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Review and Research outline for Bio-compatibility of epitaxial graphene: Next generation biological Application

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Abstract - *The objective of this proposed research is to investigate epitaxial graphene (EG) for bio-compatibility point of view, both for bio-medical equipment application as well as implantable use for biological purpose. This will overcome current barrier for biological application, such as ionic contamination effect as well replacement after a certain years. As an example, implantable pacemaker for human heart is currently made of Ti which has to replace in every 7~10 years, because of metallic contamination effect. Successful implementation of this research will enable us to a new material of pacemaker which needs not to replace ever. This proposed research will also lead to investigate blood compatibility of EG so that there would be no future effect of implantable EG based device in long life. Our investigation will focus on fibrinogen adsorption as well as cell adhesion factor analysis of EG. One possibility likes to arise of graphene oxide (GO) formation on EG based implantable device due to blood contact. So, GO effect on human cells would be investigated as well as effect for anodic oxidize EG. Comprehensive experiments would be performed to investigation GO formation on EG due to blood contact so that this material effect on human body would be documented. To attain the overall objective the following subtasks will be performed…*

(i) Very high quality epitaxial graphene growth on SiC using home built RF reactor furnace
(ii) Investigation the effect of adsorption and elutability of fibrinogen on EG
(iii) Synthesis of graphene oxide (GO) and it's effect on human cells
(iv) EG blood compatibility and investigation of GO formation while EG is in contact with blood.

Transformative form of this research would led us the pathway for next generation biological equipment development which would be more safe and longevity with the human life cycle. This will also have an impact on biomedical application with more security of human life: next generation biomedical application.

***Key Words*: Graphene, SiC, CVD, Bio-compatibility, Pacemaker**

1.INTRODUCTION

Biocompatibility refers to some specific properties of a material, suitable for particular cell type or tissue and immune response in a given organism etc. These properties determine whether the material is suitable for medical equipment application and/or medical treatment as toxics. A variety of metals are now used in prosthetic implants, such as pacemaker casings, orthopedic and reconstructive devices, dental application and joint prostheses [1,2]. Among metallic implant materials, both titanium and 316 stainless steel have been shown to create minimal inflammatory response and tissue disturbance [2,3]. However, most metal biomaterials including titanium and stainless steel will corrode permeating host tissues with metallic ions [4]. Thus there are new efforts to develop more stable forms of metallic implantable material have been an issue of research for a longtime.

In recent years, a lot of engineered nanomaterial fabricated endlessly and investigated for their application [5-10], and nanomaterial's biosafety has caused more and more attention from government and scientific communities [11,12]. Due to the combination of superior electrical properties including hardness [13], fracture toughness [14], low friction coefficient [15], high chemical resistance [16], chemically vapor deposited (CVD) diamond has been investigated as a bio-material with the comparison study with titanium and stainless steel [17]. Carbon nanotube, as the special carbon nanomaterials, have been investigated for their effects on the cells, animals and environment and evaluated for their bio-safety because of its inert behavior with the biological molecule. [18-20].

So, new material based biocompatible equipments have been a great need for a long time. Carbon is an inert material and unlikely to form bonds with other material quickly, moreover 2D form of carbon is very inert, leads us to investigate as a replacement for existing bio-material. Carbon atoms formed in a 2D form honeycomb structure called graphene, has the potential ability to be a new bio-material for next generation bio-medical application with more safety and efficient performance with the body.

1.1 Theory based justification of Graphene as a Bio-compatible material:

There are two basic required approaches to justify a biocompatible material. They are follows…

1. Justify the material for medical equipment compatibility point of view.
2. Justify the material for medical toxicity point of view.

Graphene, a two-dimensional (2D) honeycomb lattice and is a basic building block for graphitic materials of all other dimensionalities with unique physical, chemical and mechanical properties [21,22], has been used initially in sensor related applications where there is no direct interaction with the biological media. It has been suggested that the single-stranded DNA was promptly adsorbed onto graphene forming strong molecular interactions, which improved the specificity of its response to complementary DNA. It also found application in the fabrication of third-generation electrochemical biosensors, where the HRP/ss-DNA/GP/ GC electrode performed good electro-catalytic reduction for H_2O_2 with good sensitivity and stability with wide linear range [23].

It has been demonstrated that a biocompatible scaffold developed using graphene accelerates specific differentiation of human mesenchymal stem cells into bone cells without hampering the proliferation. The differentiation rate was comparable to the one achieved with common growth factors, demonstrating graphene's potential for stem cell research. Biocompatible nanographene oxides with various physical sizes has been reported which imparted aqueous stability to the NGO in buffer solutions and other biological media by covalently grafting polyethylene glycol starpolymers onto the chemically activated surfaces and edges . It has been shown that addition of graphene significantly increased the modulus of chitosan even at a very low content and the composite showed good bio-compatibility for L929 cells[23]. It has been demonstrated that macroscopic antibacterial graphene-based paper can be conveniently fabricated with superior inhibition ability to bacteria growth [23]. This could be useful in some environmentally friendly applications. However, in very recent study graphene oxides has been shown to have dose-dependent toxicity to cells and mice, such as inducing cell apoptosis and lung granuloma formation which could not be cleaned by the kidney [24]. So detail investigation of epitaxial graphene biocompatibility both for biological equipment and toxic application has become a major issue for last few years due to its exciting EG/SiC interface interaction.

Table-1 indicates a comparison of some interesting properties of EG with other existing bio-compatible material. Graphene layers have become a hot spot so far and have been actively investigated to build new composite materials. Here, we will argue for epitaxial graphene (EG) from bio-compatibility point of view with a comparison of existing bio-compatible material such as titanium, 316 stainless steel and CVD diamond and describe toxicity when graphene is in contact. We will also present initial experimental results in support of our arguments and finally we would like to conclude with the viability of graphene oxide effect on human cell.

Properties	Graphene	CVD diamond	Titanium	316 Stainless steel
Hardness (kg mm^{-2})	1800	10000	230	210
Young's modulus (Gpa)	1000	1000	120.2	215.6
Bulk Modulus (Gpa)		442	108.6	166
Thermal conductivity ($Wm^{-1}K^{-1}$)	5040	20	0.21	0.16
Thermal expansion ($X10\text{-}6k^{-1}$)	-7	1.1	8.8	17.2

Table-1: Comparative material properties of epitaxial graphene with other bio-compatible material.

1.2 Future prospect of this research:

The successful completion of this project will make advancement in biomedical/biomedicine application. Current challenges we face in this area, such as thorough and profound understanding of graphene-cell (or tissue, organ) interactions, especially the cellular uptake mechanism will be clear and have a path for next generation medical equipment material. Such knowledge certainly facilitates development of more efficient graphene based nano-platform for medical equipment, drug delivery and other applications; which is still lacking at the moment. This study will also direct us for a new class of material for heart pacemaker with more efficient and safe than those existing today.

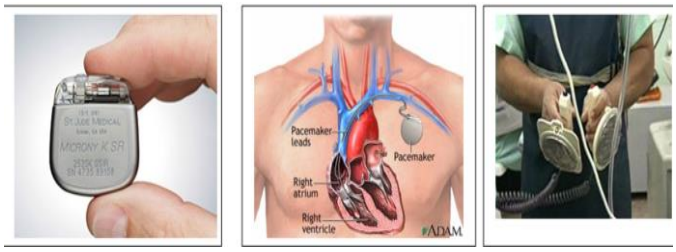

Fig-1: a) Pacemaker chip b) Pacemaker chip implanted on human heart. c) Defibrillator in bio-medical application.[source:online]

Fig-1 (a) shows an commercial pacemaker chip which is used to implant on human heart (fig-1 (b)). At this moment this chip needs to replace in every 7~10 years period, indicates an user unfriendly behavior. Successful investigation of this project with proper implementation would led to a new material built pacemaker which does not need to replace over human lifetime.A defibrillator is medical equipment, mainly used when patient is in critical moment and unable to fluent heart function. A defibrillator is effective at treating ventricular fibrillation or ventricular tachycardia (when the heart's electrical signals are too rapid and circulation is impaired). In these situations, a defibrillator can deliver a shock of about 300 joules to the heart in a very short amount of time (roughly 4 to 12 milliseconds), briefly stopping the irregular electrical impulses so that the heart's natural pacemaker can -- hopefully -- restart its regular rhythm. It is basically a two metal paddles (electrodes) with insulated handles or adhesive pads are attached to the defibrillator by insulated electrical wires called leads. The electrodes are placed on the skin of the patient's chest so that the heart is positioned roughly between them

2.Preliminary Results:

A. Graphene growth on SiC:

Epitaxial growth of large-area graphene by thermal decomposition of commercial <0001> 4H and 6H SiC substrates at high temperature and vacuum has been demonstrated [25]. This produces EG a few ML to >50 ML thick, depending on growth conditions. In Clean Energy Lab (CEL), EG was grown on commercial N+ 4H-SiC substrates, nitrogen doped ~10^{19}/cm^3. 1cmx1cm samples were degreased using Trichloroethylene (TCE), acetone and methanol respectively. They were then rinsed in DI water for three minutes. The samples were finally dipped in HF for two minutes to remove native oxide and rinsed with DI water before being blown dry. They were then set in the crucible in an inductively heated furnace where high vacuum was maintained (<10^{-6} Torr) and baked out at 1000^{0}C for 13 to 15 hours. The temperature was slowly raised to the growth temperature (1250-1400°C). All growths were performed for 60 minutes before cooling to 1000^{0}C at a ramp rate of 7~8^{0}C/min and eventually to room temperature. Slow temperature ramps were utilized to minimize thermal stress on the samples.

After growth AFM (atomic force microscopy) and Raman measurements were carried out on EG on both carbon (C) and silicon (Si) faces. Micro-Raman spectroscopy using a 632nm laser shows the G peak (~1590cm^{-1}), D peak (~1350cm^{-1}) and 2D peak (~2700cm^{-1}) characteristic of EG [26]. The ratio of intensities of the D-peak to G-peak, I_D/I_G ≤0.2 demonstrates the quality of our graphene [26]. X-ray photoelectron spectroscopy (XPS) measurements were conducted using a Kratos AXIS Ultra DLD XPS system equipped with a monochromatic Al K source will be used to find the thickness of the EG layers

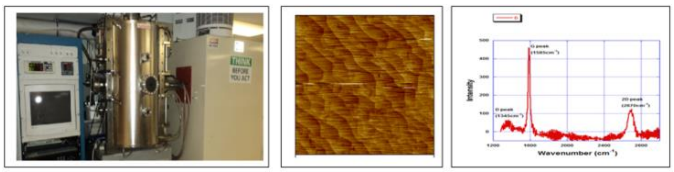

Fig-2: a) RF reactor furnace for EG growth b) AFM of EG c) Raman spectra of EG.

B. Bio-film deposition on EG:

On-axis, semi-insulating, 6H-SiC substrate was used to form epitaxial graphene on the silicon face at ~1400°C in vacuum. A slide containing a 1cm x 1cm piece of epitaxial graphene with two copper contacts sealed. Approximately 0.5cm x 0.5cm of uncovered EG was placed in the aqueous media. A baseline resistance of 1.6kΩ was measured across the graphene prior to inoculation. The system was then inoculated with ~3mL of E-coli and resistance measured throughout the growth of the bio-film. Growth of the E-coli continued for nine days total, with increases in resistance comparable to the reproduction of the bacteria in the media. Resistance of the graphene increased as high as 4.76kΩ, a difference of 3.16kΩ, before leveling off with saturation of bacteria growth. Cell death in the media due to overcrowding and starvation of some of the bacteria could explain the decrease in resistance seen after saturation Upon removal from the media the slides were preserved in 6% formaldehyde solution for further study. We predict that we will see different rates of change of resistance with different types of bacteria and surface charges, leading to the possibility of a bio-detector capable of differentiating pathogen type simply by changes observed with the graphene.

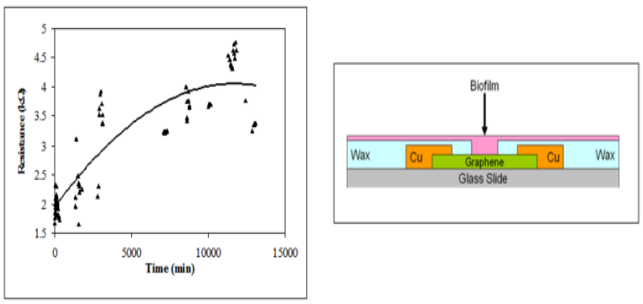

Fig-3: a) Resistivity increment of EG in bacteria. b) Cell for resistivity measurement test.

From this initial result, it is evident that EG would show the behavior of absorbed fibrinogen. EG properties are similar to CVD diamond and/or better than CVD diamond,

fibrinogen adsorption would be similar to CVD diamond but detail experiment required to establish this behavior.

3 Future research outline:

A. Research tasks:

A bio-compatible material must not alter chemical/electrical properties when it is in biological contact, such as oxide formation. For biological equipment application it must meet the following criteria
It should absorbed and denatured fibrinogen as less as possible. Fibrinogen is a soluble plasma glycoprotein, synthesized by the liver that is converted by thrombin into fibrin during blood coagulation.
There should be less cellular adhesion and activation on the surface of the material. This will keep the cells properties unchanged in response to the material.
Effect of native oxide (eg. Graphene oxide) behavior on human cell will be investigated.
Native oxide formed by that material must be investigated from biocompatible point of view.

Research methodologies:

B.1: Experimental Details for effect of fibrinogen and PMN on EG :

B.1.1: Sample preparation:

Commercial metal foils, epitaxial graphene and CVD diamond will be cut into 1X1 cm square for testing. The samples will be ultrasonicated twice in 70% ethanol for 10 minutes to remove dust and contamination from the surface. Prior to the experiments, samples will be rinsed with phosphate-buffered saline. Phosphate buffered saline (PBS) is a buffer solution commonly used in biological research. It is a water-based salt solution containing sodium chloride, sodium phosphate, and, in some formulations, potassium chloride and potassium phosphate. The buffer's phosphate groups help to maintain a constant pH. Plasma coated sample could be produced by incubating hydrated and sterilized foils, diamond and EG with fresh human heparinized plasma at room temperature in a rotary shaker (100rev/min) for 4 hour under sterile conditions.

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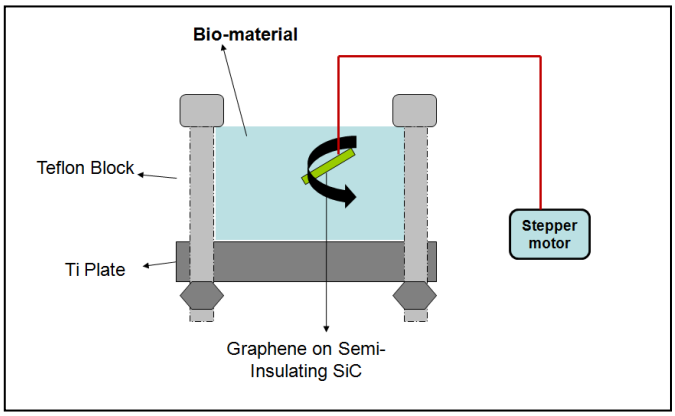

Fig-4: Schematic cell design for bio-compatibility test of EG.

B.1.2: Fibrinogen adsorption and elution:

In order to compare the steady-state concentration of surface-bound fibrinogen on different samples, adsorption and elution experiments need to be carried out. For this, samples will be incubated with human fibrinogen (final concentration 20ug/mL) for 8 hours at room temperature, rotary shake ~50rev/min. The sample will then thoroughly rinse with PBS and half will be incubated with 1% SDS for 1 hour (also shake at 100 rev/min). The SDS solution will be

used to remove elutable (non-denatured) fibrinogen loosely bound to the surface of the samples. For tetracer measurements, the amount of radioactivity on all of discs will be measured by scintillation counting.

B.1.3: Polymorphonuclear Leucocyte(PMN)-material interaction:

Purified human PMNs (Polymorphonuclear Leucocytes) will be prepared from fresh heparinized venous human blood (5 U/ml) drawn from healthy adult donars. Briefly, 40ml of heparin-anticoagulated blood will be mixed with 20ml of 6% (w/v) hydroxyethyl starch solution and the blood will be allowed to sediment for 45~55 min. The red cell-free supernatant will be centrifuged at 400g for 7~10 min to sediment the PMNs. Small numbers of contaminant red cells could be lased by addition of ice-cold distilled water for 30 s; isotonicity will then be restored by addition of an appropriate amount of 3.6% NaCl.. The cell suspension will be transferred to the top of 8 ml of Percoll (density = 1.075) and centrifuged at 200g for 30 min at 4°C. Temperature and humidity controller (incubator) will be used in this purpose. The lowest band, which contains most of the PMNs, will be aspirated into a sterile tube and the cells will be washed a few times with Ca^{2+}/Mg^{2+} free HBSS. These purified neutrophils will then be counted (by haemocytometer) and resuspended to a concentration of 2 x le6 cells/ml in HBSS containing 1.0 **mM** CaCl, and 0.5 mM MgCl[27].

Since protein adsorption is much more rapid than the transport of cells to foreign surfaces, host cells would probably interact only with the protein coating rather than with the material itself. Therefore, all samples needs to be pre-coated with human plasma for 4 h before interacting with PMNs. The purified human PMNs will be incubated with constant gentle rotary mixing (~50 rev min) with the plasma-coated samples for ~1 h and then wash a few times with PBS. Next, the materials with adherent cells will be fixed in Diff-Quick stain solution (could be bought from Baxter Healthcare Corporation, Miami, FL). The number of adsorbed PMNs on each type of material will be determined using an optical microscope and associated image processing system[23].

B.2: Analysis procedure:

B.2.1: Adsorption and elution of fibrinogen:

It is generally accepted that the initial event which indicates biocompatibility upon implantation of a material adsorption of plasma proteins from blood onto the surface **[**27]. Protein adsorption is much more rapid than the transport of cells to foreign surface. Therefore host cell probably interact with host proteins adsorbed on the material surface rather than with the foreign material itself. Thus the type and state of adsorbed proteins are critical determinants of biocompatibility [27]. The adsorption and conformational state of fibrinogen, which is major surface protein to initiate coagulation [28] and inflammation [29] can be used as an indicator of biocompatibility of many biomaterial.

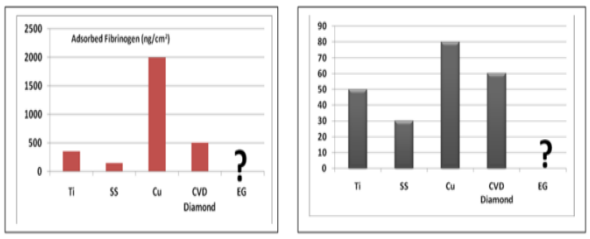

Figure-5 shows adsorbed fibrinogen (5-a) and non-elutable fibrinogen (5-b) of Ti,SS,Cu,CVD diamond. Data presented from the experiment presented in the reference [17]. It is evident that SS shows lowest adsorbed amount of fibrinogen compared to Ti, CVD Diamond and Cu.

Since surface fibrinogen will promote both coagulation reaction and inflammation, material biocompatibility must be importantly influenced by the extent of adsorption and spontaneous 'denaturation' of this protein. From this standpoint 316 stainless steel is a better choice compared to Ti, Cu and CVD diamond. But for epitaxial graphene (EG) comparative experiment required to establish this point. As CVD diamond and EG has the similar Young's modulus (~1000 Gpa) and similarity in hardness (shown in Table-1), we expect more or like the similar behavior of fibrinogen adsorption on these two material.

B.2.2:Attachments of polymorphonuclear leucocytes (PMNs):

Cellular interactions occurring at the tissue-material interface also appear to be an important determinant of the fate of biomaterials [**30**]. Polymorphonuclear leucocytes (PMNs) are the most abundant white cells in human blood, will directly adhere to the surface of implanted biomaterials [**30**]. It is thought that consequent inflammatory responses may lead to the degradation of certain implantable material such as polytherurethane. Therefore the numbers of surface adherent PMNs could reflect biocompatibility.

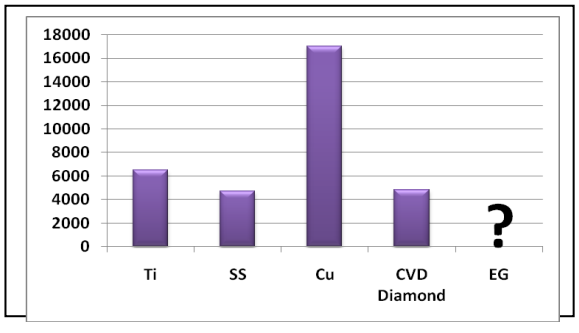

Fig:6: Number of adherent polymorphonuclear leucocytes (PMNs) while plasma-precubated.

From the above discussion it is evident that CVD diamond is comparable as a biocompatible stand point of view. However comparing the properties (Table-1), it is clear that EG is comparable to CVD diamond. So it is expected to have similar behavior of fibrinogen adsorption and PMNs attachment of EG as CVD diamond.

B.3: Experimental Details for effect of native oxide of EG (graphene oxide GO) on biological cells:

B.3.1: Synthesis of GO

Graphene oxide (GO) can be prepared from natural graphite powder by the modified Hummers method [**31**]. Graphite (2 g 500 mesh) and sodium nitrate (1 g) will be added to a 250 mL flask at 0°C. Concentrated H_2SO_4 (50 mL) will then be added slowly with stirring below 5°C. The mixture will be stirred for 30 min and 0.3 g of $KMnO_4$ with small portions below 10°C. After that the mixture will be warmed to 35 ± 3°C and stirring for 2 h, 90 mL of water will slowly dripped into the paste, causing an increase in temperature to 70°C and the diluted suspension will be stirred at this temperature for another 15~20 min. Then, it will further treated with a mixture of H_2O_2 (30%, 7 mL) and water (55 mL). The resulting suspension cake will be washed with a warm solution of 3% aqueous HCl (150 mL), followed by drying at 40°C for 24 h in vacuum. Finally, the GO will be obtained by ultrasonication of as-made graphite oxide in water for 1 h.

B.3.2: Effect of GO on human fibroblast cells:

In order to investigate the cytotoxicity of GO in vitro, we need human fibroblast cell (HDF) as the target cells to evaluate the cell viability. Every well in the plate will be planted 5,000 cells and incubate in a humidified 5% CO_2 balanced air incubator at 37°C for 24 h. Except from control wells, the contents in the remaining wells will add into medium with GO and the final concentrations were 5, 10, 20, 50, 100 μg/mL, respectively, next continued to culture from day 1 to day 5, we will measure the absorbency using the Thermo multiskan MK3 ELISA plate reader and calculated the survival rate of cells. The survival rate of cells can be calculated by the following equation[32]:

Survival rate of cells (%) = A_{570} (sample)/A_{570} (control) ×100

Where, A_{570}(sample) is absorbance intensity at 570 nm in the presence of GO, and A_{570}(control) is absorbance intensity at 570 nm in the absence of GO.

Human fibroblast cells (HDF) will be treated for 5 days with different concentrations of GO: 5, 10, 20, 50, and 100 μg/mL. In order to confirm whether GO can stimulate HDF cells secrete small molecular proteins, HDF cells will be cultured for 5 days in essential medium without 10% fetal calf serum with the aim of excluding mistaking fetal calf serum proteins as secreted small molecular proteins. HDF will be treated with 20 μg/mL GO and cultured in a humidified 5% CO2 balanced air incubator at 37°C for 24 h, then fixed cells with 2.5% glutaraldehyde solution and embedded with epoxy resin, finally made the ultrathin cell specimen and observed the specimen with TEM[32].

B.4: Analysis Procedure:

B.4.1: Effect of GO on human fibroblast (HDF) cells:

Regarding the effect of GO on HDF cells (shown in figure below et al ref [32]), GO below 20ug/mL exhibite low cytotoxicity, the cell survival rate is more than 80%, above 50ug/mL exhibited obvious cytotoxicity such as decreasing cell survival rate, inducing cell floating and cell apoptosis. As the cell culture day increased, the survival rate of cell decreased correspondingly, highly dependent on GO dose and culture time. As in figure below, GO was indeed internalized by cells and mainly located inside cytoplasm such as lysosomes, mitochondrion, and endoplasm. We also observed that, as the culture time increased, the amount of GO inside HDF cells increased accordingly, and a lot of GO appeared as black dots scattered in the cell cytoplasm around cell nuclear, a few GO located inside nucleus.

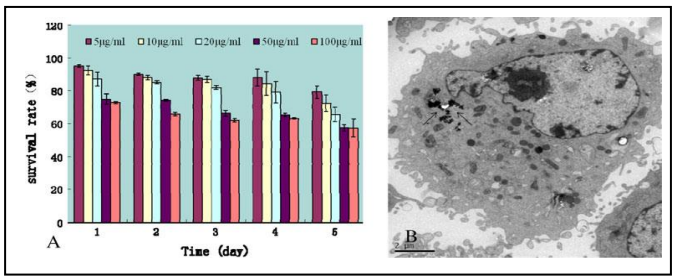

Fig-7: Effect of GO on human fibroblast cells. A) HDF survival rate at different concentration of GO on different culture time. B)TEM picture of location of GO inside HDF cells.

This process of analysis for GO effect on HDF cells would be followed on anodic oxidized EG samples. Anodic oxidation procedure of EG has been shown in ref [32]. As the possibility arises for oxide formation on EG sample, so anodic oxidized EG samples will be used to investigate the effect on HDF cells.

Effects of GO on Cell Adhesive Proteins:

The adhesive ability of GO-treated HDF cells will be evaluated with the ratio of GO-treated adhesive cell number to the control adhesive cell number after centrifuge. The cell adhesive ability supposed to decrease markedly with the increase in GO concentration and culture time. Western blot results showed that, comparing with normal cells, the expression levels of lamina, fibronectin, FAK, and cell cycle protein cyclin D3 in the HDF cells treated with GO were markedly decreased, and their expression levels in HDF cells cultured with GO decreased gradually as the amount of GO

increased, the b-actin protein expression remained unchanged in each case. There is a significant difference ($P < 0.05$) between GO-treated groups and normal control group [32].

Similar analysis techniques will be followed for anodic oxidized EG samples.

B.4.2: Blood Compatibility of Graphene:

The initial event when a material comes in contact with blood is the adsorption of proteins. The nature of protein and amount of protein adsorbed will directly influence the compatibility of the particles with the blood. One of the negative effects of the clinical application of various blood contacting materials is the activation of the platelets and complement system induced by the foreign surface. The response of blood in contact with the material depends on phisico-chemical features such as surface area, surface charge, hydrophobicity/hydrophilicity etc. The response depends directly on the surface area.

The aggregation of blood cells and activation of platelets and complement on exposure of graphene to blood will be evaluated by reported procedure ref [32]. The aggregations of the blood cells on interaction with the nanoparticles have been reported et al. [**32**]. It revealed no aggregation of blood cells on incubation of graphene a higher interaction ratio of 10mg/ ml. Polyethyeleneimine (PEI) which will be used as positive control aggregation whereas saline used as negative control did not show any aggregation [32]. The same was visible with the haemolytic property of the nano-particles. The hemolysis induced by grapheme was only 0.1 % which was well within the acceptable limits of 1% . Measuring C3a or C5a in blood or serum after contact with a material has been the most usual way of assessing complement activation. It has been claimed that a surface is biocompatible if these markers are not increased in the fluid phase [**33**].

As it has graphene is blood compatible, already reported earlier. So In this project our experimental design is to find out whether blood forms GO while in contact with EG. The schematic experimental design in below…

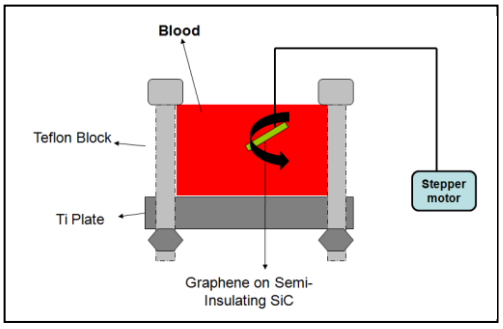

Fig-8: Schematic diagram for investigation of oxide formation while EG is in blood contact.

Grown EG will be dipped into a blood reservoir and tied with an stepper motor so that it can be revolving to have uniform blood contact at all sides of the samples. The reservoir will be in an temperature and humidity controlled incubator. This process will be continued with different types of human blood for different duration of time, starting from 1 hour to 1 month. After this graphene will be washed by DI water and Raman spectroscopy will be used to investigate GO peaks

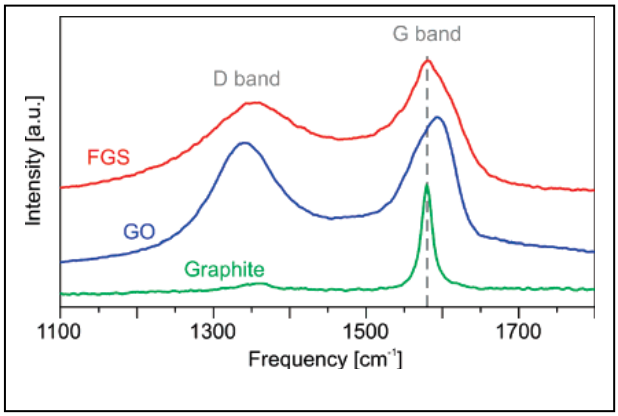

Fig-9: Raman spectra showing a clear indication of D peak position at 1593cm^{-1} for presence of graphene oxide.

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