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14. ABSTRACT
The research on mitochondria has been the focus of many scientists since the discovery of mitochondria DNA mutation as a cause of many health diseases and its major role in apoptosis. Various work has been done to measure the membrane potential of mitochondria which can inform scientists of the health state of the cells.
The objective of this study was to learn more about mitochondria's surface properties and to consequently immobilize mitochondria on graphene. The surface of graphene was coated with Polyethyleneimine (PEI),

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IMMOBILIZATION of MITOCHONDRIA on GRAPHENE

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UNIVERSITY OF CALIFORNIA,
IRVINE

IMMOBILIZATION of MITOCHONDRIA on GRAPHENE

THESIS

submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in Biomedical Engineering

by

Elaheh Shekaramiz

Thesis committee:

Peter J. Burke, Ph.D., Chair

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I am also extremely thankful of my parents, my family, and my friends for their invaluable support. I would like to particularly express my great appreciation for my mother, who has always been encouraging me to further expand my knowledge and has always been there for me in ups and downs of my life.

ABSTRACT OF THE THESIS

Immobilization of Mitochondria on Graphene

By

Elaheh Shekaramiz

Master of Science in Biomedical Engineering

University of California, Irvine, 2012

Professor Peter J. Burke, Chair

The research on mitochondria has been the focus of many scientists since the discovery of mitochondria DNA mutation as a cause of many health diseases and its major role in apoptosis. Various work has been done to measure the membrane potential of mitochondria which can inform scientists of the health state of the cells.

The objective of this study was to learn more about mitochondria's surface properties and to consequently immobilize mitochondria on graphene. The surface of graphene was coated with Polyethyleneimine (PEI), Poly-L-Lysine (PLL), Lipid bilayer, and Polyethylene glycol (PEG) to study the immobilization of mitochondria on graphene. The technique used to remove unattached mitochondria as well as the staining method were both crucial factors in adhesion of mitochondria to the surface of graphene.

CHAPTER 1: INTRODUCTION

Mitochondria

Mitochondria have important functions such as Adenosine-5'-triphosphate (ATP) production, regulation of cellular metabolism and apoptosis. Dysfunction of mitochondria may lead to a lot of diseases including cancer, and age-related diseases (Desler,C. eta al. 2011). In order to study the mitochondrial membrane potential, mitochondria should be immobilized on a surface and it must be stained. The structure and properties of the surface (graphene) as well as the staining dyes used in this study are going to be discussed in chapter 2. The surface of graphene needs to be coated so that mitochondria can adhere to it. The coating materials are discussed in chapter 3. In chapter 4, materials and methods of this experiment are proposed. Chapter 5 is the results and chapter 6 is the discussion and conclusion of this study.

Structure of Mitochondria

Mitochondria are membrane bound organelles in eukaryotic cells. Their diameter ranges from 0.5 to 1.0µm. These organelles are comprised of 4 major compartments: outer membrane, inner membrane, cristae, and matrix (Figure 1-1).

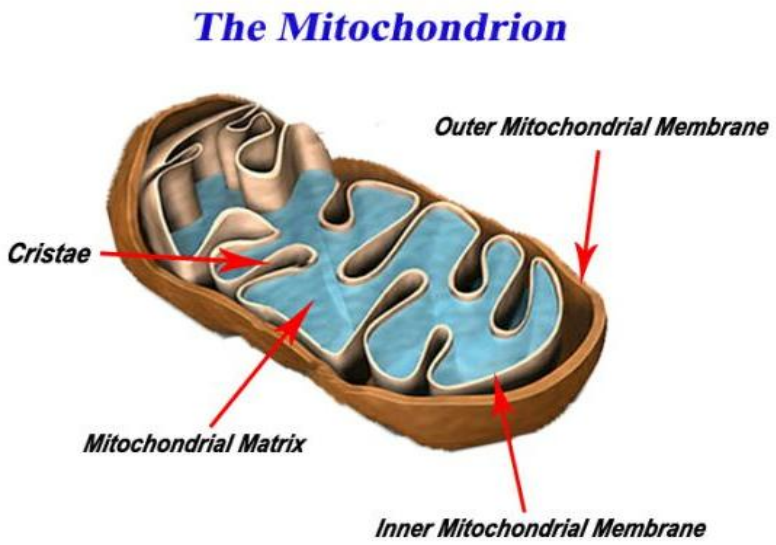

Figure 1-1. Mitochondria compartments

The outer membrane is the most external layer of mitochondria. It allows Adenosine diphosphate (ADP), Adenosine triphosphate (ATP), ions and small molecules to diffuse easily, while it is impermeable to molecules larger than 5000 Daltons. The outer membrane is equally composed of phospholipids and proteins. These proteins play a pivotal role in the maintenance of mitochondrial morphology, metabolic exchange, and communication with cytosol. For instance, the translocase of the outer membrane (TOM) complex regulates the passage of all proteins encoded in the nucleus heading to the inner mitochondria. Furthermore, the outer membrane has a sorting and assembly machinery (SAM) complex that inserts Beta barrel proteins into the outer membrane. Beta Barrel proteins are large beta-sheets that twist to form a closed structure in such a way that the first strand is hydrogen bonded to the last one. They have tasks such as passive nutrient intake, active ion transport, and defense against attack proteins (Schulz, G.E., 2000).

The inner membrane is impermeable to ions and molecules because the phospholipids in its lipid bilayer contain four fatty acids instead of two. Small molecules pass the inner membrane through the translocase of inner membrane (TIM) protein. The inner mitochondria membrane folds to create cristae, which results in a large surface area (Figure 1-1).

The cristae accommodate several vital proteins that contribute to the main functions of mitochondria including ATP synthase and cytochromes. ATP synthase is the enzyme that converts energy of protons to ATP through the following reaction:

ATP synthase + ADP + P_i → ATP Synthase + ATP

The inner membrane of mitochondria also surrounds the matrix of mitochondria. The matrix contains enzymes, ribosomes, tRNAs, and copies of mitochondrial DNA genome. It also has a role in production of ATP through the citric cycle. The enzymes of the matrix metabolize fatty acids and pyruvate to produce acetyl CoA. In addition, these enzymes oxidize acetyl CoA to produce CO2 and NADH in the citric acid cycle. NADH is later used as a carrier in the electron transport chain process to transfer the electrons across the membrane. The electron transport chain includes 4 complexes (Alberts B, et al., 2002). The transport of electrons creates a proton gradient across the inner membrane of mitochondria which is responsible for the production of ATP (Figure 1-2).

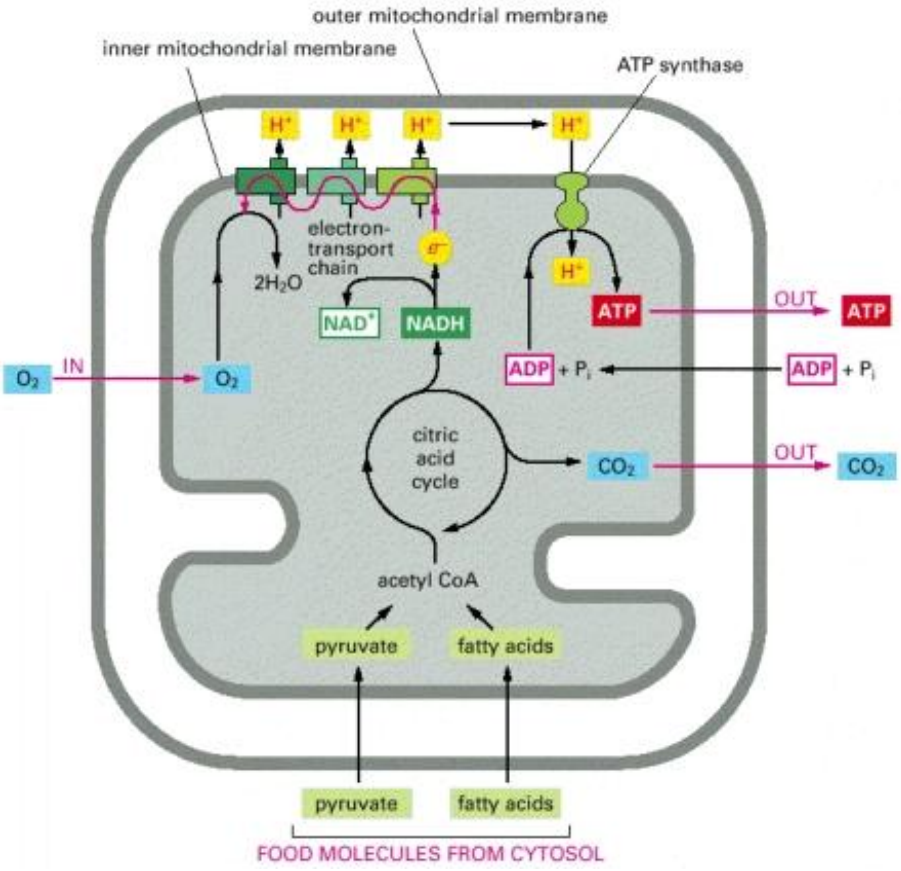

Figure 1- 2. ATP synthesis

Membrane Potential of Mitochondria

The variation in mitochondrial membrane potential has been found to be a key factor in determining whether or not mitochondria are healthy. The membrane potential is created as a result of hydrogen pumping from matrix to the intermembrane space through the 4 complexes. As discussed above, the ATP synthase utilizes these hydrogen ions to produce ATP. Thus, the membrane potential decreases as the protons are used to produce energy. However, the

membrane potential is maintained by pumping more protons across the membrane. The change in membrane potential is a signal for mitochondrial dysfunction (Lim,T. 2011).

CHAPTER 2: GRAPHENE

Graphene

Structure of Graphene

A single layered graphene is a 2D planer sheet composed of SP^2 bonded carbon atoms (Figure 2-1). Carbon atoms are bonded through in-plane σ bonds and perpendicular π orbitals(Figure 2-2). Bi-layer and few-layered graphenes have 2 or more of the 2D sheets of graphene. As can be seen in figure 2-3, Carbon atoms can be stacked as AA, AB, or ABC (Figure 2-3).

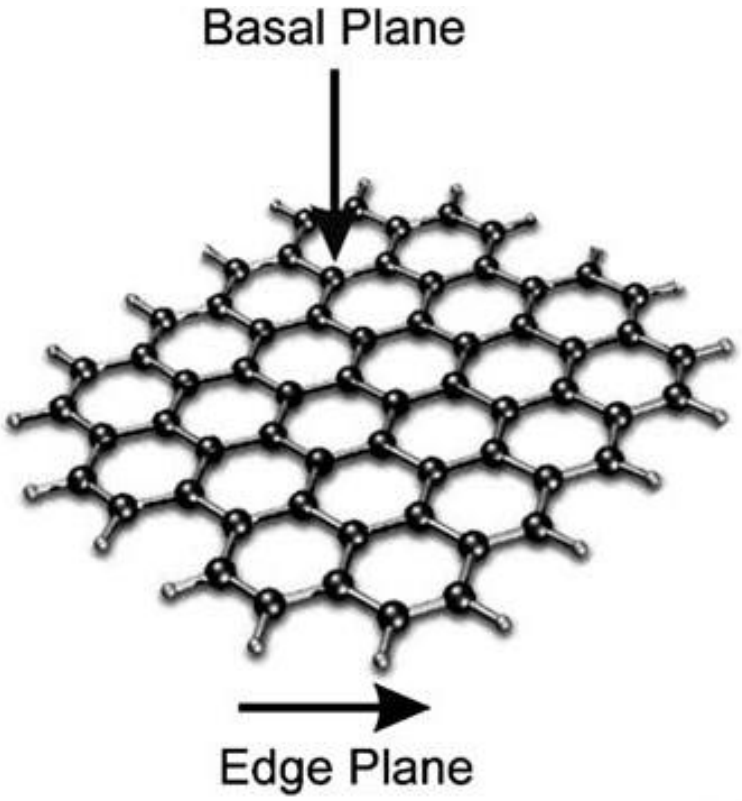

Figure 2-1. Single layer structure of graphene

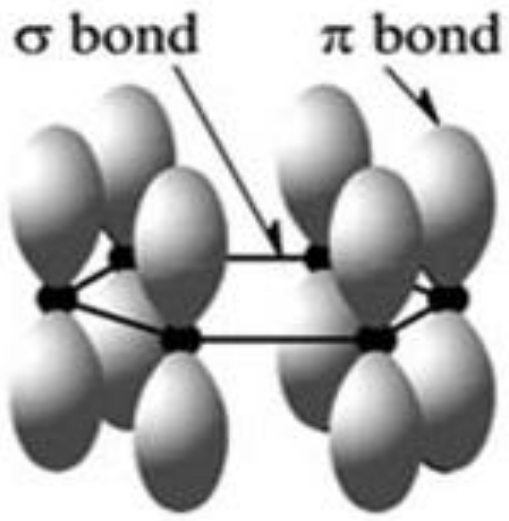

Figure 2-2. Schematic of in plane sigma bonds and perpendicular pi bonds

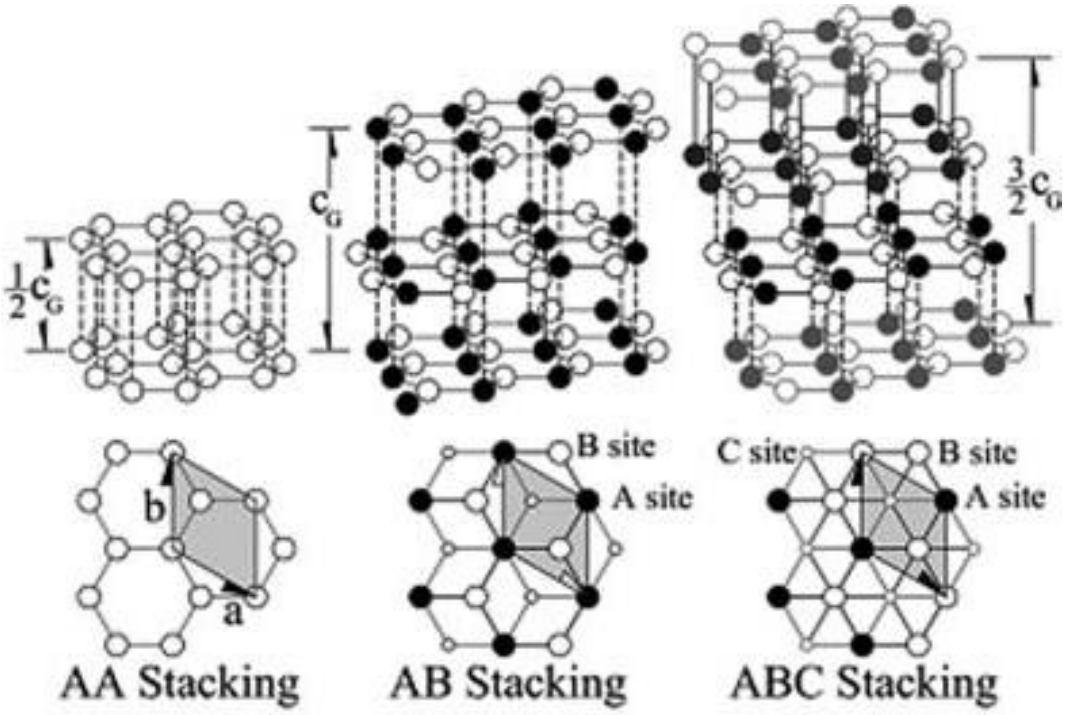

Figure 2-3. Different ways of stacking carbon atoms

Application and properties of Graphene

Graphene is synthesized in different ways depending on its application (Dale, A.C.,et al., 2010). Various methods of synthesis of graphene include exfoliation and cleavage, thermal chemical vapor deposition, plasma enhanced chemical vapor deposition, thermal deposition of silicon carbide (SiC), thermal deposition on other substrates, chemical methods, and Un-zipping carbon nanotubes (CNTs) (Choi, W.et al, 2010). However, the most common method of graphene production is through chemical modification of graphite (Kauffman, D.R., 2010). Relative to CNTs, graphene has a higher surface area (2630 $m^2 g^{-1}$) and electrical conductivity (64mS $cm^{-1)}$. Moreover, graphene has the highest electron mobility compared to carbon nanotubes or graphite (Kauffman, D.R., 2010). Graphene has numerous applications in optics, electronics (field effect transistors and transparent electrodes), and sensors (graphene based gas and biosensors). Graphene has been reported as a potential material for fabrication of glucose sensors. Using glucose oxide enzyme as a model, Shan et al. constructed a polyvinylpyrrolidone protected graphene/polyethylenimine electrochemical sensor. They measured glucose responses of up to 14mM. Further studies have shown that graphene is more effective than CNTs in sensing neurotransmitters such as dopamine in the presence of interfering agents (e.g. serotonin).

Fowler et al. have demonstrated the ability of graphene to sense gases such as NH_3, and NO_2. When NO_2 is in contact with graphene, it withdraws an electron from graphene and generates a hole conduction. NH_3 on the other hand donates an electron to graphene and induces electron conduction (Choi, W.et al, 2010).

The high electron mobility of graphene along with its excellent mechanical properties makes it a great electrode for measuring membrane potential of mitochondria (Choi, W.et al, 2010).

Dyes

JC-1 Dye

JC-1(5, 5', 6, 6'-tetrachloro-1, 1', 3, 3' tetraethylbenzimidazolylcarbocyanine iodide) is a cationic dye for measuring mitochondrial membrane potential (Figure 2-4).

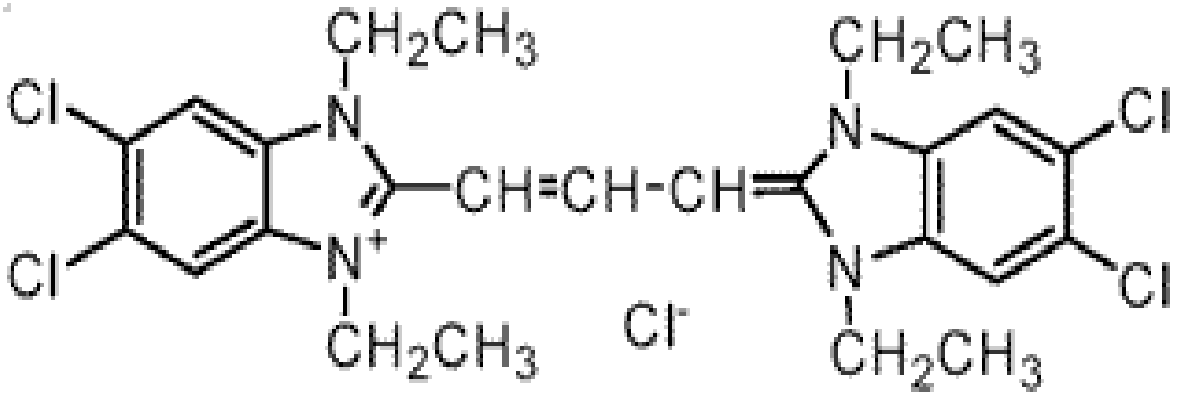

Figure 2-4. Chemical Structure of JC-1

In healthy cells, JC-1 stains the mitochondria red. Due to the negative charge of mitochondrial membrane potential, the positively charged JC-1 can enter the matrix of mitochondria and accumulate there. This buildup leads to aggregation of JC-1, which results in the red color under the fluorescent microscopy. On the other hand, when cells undergo apoptosis, mitochondria turn into green because the membrane potential of the mitochondria

collapses and JC-1 remains in the cytoplasm of the cell as monomers (Poot, M.et al., 1996**)**. Both red and green colors can be observed under the microscope simultaneously using appropriate filters. One of the disadvantages of using JC-1 dye is that it does not specifically stain mitochondria. Moreover, JC-1 has been reported to be susceptible to factors other than membrane potential such as surface/volume ratio and the presence of H_2O_2. In addition, JC-1 is sensitive to the loading concentration and time (Perry, S. W., et al., 2011).

TMRM Dye

Another commonly used dye is tetramethylrhodamine methyl (TMRM) which passes through the cell membrane, accumulates in the mitochondria, and stains the mitochondria red(Figure 2-5). However, it doesn't get aggregated in the cell membrane nor does it interact with membrane proteins. Unlike JC-1, TMRM dye is not concentration dependent (Gottlieb, E., et al., 2003).

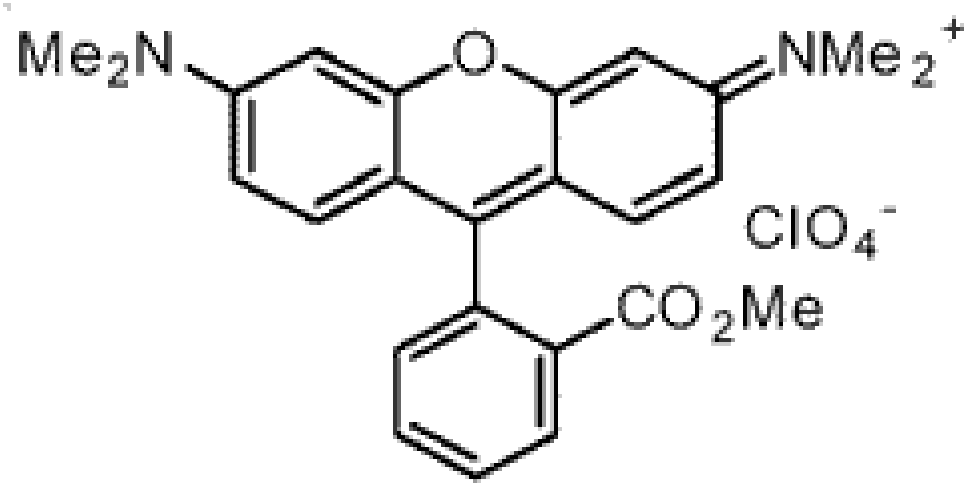

Figure 2-5. Chemical Structure of TMRM

CHAPTER 2: POLYETHYLENEIMINE (PEI), POLY-L-LYSINE (PLL), LIPID BILAYER, AND POLYETHYLENE GLYCOL (PEG)

Current Technologies in Immobilization of Mitochondria

The external surface of mitochondria is sophisticated: It contains hydrophobic, hydrophilic, cationic, and anionic sites. However, the surface of mitochondria is reported to be more negatively charged than positively charged. Moreover, in some studies, it has been reported that coating of fused-silica capillaries with hydrophilic polymers such as poly-acryloylaminopropanol (AAP) and poly-vinyl alcohol (PVA) reduces the adsorption of mitochondria on the surface (Whiting, C.E., and A. Edgar., 2006).

Some other studies have suggested that plate coating is not necessary for immobilization of mitochondria. Coating of XF24 plates with polyethyleneimine or Cell-Tak™ (a bioadhesive from a marine mussel that is a mixture of polyphenolic proteins) did not enhance the adsorption of mitochondria on the surface (Rogers, G.W., et al., 2011).

Immobilization of mitochondria on glass has also been investigated. Mitochondria (20 µl) with the concentration of 0.75–1 mg protein/ml were added to thirty-one mm glass cover slips. The cover slips were washed with 70% ethanol and water and were dried before use. They were positioned in a 700µl perfusion chamber. After 1 minute of perfusion with KCl buffer at 7ml/min rate, the mitochondria which were not attached were washed away (Vergun,O., T. V. Votyakova, and I. J. Reynolds,2003).

Another method of attaching mitochondria to glass is through coating the surface of glass with CellTak. A glass cover slip (12mm in diameter, 1mm thick) was coated with 5µg of CellTak (Farmington,CT, U.S.A.). The cover slip was cleaned first with ethanol and then with

distilled water, and was dried afterwards. Mitochondria inside incubation buffer were pipetted on top of glass. After 25min at room temperature, the glass was placed in a 700µl perfusion chamber with perfusion rate of 2.6ml/min. The clumps of mitochondria were then observed under microscope (REERS, M., R. A. KELLY and T. W. SMITH, 1989).

Other polymers such as poly-D-lysine have been used successfully to immobilize mitochondria on glass. The surface of 35mm glass dish from Ibidi GmbH Munich, Germany was coated with 20µg/ml of poly-D-lysine (MW 150,000-300,000, Sigma) for 30min at 37°C. Then, the glass was washed three times with Phosphate buffered saline (PBS). Concentration of Mitochondria was diluted to 1.0 or 5.0mg/ml using 250mM sucrose, 1 mg/ml bovine serum albumin, 10mM KH2PO4, 27mM KCl, 1mM $MgCl_2$ 40mM Hepes, and 0.5mM EGTA at pH of 7.1 (buffer B). Then, 1-5µl of mitochondria was placed on top of the coated glass. After 15-20min of incubation at 37°C, 2ml of buffer B was poured and removed gently to eliminate unstuck mitochondria (Quarato, G., et al., 2011).

Coating of glass with poly-L-lysine has also been reported for immobilization of yeast mitochondria. Coating was performed by repetitive washing of cover slips with 0.02% poly-L-lysine and de-ionized water (Kuzmenko,A., S. Tankov, B. P. English, et al., 2011).

In this study, the surface of graphene was coated with polyethyleneimine (PEI), poly-L-lysine (PLL), Polyethylene glycol (PEG), and lipid bilayer to verify the possibility of immobilization of mitochondria on graphene.

Polyethyleneimine (PEI)

Structure of PEI

Polyethyleneimine is a cationic polymer with ethylenimine motifs being accountable for positively charged backbone. PEIs could be either linear or branched. Linear polyethyleneimines (PEIs) contain secondary amines and exist in solid form at room temperature (Figure 3-1). However, branched PEIs contain all kinds of amines including primary, secondary, and tertiary, and exist in liquid form at room temperature (Figure 3-2).

Figure 3-1. Structure of Linear Polyethyleneimine

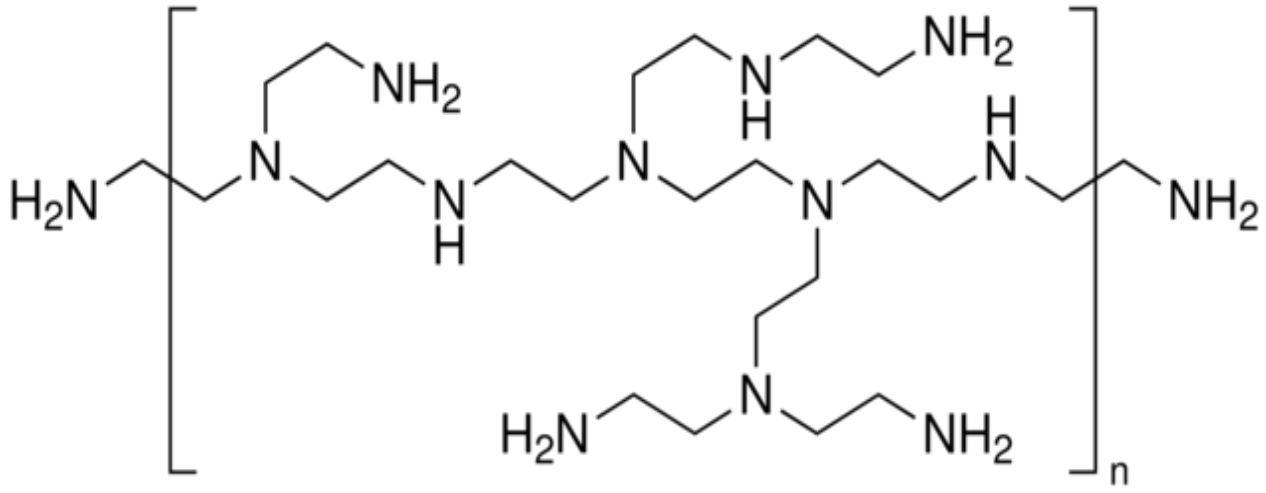

Figure 3-2. Structure of Branched Polyehyleneimine

Applications of PEI

One of the applications of PEIs is in gene delivery. This method takes advantage of the fact that PEI is positively charged. PEI condenses DNA and forms a DNA complex which can be taken up by cells (endocytosis). Endocytosis occurs because the surface of cell is negatively charged and the DNA complex is positively charged. Inside the cell, PEI protonation leads to osmotic swelling which in turn releases the DNA complex into the cytoplasm. If the DNA complex is unpacked, then it can enter the nucleus(Figure 3-3)(Lungwitz U., et al., 2005).

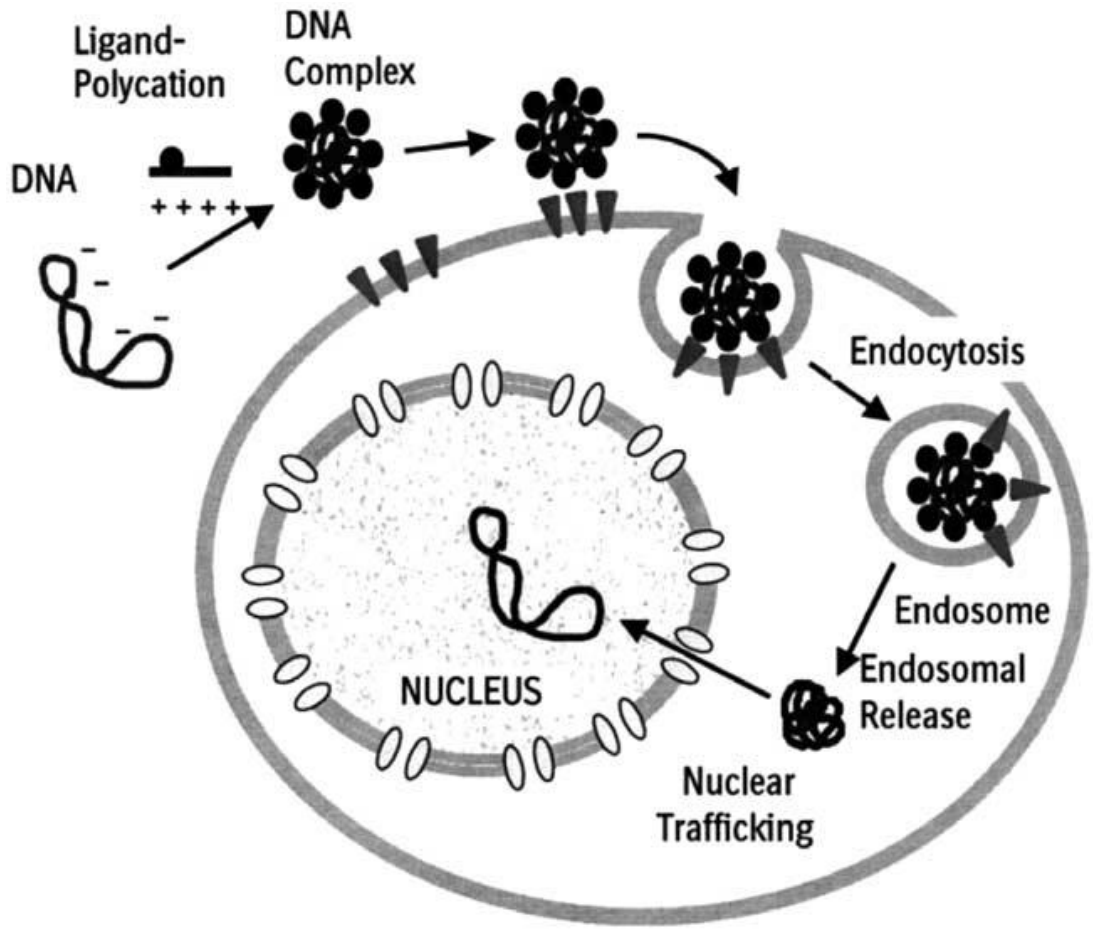

Figure 3-3. PEI application in gene delivery

One other important function of PEI is in anchoring cells to a surface. Cells attach to PEI due to ionic interactions that exists between cells and PEI. One of the drawbacks of using PEI is its cytotoxicity (Kafil,V. and Y. Omidi, 2011). The mechanism by which PEI causes cytoxicity is not known. However, linear PEIs are less toxic to cells than branched PEIs; branched PEIs have been shown to damage DNA.

In the current study, we speculated that mitochondria can be immobolized on PEI coated graphene.

Poly-L-lysine (PLL)

Structure of PLL

PLL is a cationic polymer with a molecular structure as shown in figure 3-4.

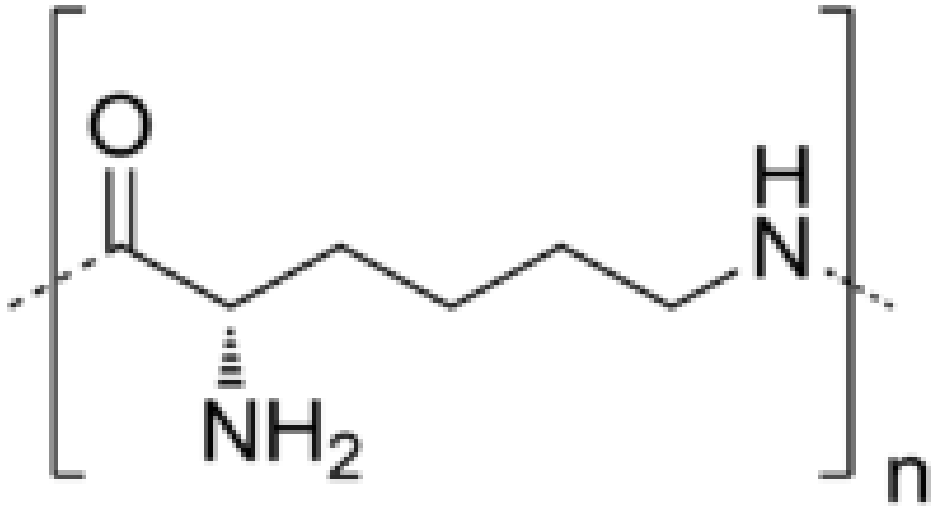

Figure 3-4. Molecular Structure of Poly-L-lysine

Applications of PLL

PLL is a production of bacterial fermentation and is used as a food preservative. In biology, PLL is used in targeting tumor cells because it binds to tumor cells with a great affinity (Jordan, C.E, et al., 1994).

Coating of electrodes with poly-l-lysine has been helpful in immobilization of DNA on electrochemical sensors (Jiang, C., et al., 2008). This attachment is a result of the electrostatic interactions between the negatively charged backbone of DNA and positively charged PLL (Figure 3-5).

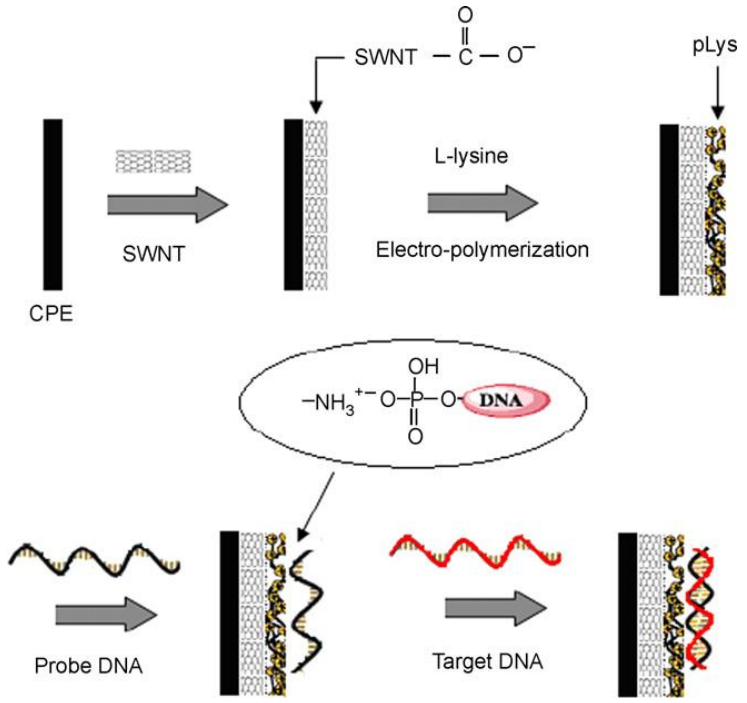

Figure 3-5. Immobilization of DNA on the PLL coated electrode

In addition, surface of tissue culture flasks have been coated with PLL for a better adherence of cells to the flask (Jordan, C.E, et al., 1994).

Lipid Bilayer

Lipid bilayer is a thin membrane composed of two flat sheets. The cell membrane of every living cell is made of lipid bilayer, which acts as a barrier around the cell. Lipid bilayer is

composed of phospholipids with hydrophilic heads and two hydrophobic tails (Figure 3-6). When phospholipids are exposed to water, they rearrange themselves so that the hydrophilic head is pointing toward water and the hydrophobic tail is pointing outwards. Inside the phospholipids, several integral and peripheral membrane proteins are located. While integral proteins interact with the hydrophobic region of the lipid bilayer, peripheral proteins interact with the hydrophilic heads of lipid bilayer. Ion channels and ion pumps are two examples of integral proteins. Ion channels are responsible for the passage of ions across the membrane passively whereas ion pumps transport ions against their concentration gradient. Peripheral proteins bind to the integral proteins and regulate cell signaling and other cell events.

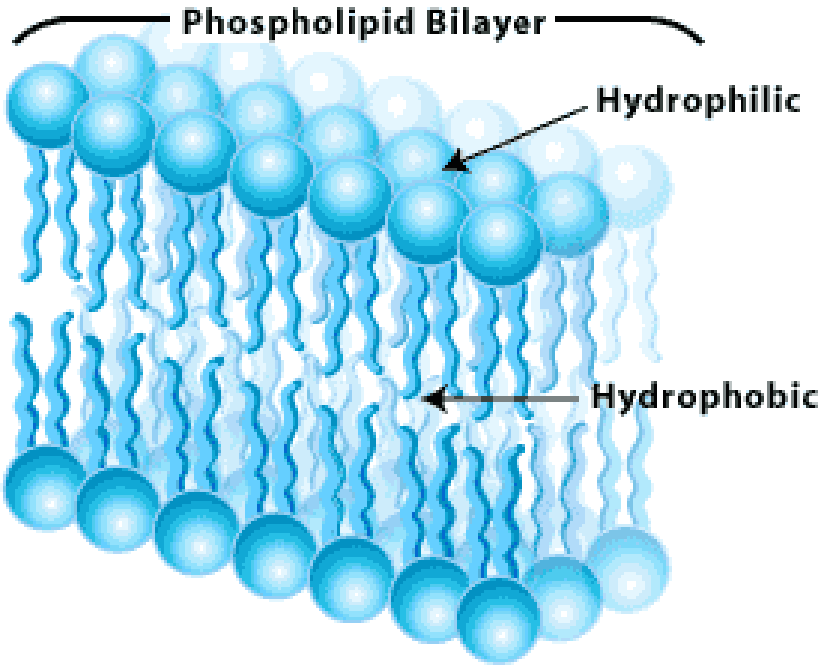

Figure 3-6. Structure of lipid bilayer

Lipid bilayers contain transmembrane ion channel proteins such as gramicidin A (gA) and α-Hemolysin(α-HL). Lipid bilayers made in the lab are called model bilayers. Commonly made lipid bilayers are Black lipid membranes (BLM), Supported lipid bilayers (SLB), Tethered Bilayer Lipid Membranes (t-BLM), and Vesicles (Castellana, E.T., and P. S. Cremer, 2006). In this study, artificial vesicles, which are called liposomes were used. Liposomes are hollow spheres of lipids with inner aqueous compartment and outer lipid bilayer (Figure 3-7). Because the membrane of liposomes is similar to plasma membrane, liposomes can fuse with the plasma membrane and release their contents. In addition, vesicles are able to fuse with other organelles inside the cell. One of the widely applications of liposomes is in drug delivery (Riaz, M., 1996).

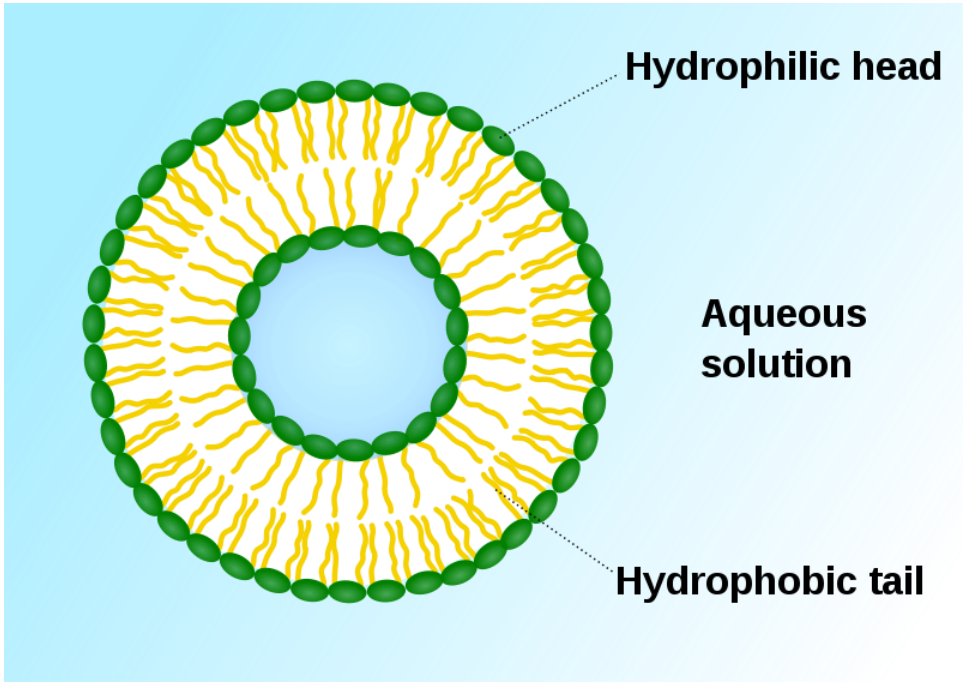

Figure 3-7. Structure of an artificial vesicle, liposome

Polyethylene glycol (PEG)

Structure of PEG

Polyethylene glycol is a linear neutral polyether, which is also recognized as polyoxyethylene, polyethylene oxid, and polyoxirane(Figure 3-8).

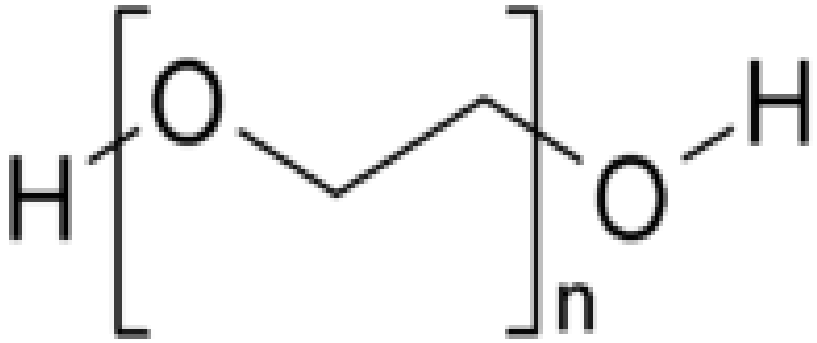

Figure 3-8. Molecular Structure of PEG

Properties of PEG

Surface coating with PEG reduces cell adhesion and protein adsorption as some studies have suggested (Alcantar, N. A., A.S. Eray, and J. N. Israelachvili, 2000). The PEG coated surfaces become hydrophilic and reject proteins. PEG coating also reduces thrombus formation and tissue damage as platelets will not adhere to the surface of PEG. PEG does not change the surface chemistry of materials, does not damage cells and active proteins, and is nontoxic. Proteins are not able to attach to PEG because of the repulsive forces. When a protein approaches PEG, the available volume for PEG polymer is reduced, thus a repulsive force is

generated. At other times, when a protein interpenetrates PEG polymer, a repulsive force is produced. PEG is biocompatible and soluble in water and other polar solvents. PEG is soluble in water because it can form hydrogen and oxygen bonds with water molecules (Alcantar, N. A., A.S. Eray, and J. N. Israelachvili, 2000).

Applications of PEG

PEG has applications in drug delivery, cell encapsulation, and reducing cell adhesion to a surface. In drug delivery, PEGylation is an established method of attaching PEG to the drug of interest (Banerjee, S.S., et al., 2012). PEGylation is known to increase solubility and circulation time of a hydrophobic drug, and is commonly used for delivering of anti-cancer drugs (Figure 3-9).

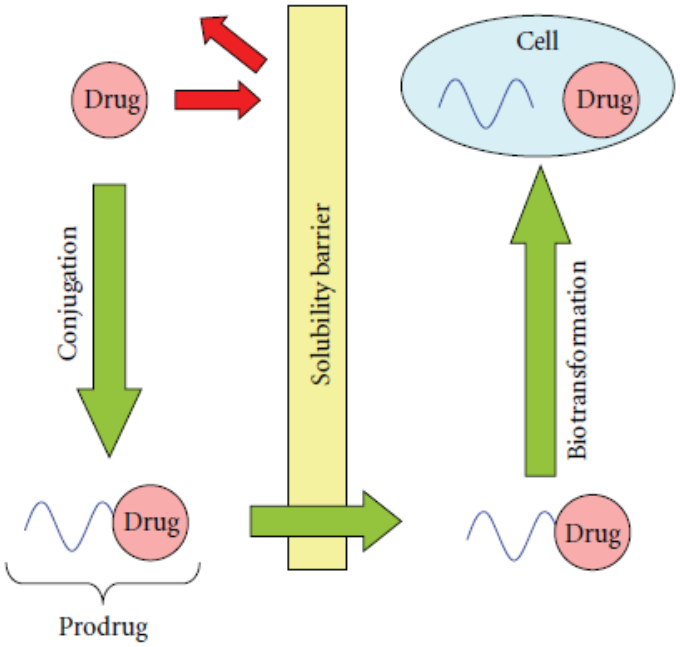

Figure 3-9. PEGylation of drugs to increase solubility

CHAPTER 4: MATARIALS AND METHODS

Materials

Glass slides with dimensions of 3˝x1˝x1.2 mm were purchased from VWR International Company. Graphene was purchased from Graphene Supermarket (Figure 4-1).

Figure 4-1. Graphene film on copper foil

All liquids including phosphate buffered saline (PBS) media were purchased from Sigma-Aldrich.

Methods

Experimental Set up

To speculate whether mitochondria can attach to the surface of graphene, six groups were defined including glass, graphene, PEG coated surface, PEI coated surface, PLL coated surface and Lipid bilayer coated surface. Mitochondria on glass, graphene, and PEG coated

surfaces were expected not to attach. However, mitochondria were anticipated to become immobilized on PEI, PLL, and lipid bilayer surfaces.

Transfer Method of Graphene film

When graphene was purchased, it was on copper foil. To obtain graphene without copper, copper had to be etched away. First, the surface of copper-graphene was spin-coated with Poly-methyl methacrylate (PMMA) in order to protect the surface of graphene. Then, PMMA was dissolved in chlorobenzene (0.8mg/ml). After the spin coating, the surface was cured at 190°C for 1 minute. Second, copper substrate was etched away by ion chloride solution (0.08g/ml) overnight. Third, the PMMA/graphene film was transferred to De-ionized (DI) water overnight. Then, PMMA/graphene film was transferred onto the target substrate (glass slide). After drying the PMMA/graphene film under the vacuum for several hours, the surface was heated at 180° C in air for 30 minutes. The PMMA surface was removed with an acetone bath (Li, X., et al., 2009).

Coating of Graphene with PEI and PLL

The surface of graphene was coated with PEI (1:15000 dilutions of 50% W/V solution in H2O) overnight. Moreover, the surface of graphene was coated with a 0.1% solution of 80,000 - 100,000 Daltons PLL in water.

Preparation of Lipid Bilayer

1mM 1, 2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC, unsaturated fatty acid) was dissolved in chloroform. 1 mM Lissamine Rhodamine Red (LR-DHPE), which is a fluorescence dye and 1mM gA solution were added to the solution. The solution was mixed at room temperature for 2 hours. The mixture was then evaporated overnight under a nitrogen stream to make the lipid suspension for the vesicle fusion. The dried lipid bilayer was rehydrated, stirred, and sonicated in 10mM phosphate buffered saline solution (PBS) at 55˚C for 1 hour to make small unilamellar vesicles (SUVs). Finally, the suspension was filtered with a 0.2µm nylon filter to obtain homogenous SUVs. The surface of graphene was coated with 20µl of lipid bilayer and was placed inside the oven for 4 hours at 60° C. The surface was slowly cooled down to room temperature over 20 minutes and was rinsed with DI water.

PDMS Preparation

A few drops of sigma cote were poured on top of the wafer in order to make the PDMS peeling easier. Silicone elastomer base were mixed thoroughly with curing agent (10:1 dilution) and the mixture was degassed for 20-30min. PDMS was then poured on top of the wafer and it was placed inside the oven (60˚C- 70˚C).

Channel Preparation

In order to determine where to look under the microscope, PDMS was cut into small squares and inside each square, a small hole was created. These channels were then placed on

top of clean glass slides. The glass slides were cleaned with 70% ethanol and then DI water, and air dried before placing PDMS chambers on top of them.

Preparation of Buffers

Before isolating mitochondria from Hela cells, incomplete H buffer, 5% BSA solution, and respiration buffer were prepared. Incomplete H buffer was a mixture of 210mM mannitol, 70mM sucrose, 1mM EGTA, and 5mM HEPES. PH of the solution was adjusted to 7.2 by adding enough KOH. The incomplete H bufferwas mixed with 5% BSA solution to make a complete H buffer (10:1 dilution).

The respiration buffer was made of 225mM mannitol, 75mM sucrose, 10mM KCl, 10mM Tris-HCl, and 5mM KH2PO4. Afterwards, the PH of the solution was adjusted to 7.2 by adding enough KOH.

Isolation of Mitochondria

Hela cells were seeded on a tissue flask 3 days before isolation. On the day of isolation, cells were washed with PBS and then trypsinized with 3ml of trypsin. 4ml of growth media was added to the mixture of cells/trypsin and was centrifuged at 3000 rpm (850xg) for two minutes. The pellet was resuspended in growth media and was centrifuged again for 2 min and the supernatant was discarded. This process was repeated, and the pellet was rinsed with ethanol/DI water and the homogenizer was pre-chilled on ice. From this point on, every step was done on ice. The pellet was resuspended in 1ml H-buffer. The resuspension was transferred to Dounce Tissue Grinder. The cells were homogenized on ice with 15-20 passes. The cells were

resuspended in 2ml H-buffer. The mixture was centrifuged at 800xg for 5 minutes. The supernatant was removed. The pellet was again resuspended in 2ml of H-buffer and was transferred back to Dounce Tissue Grinder. The cells were disrupted with 10 passes, were resuspended with 2ml of H-buffer, and were centrifuged for 5 minutes at 4˚C at the speed of 800xg twice. The supernatant was then transferred to a new tube and was centrifuged again at 10,000xg (10,300rpm) for 20 minutes at 4˚C. 1ml of incomplete buffer was added to the pellet and the mixture was centrifuged for 15 minutes at 4˚C at 10,000xg (10,300rpm). Finally, mitochondria were resuspended in 2ml of respiration buffer. The concentration of mitochondria was calculated to be around 100µg/ml.

JC-1 Preparation

JC-1 kit from Sigma Aldrich was used for staining. The content of JC-1 vial was dissolved in 25µl of DMSO and was mixed vigorously. Then, JC-1 was diluted 5-folds with DMSO to obtain a concentration of 0.2 µg/ml. JC-1 solution was kept on ice before addition of mitochondria.

TMRM Preparation

TMRM kit was purchased from Sigma-Aldrich. The content of stock vial was mixed with 100µl of DMSO. The stock solution was then diluted with 1X PBS, or cell media to achieve a 10µM concentration. Enough mitochondria in media was added to the TMRM dye to achieve a concentration of 20-200nM. Mitochondria were then incubated for 15-20 minutes at 37° C in dark.

Imaging of Mitochondria

20 µl of mitochondria was added to each of the six group (glass, graphene, PEG coated surface, PEI coated Surface, PLL coated surface, Lipid bilayer coated surface). They were incubated for 30 minutes. Then, two methods of washing were implemented. In the first method, after staining with JC-1 dye, the chambers were washed by pouring DI water on top of chamber. In the second method, the content of each chamber was pipetted out. Each chamber was washed gently by DI water using a pipette. Images were taken using a fluorescence microscope to assure that the mitochondria were still present.

CHAPTER 5: RESULTS

JC-1 Dye

The washing step was performed using a centrifuge tube and by pouring water on top of the chamber. The images with JC-1 were all taken with 60x objective (mitochondria could be seen both under red and green filter).

Glass

After 30 minutes, mitochondria could be seen on the surface of glass (Figure 5-1). However, after washing the chamber, no mitochondria were observed on the surface.

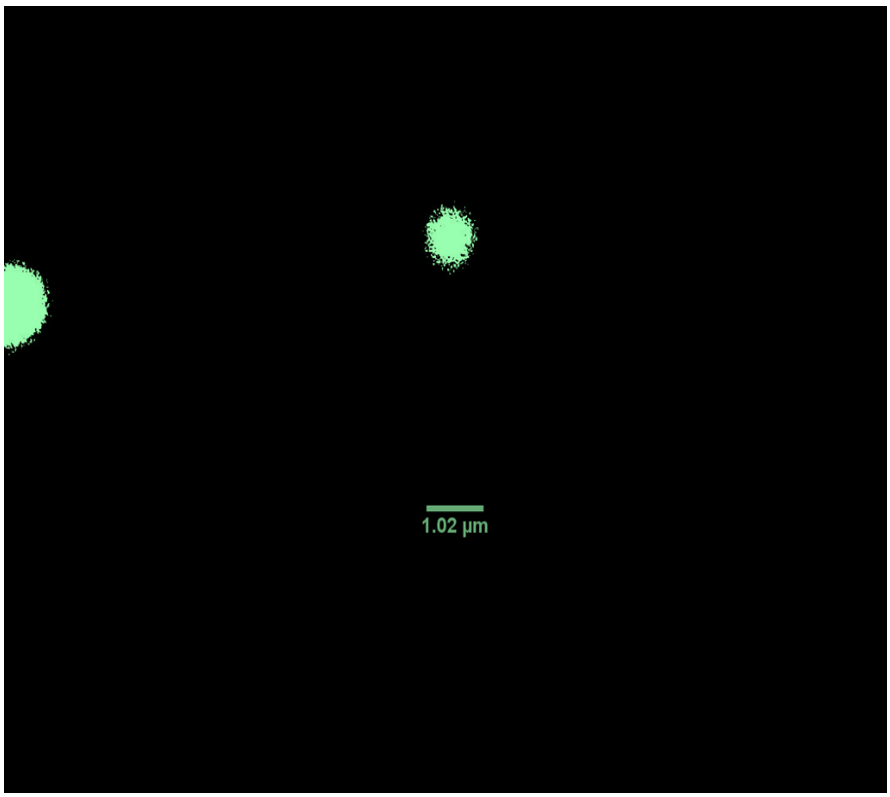

Figure 5-1. Mitochondria on Glass before washing

Graphene

On the surface of graphene, mitochondria could also be observed before washing (Figure 5-2). Once the chamber was washed with DI water no more mitochondria could be seen.

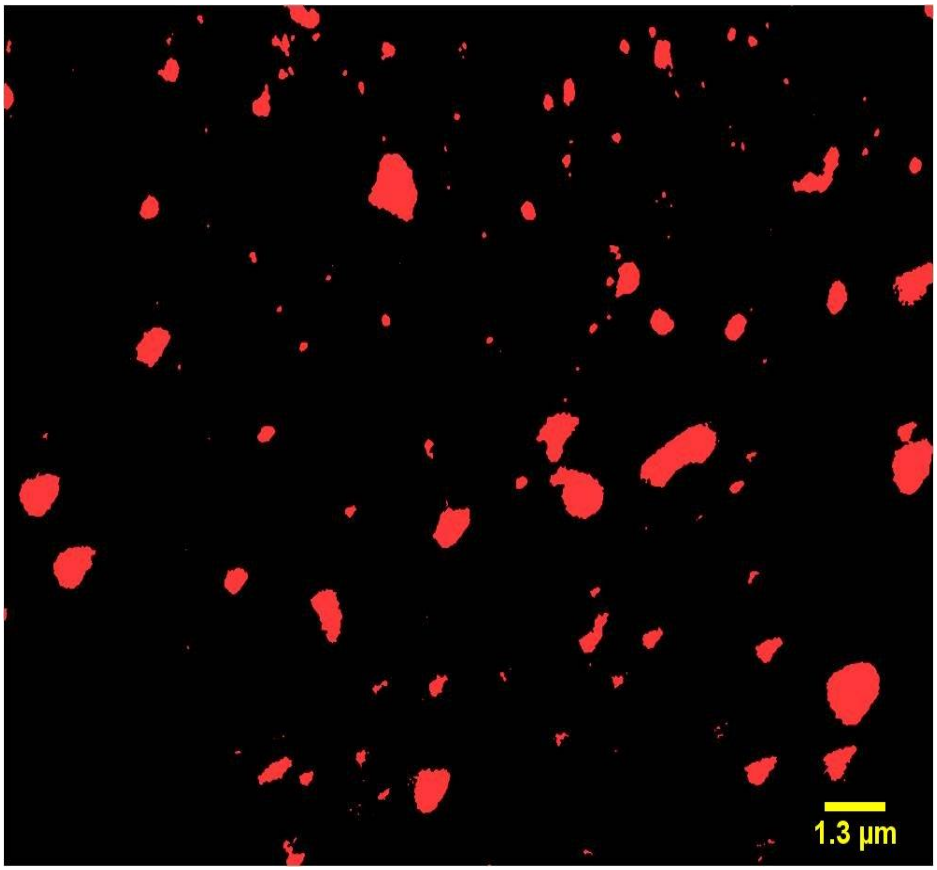

Figure 5-2. Mitochondria on graphene before washing

PLL

Mitochondria could also be detected inside the PLL chamber. In figure 5-3, some mitochondria look bigger in size than others. When the PLL chamber was washed, no mitochondria were visible.

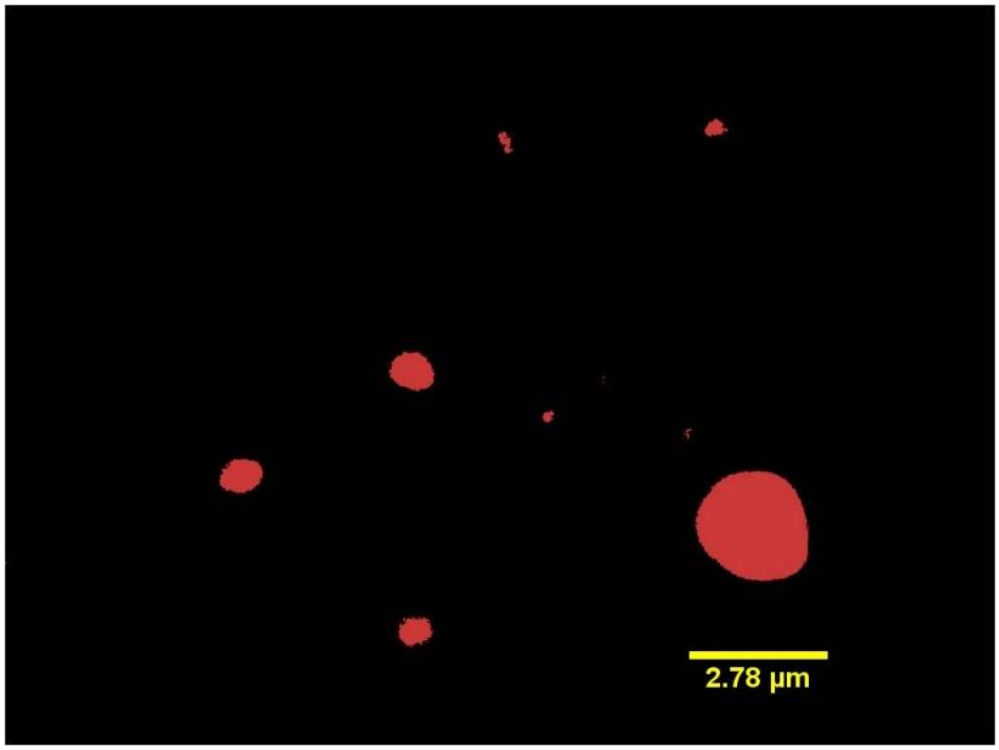

Figure 5-3. Mitochondria inside PLL coated graphene chamber

Lipid bilayer

The surface of graphene coated with lipid bilayer is depicted below (Figure 5-4).

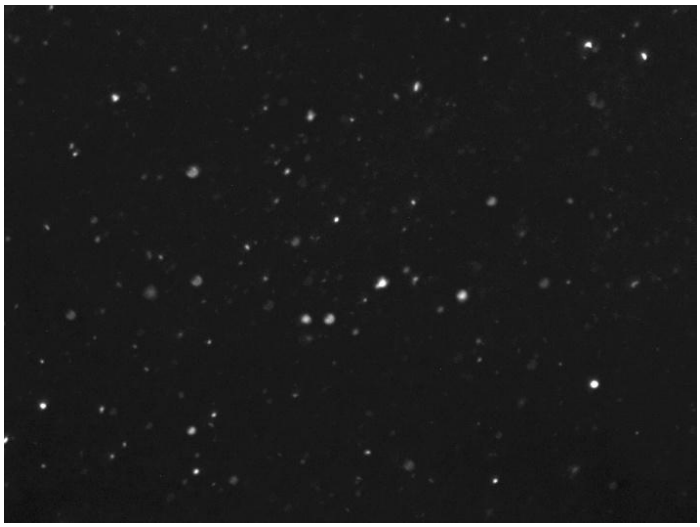

Figure 5-4. Lipid Bilayer on top of glass before addition of mitochondria

Figure 5-5 is an image of mitochondria before washing the chamber. After the wash, no mitochondria were present.

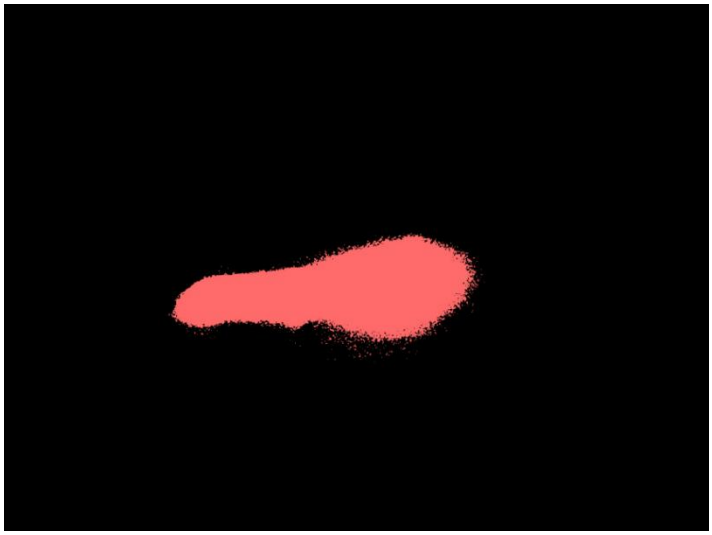

Figure 5-5. Mitochondria on Lipid bilayer coated graphene

PEG

Before washing the PEG chamber, there were some mitochondria present (Figure 5-6). On the other hand, after washing the chamber, mitochondria were absent.

Figure 5-6. Mitochondria inside PEG coated graphene chamber before washing

PEI

On PEI surface, in some of the experiments, mitochondria were detected both before and after DI washes (Figure 5-7).

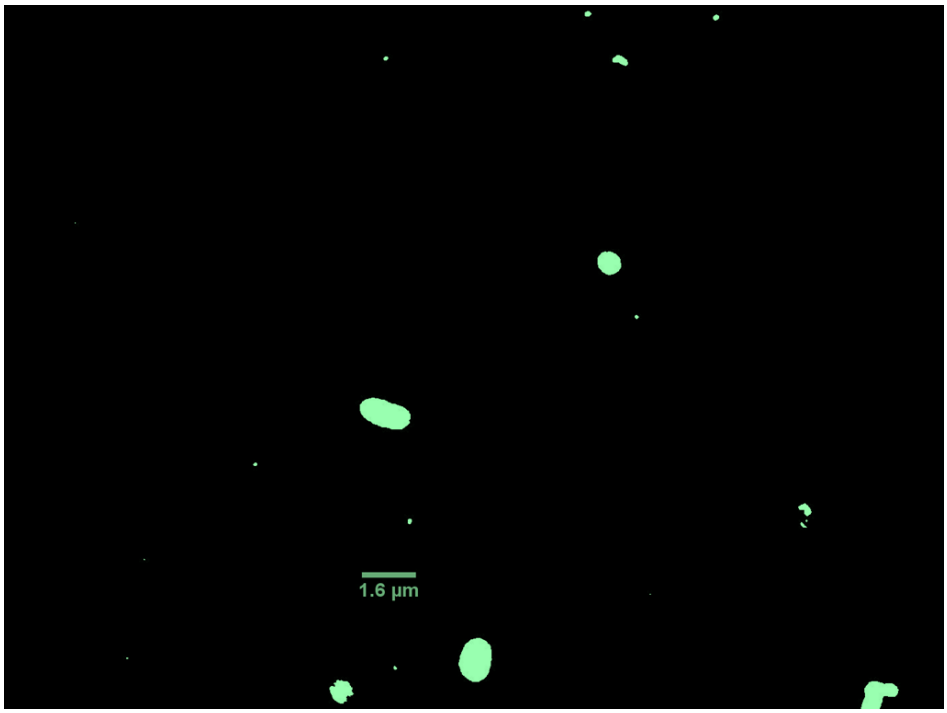

Figure 5-7. Mitochondria on PEI coated graphene

TMRM Dye

The images with TMRM dye were all taken with 40x objective. The washing was done with a pipette. This experiment was only performed once with the TMRM dye and with this method of washing.

Glass

Mitochondria could be seen inside the glass chamber before washing, but not afterwards (Figure 5-8).

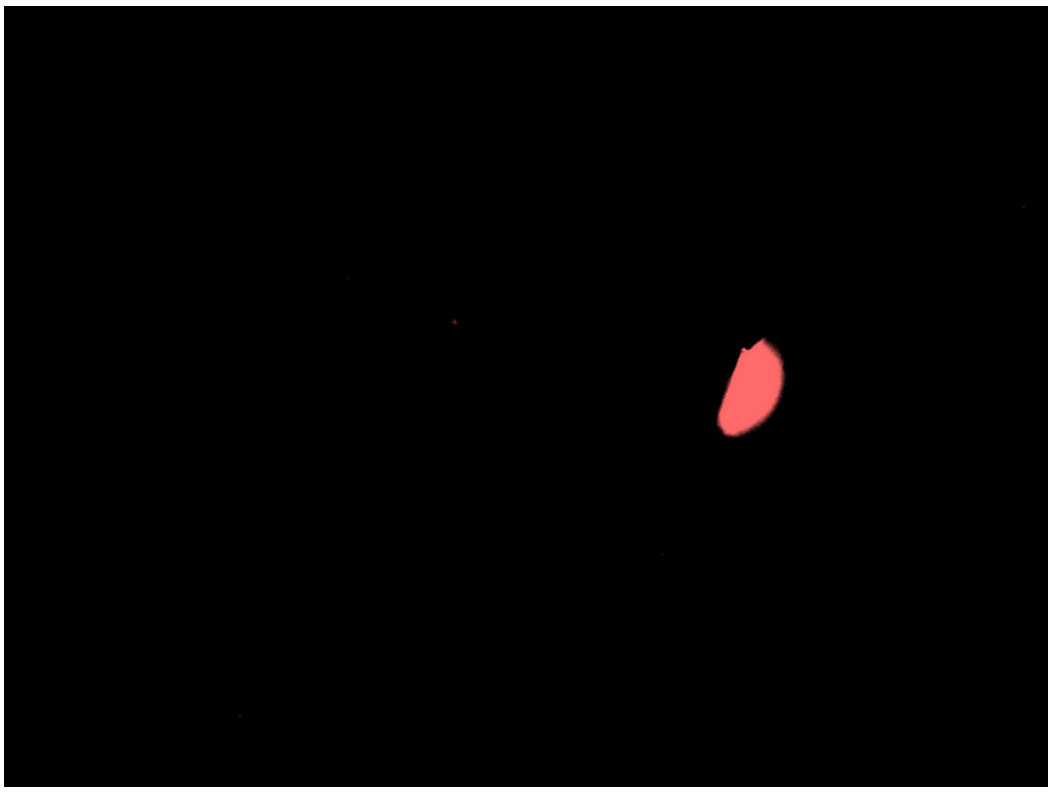

Figure 5-8. Mitochondria inside the glass chamber before washing

Graphene

Inside the graphene chamber, mitochondria were visible before washing (Figure 5-9). However, after washing, it seemed like there were no more mitochondria inside the graphene chamber.

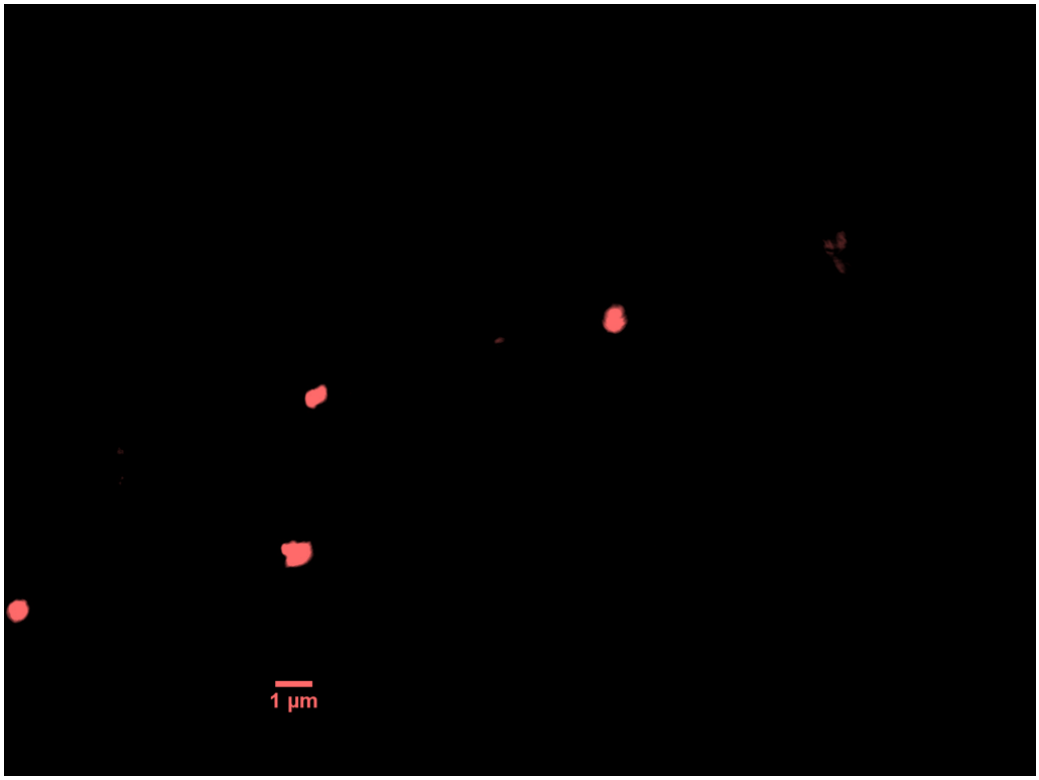

Figure 5-9. Mitochondria inside the graphene chamber before washing

PLL

Mitochondria were inside the PLL coated graphene chamber before and after washing (Figure 5-10).

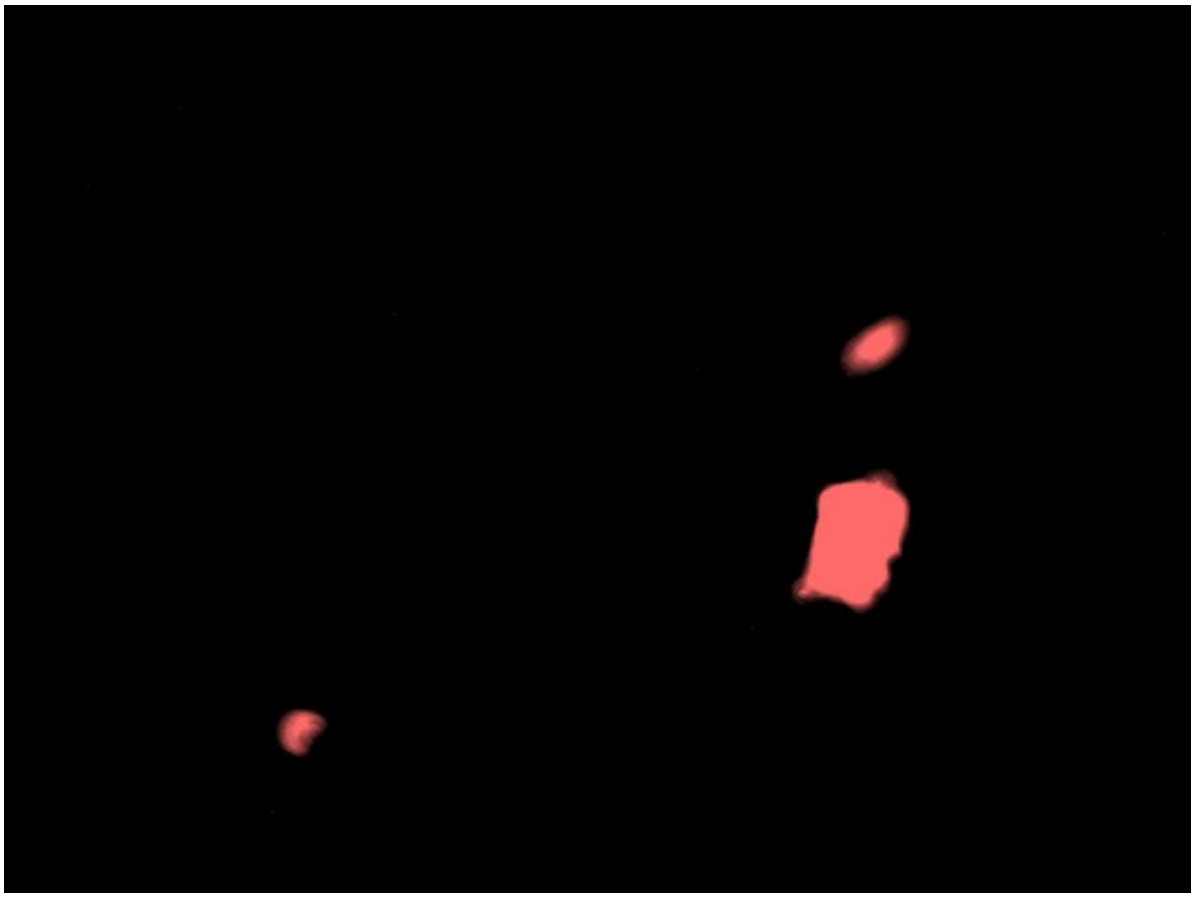

Figure 5-10. Mitochondria inside PLL coated chamber

Lipid Bilayer

Inside the chamber with coated lipid bilayer, mitochondria were visible both before and after the washing step (Figure 5-11).

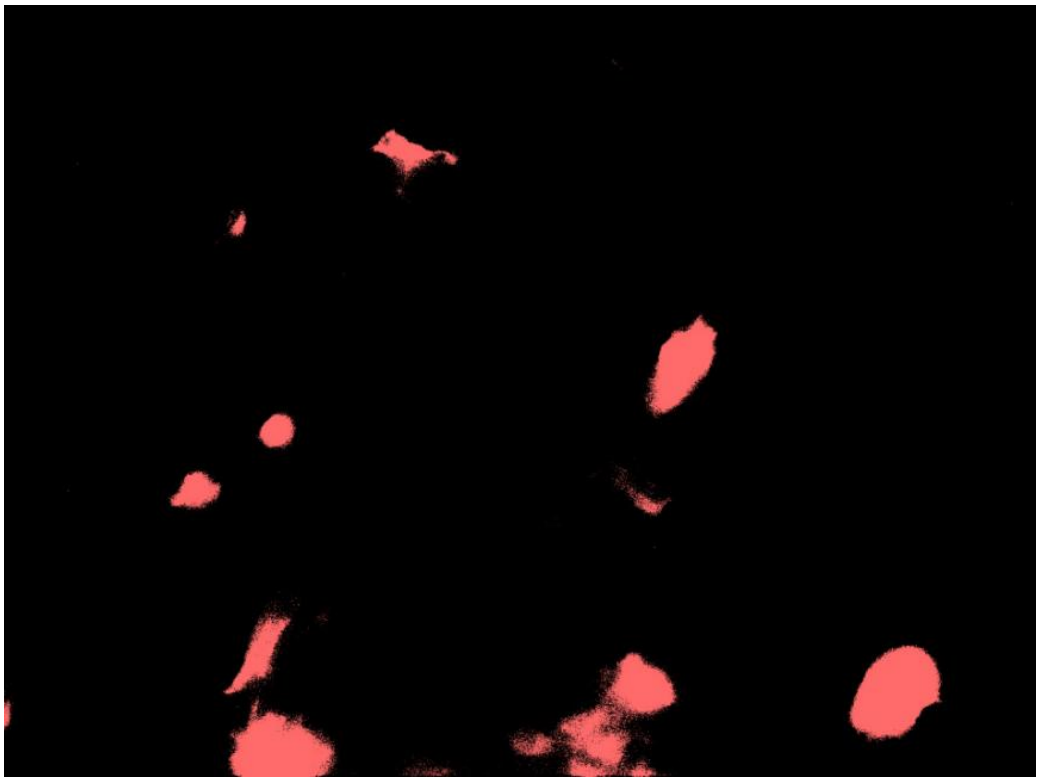

Figure 5-11. Mitochondria inside lipid bilayer coated chamber before and after washing

PEG

Mitochondria were absent after washing the chamber. However, mitochondria were observed on the surface of graphene coated with PEG before washing the chamber (Figure 5-12).

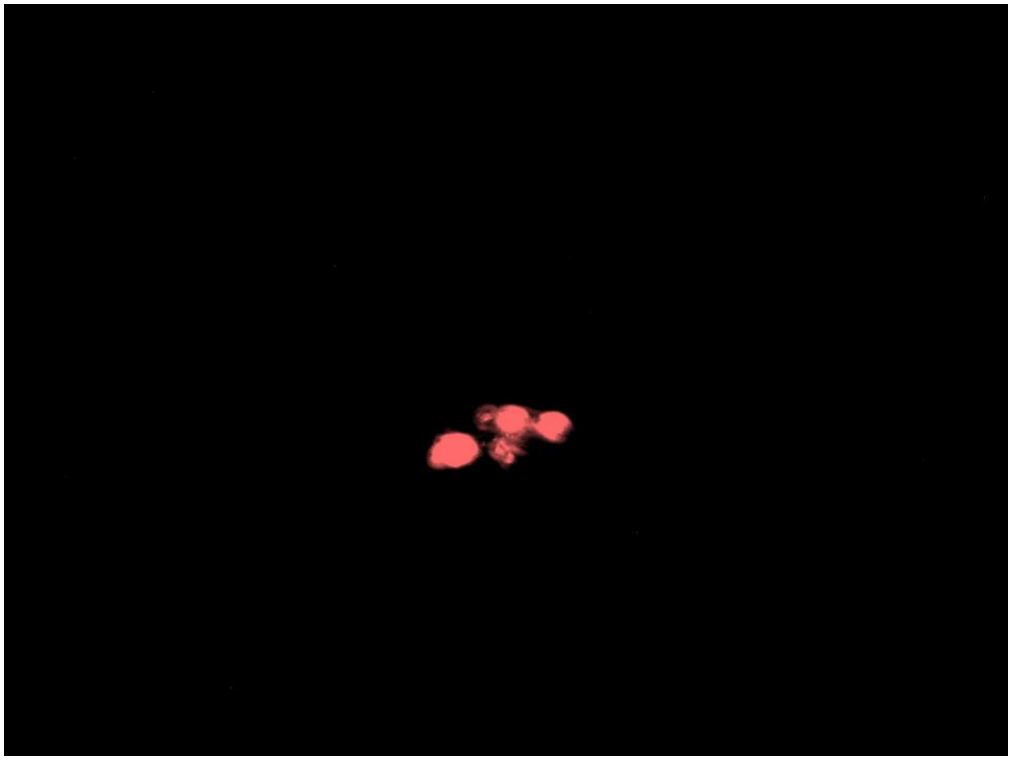

Figure 5-12. Mitochondria on PEG coated graphene surface before washing

PEI

Finally, the images of mitochondria inside the chamber coated with PEI indicated that there were mitochondria before and after washing with DI water (Figure 5-13).

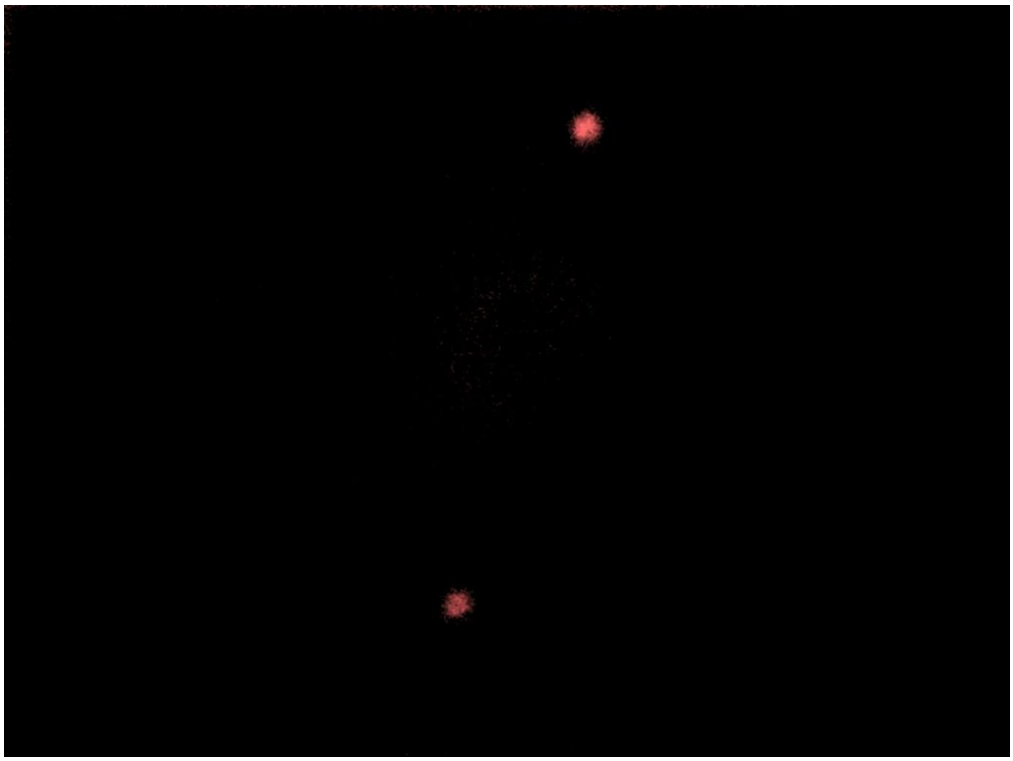

Figure 5-13. Mitochondria inside PEI coated graphene surface

The summary of the results for the JC-1 dye are presented in Table 1.

Table 5-1 Results of immobilization of mitochondria on graphene with Jc-1 dye

Groups	Glass	Graphene	PEI	PLL	Lipid bilayer	PEG
Adhesion of mitochondria	NO	NO	YES	NO	NO	NO

The summary of the results for the TMRM dye are presented in Table 2.

Table 5-2 Results of immobilization of mitochondria on graphene with TMRM dye

Groups	Glass	Graphene	PEI	PLL	Lipid bilayer	PEG on Graphene
Adhesion of mitochondria	NO	NO	YES	YES	YES	NO

CHAPTER 6: DISCUSSION AND CONCLUSION

Discussion

In the current experiment, six groups were defined to study the immobilization of mitochondria on graphene. Mitochondria were expected not to adhere to the surface of three of the groups including glass, graphene, and PEG coated graphene. As discussed before, studies have shown that the surface of mitochondria is more hydrophobic than hydrophilic and it is more negatively charged (Whiting, C.E., and A. Edgar., 2006). Since PEG has a hydrophilic surface and is not positively charged, mitochondria would not be able to attach to its surface. The surface structure of graphene is very inert for mitochondria to attach to it; graphene is not charged and contains carbon bonds. As studies have suggested, the surface of glass needs to be functionalized in order for mitochondria to adhere to it (REERS, M., R. A. KELLY and T. W. SMITH, 1989).

Mitochondria are assumed to bind to PEI, PLL, and lipid bilayer coated graphene surfaces. PEI and PLL are both positively charged polymers that have been used in numerous biological studies. Both PEI and PLL have great affinities for cells. Thus, if the surfaces of these two polymers attract cells, they should be able to attract mitochondria as well - since both cells and mitochondria have similar membrane structures. By coating the surface of graphene with lipid bilayers, it is speculated that mitochondria would attach to graphene due to the similarity of the surfaces (the surface of mitochondria is also composed of phospholipids).

In the experiment done with JC-1 dye, mitochondria were seen to be only immobilized on the PEI surface. However, with TMRM dye, the experiment was only performed once and mitochondria seemed to be bound to PEI, PLL, and lipid bilayer coated graphene surfaces.

Therefore, JC-1 dye might have influenced the results. JC-1 dye has some disadvantages over other dyes as mentioned before. Some of the drawbacks of JC-1 dye include its sensitivity to the concentration of loading and the time of loading. In addition, JC-1 dye has been known for not being specific in staining of mitochondria. In the studies of immobilization of mitochondria done by other groups, JC-1 dye has never been used for staining.

One might argue that in some images the size of mitochondria were larger than what it should be. One possibility is that the solution of mitochondria was not mixed enough and consequently, clumps of mitochondria were observed under the microscope.

Repeating the experiment with the TMRM dye, which has been demonstrated to be a more effective dye, the results were different. With TMRM dye, the results were as expected; mitochondria were immobilized on PEI, PLL, and lipid bilayer coated surfaces. As others have reported, mitochondria were attached to the surfaces of PEI and PLL coated glasses (Kuzmenko,A., S. Tankov, B. P. English, et al., 2011). However, there were some distinctions between our method and others, specifically in the washing step. Some groups have washed the mitochondria with KCl and some others with buffer B (Quarato, G., et al., 2011). Furthermore, some have placed the mitochondria inside a perfusion chamber, which might be a better environment for mitochondria (Vergun, O., T. V. Votyakova, and I. J. Reynolds, 2003). Besides, not all groups have mentioned the specifics of their washing method. In another report, buffer B was gently withdrawn to remove any unattached mitochondria (Quarato, G., et al., 2011). In our experiment, with the TMRM dye, gentle washing was done using a pipette. With JC-1 dye, the method of washing was a harsher method. Thus, the method of washing

could have had an effect on immobilization of mitochondria. To improve the results of this study, more experiments should be done with the TMRM dye. Besides, the unbound mitochondria should be washed away with KCl instead of DI water to compare the results.

Conclusion

In summary, the washing technique as well as the staining method seemed to have significant influences on immobilization of mitochondria on graphene. With JC-1 dye and the harsh washing method, the anticipated results were not achieved. However, with the TMRM dye and gentle washing, the results were as expected. More experiments should be done with the TMRM dye to find out the validity of these results.

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