmol) was added to a solution of compound **7** (400 mg, 1.54mmol) dissolved in ethanol (10 ml). The reaction was achieved by stirred and reflux overnight at 80 ºC. The reaction mixture was evaporated and extracted with Et_2O (100ml) and saturated NaCl (30ml), the organic layer was dried over (Na_2SO_4). The resulting product was purified using silica gel chromatography (DCM /MeOH (20:1)) to yield product (**8**) (215mg, 99%).

R_f: 0.38 (DCM/MeOH 20:1).

^{1}H NMR (500 MHz, $CDCl_3$): δ 4.18 (t, 2H, *J* = 5.2 Hz, C$\underline{H_2}$OH), 3.74 (t, 2H, *J* = 5.9 Hz, OC$\underline{H_2}CH_2$OH), 3.46 (t, 2H, *J* = 6.2 Hz, C$\underline{H_2}CH_2N_3$), 1.23 (t, 2H, *J* = 5.8 Hz, CH_2N_3).

^{13}C NMR (125.7 MHz, $CDCl_3$):δ 70.6, 68.3, 50.8, 29.5.

2.3.9. Synthesis of 2-(2-azidoethoxy)acetic acid (9)



Jones reagent (7ml) was added to a solution of compound **8**(250mg, 1.77mmol) dissolved in acetone (7 ml), the reaction was stirred 2 h. After that, isopropyl alcohol was added to reaction drop by drop until the green color is formed, then filtrated by silica gel and washed by DCM and evaporated to obtain the compound (**9**) with yield of 65% (180mg).

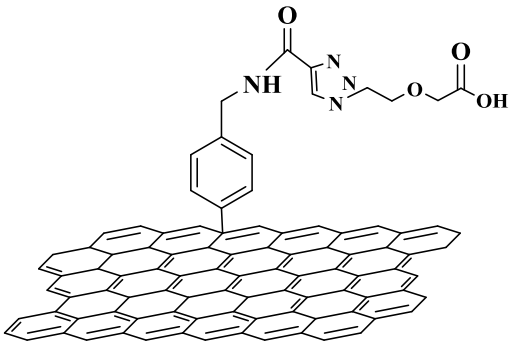
R_f: 0.4 (DCM: MeOH 9:1).

^{1}H NMR (500 MHz, $CDCl_3$): δ 4.21 (t, 2H, *J* = 4.6 Hz, CH_2COO), 3.73 (t, 2H, *J* = 5.2 Hz, CH_2O), 3.45 (t, 2H, *J* = 4.6 Hz, CH_2N_3).

^{13}C NMR (125.7 MHz, $CDCl_3$):δ173.1, 70.8, 68.1, 50.7.

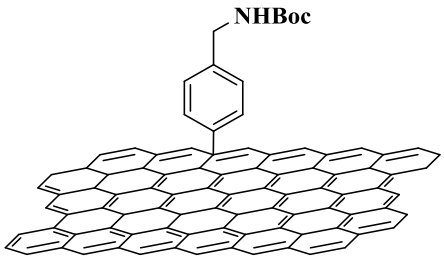
IR: 3500 cm^{-1}.

2.3.10. Graphene-COOH (10)



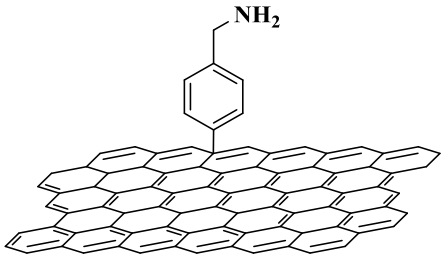
Ascorbic acid (30mg, 0.15 mmol) and anhydrous copper sulfate (75mg, 0.47 mmol) were dissolved in distilled H_2O (4 ml), then the solution was added to a sonicated solution of compound **9** (150 mg, 1.03 mmol), and graphene**3**(60mg) dissolved in DCM (4 ml) and was stirred overnight at R.T. MeOH (20 ml) was added to reaction, sonicated and a centrifugation was done for 10 min. at 15,000 rpm, supernatant was discarded followed by washing steps with MeOH (2 x 20 ml) and Et_2O (2 x 15 ml). The black powder was collected with diethyl ether and dried under vacuum. Dried black powder (70mg) was obtained.

2.3.11. Functionalized graphene (11)



Graphene powder (45 mg) and 4-[(*N*-Boc)aminomethyl]aniline (120mg, 0.54mmol) were dried under vacuum. Then 1,2-dichlorobenzene (*o*-DCB) (15ml) and acetonitrile (10ml) were added and sonicated for 10 minutes under argon and iso-amyl nitrite (435µl, 3.24mmol) was added drop-wise and stirred for 24 at 60 ºC. Functionalized graphene was distributed in eppendorfs and washed with MeOH (20ml), sonicated and a centrifugation was done for 10 min. at 15,000 rpm, the supernatant was discarded followed by washing steps with MeOH (2x15ml), DCM (2x15ml) and Et_2O (2x15ml). At the final step the product was collected with diethyl ether and dried under vacuum. Dried black powder (50mg) was obtained.

2.3.12. Graphene-amine (12)



***f*-graphene 11** (50 mg) was dissolved in DCM (9 ml) and sonicated for 5 minutes, TFA (7 ml) was added and stirred overnight. MeOH (20 ml) was

added, sonicated and a centrifugation was done for 10 min. at 15,000 rpm. Washing steps were proceeded with MeOH (2x15ml), DCM (2x15ml) and diethyl ether (2x15 ml). The product was collected with diethyl ether and dried under vacuum to obtain (45mg) of dried black powder.

2.3.13. Quantitative Kaiser test protocol [to determine the free NH_2 loading]

Three solutions of phenol, pyridine and ninhydrin were prepared as documented in the literature [68].

Three solutions were prepared separately as followed:

- Phenol solution: 40.0 g of phenol in 10 mL of absolute ethanol.
- Pyridine solution: 2 mL of potassium cyanide 1.0 mM (aqueous solution) dissolved in 98 mL of pyridine. (KCN was prepared by adding 65.0 mg in 100 mL of distilled water).
- Ninhydrin solution: 1.0 g of ninhydrin in 20 mL of absolute ethanol.

Procedure:

***f*-graphene 12** (1.2mg) was weighted in a small tube. Phenol solution (75 µl), pyridine solution (100 µl) and ninhydrin solution (75 µl) were added to the tube. The blank was prepared exactly with the same quantities of solvents but without the *f*-graphene [68].The resulting dispersion was sonicated for 5 min. and heated for 10 minutes at 120 ºC. The suspension was cooled and diluted with 60% ethanol (1ml) and filtered by glass

dropper. The tube was washed with 60% ethanol (2x 0.5ml). After that, the filtrate was analyzed by UV spectroscopy. The absorbance at λ = 570 nm was correlated to the amount of free amine functions on the Graphene surface using the equation [68]:

Concentration (mmol) = (A *d * 10^3) / (extinction coefficient* wt.)

Where A: absorbance of sample

D: Dilution is 2.25 ml

Extinction coefficient: 15000$m^{-1}cm^{-1}$

Wt.: weight of Graphene

2.4. Determination of antibacterial activities

2.4.1. Media preparation

2.4.1.1. Nutrient agar preparation

Nutrient agar was used to culture the bacteria and was also used in plate count method. According to the manufacturer, it was prepared by adding 23.0 g of powder nutrient agar up to 1.0 L distilled water followed by heating on Bunsen burner with shaking until completely dissolve. This mixture was allowed to boil for 1 min. after that it was autoclaved. Sterilization was confirmed by the control blanks and the usage of the sterilization indicator tapes. After sterilization the media were poured into sterile Petri dishes and let until solidified, then saved at 4℃ until used.

2.4.1.2. Difco™ LB Broth, Lennox (LB) broth preparation

LB broth was used to prepare the serial dilutions in order to detect the antibacterial activity. According to the manufacturer, it was prepared by mixing 20 g of LB broth powder up to 1.0 L of distilled water and dissolved by heating on Bunsen burner with shaking. This mixture was allow to boil for 1 min. and distributed to several test tubes in volumes of 4, 5 and 10 ml. LB broth tubes were autoclaved. Sterilization was confirmed by the control blanks and the usage of the sterilization indicator tapes. Sterilized LB broth was used as required for making the dilutions and preparing the bacterial cultures.

2.4.2. Bacterial strains

The antibacterial activity of graphene compounds was studied against *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) and was compared to the activity of graphene and normal saline alone as negative control.

2.4.3. Preparation of bacterial suspension

Turbidity of bacterial suspension was adjusted to be equivalent to 0.5 McFarland standard suspension. The 0.5 McFarland standard suspension was prepared by mixing 0.50mL of 1.175% (w/v) $BaCl_2.2H_2O$ and 99.5 mL of 1% (v/v) H_2SO_4. Then, the absorbance of the suspension was measured in a spectrophotometer at wavelength of 630 nm, distilled water were used as the standard blank, to obtain turbidity within 0.08 – 0.1 absorbance that

reflect bacterial concentration of about 1.5×10^8 CFU/ml. The McFarland suspension was sealed tightly to prevent evaporation and was protected it from light.

From a fresh culture plate, 3 to 4 colonies were transferred to sterile normal saline.

2.4.4. Agar diffusion disk- and well-variant methods

Both methods were used as primary technique to detect the antibacterial activities of graphene derivatives compounds. The plates were sub-cultured with bacteria from the bacterial suspension with a concentration of 1.5×10^8 CFU/ml. In agar diffusion disk- variant method, small filter paper disks were placed on inoculated plates. This was followed by addition of 20µl of the examined compound solution (0.5mg/ml) on paper disks. On the other hand, agar diffusion well-variant methods involved making wells with distance of 5 mm in agar to which 100µl of examined compound was added. After the drying of the wells and disk, the plates were incubated at 37℃ for 24 hours. In both methods, each compound was examined in duplicate [69, 70].

2.4.5. Detection of reduction in bacterial concentration

Fresh bacterial cells suspensions (1.5×10^8CFU/ml) were diluted in a sterile 0.9% normal saline to 10^6 colony-forming units (CFU)/ml. Graphene derivatives were prepared at concentration of 500µg/ml as stock solution in sterile 0.9% NaCl. From the stock solution 500µl was mixed with equal

volume of diluted bacterial suspension and the final concentration of graphene derivative was 250µg/ml. The bacterial cells were exposed to suspended graphene compound for 3 hours under constant agitation at room temperature. In parallel, normal saline without graphene compounds was used as a negative control.

At the end of the exposure period the mixture was vigorously shaken by vortex to break any aggregates, then it was serially diluted using a sterile 0.9% NaCl solution. Then 100µl of each dilution was placed on nutrient agar media immediately after vortex and spread by sterile bent glass rod. The plates were incubated overnight at 37 °C [70].

2.4.6. Broth Microdilution method

This technique was used in order to determine the minimum inhibitory concentration (MIC) for each compound. The used protocol was according to that of CLSI [69-71].

Graphene compounds were dissolved in 0.9% NaCl to obtain a concentration of (250 µg/mL) for each compound. McFarland concentration 10^7 CFU/ml of *S.aureus* and *E. coli* was prepared in LB broth.

100µl LB broth was pipetted in each well of 96-well plate. After that 100µl of graphene compound was pipetted in the first well from which 100µl was transferred to next well. Then 1µl of bacterial suspension (10^7 CFU/ml) was added to each well except number 11 (negative control), after

inoculation of bacteria, the plates were incubated for 24 hours at 37 ℃. Broth microdilution method was performed in duplicate for each compound. The lowest concentration of each compound that did not allow any visible bacterial growth in the inoculated wells was considered the minimal inhibitory concentration (MIC).

2.5. Glutathione experiment

Followed a documented procedure [72], the glutathione experiment was conducted as follow: graphene and its derivatives (graphene-alkyne **3**, graphene-TEG **6**, graphene-COOH **10**, graphene-amine **12**) dispersions (225 µl at 100 µg/ml) in 50mM $NaHCO_3$ buffer (pH 8.6) was added into 225 µl of GSH (0.8 mM in the $NaHCO_3$ buffer). All samples were prepared in duplicate. The mixtures were transferred into eppendorfs. The eppendorfs were preserved in dark and incubated in a shaker at 250 rpm for 2 h. After that, 785 µl of 0.05 M Tris-HCl and 15 µl Ellman's reagent [73] were added to produce a yellow product followed by a filtration using a 0.45 µm filter. An aliquot of each sample was taken and its absorbance was measured at 405 nm. Sample without graphene was used as a negative control. Sample with H_2O_2 (10 mM) was used as a positive control. The loss of GSH activity was measured as follow: loss of GSH % = (abs. of negative control - abs. of sample)/abs. of negative control) x 100 [72].

2.6. Hemolysis assay

5ml of fresh human blood sample was collected with heparin tubes for testing the hemolytic activity of our compounds [74]. Centrifugation at

1000 rpm for 20 min at 4 °C was done for the sample to obtain Human Red Blood Cells (HRBCs), the supernatant discharged and washed five times with PBS. HRBCs were diluted 10 times with PBS buffer. Positive control (complete hemolysis) was prepared by incubating H_2O (1 ml) with HRBC suspension (100 µl). Negative control (no hemolysis) was also prepared by incubating PBS (1 ml) and HRBC suspension (100 µl). For hemolytic activity of the prepared graphene compounds, HRBC suspension (100 µl) was incubated with (1 ml) of various concentrations of graphene compounds solutions (0.2, 0.4 and 0.6 mg/ml in PBS) as testing samples. After gentle shaking, the mixtures were incubated at room temperature in duplicate for 2 and 24 hours. The tubes were centrifuged at 5000 rpm for 1 min. The absorption of the supernatants were read at 541 nm [74].

The hemolysis percentage was calculated as follows:

% of Hemolysis = (abs. of sample – abs. of negative control)/ (abs. of positive control – abs. of negative control)

Chapter Three
Results & Discussions

Graphene has a small size, sharp edges, high surfaces area (2600 m^2/g) which means high capacity of loading and conjugation. These properties increase its biomedical applications such as; drug or gene delivery, biosensors, tumor imaging, adsorption of enzymes, and cancer photo-thermal therapy [59].

However, as graphene is a hydrophobic material (has a low solubility and dispersability in physiological solution) which leads to aggregation and precipitation in cell tissue and causes toxicity for different type of cells. The toxicity may come from graphene synthesis, because it is synthesized by various methods, e.g., mechanical or chemical exfoliation, chemical vapor deposition… etc., this lead to include some hazardous chemical impurities in graphene such as nitrate, sulfate, and peroxide [59, 75].

Therefore, functionalization of graphene (covalently or noncovalently) has improved its solubility and dispersability in water, biocompatibility and drug delivery efficiency [59], water purification (may be used as a sensors for detecting contamination), and reduce the toxic effects in human cells [59].

According to literature there are different anti-bacterial mechanism for *f*-graphene like; initial cell deposition on, significant membrane stress caused by direct contact with sharp edges, superoxide anion-independent oxidation

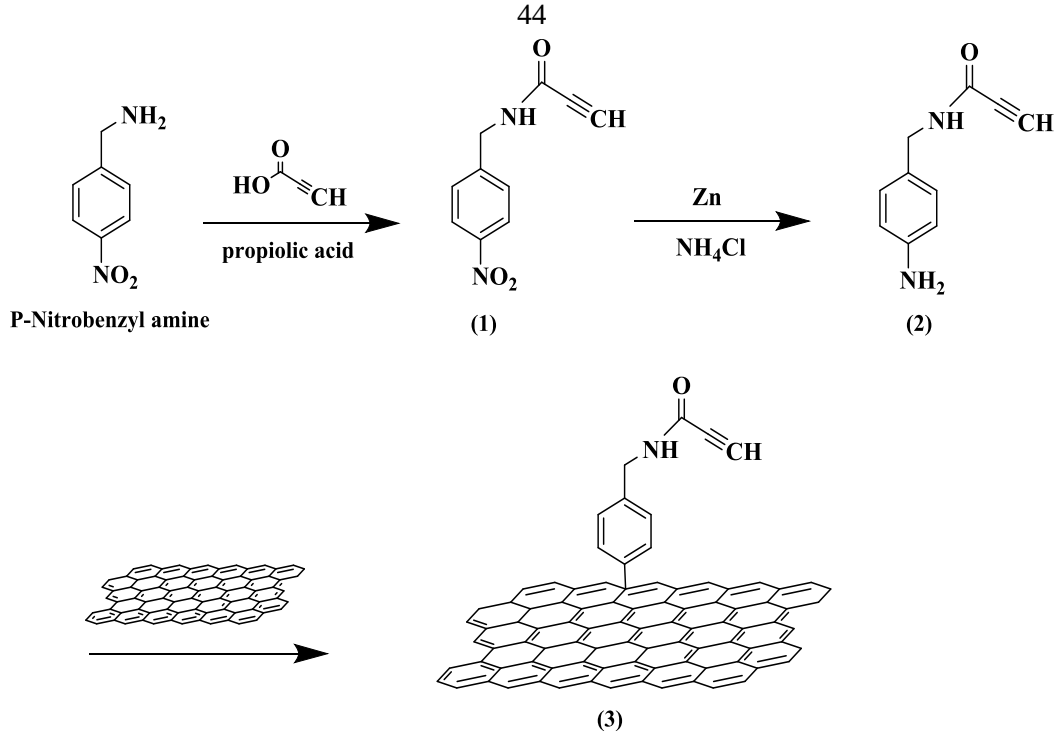
and oxidative stress-mediated antibacterial activity of *f*-graphene in additional bacterial strains [59].

However, upon our knowledge there is no report has studied the effects of the various charged groups based graphene (positive, negative and neutral) on the interaction with the bacteria and the antibacterial activity and determine if the charge has effect on the antibacterial activity of graphene or not.

3.1. Synthesis and functionalization of graphene

3.1.1. Graphene-alkyne

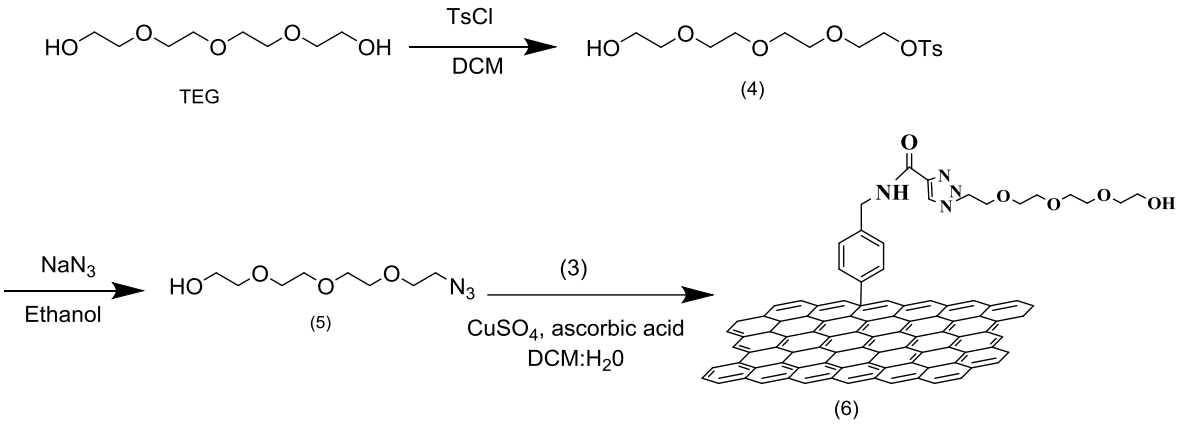
Graphene sheets were functionalized with alkyne group through multi stage process started with synthesis of compound **(1)** from P-Nitrobenzyl amine as a starting material reacted with propiolic acid using TBTU as a coupling agent and DIPEA as Hünig's base. Then, the nitro group in compound **(1)** was reduced to amine group with Zinc metal and ammonium chloride to produce compound **(2)**. After that, a Tour reaction was used in order to functionalize the graphene sheets since it is one of the most effective reactions to produce graphene sheets with high yield, purity and stability. Therefore and through Tour reaction, the compound **(2)** was attached to graphene sheets to get the product graphene-alkyne **(3)** as shown in scheme 2.



Scheme 2: Synthesis of graphene-alkyne

3.1.2. Graphene-TEG

Synthesis of graphene-TEG was started with synthesis of linker **(4)** from tetraethyelene glycol (TEG) through converting selectively the OH group of TEG to tosyl group to obtain TEG-Tosyl linker **(4)**. The linker **(4)** was reacted with NaN_3 in ethanol to replace tosyl group with azide to get TEG-N_3 linker **(5)**. Functionalized graphene **(3)** was linked with linker **(5)** through click reaction to produce graphene-TEG **(6)** as shown in scheme 3.



Scheme 3: Synthesis of graphene-TEG**.**

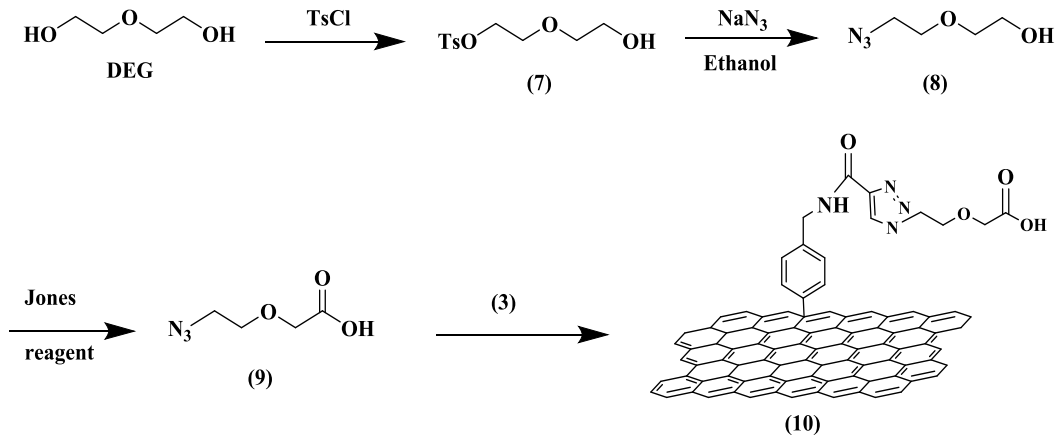
- **Click reaction:**

The term Click reaction (chemistry) describes the reactions that combines the biocompatible molecules (biomolecule) or small ones together to obtain a high yield of product. This reaction was introduced by K. Barry Sharpless in 1998 who had noble prize in chemistry in 2001. The by-product of this reaction easily to remove by using solvents without needs to use a chromatography or other separation methods. This reaction has an interested used in pharmacological and various biomimetic applications and other industries in capabilities for generating large libraries of compounds for screening in discovery research [76].

3.1.3. Graphene-COOH

Synthesis of graphene-COOH was started with synthesis of linker **(7)** from diethylene glycol (DEG) through reacting OH group of DEG with tosyl chloride to obtain DEG-Tosyl linker **(7)**. The linker **(7)** was reacted with sodium azide in ethanol to get OH-DEG-N_3 linker **(8)**, then oxidized by John's reagent to produce the linker **(9)**. Functionalized graphene **(3)** was

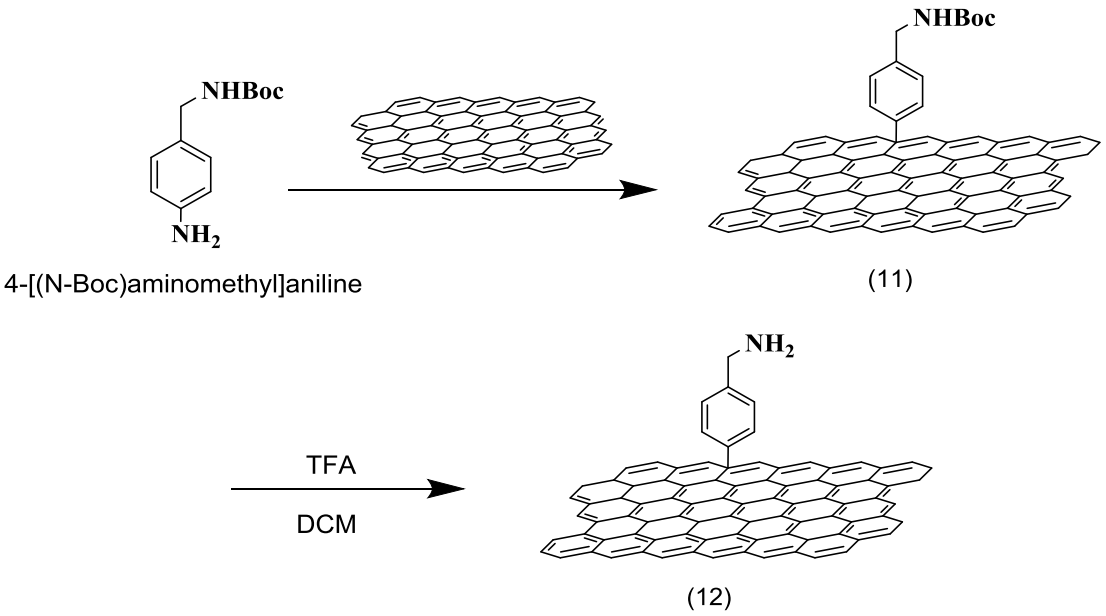
linked with linker **(9)** through click reaction to obtain graphene-COOH **(10)** as shown in scheme 4.



Scheme 4: Synthesis of graphene-COOH.

3.1.4. Graphene-amine

Graphene-amine was synthesized through the covalent functionalization of graphene sheets with 4-[(*N*-Boc)aminomethyl]aniline through tour reaction in presence of *o*-DCB, acetonitrile and iso-amyl nitrite to get product **(11)**. To obtain the free amine; a deprotection reaction was conducted to remove Boc group in the presence of trifluoroacetic acid in DCM to obtain graphene-amine **(12)** as shown in scheme 5.



Scheme 5: Synthesis of graphene-amine

In order to determine the free NH_2 loaded, Kaiser test was done as mentioned in literature [68], obtaining the total amine loaded was 0.72 mmol per gram of graphene.

3.2. Characterization of functionalized graphene

3.2.1. Dispersability & Morphology of *f*-graphene

Graphene sheets are considered hydrophobic in character and have low water solubility with large aggregations due to Van der Waals interactions between the sheets as observed in figure 3 A.I. Moreover, under the transmission electron microscope the aggregations of the graphene sheets are observed as in (figure 3 A.II). However, upon their functionalization with the different functional groups (TEG, COOH, amine) the graphene becomes more dispersed in water as shown in figure 3 (B.I, C.I, D.I). In addition, the TEM images of the three different functionalized graphene

demonstrate the separation of the graphene sheets and it can be observed single sheet of graphene with a diameter range from 0.6-0.8 µm as shown in figure 3 (B.II, C.II, D.II).

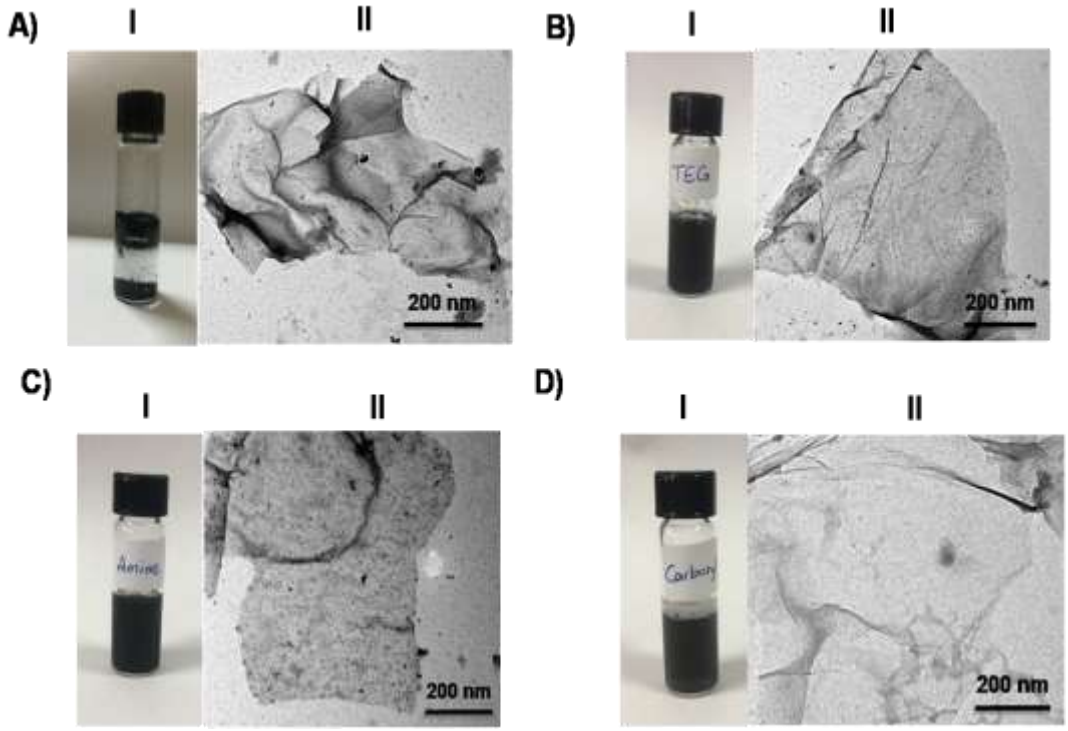


Figure 3.1: **A) I.** Photographic vial of graphene in water, **II.** TEM image of graphene. **B) I.** Photographic vial of graphene-TEG in water, **II.** TEM image of graphene-TEG. **C) I.** Photographic vial of graphene-amine in water, **II.** TEM image of graphene-amine. **D) I.** Photographic vial of graphene-COOH in water, **II.** TEM image of graphene-COOH.

3.2.2. IR spectroscopy

Infrared spectra are conducted in order to examine the presence of the different functional groups of the functionalized graphene (graphene-TEG, graphene-amine, and graphene-COOH). As shown in figure 3.2, the spectrum of graphene-COOH confirms the presence of the carboxyl group as the broad band at 3500 cm^{-1} refers to the OH and the sharp peak at 1730 cm^{-1} refers to the carbonyl. The sharp peak at 3660 cm-1 in the spectrum of

graphene-amine confirms the presence of the amine group and the broad peak at 3410 cm-1 in graphene-TEG spectrum prove the existence of the alcohol group.

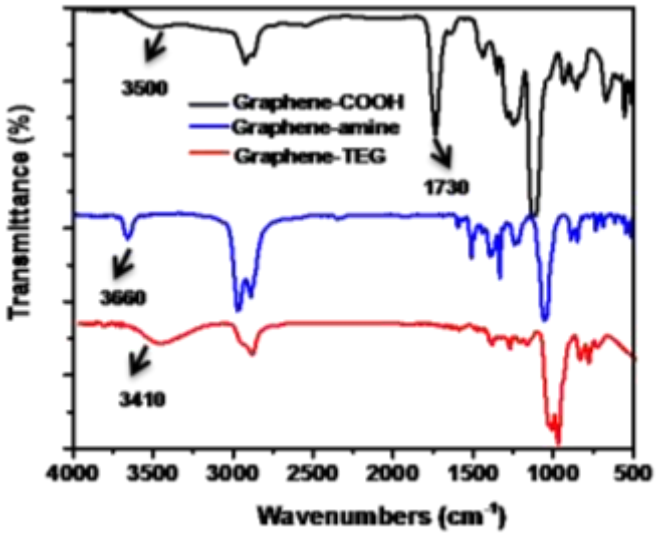


Figure 3.2: IR spectra of graphene-COOH, graphene-amine, graphene-TEG.

3.2.3. Thermogravimetric analysis (TGA)

Thermogravimetric analysis was conducted in order to determine the degree of the functionalization on the surface of graphene sheets. The mass loss of the functionalized graphene was compared with graphene alone at 700º C as shown in figure 3.3. As can be observed, graphene alone is a thermo-stable material and loses less than 10% of its weight. However, the functionalized graphene in the case of the graphene-TEG was 32% the degree of functionalization and approximately the same degree was obtained in the case of graphene-amine (33%). While graphene-COOH gave the highest degree of functionalization with a percentage of 47%. As seen, the percentage of weight loss is proportional with the amount of the

added functional groups on the surface of the graphene sheets. Moreover, the TGA analysis confirms the successful functionalization of the graphene sheets quantitatively.

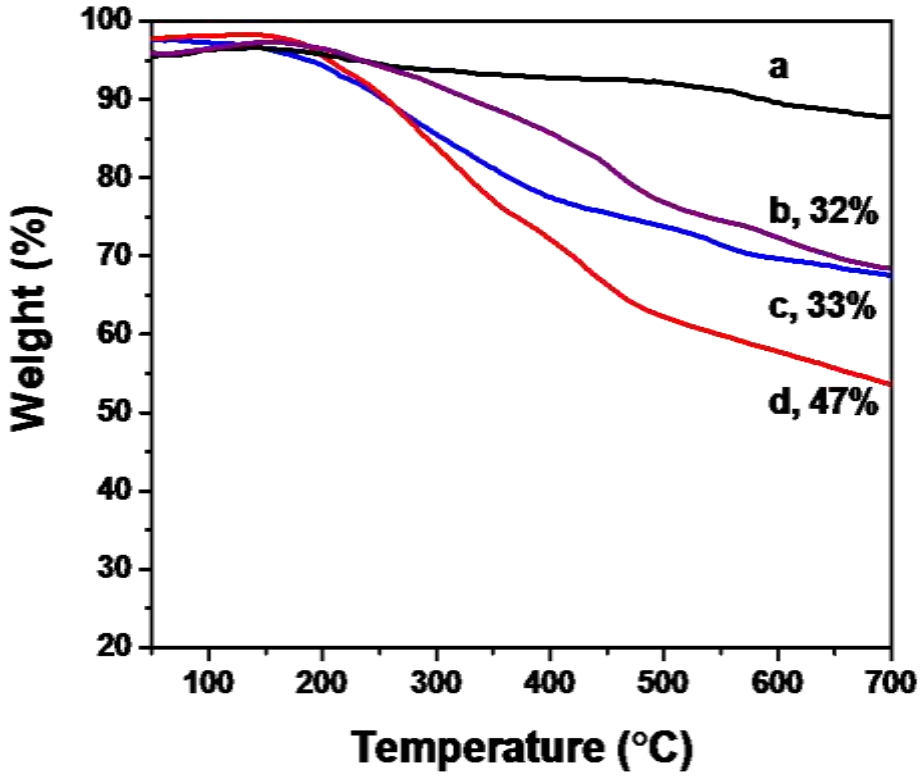


Figure 3.3: TGA analysis of (a) graphene, (b) graphene-TEG, (c) graphene-amine, (d) graphene-COOH.

3.3. Antibacterial activity

Graphene showed an interesting antibacterial activity through various mechanism of actions. However, there is no report regarding the charge effects on the functionalized graphene on the antibacterial activity. Here, the graphene sheets were functionalized with negative charge (carboxyl group), neutral charge (tetraethylene glycol) and the positive charge (amine

group). The antibacterial activity were determined using different techniques such as the agar diffusion disk, the reduction of the colony forming units and the determination of the minimum inhibitory concentration (MIC).

3.3.1. Agar diffusion disk- and well-variant methods

Firstly the disc and well diffusion methods were used to assess the antibacterial activity of each compound and compare the results qualitatively with the graphene alone. There was no observed antibacterial effect in case of disc diffusion method. In well method, the zone of inhibition was increased for *f*-graphene over the graphene. The zone of inhibition was determined on Gram-positive bacteria (*S. aureus*) and Gram-negative bacteria (*E .coli*). As can be observed in figure 3.4 and table 1, it was obvious the enhancement of the antibacterial activity of the three *f*-graphene in comparison to the graphene and DMSO as the zone of inhibition was increased in all cases.

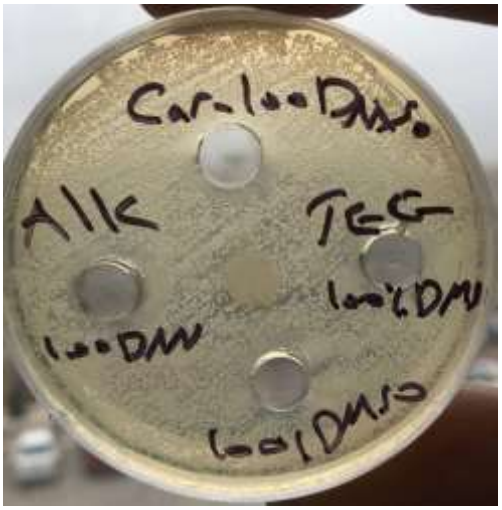


Figure 3.4: Well diffusion method for S. aureus.

Table 1: Inhibition zone in well diffusion method.

Zone of inhibition value in cm					
Bacteria species	Graphene	DMSO	Graphene-TEG	Graphene-COOH	Graphene-amine
E.coli	0.1	0.2	0.3	0.5	0.4
S. aureus	0.1	0.2	0.4	0.6	0.3

3.3.2. Broth microdilution method

The broth microdilution technique was used in order to detect and determine the minimum inhibition concentration (MIC) of the graphene and the *f*-graphene (graphene-COOH, graphene-amine and graphene-TEG) as described in the methodology section. As can be observed in figure 3.5 and table 2, the MIC of the graphene was 500µg/ml in both bacteria. However, it was 250µg/ml in the case of graphene-amine and graphene-TEG at both bacteria. On the other hand, the best antibacterial activity with the lowest MIC was in the case of graphene-COOH with a concentration of 125µg/ml at the both types of bacteria.

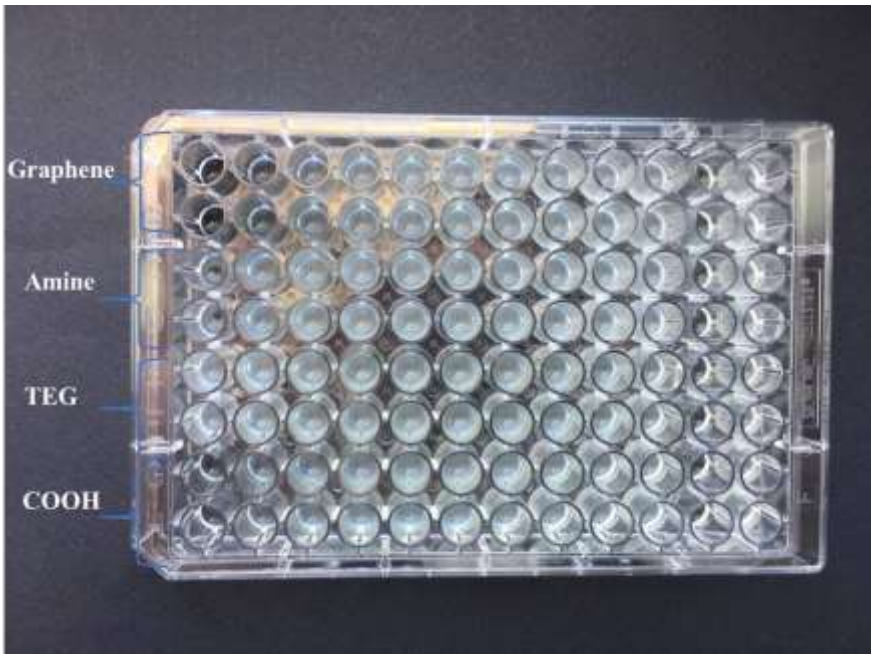


Figure 3.5: Broth microdilution method.

Table 2: Minimum inhibitory concentration (MIC).

	MIC (µg/ml)			
Bacterial species	Graphene	Graphene-COOH	Graphene-amine	Graphene-TEG
S. aureus	No effect	125	250	250
E.coli	No effect	125	250	250

3.3.3. Reduction in bacterial colony concentration (CFU)

The reduction of bacterial growth was detected as described earlier. We investigated the effects of different surface functional groups with a different charges (graphene-TEG (-OH) is neutrally charged, graphene-COOH (-COOH) is negatively charged and graphene-amine ($-NH_2$) is positively charged). By using these compounds it would be possible to determine if these charges have an effect on antibacterial activity.

The reduction of the bacterial concentration expressed in CFU/ml was determined by incubating the bacteria *E. coli* and *S. aureus* with the different graphene derivatives at a concentration of 250µg/ml of each material (normal saline solution was used as a negative control). As shown in table 3, figure 3.6 and 3.7, grater reduction in bacterial growth was observed in the samples treated with *f*-graphene in comparison with normal saline or graphene alone. It was noticed that the growth of *S. aureus* and *E. coli* by 13% and 45% respectively. However, a complete reduction in the growth of both types of bacterial by graphene-COOH. In the case of graphene-amine the percentage of reduction was 50% for *S. aureus* and 85% in the case of *E. coli*. Finally, the percentage of reduction in the case of graphene-TEG was 65% for *S. aureus* and 70% for *E. coli*. Therefore, all the synthesized and functionalized graphene showed an improvement of the antibacterial activity in comparison to the graphene and the negative control.

Table 3: CFU per ml percentage average.

Bacterial species	**Control normal saline**	**Graphene**	**Graphene-amine**	**Graphene-TEG**	**Graphene-COOH**
E. coli	123	84.6	46.2	38.5	1.5
S. aureus	100	92.3	53.8	30.8	0

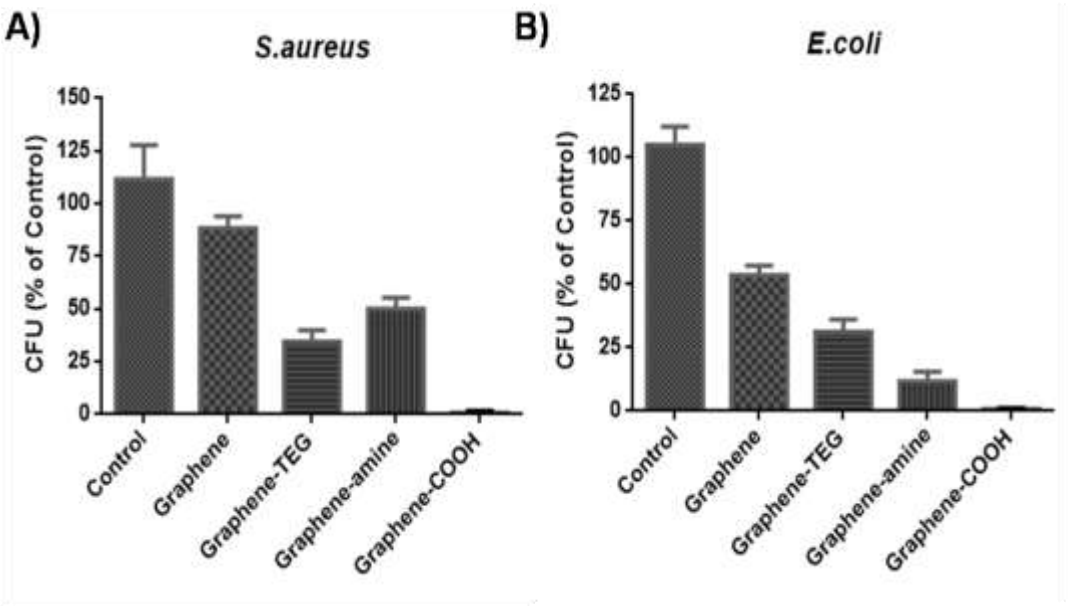


Figure 3.6: Percentage of bacterial concentration (CFU/ml) reduction for: A) *S. aureus*, B) *E.coli*.

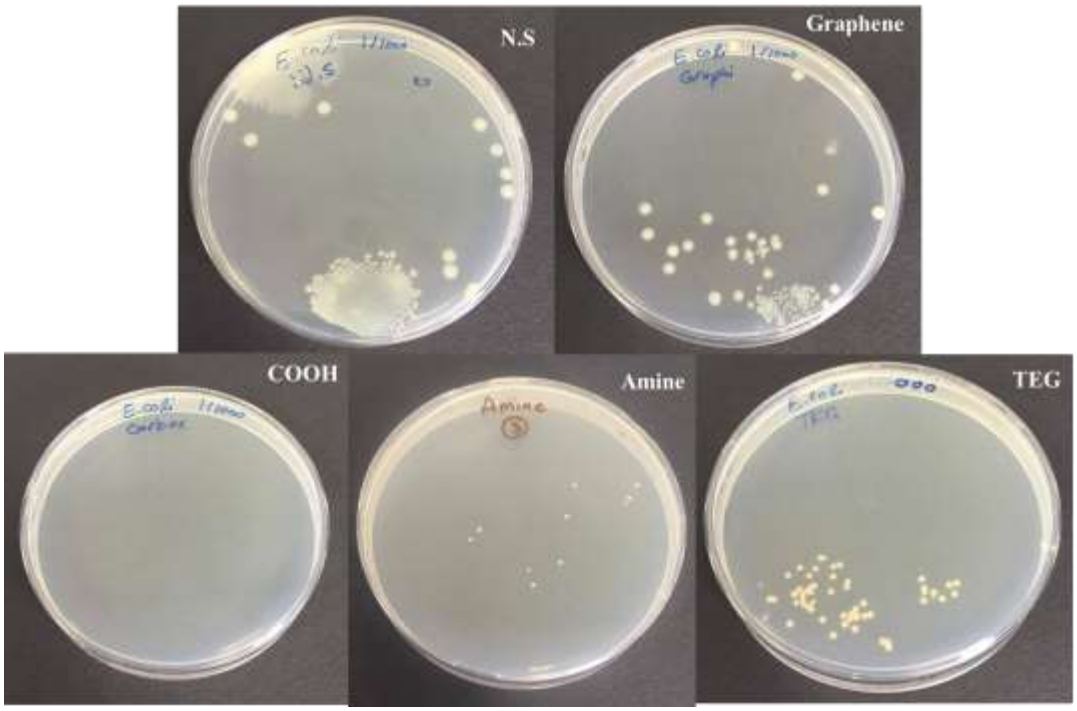


Figure 3.7: Reduction in bacterial colony concentration for E.coli.

3.4. Oxidative stress by Glutathione

Previous studies have shown that the graphene antibacterial mechanism could be due to the formation of reactive oxygen species (ROS) which increases the bacterial cell damage. Therefore, in this study the oxidative stress mechanism was investigated by *in vitro f*-graphene-mediated oxidation of glutathione.

Ellman's assay was used in order to study the percentage of the loss of glutathione (GSH %) activity upon exposure to graphene materials. Table 4 and figure 3.8 demonstrates different percentages for the loss of glutathione activity which was with the highest achieved by graphene-COOH material (83%) comparable with the positive control (H_2O_2, 90%). Whereas graphene-amine and graphene-TEG with percentages of 58% and 53% respectively.

Table 4: loss of glutathione (%)

Control	H_2O_2	Graphene	Graphene-amine	Graphene-COOH	Graphene-TEG
2	90.55	25.57028	52.97189	83.05221	45.74699

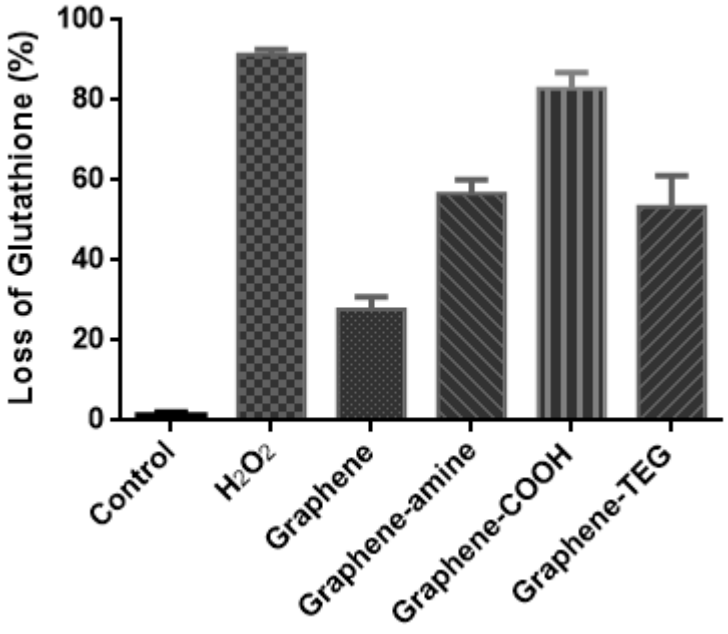


Figure 3.8 loss of glutathione (%) by graphene and the *f*-graphene materials**.**

All *f*-graphene showed better loss of glutathione activity in comparison to the graphene due to the formation of the reactive oxygen species which might explain the better antibacterial activity of all *f*-graphene. The extent of antioxidant activity correlates well with the antibacterial activity of graphene species. Moreover, the graphene-COOH showed the highest loss of glutathione activity which corresponds to the highest antibacterial activity due to the formation of ROS species.

3.5. Hemo-compatibility of *f-graphene*

One of the important concepts in order to apply the nanomaterials in vivo is the hemocompatibility especially with materials that will interact directly with the blood. The hemolytic activity of graphene and the functionalized graphene nanomaterials were studied on human blood sample. The

hemolytic behavior was conducted at various concentrations (200, 400 and 600 µg/ml) and at two interval times (2 and 24 hours) and was calculated and shown in figure 3.9 which demonstrates the hemolytic activity of the graphene and the *f*-graphene nanomaterials.

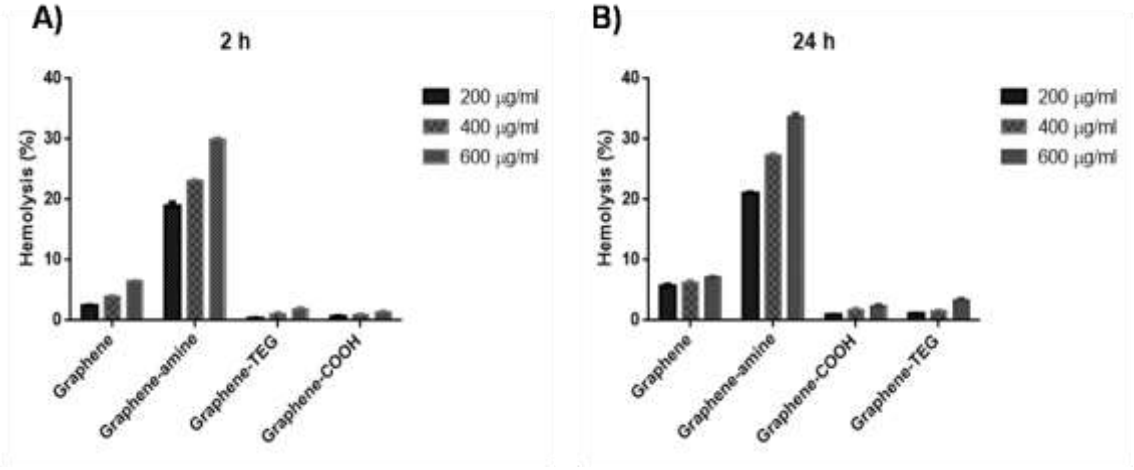


Figure 3.9: Percentage of hemolysis at three different concentrations (200, 400, and 600 µg/ml) after: A) 2 hours and B) 24 hours.

The permissible percentage of the hemolytic activity should be less than 5% in order to consider the material is hemocompatible [77]. Accordingly, the graphene, graphene-TEG and graphene-COOH showed acceptable level of hemo-toxicity at the three different concentrations (200, 400 and 600 µg/ml) for up to 24 hours. However, graphene-amine showed a significant hemolytic activity with a maximum percentage of 33% with highest concentration of 600µg/ml after 24 hours. An explanation for this amino toxicity in blood cells might be its reaction with cysteine-β-93 amino acid and causes hemoglobin toxicity [78]. Table 5 summarizes the percentage of the hemolysis for various functionalized graphene nanomaterials.

Table 5: summarize the percentage of hemolytic activity of *f*-graphene at 2 & 24 hrs.

Conc. (µg/ml)	Graphene-COOH		Graphene-TEG		Graphene-amine	
	2hr	**24hr**	**2hr**	**24hr**	**2hr**	**24hr**
200	0.75 %	1 %	0.5 %	1.5 %	19%	19 %
400	1 %	2 %	1 %	2 %	26 %	27%
600	2 %	4 %	3 %	5 %	38%	38 %

Therefore, it can be concluded that graphene-TEG and graphene-COOH demonstrate an excellent antibacterial activity with a negligible hemolytic activity over concentration range of (200-600µg/ml), give them a good biocompatibility and could be facilitates their use in medical application.

Conclusion

Graphene have been successfully functionalized covalently with different functional groups carrying different type of charges (positive, negative and neutral). The functionalization of graphene sheets were confirmed by transmission electron microscopy and IR spectroscopy. The functionalization demonstrates good dispersability of the functionalized graphene in normal saline. The degree of functionalization was quantified using the TGA obtaining (32%) of the functionalization in the case of graphene-TEG, (33%) of graphene-amine and (47%) of graphene-COOH.

Regarding the antibacterial activity was tested against *E.coli* & *S.aureus*, agar diffusion well-variant method used as primary technique to determine the antibacterial activities of graphene derivatives compounds. The MIC was determined for each *f*-graphene through the broth microdilution method obtaining 250µg/ml for graphene-amine and graphene-TEG and 125µg/ml for graphene-COOH. The reduction of bacterial growth was detected colony forming unit/ml method, the result shows that graphene-COOH completely reduced the growth of types of tested bacteria and gives the highest antibacterial activity. The mechanism of the antibacterial activity has been investigated through the formation of reactive oxygen species, which was confirmed by testing the loss of the glutathione activity. The hemolytic behavior of the functionalized graphene showed an acceptable level of hemo-toxicity at various concentrations of graphene-COOH and graphene-TEG for up to 24 hours, whereas the graphene-amine showed hemolytic toxicity. In conclusion, the functionalized graphene with

the carboxyl group demonstrates the highest antibacterial activity with a good biocompatibility. Therefore, we recommend to further *in vitro* & *in vivo* studies for this interesting nano-drug and go forward *in vivo* tests.

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جامعة النجاح الوطنية

كلية الدراسات العليا

تفعيل صفائح الجرافين وتاثيرها المضاد للبكتيريا

إعداد

ديما غالب حسني السوقي

إشراف

د. محي الدين العسالي

د. معتصم المصري

قدمت هذه الأطروحة استكمالاً لمتطلبات الحصول على درجة الماجستير في العلوم الصيدلانية بكلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس- فلسطين.

2017

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الملخص

الجرافين هو عبارة عن صفائح رقيقة مكونة من طبقة واحدة من ذرات الكربون تترتب فوق بعضها البعض على شكل مكعبات ثنائية الإتجاهات على هيئة خلايا النحل. يعتبر الجرافين من اكثر المواد التي تتم عليها الدراسات حديثا بسبب تمتعها بمزايا فريدة من نوعها وخصوصا مساحتها السطحية الكبيرة. الجرافين هو من المواد التي لها ذائبية قليلة في الماء وفي السائل الحيوي في جسم الإنسان مما يؤدي إلى ترسبه في الخلايا مسببا السميّة لتلك الخلايا. لهذا يهدف تفعيل الجرافين إلى تحسين الذائبية في الماء وبالتالي تقليل السمية لكي يتوافق مع الإستخدامات العديدة له في المجال الطبي.

الهدف الاساسي لهذا المشروع هو التفعيل التساهمي لصفائح الجرافين بمجموعات فعاله مختلفة الشحنات (الموجبة، السالبة والمتعادلة) لتحسين الذائبية بالماء ولدراسة تاثيرها المضاد للبكتيريا وتحديد تأثير الشحنات المختلفة عليها.

لقد تم بنجاح تفعيل صفائح الجرافين بشكل تساهمي بثلاث مجموعات مختلفة (مجموعة الكاربوكسيل، الأمين ورباعي إيثيلين الجلايكول). لتأكيد نجاح التفعيل تم فحصه بواسطة الأشعة تحت الحمراء والمجهر الإلكتروني الناقل.

نتيجة لتفعيل الجرافين أصبحت ذائبيته بالماء أفضل ولدراسة كمية التفعيل على الصفائح تم استخدام جهازالتحليل الحراري للكتلة والذي أعطى نسبة (32%) في حالة الجرافين–رباعي ايثيلين الجلايكول، و(33 %) في حالة الجرافين–أمين و(47%) في حالة الجرافين–كاربوكسيل.

تم دراسة تأثير الجرافين المفعل ضد البكتيريا بواسطة الانتشار على الأقراص والخانات على وسط من الاغار وتم إيجاد تأثيرمضاد للبكتيريا من جميع المركبات، ولدراسة التأثير الكمي تم دراسته بواسطة طريقة حساب التركيز الأدنى لتثبيط النشاط البكتيري، وأظهرت النتائج ان تركيز التثبيط بلغت (250 مايكروغرام / مل) في حالة الجرافين–أمين والجرافين–رباعي ايثيل الجلايكول و(125 مايكروغرام/ مل) في حالة الجرافين–كاربوكسيل. التقليل من النمو البكتيري تم دراسته بواسطة تحليل عدد المستعمرات البكتيرية وأظهرت الدراسة أن عدد المستعمرات قلت في حالة الجرافين المفعل لكل المركبات وعدم وجود أي نمو للخلايا في حالة الجرافين–كاربوكسيل.

تم فحص الية السمية للجرافين عن طريق أكسدة الجلوتاثيون، وأظهرت النتائج أن الجرافين–كاربوكسيل قلل من نشاط الجلوتاثيون بنسبة (83%). وأخيرا جرافين–كاربوكسيل وجرافين–رباعي ايثيل جلايكول أظهر ملاءمته لخلايا الدم مما يعزز الدراسات المستقبليه له.