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Design and fabrication of graphene/lipid nanostructured interfaces for applications in membrane protein biosensing

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Come l'amore cresce dentro di te, così cresce la bellezza.

Perché l'amore è la bellezza dell'anima.

Sant'Agostino

Il per sempre è composto da molti adesso

Emily Dickinson

Contents

Introduction	1
1 Graphene-based biosensors	3
1.1 Introduction	3
1.1.1 Graphene-based electrochemical biosensors	6
1.1.2 Graphene-based field-effect transistor (FET) biosensors	6
1.1.3 Graphene-based optical biosensors	7
1.2 Graphene	7
1.2.1 Overview on the synthesis of graphene	8
1.2.1.a "Top-down"	9
1.2.1.b "Bottom-up"	9
1.2.2 Graphene properties	10
1.2.3 Surface chemistry	11
1.3 Cell membrane	12
1.3.1 Properties of cell membrane	12
1.3.2 Membrane proteins	14
1.3.3 Liposomes and supported lipid bilayers	15
1.4 Graphene-lipid interaction: state-of-the-art	15
2 Materials and methods	21
2.1 Materials	21
2.1.1 Synthesis and transfer of graphene	21
2.1.1.a Synthesis	21
2.1.1.b Transfer	25
2.1.2 Preparation of liposome solutions	27
2.2 Characterization methods	32

2.2.1	Micro-Raman spectroscopy	32
2.2.2	Water contact angle measurements	35
2.2.3	Scanning electron microscopy	36
2.2.4	X-ray photoelectron spectroscopy	39
2.2.5	Atomic force microscopy	45
2.2.6	Electrochemistry	47
2.2.6.a	The concept of impedance	48
2.2.6.b	Electrochemical impedance spectroscopy	50
2.2.6.c	Equivalent circuit	53
3	Synthesis and transfer of graphene	57
3.1	Introduction	57
3.2	Results and discussion	58
3.2.1	Synthesis	58
3.2.1.a	Copper foil surface treatment	58
3.2.1.b	Copper foil geometry	62
3.2.1.c	Quartz furnace	62
3.2.2	Transfer	67
3.2.2.a	Etching solution	68
3.2.2.b	Cyclododecane: an alternative to PMMA	70
3.2.2.c	The influence of the substrate	70
3.3	Conclusions	74
4	Characterization and surface treatment of graphene	75
4.1	Introduction	75
4.2	Results and discussion	75
4.2.1	Characterization of graphene as-transferred	75
4.2.1.a	SEM observation	75
4.2.1.b	Micro-Raman spectroscopy	79
4.2.2	Surface treatments of graphene and characterization	80
4.2.2.a	Water contact angles	83
4.2.2.b	MicroRaman spectroscopy	84
4.2.2.c	AFM	89
4.2.2.d	XPS	91
4.3	Conclusions	94

5	Electrochemistry of supported lipid membranes on CVD-G	97
5.1	Introduction	97
5.2	Results and discussion	97
5.2.1	Preparation of the samples for EIS measurements	97
5.2.2	Electrochemical cell	98
5.2.3	EIS measurements	99
5.2.4	Equivalent circuit	114
5.3	Conclusions	118
6	Conclusions and future perspectives	121
	Bibliography	125
	List of publications	134

List of Figures

1.1	Scheme of a graphene-based biosensor. The biosensor consists of a receptor layer, which is a biomolecule (e.g., DNA, protein and so on), and a transducer, which is based on graphene material. [1]	4
1.2	Scheme of the graphene-based electrochemical biosensor with ion channel protein in supported lipid membrane.	5
1.3	The carbon atomic orbitals in the sp^2 honeycomb lattice.	8
1.4	Schematic diagram of graphene preparation strategies: "top-down" and "bottom-up".	8
1.5	Schematic diagram of the formation of graphene by CVD.	10
1.6	The cell membrane structure.	13
1.7	The parts of a typical phosphatidylcholine molecule, represented (a) schematically, (b) by formula, and (c) as a computer-generated model.	13
1.8	Schematic representation of membrane protein functions.	15
1.9	Schematic representation of a (a) vesicle and (b) SLB.	16
1.10	Molecular dynamics simulations showing fusion of lipid-coated monolayer graphene with a lipid bilayer, leading to localization of the sheet in the interleaflet hydrophobic core. [2]	17
1.11	Schematic of the structure of lipid membranes on rGO/SiO ₂ . [3]	18
1.12	Scheme of the lipid writing process with dip-pen nanolithography. [4]	19
1.13	Schematic illustration of three model membrane systems: (a) supported monolayer, (b) bilayer, and (c) intact vesicles. [5]	19
2.1	Schematic diagram (a) and photo (b) of the tube-furnace CVD system at CNR-IMM Bologna.	22
2.2	Schematic diagram of the formation of graphene after H ₂ annealing.	24

2.3	Schematic diagram of the transfer of graphene with PMMA as media. Adapted from [6,7].	26
2.4	Chemical structure (a) and 3D view (b) of cyclododecane.	27
2.5	Schematic diagram of transfer of graphene using cyclododecane. . .	28
2.6	Chemical structure (a) and 3D structure (b) of DOPC.	29
2.7	Chemical structure (a) and 3D structure (b) of DOPS.	29
2.8	Chemical structure (a) and 3D structure (b) of EPC.	30
2.9	Mini-Extruder from Avanti Polar Lipids.	31
2.10	Lipid solution (left) and liposome solution after the extrusion through a membrane with pore size of 50 nm.	31
2.11	Schematic representation of the elastic (Rayleigh) and inelastic scattering (Stokes on the right and anti-Stokes on the left) related to a single vibrational mode.	33
2.12	Raman spectra from crystalline monolayer graphene.	34
2.13	Micro-Raman system at CNR-ISMN Bologna.	35
2.14	Illustration of contact angle formed by sessile liquid drop on a smooth homogeneous solid surface.	36
2.15	(a) Digidrop GBX Model D at CNR-ISMN Bologna and (b) Data-Physics OCA 15Pro at DICMA - Rome.	37
2.16	Mechanisms of emission from the interaction volume of the sample. .	38
2.17	SEM Zeiss Gemini 1530.	39
2.18	(a)Photo of the XPS spectrometer assembled on the UHV analysis chamber; (b) scheme and (c) photograph of the inside of the analysis chamber xz view point. Both the X-ray source and the analyzer are aligned with the upper surface of the sample holder.	41
2.19	Comparison XPS C 1s spectra of (a) CVD graphene on nickel foil after thermal annealing and (b) oxidized CVD graphene on iridium. Peak labels in (b) are: sp^2 aromatic carbon; C1 epoxy; C2 defects; C3 hydroxyl; C4 carbonyl; C5 carboxyl; C6, C-Ir bond, sp^2 aromatic carbon. Reproduced with permission from (a) ref [8] and (b) [9]. .	42

2.20	Detail of hard-X-ray photoemission spectroscopy C 1s spectrum in graphitized carbon. The spectrum is fitted using an asymmetric line-shape for the sp^2 component and a symmetric one for the other components. Functional groups: sp^2 regions (black curve), defect due to metals (D2), sp^3 and other topological defects (D1), hydroxyl (C–OH), epoxy (C–O), carbonyl (C=O), carboxyl (–COOH) and shake-up ($\pi - \pi^*$) [6].	43
2.21	Schematic presentation of AFM.	46
2.22	NT-MDT AFM (model NTEGRA Spectra).	47
2.23	Phasor diagram for an alternating voltage.	48
2.24	Phasor diagram showing the relationship between alternating current and voltage signals at frequency ω	49
2.25	Bode plots of modulus (a) and phase (b) for a series RC circuit; (c) Nyquist plot for a parallel RC circuit.	51
2.26	Scheme of a three-electrode electrochemical cell and potentiostat. In a three-electrode cell potential and current measurements are separated into an RE and a CE loop. The potential of the working electrode (WE) is measured with respect to the reference electrode (RE). The input of RE has a high-impedance forcing all current to flow through the counter electrode (CE).	52
2.27	Gamry Reference 600 TM	54
2.28	Behavior of charged molecules at the electrode surface.	55
3.1	Copper foil without etching: (a) SEM image; (b) optical and (c) AFM surface profile image; (d) SEM image of transferred graphene grown on this type of copper foil.	59
3.2	Copper foil after etching: (a) AFM surface profile image and (b) SEM image.	60
3.3	Copper foil after annealing: (a) AFM surface profile image and (b) SEM image; (c) SEM image of transferred graphene grown on the treated copper foil.	61
3.4	Copper foil geometries (left) and corresponding SEM images of transferred graphene (right).	63

3.5	SEM images of a heavily contaminated graphene film grown by CVD with an aged quartz tube, on copper (a,b) and after transfer on SiO ₂ /Si 300 nm (c). Copper surface reconstruction and graphene folds are clearly visible.	65
3.6	(a) Schematics of the modified configuration of the low-pressure CVD system with a coaxial alumina screen tube. The boat is inserted and extracted from the hot zone as in the original configuration. (b) Graphic design and (c) photographic image of the tantalum boat with lid (12 cm in length).	66
3.7	SEM micrographs at different magnification of graphene films grown in the optimized CVD reactor using the tantalum boat on copper (a,b) and after transfer onto a SiO ₂ /Si substrate (c). No growth contaminants are visible at any magnification.	67
3.8	SEM images of transferred graphene using different solutions.	69
3.9	Graphene transferred using cyclododecane.	71
3.10	Differences between SiO ₂ by thermal oxidation and LTO film deposited by LPCVD.	73
3.11	Transferred graphene on a SiO ₂ /Si wafer.	74
4.1	SEM images of CVD-graphene sample CVD-G-01.	77
4.2	SEM images of CVD-graphene sample CVD-G-02.	77
4.3	SEM images of CVD-graphene sample CVD-G-03.	77
4.4	SEM images of CVD-graphene sample CVD-G-04.	77
4.5	SEM images of CVD-graphene sample CVD-G-05.	77
4.6	SEM images of CVD-graphene sample CVD-G-06.	77
4.7	SEM images of CVD-graphene sample CVD-G-07.	78
4.8	SEM images of CVD-graphene sample CVD-G-08.	78
4.9	SEM images of CVD-graphene sample CVD-G-09.	78
4.10	SEM images of CVD-graphene sample CVD-G-10.	78
4.11	SEM images of CVD-graphene sample CVD-G-11.	78
4.12	Raman spectrum of sample CVD-G-08.	79
4.13	Raman maps of (a) I_{2D}/I_G and (b) I_D/I_G ratios of sample CVD-G-08.	81
4.14	Raman maps of (a) I_{2D}/I_G and (b) I_D/I_G ratios of sample CVD-G-09.	82
4.15	Photo of the Cold Walls Reactor.	83

4.16	Raman maps of (a) I_{2D}/I_G and (b) I_D/I_G ratios of sample CVD-G-08 after surface treatment ST-A.	86
4.17	Raman maps of (a) I_{2D}/I_G and (b) I_D/I_G ratios of sample CVD-G-08 after surface treatment ST-B.	87
4.18	Raman maps of (a) I_{2D}/I_G and (b) I_D/I_G ratios of sample CVD-G-09 after surface treatment ST-C.	88
4.19	AFM (a) and SEM (b) images of CVD-G-08 as is.	89
4.20	AFM (a) and SEM (b) images of CVD-G-08 after surface treatment ST-A.	90
4.21	AFM (a) and SEM (b) images of CVD-G-08 after surface treatment ST-B.	90
4.22	AFM and SEM images of CVD-G-09: (a), (b) after surface treatment ST-C.	91
4.23	XPS spectra of (a) CVD-G as is and (b) after ST-B.	92
4.24	(a) O/C calculated after PMMA subtraction; (b) % of PMMA; (c) % of copper after etching on CVD-graphene as-transferred and after thermal surface treatments.	93
5.1	Stencil mask used for the evaporation of nickel contacts (a); design of the nickel contact on the graphene sample (b); photo of one of the samples in which graphene is highlighted by red (c).	98
5.2	Electrochemical cell made of PTFE: (a) schematic view of the three elements of the cell and position of the electrodes; (b) photograph of the fabricated and assembled cell.	99
5.3	Bode plots of modulus (a) and phase (b) and Nyquist plot (c) of gold-electrode in comparison with CVD-G-01.	100
5.4	Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-100), and Nyquist plot (c) of CVD-G-01.	103
5.5	Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-100), and Nyquist plot (c) of CVD-G-02.	104
5.6	Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-50), and Nyquist plot (c) of CVD-G-03.	105

5.7	Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-50), and Nyquist plot (c) of CVD-G-04.	106
5.8	Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-50), and Nyquist plot (c) of CVD-G-05.	107
5.9	Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-100), and Nyquist plot (c) of CVD-G-06.	108
5.10	Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-50), and Nyquist plot (c) of CVD-G-07.	109
5.11	Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-100), and Nyquist plot (c) of CVD-G-08.	110
5.12	Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-50), and Nyquist plot (c) of CVD-G-09.	111
5.13	Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC/EPC-50), and Nyquist plot (c) of CVD-G-10.	112
5.14	Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC/DOPS-50), and Nyquist plot (c) of CVD-G-11.	113
5.15	Equivalent circuit.	114
5.16	Graph bars of R_g (a), Y_g (b) and W (b) when using DOPC-100 solution, with different ST.	115
5.17	Graph bars of R_g (a), Y_g (b) and W (b) when using DOPC-50 solution, with different ST.	116
5.18	Graph bars of R_g (a), Y_g (b) and W (b) when using DOPC/EPC-50 (left) and DOPC/DOPS-50 (right) solution, with the same surface treatment (ST-D).	118

List of Tables

2.1	Characteristics of the CVD system.	23
2.2	Characteristics of copper foil from GoodFellow	24
2.3	Properties of DOPC, DOPS and EPC lipids, as given by Avanti Polar Lipids Inc.	30
2.4	Characteristics of SEM Zeiss Gemini 1530.	39
2.5	List of used fitting curves for the various functional groups and corresponding chemical shifts.	44
2.6	Characteristics of Gamry Reference 600 TM	53
4.1	Characteristics of the Cold Walls Reactor.	80
4.2	Surface treatment of CVD-graphene samples.	84
4.3	Mean values and standard deviation of WCAs of CVD-graphene before and after surface treatments.	85
4.4	XPS of CVD-graphene before and after surface treatments.	92
5.1	Mean values and standard deviation of WCAs of samples and the type of liposome solution used.	101

Introduction

Plasma membranes, or cytoplasmatic membranes, are the reaction fields of many biological reactions involved in diseases, and an active barrier for the transportation of information and materials between the inside and outside compartments of cells. Lipid bilayers are the fundamental structure of cell membranes. Examining the functional behavior of lipids and proteins and their ensemble by using artificial lipid bilayers integrated in biosensors is important in order to determine the physical and chemical processes involved in cell membrane reactions, that are relevant in a wide range of biomedical applications. By using supported lipid bilayers (SLBs) on graphene-based substrates, it is possible to develop novel biosensors able to obtain molecular information from membrane proteins embedded in lipid bilayers: the graphene acts as the transducer of the electrical signals and the phospholipidic bilayers with embedded molecular proteins form the receptor layer.

The main objectives of this thesis are the design and optimization of the interactions between graphene and lipid bilayers, through the analysis of the fabrication process of graphene, its surface treatments and how these affects the formation of supported lipid membranes. These are essential steps aiming at manufacturing a novel properly designed biosensing platform with integrated membrane proteins. Nanomaterials can play a very significant role in the development of the next generation of biosensors, since they can help to address some of the key issues in the development of biosensors. Graphene is a carbon-based nanomaterial that has recently attracted most interest due to its unique electronic properties, arising from its crystal structure, which result in a extremely low noise level and an increased signal-to-noise ratio. The fabrication of biosensors based on SLBs on graphene requires the study and optimization of the building blocks of the sensor and of the interface between them. The formation of SLBs is strongly influenced by the physical and chemical properties of the underlying substrates. Then, in order to

induce the formation of lipid bilayers possessing a sufficient stability for practical biosensing applications, the wetting properties of the supporting layer should be investigated. Since graphene is a hydrophobic material, the introduction of defects on the surface both by heat treatments or functionalization have been investigated.

Chapter 1 describes biosensors and their importance for studying biological process at a cellular scale. In particular, it focuses on biosensors at the nanoscale, with a special attention to carbon nanomaterials, that play a very significant role in the development of the next generation of biosensors. This chapter presents a brief state-of-the-art section on the interaction of graphene-based materials with lipids membranes, both mono- and bi-layers. Chapter 2 describes the procedure of synthesis and preparation of all the materials used in this study (e.g. CVD-graphene surfaces and liposome systems), and also the methods of characterization used to study these materials and their interaction. Chapter 3 presents the results of synthesis and transfer of CVD-graphene sample, with a particular focus on several issues. Chapter 4 presents the characterization of CVD-graphene samples and describes the surface treatments performed in order to improve its compatibility towards SLBs. Chapter 5 reports the study of the interaction of graphene treated surfaces with liposomes by means of electrochemical measurements.

The synthesis of graphene by catalyzed chemical vapor deposition (C-CVD), the characterization of graphene through scanning electron microscopy (SEM), the surface heat treatments in high vacuum, the transfer of graphene and the preparation of the samples with nickel contact evaporation were carried out at CNR-IMM Bologna, while the characterization of graphene using micro-Raman spectroscopy and water contact angle measurements were carried out at CNR-ISMN Bologna. The X-ray photoelectron spectroscopy (XPS) and atomic force microscopy (AFM) measurements were performed at CNR-ISOF Bologna. The formation of the SLBs from incubation in liposome solutions obtained via the extrusion procedure, the design and fabrication of the electrochemical cell for electrochemical impedance spectroscopy (EIS), and the analysis of the interaction of graphene with SLBs were carried out at the Department of Chemical, Materials and Environmental Engineering of Sapienza University of Rome.

Chapter 1

Graphene-based biosensors

1.1 Introduction

IUPAC defines *biosensor* as follows: "A device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals". [10] The key component of any biosensor is the biorecognition layer. The surface, on which this biorecognition layer is deposited, is of crucial importance in order to obtain a highly sensitive and selective biosensor. Thus, the transducing material should possess a high conductivity and a preferably low electron transfer resistance on its surface. Furthermore, a large surface area for immobilizing biomolecules is also beneficial.

For these reasons, there is emerging interest in nanostructured surfaces to be used in detection, and in particular graphene-based nanomaterials has captured great interest among physicists, chemists and materials scientists. Graphene-based biosensing performance heavily depends on purity degree, lateral size, number of layers, oxidation level, distribution of oxygen-containing moieties, and/or defects of the employed graphene derivatives, since these features often modulate electrical conductivity, fluorescence-quenching capabilities, photoluminescence, binding interactions (between graphene derivatives and biomolecules) and kinetics, and capacity of biomolecules adsorption. In comparison with other electrode materials, graphene-based nanomaterials exhibit improved features (e.g., high electrical conductivity, very fast heterogeneous electron transfer, and large surface area). [11]

Various detection methodologies, including optical, magnetic, plasmonic, and

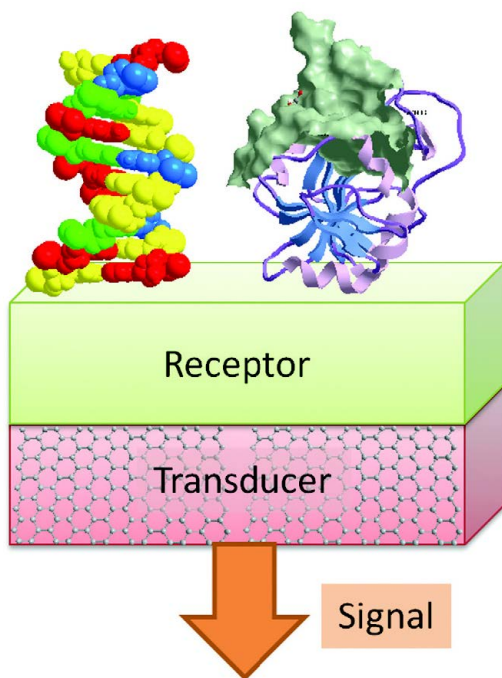


Figure 1.1. Scheme of a graphene-based biosensor. The biosensor consists of a receptor layer, which is a biomolecule (e.g., DNA, protein and so on), and a transducer, which is based on graphene material. [1]

acoustic analyses, have been developed for the rapid and accurate discrimination of low concentrations of target molecules. Although such assays have attractive sensing mechanisms, they also have critical limitations, such as their time-consuming operation, high costs, low resolution, and noisy backgrounds. The observation of changes in electrical signals can provide more specific and precise information about target molecules, and electrical sensing devices also offer label-free and real-time measurements.

Graphene is thought to become especially widespread in biosensors and diagnostics. The large surface area of graphene can enhance the surface loading of desired biomolecules, and excellent conductivity and small band gap can be beneficial for conducting electrons between biomolecules and the electrode surface. Biosensors can be used, among other things, for the detection of a range of analytes like glucose, glutamate, cholesterol, hemoglobin and more. Graphene also has significant potential for enabling the development of electrochemical biosensors,

based on direct electron transfer between the enzyme and the electrode surface.

The studies on the electrochemical properties of graphene and its derivatives provide the opportunity to develop novel, simple, and efficient electrochemical biosensors. [12] Thus the aim of the present study is to develop a new kind of electrochemical biosensor, which uses graphene as transducer, and the membrane proteins as the recognition element; a scheme of the biosensor is shown in Figure 1.2. Those membrane proteins are embedded in the lipid bilayer, which are the fundamental structure of cell membranes. [13] Examining the functional behavior of lipids and proteins, and their ensemble using artificial lipid bilayers integrated in biosensors is important to determine the physical and chemical processes involved in cell membrane reactions. Supported lipid bilayers (SLBs) formed by the spontaneous deposition of liposomes in solution have already found widespread use as mimics of cell membranes, for the functional investigation of membrane proteins, and as building blocks for biosensors. [14]

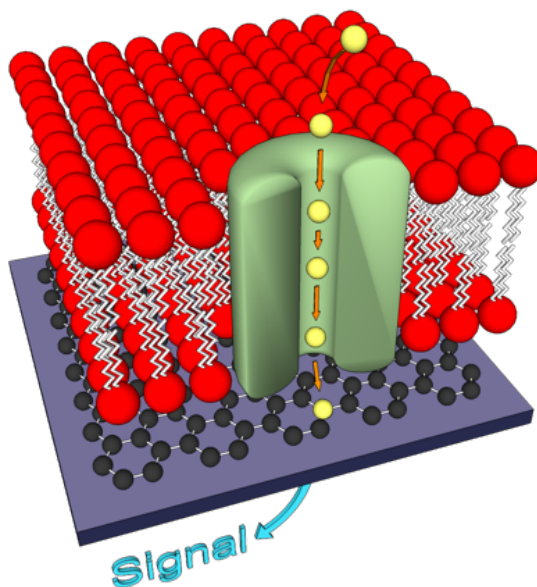


Figure 1.2. Scheme of the graphene-based electrochemical biosensor with ion channel protein in supported lipid membrane.

In view of the complexity and diversity of the functions performed by the different proteins embedded in a biomembrane, it has been found convenient to incorporate single integral proteins (melittin) into experimental models of biological

membranes, so as to isolate and investigate its functions.

1.1.1 Graphene-based electrochemical biosensors

Electrochemical detection is highly sensitive to electroactive molecules and also offers detection selectivity, as different molecules can be oxidized/reduced at different potentials.

Graphene is an excellent conductor of electrical charge and heterogeneous electron transfer occurs at the edges or at defects in the basal plane. The high surface area of graphene facilitates large amounts of defects and a large number of electroactive sites. Electrodes made from graphene have more uniform distribution of electrochemically active sites than do those made from graphite. The entire volume of graphene is exposed to the surroundings due to its 2D structure, making it very good in detecting adsorbed molecules on the electrode. Since graphene has a large electrochemical potential window (approximately 2.5 V in 0.1 mM phosphate buffer saline solution), detection of molecules that have high oxidation or reduction potential (e.g., nucleic acids) becomes feasible. [15]

1.1.2 Graphene-based field-effect transistor (FET) biosensors

Field-effect transistors (FET) have received a great deal of interest in the area of biosensing because they can be fully integrated into the electronic chips produced by semiconductor companies. Therefore, it is not only academia that is fascinated by these devices, but there is a strong interest (and investment) from industry as well. Field-effect transistor-based biosensors rely on biorecognition events between molecules at the gate of the FET. Upon biorecognition between the probe and the target biomolecules, the electric charge distribution changes the charge carrier density at the biorecognition layer and thus, the conductivity of the channel between the source and drain. Graphene is an ideal material for the construction of FET biosensors because it is a zero-band gap semiconductor, and the band gap can be tuned by surface modification. As mentioned above, FET transistors are ideal for sensing charged molecules. Therefore, graphene-based FETs can be employed for DNA sensing, since DNA has a charged phosphate backbone. [16–18]

1.1.3 Graphene-based optical biosensors

In optical biosensors, optical properties such as absorbance, reflectance or fluorescent emissions and their change in intensity, decay time, anisotropy, quenching efficiency or luminescence energy transfer are measured in the ultraviolet (UV), visible or near infrared (NIR) ranges upon interaction of biomolecules with the target analyte. Optical sensors offer advantages of low cross-sensitivity, long life and lower contamination sensitivity. The latter is particularly helpful in the fabrication of high-performance biocompatible biosensors. One way of optically detecting biomolecules using graphene is by attaching a fluorophore, which can exhibit different photophysical properties because of its interaction with graphene. When a fluorophore binds to graphene and is then excited by an external radiation, the emission from the fluorophore is quenched, resulting in a phenomena known as fluorescence resonance energy transfer (FRET). [19–21]

1.2 Graphene

Graphene consists of a single-layer sheet of sp^2 -hybridized carbon atoms and is thus classified as a 2D nanomaterial. Compared to one-dimensional carbon nanotubes (CNTs) and zero-dimensional fullerenes, 2D graphene derivatives are more promising owing to their reproducibility and homogeneous functionalizations. In the sp^2 configuration, the 2s, $2p_x$ and $2p_y$ orbitals hybridize forming three sp^2 covalent σ bonds, 120° from each other in the xy plane, binding each carbon atom to its three neighbors. The p_z electrons that remain perpendicular to the hexagonal network form delocalized π electron states that collectively form valence and conduction energy bands. For this reason, sp^2 carbons are also called π -electron materials. The delocalized electronic states in monolayer graphene are highly unusual, because they behave like relativistic Dirac fermions, that is, these states exhibit a massless-like linear energy-momentum relation (like a photon), and are responsible for unique transport (both thermal and electronic) properties at sufficiently small energy and momentum values. This unusual electronic structure is also responsible for unique optical phenomena.

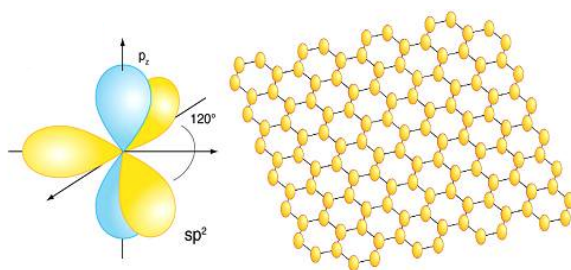


Figure 1.3. *The carbon atomic orbitals in the sp^2 honeycomb lattice.*

1.2.1 Overview on the synthesis of graphene

The preparation methods of graphene, can be divided into "bottom-up" where the carbon network is assembled from some source of carbon atoms and "top-down", where a larger layered structure (i.e., graphite) is broken into individual graphene layers.

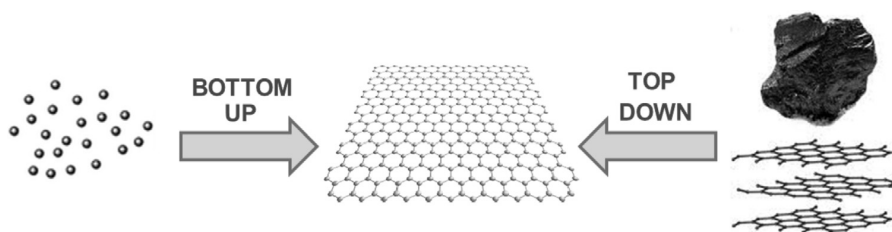


Figure 1.4. *Schematic diagram of graphene preparation strategies: "top-down" and "bottom-up".*

Each approach has different advantages. In the "top-down" approaches large quantities of graphene can be easily prepared. However, these methods have a serious drawback: it is almost impossible to produce single-layer graphene with controlled size. In principle, "bottom-up" approaches are very simple, but graphene is prepared using high temperature and low pressure processes. Compared with the "top-down" methods, the "bottom-up" approaches can produce graphene sheets of better quality and, more importantly, larger surface areas via growth on certain substrates.

1.2.1.a "Top-down"

Micromechanical exfoliation is one of the most widespread processes for preparing graphene. The standard micromechanical exfoliation method uses Scotch tape: this was the method first used to experimentally isolate graphene by Andre Geim and Konstantin Novoselov. [22] Graphene prepared by the Scotch tape method is of high quality, without any defects, but the method requires a hard laboratory work. Therefore, various alternative methods have been developed to overcome this drawback of micromechanical exfoliation. Versatile intercalants for graphite exfoliation have been designed, and their ability to physically separate graphene from graphite has been examined.

Graphene oxide (GO) has been used as a precursor to produce graphene. This chemical approach is the most popular method for preparing graphene in large quantities. GO is usually obtained by the Hummers and Offeman method, which utilizes concentrated acids and strong oxidants. [23] Graphite, which consists of a layered structure with various functional groups (hydroxyl, epoxy, and carboxylic groups) on the basal planes and at the sheet edges, can be converted into graphite oxide by chemical treatment, resulting in graphite oxide with hydrophilic properties. Subsequent exfoliation by thermal treatment or sonication in water, followed by the reduction of the GO, gives a solution of GO sheets in water, that have to be reduced to produce graphene sheets. The resulting material is called reduced graphene oxide (rGO). GO is electrically insulating; in most cases, rGO still contains residual defects, consequently efficient reduction processes are highly important for graphene-based electronics.

1.2.1.b "Bottom-up"

Silicon carbide (SiC) crystals can be used as a substrate for epitaxial growth; graphene has been prepared on this substrate by high temperature processes with or without hydrogen flow. [24] Interestingly, single- or bilayer graphene can be prepared on the Si-face of the crystal, whereas few-layer graphene grows on the C-face depending on the heating rate, pressure and cooling rate. In the case of epitaxial growth, the graphene partially forms on the silicon carbide crystal, which renders it heterogeneous because of defects or grain boundaries. Moreover, its crystalline quality and size are very low and irregular, like those of rGO.

The alternative chemical vapor deposition (CVD) method is a well-known

process that can fabricate size-controlled and high-quality graphene via exposure to gaseous compounds. Graphene can be grown on various substrates in different ways, depending on the temperature, gases and carbon sources used. [25] After coating a polymeric support on the top surface of graphene, the graphene/polymer support can be transferred onto various substrates, such as glass, silicon wafer, and polymer films. The support is then easily removed by dissolution.

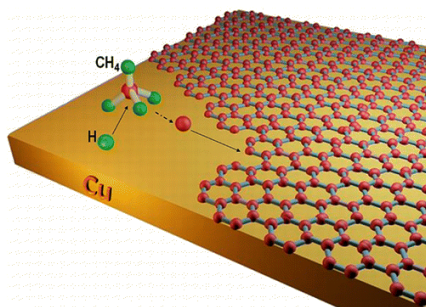


Figure 1.5. *Schematic diagram of the formation of graphene by CVD.*

1.2.2 Graphene properties

Numerous experiments and theoretical calculations have shown that the graphene has an extremely high thermal conductivity, $3000 \frac{\text{W}}{\text{m K}}$ to $5000 \frac{\text{W}}{\text{m K}}$, which is close to or exceeds that of diamond. [26] This ensures a fast heat dissipation in large scale integrated electronics. Graphene has been proved to be one of the strongest materials ever encountered in nature, with a Young modulus of 1 TPa and tensile strength of 100 GPa, which is very similar to those of high quality carbon nanotubes. [27]

Monolayer graphene represents an extreme case, in which every atom lies on the surface, and in fact, each atom is exposed to the surrounding medium on two sides, giving rise to the theoretical maximum surface area of an sp^2 -hybridized carbon sheet of about ($2630 \text{ m}^2/\text{g}$). This is at least an order of magnitude higher than the surface area of most of the other nanomaterials studied in biological systems.

Graphene has excellent electrical and electronic properties, such as extremely high charge carrier mobility and the ambipolar field effect and quantum Hall effect. Graphene has been reported to have a large theoretical surface area , large

potential window (approximately 2.5 V in 0.1 mM phosphate-buffered saline), low charge-transfer resistance, excellent electrochemical activity, and a fast electron transfer rate, and graphene transistors have demonstrated high carrier mobilities ($200\,000 \frac{\text{cm}^2}{\text{Vs}}$). [15] Due to these properties, graphene-based nanoelectronics can pave the way for rapid, accurate, and portable sensing technologies for chemical/bio-hazardous substances, such as cancers, infectious diseases, super-bacteria, and hormones.

It has been noted that these exceptional electronic, mechanical, and thermal properties are only achievable in graphene of very high quality and could be greatly impaired if structural defects are introduced into the honeycomb lattice.

1.2.3 Surface chemistry

Surfaces play a central role in the biological interactions of nanomaterials. Pristine graphene is highly hydrophobic (water contact angle near 90°), which has limited its applications in biological systems. Thus, a major goal in graphene chemistry has been the bio-functionalization, aimed at exploiting novel graphene-based materials in biological and medical applications. Functionalized graphene materials are expected to conjugate with various recognition molecules and can combine with other functional materials for electrochemical and electronic bioanalysis. Graphene can also be functionalized by both non-covalent and covalent processes to enhance the specific sensing capabilities of biosensors. Chemical moieties, commonly, carboxylic ($-\text{COOH}$) and hydroxyl ($-\text{OH}$) groups, can be covalently created on the graphene surface using strong acids and/or oxidants. Although covalent strategies can effectively, stably and specifically introduce functionalities, they unavoidably alter the native electronic structure and physical properties of graphene by converting sp^2 carbons to sp^3 ones, causing severe decrease in carrier mobility. In view of this problem, non-covalent modifications have been employed in order to preserve the intrinsic properties of the original graphene materials. Various molecules can physically adsorb onto graphene materials without the need of any coupling reagents. Graphene can be viewed as a giant (the largest) aromatic molecule. It can firmly interact with any molecules with aromatic ring(s) on the surface. π - π stacking interactions have been extensively utilized to modify graphene because the formed bonds are strong, the modification process is simple to achieve and the modification reaction is nondestructive. To strengthen π - π interactions, the π

systems of graphene and the functional molecule must have very similar electronic systems (i.e., electron densities).

1.3 Cell membrane

One of the key features of living organisms lies in their possession of biological membranes. They are thin, dynamic structures that keep a cell separated from the external environment and control the traffic of ions, molecules, and signals between the cell and the external environment. These biological membranes are sheet-like structures, which increase the surface area available for biological activities. They are typically 3 nm to 5 nm thick in animal cells.

The plasma membrane is a barrier with selective permeability between the inside and outside of the cell. This allows the cell to maintain a homeostatic condition appropriate to the biochemical reactions necessary to sustain life. The plasma membrane also acts as a responsive barrier allowing the cell to react to its environment. Specific receptors and transducing proteins in the membrane enable the cell to respond to signals generated by other cells.

All cell membranes contain two principal components: lipids and proteins. A membrane may be regarded as a two-dimensional array of lipid molecules in which protein molecules float. These are associated non-covalently and vary in their relative compositions depending on the origin and function of the membrane. A schematic representation of the cell membrane and its components is shown in Figure 1.6.

Membrane lipids occur as a double layer or bilayer. All the major lipids found in cell membranes are amphipathic: their structure has a water-soluble hydrophilic region (the polar headgroup) and a water-insoluble hydrophobic region (the hydrocarbon tail). Examples of membrane lipids are the phospholipids, such as phosphatidylcholine, whose molecular structure is reported in Figure 1.7.

1.3.1 Properties of cell membrane

Experimental investigations on liposomes composed of different phospholipids suggest two possible consistencies for the lipid bilayer: gel-like, where the acyl chains of the polar lipids are packed tightly in a crystalline array; and fluid-like - proposed by Singer and Nicolson in 1972 [28] - where the fatty acyl chains

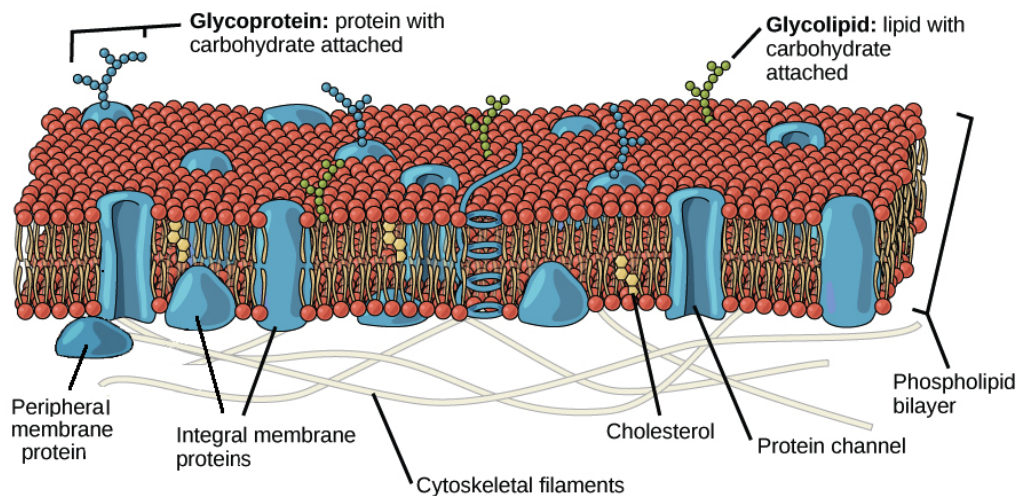


Figure 1.6. The cell membrane structure.

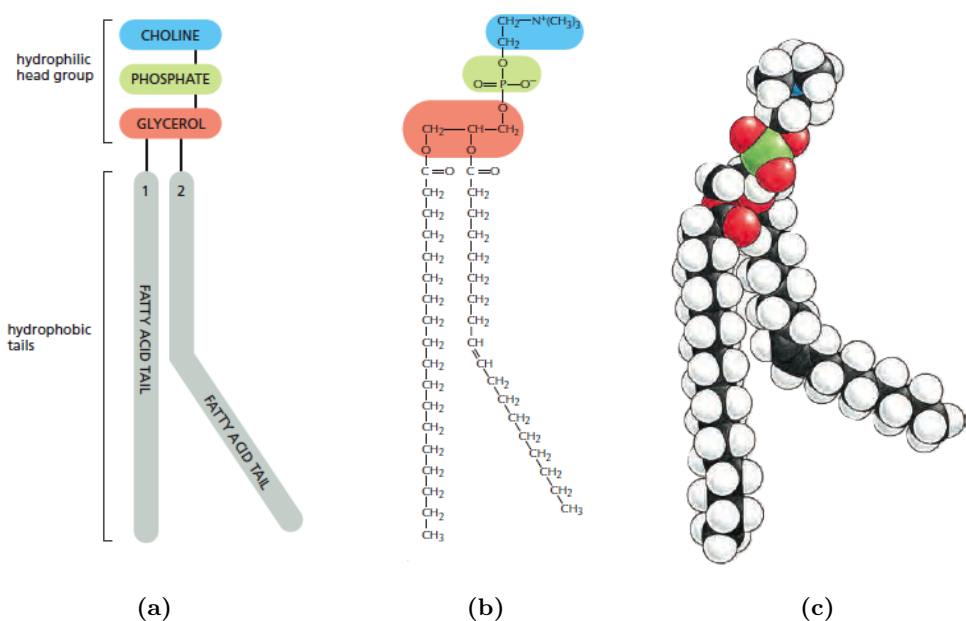


Figure 1.7. The parts of a typical phosphatidylcholine molecule, represented (a) schematically, (b) by formula, and (c) as a computer-generated model.

have increased motion and assume a more random configuration. This change in consistency or phase transition may be explained by a *melting* of the fatty acyl

chains. The temperature at which this occurs depends on a number of factors. In general, fluidity is favored by the presence of fatty acyl chains with short chain lengths or one or more *cis* double bonds. However, biological membranes contain a mixture of fatty acids and so do not show the distinct transition temperatures associated with artificial bilayers. Cells adjust their fatty acid content and maintain membrane fluidity appropriate to their environment.

In the cell membrane, both lipid and protein molecules are arranged in a tightly packed, water excluding mosaic. Interactions occur between the hydrophobic regions of both lipids and proteins in the core of the membrane, although the hydrophobic portions of proteins appear to terminate with positively charged residues at the phospholipid surfaces. At the surfaces, the hydrophilic regions of the amphipathic molecules are in contact with aqueous environments.

Cholesterol, a major component of mammalian plasma membranes, can affect membrane fluidity. By intercalating within the lipid bilayer with its polar-hydroxyl group in contact with the aqueous environment, the rigid steroid nucleus interacts with the fatty acyl chains near their polar-head groups. This dampens the random movement of the fatty acyl chains and also prevents them from making stable interactions with one another. Cholesterol therefore prevents the fluid-to-gel phase transition of the membrane that is normal at low temperatures but, in addition, has an overall dampening effect on membrane fluidity. Both lipids and proteins are free to move laterally in the plane of the membrane, although recent studies suggest that this freedom is to some extent limited by interactions with components of the cytoskeleton, the network of filaments that extends throughout the cytoplasm and supports the cell membrane.

1.3.2 Membrane proteins

Membrane proteins have diverse functions. For example, they function as transport proteins, carrying substances in and out of the cell; receptor proteins, transmitting signals from the exterior to the interior of the cell; or anchor proteins, for the attachment of structural proteins both inside and outside the cell.

Membrane proteins are peripheral when they are bound to the surface of the bilayer or integral when they are embedded in the lipid bilayer. In this latter case, membrane proteins are amphipathic: they have hydrophilic portions which extend from the membrane and hydrophobic or non-polar portions which are associated

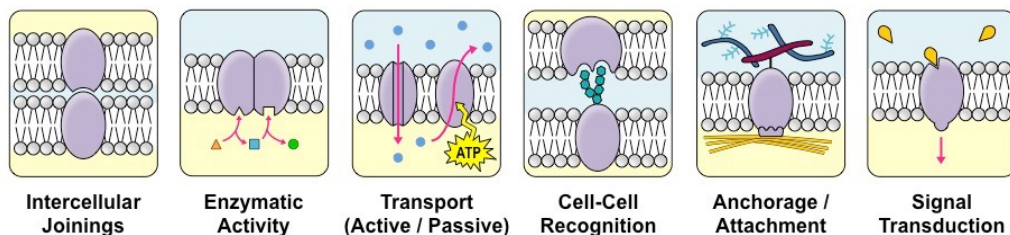


Figure 1.8. *Schematic representation of membrane protein functions.*

with or embedded in the lipid bilayer.

1.3.3 Liposomes and supported lipid bilayers

The basic component of a cell membrane is the lipid bilayer structure. A lipid bilayer exists stably in an electrolyte solution as a vesicle-like structure with an aqueous core, which is named liposome (Figure 1.9(a)). These structures form because hydrophobic interactions occur between the fatty acyl chains, and they are further stabilized by electrostatic and hydrogen-bonding interactions between the polar-head groups of the lipid molecules. Ultrasonication or size reduction by extrusion can produce vesicles having only one bilayer of lipid molecules. Another form of the lipid bilayer is the planar structure that can originate from assembly of lipids on flat surfaces. This structure is called supported lipid bilayer (SLB) (Figure 1.9(b)). Under certain circumstances, the SLB on a solid surface maintains its fluidic properties, and so it can be used as a model of cell membrane. In order to support a high quality membrane (i.e. little or no defects and high lipid mobility) the supporting surface should be hydrophilic, smooth, and clean. [29]

Since plasma membranes allow for incorporation of selected functional components, artificial model membranes are advantageous for the investigation of biomembranes and their physiological functions.

1.4 Graphene-lipid interaction: state-of-the-art

The literature on the biological interactions of graphene nanomaterials is growing rapidly and includes studies primarily motivated by biomedical applications (i.e. graphene-based biosensors, cancer therapy, drug delivery, fluorescence imaging

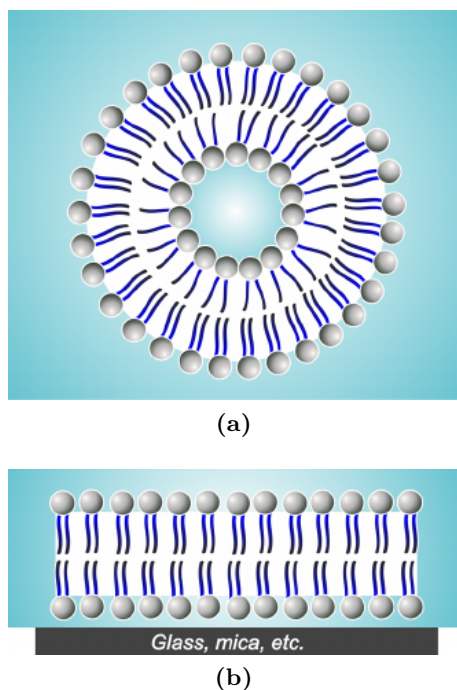


Figure 1.9. *Schematic representation of a (a) vesicle and (b) SLB.*

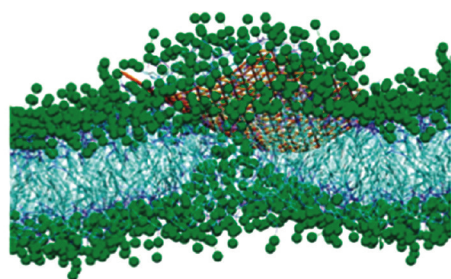
probes, tissue engineering scaffolds), and environmental health and safety. [30] The biomedical and environmental health and safety fields share a common scientific goal: to understand the fundamental interactions of new ultrathin carbon forms with biological molecules.

Biomolecules maintain their activity only in aqueous environment, and target molecules are detected through their adsorption or immobilization to the graphene surfaces supported by the substrates in liquid environment. In biosensing, a number of receptor molecules that could impart selectivity to graphene would work best if they existed in their native environment, for instance, in a phospholipid cell membrane. In many cases, direct covalent or non-covalent binding of functional groups on graphene can often suppress their behavior or even denature them. When interfacing graphene with biological systems such as cells or in using it as transducer for biological sensing, a layer mimicking cellular membranes over graphene could provide a more native environment.

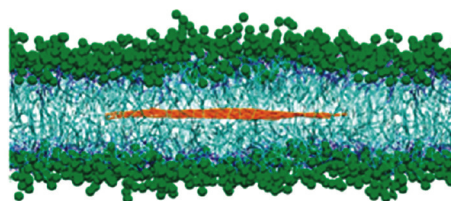
The two-dimensional geometry of graphene may lead to unique interactions with lipid assemblies. Nowadays a large number of graphene-related materials

are fabricated with different methods of production: mono- to few-layer graphene, ultrathin graphite, graphene oxide (GO), reduced graphene oxide (rGO), and porous graphene (PG).

The characteristics of graphene-based materials and their interactions with lipids were first investigated using computer simulations. Titov *et al.* used coarse-grained molecular dynamics simulations to study the interaction of monolayer to few-layer graphene (up to 8 layers) with lipid bilayers. [2] The graphene monolayers were observed to localize in the hydrophobic core (Figure 1.10) with minimal perturbation of the overall bilayer thickness when the graphene layers number is small. This work suggests the ability to form stable and functional graphene-lipid hybrid structures. [30]



(a) Intermediate stage of fusion and entry.



(b) Stable embedding in the hydrophobic core.

Figure 1.10. Molecular dynamics simulations showing fusion of lipid-coated monolayer graphene with a lipid bilayer, leading to localization of the sheet in the interleaflet hydrophobic core. [2]

Phan *et al.* investigated the van der Waals interaction between a lipid membrane and a substrate covered by a graphene sheet using the Lifshitz theory. [31] The calculations showed that a graphene-covered substrate can repel the biological membrane in water. This was attributed to the significant changes in the response properties of the system due to the monolayer graphene. It was also found that the van der Waals interaction is mostly dominated by the presence of graphene, while the role of the particular substrate is secondary. Porous graphene (PG), a graphene sheet with micropores originating from the removal of some sp^2 carbon atoms from the plane, was used in molecular dynamics simulations to characterize DNA sequencing. [32]

Preliminary studies were carried out using several kinds of graphene-based

materials. Tsuzuki, Tero *et al.* described the formation of lipid membranes on reduced GO (rGO), which has amphipathic properties because of the co-existence of hydrophilic areas containing hydroxyl ($-\text{OH}$), carboxyl ($-\text{COOH}$), and epoxide groups ($\text{C}-\text{O}-\text{C}$), and hydrophobic areas similar to perfect graphene. [3] The elimination of these hydrophilic functional groups by reduction (chemical or thermal) increases the hydrophobicity of the GO surface. In the work, the authors showed that continuous and fluid planar lipid membranes were formed on rGO as well as on SiO_2 , and they proposed a structural model of lipid bilayer/lipid monolayer/rGO based on the AFM topography, as showed in Figure 1.11.

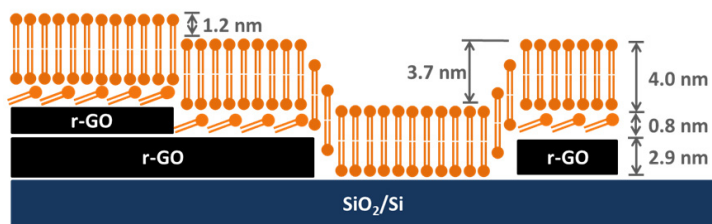


Figure 1.11. Schematic of the structure of lipid membranes on rGO/SiO₂. [3]

Liu, Wang *et al.* reported a method for assembling ordered phospholipid membrane architectures on GO surface to prepare a lipid membrane–graphene hybrid material in aqueous solution. [33] Homogeneous aqueous suspensions of liposome stabilized graphene were prepared through chemical reduction of GO in the presence of liposomes. Different types of liposomes, neutral, anionic and cationic, were prepared and used to investigate their solubilization to graphene sheets. It was found that the neutral (zwitterionic) liposomes are difficult to spread on graphene in aqueous solution, which is likely due to their weakly charged headgroups. However, liposomes formed by positively and negatively charged lipids, could spread well on graphene sheets in aqueous solution. This indicates that the electrostatic repulsion, originating from large amounts of charged liposomes, plays an important role in the dispersion on graphene.

Okamoto *et al.* prepared supported lipid bilayers on GO, to obtain detailed information of biochemical molecules on and in plasma membranes. [34] The GO flakes were deposited on SiO_2/Si surfaces. They found that planar lipid bilayers were formed in a reproducible way on the GO/SiO₂/Si surface in the presence of Ca^{+2} ions, while unruptured vesicles remained on the GO surface without Ca^{+2} ion.

Hirtz et al. [4] reported formation of a lipid monolayer on graphene flakes by dip-pen lithography, using the method shown in Figure 1.12, as confirmed by direct morphological measurements with atomic force microscopy.

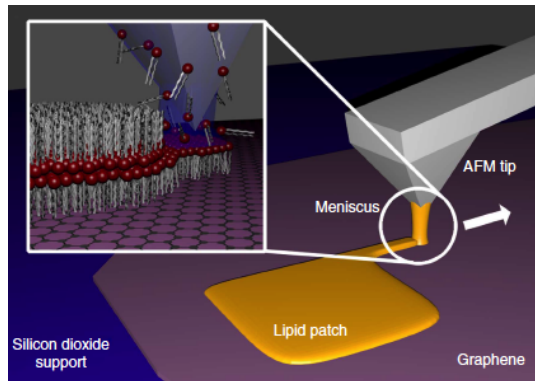


Figure 1.12. Scheme of the lipid writing process with dip-pen nanolithography. [4]

Recently Tabaei *et al.* [5] investigated how the surface properties of graphene affect the self-assembly of phospholipid molecules on graphene, and compared the results with those obtained from oxygen-plasma treated graphene, which is characterized by an increase of surface hydrophilicity. Two fabrication strategies were chosen, the vesicle fusion and the self assembled lipid bilayer methods, and the corresponding membrane formation processes were tracked by quartz crystal microbalance with dissipation monitoring. Depending on the preparation conditions, graphene can support the formation of a lipid monolayer, a lipid bilayer, or intact vesicles, as shown in Figure 1.13.

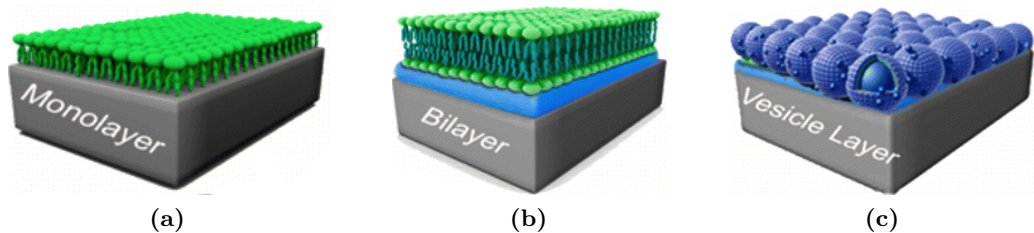


Figure 1.13. Schematic illustration of three model membrane systems: (a) supported monolayer, (b) bilayer, and (c) intact vesicles. [5]

In conclusion the formation of supported lipid membranes is strongly influenced by the physical and chemical properties of the underlying substrates. Hydrophilicity

of the substrate surface is one of the critical factors for the formation and stability of supported lipid bilayer, because the lipid bilayer is a self-assembled structure sustained by hydrophilic/hydrophobic interactions. Generally, complete lipid bilayers form on more hydrophilic surfaces, whereas single lipid monolayers adhere onto more hydrophobic surfaces. The simplest way to evaluate the hydrophilicity (or hydrophobicity) of graphene is through water contact angle (WCA) measurements: this value is of the order of 95° to 100° for a monolayer of graphene [35]. Since graphene is a hydrophobic material, surface treatments are necessary to induce the formation of supported lipid bilayers.

Chapter 2

Materials and methods

2.1 Materials

2.1.1 Synthesis and transfer of graphene

2.1.1.a Synthesis

Chemical vapor deposition (CVD) is a technique that enables deposition of thin solid film on substrates from the vapor species through chemical reactions. Figure 2.1 shows the schematic drawing and a photo of the tube-furnace CVD system for graphene growth at CNR-IMM Bologna. It is composed of a gas delivery system, a reactor and a gas removal system. During the CVD process, reactive gas species are fed into the reactor by the gas delivery system and gas flow is controlled by digital mass flow meters.

The reactor is a quartz tube about 2" in diameter where the chemical reaction takes place and the solid materials are deposited on substrates. Heaters are placed surrounding the reactor to provide high temperatures. The system can operate both at atmospheric (AP) and low pressure (LP). The by-products of the reaction and non-reacted gases are removed by the pump, which is necessary for the LP-CVD configuration. Table 2.1 shows the characteristics of the CVD system.

CVD is far from an easy-to-control process and for graphene synthesis a catalyzed - CVD (C-CVD) technique is used, in which the decomposition of gas molecules is activated by the presence of a catalyst substrate, allowing to reduce the reaction temperature. In addition to the control of parameters such as temperature,

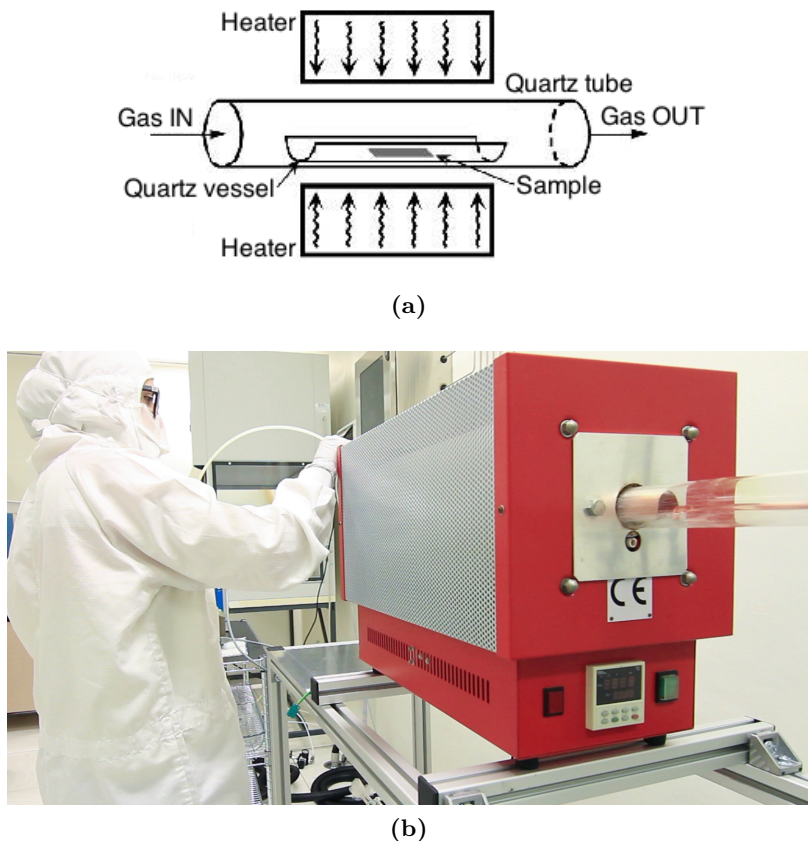


Figure 2.1. Schematic diagram (a) and photo (b) of the tube-furnace CVD system at CNR-IMM Bologna.

pressure, time that other deposition techniques also inevitably encounter, other factors need to be considered carefully to control a CVD process. The transport kinetics of gas species, depending on gas velocity, temperature and pressure, tends to be complicated, with convection and diffusion dominating in different regions of the reactor. The chemical reaction usually involves many intermediate steps, which are not well controlled yet.

The popular recipe for monolayer graphene growth that is currently adopted was first introduced by Li *et al.* and employs a low-pressure mixture of methane and hydrogen flowing over a copper substrate heated to a temperature slightly below its melting point (1000 °C). [36] Because of the low solubility of carbon in copper (<0.001 atomic % at 1000 °C), the growth is restricted to the surface of the catalyst,

Table 2.1. *Characteristics of the CVD system.*

Maximum temperature	1200 °C
Power	1450 W
Process gases	Ar, N ₂ , H ₂ , NH ₃ , CH ₄ , C ₂ H ₂ , O ₂

thus allowing formation of single layer graphene and the process is believed to be self-limiting. Graphene deposition can be divided into two parts: first the pyrolysis of precursor (methane - CH₄) to carbon, and then the formation of graphitic structure from dissociated carbon atoms. The precursor dissociation should occur only on the surface of the catalyst to avoid the precipitation of carbon clusters in the gas phase. The copper catalyst has the function to lower the energy barrier not only of the reaction, thus allowing to operate at lower temperature (1000 °C instead of 2500 °C), but also formation of the graphitic structure. Graphene growth is also strongly dependent on the hydrogen contribution during growth time, where it seems to act both as an activator for the formation of surface-bound carbon and as an etching reagent controlling the grains shape and dimension by etching away the weak carbon-carbon bonds. Hydrogen is also used during the heating and annealing steps for preparing the copper catalyst surface, in order to remove residual copper oxide, and to increase the crystalline grain dimensions of copper. [37]

The overall CVD process is shown in Figure 2.2 and in conclusion it is a multistep reaction involving:

1. binding of active hydrogen atoms to the surface of copper;
2. transport and adsorption of the carbon species on the surface;
3. dissociation of surface-bound methane as a form of active carbon species;
4. graphene nucleation and carbon incorporation into the growing graphene layer;
5. etching of the as-formed graphene.

We use the hot walls reactor shown in Figure 2.1(b), which is connected to a rotary pump allowing to reach the pressure of about 0.1 Torr. Our process is similar to the one previously described, and it can be summarized in:

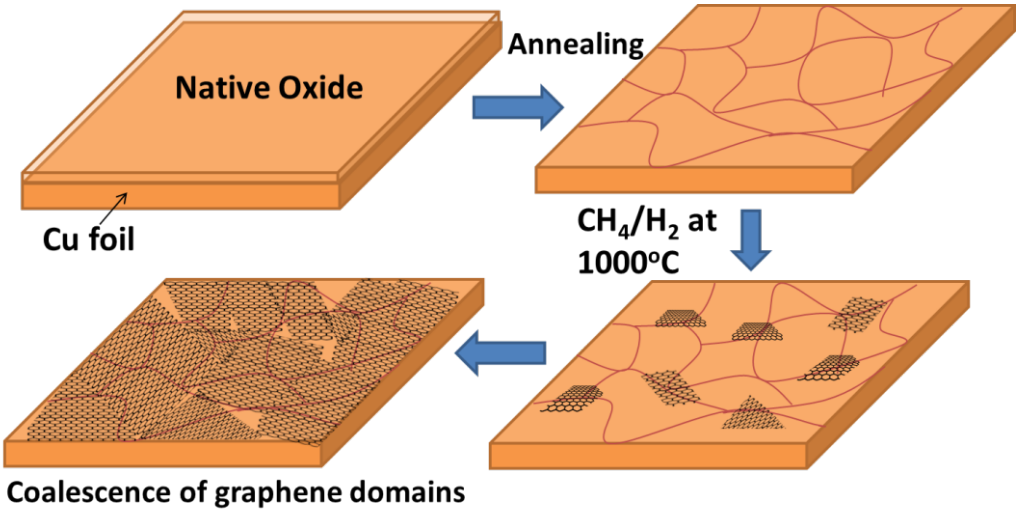


Figure 2.2. Schematic diagram of the formation of graphene after H_2 annealing.

- insert of a oxygen-free high thermal conductivity (OFHC) copper foil (GoodFellow - with characteristics summarized in Table 2.2) with the dimensions of 7 cm $\times$ 6 cm into the quartz furnace;
- start of the rotary pump, in order to reduce the pressure;
- start of a controlled thermal ramp with 10 s.c.c.m. of hydrogen, in order to reach 1000 °C;
- start of the *annealing*, with 50 s.c.c.m. of hydrogen, with constant temperature;
- introduction of methane, with the ratio methane:hydrogen of 50:500 s.c.c.m. for 10 minutes;
- cool down under a flow of 150 s.c.c.m. of argon.

Table 2.2. Characteristics of copper foil from GoodFellow

Purity	99.95 %
Thickness	0.025 mm
Density	8.94 $\frac{\text{g}}{\text{ml}}$

2.1.1.b Transfer

After synthesis, the graphene film is transferred: in order to use graphene as a highly conductive substrate for biosensing applications, it must be removed from the conducting catalyst surface and transferred onto an insulating surface. The one-atom thick and highly hydrophobic layers of graphene can be easily fractured or folded, making the post-growth processing a delicate task, especially for device fabrication. Graphene transfer is carried out - using poly(methylmethacrylate) (PMMA) as a supporting layer - by removing the graphene layer on the bottom surface of the catalyst and etching the underlying catalyst. Figure 2.3 shows a schematic diagram of the transfer of graphene with PMMA as media.

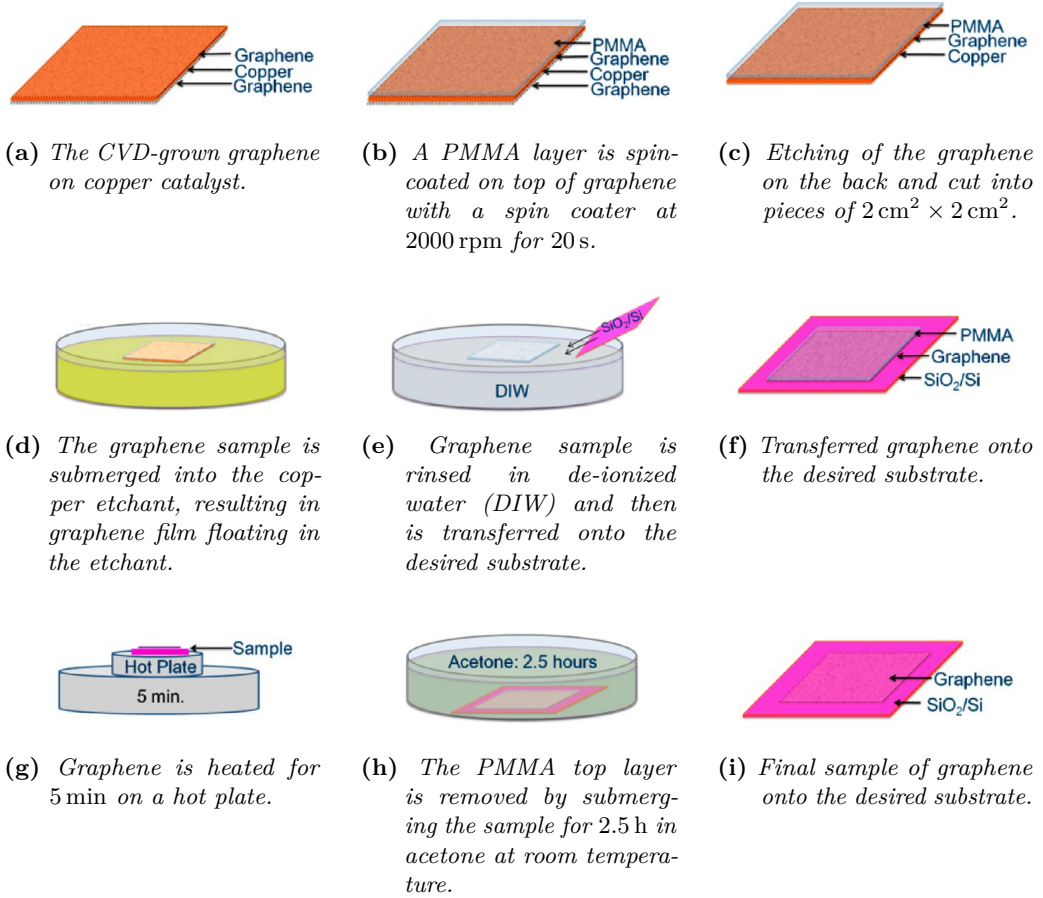


Figure 2.3. Schematic diagram of the transfer of graphene with PMMA as media. Adapted from [6, 7].

Although PMMA is highly effective in providing mechanical strength to the film and keeping it intact, its residues are known to induce defects on graphene and/or affect its electronic mobility. [38] A thin layer (1 nm) of PMMA remain adsorbed on graphene even after wash-off in acetone, giving the film a mild p-type doping. [39] In our research we use cyclododecane as supporting material for the transfer and handling of few-layer graphene films. [40] Cyclododecane ($C_{12}H_{24}$) is a cyclic hydrocarbon, with the chemical structure shown in Figure 2.4, appearing as a white waxy solid made of large translucent crystals. It is readily soluble in non-polar and aromatic solvents, while it is insoluble in polar solvents such as water, being highly hydrophobic. This non-toxic and eco-friendly organic compound is solid at room temperature, has a low density (0.82 g/cm^3 at 80°C) and a high vapor pressure (1.33 kPa at 100°C). Its melting point is 60.7°C , while the boiling point is 247°C . Thanks to these characteristics, cyclododecane completely sublimates upon air exposure.

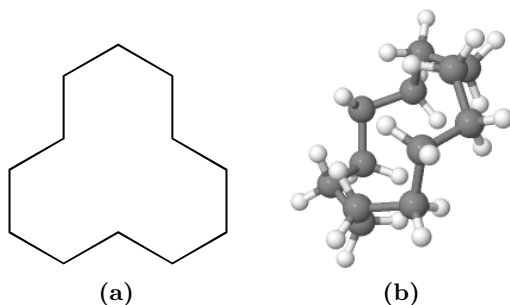


Figure 2.4. Chemical structure (a) and 3D view (b) of cyclododecane.

Figure 2.5 shows a schematic diagram of the steps to transfer graphene using cyclododecane: the process is quite similar to the one using PMMA and cyclododecane must be highly pure to avoid any residue. It could be also beneficial in the fabrication of graphene-based biosensors with supported lipid membranes, since it can be diluted in chloroform, which is the solvent in which also liposomes can be diluted.

2.1.2 Preparation of liposome solutions

Liposome extrusion is currently one of the most common procedures for producing unilamellar liposomes (also known as vesicles) on a research scale. The

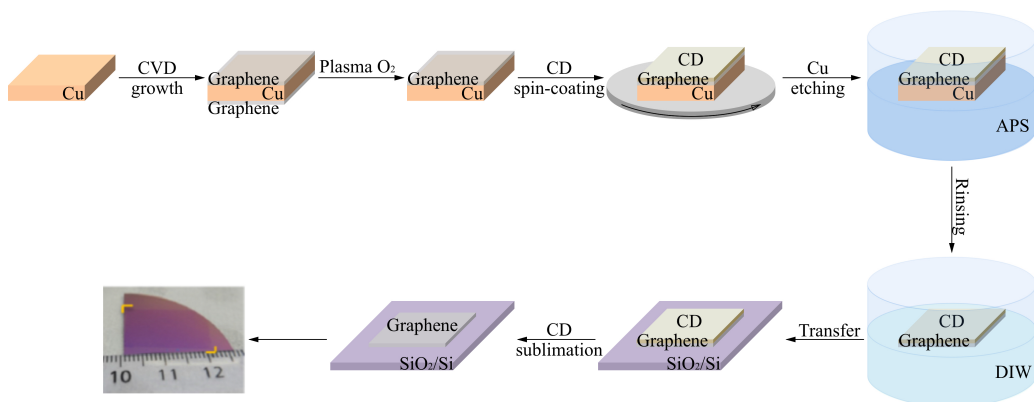


Figure 2.5. *Schematic diagram of transfer of graphene using cyclododecane.*

advantages of the extrusion technique are the following: it can be applied to a wide variety of lipid species and mixtures, it works directly from multilamellar vesicles (MLVs), and it is rapid. Problems related to the removal of organic solvents or detergents from final preparations, common in other methods, are eliminated, and it is possible to prepare homogeneous populations of unilamellar liposomes in the desired size range. Multilamellar vesicles are forced through polycarbonate filters with defined pore sizes to obtain a liposome population with a mean diameter that reflects the diameter of the filter pore: as the concentric layers of the multilamellar liposome deform to pass through the pore, a breaking and resealing of the membranes occurs multiple times, which favors the formation of unilamellar vesicles. [41]

The liposome extrusion procedure starts from the choice of phospholipid: in this study 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) and 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (EPC), whose chemical structure is shown respectively in Figure 2.6, Figure 2.7 and Figure 2.8, were chosen. DOPC, DOPS and EPC were purchased in form of powder from Avanti Polar Lipids and kept frozen at -20°C until use. Table 2.3 shows their characteristics.

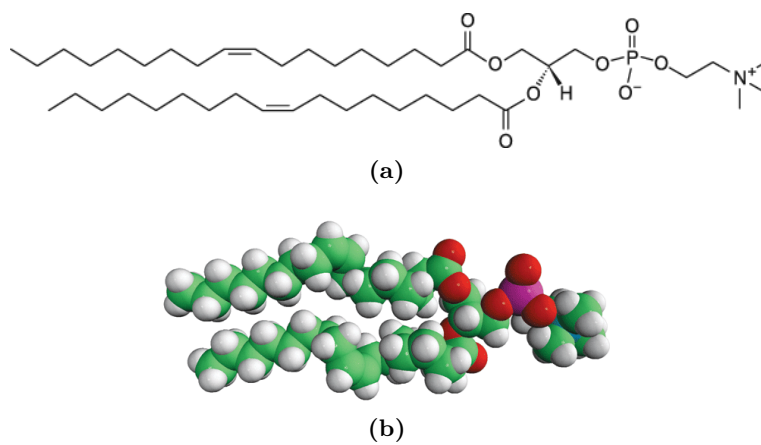


Figure 2.6. Chemical structure (a) and 3D structure (b) of DOPC.

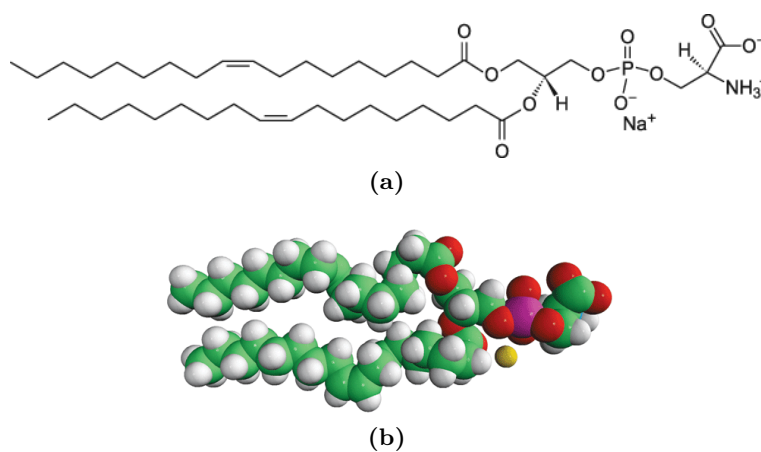


Figure 2.7. Chemical structure (a) and 3D structure (b) of DOPS.

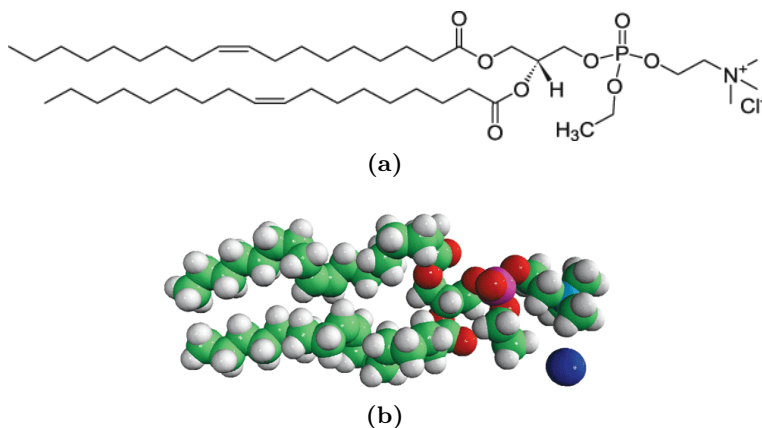


Figure 2.8. Chemical structure (a) and 3D structure (b) of EPC.

Table 2.3. Properties of DOPC, DOPS and EPC lipids, as given by Avanti Polar Lipids Inc.

Product name	DOPC	DOPS	EPC
Molecular weight	786.113	810.025	850.628
Chemical formula	$C_{44}H_{84}NO_8P$	$C_{42}H_{77}NO_{10}PNa$	$C_{46}H_{89}NO_8PCl$
Transition temperature	$-17^{\circ}C$	$-11^{\circ}C$	$-63^{\circ}C$

DOPC is a zwitterionic lipid in nature, i.e. it has a zero net charge in commonly used buffers, while DOPS has a net negative charge, and EPC has a net positive charge. Their transition temperatures, reported in Table 2.3, are far below room temperature, which allows the extrusion procedure to be conducted at room temperature. In this work, DOPC is hydrated using a phosphate buffered saline (PBS) solution at physiological pH (7.4); we also prepared a solution made up of a mixture of lipids, in particular DOPC:DOPS (2:1) and DOPC:EPC (2:1) by hydrating and mixing them in chloroform, then we let the chloroform evaporate in a controlled environment and then diluted them in PBS. The obtained solutions ($1 \frac{mg}{ml}$) are stirred for about 1 h before extrusion. PBS is purchased from Sigma-Aldrich, and dissolved in ultrapure water ($18.2 M\Omega$, <5 ppb total organic carbon) produced by a Millipore Direct-Q UV water purification system. The second step is the extrusion of liposomes starting from the solution previously described: the Avanti Mini-Extruder is used to prepare small, unilamellar vesicles, with sequential extrusion through two different filters with decreasing pore size. The Avanti Mini-Extruder is shown in Figure 2.9; it is essentially composed of two syringes, a

polycarbonate membranes assembled in a central fitting piece and a holder/heating block.



Figure 2.9. *Mini-Extruder from Avanti Polar Lipids.*

To obtain a narrow size distribution of the liposome population, it is necessary to perform the extrusion of the liposome suspension several times through the filter membrane. In this study, small unilamellar vesicles are formed by pushing the suspension 5 times through a membrane with pore size of 800 nm and 31 times through a membrane with the desired pore size (50 nm and 100 nm). The size distribution of the liposome population after extrusion was previously determined by dynamic light scattering measurements, confirming the homogeneous size distribution. [42] After the final extrusion, the lipid solution is stored at 4 °C for a maximum of 7 days.



Figure 2.10. *Lipid solution (left) and liposome solution after the extrusion through a membrane with pore size of 50 nm.*

2.2 Characterization methods

Since the properties of graphene are largely determined by its quality and number of layers, the characterization of graphene is an important issue.

2.2.1 Micro-Raman spectroscopy

Vibrational spectroscopy is commonly used to study a very wide range of materials and can be carried out both for a simple identification test (by observing some peculiar peaks) and for an in-depth, qualitative and quantitative analysis on a complete spectrum. Vibrational spectroscopy includes several different techniques, among which the most important are mid-infrared (IR), near-IR, and Raman spectroscopy. All these techniques have various advantages and disadvantages with respect to instrumentation, sample handling, and applications.

Raman spectroscopy involves the study of the interaction of radiation with molecular vibrations, that is the manner in which photon energy $E = h \cdot \nu$ is transferred to the molecule by changing its vibrational state. A photon excites the molecule from the ground state to a virtual energy state: when the molecule relaxes it emits a photon. The scattered light photons include mostly the dominant Rayleigh scattering component and the very small amount of Raman scattered photons. No energy is lost for the elastically scattered Rayleigh light while the Raman scattered photons lose some energy relative to the exciting energy to the specific vibrational coordinates of the sample. Rayleigh scattering is the most probable event and the scattered intensity is about 10^{-3} less than that of the original incident radiation. This scattered photon results from a transition from the virtual state back to the ground state and is an elastic scattering of a photon resulting in no change in energy. Raman scattering is far less probable than Rayleigh scattering with an observed intensity that is approximately 10^{-6} that of the incident light for strong Raman scattering. This scattered photon results from a transition from the virtual state to the first excited state of the molecular vibration. This is described as an inelastic collision between photon and molecule, since the molecule acquires different vibrational energy and the scattered photon now has different energy and frequency. As shown in Figure 2.11 two types of Raman scattering exist: Stokes and anti-Stokes. Molecules initially in the ground vibrational state give rise to Stokes Raman scattering while molecules initially in

vibrational excited state give rise to anti-Stokes Raman scattering. The intensity ratio of the Stokes relative to the anti-Stokes Raman bands is governed by the absolute temperature of the sample, and the energy difference between the ground and excited vibrational states.

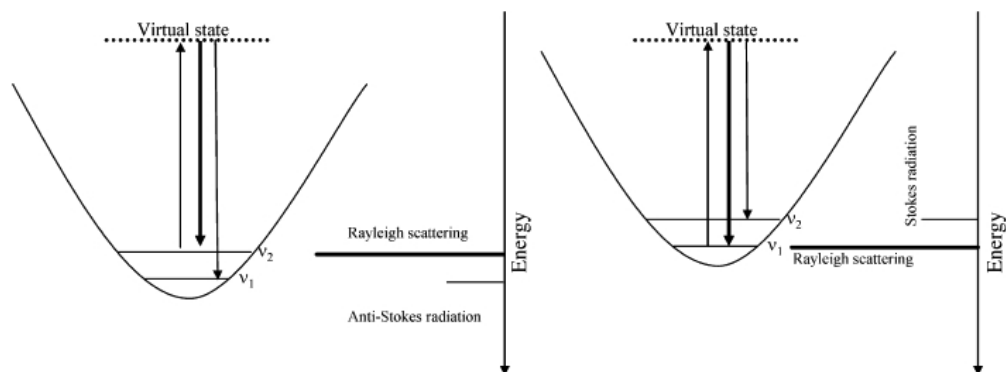


Figure 2.11. *Schematic representation of the elastic (Rayleigh) and inelastic scattering (Stokes on the right and anti-Stokes on the left) related to a single vibrational mode.*

The Raman vibrational bands are characterized by their frequency (energy), intensity (polar character or polarizability), and band shape (environment of bonds). Since the vibrational energy levels are unique to each molecule, the Raman spectrum provides a *fingerprint* of a particular molecule. The frequencies of these molecular vibrations depend on the masses of the atoms, their geometric arrangement, and the strength of their chemical bonds. Raman spectroscopy has historically played an important role in the study and characterization of graphitic materials. For sp^2 nanocarbons, Raman spectroscopy can give information about crystallite size, clustering of the sp^2 phase, the presence of sp^3 hybridization and chemical impurities, mass density, optical energy gap, elastic constants, doping, defects and other crystal disorder, edge structure, strain and number of graphene layers. Figure 2.12 shows a typical Raman spectra from monolayer graphene. The two most prominent features are the G band at about 1585 cm^{-1} and the 2D band at about 2700 cm^{-1} . The G band corresponds to a vibrational mode common to sp^2 carbon materials. The 2D band is related to a second-order two-phonon¹

¹A phonon is a quantum mechanical description of an elementary vibrational mode in which a lattice of atoms uniformly oscillates at a single frequency; in classical mechanics this is known

process (in-plane optical mode) and exhibits a strong frequency dependence on the energy of the excitation laser. In addition to the G band and 2D band peaks, a low intensity peak is observed at about 1346 cm^{-1} corresponding to the D band, which is associated with structural defects: the I_D/I_G intensity ratio gives informations about the amount of defects in the lattice; the nature of those defects will be further discussed. The I_G/I_{2D} intensity ratio gives informations about the number of layers: 2D band in monolayer graphene is roughly 4 times more intense than the G band, and the intensity decreases with increasing the number of graphene layers. Thus Raman spectroscopy can distinguish a single layer, from a bilayer or few layers. [43–48]

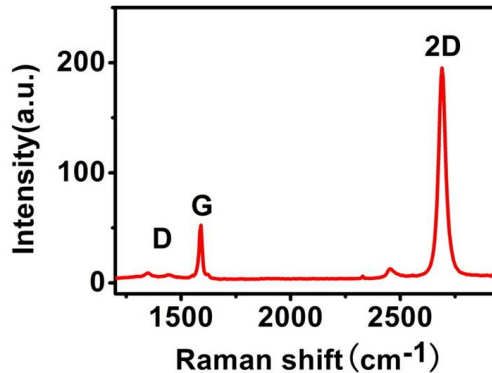


Figure 2.12. *Raman spectra from crystalline monolayer graphene.*

Micro-Raman spectroscopy involves the association of an optical microscope with a Raman spectrometer: the excitation laser beam is focused onto the same surface through the objective of the optical microscope, which allows a reduction of the light probe to $1\text{ }\mu\text{m}$ size and the radiation scattered from the sample, collected by the microscope, is analyzed by the spectrometer. In this study, Raman spectroscopy was carried out at CNR-ISMN (Institute for Nanostructured Materials) Bologna using the system shown in Figure 2.13, which is composed by a Leica DM/LM microscope and a Renishaw spectrometer, with an excitation laser beam of wavelength $\lambda = 488\text{ nm}$ and a laser spot size of $1\text{ }\mu\text{m}$. The spectrometer is equipped with a computer controlled $x - y$ stage for mapping the Raman signal

as a normal mode. Normal modes are important because any arbitrary lattice vibration can be considered as a superposition of these elementary vibrations.

on the sample.



Figure 2.13. *Micro-Raman system at CNR-ISMN Bologna.*

2.2.2 Water contact angle measurements

The term *wetting* describes the contact between a liquid and a solid surface, which depends on intermolecular interactions when the two are brought together. The amount of wetting depends on the energies (or surface tensions) of the interfaces involved such that the total energy is minimized. The degree of wetting of a surface or interface can be described by the contact angle, that is the angle at which the liquid/vapor interface meets the solid/liquid interface. For flat surfaces the contact angle is measured from a drop of a suitable liquid resting on a surface (sessile drop method). If the liquid is very strongly attracted to the solid surface, as for example, water on a hydrophilic solid, the droplet will completely spread out on the solid surface and the contact angle will be close to 0° . Less strongly hydrophilic solids will have a contact angle up to 90° . If the solid surface is hydrophobic, the contact angle will be larger than 90° . The contact angle thus directly provides information on the interaction energy between the surface and the liquid. The theoretical description of contact arises from the consideration of a thermodynamic equilibrium between three phases as shown in Figure 2.14: the liquid phase (L), the solid phase (S), and the gas/vapor phase (V) (the latter will be a mixture of ambient atmosphere and an equilibrium concentration of the liquid vapor). As first described by Thomas Young in 1805, the contact angle of a liquid drop on an ideal solid surface is defined by the mechanical equilibrium of the drop under the

action of three interfacial tensions. [49]

$$\gamma_{LV} \cdot \cos \theta = \gamma_{SV} - \gamma_{SL} \quad (2.1)$$

γ_{LV} , γ_{SV} , and γ_{SL} represent the liquid-vapor, solid-vapor, and solid-liquid interfacial tensions, respectively, and θ is the contact angle. Equation (2.1) is usually referred to as Young's equation, and θ is Young's contact angle. [50–52]

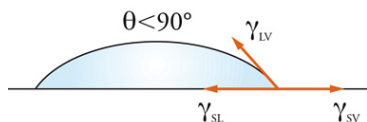


Figure 2.14. *Illustration of contact angle formed by sessile liquid drop on a smooth homogeneous solid surface.*

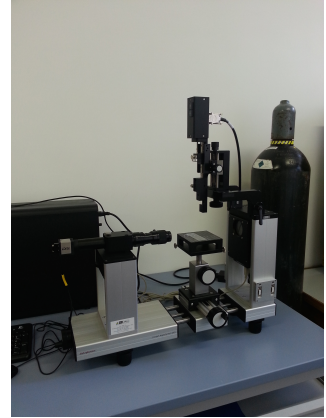
In this work we measure the contact angles using a Digidrop GBX Model D at CNR-ISMN Bologna, shown in Figure 2.15(a) and with the DataPhysics OCA 15Pro at Department of Chemical Engineering, Materials and Environment (DICMA) of Sapienza University of Rome, shown in Figure 2.15(b). They both measure the angle formed between the liquid/solid interface and the liquid/vapor interface using a high-resolution camera and software to capture and analyze the contact angle. The Digidrop GBX Model D can measure contact angle ranging between 0° and 180° with an accuracy of $\pm 0.1^\circ$ and surface energy between $0 \frac{\text{mN}}{\text{m}}$ and $1000 \frac{\text{mN}}{\text{m}}$. The DataPhysics OCA 15Pro can measure contact angle ranging between 0° and 180° with a $\pm 0.1^\circ$ measuring precision, and surface energy between $0.01 \frac{\text{mN}}{\text{m}}$ and $2 \frac{\text{mN}}{\text{m}} \times 10^3 \frac{\text{mN}}{\text{m}}$ with a resolution of $\pm 0.01 \frac{\text{mN}}{\text{m}}$. The water contact angle of water on a monolayer of graphene is in the order of 95° – 100° . [35]

2.2.3 Scanning electron microscopy

A scanning electron microscope (SEM) is an electron microscope that produces images of a sample by scanning it with a focused beam of electrons. An electron microscope has greater resolving power than a light microscope (resolving power is inversely proportional to wavelength, and electrons wavelength is lower than the one of photons). The electron microscope uses electrostatic and electromagnetic lenses to control the electron beam and focus it to a spot with a diameter of



(a)



(b)

Figure 2.15. (a) *Digidrop GBX Model D at CNR-ISMN Bologna and (b) Data-Physics OCA 15Pro at DICMA - Rome.*

nanometer-size to form an image. These electron optical lenses are analogous to the glass lenses of a light optical microscope.

When the primary electron beam interacts with the sample, the electrons lose energy by repeated random scattering and absorption within a teardrop-shaped volume of the specimen known as the interaction volume, as can be seen in Figure 2.16.

The size of the interaction volume depends on the landing energy of electrons, the atomic number of the specimen and the density of the specimen. The energy exchange between the electron beam and the sample results in the reflection of high-energy electrons by elastic scattering, emission of secondary electrons by inelastic scattering and the emission of electromagnetic radiation, which can be detected by specialized detectors.

As a powerful technique, SEM has been extensively used for imaging new materials, especially at micro- and nanoscales. In particular, SEM is a rapid, non-invasive and effective manner for imaging the morphologies of graphene films. SEM has the advantages of detecting impurities, ruptures, folds, voids and discontinuities of synthesized or transferred graphene on a variety of substrates. However, SEM imaging of graphene is difficult mainly because of the following reasons: first, acquiring images of an atomic-thick layer (e.g. graphene) is quite challenging due to the resolution limitation of SEM; secondly, the ultra-thin graphene layer is

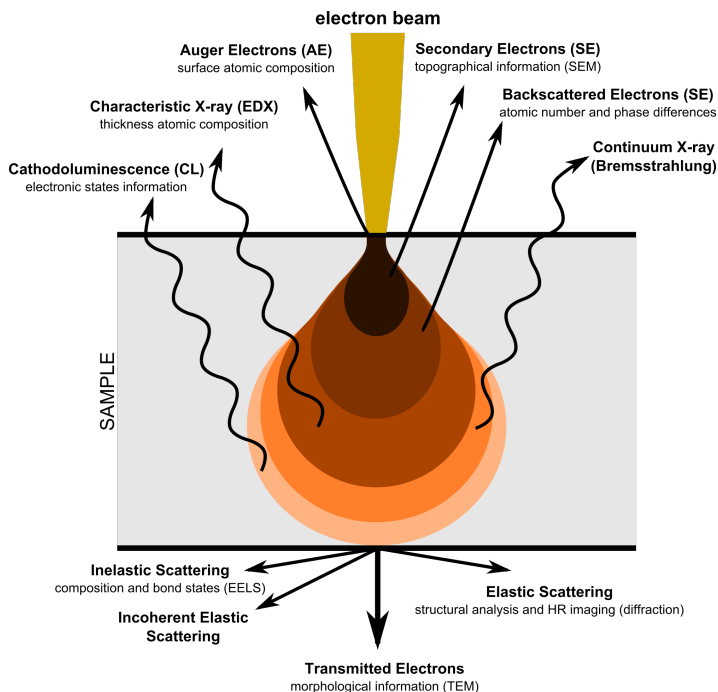


Figure 2.16. *Mechanisms of emission from the interaction volume of the sample.*

"transparent" to high energy electron beams. Hence SEM images easily display the morphologies of the substrate beneath the graphene, not the graphene itself. Furthermore, the graphene films are too smooth to generate sufficient contrasts in SEM imaging and it can be observed only if folds or wrinkles are present. Although such a weak contrast can be enhanced by digital imaging processing, this process also magnifies the noise level simultaneously and may lead to an unacceptable image quality. SEM imaging of graphene is based on three typical contrasts: surface roughness contrast, edge contrast, and thickness contrast. [53]

In this study SEM images were acquired using SEM Zeiss Gemini 1530 (Figure 2.17), which is equipped with a field emission gun as electron source. This kind of emitter is superior to thermoionic source because the electron beam is smaller in diameter, more coherent and with up to three orders of magnitude greater current density (or brightness) than can be achieved with conventional thermoionic emitters, thus resulting in a significantly improved signal-to-noise ratio and spatial resolution, and greatly increased emitter life and reliability compared with thermoionic devices.



Figure 2.17. SEM Zeiss Gemini 1530.

Table 2.4. Characteristics of SEM Zeiss Gemini 1530.

Accelerating voltage	0.2 kV–30 kV
Type of emitter	Schottky field emitter
Resolution at 20 kV	1 nm
Resolution at 1 kV	2.1 nm

2.2.4 X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) is the study of the energy distribution of the electrons emitted from X-ray irradiated materials. In principle all electrons, from the core to the valence levels, can be studied. In this respect the technique differs from ultraviolet photoelectron spectroscopy, in which only the valence electrons can be studied. The relevance of photoelectron spectroscopy to the understanding of chemical structure became evident with the discovery of shifts in the K-shell photolines of different compounds of the same element by a group from Uppsala. [54]

The photoelectric interaction between monoenergetic X-rays and a solid or

gaseous sample causes electrons to be ejected with discrete kinetic energies. The observable quantities in photoelectron spectroscopy are thus the kinetic energies corresponding to discrete electron "lines". Electron that suffer energy losses in traversing the sample are seen in the spectral background.

Energy conservation for the photoemission process can be expressed by the equation

$$E_{h\nu} = E_k + E_\Phi + E_B(i) \quad (2.2)$$

where $E_{h\nu}$ is the X-ray energy, E_k is the photoelectron kinetic energy, E_Φ is a small correction for solid effects (work function, etc.), and $E_B(i)$ is the electron binding energy on the i^{th} level. An electron energy analyzer is used to scan the kinetic energy spectrum and to record the E_k values of the discrete photolines. The value of E_Φ is either known or is assumed to be constant for a given system. Thus binding energies $E_B(i)$ can be determined by using the energy conservation equation.

The power of the X-ray photoelectron method lies in the fact that the measured quantity, the electron binding energy of an atom, is a function of the chemical environment of the atom.

The XPS spectrometer in the Nanochemistry Labs at the ISOF Institute in the CNR Bologna Research Area is mounted on a pre-existing Ultra-high-vacuum (UHV) system which had previously hosted an Omicron scanning tunnel microscope (STM) and it is shown in Figure 2.18(a). The XPS spectrometer operates at a working pressure of 10^{-10} mbar, with a depth of analysis of 3 nm and an analyzed area of $2.5 \text{ mm} \times 7 \text{ mm}$.

One of most critical contaminants of graphene is water, so all samples were heated in the UHV preparation chamber at 200°C for 1 h. Figure 2.19 shows the XPS C 1s EDCs of (a) pure CVD graphene directly grown on nickel [8], where only the sp^2 component is present, and, for comparison, (b) oxidized CVD graphene directly grown on iridium (Ir) [9].

Figure 2.19(b) shows a much more complex structure of the C 1s signal with respect to that of pure CVD graphene due to the contribution of different bonded carbon species such as sp^2 aromatic or sp^3 defective carbon, or C–O functionalizations including hydroxyl (C–OH), epoxy (CO–C), carbonyl (C=O) and carboxyl (O=C–O). The spectrum taken from [9] is one of most accurate studies on the

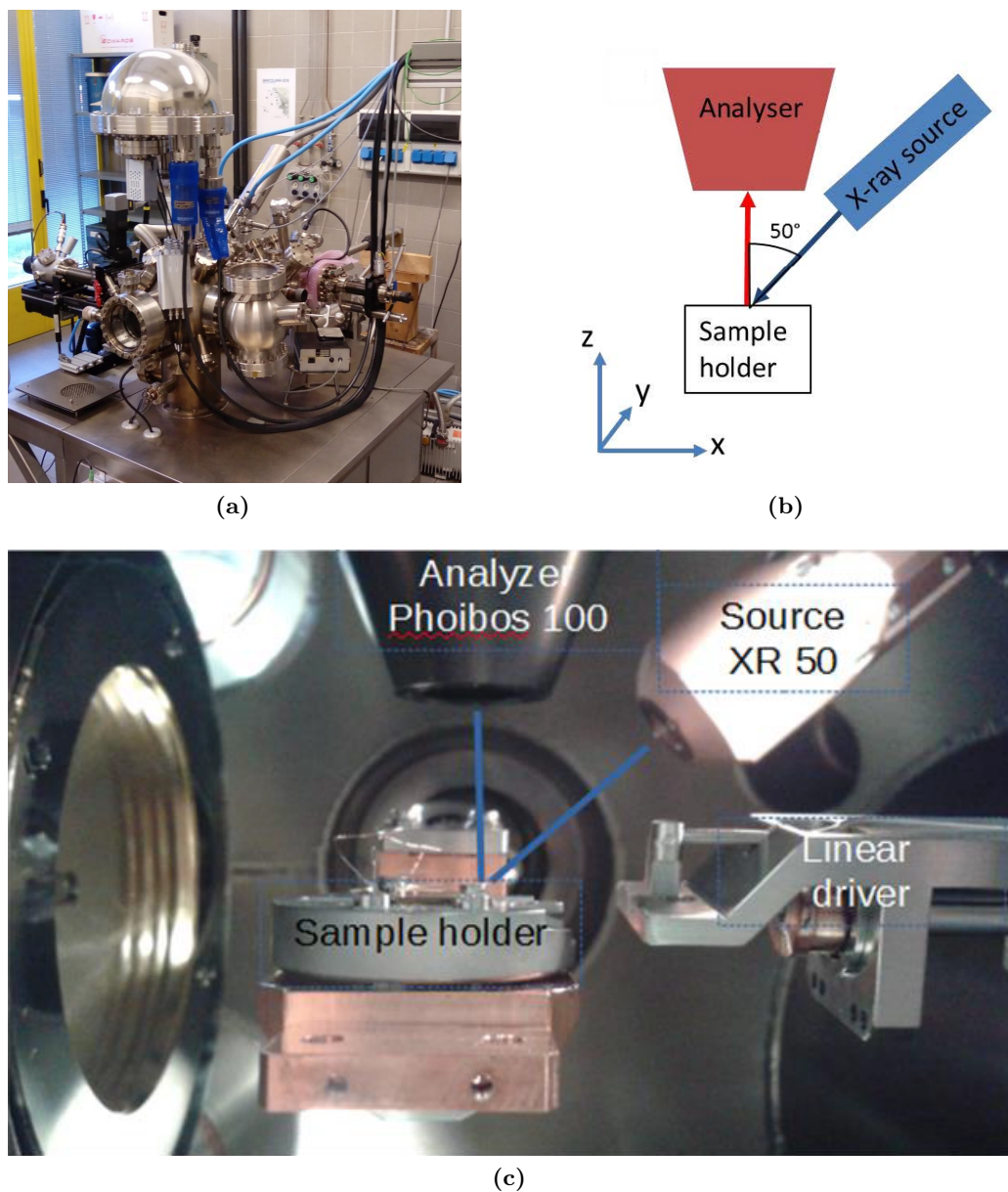


Figure 2.18. (a) Photo of the XPS spectrometer assembled on the UHV analysis chamber; (b) scheme and (c) photograph of the inside of the analysis chamber xz view point. Both the X-ray source and the analyzer are aligned with the upper surface of the sample holder.

oxidation of CVD graphene under extremely well-controlled conditions, pristine graphene CVD on iridium is not shown as it is similar to that obtained on nickel

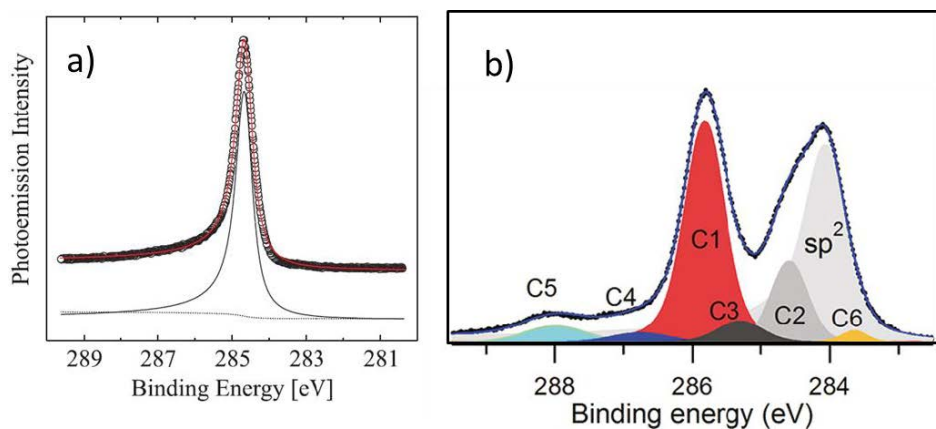


Figure 2.19. Comparison XPS C 1s spectra of (a) CVD graphene on nickel foil after thermal annealing and (b) oxidized CVD graphene on iridium. Peak labels in (b) are: sp^2 aromatic carbon; C1 epoxy; C2 defects; C3 hydroxyl; C4 carbonyl; C5 carboxyl; C6, C-Ir bond, sp^2 aromatic carbon. Reproduced with permission from (a) ref [8] and (b) [9].

or copper.

Due to the complexity of the XPS spectrum, a detailed analysis requires the use of a robust fitting procedure which takes into accounts the physico-chemical properties of the surface. In particular there are two main issues: chemical shift and peak line shape.

Carbon-carbon bond distributions can be described using two components: (sp^2) aromatic and (sp^3) defects having different energies. All the chemical shifts are referred to the position of the sp^2 C 1s signal. The sp^3 contribution derives from the presence of topological defects (single atoms or nanometric holes) where different carbon bonding is present. The resolution of a standard XPS spectrometer (almost 0.9 eV) does not allow such fine distinctions between the various different defects. For this reason, the sp^3 contribution is considered here as a single peak shifted by +0.6 eV with respect to that from sp^2 .

Peaks deriving from sp^2 and sp^3 carbons show different line-shapes. The sp^2 hybridization is associated with the semi-metallic nature of graphene. In perfect graphene or in pristine graphite, it is the only chemical state of carbon and the photoemission spectrum of this group has a typical asymmetric line-shape usually associated with shake-up in metallic-like materials [55–57], as also shown in Figure 2.19(a). On the other hand, the sp^3 carbons and all the other functional

groups are described by simple Lorentzian (L) curves.

The use of the asymmetric line-shape in the fitting procedure is crucial because the long tail of the asymmetric curve (see Figure 2.20) strongly influences interpretation of the data to estimate the relative abundance of the other functional groups. The incorrect use of a symmetric curve to describe the sp^2 component corresponds to an underestimation when measuring higher relative binding energies. In the fitting procedure, this underestimation is compensated by a systematic overestimation of all the other components.

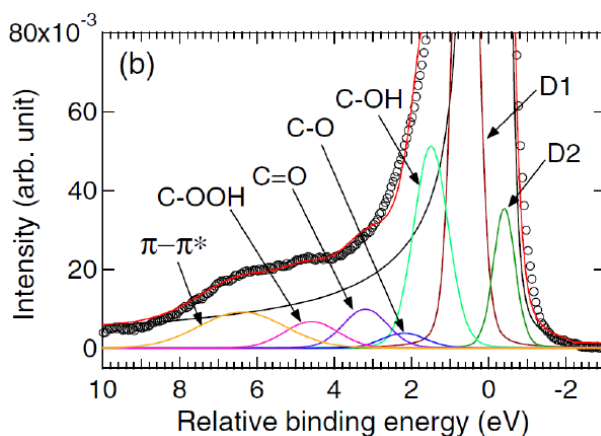


Figure 2.20. Detail of hard-X-ray photoemission spectroscopy $C\ 1s$ spectrum in graphitized carbon. The spectrum is fitted using an asymmetric line-shape for the sp^2 component and a symmetric one for the other components. Functional groups: sp^2 regions (black curve), defect due to metals (D2), sp^3 and other topological defects (D1), hydroxyl (C-OH), epoxy (C-O), carbonyl (C=O), carboxyl ($-COOH$) and shake-up ($\pi - \pi^*$) [6].

We fitted the measured XPS spectra using the so-called Asymmetric Pseudo-Voigt (LF): the asymmetry is described by considering separately the two sides of the peak using two damped Lorentzian functions having the same width.

Summarizing, all the fitting procedures were carried out using the CasaXPS software, by using a set of two functions, as reported in Table 2.5:

- Voigt function, to describe the symmetric peaks;
- LF function, to describe the asymmetric sp^2 peak.

One of the most commonly used parameters to compare the chemical properties

Table 2.5. *List of used fitting curves for the various functional groups and corresponding chemical shifts.*

Functional Group	Chemical shift [eV]	Line-shape (fitting procedure)
C=C (sp ² aromatic)	-	LF
C–C (defects)	0.6	V
C–OH	1.3	V
C–O–C	2.3	V
C=O	3.6	V
O–C=O	4.7	V

of different graphene-based materials is the oxygen-to-carbon ratio (O/C). In general, the O/C ratio can be calculated using two methods:

- Comparing the areas of O 1s and C 1s peaks;
- Fitting analysis of the C 1s signal.

The first consists of estimating the ratio between the areas of oxygen and C 1s signals, by taking into account the photoemission cross-section and the transmission of the analyzer. This is the most accurate and effective method, where no systematic errors are present. This procedure can be applied only in the case of oxygen-free substrates. For example, this procedure cannot be used when graphene is supported on a common substrate such as silicon, because of the native oxide layer (about 2 nm thick).

The second method consists of a quantitative analysis of the C 1s signal using the fitting approach described above. In this case, both the photoemission cross-section and the analyzer transmission are constants. The total area (A_{tot}) of all the measured peaks due to the chemical shifts is proportional to the number of carbon atoms. The area of a single peak (A_i) is proportional to the number of carbon atoms associated with each group. In this way, it is possible to associate the relative amounts of oxygen atoms with respect to carbon (O/C): hydroxyl (1 to 1), epoxy (1 to 2), carbonyl (1 to 1) and carboxyl (2 to 1).

$$(O/C)_{fit} = \sum_i \frac{n_i \cdot A_i}{A_{tot}} = \frac{A_{C-OH} + A_{C=O} + \frac{1}{2} \cdot A_{C-O-C} + 2 \cdot A_{O-C=O}}{A_{tot}} \quad (2.3)$$

Using this method it is not necessary to monitor the oxygen photoemission peak and thus, it can be applied to all kinds of substrates, even in the case of oxygen-rich substrates such as those in devices or thin films. However, this method can be strongly affected by the over/under estimation of the various contributions to the C 1s envelope and the experimental error (up to 10%) is usually larger than in the case of the first method. For this reason, correct application of the fitting procedure is crucial when using the second method. [58]

2.2.5 Atomic force microscopy

Conventional optical microscopes have a resolution limit which is determined by the aperture of the object lens, and a resolution better than about one-half the wavelength of the light used can principally not be achieved. Electron microscopes typically have resolutions of 20 nm vertical and 1 nm lateral, but their known disadvantage is that most surfaces are severely damaged due to the high energies of the electron beam required in achieving a high resolution, to the high vacuum and to the conductive samples.

The AFM technique was introduced in 1986 by Binnig, Quate, and Gerber with the intention of overcoming the drawbacks of its predecessor, the scanning tunneling microscope (STM). [59,60] The most prominent upgrades are the capability of AFM to also measure non-conductive samples and to measure the surface topography of samples at sub-nanometric resolution. [61] A schematic representation of the basic AFM set-up is shown in Figure 2.21. When using AFM, a tip attached to a flexible cantilever moves across the sample surface, measuring the surface morphology on the atomic scale. The principle underlying the microscope is based on the insight that if atoms are approached to one another so closely that their electron clouds touch (i.e., that there is a low-level overlap of the wave functions of the atom at the apex of a sharp tip with the surface atoms of the sample to be inspected), interatomic forces occur. There are a number of ways to detect the deflection of the cantilever in an AFM. The most common method is using a laser beam. A diode laser is focused onto the back reflective surface of the cantilever, and reflects onto a photodetector. This is position sensitive, and usually has four sectors. The vertical deflection of the cantilever is determined by the difference in light intensity measured by the upper and lower sectors. [62]

Atomic Force Microscopy measurements were performed with a NT-MDT AFM

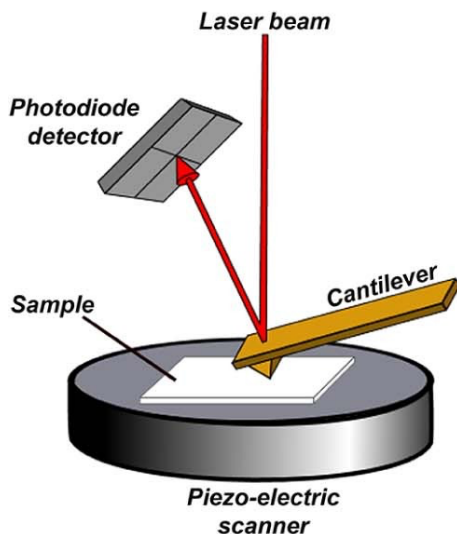


Figure 2.21. *Schematic presentation of AFM.*

(model NTEGRA Spectra), shown in Figure 2.22, using cantilevers in semi-contact mode (Bruker RTESP tips, material: 1 W cm to 10 W cm phosphorus (n)-doped silicon, $f_0 = 27 \text{ kHz}$ to 309 kHz , $k = 20 \text{ Nm}^{-1}$ to 80 Nm^{-1}). In semi-contact (or dynamic force or tapping mode), the tip periodically touches or taps the surface of the samples. This is achieved with the aid of a piezoelectric crystal that causes the cantilever to oscillate in a vertical direction near its resonance frequency. A feedback system is responsible for maintaining the oscillation amplitude constant. When the tip passes over a depression on the surface, the cantilever has more space to oscillate and the amplitude increases. In a similar way, the amplitude decreases when the tip passes over a bump. The changes in oscillation amplitude are used to identify and measure surface features. The alternating contact with the sample provides a higher resolution compared to the other scanning modes. It is also appropriate for fragile and soft samples that could be easily damaged by the shear or adhesion forces applied in the other modes. Raw AFM data were treated by using histogram flattening procedures to remove the experimental artifacts due to the piezo-scanners.



Figure 2.22. *NT-MDT AFM (model NTEGRA Spectra).*

2.2.6 Electrochemistry

The word electrochemistry derives from the terms electricity and chemistry. Ordinary dictionaries define it as the science which describes the interactions between chemistry and electricity, or the chemical phenomena that are coupled with reciprocal exchanges of electric energy.

More precisely, it is a science that analyses and describes the transformations of matter on the atomic scale by using the shifts of electronic charge which can be controlled by means of electric devices. Such transformations are called oxidation-reduction reactions.

One of the advantages of electrochemistry over chemistry lies in the additional, adjustable degree of freedom offered by the application of a voltage or a current. Indeed, it is possible to vary the energy of the active species in a continuous and controlled manner, and also to attain a highly selective reactivity with acute control of the reaction and of its extent.

Electrochemical behavior could be analyzed through a wide range of techniques: for our research, the chosen approach is to perturb the system, which is immersed in a conductive electrolyte, with an alternating signal of small magnitude and to

observe the way in which the system follows the perturbation at steady state.

It allows to get an experimental ability to make high-precision measurements because the response may be indefinitely steady and can therefore be averaged over a long term, to treat the response theoretically by linearized (or pseudo-linearized) current-potential characteristics, and to measure over a wide time (or frequency) range. [63,64]

2.2.6.a The concept of impedance

A purely sinusoidal voltage can be expressed as

$$e = E \cdot \sin(\omega t) \quad (2.4)$$

where ω is the angular frequency, which is 2π times the conventional frequency in Hz. It is convenient to think of this voltage as a rotating vector (or phasor) as seen in Figure 2.23. Its length is the amplitude E and its frequency of rotation is ω . The observed voltage at any time, e , is the component of the phasor projected on some particular axis (normally for $\omega t = 0^\circ$).

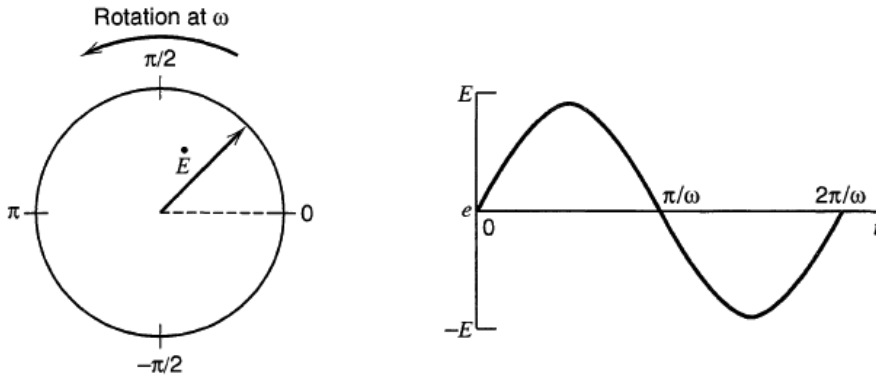


Figure 2.23. *Phasor diagram for an alternating voltage.*

It is possible to consider the relationship between two related sinusoidal signals, such as the current, i , and the voltage, e . Each is then represented as a separate phasor, $\dot{I}$ or $\dot{E}$, rotating at the same frequency. As shown in Figure 2.24, they generally will not be in phase; thus their phasors will be separated by a phase angle, ϕ . One of the phasors, usually $\dot{E}$, is taken as a reference signal, and ϕ is measured with respect to it. In the figure, the current lags the voltage. This can

be expressed generally as

$$i = I \cdot \sin(\omega t + \phi) \quad (2.5)$$

where ϕ is a signed quantity, which is negative in this case.

The relationship between two phasors at the same frequency remains constant as they rotate; hence the phase angle is constant. Consequently, it is possible to study the relationships between phasors simply by plotting them as vectors having a common origin and separated by the appropriate angles.

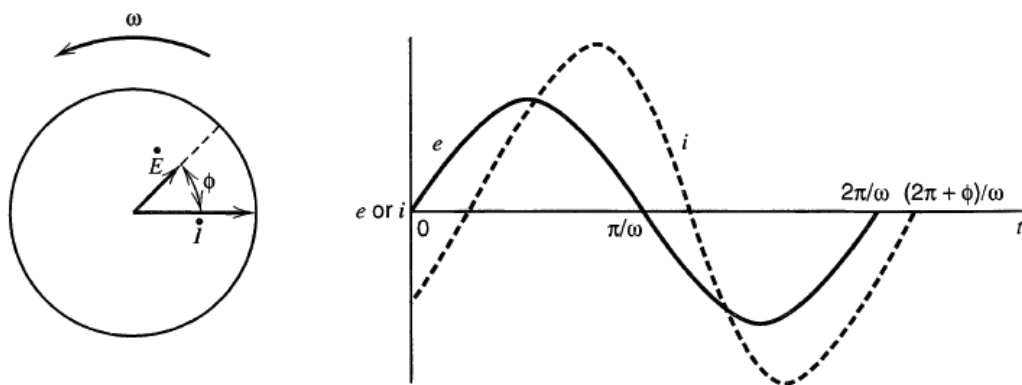


Figure 2.24. Phasor diagram showing the relationship between alternating current and voltage signals at frequency ω .

These concepts can be applied to the analysis of some simple circuits: considering first a pure resistance, R , across which a sinusoidal voltage $e = E \cdot \sin(\omega t)$ is applied. By applying the Ohm's law, the current can be written in phasor notation as

$$\dot{I} = \frac{\dot{E}}{R} \quad (2.6)$$

The impedance is a kind of generalized resistance, and Equation (2.6) is a generalized version of Ohm's law. The phase angle expresses the balance between capacitive and resistive components in the series circuit. For a pure resistance, $\phi = 0$; for a pure capacitance, $\phi = \frac{\pi}{2}$; and for mixtures, intermediate phase angles are observed. The variation of the impedance with frequency is often of interest and can be displayed in different ways.

There are many ways of visualizing impedance spectra: in this study we use

the Nyquist plot and the Bode plots for both modulus and phase.

The Nyquist plot has several advantages: the primary one is that the plot format makes it easy to see the effects of the ohmic resistance. If data are taken at sufficiently high frequencies, it is easy to extrapolate the obtained curve toward the left, down to the x axis to read the ohmic resistance. The shape of the curve (a semicircle for RC circuit, as shown in Figure 2.25(c)) does not change when the ohmic resistance changes. Another advantage of this plot format is that it emphasizes circuit components that are in series. The Nyquist plot format has also some disadvantages. For example, frequency does not appear explicitly. Secondly, although the ohmic resistance and charge transfer resistance can be easily read directly from the Nyquist plot, the electrode capacitance can be calculated only after the frequency information is known.

Another format for evaluating electrochemical impedance data is known as Bode plot, in which $Z(\omega)$ and ϕ are plotted versus $\log \omega$. The Bode plot has the advantage of showing easily the dependence of the impedance from the frequency. The plot uses the logarithm of frequency to allow a very wide frequency range to be plotted on one graph, but with each decade given equal weight. The Bode plot also shows the magnitude on a log axis so that wide impedance ranges can be easily plotted on the same set of axes. This can be an advantage when the impedance depends strongly on the frequency, as in the case including a capacitor. The Bode plot is a useful alternative to the Nyquist plot, but it also has some disadvantages. The greatest one is that the shape of the curves can change if the circuit values change. Plots for a series RC circuit are shown in Figure 2.25(a) and (b).

More complex circuits can be analyzed by combining impedances according to rules analogous to those applicable to resistors. For impedances in series, the overall impedance is the sum of the individual values (expressed as complex vectors). For impedances in parallel, the inverse of the overall impedance is the sum of the reciprocals of the individual vectors.

2.2.6.b Electrochemical impedance spectroscopy

The electrochemical impedance spectroscopic (EIS) approach, which is largely based on similar methods used to analyze circuits in electrical engineering practice, was developed by Sluyters *et al.* [65] and later extended by others.

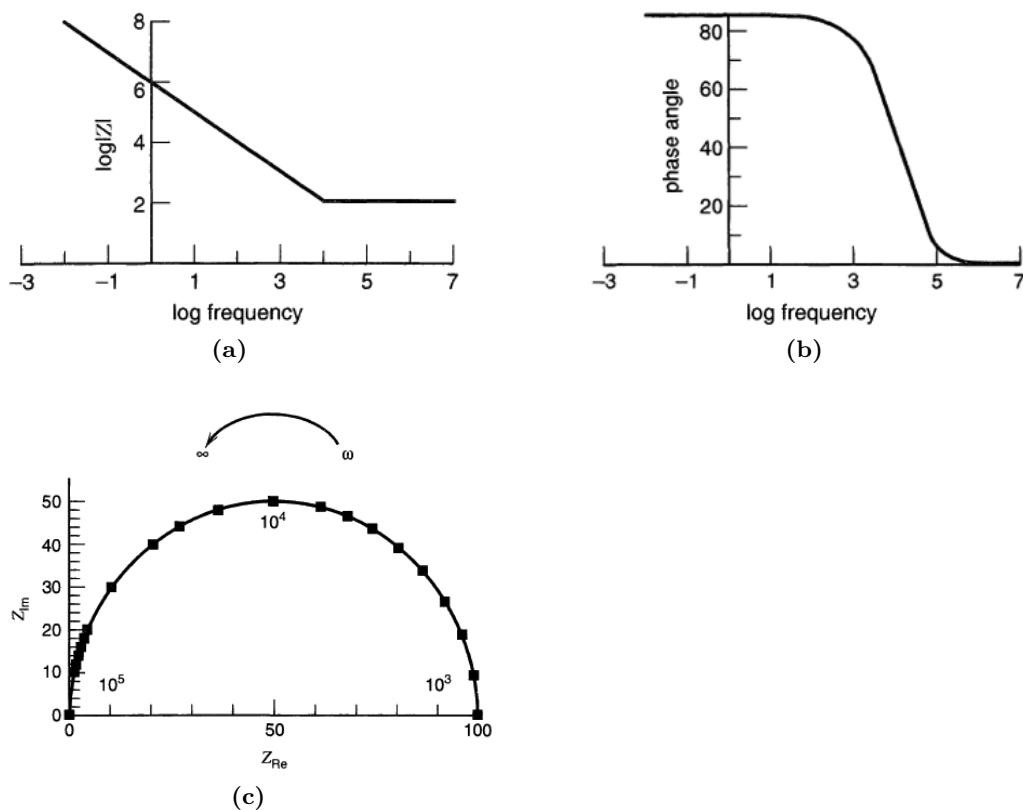


Figure 2.25. Bode plots of modulus (a) and phase (b) for a series RC circuit; (c) Nyquist plot for a parallel RC circuit.

The mathematical approach of electrochemical impedance data is based on Ohm's law, i.e. on the linear interdependency between potential perturbation and current response or vice-versa. However, the potential-current dependencies of electrochemical systems in general are non-linear. On the other hand, it is possible to extract a small fraction of this dependence, in a voltage range where it can be approximated as linear, e.g. in the range of 5 mV to 10 mV.

The name impedance "spectroscopy" derives from the fact that the impedance is generally determined at different frequencies rather than just one.

A standard potentiostat used for electrochemical measurements will contain a working electrode (WE), a counter electrode (CE) and a reference electrode (RE), as seen in Figure 2.26.

EIS can be successfully applied for the characterization of biosensing surfaces

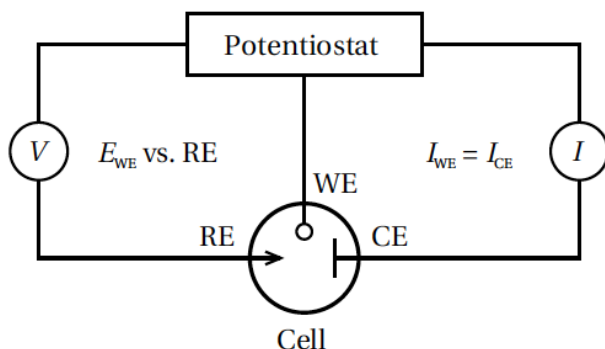


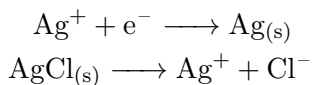
Figure 2.26. Scheme of a three-electrode electrochemical cell and potentiostat. In a three-electrode cell potential and current measurements are separated into an RE and a CE loop. The potential of the working electrode (WE) is measured with respect to the reference electrode (RE). The input of RE has a high-impedance forcing all current to flow through the counter electrode (CE).

and/or in evaluation of bioanalytical signals generated by biosensors.

The usefulness of impedance spectroscopy lies in the ability to distinguish the dielectric and electric properties of individual contributions of components under investigation. Impedance spectroscopy is a non-destructive technique, that can provide quantitative information about the processes at the electrodes and about complex interfaces, and can extract some characteristics of materials including high resistance materials.

The three-electrode cell setup is the most common used in electrochemistry and proper cell design is essential. Three electrodes are necessary to study the behavior of analyte at the electrode/electrolyte interface. The system must be perturbed and this can be done by polarizing the working electrode on which the reaction of interest is occurring, by simply applying a potential or a current to it. In order to apply a potential difference, a reference electrode, whose potential is almost constant, is required. The reference electrode is an electrode which has a stable and well-known electrode potential: it is used as a point of reference in the electrochemical cell for the potential control and measurement, and to isolate the impedance of cell components. The high stability of the electrode potential is reached by employing a redox system: in this study the reaction is between the silver metal (Ag) and its salt - silver chloride (AgCl). The exact standard potential can be found in the literature and is 0.222 49 V, with a standard deviation of 0.13 mV at 25 °C. [66] The equations - corresponding to the redox reaction - can

be presented as follows:



The flowing current through the reference electrode is kept close to zero (ideally, zero): this can be achieved by using a third electrode to close the current circuit in the cell, the counter or auxiliary electrode, together with a very high input impedance of the instrument. After the perturbation, the current must be recorded: if the reference electrode is used for this purpose, its potential will change, and it will be no more a stable reference for all the system. Counter electrode is usually made of an inert material (platinum Pt) and usually it does not participate in the electrochemical reaction. Because the current is flowing between the working electrode and the counter electrode, the total surface area of the latter must be higher than the area of the working electrode, so it will not be a limiting factor in the kinetics of the electrochemical process under investigation. In this study, EIS was carried out using the Gamry Reference 600TM, shown in Figure 2.27, which is a potentiostat with an impedance analyzer; its characteristics are summarized in Table 2.6. [64] Gamry Reference 600TM accurately controls the potential of the counter electrode against the working electrode and the current that flows between them, so that the potential difference between the working electrode and the reference electrode is well defined, and corresponds to the value specified by the user.

Table 2.6. *Characteristics of Gamry Reference 600TM.*

Max. Current	±600 mA
Min. Voltage Resolution	1 µV
Min. Current Resolution	20 aA
Rise time	<250 ns
Noise and Ripple	<2 µV
Frequency Range	10 µHz ÷ 1 MHz
Input Impedance	>10 ¹⁴ Ω

2.2.6.c Equivalent circuit

In a general sense, the response of an electrochemical cell can be considered as the response of an impedance to a small sinusoidal excitation. The performance

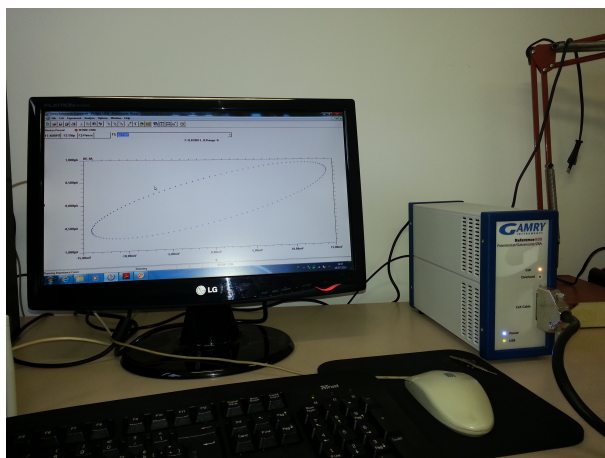


Figure 2.27. *Gamry Reference 600TM.*

of an electrochemical cell can be represented by an **equivalent circuit** made of resistors and capacitors where the current flows with the same amplitude and phase angle as it does in the real cell under a given excitation.

EIS data are commonly analyzed by fitting them using an equivalent electrical circuit model consisting of passive elements, that do not generate current or potential, such as resistors (R), capacitors (C), and inductors (L). To be useful and rational, the model should be built with elements having a physical meaning in the electrochemistry of the system.

A way of interpretation is to use an equivalent circuit, whose choice is mostly heuristic. Then, its parameters are determined from the best fitting of theoretically calculated impedance plots to experimental ones and the results of this analysis are accepted if the deviation is sufficiently small. [64] The reactions occurring consequently are modeled by elements or circuits connected in series, whereas simultaneously occurring processes are modeled by elements or circuits connected in parallel. This analysis is performed for each set of impedance data, $Z(\omega)$, measured for different values of external parameters of the system: bias potentials, bulk concentrations, temperature, and so on. The equivalent circuit is considered as appropriate for this system if the parameters defining the elements of the circuit show the expected dependencies on the external parameters. The fitting of the model to the experimental data is performed using the Levenberg-Marquardt algorithm. The steps to build an equivalent circuit are:

- predict the system elements that will play a part in the cell's impedance;
- build these elements into an equivalent circuit model;
- use of a non-linear least squares fitting program to fit the model to the experimental data to minimize the deviation between a spectrum of the model and experimental data spectrum.

An electrode/electrolyte interface may be simplified as shown by the schematic diagram in Figure 2.28. When a potential is applied to the electrode, solvated counter ions form an electrical double layer by aligning along the electrode surface, which is represented by the so-called double layer capacitor with a capacitance of (C_d). It is measured in solution-containing background electrolyte only, for example, aqueous salt solutions, where no Faradaic process is occurring.

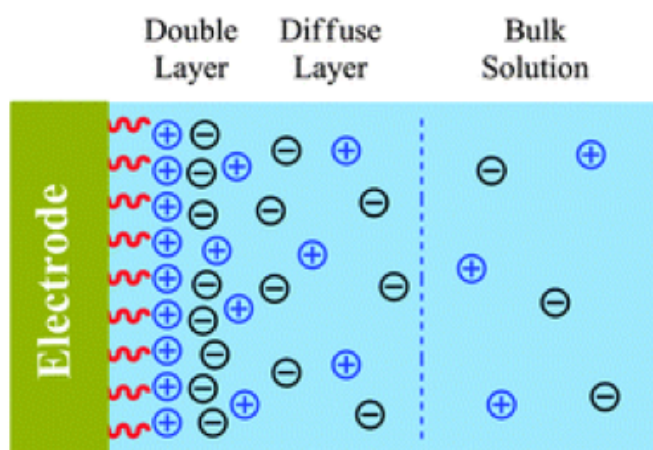


Figure 2.28. *Behavior of charged molecules at the electrode surface.*

However, most impedance studies are now carried out at solid electrodes. At these electrodes the double-layer capacitance is not purely capacitive and often displays a certain frequency dispersion. Such behavior cannot be modeled by a simple circuit consisting of R, L, and C elements. To explain such behavior, a constant phase element (CPE) is usually used. Replacing the capacitor by the CPE but preserving the resistor in the circuit has the practical advantage that the d.c. conductivity can still be determined by directly fitting the parameter R. Mathematically, a CPE's impedance is given by

$$\frac{1}{Z} = Q_O(j\omega)^n \quad (2.7)$$

A consequence of this simple equation is that the phase angle of the CPE impedance is independent of the frequency and has a value of $-(90 \cdot n)^\circ$. When $n = 1$, the CPE has the same equation of a capacitor. When n is close to 1, the CPE resembles a capacitor, but the phase angle is not 90° : it is constant but less than 90° .

Once the electron transfer gets started, the Warburg impedance (W) due to the mass transport begins to play a role in determining the electrode kinetics. The Warburg impedance can be used to model a semi-infinite linear diffusion because it is only the linear distance from the electrode that matters. The Warburg impedance is an example of a constant phase element for which the phase angle is a constant 45° and independent of frequency. The magnitude of the Warburg impedance is inversely proportional to the square root of the frequency ($1/\omega^{1/2}$), as you would expect for a CPE with an n -value of 0.5. The equation for a Warburg's impedance (Z_w) is a simple one, but the equation for the Warburg constant (σ) is a bit more complicated.

$$\begin{aligned} Z_w &= \frac{\sigma}{\omega^{1/2}} - j \frac{\sigma}{\omega^{1/2}} \\ |Z_w| &= \sqrt{2} \frac{\sigma}{\omega^{1/2}} \end{aligned} \quad (2.8)$$

Chapter 3

Synthesis and transfer of graphene

3.1 Introduction

As seen in Section 2.1.1.a, we performed graphene synthesis by catalyzed-CVD (C-CVD) technique, which enables the synthesis of single- to few-layer graphene membranes, at lower temperatures than gas pyrolysis, by introducing a catalytic support. The solid catalyst surface allows the decomposition of the carbon feedstock species used in the process at a lower energy per molecule, and therefore at a lower process temperature. C-CVD is far from an easy-to-control process: in addition to the standard parameters such as pressure, temperature and gas flow rate, the combination of catalyst type and gas mixture, the catalyst surface preparation, the type of carrier or etching gases in the mixture, and the flow velocity should be carefully controlled. However, in order to obtain a defect-free and clean graphene surface, in this study we had to face several issues, not only related to the synthesis process, but also to the transfer of graphene on the final substrate.

Wrinkles formation during the CVD growth process is a harmful unavoidable and uncontrollable phenomenon. It is widely accepted that they are related to the compressive strain induced during cooling due to the difference in the thermal expansion coefficient of graphene and the copper substrate. Moreover, the copper substrate used during the growth process can contain many imperfections such

as steps, which can produce corrugations, folds and cracks during the transfer process. [67, 68] When graphene is grown in quartz furnaces, it is also common to end up with samples contaminated by heterogeneous particles, which alter the growth mechanism and affect graphene properties.

In this work we investigated the morphology and the geometry of the copper foil when inserted in the quartz tube of the furnace, to better understand its influence on graphene synthesis and try to overcome some of the related issues. We also discuss the contamination issue, presenting a practical solution to solve it and obtain clean graphene. Although quality improvements can be made during the synthesis steps, by tuning the synthesis parameters or by treating the copper foil, it is important to note that most of the degradation of graphene quality occurs during the transfer process due to tearing and ripping of the graphene sheets. In this work we investigate three different aspects of graphene transfer: the choice of the etching solution used for the catalyst foil, an alternative to PMMA as the supporting media for the transfer process, and the influence of the final substrate on the adhesion and continuity of graphene.

3.2 Results and discussion

3.2.1 Synthesis

3.2.1.a Copper foil surface treatment

The copper substrate influences graphene nucleation and growth significantly. Therefore, we investigated the effect of the copper foil on graphene morphology and quality of the morphology using SEM, optical microscopy and profilometry. The copper foil as purchased, after a surface treatment, and after annealing with H_2 prior to the deposition were studied.

As previously mentioned, the growth substrates are a commercial laminated copper foil and an oxygen-free copper foil as an alternative: since the last one is already free from a copper oxide layer, it is not necessary to perform the oxide etching treatment before the synthesis. The thickness of both copper foils is 25 μm .

The optical micrograph in Figure 3.1(b) shows that the surface is crossed by repeated and dense straight ripples along one direction. This is due to the industrial lamination technique used for producing the thin copper foils. The color of the surface is not uniform, but scattered with dark areas covered with copper

oxide. The morphology of transferred graphene is highly affected by that of the copper substrate, as can be seen in Figure 3.1(d), with a lot of cracks and holes in the lamination direction.

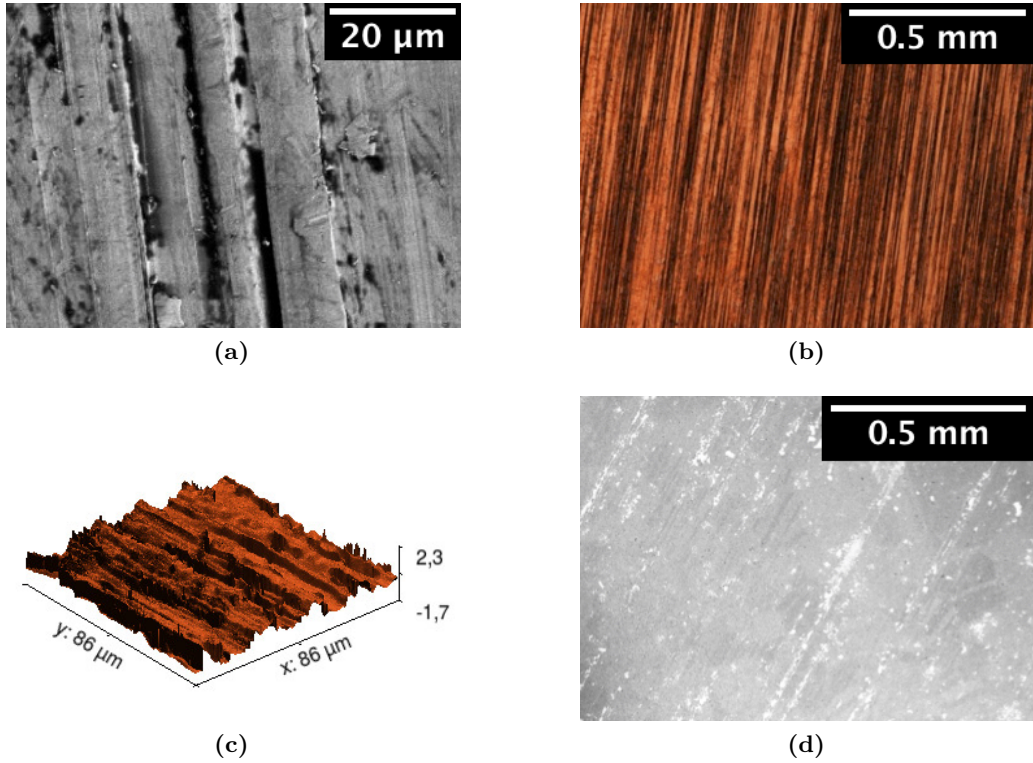


Figure 3.1. *Copper foil without etching: (a) SEM image; (b) optical and (c) AFM surface profile image; (d) SEM image of transferred graphene grown on this type of copper foil.*

A copper foil etching treatment was introduced to smooth the surface, to remove impurities, and to remove the native oxide: the steps are listed below.

- 60 s in acetic acid
- Rinsing in deionized water
- 10 s in nitric acid (dil. 1:2)
- Rinsing in deionized water
- 10 s in acetic acid

As shown in Figure 3.2, oxidized areas are reduced and the surface seems to be more homogeneous and smooth (with lower roughness). The lamination ripples are still present, but on average their height is lower.

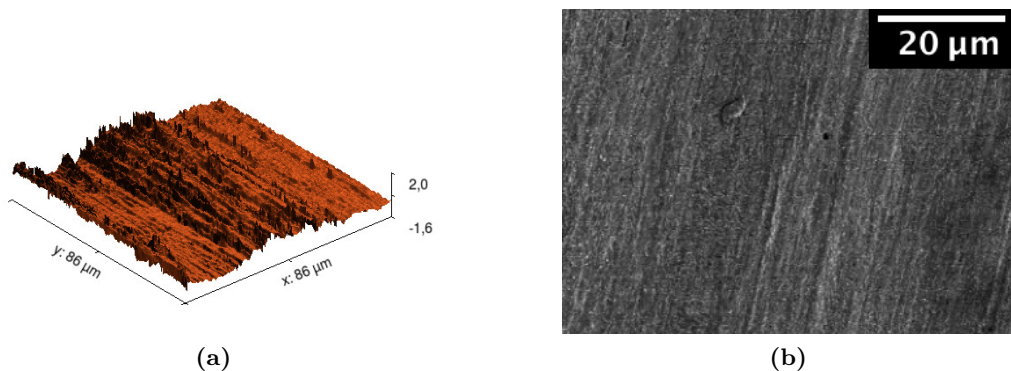


Figure 3.2. *Copper foil after etching: (a) AFM surface profile image and (b) SEM image.*

The commercial copper foil is polycrystalline, made of grains with different sizes and orientations. It is known from literature that the Cu(100) surface causes slow, multilayer graphene growth, while high index copper facets cause compact graphene island formation, with growth rates even faster than those on Cu(100). The Cu(111) surface promotes fast, monolayer graphene growth with few defects. [69,70] The annealing at high temperatures with high hydrogen concentration increases the size of grains with deep grain boundaries, reduces the nucleation sites, and promotes the reorganization of crystalline planes. As it can be noted in Figure 3.3(a) and (b), the copper foil still has the lamination lines, even after the annealing. As a result, the graphene grown using a treated copper foil, after the annealing, is continuous and almost free from holes, as seen in Figure 3.3(c).

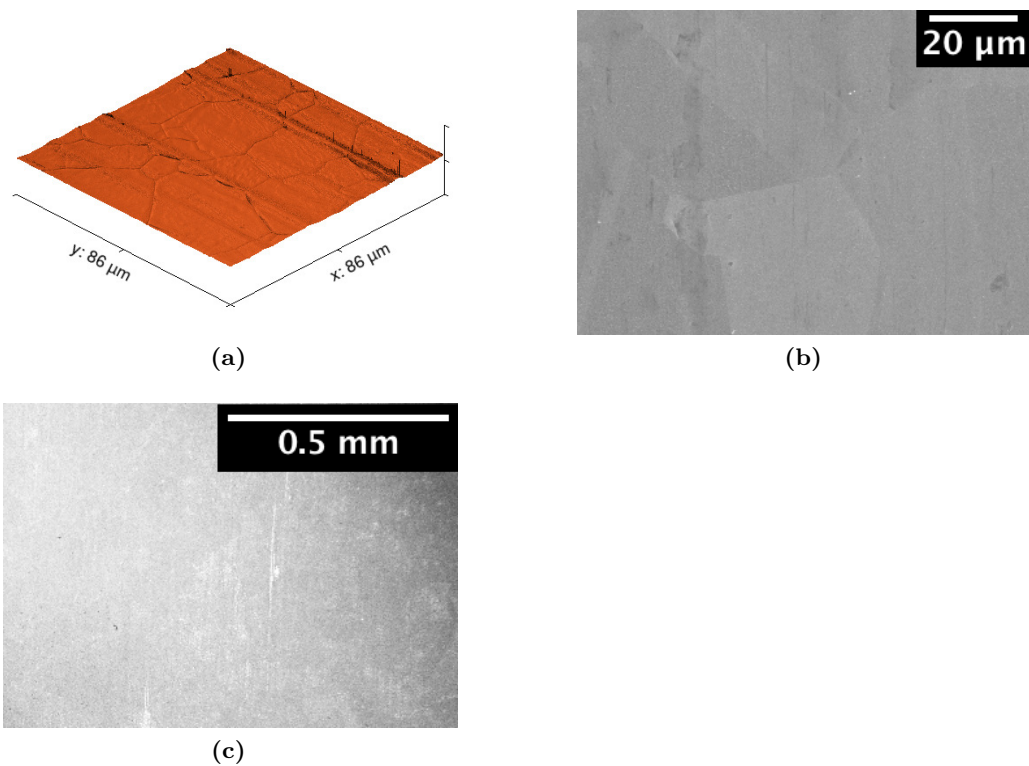


Figure 3.3. Copper foil after annealing: (a) AFM surface profile image and (b) SEM image; (c) SEM image of transferred graphene grown on the treated copper foil.

3.2.1.b Copper foil geometry

The copper foil geometry chosen for the synthesis is critical in order to avoid contamination from the quartz tube on the graphene surface, but also to avoid mechanical stress to graphene during the transfer process. In this study different copper foil shapes have been considered; the graphene grown on these foils is transferred after the synthesis and its morphology is studied using SEM imaging.

We first used a flat piece of copper foil: Figure 3.4(a) and (b) show the flat piece of copper and the transferred graphene coming from that type of shape. The SEM image evidences a lot of impurities, but the graphene layer is continuous.

We then modified the shape as seen in Figure 3.4(c): the new geometry is made up of one bended piece of copper, including a flat piece similar to the previous one and a connected arch-shaped part. The arcuate part has the advantage of protecting the flat piece from the contaminations deriving from the quartz tube. However, in order to transfer the graphene grown on it, this kind of shape is not so convenient, because it needs to be flattened out before starting the transfer procedure. This process induces mechanical stress, as can be seen in the SEM image of transferred graphene (Figure 3.3(d)), which shows a lot of cracks and holes, but is free from impurities. It is possible to conclude that handling the copper foil after the synthesis induces mechanical stress, which corresponds to holes and cracks in the graphene lattice.

The third copper bending geometry, resulting in a shape similar to the previous one, includes two separated pieces, the flat and the arch-shaped parts (Figure 3.4(e)). This geometry has brought a remarkable improvement: two separated pieces of copper allow to avoid handling of the copper foil after synthesis, thus reducing the damage in the graphene, but preserving the protection of the graphene grown in the internal surface of the foil from the contaminations coming from the quartz tube during the synthesis. The transferred graphene shown in Figure 3.4(f) is continuous and free from contaminations.

3.2.1.c Quartz furnace

More details on Section 3.2.1.c can be found in the published article by N. Lisi, T. Dikonimos, F. Buonocore, M. Pittori, R. Mazzaro, R. Rizzoli, S. Marras, A. Capasso - *Contamination-free graphene by chemical vapor deposition in quartz furnaces* - Scientific Reports - 2017 - 7:9227

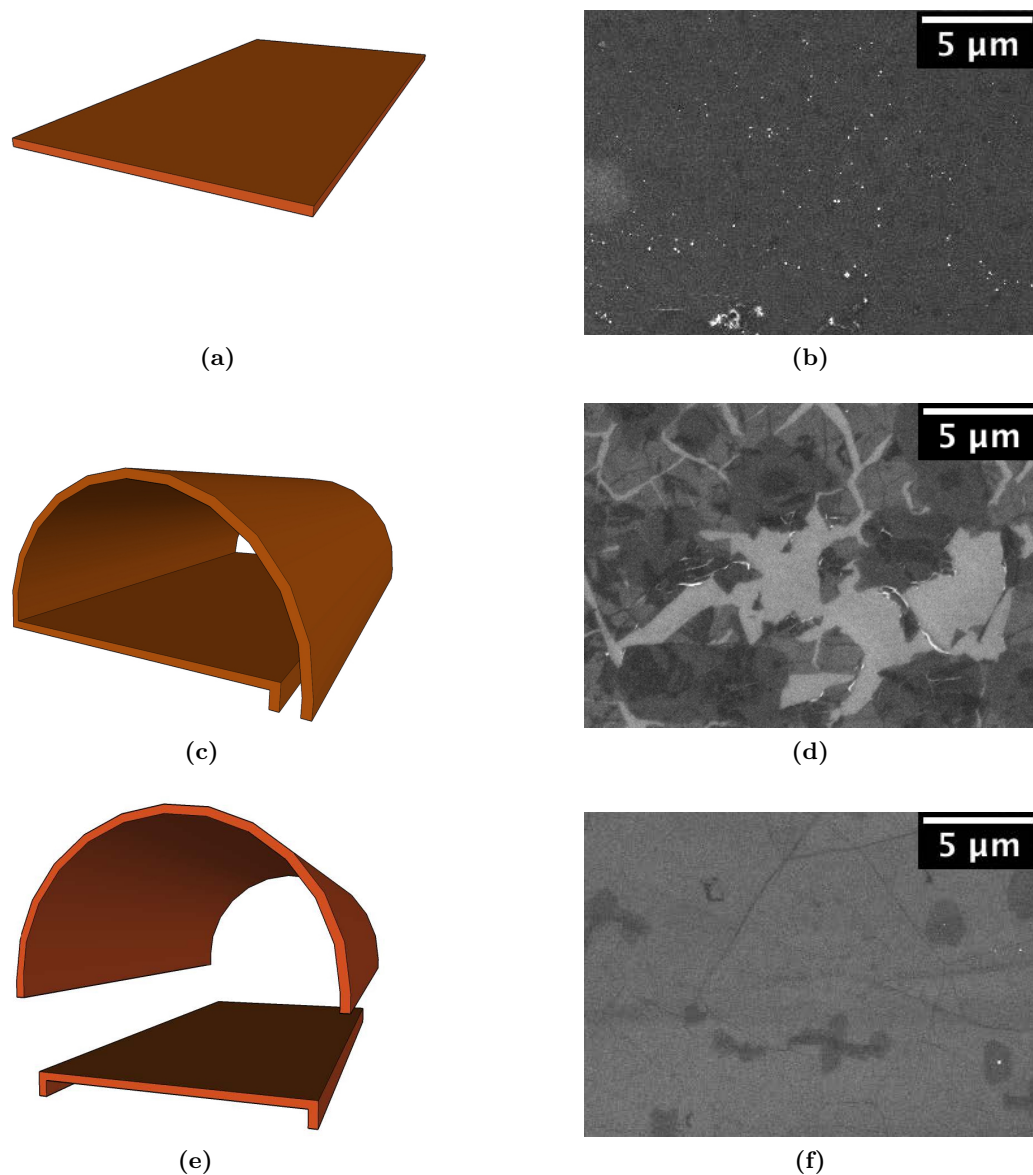


Figure 3.4. *Copper foil geometries (left) and corresponding SEM images of transferred graphene (right).*

CVD can be performed either in cold-wall metal chamber reactors or in hot-wall reactors. The latter kind of reactors are the most widespread mainly for the C-CVD process on copper foil, because of the steady-state, isothermal, clean gas flow conditions they offer, which can be achieved by simple and cheap experimental

systems even for the processing of large area copper foils. A hot-wall reactor usually consists of a quartz-tube furnace (i.e., amorphous SiO_2). Fused quartz contains no heterogeneous elements and then has higher working and melting temperatures [71], with negligible vapor pressure at 1000°C . A tube-furnace made of electrically fused quartz of the highest grade can operate in vacuum up to 1160°C . For these reasons most of the research focusing on the CVD growth of graphene on copper has been carried out in hot-wall quartz furnaces.

Although excellent in terms of crystalline quality, the graphene samples grown with this kind of CVD systems may often show contaminations on their surface. These contaminations appear as white particles (with various density) sitting on top of the graphene in the reported SEM images (either on copper or on other transfer substrates). The contaminations are found when graphene is grown in a CVD system with a quartz tube (either at low or atmospheric pressure). [37, 70, 72–77]

Figure 3.5(a) and (b) show SEM micrographs of graphene samples on copper substrates after the growth: the contaminations appear as small, rounded white dots over the surface. They are preserved after transferring the graphene films onto SiO_2/Si , as visible in Figure 3.5(c). Such a substantial degree of contamination is found to be connected to the working age of the quartz tube. A proof of this is the fact that no contaminations are usually found on graphene when installing a new tube (along with a new quartz boat) in the CVD system; with a new tube, uncontaminated graphene samples can be produced until a given number of growth processes are done. The microscopic analysis of the graphene samples transferred on SiO_2/Si shows a degree of contamination increasing with the working age of the tube.

In order to overcome the practical and cost limitations arising from replacing the quartz tubes every few growth processes, we developed a novel reactor configuration by adding an alumina tube coaxial to the tube furnace as a screen for copper (from the substrates to the tube) and SiO (from the tube to the substrate) vapors contaminants during growth (schematics in Figure 3.6). An alumina boat was used instead of the quartz one in order to suppress its contribution to the sample contamination. Alumina ceramic possesses a remarkably higher thermo-chemical stability and its melting temperature (2070°C) is considerably higher than that of quartz. [78] Conversely, a tube made of alumina would be less proficient in a CVD furnace configuration due to many practical drawbacks with respect to one

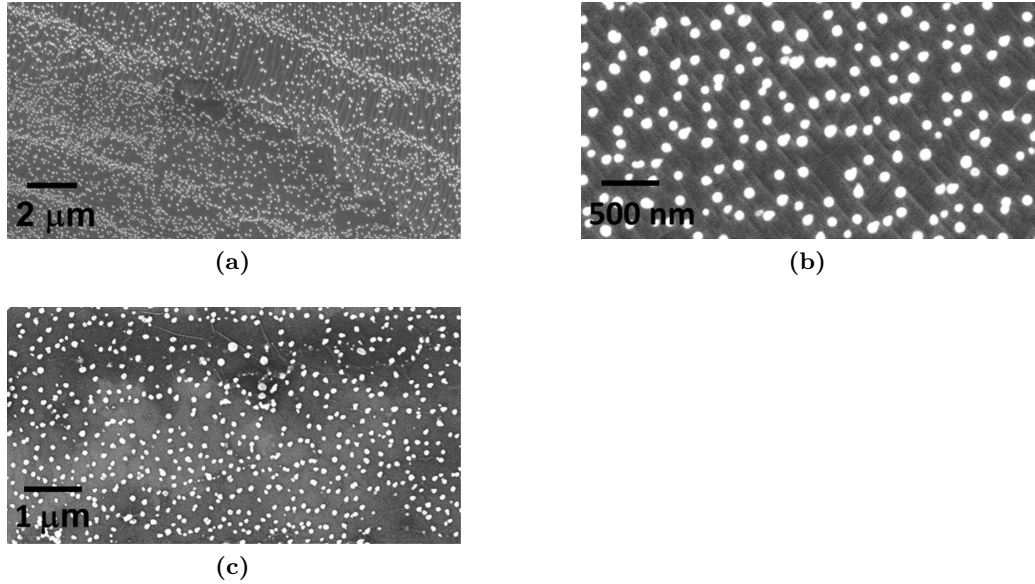


Figure 3.5. SEM images of a heavily contaminated graphene film grown by CVD with an aged quartz tube, on copper (a,b) and after transfer on SiO_2/Si 300 nm (c). Copper surface reconstruction and graphene folds are clearly visible.

made of quartz: the higher thermal conductivity of alumina would increase the heat flow along the tube axis; its higher sensitivity to thermal shocks could cause mechanical failures and its rough, microcrystalline surface would make vacuum head seals more difficult to realize. We avoid all these issues by using a larger quartz tube (60 mm outer diameter - OD - and 55 mm inner diameter - ID) fitted with high vacuum seals and a smaller coaxial alumina tube (40mm OD, 30mm ID), bridging the two reactor cold ends and acting as a screen (Figure 3.6). In this geometry, the SiO vapors cannot travel directly from the inner quartz tube surface towards the hot samples since they would readily condense onto the colder alumina tube edges, nor copper vapors could follow the opposite route to coat and degrade the quartz walls. Alumina is not impervious to copper, which at high temperature can diffuse into its bulk, [79] however the diffusion coefficient is considerably lower than that in SiO_2 . [80]

No direct evidence of a detectable contamination arising from the alumina boat was obtained, but it was observed that it is subjected to cracking upon cooling after a few cycles. It has to be considered that the boat is not exposed to air while hot,

unlike the furnace tube. Therefore, it seems more appropriate to substitute it with a boat made of a refractory metal to further avoid possible contamination sources upon aging. As expected, the contaminants were observed to be fully suppressed by substituting the alumina boat with a tantalum (Ta) boat with a lid, built with an open matchbox geometry which allows the free circulation of the process gases over the copper foils, as schematized in Figure 3.6. Tantalum is a refractory metal with an extremely high melting point of 2990 °C, which reaches a value of 3850 °C in case of its carburized form (TaC). [78] Other than having high thermal stability, tantalum is also not miscible with copper (although miscible with silicon) and can efficiently act as a strong oxygen binding metal ("oxygen getter"), possibly further reducing SiO vapors, and can thus guarantee an unpolluted, stable CVD gaseous environment.

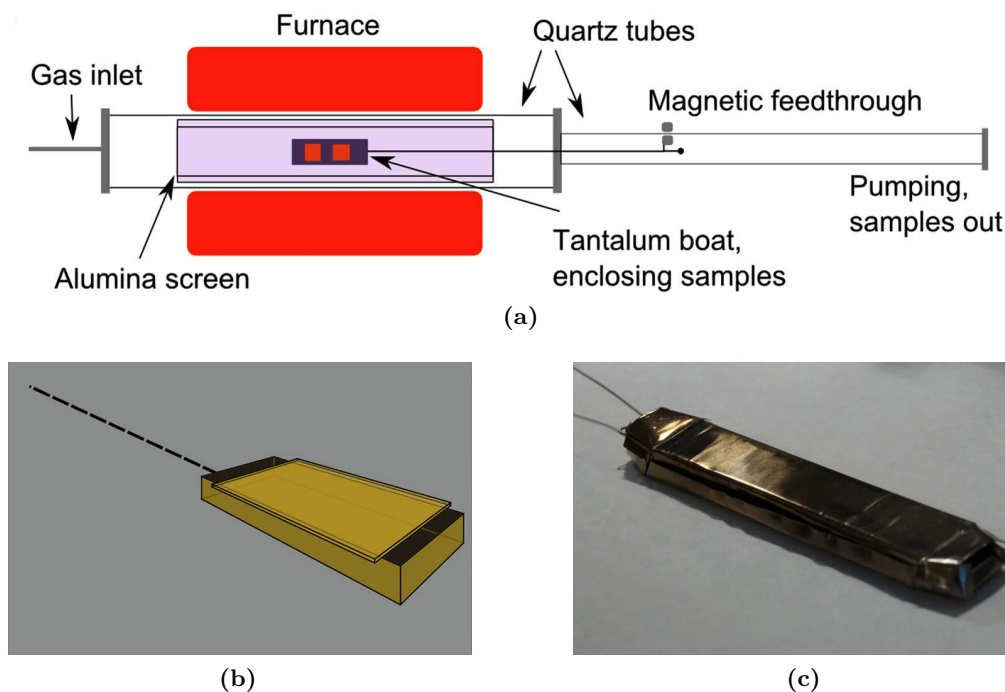


Figure 3.6. (a) Schematics of the modified configuration of the low-pressure CVD system with a coaxial alumina screen tube. The boat is inserted and extracted from the hot zone as in the original configuration. (b) Graphic design and (c) photographic image of the tantalum boat with lid (12 cm in length).

Figure 3.7 shows SEM micrographs of graphene samples grown in the modified CVD system with the tantalum boat. No white dots can be found anywhere contaminating the samples. A few secondary graphene domains are visible, but they are now randomly-oriented, proving that the nucleation is not driven by the presence of preferential sites, such as defects or contaminations.

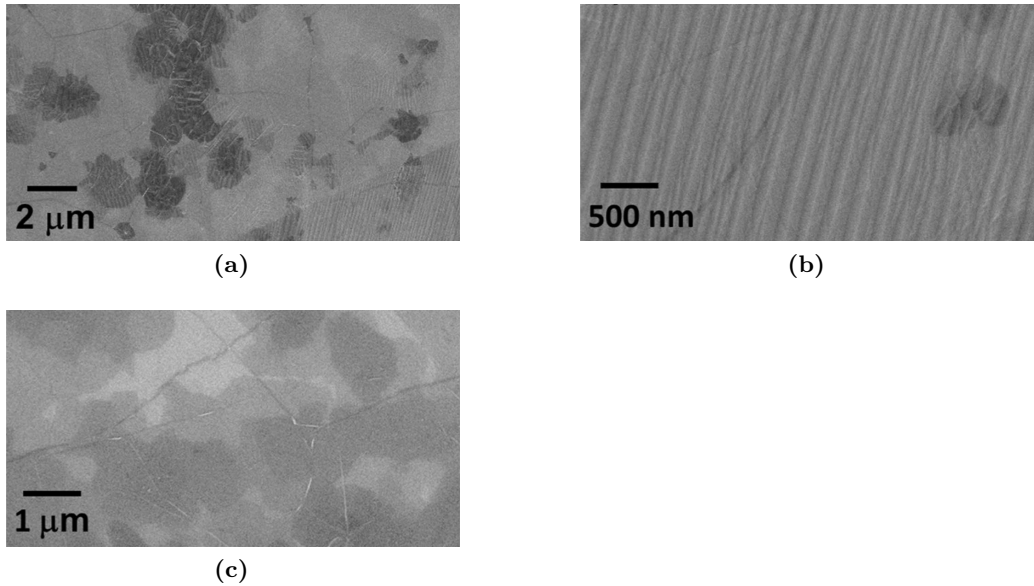


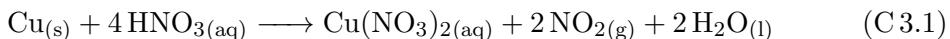
Figure 3.7. SEM micrographs at different magnification of graphene films grown in the optimized CVD reactor using the tantalum boat on copper (a,b) and after transfer onto a SiO₂/Si substrate (c). No growth contaminants are visible at any magnification.

3.2.2 Transfer

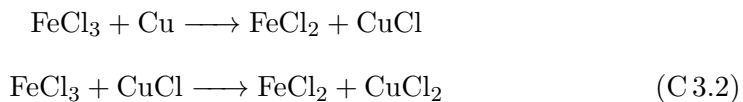
Another key issue in graphene processing is the transfer of graphene on the desired substrate. In this work we investigated how the copper etching solution influences the cleanliness of graphene; we then used an alternative to PMMA, as described in Section 2.1.1.b, and the influence of the final substrate on the adhesion and continuity of graphene.

3.2.2.a Etching solution

The metal substrate used for graphene synthesis has only a catalytic purpose and is typically undesired after the synthesis is complete. In fact, for transferring graphene the metal should be removed. Copper is usually etched away by nitric acid (HNO_3) following the reaction:

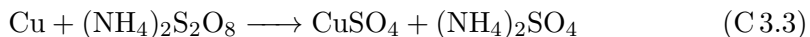


The nitric acid reaction with copper results in a colored solution of copper nitrate and brown vapors of nitrous dioxide (NO_2), resulting in bubbles which damage graphene. This reaction has been also used to preclean the copper foil before graphene growth. [81] As an alternative, iron-chloride (FeCl_3) can be used, following the reaction:



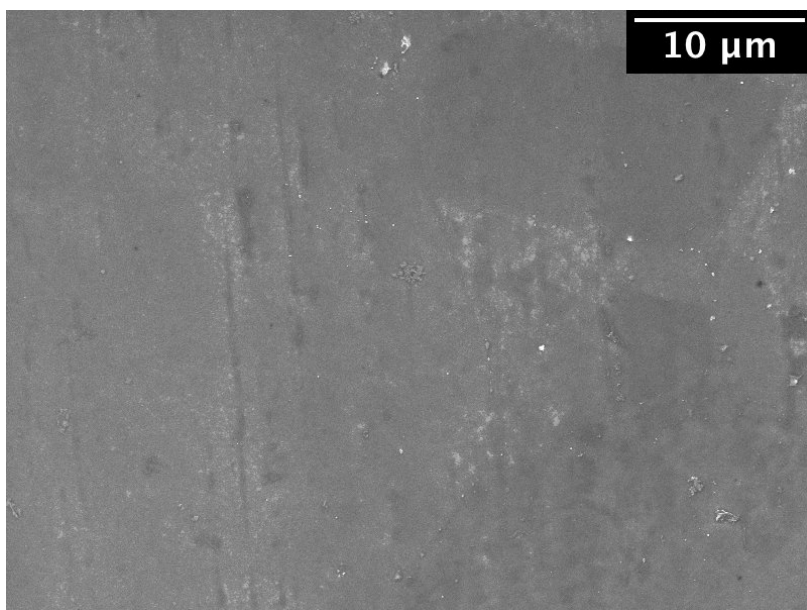
This reaction does not produce gas, but its drawback is that it could leave residues on graphene, even after rinsing it with water, which can alter its properties.

The best solution is to use ammonium persulfate ($(\text{NH}_4)_2\text{S}_2\text{O}_8$), following the reaction:

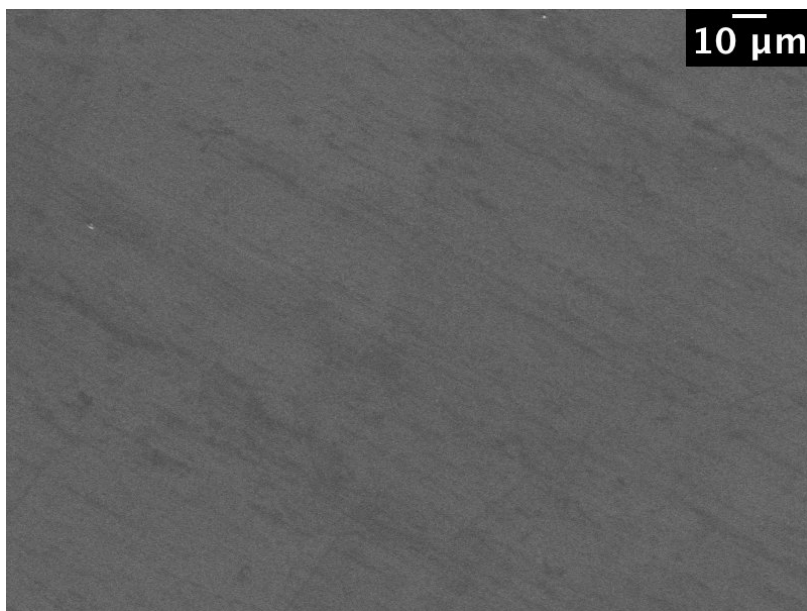


This reaction does not leave residues and does not generate bubble: the etching of copper is more gentle.

Figure 3.8 shows the difference between two samples of graphene transferred using iron chloride and ammonium persulfate: (a) shows the presence of small clusters, probably made of copper-iron salts, that can not be removed after the synthesis; (b) evidences that, using the ammonium persulfate etching solution, the transferred graphene is free from residues on the surface.



(a) *Transferred graphene using iron chloride solution.*



(b) *Transferred graphene using ammonium persulfate solution.*

Figure 3.8. *SEM images of transferred graphene using different solutions.*

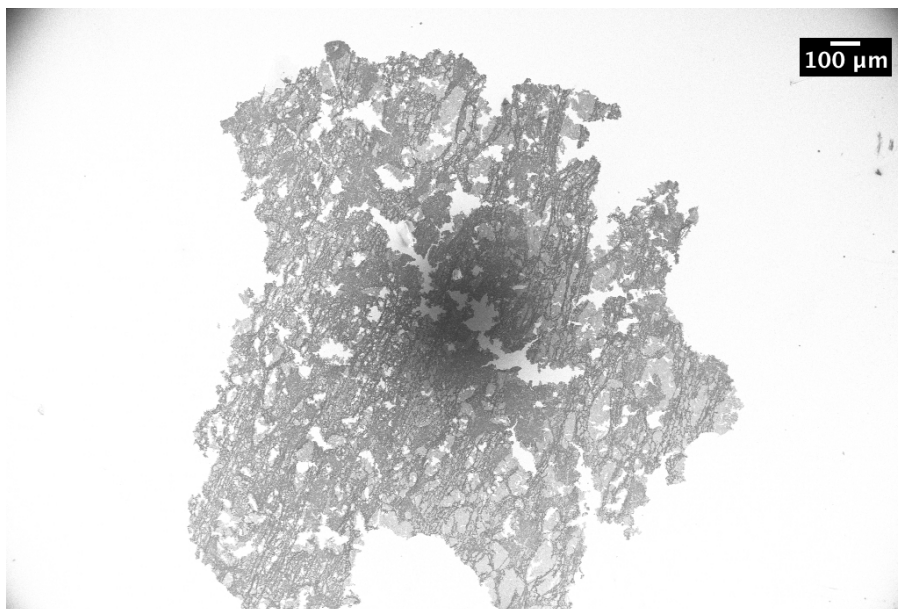
3.2.2.b Cyclododecane: an alternative to PMMA

As seen in Section 2.1.1.b, we used cyclododecane, a waxy solid compound, as an alternative to PMMA supporting layer. Two drops of the solution made of cyclododecane diluted in chloroform was spin-coated over the sample with a spin-coater (1500 rpm for 60s) and copper was etched using a solution made of ammonium persulfate.

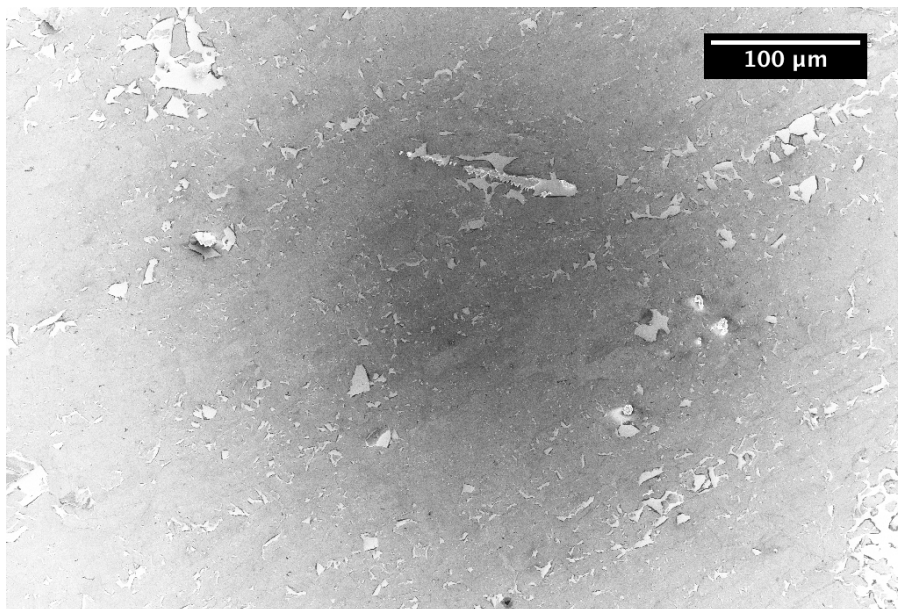
We had to face some issues during the first trials in the use of cyclododecane, for instance, it was hard to remove the graphene grown on the back surface of the copper foil without damaging the film, since the cyclododecane film is stiffer than the PMMA film and thus subjected to cracking. The graphene grown on the back surface of the copper foil was removed by gently rubbing with a cotton cloth. In addition, it was necessary to find the best concentration of cyclododecane solution and we succeeded in transferring graphene with a solution 50% w/w. The cyclododecane was observed to completely sublime overnight, leaving the graphene film intact and cleaner than using PMMA, as evident in Figure 3.9. The film in (a) shows mechanical damages, with small and non continuous graphene islands, while the film in (b) is continuous and with a small amount of tears or cracks, and no residues. However, due to the larger stiffness of cyclododecane film with respect to PMMA, we concluded that this transfer process is suitable for small pieces of graphene, and cyclododecane seems to provide a valid mechanical support to graphene during the transfer process, maintaining the atomically thin film intact. We needed to transfer graphene with larger dimensions than the ones obtained using cyclododecane, therefore we decided to transfer graphene with PMMA, by controlling the amount of residues left on the surface. The cyclododecane transfer procedure will be resumed and optimized for large samples: this could be useful for the functionalization of graphene, since cyclododecane is soluble in chloroform, which is the solvent in which lipids are soluble and can be mixed.

3.2.2.c The influence of the substrate

Another key point for the practical application of graphene in biosensors is the study of its adhesion to the desired substrate. In the case of SiO₂/Si wafer substrate, the different surface roughness of the oxide, depending on whether it has been obtained by thermal oxidation of silicon at high temperature (hereinafter referred to as thermal oxide) or by silicon oxide film deposition by LPCVD (low-



(a) *First trial.*



(b) *Successfully transferred graphene.*

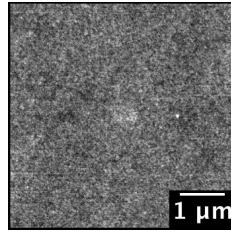
Figure 3.9. *Graphene transferred using cyclododecane.*

temperature oxide - LTO - film growth), strongly affects the adhesion of graphene. To evaluate its hydrophilicity, the two types of substrates were analyzed with

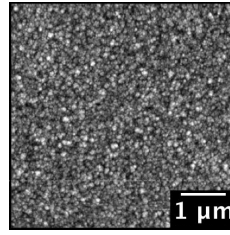
contact angle measurements, while their roughness was mapped through the use of AFM imaging (Figure 3.10). We transferred graphene on the two substrates with different surface morphology and properties by keeping the same transfer conditions, and we investigated the adhesion differences depending on the target substrate roughness. The average roughness can be evaluated from AFM, and we obtain:

- Silicon dioxide by thermal oxidation: 0.2 nm
- Silicon dioxide by LTO: 2.36 nm

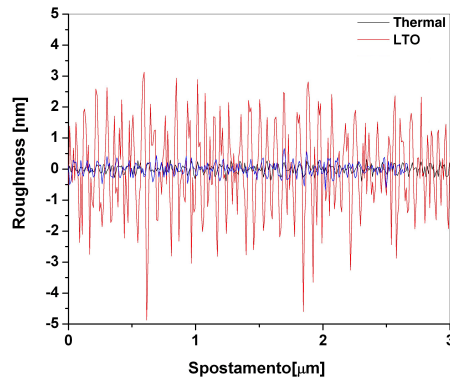
The average WCA of silicon dioxide by thermal oxidation is 59° , while the average WCA of the one obtained by LTO is 29° , but we realized that using our wet transfer process, it was hard to transfer continuous graphene layers on LTO films, while very good results were obtained on thermal SiO_2 . The most probable hypothesis, explaining this behavior during wet transfer, is that the adhesion of the graphene/PMMA membrane to the substrate is more influenced by the effect of substrate roughness than by its hydrophilic/hydrophobic nature. The water gets trapped between graphene and the substrate and causes the slip-off of graphene during the transfer procedure, resulting in cracks and holes when water evaporates. The SiO_2/Si wafer with the thermal oxide is proven to be the best substrate for supporting graphene rather than the LTO one.



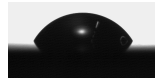
(a) AFM image of SiO_2 by thermal oxidation.



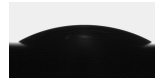
(b) AFM image of SiO_2 by LTO.



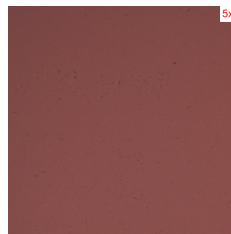
(c) Average roughness.



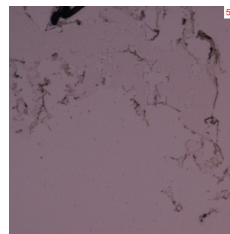
(d) WCA of SiO_2 by thermal oxidation.



(e) WCA of SiO_2 by LTO



(f) Optical micrograph of transferred graphene on SiO_2 by thermal oxidation.



(g) Optical micrograph of transferred graphene on SiO_2 by LTO

Figure 3.10. Differences between SiO_2 by thermal oxidation and LTO film deposited by LPCVD.

3.3 Conclusions

In conclusion we succeeded in synthesizing a graphene layer which is continuous and free of defects, by first treating the copper foil with acetic acid and nitric acid, by choosing its proper shape, and by modifying the hot-wall CVD system. Moreover, after the synthesis, the graphene transfer procedure was also improved by using cyclododecane, instead of PMMA, and ammonium persulfate, instead of iron chloride, for copper etching. The cyclododecane based method, which is more suitable for transferring small samples of graphene, due to its larger stiffness with respect to PMMA, and the combined ammonium persulfate etching step result in a graphene layer which is free from residue. However, for developing the electrochemical biosensors based on graphene supported phospholipidic layer, large area graphene samples on an insulating substrates are required. For this kind of samples, the cyclododecane based method should be optimized and needs further developments. $2\text{ cm} \times 2\text{ cm}$ graphene layers were transferred on SiO_2/Si wafer, because the thermal silicon oxide was proven to be more suitable for supporting graphene than the LTO one.

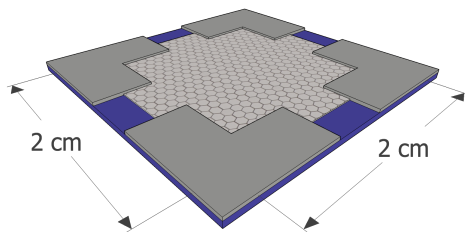


Figure 3.11. *Transferred graphene on a SiO_2/Si wafer.*

Chapter 4

Characterization and surface treatment of graphene

4.1 Introduction

The transferred CVD-graphene samples have been characterized by SEM, AFM, Raman and XPS spectroscopy and water contact angles measurements. For our experiment, we prepared large area samples of $2\text{ cm} \times 2\text{ cm}$ covered with mono- to few-layer of graphene. Those samples were then heated in high-vacuum and in oxygen atmosphere in order to introduce defects in the lattice, to promote the adhesion of SLB.

4.2 Results and discussion

4.2.1 Characterization of graphene as-transferred

4.2.1.a SEM observation

The presented graphene samples were synthesized with the best procedure described in Section 3.3, using both the treated copper foil with acetic acid and nitric acid (CVD-G-01, -02, -03, -04, -05, -06, -07), and using the oxygen-free copper foil (CVD-G-08, -09, -10, -11), by choosing the proper shape; all the samples were transferred using PMMA as supporting media for graphene and ammonium persulfate as the etching solution, giving the best results.

The morphology of the CVD-graphene layers transferred on SiO₂/Si substrates was observed by SEM. The purpose of this analysis was to determine whether graphene had entirely covered the SiO₂/Si substrate or not and to roughly evaluate the presence of a overlayers. The SEM images of Figures 4.1 to 4.11 at different magnification confirm that graphene fully covers the SiO₂/Si substrates and it has small islands of overgrowth, which are well described in literature as multilayer islands with an inverted wedding cake (IWC) structure. [82] It is also possible to notice the presence of PMMA residuals, which are not fully removed by acetone. This is still a big issue - as reported in the literature - which needs to be solved in order to obtain clean graphene surfaces, for applications in electronic devices and sensors. In the SEM images we highlighted the presence of overgrowth, and also the PMMA residuals: the latter modify the electrical properties of graphene.

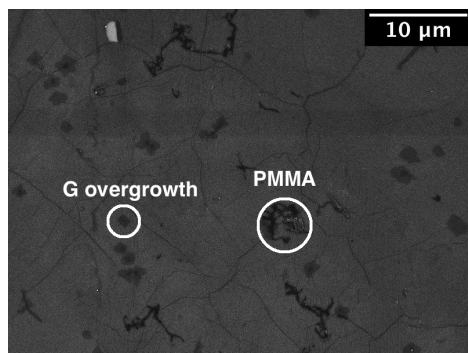


Figure 4.1. SEM images of CVD-graphene sample CVD-G-01.

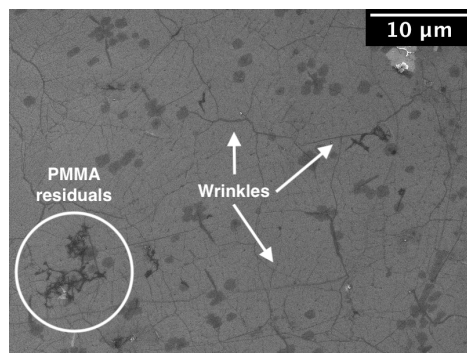


Figure 4.2. SEM images of CVD-graphene sample CVD-G-02.

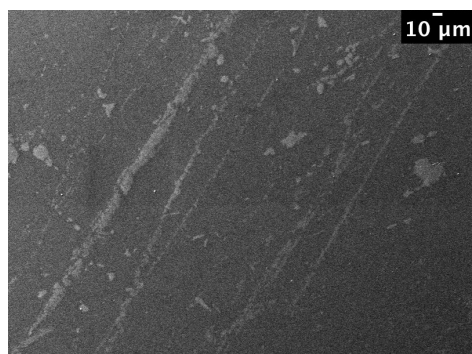


Figure 4.3. SEM images of CVD-graphene sample CVD-G-03.

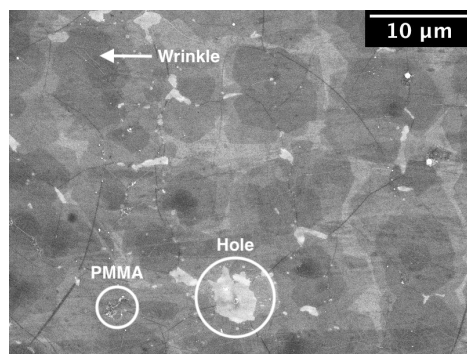


Figure 4.4. SEM images of CVD-graphene sample CVD-G-04.

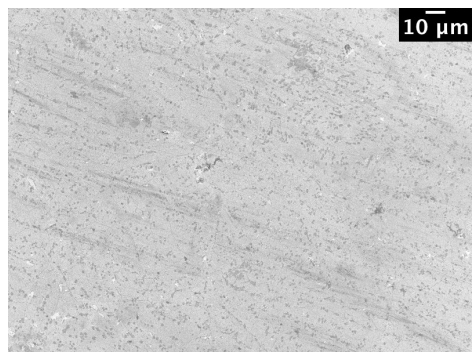


Figure 4.5. SEM images of CVD-graphene sample CVD-G-05.

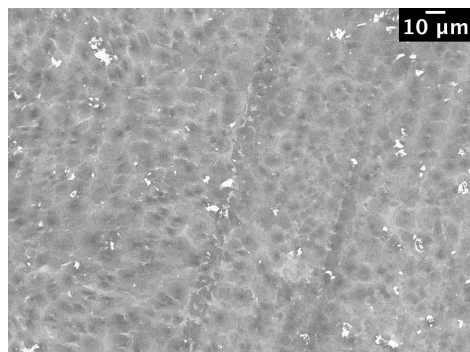


Figure 4.6. SEM images of CVD-graphene sample CVD-G-06.

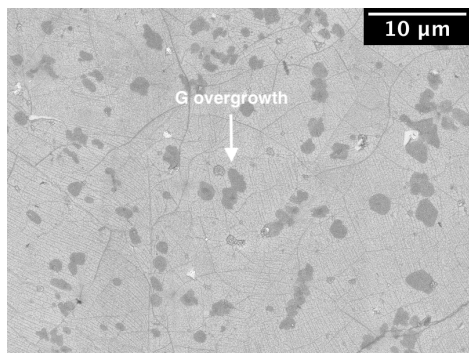


Figure 4.7. SEM images of CVD-graphene sample CVD-G-07.

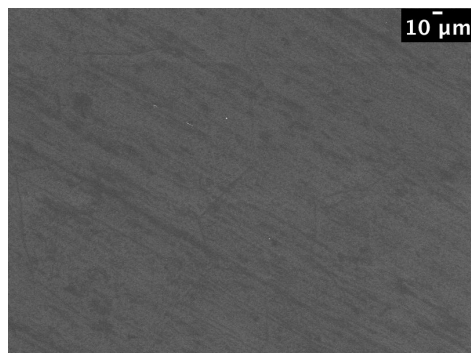


Figure 4.8. SEM images of CVD-graphene sample CVD-G-08.

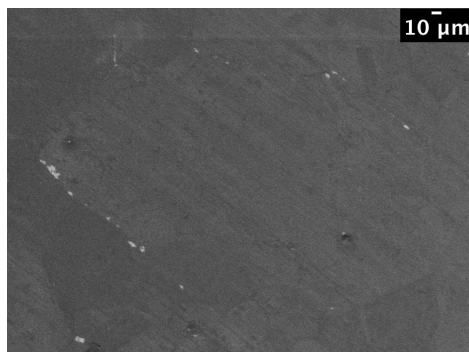


Figure 4.9. SEM images of CVD-graphene sample CVD-G-09.

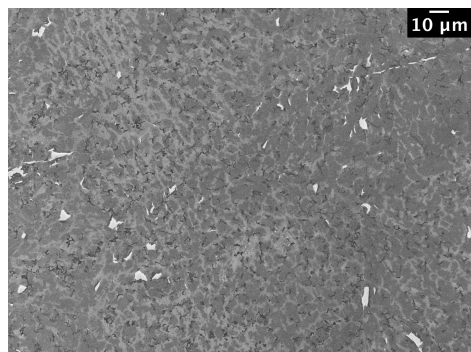


Figure 4.10. SEM images of CVD-graphene sample CVD-G-10.

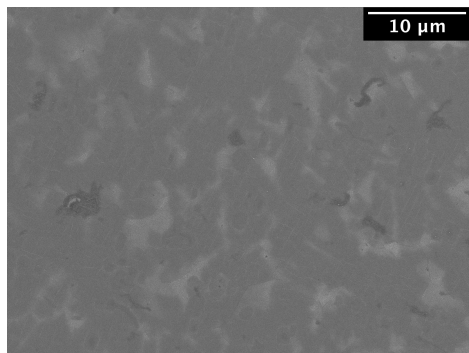


Figure 4.11. SEM images of CVD-graphene sample CVD-G-11.

4.2.1.b Micro-Raman spectroscopy

Micro-Raman spectra were collected with the instrument described in Section 2.2.1. In this study the laser was focused on the sample under test using a 100x objective. The sample was then mapped with a grid 8×7 and a step of $1\mu\text{m}$, resulting in 56 Raman spectra for each sample. Each spectrum make it possible to evaluate the quality and number of graphene layers at the point of the sample under investigation. The value of the $I_{2\text{D}}/I_{\text{G}}$ ratio (that is the ratio between the intensities of the 2D peak and of the G peak) together with the $I_{2\text{D}}$ and the 2D-peak full width at half maximum are the key parameters to evaluate the quality and the number of layers in graphene films. Figure 4.21 shows one of the Raman spectra of the map acquired on the CVD-G-08 sample, having the higher $I_{2\text{D}}/I_{\text{G}}$ ratio. The I_{D} is small, while the $I_{2\text{D}}$ seems to be almost 1.7 times I_{G} , therefore it is possible to deduce that graphene is mono- to few-layer.

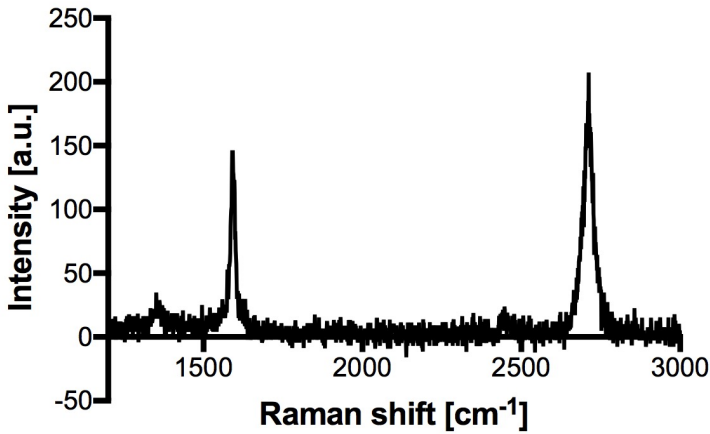


Figure 4.12. *Raman spectrum of sample CVD-G-08.*

For a quick check of the samples, from the measured spectra it is possible to create the maps of the $I_{2\text{D}}/I_{\text{G}}$ and $I_{\text{D}}/I_{\text{G}}$ ratios. If $I_{2\text{D}}/I_{\text{G}}$ is around 4 and the $I_{\text{D}}/I_{\text{G}}$ is very low, then that area of the sample is covered by monolayer graphene. Raman spectra were extracted with a MATLAB script and colored maps were created with OriginPro in order to get a faster and intuitive representation of the quality and number of layers in the graphene film of each sample. Figures 4.13 and 4.14 show the maps obtained on the sample CVD-G-08 and on the sample CVD-G-09, for (a) the $I_{2\text{D}}/I_{\text{G}}$ and (b) the $I_{\text{D}}/I_{\text{G}}$ ratios. The intensity of blue and red are related to

the intensity of the main peaks of the spot of the sample under investigation: the more intense blue is related to the highest value of I_{2D}/I_G , indicating that graphene is mono- to few-layer, while the more intense red is related to the highest I_D/I_G value, thus indicating the presence of defects in the lattice. These maps show that before the surface treatment the CVD-graphene samples under investigation are mono- to few-layer, with a small amount of defects.

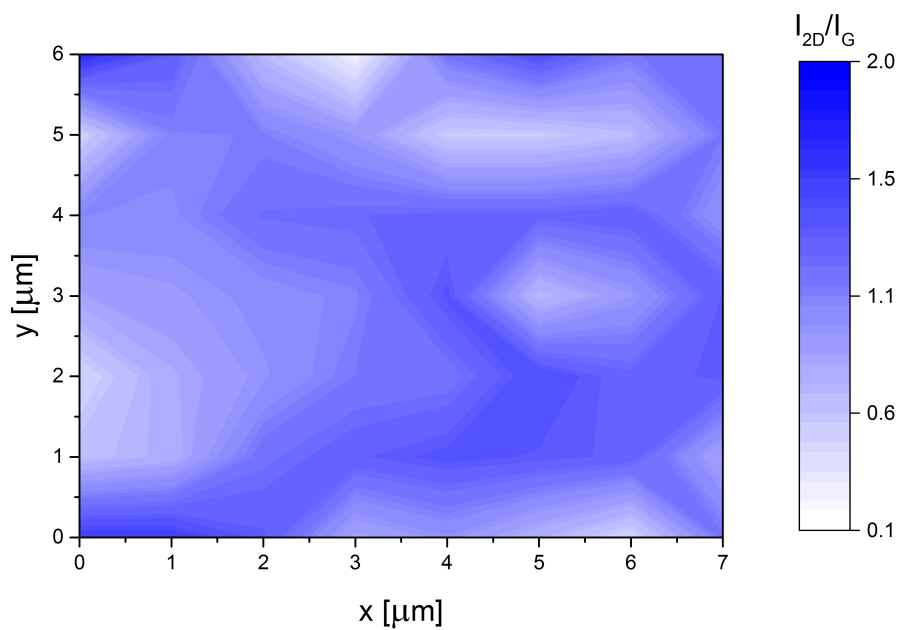
4.2.2 Surface treatments of graphene and characterization

It is well established that bilayer lipid membranes are held in place above a solid support by a combination of van der Waals, electrostatic, hydration and steric forces. On the other hand, CVD-produced graphene is highly hydrophobic, thus making it necessary to perform surface treatments in order to allow the interaction between the lipid bilayers and the graphene surface. This can be achieved through a controlled mild oxidation of graphene, which introduces carboxyl ($-\text{COOH}$), hydroxyl ($-\text{OH}$) and epoxide ($\text{O}-\text{C}-\text{O}$) groups in the lattice. These groups promote the interaction of graphene with the lipid bilayer, thus forming a good platform for the deposition of the lipidic layer. In this study we use a thermal annealing process in oxidizing atmosphere with a low partial pressure of O_2 , using a Cold Walls CVD system by Elettrorava, which is shown in Figure 4.15, with the characteristics summarized in Table 4.1. The system was designed for the deposition of carbon nanotubes and graphene on flat substrates with a 3" diameter. The walls of the reaction chamber are water-cooled and only the sample is put in contact with the heater. The process is computer controlled.

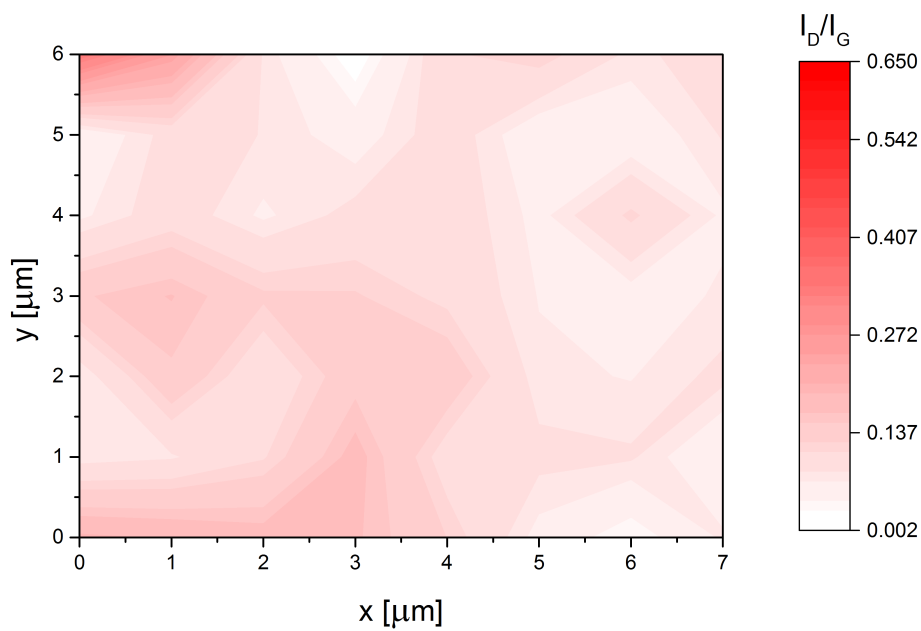
Table 4.1. *Characteristics of the Cold Walls Reactor.*

Temperatures	Up to 1000°C
Base vacuum	$<10^{-7}$ mbar
Process gases	Ar, N_2 , H_2 , NH_3 , CH_4 , C_2H_2 , O_2

Samples were treated with a constant flow of O_2 (20 s.c.c.m.) at varying temperatures and exposure times, as summarized in Table 4.2. A pre-annealing step for 30 min at 500°C under high vacuum (HV) was carried out on the samples, in order to obtain a better cleaning of the graphene surface, getting rid of residues from PMMA or other sources. The surface treatments (ST) are divided into the following type of processes:

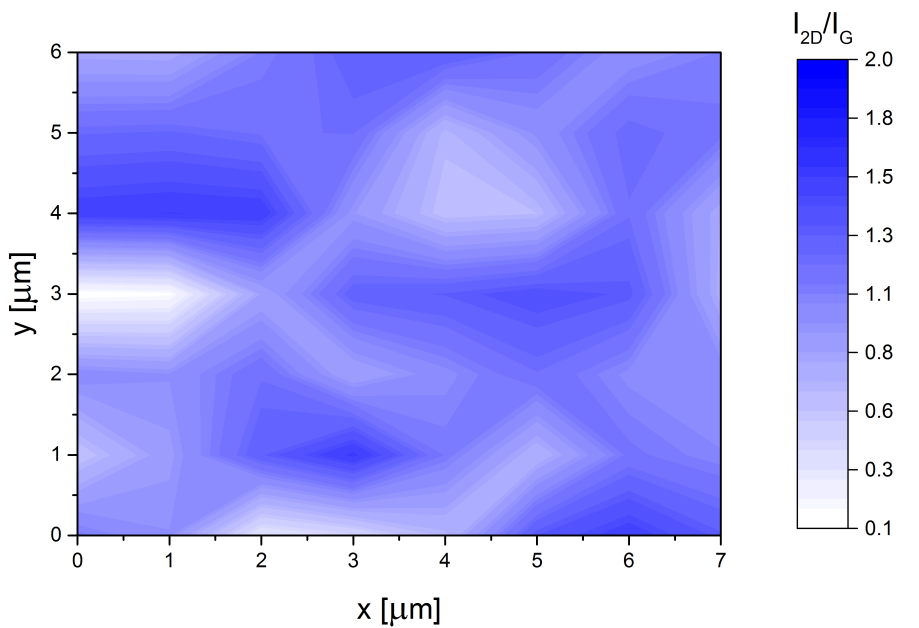


(a)

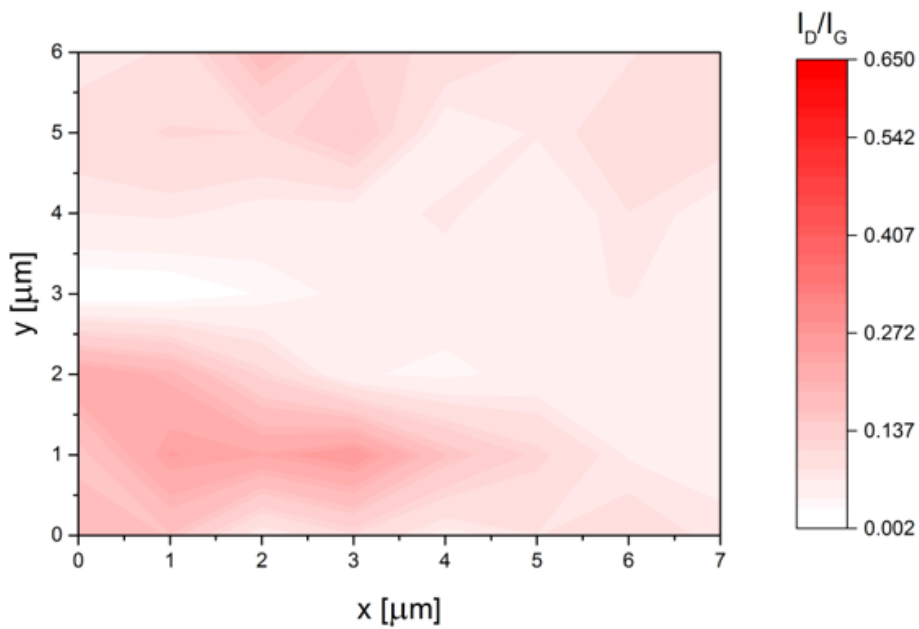


(b)

Figure 4.13. Raman maps of (a) I_{2D}/I_G and (b) I_D/I_G ratios of sample CVD-G-08.



(a)



(b)

Figure 4.14. Raman maps of (a) I_{2D}/I_G and (b) I_D/I_G ratios of sample CVD-G-09.



Figure 4.15. *Photo of the Cold Walls Reactor.*

- ST-A: 4 h at 300 °C;
- ST-B: 4 h at 300 °C + 2 h at 350 °C;
- ST-C: 3 h at 350 °C;
- ST-D: 4 h at 300 °C + 3 h at 350 °C

4.2.2.a Water contact angles

Water contact angles (WCAs) were measured as described in Section 2.2.2. Ultrapure water (18.2 M Ω , <5 ppb total organic carbon) was used as probe liquid, with drops of 1 μ L for each measurement. The analysis was performed with the software provided with the instrument. Table 4.3 shows the mean WCA and standard deviation for each sample before and after surface treatments. These results show the hydrophobicity of graphene, with WCAs ranging from 77° to 96°. The variability of WCAs before the surface treatments arise from the presence of

Table 4.2. *Surface treatment of CVD-graphene samples.*

Sample	T _{set} for annealing (O ₂)	Time	ST
	°C	h	
CVD-G-01	-	-	-
CVD-G-02	-	-	-
CVD-G-03	300	4	ST-A
CVD-G-04	300	4	ST-A
CVD-G-05	300	4	ST-B
	350	2	
CVD-G-06	300	4	ST-B
	350	2	
CVD-G-07	300	4	ST-B
	350	2	
CVD-G-08	300	4	ST-B
	350	2	
CVD-G-09	350	3	ST-C
CVD-G-10	300	4	ST-D
	350	3	
CVD-G-11	300	4	ST-D
	350	3	

PMMA residuals on the graphene surfaces. After the graphene surface treatments, the WCAs decrease while increasing the temperature or time of the treatments. Moreover, the decrease of the standard deviation values after annealing is attributed to the reduction of the amount of PMMA residuals on the graphene surface, giving a better uniformity of WCA measurements. The decrease in WCAs after the annealing treatments implies that some defects are introduced in the lattice, and this can be confirmed by using other characterization techniques, as described in the following sections.

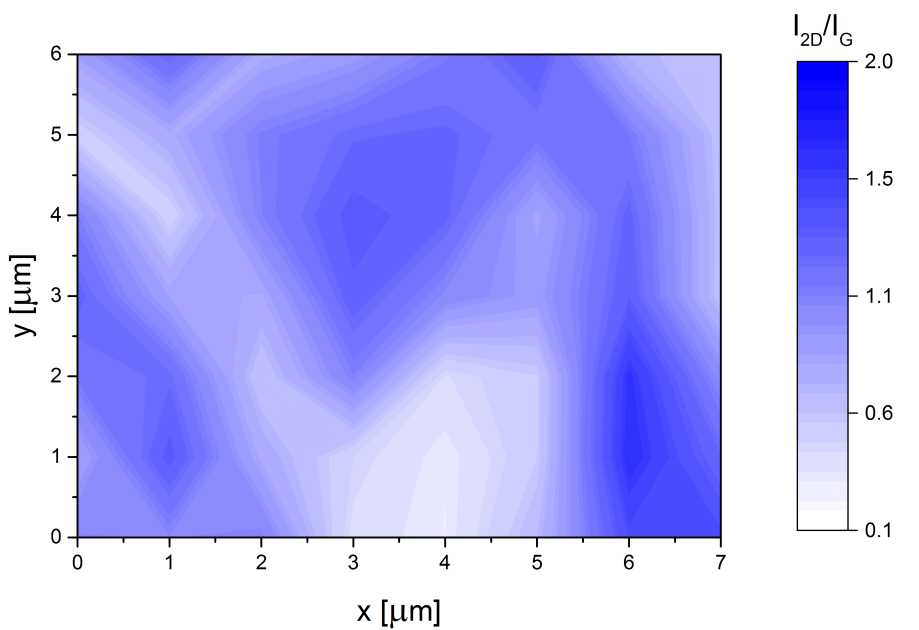
4.2.2.b MicroRaman spectroscopy

After the surface treatments, we acquired new Raman spectra, as described in Section 2.2.1, and we obtained the corresponding maps, reported in Figures 4.16 to 4.18. As can be seen in the red maps, the ratio of the intensity of the D peak to G peak is sensibly increased (the red intensity is higher), with respect to the corresponding maps of the same samples before the heat treatment. This means that the heat treatments introduces new defects in the lattice. The blue map,

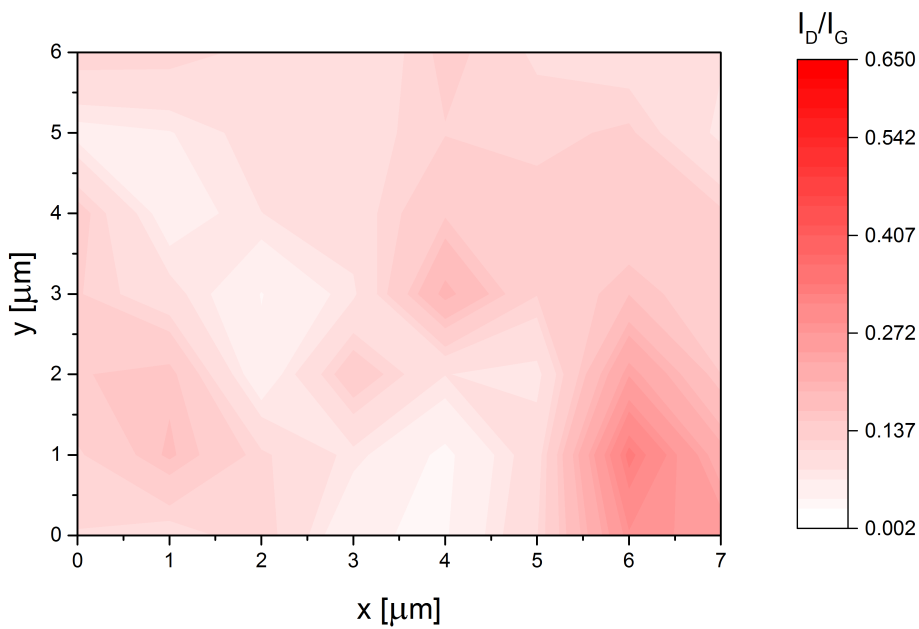
Table 4.3. *Mean values and standard deviation of WCAs of CVD-graphene before and after surface treatments.*

Sample	WCA before	WCA after ST	ST
CVD-G-01	$86^\circ \pm 4^\circ$		
CVD-G-02	$91^\circ \pm 2^\circ$		
CVD-G-03	$96^\circ \pm 1^\circ$	$88^\circ \pm 2^\circ$	ST-A
CVD-G-04	$80^\circ \pm 3^\circ$	$86^\circ \pm 1^\circ$	ST-A
CVD-G-05	$78^\circ \pm 2^\circ$	$68^\circ \pm 1^\circ$	ST-B
CVD-G-06	$77^\circ \pm 2^\circ$	$64^\circ \pm 1^\circ$	ST-B
CVD-G-07	$77^\circ \pm 2^\circ$	$67^\circ \pm 1^\circ$	ST-B
CVD-G-08	$87^\circ \pm 4^\circ$	$88^\circ \pm 1^\circ$	ST-B
CVD-G-09	$83^\circ \pm 2^\circ$	$72^\circ \pm 1^\circ$	ST-C
CVD-G-10	$77^\circ \pm 1^\circ$	$69^\circ \pm 1^\circ$	ST-D
CVD-G-11	$77^\circ \pm 2^\circ$	$68^\circ \pm 1^\circ$	ST-D

showing the ratio of the intensity of the 2D peak to G peak, shows that graphene surfaces remain uniform, and is still made of mono- to few-layer graphene.

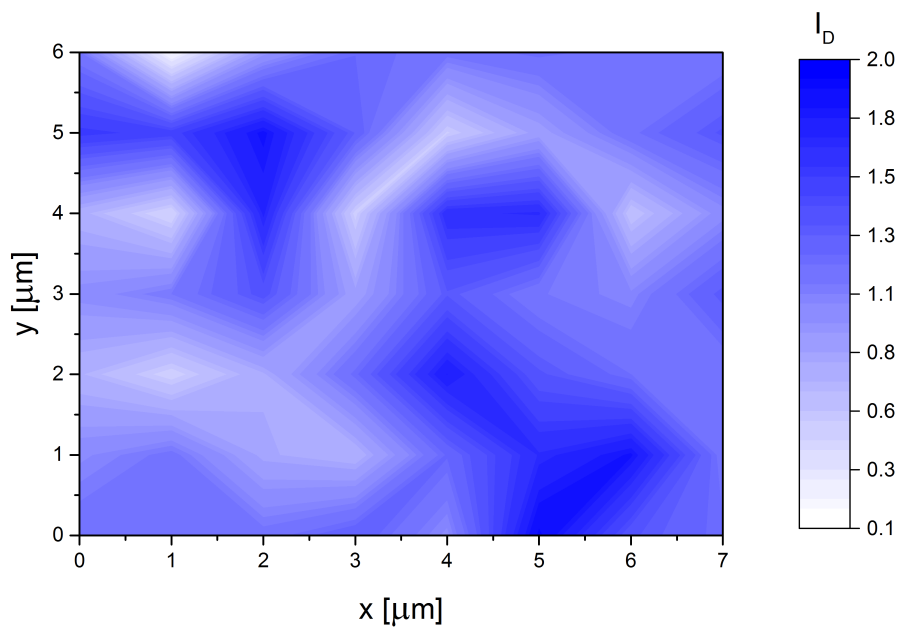


(a)

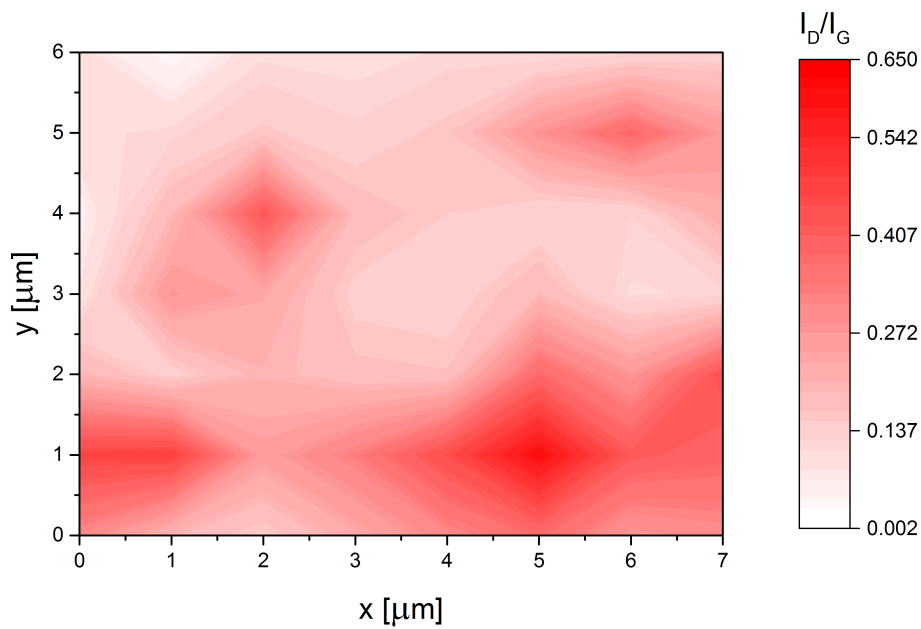


(b)

Figure 4.16. Raman maps of (a) I_{2D}/I_G and (b) I_D/I_G ratios of sample CVD-G-08 after surface treatment ST-A.

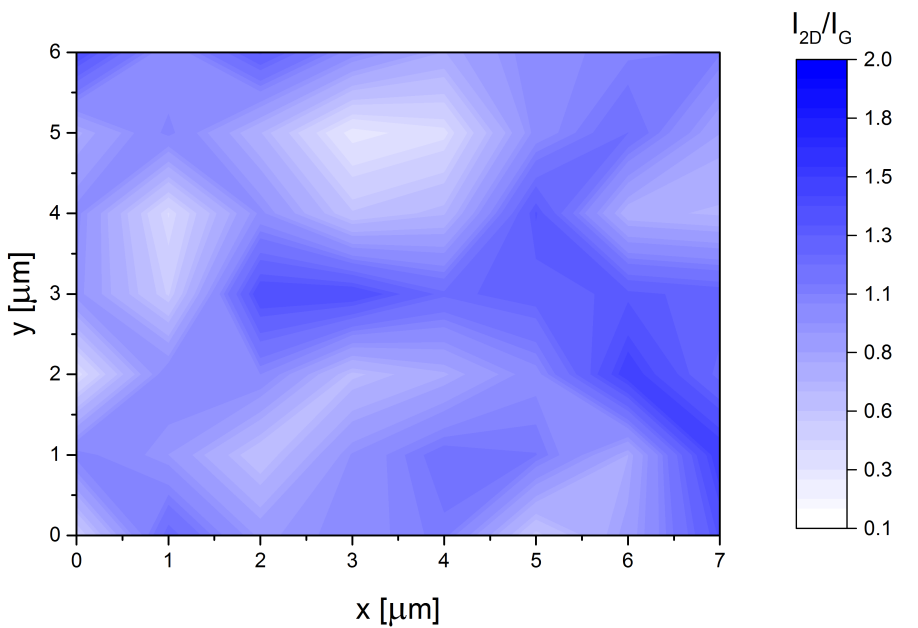


(a)

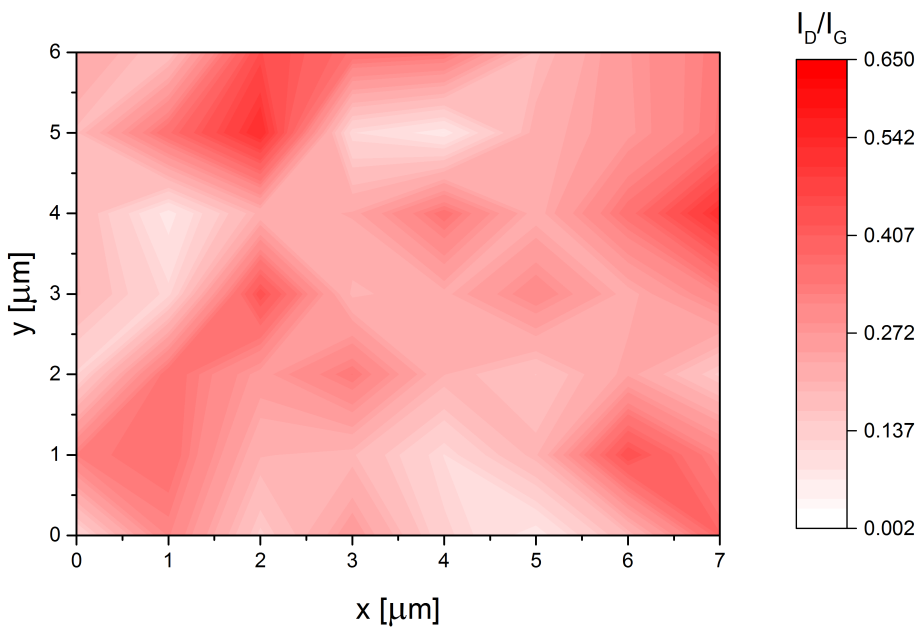


(b)

Figure 4.17. Raman maps of (a) I_{2D}/I_G and (b) I_D/I_G ratios of sample CVD-G-08 after surface treatment ST-B.



(a)



(b)

Figure 4.18. Raman maps of (a) I_{2D}/I_G and (b) I_D/I_G ratios of sample CVD-G-09 after surface treatment ST-C.

4.2.2.c AFM

AFM characterization was performed as described in Section 2.2.5. In order to avoid contamination on the final samples, which could alter the interaction between the graphene surface and the lipids, we acquired the AFM images on smaller test samples ($1\text{ cm} \times 1\text{ cm}$) of graphene from the same synthesis; we then performed the same surface treatments on those samples and acquired AFM images again. Before surface treatments it is possible to notice the presence of PMMA residues on the graphene surface (white spots in Figure 4.19(a)). The surface treatment seems to reduce the amount PMMA, but it has the effect of increasing the wrinkles, and consequently the roughness of the graphene layer, although it remains continuous. AFM images, WCAs measurements, Raman maps and SEM images show that the best graphene samples are the one with the ST-B and ST-C surface treatments, since graphene is still continuous, almost free from PMMA residues and with an increased presence of defects in the lattice, which could be a good platform for the interaction with liposomes.

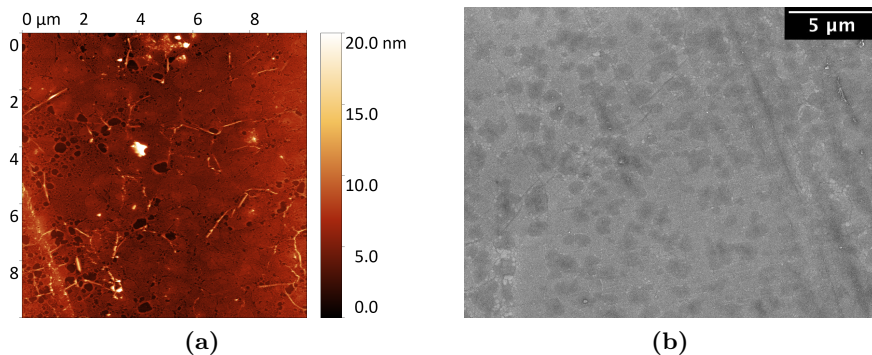


Figure 4.19. AFM (a) and SEM (b) images of CVD-G-08 as is.

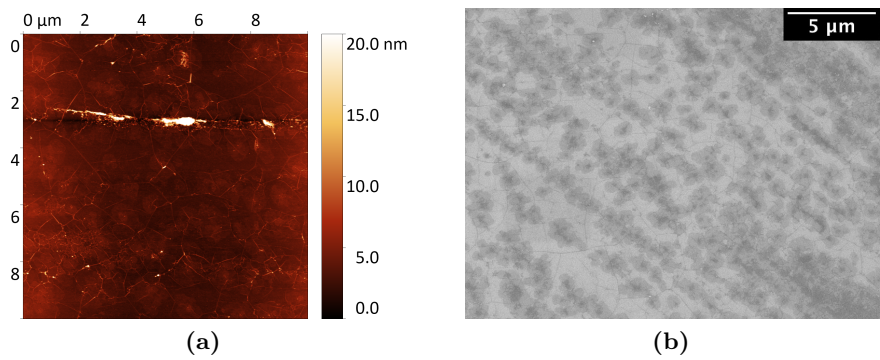


Figure 4.20. AFM (a) and SEM (b) images of CVD-G-08 after surface treatment ST-A.

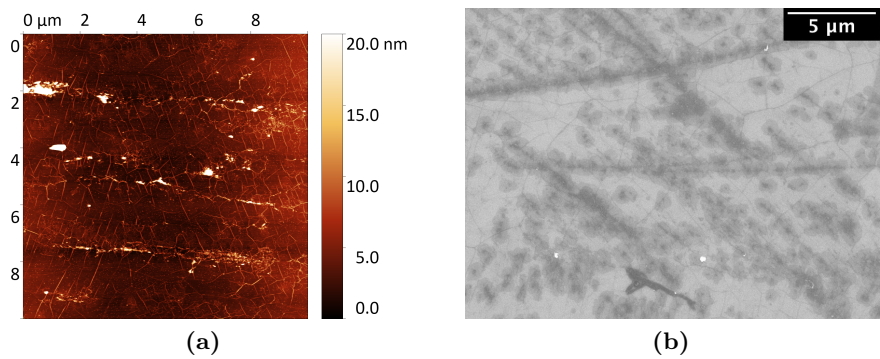


Figure 4.21. AFM (a) and SEM (b) images of CVD-G-08 after surface treatment ST-B.

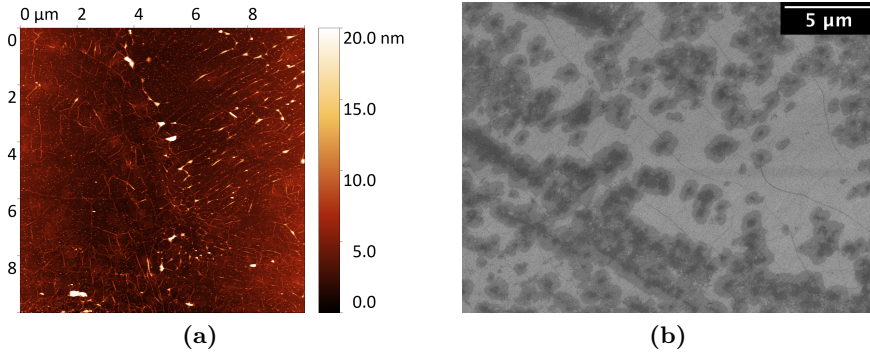


Figure 4.22. *AFM and SEM images of CVD-G-09: (a), (b) after surface treatment ST-C.*

4.2.2.d XPS

XPS was performed using the apparatus described in Section 2.2.4. Two resulting spectra in the binding energy (BE) range between 294 eV and 280 eV, characteristic of the C 1s peak, are shown in Figure 4.23: (a) shows the spectrum of graphene as is, while (b) shows the spectrum of graphene after surface treatment (in particular ST-B). The dotted line represents the whole C 1s peak, the peaks filled in grey, displayed at the bottom of the Figure 4.23(a) and (b), represent the PMMA contribution, which is subtracted from the spectrum, in order to obtain the only contribution from graphene (the sum of the peaks in red and green). The green peak represents the sp^2 carbon, while the three red peaks represent the C=O, C–O–C bonds and the sp^3 carbon, respectively.

The O/C ratio was calculated after PMMA quantification and subtraction as described in Section 2.2.4; we also measured the amount of copper, which is still present after the etching of the substrate and is in agreement with what can be found in the literature, that is to say a copper $Cu\% < 0.5\%$. The O/C ratio, the PMMA and copper percentages are shown in Figure 4.24(a), (b) and (c) and are summarized in Table 4.4. It is possible to notice that the amount of PMMA decreases significantly after surface treatments, thus indicating that the surface treatments clean the surface. The O/C ratio indicates that the amount of oxygen does not increase after surface treatments: this could imply that the surface heat treatments in oxygen have the only effect of cleaning the surfaces and increasing

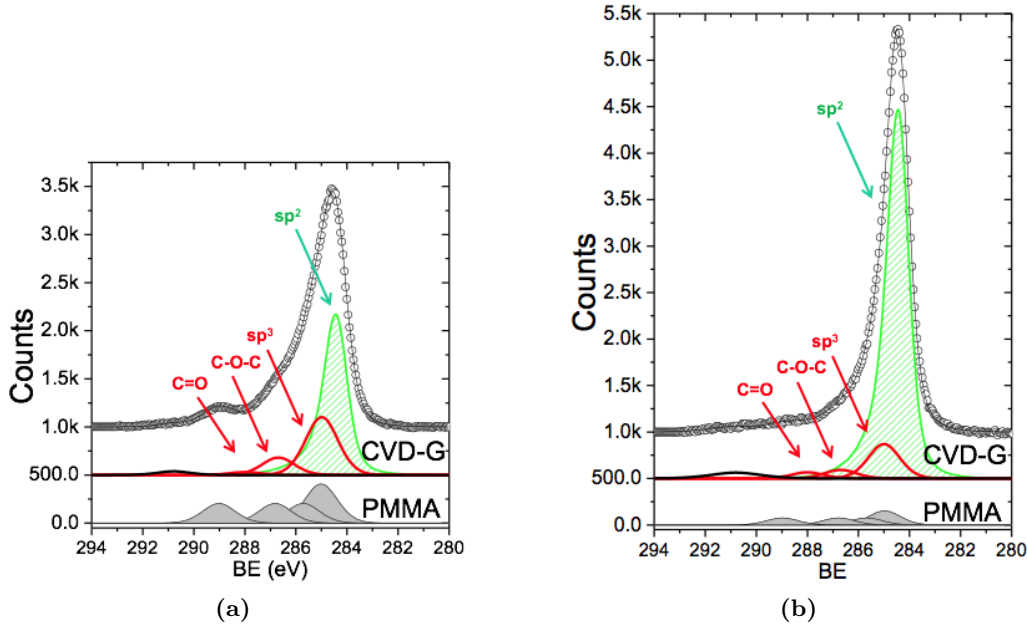


Figure 4.23. XPS spectra of (a) CVD-G as is and (b) after ST-B.

the wrinkles on graphene. The amount of copper displayed in Figure 4.24(c) is always lower than 0.5%, therefore it is in the range of the copper contamination reported in the literature after copper etching. Thus, the copper content is only related to the variyin amount of copper loft on the different graphene samples after the copper etching step, and no effect of the heat treatments on the Cu% can be inferred.

Table 4.4. XPS of CVD-graphene before and after surface treatments.

Sample	ST	PMMA	O/C	Cu
CVD-G-08	-	61 ± 4	0.034 ± 0.009	0.057 ± 0.030
CVD-G-08	ST-A	9 ± 2	0.029 ± 0.009	0.157 ± 0.030
CVD-G-08	ST-B	11 ± 2	0.031 ± 0.009	0.154 ± 0.050
CVD-G-09	ST-C	12 ± 2	0.024 ± 0.009	0.395 ± 0.080

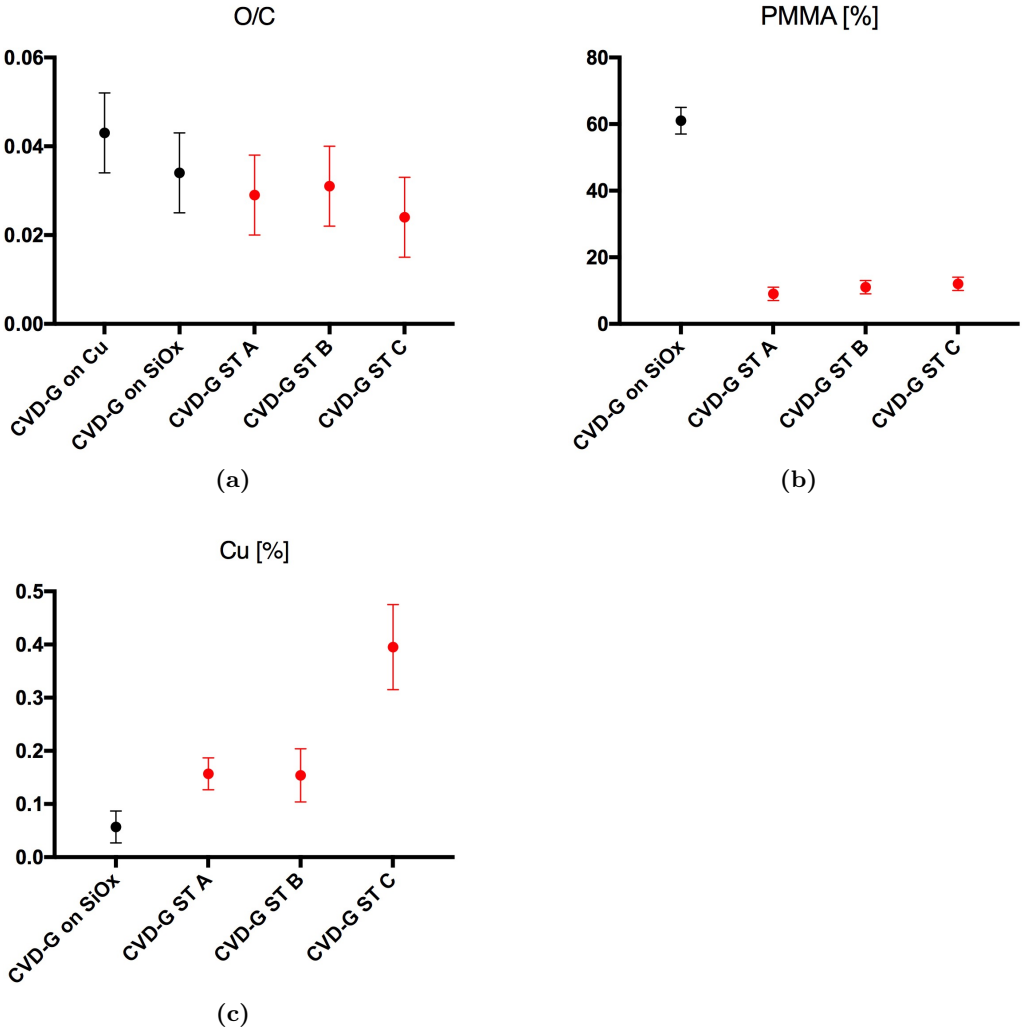


Figure 4.24. (a) O/C calculated after PMMA subtraction; (b) % of PMMA; (c) % of copper after etching on CVD-graphene as-transferred and after thermal surface treatments.

4.3 Conclusions

In conclusion, SEM images confirm that graphene fully covers the SiO₂/Si substrates and that it has small islands of overgrowth. It is also possible to notice the presence of residuals of PMMA, which is not fully removed by acetone: unluckily it is still a big issue, as discussed in the literature, which needs to be solved in order to obtain clean graphene. We also used an alternative to PMMA for the transfer of graphene (Section 3.2.2.b), but the cyclododecane based method is more suitable for transferring small samples of graphene (max. 1 cm × 1 cm), due to its larger stiffness with respect to PMMA.

The result of WCA measurements, before and after the surface treatments, show the hydrophobicity of graphene, with values in the range from 77° to 96°. The variability of WCAs before surface treatments arises from the presence of PMMA residuals on the graphene surface. After the heat surface treatments the WCAs of graphene decrease while increasing the temperature or time of the surface treatments; the lower standard deviation after the annealing is attributed to the decreasing amount of PMMA residues, leaving a more homogeneous surface characterized by a lower spread of the WCAs values.

Raman maps show that the CVD-graphene samples under investigation are mono- to few-layer, with a small amount of defects; after surface treatments, the ratio of the intensity of D peak to G peak increases confirming the presence of defects in the lattice, as can be seen from the higher intensity of color in the maps.

AFM images show the presence of residues on the graphene surface before surface treatments. The surface treatment seems to reduce the amount PMMA, but it has the effect of increasing the wrinkles, and consequently the roughness of the graphene layer, although the layer remains continuous. From the literature it is known that these irregularities are site that could act to favor the interaction of the graphene surface with liposomes by inducing liposome rupture and lipid membrane formation. [83]

XPS spectra show that the surface treatments remove PMMA residuals from the surface. The O/C ratio indicates that the amount of oxygen does not increase after surface treatments. On the other hand, upon thermal treatment, the different thermal expansion coefficient of graphene on SiO₂ gives rise to an increase of the wrinkles on graphene layers, as observed by SEM and AFM characterization. The increased amount of defects observed by micro-Raman spectroscopy is then related

to the formation of wrinkles, which are source of defects.

The graphene samples after surface treatments were chosen as potential platforms to study the interaction of graphene and lipid membranes, which are preferentially formed on more hydrophilic substrates ($\text{WCA} < 50^\circ$).

Chapter 5

Electrochemistry of supported lipid membranes on CVD-G

5.1 Introduction

After the surface treatments of graphene, to improve its compatibility towards supported lipid membranes, we performed the analysis of the interaction with liposomes of different sizes and composition using electrochemical impedance spectroscopy (EIS). We present here the Bode and Nyquist plots; in order to give a clear and intuitive visualization of the results, in Section 5.2.4 the results of the EIS data fitting using an equivalent circuit model are presented.

5.2 Results and discussion

5.2.1 Preparation of the samples for EIS measurements

After surface treatments and characterization of graphene, nickel contacts , approximately 300 nm thick, were deposited in order to improve the contact to graphene and avoid its damage by scratching when using alligator clips during EIS measurements. Contacts were deposited by thermal evaporation using a Edwards E306 belljar evaporator system equipped with a rotating source holder with 4 positions (allowing up to 4 different materials to be deposited in sequence in the same vacuum cycle), a thickness monitor and a pumping system with a rotary and turbo pump. The evaporation of nickel was carried out through a specifically

designed stencil mask, shown in Figure 5.1(a). One of the resulting graphene samples is shown in Figure 5.1(b), where the red square highlight the graphene layer edges. Moreover, the deposition of the 4 contacts at the edges of graphene was designed to ensure an anchoring of the graphene to SiO_2 , which is useful in view of the following incubation with liposome solutions and EIS measurements, both performed in wet environment.

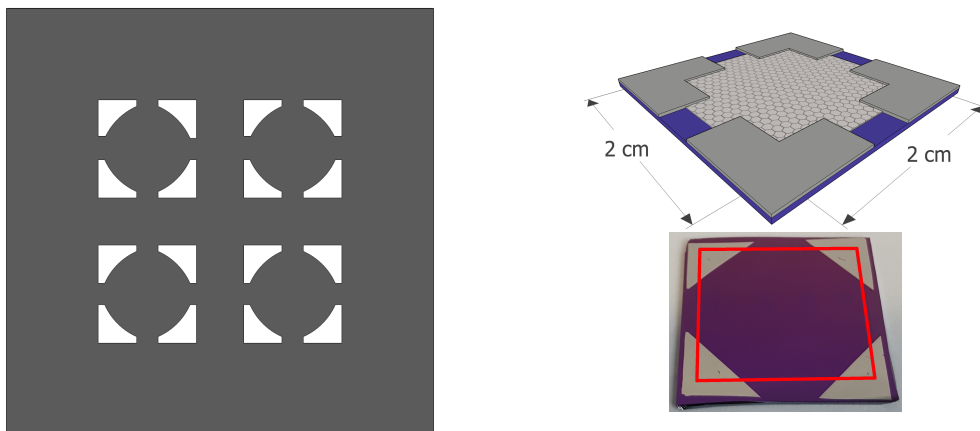


Figure 5.1. *Stencil mask used for the evaporation of nickel contacts (a); design of the nickel contact on the graphene sample (b); photo of one of the samples in which graphene is highlighted by red (c).*

5.2.2 Electrochemical cell

Graphene surfaces constitute the working electrode of the electrochemical cell: SiO_2/Si wafer has a dimension of $2.5\text{ cm} \times 2.5\text{ cm}$, and CVD graphene $2\text{ cm} \times 2\text{ cm}$, thus it was necessary to design a customized electrochemical setup with proper dimension. The electrochemical cell is made of polytetrafluoroethylene (PTFE), which has low chemical reactivity and can be thoroughly cleaned in acid bath. The electrochemical cell consists of three different parts sealed by two O-rings: the first one is the support on which the working electrode (i.e. CVD graphene on SiO_2/Si wafer) is mounted; the second one contains a 1 cm wide circular chamber where graphene gets in contact with the liposome solution; the third one is where the counter and the reference electrode are held in place. The central element of the electrochemical cell is provided with two holes for connection of the inner chamber

with rinsing tubes. The system allows for the injection of the liposome solution as well as the elimination of the non-interacting liposomes out of the measuring cell at the end of the incubation time. We designed and fabricated the electrochemical cell, taking into account the dimension of the graphene working electrode and the geometry of contacts and having the proper reaction volume of the sample chamber (1 ml). Figure 5.2(a) shows the design of the electrochemical cell, and (b) shows a real picture of the electrochemical cell: in both images, all the electrodes are highlighted.

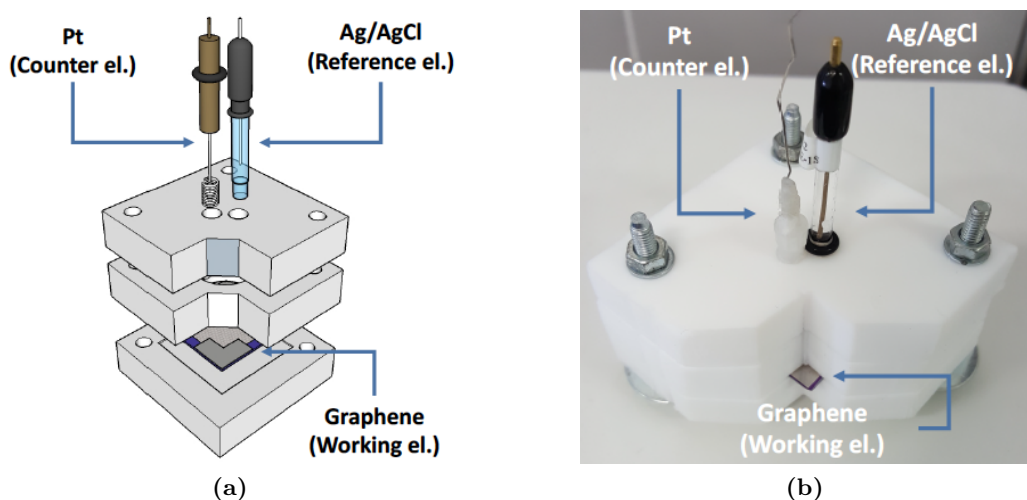


Figure 5.2. *Electrochemical cell made of PTFE: (a) schematic view of the three elements of the cell and position of the electrodes; (b) photograph of the fabricated and assembled cell.*

5.2.3 EIS measurements

First of all we carried out EIS measurements on a gold chip, which is one of the most common used working electrode, and we compared the resulting plots with those of CVD-G-01, as shown in Figure 5.3. Using the Gamry Reference 600, three potentiostatic EIS measurements were carried out in PBS solution at room temperature (25 °C) with an excitation amplitude of 10 mV rms and a frequency range from 0.01 Hz to 10^5 Hz. The modulus curve - Figure 5.3(a) - shows an order of magnitude of difference between gold and graphene electrode, both at high and low frequencies, thus indicating a great difference between those two electrodes.

The behavior of graphene electrode at high frequencies is characteristic and it closely resembles what is found in the literature in the case of a coated surface and therefore it is possible to deduce that some kind of coating, even though it can be not continuous, is presente on the graphene electrode (for example PMMA residuals). [84–87]

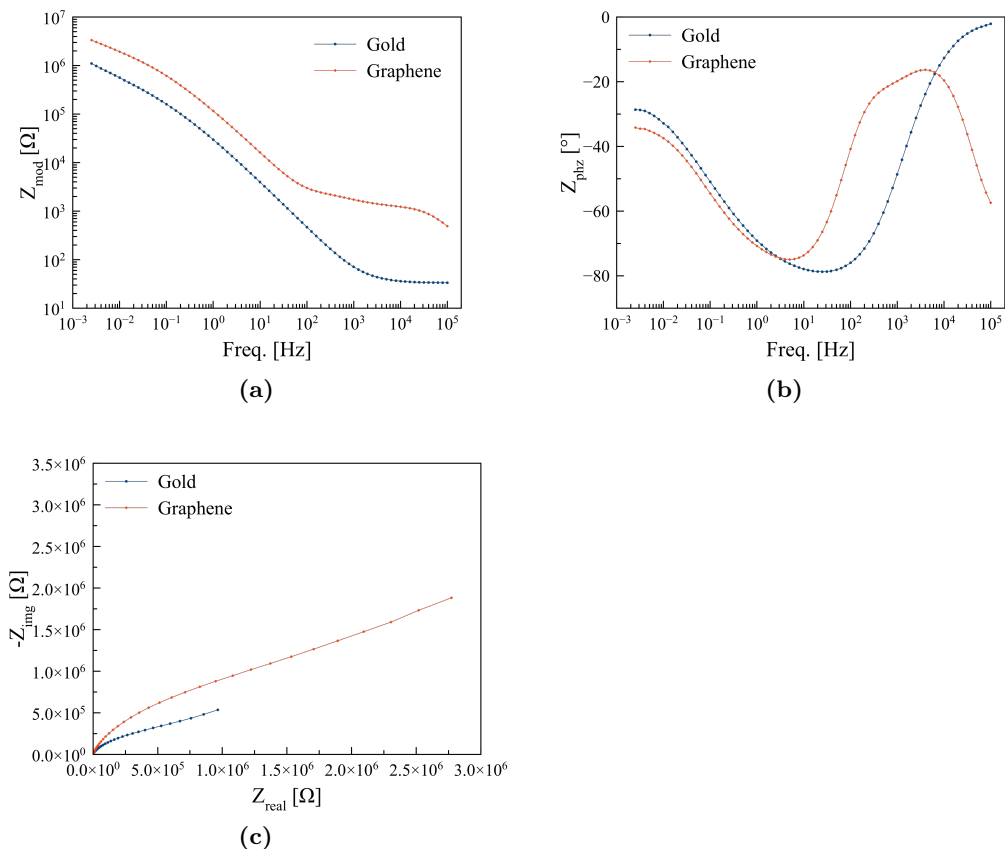


Figure 5.3. Bode plots of modulus (a) and phase (b) and Nyquist plot (c) of gold-electrode in comparison with CVD-G-01.

EIS experiments of the interaction of graphene with liposomes were carried out on the CVD-graphene samples as summarized in Table 5.1, in which the WCAs after the surface treatments and the type of solution are also reported. After these measurements, the PBS solution in the sample chamber of the electrochemical cell was replaced with the PBS solution containing liposomes. The incubation was conducted overnight, after which the non-adsorbed liposomes were rinsed off using

Table 5.1. *Mean values and standard deviation of WCAs of samples and the type of liposome solution used.*

Sample	WCA	Surface treatment	Liposome solution
CVD-G-01	$86^\circ \pm 4^\circ$		DOPC-50
CVD-G-02	$91^\circ \pm 2^\circ$		DOPC-100
CVD-G-03	$88^\circ \pm 2^\circ$	ST-A	DOPC-50
CVD-G-04	$86^\circ \pm 1^\circ$	ST-A	DOPC-50
CVD-G-05	$68^\circ \pm 1^\circ$	ST-B	DOPC-50
CVD-G-06	$64^\circ \pm 1^\circ$	ST-B	DOPC-100
CVD-G-07	$67^\circ \pm 1^\circ$	ST-B	DOPC-50
CVD-G-08	$88^\circ \pm 1^\circ$	ST-B	DOPC-100
CVD-G-09	$72^\circ \pm 1^\circ$	ST-C	DOPC-50
CVD-G-10	$69^\circ \pm 1^\circ$	ST-D	DOPC/EPC-50
CVD-G-11	$68^\circ \pm 1^\circ$	ST-D	DOPC/DOPS-50

PBS buffer (at least 5 ml), and three new measurements were performed, using the same parameters as before. As a result, three different plots were extracted (each one showing the mean value of the three measurements): two Bode plots, one with the modulus and one with the phase of the impedance, and a Nyquist plot, showing the real and imaginary parts of the impedance.

The measurements were carried out using different liposome solutions, whose preparation is described in Section 2.1.2:

- Solution 1 (DOPC-100): liposomes made up of DOPC lipids with average diameter of 100 nm;
- Solution 2 (DOPC-50): liposomes made up of DOPC lipids with average diameter of 50 nm;
- Solution 3 (DOPC/EPC-50): liposomes made up of a mixture of DOPC and EPC (2:1) with average diameter of 50 nm;
- Solution 4 (DOPC/DOPS-50): liposomes made up of a mixture of DOPC and DOPS (2:1) with average diameter of 50 nm.

Figures 5.4 to 5.14 show respectively the Bode plots of the modulus, the Bode plots of the phase and the Nyquist plots of graphene samples with different surface treatments before and after the interaction with different liposome solution.

The contribution of the interaction of graphene with SLB can be found at low frequencies, and causes the increase of the (incomplete) semicircle curve shape in the Nyquist plot, but it is difficult to evaluate it only from these plots: we will discuss the results by using the fitting with an equivalent circuit (EC), as described in Section 2.2.6.c.

CVD-G-01

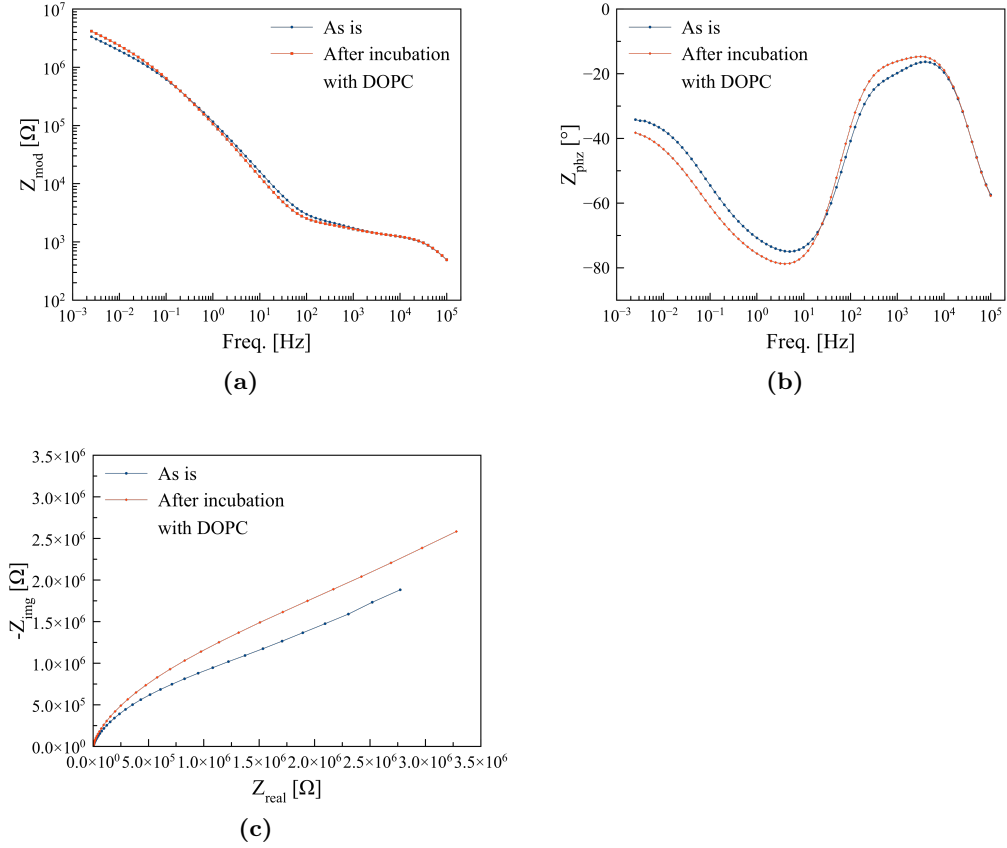


Figure 5.4. Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-100), and Nyquist plot (c) of CVD-G-01.

CVD-G-02

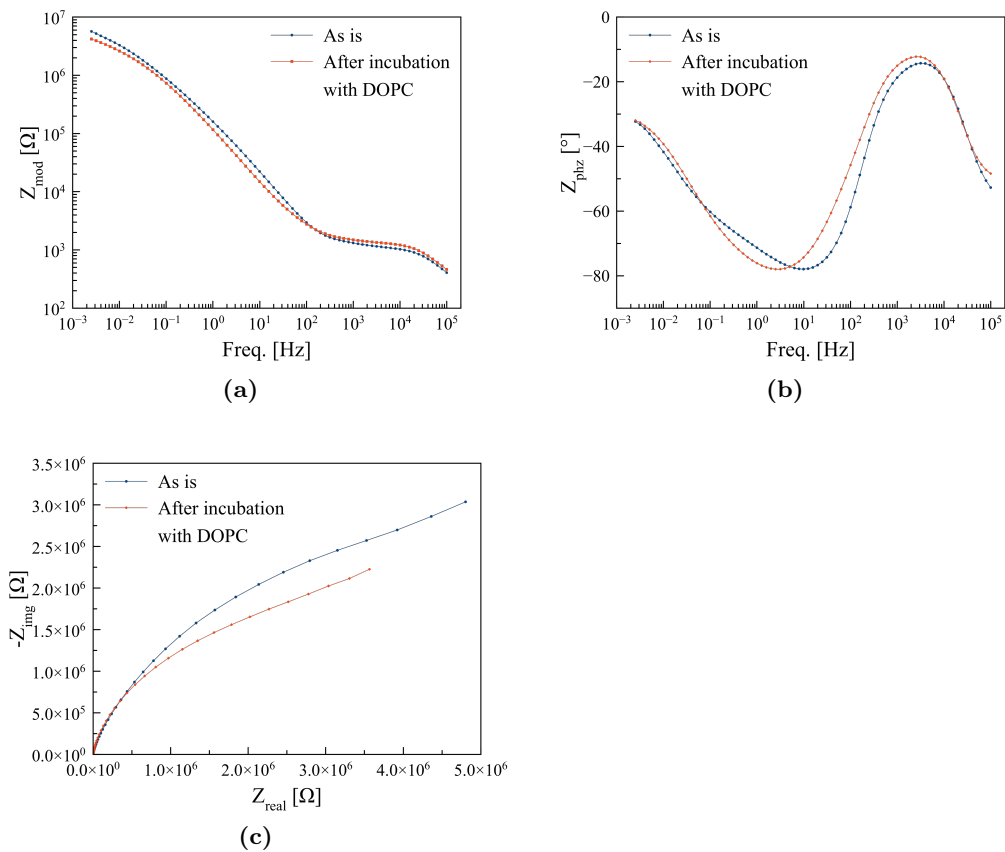


Figure 5.5. Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-100), and Nyquist plot (c) of CVD-G-02.

CVD-G-03

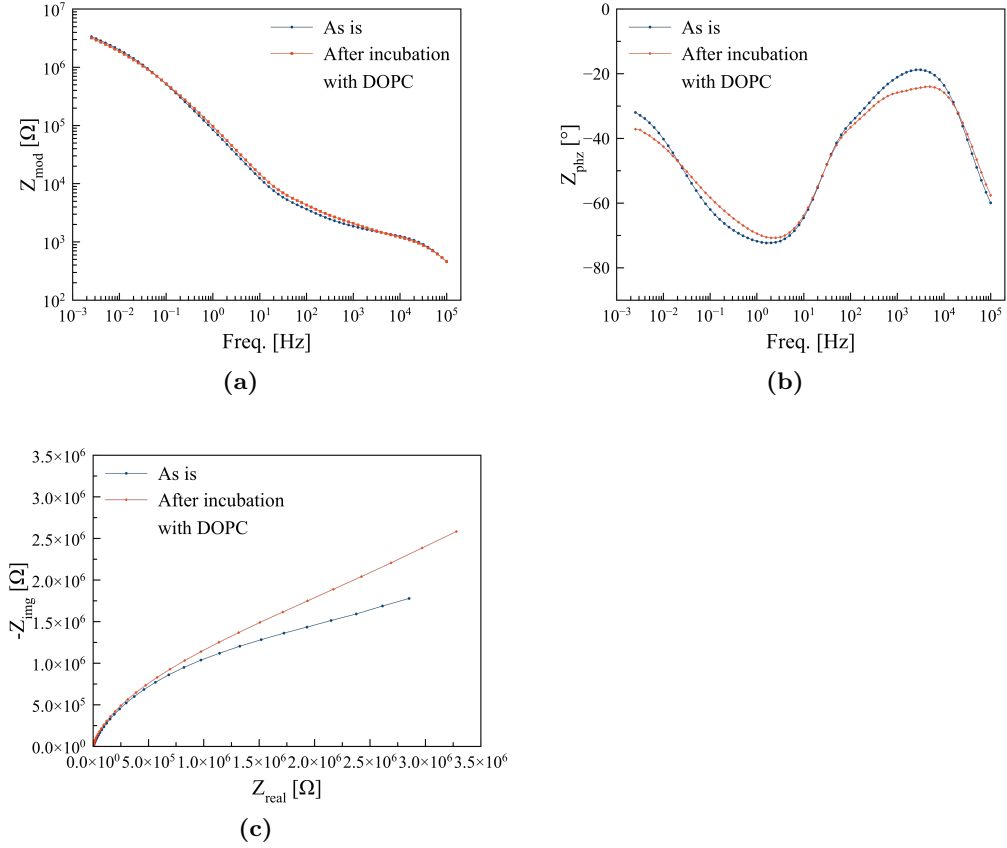


Figure 5.6. Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-50), and Nyquist plot (c) of CVD-G-03.

CVD-G-04

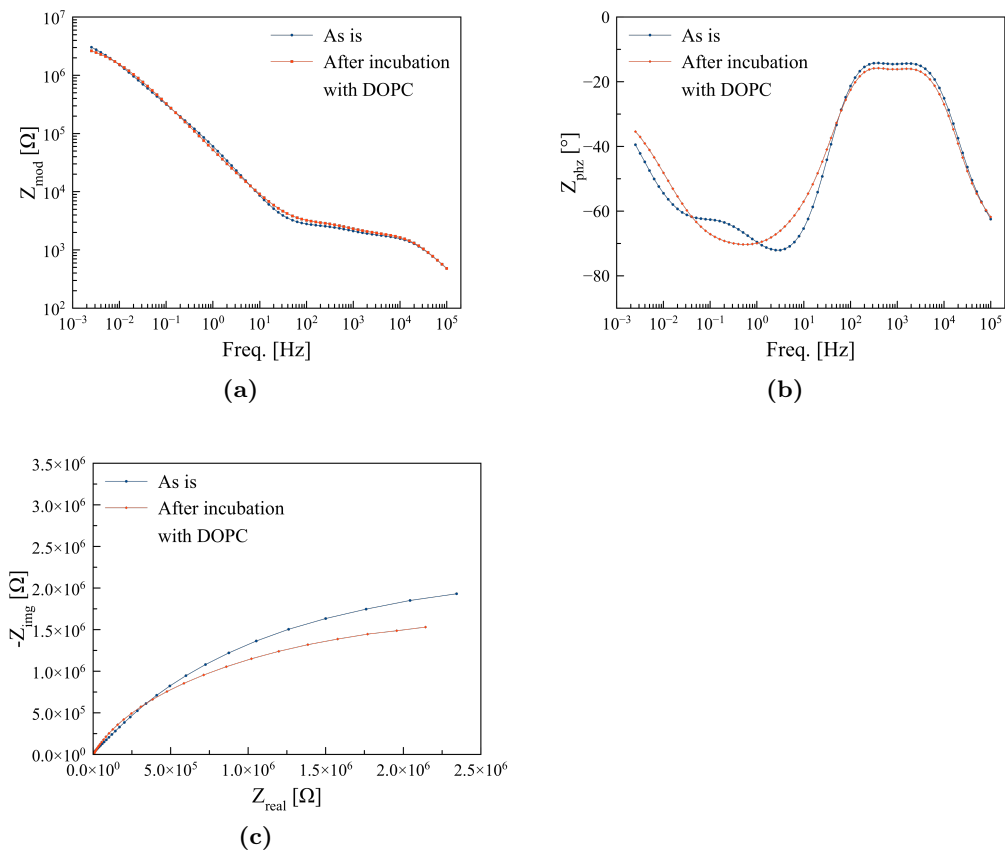


Figure 5.7. Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-50), and Nyquist plot (c) of CVD-G-04.

CVD-G-05

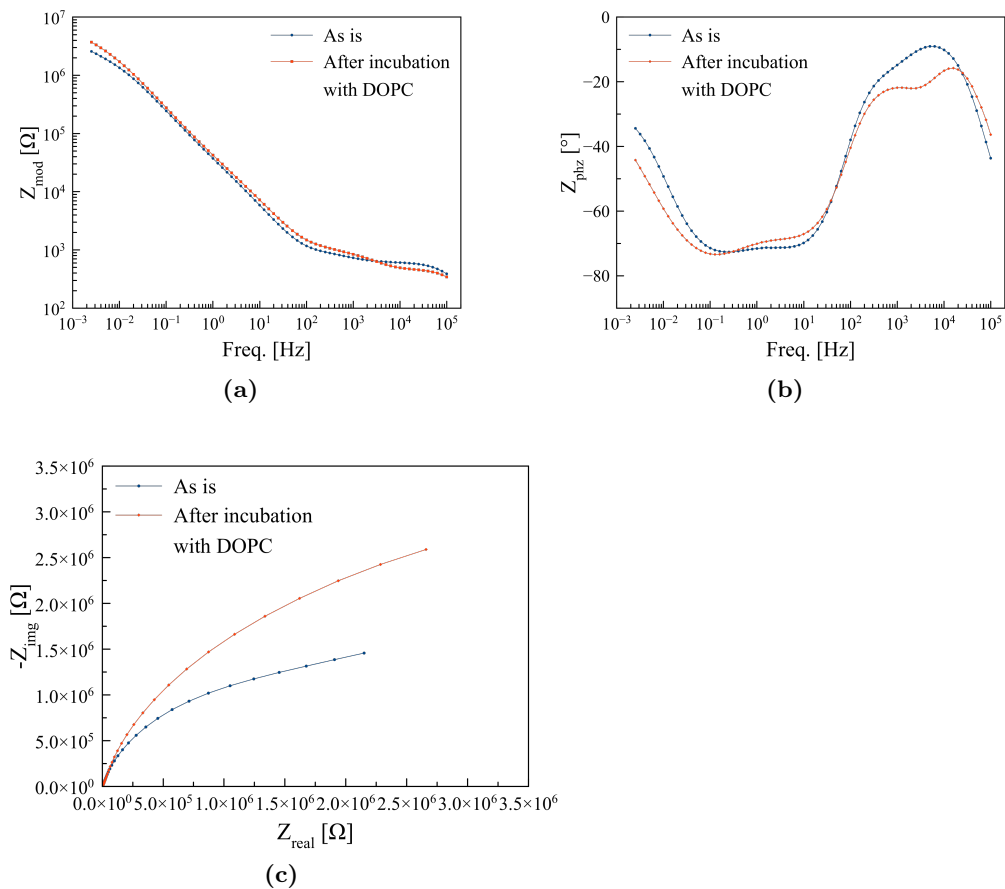


Figure 5.8. Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-50), and Nyquist plot (c) of CVD-G-05.

CVD-G-06

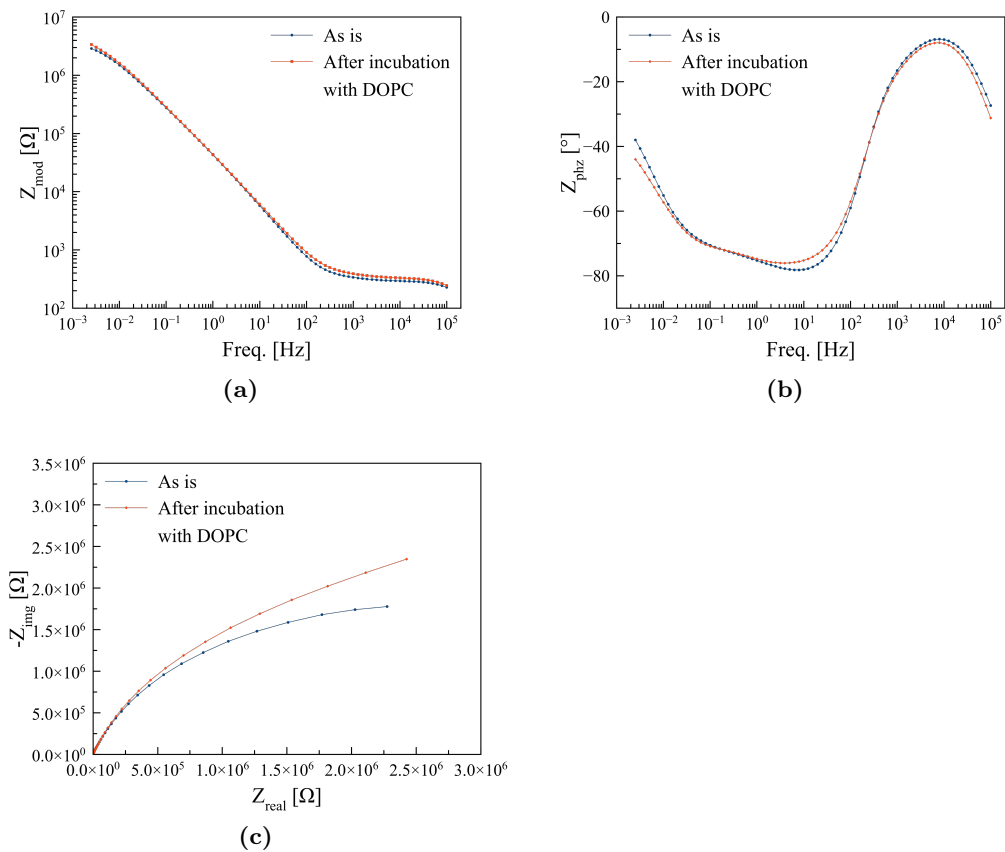


Figure 5.9. Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-100), and Nyquist plot (c) of CVD-G-06.

CVD-G-07

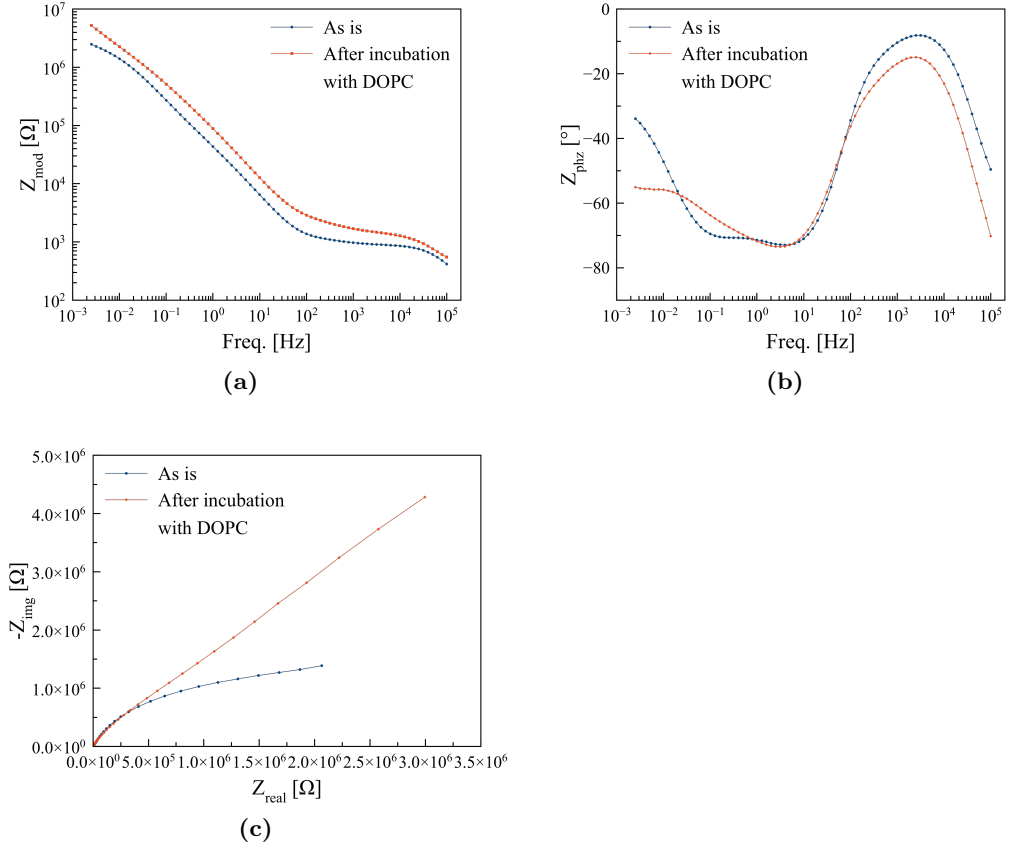


Figure 5.10. Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-50), and Nyquist plot (c) of CVD-G-07.

CVD-G-08

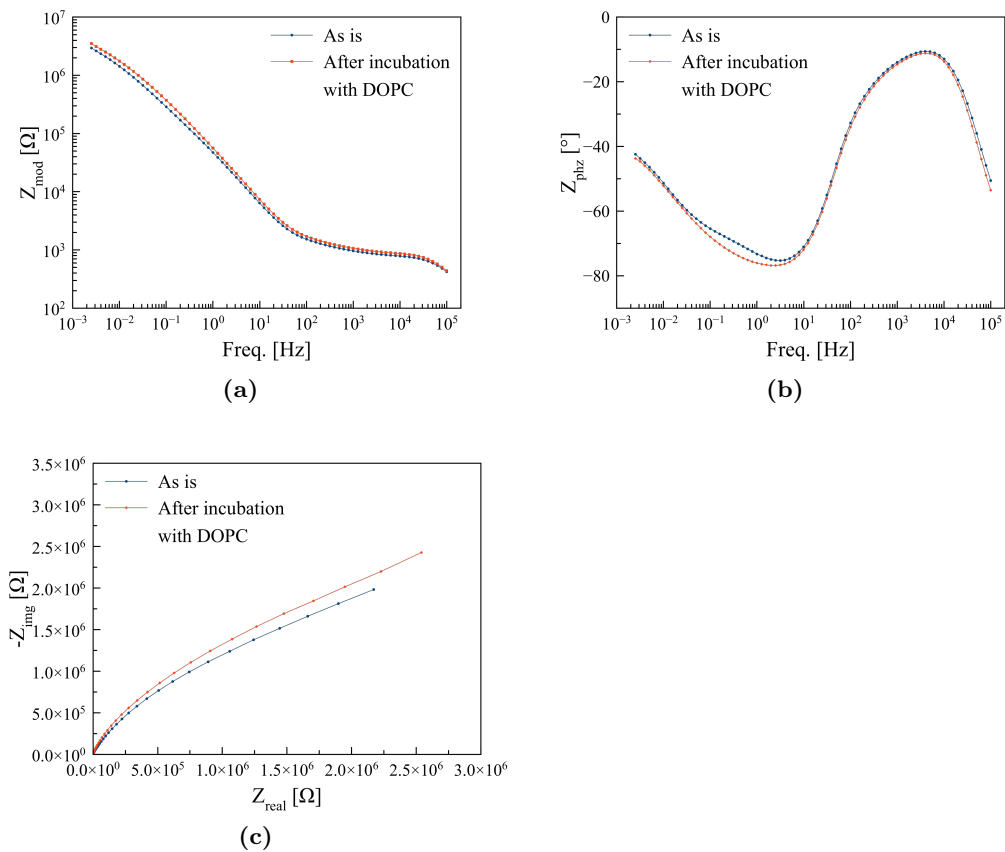


Figure 5.11. Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-100), and Nyquist plot (c) of CVD-G-08.

CVD-G-09

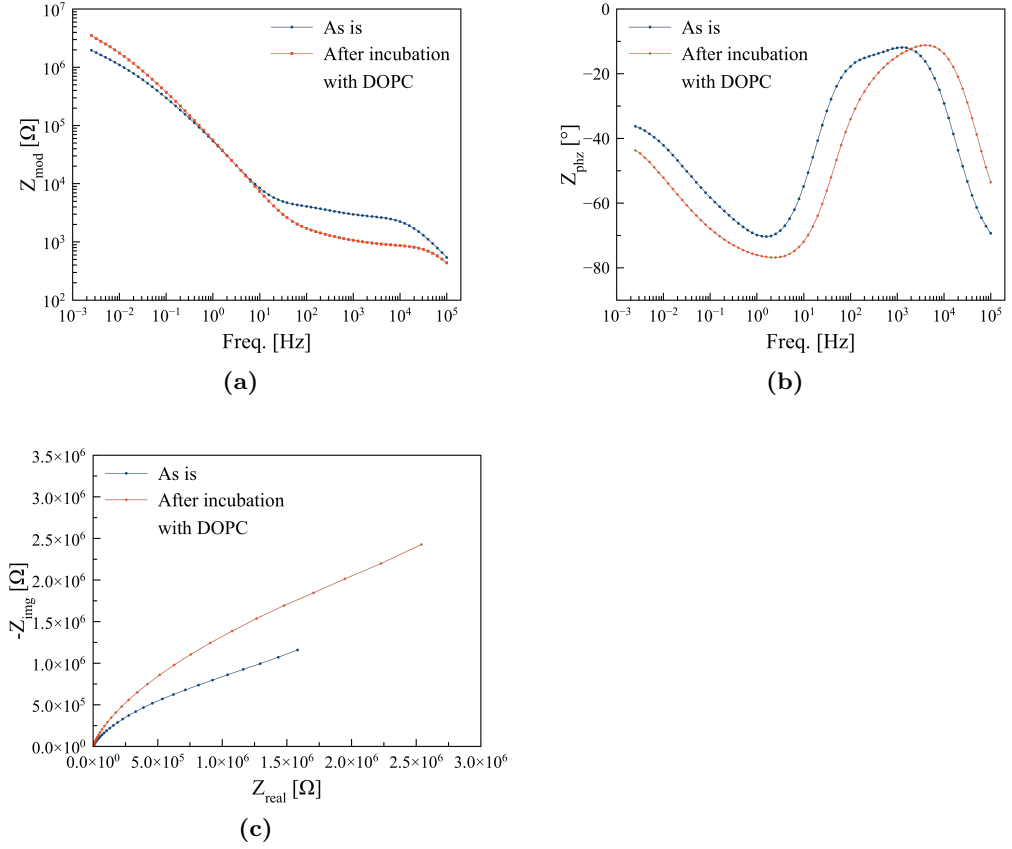


Figure 5.12. Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC-50), and Nyquist plot (c) of CVD-G-09.

CVD-G-10

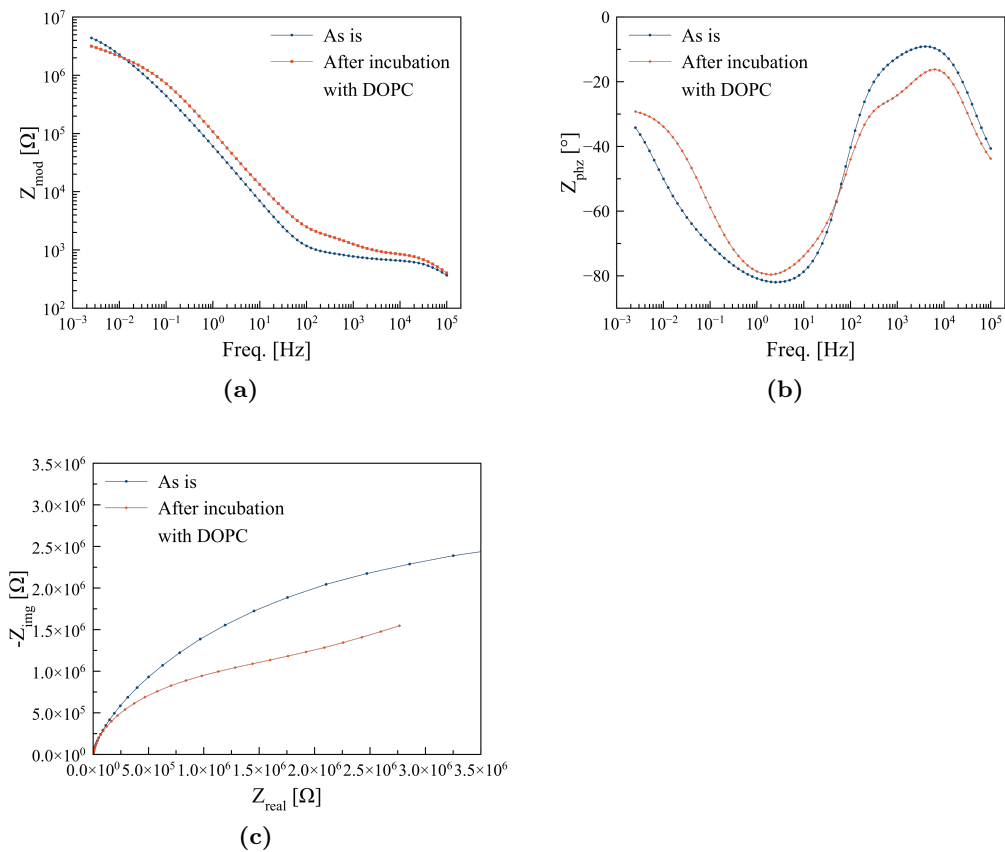


Figure 5.13. Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC/EPC-50), and Nyquist plot (c) of CVD-G-10.

CVD-G-11

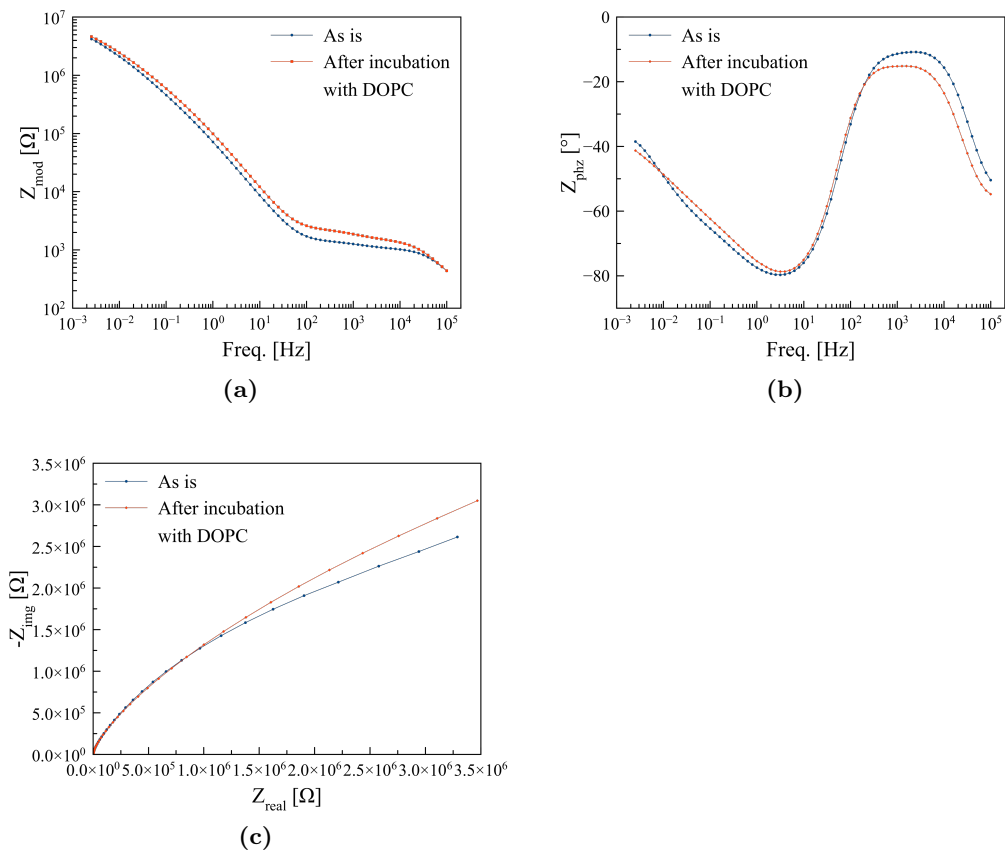


Figure 5.14. Bode plots of the modulus (a) and phase (b) of the impedance before (blue) and after (red) incubation with liposome solution (DOPC/DOPS-50), and Nyquist plot (c) of CVD-G-11.

5.2.4 Equivalent circuit

EIS data were analyzed by fitting them using an equivalent electrical circuit model as described in Section 2.2.6.c. Several circuits were designed ad hoc assembling different elements in the effort to fit impedance data at both ends of the frequency range. Figure 5.15 shows the EC that was finally selected for this study: R_s represents the ohmic resistance (solution, wires and other uncompensated resistance), C_{dl} represents the density of conductive pathways, R_d represents the presence of non-conductive "defects" in the graphene lattice (pores, molecules, etc.), R_g , W and CPE (characterized by Y_g and n_g) represents the graphene surface. R_d and C_{dl} are related to the non-ideality of the graphene lattice.

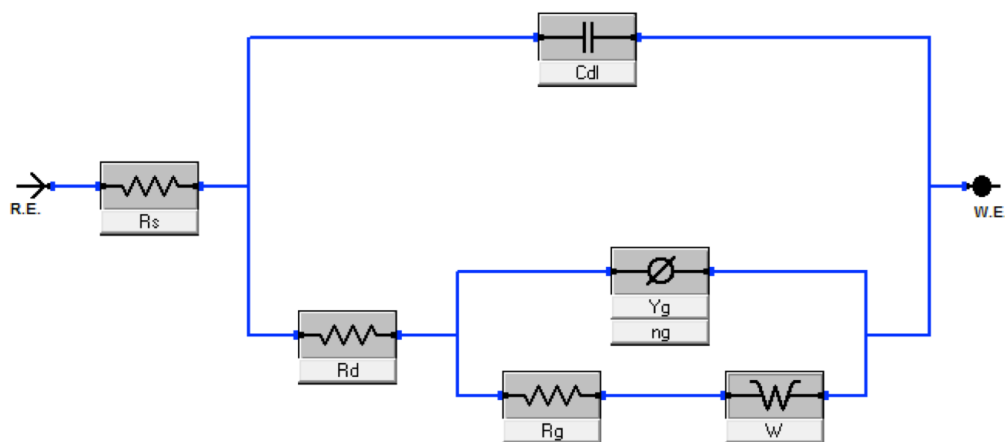


Figure 5.15. *Equivalent circuit.*

After choosing the EC, we fitted the three measured EIS curves, using the software provided by Gamry, before incubation; R_s , R_d and C_{dl} are fixed, using the mean values coming from the fit of the three curves before the incubation with the liposome solution; those values are fixed since the characteristics of the graphene surface are not modified by incubation with the liposomes. We fitted the three curves after the incubation with the liposome solution and rinsing, and compared the obtained values with the values before incubation. The contribution of the supported lipid membrane can be thought to be part of R_g , CPE and W , that are shown in Figures 5.16 to 5.18; the blue and red bar shows respectively the value before and after the incubation with the solution.

Figure 5.16(a), (b) and (c) show the resulting graph bars when using the DOPC-100 liposome solution: R_g increases in all samples, while W decreases in two samples of three. The increase of R_g and the decrease of W imply the presence of an insulating layer (i.e. lipid) on graphene. The standard deviation of R_g in the samples increases while increasing the time of annealing: the surface "changes" during electrochemical measurements and this can be related to the fact that the surfaces, modified by the annealing processes with the introduction of defects in the lattice, are then modified during electrochemical measurements (as can be seen for graphene oxide, whose reduction can be electrochemically performed [88]).

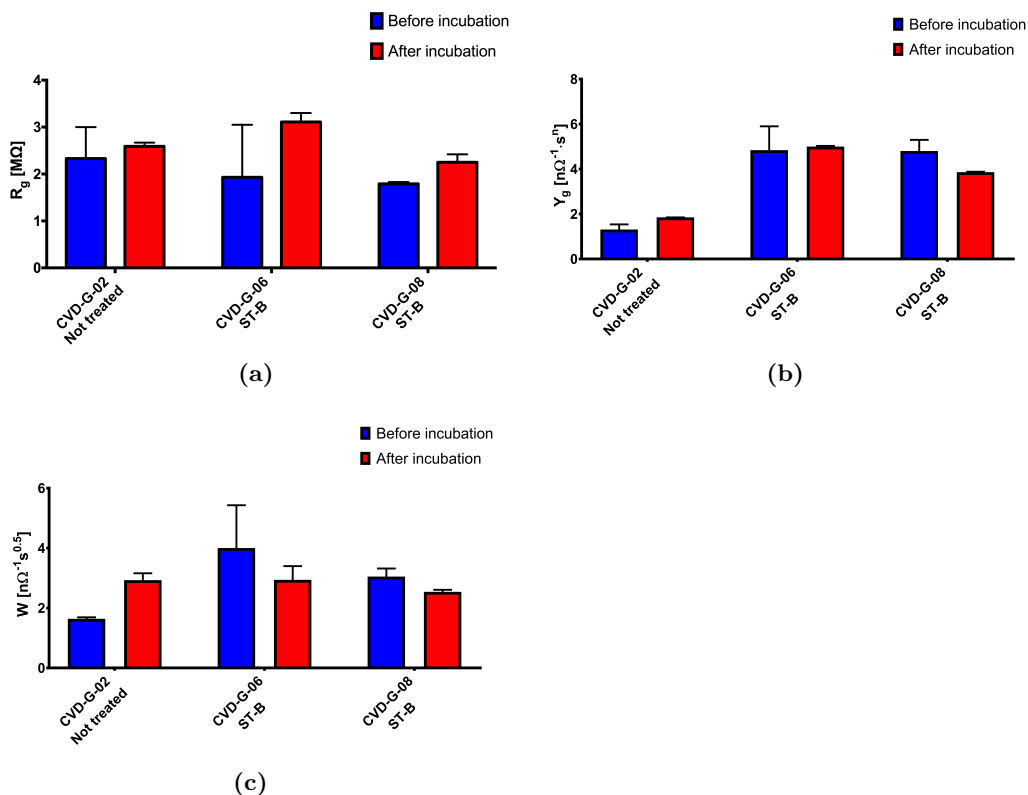


Figure 5.16. Graph bars of R_g (a), Y_g (b) and W (b) when using DOPC-100 solution, with different ST.

Figure 5.17(a), (b) and (c) show the resulting graph bars when using DOPC-50 liposome solution: R_g increases in five samples of six, while W decreases in three samples of six: the decrease of W is related to the lowest diffusion of ions, probably

due to the presence of an insulating layer over graphene. The increase of R_g and the decrease of W is attributed to the presence of an insulating layer (i.e. SLB) on graphene. The increase of R_g is higher when using DOPC-50 solution than the increase of R_g when using DOPC-100 solution: liposomes with smaller dimensions (50 nm) are more unstable than the larger one (100 nm) due to their higher degree of curvature and tend to rupture more easily, thus forming an insulating layer on an adequate substrate.

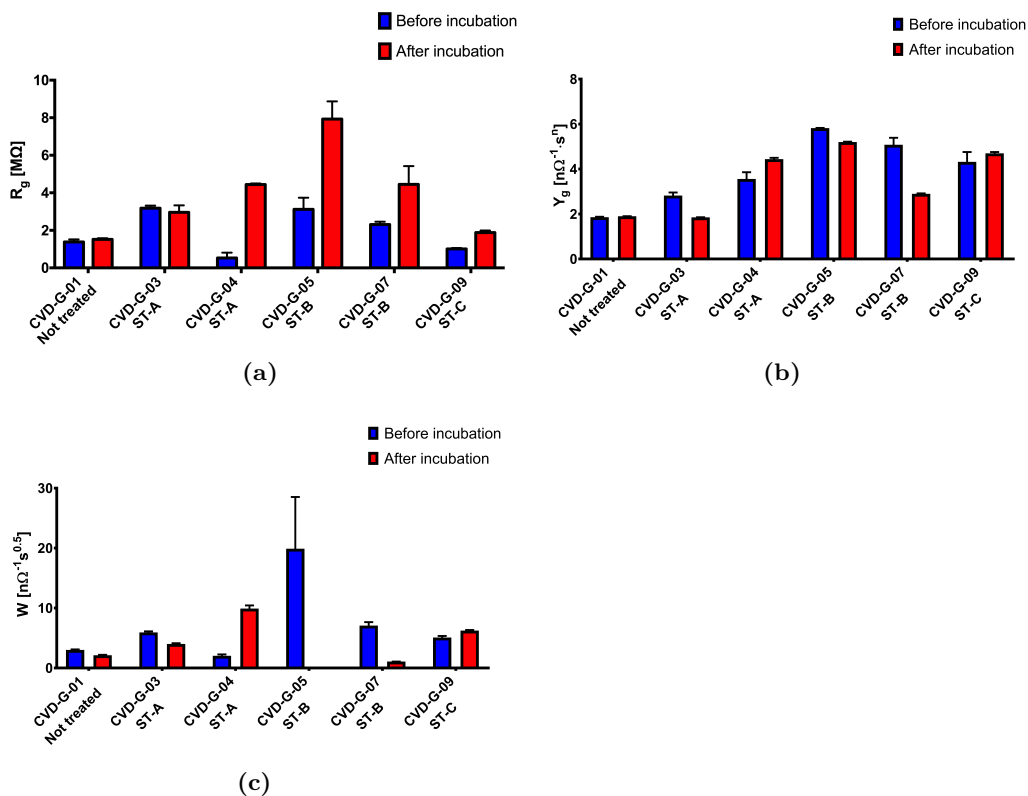


Figure 5.17. Graph bars of R_g (a), Y_g (b) and W (b) when using DOPC-50 solution, with different ST.

Figure 5.18(a), (b) and (c) show the resulting graph bars when using DOPC/EPC-50 and DOPC/DOPS-50 liposome solutions (e.g. using a mixture of charged and zwitterionic lipids). Both samples are ST-D treated. From these bar graphs, it is possible to deduce that the negative charged liposomes (DOPC/DOPS-50) seem to be more attracted by graphene than the positive charged ones (DOPC/EPC-50).

The variation of R_g , Y_g and W is much higher for DOPC/EPC-50 than those for DOPC/DOPS-50, thus indicating a poor interaction between graphene and the positive charged liposomes, which do not spread on the surface, but tend to lay on the graphene surface, even if it has WCAs lower than that of neat CVD-graphene.

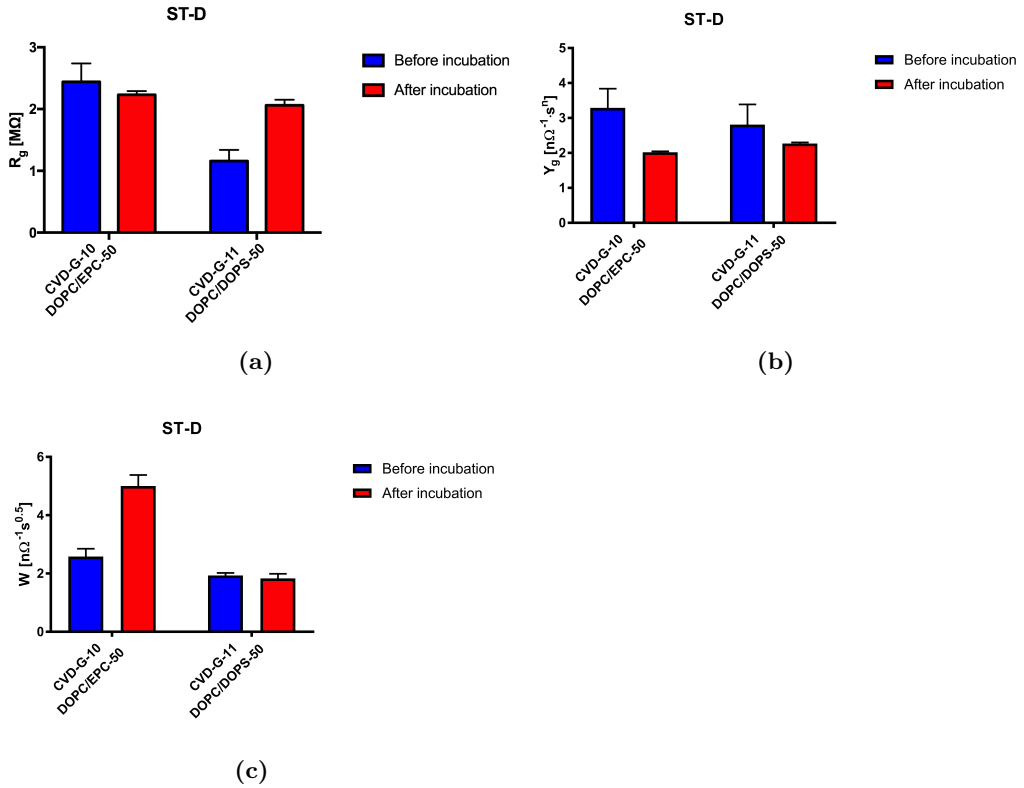


Figure 5.18. Graph bars of R_g (a), Y_g (b) and W (b) when using DOPC/EPC-50 (left) and DOPC/DOPS-50 (right) solution, with the same surface treatment (ST-D).

5.3 Conclusions

After the surface treatments of graphene, to improve its compatibility towards supported lipid membranes, we performed the analysis of the interaction with liposomes solution using electrochemical impedance spectroscopy (EIS). These results show that the electrochemical response of the CVD-graphene surface (after thermal annealing in O_2) before and after incubation in four liposome solution is different: the impedance spectrum and the Nyquist plot vary significantly after incubation in the liposome solution. EIS data are analyzed by fitting them using an equivalent electrical circuit model: the so-obtained variation of R_g , Y_g and W of the system shows that the best interaction is obtained when using a solution

made of liposome extruded through a 50 nm pore size membrane composed by only DOPC lipids and using graphene with low WCAs, whose decrease is related to the time and temperature of the surface treatments. However, the variation of those parameters is not consistent with a good, that is fully sealing, lipid membrane on the conductive surface: this variation is usually of the order of hundreds of $M\Omega$. Next, other types of functionalizations of the graphene surface, including bioconjugation of hydrophilic molecules via chemical bonding (e.g. using diazonium salt), will be investigated to improve the biocompatibility of the hydrophobic graphene surface towards supported lipid membrane systems.

Chapter 6

Conclusions and future perspectives

In the first part of the thesis, the synthesis by CVD, the transfer and characterization of graphene, which constitutes the conductive layer of the biosensor assembly, was investigated. We performed graphene synthesis by catalyzed-CVD (C-CVD) technique, which enables the synthesis of single- to few-layer graphene membranes, at lower temperatures than gas pyrolysis, by introducing a catalytic support. In order to obtain a defect-free and clean graphene surface, in this study we had to face several issues, not only related to the synthesis process, but also to the transfer of graphene on the final substrate. We succeeded in synthesizing a graphene layer which is continuous and free of defects, by first treating the copper foil with acetic acid and nitric acid, by choosing its proper shape, and by modifying the hot-wall CVD system. Moreover, after the synthesis, the graphene transfer procedure was also improved by using cyclododecane, instead of PMMA, and ammonium persulfate, instead of iron chloride, for copper etching. The cyclododecane based method, which is more suitable for transferring small samples of graphene (max. $1\text{ cm} \times 1\text{ cm}$), due to its larger stiffness with respect to PMMA, and the combined ammonium persulfate etching step resulted in a graphene layer which is free from residue. For developing the electrochemical biosensors based on graphene supported phospholipidic layer, larger area graphene samples on insulating substrates, which will serve as the working electrode in the custom-made electrochemical cell, were investigated. $2\text{ cm} \times 2\text{ cm}$ graphene layers

were transferred on SiO₂/Si wafer, because the thermal silicon oxide was proven to be more suitable for supporting graphene in terms of adhesion. As the sample area is quite large, the transferring method based on PMMA was used, while trying to optimize that based on cyclododecane for larger samples.

The properties of the transferred CVD-graphene were characterized by several techniques, including SEM, AFM, Raman and XPS spectroscopy and water contact angles measurements. In order to promote the adhesion and stability of support lipid membranes, the transferred CVD graphene was subjected to several surface treatments, in particular heating in high-vacuum and oxygen atmosphere, with the aim to modify the lattice of graphene and to reduce its natural hydrophobic nature. SEM images confirm that graphene fully covers the SiO₂/Si substrates and that it has small islands of overgrowth. It is also possible to notice the presence of residuals of PMMA, which is not fully removed by acetone. This is a renown issue, a clean graphene surface is hard to attain and many authors from the literature have been investigating different approaches to find a solution. The result of WCA measurements, before and after the surface treatments, show the hydrophobicity of graphene, with values in the range from 77° to 96°. The variability of WCAs before surface treatments arises from the presence of PMMA residuals on the graphene surface. After the thermal treatments, the WCAs of graphene decrease while increasing the temperature or time of the surface treatments; the lower standard deviation after the annealing is attributed to the decreasing amount of PMMA residues, leaving a more homogeneous surface characterized by a lower spread of the WCAs values. Raman maps show that the CVD-graphene samples under investigation are mono- to few-layer, with a small amount of defects; after surface treatments, the ratio of the intensity of D peak to G peak increases confirming the presence of defects in the lattice, as can be seen from the higher intensity of the color in the I_D/I_G maps. AFM images show the presence of residues on the graphene surface before surface treatments. Thus, these results lead us to the conclusion that the applied surface treatment reduces the amount of PMMA, but it has the effect of increasing the wrinkles, and consequently the roughness of the graphene layer, although the layer remains continuous. From the literature it is known that these irregularities are site that could favour the interaction of the graphene surface with liposomes by inducing liposome rupture and lipid membrane formation. XPS spectra show that the surface treatments remove PMMA residuals

from the surface. The O/C ratio indicates that the amount of oxygen does not increase after surface treatments. On the other hand, upon thermal treatment, the different thermal expansion coefficient of graphene on SiO₂ gives rise to an increase of the wrinkles on graphene layers, as observed by SEM and AFM characterization. The increased amount of defects observed by micro-Raman spectroscopy is then related to the formation of wrinkles, which are source of defects. The graphene samples after surface treatments were chosen as potential platforms to study the interaction of graphene and lipid membranes, which are preferentially formed on more hydrophilic substrates (WCA < 50°).

After the optimization of the CVD synthesis of the graphene layer and its full characterization, we performed the analysis of the interaction of graphene surfaces with liposomes solution using electrochemical impedance spectroscopy (EIS). Four different liposome solutions, in terms of composition and size, were investigated. EIS results showed that the electrochemical response of the surface of CVD grown graphene (after thermal annealing in O₂) before and after incubation is different: the impedance spectrum and the Nyquist plot vary significantly after incubation in the liposome solution. EIS data were analyzed by fitting them using an equivalent electrical circuit model: the so-obtained variation of key parameters (R_g , Y_g and W) of the system shows that the best interaction is obtained when using a solution made of liposome extruded through a 50 nm pore size membrane composed by only DOPC lipids and using graphene with low WCAs, whose decrease is related to the time and temperature of the surface treatments. However, although we observe an increase of resistance and decrease of capacitance after liposome incubation, which are consistent with the formation of an insulating layer on the conductive graphene substrate, the variation of those parameters is likely not sufficient to ensure a fully sealing lipid membrane on the large scale graphene surface considered in this study.

In future, it would be interesting to investigate other types of functionalizations of the graphene surface, including bio-conjugation of hydrophilic molecules via chemical bonding (e.g. using diazonium salt), to improve the biocompatibility of the hydrophobic graphene surface towards supported lipid membrane systems. The cyclododecane transfer procedure could be resumed and optimized for larger samples, because the use of this waxy organic compound could be useful for the functionalization of graphene. Cyclododecane is soluble in chloroform, in which

also the lipids are soluble. Thus, the cyclododecane and lipids can be mixed and spun over the graphene surface, allowing the formation both of the supporting layer for graphene transfer and, as soon as the cyclododecane sublimates, of the lipid layer.

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List of publications

- M. Pittori, L. Ortolani, D. Gentili, V. Morandi, R. Rizzoli, M. G. Santonicola
- *Graphene-lipids interaction: towards the fabrication of a novel sensor for biomedical uses* - Proceedings of IEEE Nano 2015 - 27-30 Jul. 2015 - Rome (IT) - DOI: 10.1109/NANO.2015.7388746
- N. Lisi, T. Dikonimos, F. Buonocore, M. Pittori, R. Mazzaro, R. Rizzoli, S. Marras, A. Capasso - *Contamination-free graphene by chemical vapor deposition in quartz furnaces* - Scientific Reports - 2017 - Vol. 7(1) - 9927:1 - DOI: 10.1038/s41598-017-09811-z

Conference proceedings

- M. Pittori, L. Ortolani, D. Gentili, V. Morandi, R. Rizzoli, M. G. Santonicola
- *Synthesis of smooth graphene surfaces by CVD for electrochemical biosensors with supported lipid membranes* - Proceedings of GraphITA 2015 - 14-18 Sep. 2015 - Bologna (IT)
- M. Pittori, L. Ortolani, D. Gentili, V. Morandi, R. Rizzoli, M. G. Santonicola
- *Improving the cleanliness of graphene grown on copper by chemical vapor deposition for biosensing applications* - Proceedings of Graphene2016 - 19-22 Apr. 2016 - Genova (IT)
- M. Pittori, L. Ortolani, D. Gentili, V. Morandi, R. Rizzoli, M. G. Santonicola
- *Graphene-Supported lipid bilayer interaction for the fabrication of novel electrochemical biosensors* - Proceedings of E-MRS Fall Meeting - 19-22 Sep. 2016 - Warsaw (PL)

- G. D'Arrigo, R. Rizzoli, P. Musumeci, F. Gregory, M. Pittori, V. Morandi, C. Bongiorno, M. Calabretta, E. Rimini - *Mechanical and electrical characterization of CVD-grown Graphene transferred on chalcogenide $Ge_2Sb_2Te_5$* - Proceedings of EPCOS 2016 - 4-6 Sep. 2016 - Cambridge (UK)
- M. Pittori, L. Ortolani, D. Gentili, V. Morandi, R. Rizzoli, M. G. Santonicola - *Study of Graphene - Supported Lipid Bilayers interaction for applications in novel electrochemical biosensors* - Materials.it 2016 - 12-16 Dec. 2016 - Aci Castello (IT)
- G. D'Arrigo, R. Rizzoli, V. Morandi, C. Bongiorno, M. Calabretta, E. Rimini, M. Pittori - *Mechanical and electrical characterization of CVD-grown graphene transferred on chalcogenide $Ge_2Sb_2Te_5$* - Materials.it 2016 - 12-16 Dec. 2016 - Aci Castello (IT)
- M. Pittori, L. Ortolani, D. Gentili, A. Kovtun, F. Liscio, V. Morandi, R. Rizzoli, M. G. Santonicola - *Improvement of CVD-graphene interaction with supported lipid bilayer for the application in novel electrochemical biosensors* - Graphene2017 - 28-31 Mar. 2017 - Barcellona (SP)

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