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Effects of Graphene on lipid vesicles: a combination of experiments and simulations.

Efecte del grafè en vesícules lipídiques: combinació d'experiments i simulacions.

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B:KC Barcelona Knowledge Campus
Campus d'Excel·lència Internacional

I am among those who think that science has great beauty.

Marie Curie

M’agradaria agrair al Ramon la seva màxima implicació i dedicació per tal que em quedi el millor treball possible. També agrair als companys del laboratori 4017 per ajudar-me sempre que ho he necessitat i per fer les hores de treball més amenes.

REPORT

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1. SUMMARY

Graphene is a carbon-based nanomaterial which has gained popularity in the field of biomedicine and biotechnology because of its exceptional properties. This new interest also unleashed concerns about the possible toxic effects of graphene on living beings.

This work is focused on analyzing the interaction between graphene and the plasma membrane of eukaryotic cells. For this purpose, simple *in vitro* lipid vesicles mimicking cell membranes are produced to perform experiments where the interaction can be observed through an optical microscope. Molecular Dynamics simulations have also been performed to understand the interaction at a molecular level.

Two types of artificial vesicles have been produced for different purposes. The first type is composed of a binary mixture of lipids (POPC and cholesterol) and it is used to study the interaction with graphene. The results of both experiments and simulations show an attractive interaction and subsequent insertion of graphene inside the lipid bilayer which indicates that the bilayer makes a good solvent for graphene and, thus, a site of graphene bioaccumulation.

The second type is composed of a ternary mixture of lipids (DOPC, DPPC and cholesterol), which leads to vesicles with a segregation of two phases that can be distinguished through fluorescence microscopy. It is well known that cell membranes are not homogeneous, in fact, they are composed of domains: a liquid-ordered phase formed by saturated lipids and cholesterol and a liquid-disordered phase formed by unsaturated lipids. The biological complexity of the domains in the cell membrane can be approached by means of the two-phase vesicles which are used to test if graphene has a preference for a specific phase. MD simulations were also performed to study the insertion process of graphene in each phase. Both the experiments and simulations show that graphene has no preference for any of the phases. Molecular dynamics simulations reveal that the graphene insertion in the liquid-ordered phase takes place in twice the time of the insertion in the liquid-disordered phase.

Keywords: graphene, graphene oxide, lipid bilayer, vesicles, electroformation, molecular dynamics, liquid-ordered phase, liquid-disordered phase.

2. RESUM

El grafè és un nanomaterial basat en carboni el qual ha guanyat popularitat en els camps de la biomedicina i biotecnologia per les seves excepcionals propietats. Aquest nou interès ha despertat alhora preocupacions sobre els la possible toxicitat del grafè per organismes vius.

Aquest treball te com a objectiu analitzar la interacció entre el grafè i la membrana plasmàtica de les cèl·lules eucariotes. Per aquesta raó, s'han generat vesícules *in vitro* simples que imiten la membrana cel·lular: així s'han pogut realitzar experiments en els quals s'ha observat la interacció a través d'un microscopi òptic. També s'han realitzat simulacions de Dinàmica Molecular per tal d'entendre la interacció a nivell molecular.

S'han produït dos tipus de vesícules amb diferents propòsits. El primer tipus està compost d'una mescla binària de lípids (POPC i colesterol) i s'ha emprat per estudiar la interacció amb el grafè. Els resultats dels experiments i les simulacions mostren una interacció atractiva i una consegüent inserció del grafè dins la bicapa lipídica. Això indica que la bicapa lipídica és un bon dissolvent per al grafè i, per tant, un lloc on es bioacumula el grafè.

El segon tipus està compost per una mescla ternària de lípids (DOPC, DPPC i colesterol), que porta a vesícules amb una segregació en dues fases que poden ser percebudes amb microscòpia fluorescent. És ben conegut que les membranes plasmàtiques no són homogènies. Fet i fet, estan compostes de dominis: una fase líquida-ordenada formada per lípids saturats i colesterol, i una segona fase líquida-desordenada formada per lípids insaturats. La complexitat biològica dels dominis a la membrana cel·lular es pot aproximar mitjançant vesícules de dues fases, que seran utilitzades per determinar si el grafè té preferència per una fase o una altra. També s'han realitzat simulacions de Dinàmica Molecular per estudiar el procés d'inserció del grafè en cada fase. Els experiments i simulacions mostren que el grafè no te preferència per cap de les dues fases. Les simulacions revelen que la inserció del grafè a la fase líquida-desordenada requereix el doble de temps que la inserció a la fase líquida-desordenada.

Paraules clau: grafè, òxid de grafè, vesícules, bicapa lipídica, electroformació, dinàmica molecular, fase líquida-ordenada, fase líquida-desordenada.

3. INTRODUCTION

Graphene is a nanomaterial formed by one single layer of carbon atoms arranged in a hexagonal array provided by their sp^2 hybridization (Figure 1). It possesses exceptional electrical, mechanical, optical and thermal properties [1] owing to its structure. Consequently, many researchers from different fields in science have taken interest in this extraordinary material. In the past decade there has been a good deal of research focused on developing new applications for graphene. Since it is a carbon-based material, many possibilities emerge in fields like biomedicine and biotechnology. Some promising applications that are being explored include drug delivery vehicles [2], analytical and sensing devices [3], cell imaging [4], tissue engineering [5] and antibacterial agents [6].

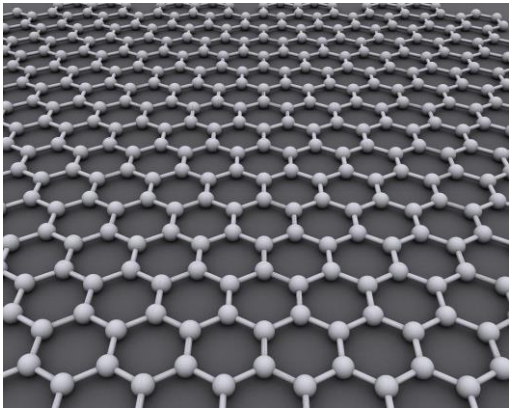

Figure 1. Structure of Graphene.

(Alexander AIUS, 21/04/18 via Wikimedia Commons, Creative Commons Attribution)

Meanwhile, the toxicity of carbon based nanomaterials, in particular of graphene, is also being investigated [7]. It is a serious concern to address occupational [8], environmental [9] and biomedical exposures. Recently, graphene has been reported to exhibit toxicity effects on bacteria

[10] and human cell lines [11]. Moreover, the size of graphene nanoparticles falls into the category of potentially respirable materials. This fact may be relevant in the workplace during several steps of the manufacturing process. Likewise, in biomedical applications graphene may be deliberately implanted or injected. As a result, there is an urgent need to understand potential physiological and pathological reactions of living beings to the exposure of graphene. Research in this direction is crucial for the future of many applications of graphene and for dealing with its toxicity to assure safety to living beings and the environment.

The first step to study the effects of graphene on living beings is the interaction with the cell membrane. A cell membrane is the boundary that separates the interior of cells from the outside environment which is formed by a matrix of lipids where proteins are embedded. The structure of a lipid generally consists of two hydrophobic fatty acid "tails" and a hydrophilic "head", all joined together by a glycerol molecule. Figure 2 shows the structure of a phospholipid, a lipid that contains a phosphate group. Due to its amphiphilic structure, they can organize themselves in a structure known as a bilayer. In the bilayer, the hydrophobic tails are facing inwards whereas the hydrophilic heads face outwards. The proteins inserted in the membrane are responsible for various biological activities such as transporting substances across the membrane, signaling processes or enzymatic activity. In addition of being a complex system, the cell membrane is dynamic meaning that is in constant change because of the diffusion of lipids through the membrane and the biological activity of the cell carried out by membrane proteins.

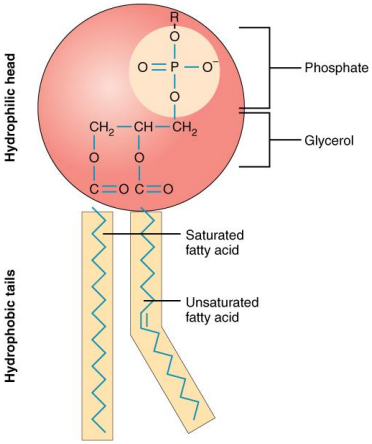

Figure 2. Schematic structure of a phospholipid (OpenStax, *15/05/18 via Wikimedia Commons, Creative Commons Attribution)*

To successfully provide insights on the nonspecific interactions between graphene and biological lipid bilayers, a simple membrane vesicle model system is needed (Figure 3). With this purpose, simple *in vitro* lipid vesicles mimicking the cell membrane are used in this work. These vesicles are composed by 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) and a 30 mol% of cholesterol. Cholesterol is a key element present in all eukaryotic cell membranes to build and maintain its structure, fluidity and permeability. The cell membrane has very specific properties that are due to cholesterol, for instance it increases membrane packing, its tetracyclic ring contributes to the fluidity of the membrane and it also reduces the permeability to specific molecules [13]. Hence, by adding cholesterol, the physical properties of our experimental vesicles are closer in many aspects to the ones in real cell membranes.

The electroformation method proposed by Almendro Vedia et al. [14] is used to produce the vesicles and the observation process is performed through an optical microscope. Experimental work regarding the interaction between graphene and in vitro vesicles has already been done with other techniques. These studies have unveiled some of the characteristics of graphene-membrane interactions that may eventually lead to graphene adsorption, disruption and other interaction modes [15]–[20].

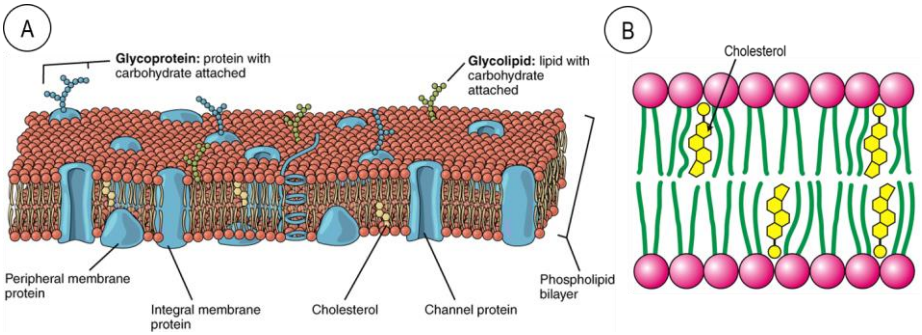

Figure 3. (A) Cell membrane (OpenStax, *15/05/18 via Wikimedia Commons, Creative Commons Attribution)* (B) Schematic structure of the lipid bilayer of a vesicle. *(Extracted from Biochemistry, Seventh Edition, 2012 W.H. Freeman and company)*

The plasma membrane of eukaryotic cells is formed by highly diverse lipid species and proteins, and according to the fluid mosaic model, it can be considered as a two-dimensional liquid in which lipids and proteins diffuse. The principal constituents of the lipid mass are

phospholipids, and cholesterol is usually present in a molar fraction of 10-30 mol%. The plasma membrane also contains a large quantity of proteins as well as structures formed by the actin-based cytoskeleton. Lipids and proteins are arranged in domains of structural and compositional heterogeneity. It is well known how artificial membranes composed by saturated and unsaturated lipids become separated into a liquid-ordered (L_o) phase and a liquid-disordered (L_d) phase once the cholesterol is added to the membrane mixture. The segregation of the two phases due to preferential association of saturated lipids and cholesterol may explain the formation of nano-sized domains, referred as rafts, in biological cell membranes [21] (Figure 4). This lateral organization in rafts is very important for various cellular processes, such as membrane fusion, protein trafficking, and signal transduction [22], [23].

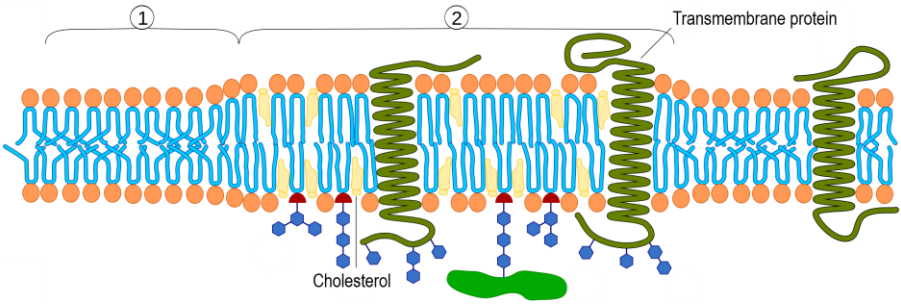

Figure 4. Example of a plasma membrane. (1) Liquid-disordered phase composed of unsaturated lipids. (2) Liquid-ordered phase composed by cholesterol and saturated lipids, also called lipid raft.

(Artur Jan Fijałkowski, 06/05/18 via Wikimedia Commons, Creative Commons Attribution)

Consequently, in order to approach the biological complexity, it is important, then, to reproduce the lipid L_o/ L_d heterogeneity of the plasma membrane in our artificially made vesicles. For this purpose, ternary mixtures of lipids have been also used to obtain membranes displaying segregation in L_o/L_d phases. The specific composition of these vesicles is 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) 1:1 with a 30 mol% of cholesterol [13]. DPPC is a saturated lipid, and therefore it will form, together with cholesterol, the liquid-ordered phase, and DOPC which is unsaturated will form the liquid-disordered phase. Moreover, a fluorescent probe (NBD-DMPE) is used to observe the different phases. The structure of this compound consists of a saturated phospholipid attached to the NBD

group, which is the fluorophore. This type of lipid probe will be preferentially placed in the liquid-ordered phase.

In addition to experiments with in vitro lipid vesicles, Molecular Dynamics (MD) simulations are performed to unveil the molecular details of the graphene-membrane interaction. Two flat membrane models are used for the simulations: the first is a homogeneous bilayer composed by POPC and 30 mol% cholesterol, and the second is a heterogeneous bilayer composed by dilinoleoyl-phosphatidylcholine (DUPC), DPPC and 30 mol% cholesterol. In this case, DUPC is used instead of DOPC because it contains more unsaturations and the separation of the system in two phases is clearer.

The study of the interaction between graphene and a lipid bilayer by means of MD simulations has recently been attempted using different approaches. These works are starting to provide insights at the molecular level on how graphene favorably interacts with lipid membranes [24], [25]. In the present work, direct optical observation of graphene-vesicle interaction is presented. An important innovation is reported by characterizing the interaction in the two L_o/L_d phases of vesicles mimicking the raft organization in cell membranes.

4. OBJECTIVES

The scope of this study is the characterization of the interaction between graphene and lipid bilayers mimicking cell membranes. The approach of this study will be both experimental and through simulations. The specific objectives are detailed as follows:

To generate through the electroformation method, vesicle dispersions of both homogeneous (POPC/cholesterol) and heterogeneous (DOPC/DPPC/cholesterol) lipid vesicles.

To identify through fluorescence microscopy the liquid-ordered and liquid-disordered phases in the two-phase vesicles.

To observe and characterize through an optical microscope the interaction between graphene and homogeneous lipid vesicles.

To observe and characterize through fluorescence microscopy the interaction between graphene and the two phases of heterogeneous vesicles.

To unveil the molecular details of the interaction between graphene and the lipid bilayer through Molecular Dynamics simulations.

To find out whether graphene particles prefer one specific phase or not by a combination of molecular dynamics simulations and experiments with heterogeneous vesicles.

5. METHODOLOGIES

This section explains in detail the procedure of the experiments and simulations performed in this work.

5.1. EXPERIMENTS

The experiments in the laboratory will be described step by step, starting with the method used for obtaining vesicles to the observation process and the way graphene is added to the system.

5.1.1. Solutions and material

In the present work the vesicles are obtained by the following electroformation method reported in Almendro Vedia et al [14]. It was the chosen method because of its simplicity: it is inexpensive and requires very little material. It has also been proved to work well to massively produce vesicles.

The solutions needed to proceed with the electroformation are two: a sucrose solution and a lipids solution. The sucrose solution is 200 mM in water and the lipids solution is composed by two or more lipids dissolved in chloroform. In this work two different lipids solutions have been used for different purposes. The first solution is POPC + cholesterol (0,5 mg/ml POPC + 30 mol% cholesterol) and the second one is DPPC + DOPC + cholesterol (PC 1:1 + 30 mol% cholesterol). From now on, the first solution will be called “two-lipid solution” and the second one “ternary-lipid solution”. The vesicles formed with each one of the solutions will be called “one-phase vesicles” and “two-phase vesicles” because they form a single homogeneous phase and two segregated phases, respectively. The existence of two phases will be discussed further on in the Results section.

The material required to perform the electroformation process is the following: two sheets of ITO glass (a glass coated with Indium Tin oxide, which is an electrical conductor; dimensions: 3,75 x 2,5 cm), adhesive putty, two copper strips, two thin wires, plastic strips and two clips. This material will be used to build a cell (Figure 5) where the electroformation will take place. The procedure to build the electroformation cell consists in the following steps:

- Wash the sheets of ITO glass with Milli-Q water and dry them. With the help of a multimeter check which is the conducting side.
- Wash again the conducting surface with chloroform and dry.
- With a micropipette place two drops of 5 μL of the lipid solution on one of the two ITO glass sheets and let the chloroform evaporate.
- Make a thin cylinder with the putty and place it forming a “U” around the two drops of lipids leaving one end larger than the other.
- Place the second ITO glass on the putty and press a little.
- Cut two strips of copper that fit the space outside the putty and some plastic strips to put under the copper. The copper and plastic strips must be 1 mm tall. Then, with some zeal stick the wires to the copper.
- Put the copper and plastic strips in the cell and fix it with clips.
- Inside the cell put the sucrose solution until full. Then, bend the putty to seal the cell.

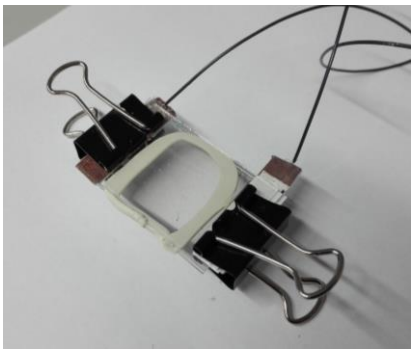

Figure 5. Electroformation cell.

5.1.2. Vesicle electroformation process

The electroformation cell is then connected to an alternate current of ±1 Volts with a frequency of 2 Hz. This current goes on for about 12 hours or more. Figure 6 shows a photo taken during this process. The photo shows the edge of a lipid rich zone of the electroformation cell where both lipids (dark dots in the bottom left) and already formed vesicles can be seen. After the process, the vesicles are collected in an Eppendorf flask and stored in the fridge. The vesicle dispersion system can last for about two weeks.

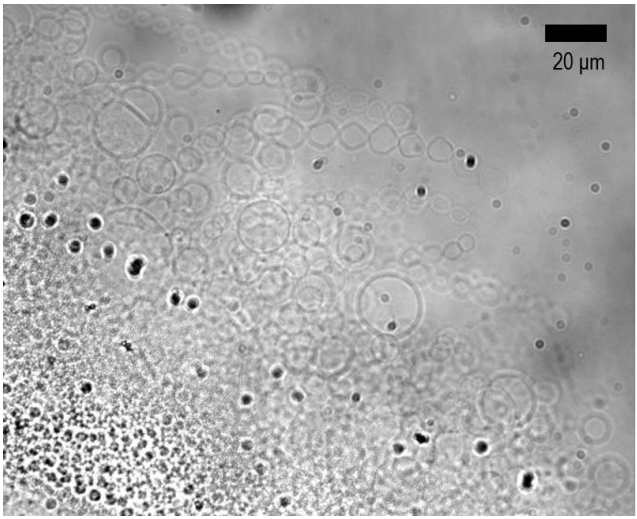

Figure 6. Electroformation process of vesicles.

To explain what happens in the process of electroformation is essential to look at the structure of the lipids. Lipids are formed by a polar head and apolar tails. Because of this, when lipids are placed in an aqueous solution they tend to hide the hydrophobic tail by sticking it to the glass. The system will not change unless some energy is applied to overcome the activation energy required to form stable bilayer vesicles. This energy is provided here by the oscillation of the electric field between the glass sheets. The lipids used in this work belong to a family called phosphatidylcholines (PC) and their main feature is the presence of choline in the headgroup. The head of a phosphatidylcholine is a dipole because of the positive charge of the choline in contrast with the negative charge of the phosphate group. The behavior of a dipole in an AC is

clear: the dipole changes its orientation to align with the electric field (Figure 7). This movement allows the lipids to arrange themselves as a more stable bilayer structure forming vesicles.

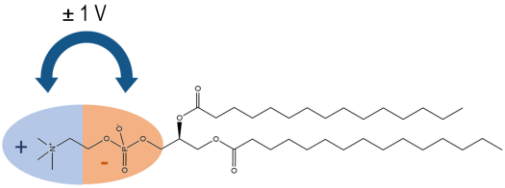

Figure 7. Movement of a dipole in the presence of the alternate current applied at the electroformation cell.

5.1.3. Vesicle deposition and observation

The observation of the vesicles is done with an optical microscope. The support for the observation is a slide with a handmade cylindrical pool (8 mm diameter x 3 mm depth) of polydimethylsiloxane (PDMS), a silicon-based organic polymer. The procedure consists in placing the vesicle dispersion in the pool and then adding a solution of sodium chloride (100 mM). The addition of this solution will make the vesicles fall to the bottom of the pool so that all of them can be observed in a sole 2D plane. The mechanism of this process is based in the osmolality and weight of the ions of sodium and chloride and the molecules of sucrose. The solutions of sucrose and sodium chloride have the same osmolarity in order to prevent the vesicles from exploding or shrinking. The overall osmolarity after adding the sodium chloride solution will be the same as before, but the solution inside the vesicles will be denser than the outer solution rich in sodium chloride (M (sucrose) = 342,30 g mol^{-1} > M (NaCl) = 58,44 g mol^{-1}). Consequently, the vesicles will be heavier than the rest of the solution and fall at the bottom of the pool.

Two different cameras have been used for the observation process. The Pixelink camera was used on the one-phase vesicles and the Andor iXon Ultra 897 camera on the two-phase vesicles. The segregation in two phases is observed by fluorescence microscopy. The sample is illuminated with an intense, near-monochromatic light provided by a high-power LED which is absorbed by the fluorophores, causing them to emit light of higher wavelength. NBD-DMPE, a phospholipid with a fluorophore group in its head, is used as a fluorescent probe in a proportion of 1% in the ternary-lipid solution. The Andor iXon Ultra 897 camera is able to generate an image

from the emitting light of the fluorophore in the sample. This compound is predicted to be present in the L_o phase, so this L_o regions will be illuminated in the generated image.

5.1.4. Addition of graphene

After the vesicles have been deposited, a dispersion of graphene is poured in the upper part of the observation pool. In this way, graphene particles slowly fall to the bottom of the solution where the vesicles are. To make the dispersion, 1 mg of graphene powder provided by Graphenea was dispersed in 25 mL of a sodium chloride solution (100 mM) using an ultrasonic bath. This way, the osmolality of the solution in the observation pool is maintained. Graphene particles used in these experiments are 20% oxidized, otherwise the dispersion would not be possible. The oxidized graphene, also called graphene oxide (GO), can be transiently dispersed in water due to the presence of epoxy, hydroxyl, and carboxyl functional groups [26]. In most biological and biomedical studies, the use of GO is common due to the better affinity to aqueous medium than non-oxidized graphene. When the GO dispersion is observed with the microscope, the graphene particles are seen as dark flakes, which are composed of graphene sheets bended and folded. The graphene flakes have a size between 2 and 3 times the width of the lipid bilayer of the vesicles, on average 5 μm.

To successfully observe the interaction with deposited vesicles, the procedure of the experiments was to locate in the microscope a region containing several medium or large vesicles and make captures every 15 seconds during 30 minutes to 1 hour. Then, the sequence of photos is studied to observe motion of both graphene flakes and vesicles. These dynamics have been studied to characterize the interaction between them.

5.2. MOLECULAR DYNAMICS SIMULATIONS

Molecular dynamics (MD) is a numerical simulation method that calculates the time dependent behavior of a molecular system, which gives a view of its dynamic evolution. A MD simulation begins with the initial conditions of the studied system, which are the number and type of particles, their positions and velocities and the simulation box settings. Once these parameters are fixed, the simulation can be run using the GROMACS simulation package [27]. The algorithm for a MD simulation starts calculating the potential energy of each particle, which is a function of

the positions of all the particles in the system. Next, it solves the derivative of the potential energy with respect to the positions to obtain the forces. Subsequently, the simulation package integrates the Newton's motion equation for every particle for a fixed time-step. The calculated acceleration gives new positions and velocities for each particle which the program updates. The simulation package continues to perform the number of iteration steps fixed by the user.

The calculation of the potential energy is done with a force field, which in molecular modeling is defined as a parametrization setting that contains specific characteristics and interactions of atoms, bonds, angles and dihedrals for different molecules and it is contrasted with experimental data. The parametrization allows to calculate the different contributions of the interaction potential energy between pairs of particles. These contributions are described in Figure 8 as intramolecular and intermolecular interactions.

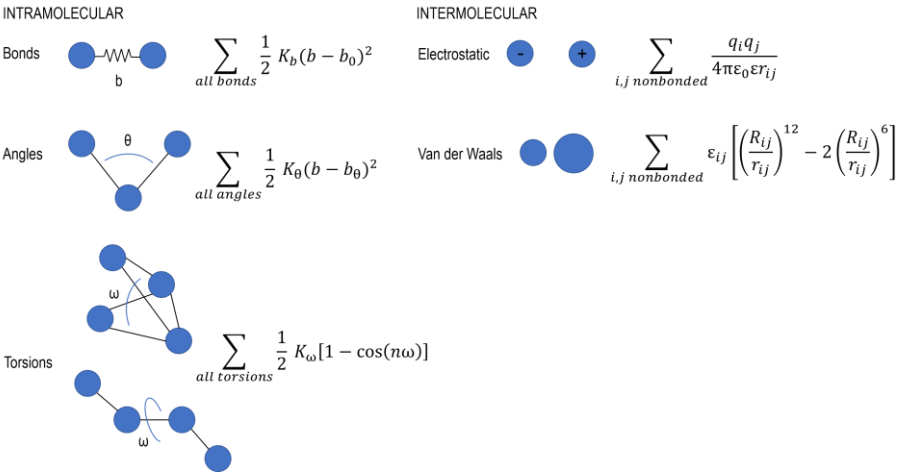

Figure 8. The components of the total potential energy defined by the MARTINI force field.

There exist different types of force fields based on two molecular models: the atomistic model and the coarse-grained model. The atomistic model represents every atom in the system while the coarse-grained model reduces the number of particles by mapping several heavy atoms to one coarse-grained interaction site or bead. In this work, the studied system consists of a lipid bilayer formed by a great number of large molecules, and this fact makes the calculations in the atomistic model too time-demanding. Thus, the MARTINI force field [28], which follows a 4-to-1

coarse-graining, is used in this work. The MARTINI force field beads are described by their acid-base properties and polarity. The resulting simulations with this particular model are called Coarse-Grained Molecular Dynamics (CGMD).

Two kinds of lipid bilayers which differ in composition are used in this work. The first is formed by POPC and a 30 mol% of cholesterol and the second one is formed by DUPC, DPPC 1:1 and a 30 mol% of cholesterol. The coarse-grain mapping for these molecules is as shown in Figure 9. These mixtures of lipids form a lipid bilayer in a previous self-assembly MD simulation. When the bilayer is properly equilibrated, a graphene sheet is placed in the simulation box with its corner pointing the surface of the lipid bilayer.

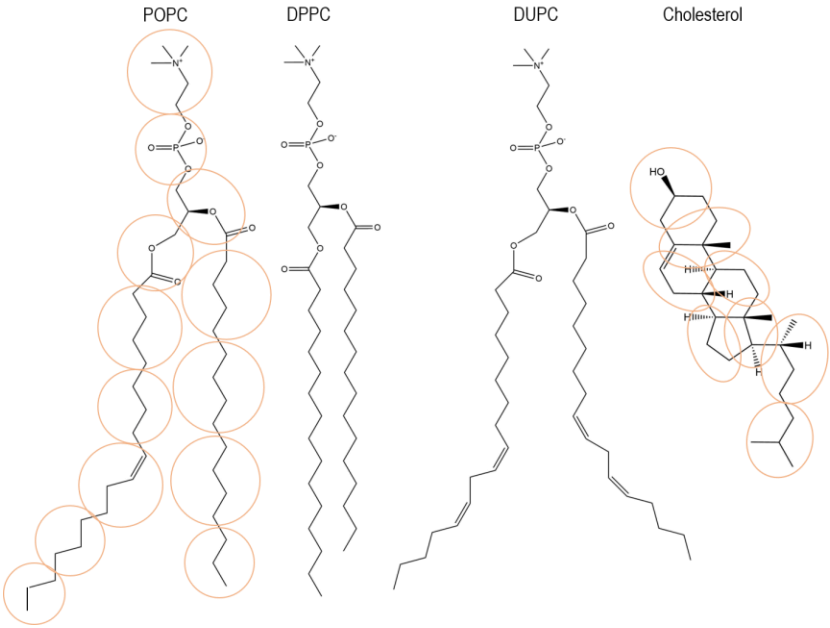

Figure 9. The molecules that compose the two types of lipid bilayers. In POPC and cholesterol the coarse graining is described as examples.

The graphene sheet is square-shaped and composed of 120 coarse-grained beads which altogether represent an area of 10,5 nm^2. Each coarse-grained bead corresponds to 4 carbon atoms that follow a hexagonal arrangement. When the graphene sheet is added, a minimization

simulation is needed to avoid overlapping of graphene with the other atoms and molecules. The minimization will randomly move the overlapped particles until the forces have diminished at normal levels. In short, the simulation box is composed by the lipid bilayer, which has a planar square-shape, water molecules and a graphene sheet (Figure 10).

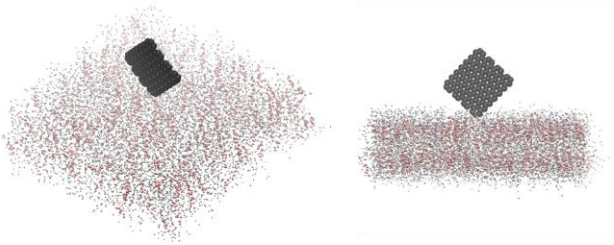

Figure 10. The one-phase lipid bilayer from two perspectives. POPC is shown in silver, cholesterol is pink and graphene is black. Water is not plotted to gain simplicity.

The simulation box has periodic boundaries, which means that when a molecule passes through one side of the box, re-appears on the opposite side. The simulation box has also programmed a specific ensemble. An ensemble is a collection of all possible systems which have different microscopic states but have an identical macroscopic or thermodynamic state. There exist different ensembles with different characteristics. The one used in this work is the Isobaric-Isothermal Ensemble (NPT): a fixed number of atoms, N, a fixed pressure, P, and a fixed temperature, T. This means that the volume of the box may change to keep the pressure at a constant value and the kinetic energy of the particles may have to be adjusted in order to maintain the temperature. Other settings that the user must fix are the time-step and total number of steps. The time-step refers to the interval of time for which the simulation package will calculate the trajectory of each particle. The total number of steps determines the total simulation time.

As was mentioned before, two kinds of lipid bilayers are used in this work. The mixture of two lipids leads to a one-phase lipid bilayer whereas the ternary mixture of lipids leads to a bilayer composed of two phases. This outcome will be further explained in the Results section. Two simulations are performed with the two-phase lipid bilayer: one where the graphene sheet is placed close the L_o phase and another one where it is placed close to the L_d phase. For the one-

phase lipid bilayer there is only one possibility, and thus, one simulation. The initial conditions and parameters of the MD simulations are described in table 1 and 2.

One-phase lipid bilayer	
POPC molecules	576
Cholesterol molecules	248
Water molecules	16000
Graphene size	10,5 nm^2
Graphene oxidation	20%
Pressure	1 bar
Temperature	310K
Simulation time-step	0,01 ps
Total simulation time	1000 ns

Table 1. Specifications of the MD simulation performed with the one-phase lipid bilayer.

Two-phase lipid bilayer	
DUPC molecules	828
DPPC molecules	540
Cholesterol molecules	32000
Graphene size	10,5 nm^2
Graphene oxidation	20%
Pressure	1 bar
Temperature	295K
Simulation time-step	0,01 ps
Total simulation time	1000 ns

Table 2. Specifications of the two MD simulations performed with the two-phase lipid bilayer.

6. RESULTS AND DISCUSSION

This section is dedicated to explaining the results, interpretation and discussion of the experiments and simulations.

6.1. EXPERIMENTS

The results of the experiments with one-phase and two-phase vesicles will be described in the following sections.

6.1.1. One-phase vesicles

The one-phase vesicles are composed of POPC and 30 mol% of cholesterol. They have been observed and characterized by capturing frames of the observation pool through an optical microscope as shows the capture in Figure 11. The diameter of 300 vesicles has been measured to analyze the size distribution of the vesicles. A histogram has been elaborated with this data as shows Figure 12. The highest frequencies correspond to vesicles from 7 to 13 μm in diameter and the average size of the vesicles is 13 μm. The vesicles that have been selected to characterize the graphene interaction are those larger than the average size, of an approximate range from 15 to 25 μm.

To observe the interaction between graphene and the vesicles a small volume of the graphene dispersion is added to the observation pool. The graphene flakes slowly fall to the bottom of the pool where the vesicles are. Then, a favorable interaction has been observed between graphene and vesicles. This attractive interaction is due to intermolecular Van der Waals forces and strongly depends on the distance between interacting molecules. This weak interaction will only be present if the distance is very short and, in this case, there will be also an insertion of the graphene flake into the bilayer. The insertion will be further explained at the molecular level through MD simulations.

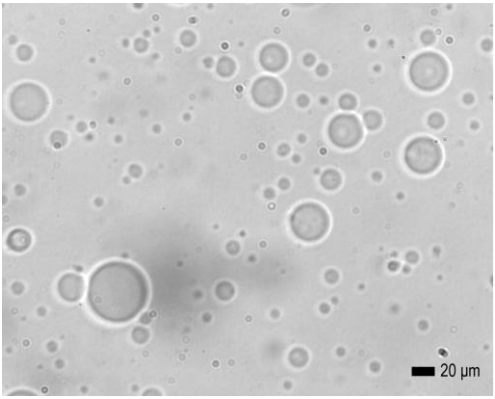

Figure 11. Image of the vesicles taken with the Pixelink camera.

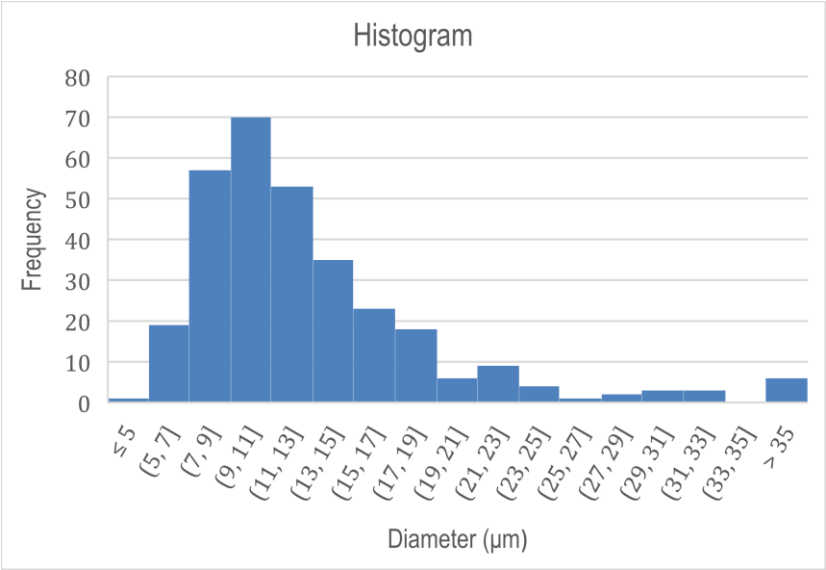

Figure 12. Size distribution of the one-phase vesicles in the sodium chloride solution.

It has been observed that, once inserted, the graphene flake can move laterally on the membrane plane, which matches with the MD simulations results reported in the next section. This movement confirms the existence of an insertion of the graphene in the bilayer. It has also been observed that the dispersion in the pool is dynamic, so vesicles and graphene flakes often rotate and move. This motion in the pool may bring graphene and vesicles together and lead to

an interaction. Figure 13 and 14 show a sequence in time where the movement of the inserted graphene flakes on these vesicles can be observed. Some vesicles have an internal structure due to the presence of small vesicles inside them. Consequently, this kind of vesicles are more fragile and when a graphene flake falls on them, they easily explode liberating the small vesicles. Figure 15 displays the explosion of a vesicle with internal structure due to the interaction with graphene.

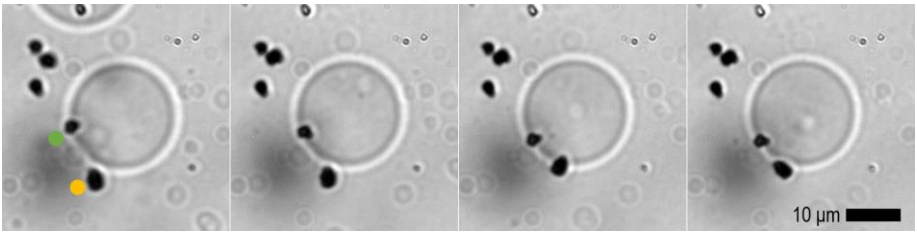

Figure 13. Sequence in time of two moving particles of graphene (in yellow and green) inserted on a vesicle.

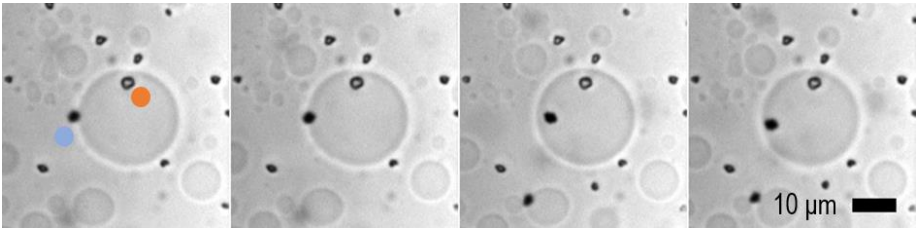

Figure 14. Sequence in time of two moving particles of graphene (in blue and orange) inserted on a vesicle.

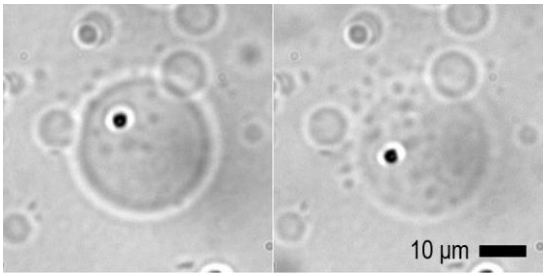

Figure 15. Sequence in time of a vesicle with internal structure that eventually explodes due to the presence of graphene.

The attractive Van der Waals forces lead to an insertion of the graphene flakes on the lipid bilayer of the vesicles. In the system studied in this work, the lipids forming the vesicles are stabilized and in equilibrium. On the contrary, the particles of graphene are apolar and, therefore, extremely *uncomfortable* in a polar aqueous solution. For graphene, the only way to minimize its energy is to get inside of a vesicle's lipid bilayer where the apolar tails of the lipids are. In short, an insertion will happen when the graphene flake and the lipid bilayer are close, and the Van der Waals forces are present. This means that lipid bilayers are good solvents for graphene particles as will be confirmed in the MD simulations results.

However, in this system the total insertion of graphene is difficult due to the size of the graphene flakes. These particles have an average size of 5 μm in diameter, which is far greater than the width of the lipid bilayer. Moreover, the particles are not a clean single layer of carbon atoms but an oxidized large bended and folded graphene sheet. Therefore, the graphene flake may insert only partially. Nevertheless, the insertion takes place because is the most favorable situation for graphene.

6.1.2. Two-phase vesicles

The experiments performed in this work were held with two types of vesicles which differ in composition. The binary mixture of lipids (POPC/cholesterol) leads to a one phase lipid bilayer and the ternary mixture of lipids (DPPC/DOPC/Chol) leads to a segregation of two phases. The temperature and the lipid ratio of the ternary mixture play an important role to determine whether the system will have one or two phases. The physical state of the mixture in the bilayer is also a relevant factor and can as well vary depending on the temperature.

In this work, the mixture of DPPC/DOPC/Chol was made in a 1:1:1 ratio based on the data provided by Sarah L. Veatch et al. [29]. In their work, they explain that membranes that exhibit micron-scale liquid immiscibility contain a minimum of three components: a high melting temperature (saturated, DPPC) lipid, a low melting temperature (unsaturated, DOPC) lipid, and cholesterol. The miscibility temperature for the mixture is 29ºC, which means two liquid phases will be present at room temperature. The component that is responsible for the segregation of phases is cholesterol as it strongly prefers to be surrounded by saturated lipids rather than unsaturated lipids (Figure 16). Thus, cholesterol recruits saturated lipids and forms a liquid-ordered phase separated from a liquid-disordered phase. The L_o phase, despite being a liquid

phase, has a more compact structure than the L_d phase, which displaces more spaces between lipids because of the disordered nature of unsaturated lipids. The unsaturations of the hydrocarbon chain cause a major structural change on the lipid increasing its area.

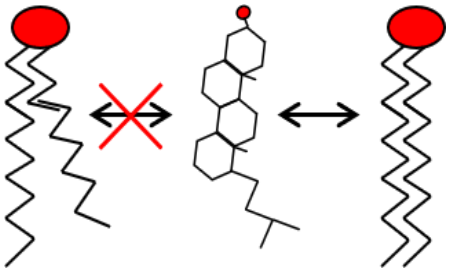

Figure 16. Preference of cholesterol for saturated phospholipids

The fluorescent probe (NBD-DMPE), which is a saturated phospholipid with a fluorophore in its head will be present in the liquid-ordered phase. This fact implies that in fluorescence microscopy, the bright region corresponds to the L_o phase whereas the dark region corresponds to the L_d phase. The phases completely separate into a single dark region and a single bright region. However, not all the obtained vesicles have two phases because some vesicles were formed by only one of the two lipid phases. Consequently, there can be observed one phase vesicles that are completely dark or bright and two-phase vesicles that show a dark and a bright region (Figure 17). It has been observed too that some vesicles display bulged regions (Figure 18). This bulged regions eventually disappear to minimize the phase boundary and form a single sphered vesicle.

The experiments with the one-phase vesicles have shown an insertion of the graphene flakes in the membrane. The objective with the two-phase vesicles is to observe whether if there is a preference of insertion for one of the two phases or not. The two phases can be seen through fluorescence microscopy, and the graphene flakes can be observed through optical microscopy the same way that with the one-phase vesicles. That is why, the study is performed combining both fluorescence and optical microscopy.

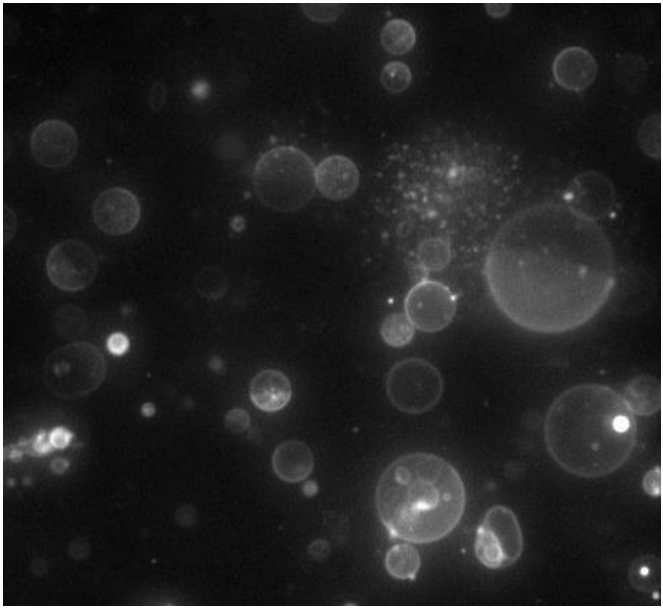

Figure 17. Fluorescence microscopy of the vesicles where the L_d and L_o phases can be distinguished. Frame captured by the Andor iXon Ultra 897 camera.

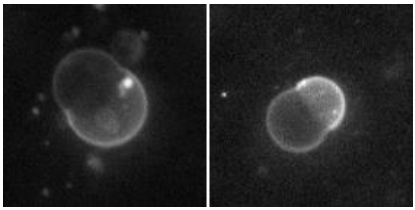

Figure 18. Vesicles displaying bulged regions

To address this question, 75 vesicles that presented both L_d and L_o phases have been studied by analyzing the number of inserted graphene flakes in each phase of every vesicle. A total of 165 graphene flakes were counted and, as a result, 48% of graphene flakes were inserted to the L_o phase and the other 52% was inserted in the L_d phase. This ratio indicates no preference of the graphene for any of the phases whatsoever. Figure 19 presents some of the studied vesicles.

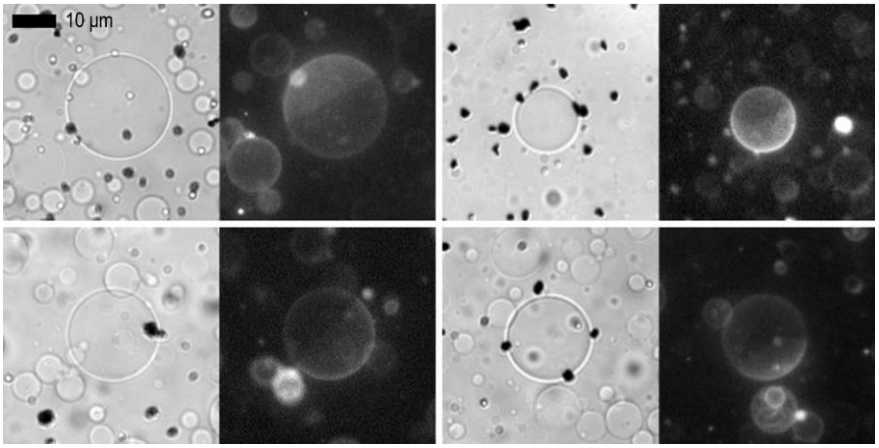

Figure 19. Four montages combining optical and fluorescence microscopy images. On the left of each panel there are the optical microscopy images where the graphene particles can be seen. On the right of each panel there are the fluorescence microscopy images where the phase separation can be seen.

6.2. MOLECULAR DYNAMICS SIMULATIONS

The aim of performing the MD simulations is to gain insights of the interaction between graphene and lipid membranes on a molecular detail. The lipid bilayer of the simulations emulates the membrane of the vesicles in the experimental section. The one-phase lipid bilayer composed by POPC and cholesterol is equivalent to the one-phase vesicles whereas the two-phase lipid bilayer composed by DUPC, DPPC and cholesterol is equivalent to the two-phase vesicles formed by DOPC, DPPC and cholesterol. DOPC is substituted for DUPC in the simulations because it has a high degree of unsaturation that in contrast with the saturated DPPC provides a better separation of the L_d and L_o phases.

In this section, the performed MD simulations will be exposed and discussed in detail. Three simulations have been conducted regarding several factors. The most important factor is the type of lipids that compose the membrane which determines whether one or two phases will be present. In the case of the two-phase lipid bilayer, the initial position of the graphene sheet is another factor. In all the simulations, the graphene sheet is initially placed with its corner perpendicularly pointing to the lipid bilayer.

6.2.1. One-phase lipid bilayers

The simulation with the one-phase lipid bilayer shows how graphene easily penetrates the membrane. The full insertion time is of approximately 400 ns and once the graphene sheet is inside the bilayer (Figure 20), it rotates and moves laterally on the membrane plane. It is observed that the graphene sheet stays inside the bilayer during the total time of the simulation. As discussed in the experimental section, the apolarity of the graphene sheet makes it not compatible with the aqueous solution, therefore graphene becomes fully protected from the water molecules and achieves a state of equilibrium inside the lipid bilayer. This indicates that the bilayer is a good solvent for graphene, which is a relevant fact for studies held in the field of biomedicine and biotechnology to investigate new applications and toxicity of carbon-based nanomaterials.

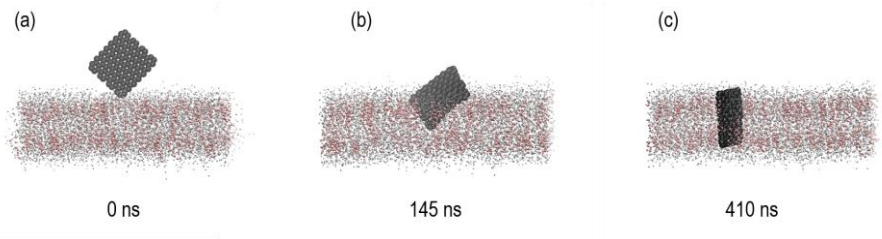

Figure 20. MD simulation for the one-phase lipid bilayer.

6.2.2. Two-phase lipid bilayers

Two simulations have been performed for the two-phase lipid bilayer: the first has the graphene sheet initially placed close to the L_d phase and the second close to the L_o phase. The two-phase lipid bilayer without the graphene sheet is as shows in Figure 21, where the L_d phase, mainly composed by unsaturated DUPC, is colored in grey and the L_o phase, mainly composed by saturated DPPC, is colored in green. The L_o phase is enriched in cholesterol, which corresponds to the pink beads.

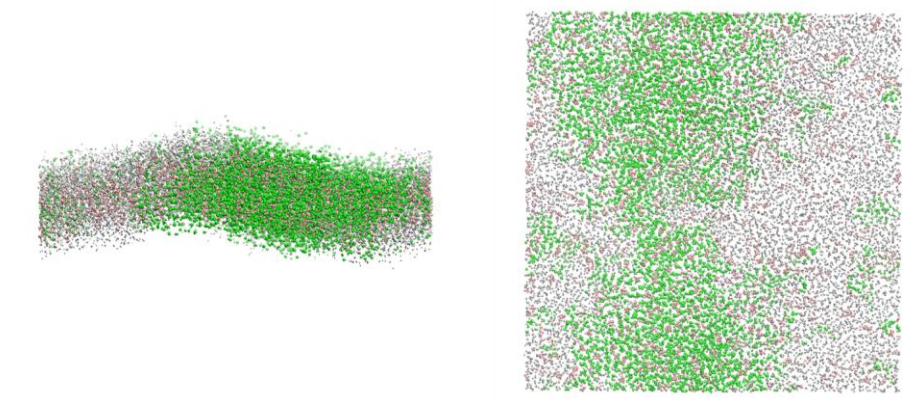

Figure 21. The two-phase lipid bilayer seen from a lateral (left) and top (right) perspective. DUPC is colored in grey, DPPC is colored in green and cholesterol is colored in pink.

Both simulations have showed an insertion of the graphene sheet inside the bilayer. Figure 22 shows a sequence of the simulation on the L_d phase and Figure 23 shows a sequence of the simulation in the L_o phase. However, the graphene sheet enters the lipid bilayer at two different rates depending on the phase. Actually, the insertion in the L_o phase is still not complete after 1000 ns. The total insertion of the graphene sheet in the membrane is not known but it surely would be more than twice of the insertion time in the L_d phase. An increase of the simulation time would be needed to obtain more precise results about the insertion process in the L_o phase.

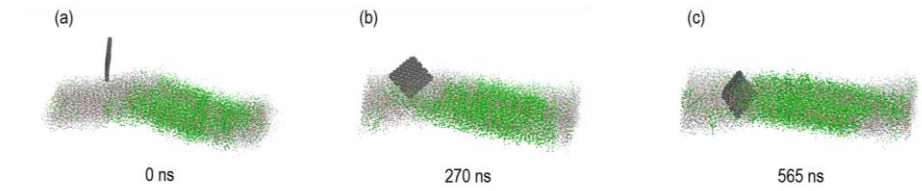

Figure 22. MD simulation for the two-phase lipid bilayer where the graphene sheet is initially placed close to the L_d phase

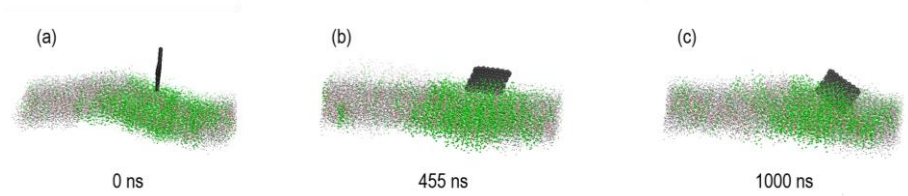

Figure 23. MD simulation for the two-phase lipid bilayer where the graphene sheet is initially placed close to the L_o phase.

The reason of the difference in the insertion rates lies in the structure of the phases: the L_o phase is more compact than the L_d phase, thus, the penetration is slower. This difference may suggest a preference for the L_d phase, and that differs with the experimental results. However, if the data is properly analyzed, the results of both experiments and simulations go in the same way. The MD simulations show that the L_d phase displays more spaces between lipids and for this reason, it is easier for graphene to penetrate the bilayer whereas the compact structure of the L_o phase makes it more difficult. Despite this difference in the kinetics of the graphene absorption, both experimental and simulations show that the insertion of graphene takes place without any preference for any of the two phases. Above all, graphene is *uncomfortable* in an aqueous solution and tries to find a better solvent, like the lipid bilayer.

Even though the insertion time in the L_o phase is larger, once the graphene sheet is inside the bilayer, one could expect a more favorable and attractive interaction than in the L_d phase. It could be due to the compact structure of the phase, given by the saturated lipids, which makes a better solvent than the unsaturated ones present in the L_d phase. It would take more simulations to confirm this conjecture and a more exhaustive analysis of the free energy of the system.

10. CONCLUSIONS

The vesicle electroformation method has been proved to work well to successfully obtain vesicles in an average size range between 5 and 20 μm. This method has also been used for the first time to obtain heterogeneous vesicles.

The deposition of the vesicles in the observation pool and the subsequent addition of graphene is a good method to observe the graphene-vesicles interaction.

In the experiments, it has been observed an attractive interaction between graphene and vesicles. The Van der Waals forces lead to an insertion of graphene flakes inside the lipid bilayer.

The MD simulations provided molecular detail on the insertion of graphene and showed that once inside the membrane, graphene moves laterally in the bilayer plane in a random way. It also shows how graphene stays in the membrane indicating its affinity: the lipid bilayer is a good solvent for graphene.

The experiments with the two-phase vesicles showed no preference of graphene for any of the phases. The MD simulations show that the rates of insertion of graphene into the bilayer are different in each phase: the insertion in the L_o phase takes twice the time of the L_d phase insertion. The difference in insertion rates is due to the structure of the phases which is provided by the degree of unsaturation of the lipids that compose it.

The main conclusion of this work is that the lipid bilayer is a good solvent for graphene. This fact has important relevance in the fields of biomedicine and biotechnology where they are studying possible applications for graphene and its toxicology. A basic step in these investigations is to know the specifics of the bioaccumulation of this material. Bioaccumulation refers to the accumulation of substances, in this case graphene, in a living organism. This work provides prove that graphene may accumulate inside the plasma membranes of eukaryotic cells.

11. REFERENCES AND NOTES

[1] K. S. Novoselov, "Nobel Lecture: Graphene: Materials in the Flatland," *Rev. Mod. Phys.*, vol. 83, no. 3, pp. 837–849, 2011.

[2] Z. Liu, J. T. Robinson, X. Sun, and H. Dai, "PEGylated Nano-Graphene Oxide for Delivery of Water Insoluble Cancer Drugs (b)," *J Am Chem Soc*, vol. 130, no. 33, pp. 10876–10877, 2008.

[3] Y. Liu, X. Dong, and P. Chen, "Biological and chemical sensors based on graphene materials," *Chem. Soc. Rev.*, vol. 41, no. 6, pp. 2283–2307, 2012.

[4] X. Sun *et al.*, "Nano-graphene oxide for cellular imaging and drug delivery," *Nano Res.*, vol. 1, no. 3, pp. 203–212, 2008.

[5] S. Sayyar, E. Murray, B. C. Thompson, S. Gambhir, D. L. Officer, and G. G. Wallace, "Covalently linked biocompatible graphene/polycaprolactone composites for tissue engineering," *Carbon N. Y.*, vol. 52, pp. 296–304, 2013.

[6] H. Yuan, C. Huang, J. Li, G. Lykotrafitis, and S. Zhang, "One-particle-thick, solvent-free, coarse-grained model for biological and biomimetic fluid membranes," *Phys. Rev. E - Stat. Nonlinear, Soft Matter Phys.*, vol. 82, no. 1, pp. 1–8, 2010.

[7] A. B. Seabra, A. J. Paula, R. De Lima, O. L. Alves, and N. Durán, "Nanotoxicity of graphene and graphene oxide," *Chem. Res. Toxicol.*, vol. 27, no. 2, pp. 159–168, 2014.

[8] T. A. J. Kuhlbusch, C. Asbach, H. Fissan, D. Göhler, and M. Stintz, "Nanoparticle exposure at nanotechnology workplaces: A review," *Part. Fibre Toxicol.*, vol. 8, pp. 1–18, 2011.

[9] V. C. Sanchez *et al.*, "Biopersistence and potential adverse health impacts of brous nanomaterials: what have we learned from asbestos?," *Pathology*, 2009.

[10] O. Akhavan and E. Ghaderi, "Toxicity of graphene and graphene oxide nanowalls against bacteria," *ACS Nano*, vol. 4, no. 10, pp. 5731–5736, 2010.

[11] K. H. Liao, Y. S. Lin, C. W. MacOsko, and C. L. Haynes, "Cytotoxicity of graphene oxide and graphene in human erythrocytes and skin fibroblasts," *ACS Appl. Mater. Interfaces*, vol. 3, no. 7, pp. 2607–2615, 2011.

[12] G. M. Cooper, *The Cell: A Molecular Approach*. 2000.

[13] M. D. Houslay and K. K. Stanley, *Dynamics of biological membranes : influence on synthesis, structure, and function*. Chichester [etc.] : Wiley, 1982.

[14] V. G. Almendro Vedia, P. Natale, S. Chen, F. Monroy, V. Rosilio, and I. López-Montero, "iGUVs: Preparing Giant Unilamellar Vesicles with a Smartphone and Lipids Easily Extracted from

Chicken Eggs," *J. Chem. Educ.*, vol. 94, no. 5, pp. 644–649, 2017.

[15] L. Rui *et al.*, "Reduced graphene oxide directed self-assembly of phospholipid monolayers in liquid and gel phases," *Biochim. Biophys. Acta - Biomembr.*, 2015.

[16] Y. Li *et al.*, "Graphene microsheets enter cells through spontaneous membrane penetration at edge asperities and corner sites," *Proc. Natl. Acad. Sci.*, vol. 110, no. 30, pp. 12295–12300, 2013.

[17] P. J. J. Huang, F. Wang, and J. Liu, "Liposome/Graphene Oxide Interaction Studied by Isothermal Titration Calorimetry," *Langmuir*, vol. 32, no. 10, pp. 2458–2463, 2016.

[18] X. Liu and K. L. Chen, "Interactions of graphene oxide with model cell membranes: Probing nanoparticle attachment and lipid bilayer disruption," *Langmuir*, 2015.

[19] R. Frost, S. Svedhem, C. Langhammer, and B. Kasemo, "Graphene Oxide and Lipid Membranes: Size-Dependent Interactions," *Langmuir*, vol. 32, no. 11, pp. 2708–2717, 2016.

[20] L. Wu, L. Zeng, and X. Jiang, "Revealing the Nature of Interaction between Graphene Oxide and Lipid Membrane by Surface-Enhanced Infrared Absorption Spectroscopy," *J. Am. Chem. Soc.*, vol. 137, no. 32, pp. 10052–10055, Aug. 2015.

[21] K. Simons and E. Ikonen, "Functional rafts in cell membranes," *Nature*, vol. 387, no. 6633, pp. 569–572, 1997.

[22] K. Simons and D. Toomre, "Lipid rafts and signal transduction," *Nat. Rev. Mol. Cell Biol.*, vol. 1, no. 1, pp. 31–39, 2000.

[23] A. D. Douglass and R. D. Vale, "Single-molecule microscopy reveals plasma membrane microdomains created by protein-protein networks that exclude or trap signaling molecules in T cells," *Cell*, vol. 121, no. 6, pp. 937–950, 2005.

[24] A. V. Titov, P. Král, and R. Pearson, "Sandwiched Graphene- Membrane Superstructures," *ACS Nano*, vol. 4, no. 1, pp. 229–234, 2009.

[25] J. Wang, Y. Wei, X. Shi, and H. Gao, "Cellular entry of graphene nanosheets: the role of thickness, oxidation and surface adsorption," *RSC Adv.*, vol. 3, no. 36, p. 15776, 2013.

[26] D. R. Dreyer, S. Park, C. W. Bielawski, and R. S. Ruoff, "Graphite oxide," *Chem. Soc. Rev.*, vol. 39, no. 1, pp. 228–240, 2010.

[27] H. J. C. Berendsen, D. van der Spoel, and R. van Drunen, "GROMACS: A Message-Passing Parallel Molecular Dynamics Implementation," *Comput. Phys. Comm.*, vol. 91, pp. 43–56, 1995.

[28] S. J. Marrink, H. J. Risselada, S. Yefimov, D. P. Tieleman, and A. H. de Vries, "The MARTINI Force Field: Coarse Grained Model for Biomolecular Simulations," *J. Phys. Chem. B*, vol. 111, pp. 7812–7824, 2007.

[29] S. L. Veatch and S. L. Keller, "Separation of Liquid Phases in Giant Vesicles of Ternary Mixtures of Phospholipids and Cholesterol," *Biophys. J.*, 2003.

12. ACRONYMS *(in order of appearance)*

POPC: 1-palmitoyl-2-oleoylphosphatidylcholine

L_o: liquid-ordered

L_d: liquid-disordered

DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine

DOPC: 1,2-dioleoyl-sn-glycero-3-phosphocholine

NBD-DMPE: [O-(1-O,2-O-Dimyristoyl-L-glycero-3-phospho)-2-(7-nitro-2,1,3-benzoxadiazole-4-ylamino) ethanol] anion

DUPC: dilinoleoyl-phosphatidylcholine

ITO: Indium tin oxide

PC: Phosphatidylcholine

AC: Alternate Current

PDMS: Polydimethylsiloxane

LED: Light Emitting Diode

GO: Graphene Oxide

MD: Molecular Dynamics

GROMACS: Groningen Machine for Chemical Simulations

CGMD: Coarse-Grained Molecular Dynamics