An Investigation on the optimisation technique of Graphene Oxide synthesis for Biomedical applications.

A Thesis submitted in partial fulfilment of the requirements for the degree of Master of Technology

In

BIOTECHNOLOGY

By

R. KRISHNA MURTHY

213BM2029

Under The Supervision of

DR. SUBHANKAR PAUL

Department of Biotechnology & Medical Engineering
National Institute of Technology
Rourkela-769008, Orissa, India
2015

National Institute of Technology, Rourkela

CERTIFICATE

This is to certify that the thesis entitled "**An Investigation on the optimisation technique of Graphene Oxide synthesis for Biomedical applications.**" by **R. KRISHNA MURTHY (213BM2029)** submitted to the National Institute of Technology, Rourkela for the award of Master of Technology in Biotechnology during the session 2013-2015 is a record of bonafide research work carried out by him in the Department of Biotechnology and Medical Engineering under my supervision and guidance.

To the best of my knowledge, the matter embodied in the thesis has not been submitted to any Other University / Institute for the award of any Degree or Diploma.

Place: NIT Rourkela

Date:

Dr. Subhankar Paul
(Associate Professor)
Department of Biotechnology & Medical Engineering
National Institute of Technology
Rourkela-769008

ACKNOWLEDGEMENTS

"In order to attract more of the blessings that life has to offer, you must truly acknowledge what you already have."- **Ralph Marston**

Through this acknowledgment, I would like to express my sincere gratitude to Dr. Krishna Pramanik, HOD, Dept. of Biotechnology and Medical Engineering, NIT Rourkela for her valuable insights throughout the project.

I would like to extend my deepest gratitude to Dr. Subhankar Paul (Associate Professor, Dept. of Biotechnology and Medical Engineering, NIT Rourkela), for giving me the opportunity to do the project under his supervision, for taking keen interest and supporting me in each of my endeavours. Without his guidance and support, it would not have been possible for me to complete this research work within the allotted time period.

I would like to express my gratitude towards Mr. Deependra Kumar Ban and Mr. Sailendra Kumar Mahanta for all the technical guidance extended towards me during the course of this project work. Also I would like to thank my batchmates Prashant Kumar, Ram Sahu and Usha Pandey for their precious suggestions that helped me to perform my project work in a smooth manner.

I am grateful to Dr. B. P. Nayak and Dr. Sirsendu Sekhar Ray, for collection of blood for my research purpose.

I would like to thank Dr. Anushree Roy, Prof, IIT Kharagpur and Barnita Paul for their help with Raman Spectroscopy.

Lastly, I would like to thank the non-teaching staff of Dept. of BME, NIT Rourkela for the successful completion of the project.

Date: R. Krishna Murthy

Place: NIT Rourkela **Roll No**-213BM2029

Contents

ACKNOWLEDGEMENTS iii

LIST OF FIGURES v

ABSTRACT vii

INTRODUCTION 1

REVIEW OF LITERATURE 6

MATERIAL & METHODS 15

RESULTS & DISCUSSION 19

CONCLUSION 28

REFERENCES 30

LIST OF FIGURES

Fig no.	Figure Caption	Page No.
1	Various biomedical applications of Graphene Oxide.	4
2	GO samples synthesized by modified Marcano's method after purification.	20
3	Plot showing variation in GO Yield according to change in Initial graphite concentration.	20
4	UV-Vis spectrum of 0.05mg/ml GO solutions in wavelength range 200-800nm.	21
5	FESEM Micrographs of GO Samples.	22
6	XRD pattern and characteristic peaks of GO Samples. Plots showing Percentage Hemolysis (as compared to 1% Triton X solution) of GO Samples.	23
7	Raman spectrum of GO samples at 488nm LASER.	24
8	Plots showing effect of GO on the growth of *E. coli* and *P. aeruginosa*.	26
9	Plots showing Percentage Hemolysis (as compared to 1% Triton X solution) of GO Samples.	27

Abbreviations

M	Molar
mg	Milligram
L	Litre
µg	Microgram
nm	Nanometre
GO	Graphene Oxide
E. coli	*Escherichia coli*
P. aeruginosa	*Pseudomonas aeruginosa*
Cps	Counts per second
Arb. U.	Arbitary Units

Abstract

Graphene Oxide (GO) demonstrates various properties suitable for a plethora of applications. Till now the most common method for GO synthesis has been Hummers method. Yet this method has many disadvantages like risk of explosion, inability to scale up etc. Hence, here we have used Marcano's synthesis method with few modifications such as reduction in initial quantity of graphite, duration of heat treatment and decreasing the number of filtration steps. Further, we analysed the effect of a range of graphite concentrations as the reactant on the yield of GO synthesized. We characterized the GO by UV-Vis spectrophotometry, Field Emission Scanning Electron Microscopy (FESEM) analysis, X-Ray Diffraction and Raman Spectroscopy techniques. X-Ray Diffraction and Raman analyzes identified the nanostructured GO. UV-Vis Spectroscopy confirmed the formation of GO by showing the characteristic peak close to 230nm. FESEM imaging showed the formation of both two-dimensional and three-dimensional GO nanostructures. Suitability for biomedical applications of the synthesized GO was also analyzed by experiments such as hemolytic assay and its effects on bacterial growth. It was found that at higher concentration of GO, hemolysis was induced in a dose-dependent manner and it was within 10% as compared to Triton-X detergent. Moreover, GO was found to enhance the growth of both *E.coli* and *P. aeruginosa* in a concentration-dependent manner.

Keywords: Graphene Oxide, Hemolysis, Biocompatibility, Nanobiotechnology

Chapter 1

INTRODUCTION

A major discovery in the field of nanomaterials occurred in 2004 when Novoselov et al. discovered a way to peel off atomically thin layers of graphite consisting entirely of 2D inter-bonded carbon atoms and thus, Graphene was born [Novoselov et al., 2004]. Since then graphene has revolutionized the usage of nanomaterials in all fields of science. Graphene has potential uses in electronics [Palacios, 2011], photo-optics [Schall et al., 2014], semiconductor devices [Sorokin and Chernozatonskii, 2013], biosensors [Kuila et al., 2011], tissue engineering [Goenka et al., 2014], ultrafiltration [Huang et al., 2015], energy storage [Raccichini et al., 2015] etc. Two of the many properties of graphene that makes it useful are its strength and its conductivity. Graphene is highly conducting of electricity unlike its parent material graphite. Its high conductivity results from free flowing electron cloud over the hexagonally bonded carbon skeleton [Novoselov et al., 2004]. This same hexagonal bonding also imparts graphene with its incredible strength [Lee et al., 2008]. There are many methods of graphene synthesis such as mechanical exfoliation [Geim and Macdonald, 2007], chemical vapour deposition [Zhang et al., 2011], epitaxial growth [Riedl et al., 2009] etc. although these methods are the initial methods but most preferred hassle-free approach has been by reduction of graphene oxide [Eigler et al., 2013].

Graphene Oxide (GO) an oxidized form of graphene is most commonly used derivative of graphene. Its first synthesis was reported in 1859 by Brodie [Brodie, 1859]. He synthesized GO from treating graphite with $KClO_3$ and fuming HNO_3. Major breakthrough in GO synthesis came in 1957 when Hummers synthesized GO by adding H_2SO_4, $NaNO_3$ and $KMnO_4$ to graphite [Hummers and Offeman, 1958]. All these methods can be characterized as “top-down” approaches for GO synthesis, but another method called Tang – Lau method produces GO from thermal treatment of Glucose, this method is a “Bottom-Up” method, this results in easy and cheap production of GO with modifiable properties [Tang et al., 2012]. Another bottom-up strategy utilizes citric acid as the pyrolysed agent instead of glucose [Dong et al., 2012]. GO varies a lot in its properties according to method of synthesis and due to extent of oxidation and hydration. Interest in GO rose, as it was touted as the easiest route to graphene synthesis on a large scale. It reduces easily in liquid medium to yield highly conductive graphene flakes [Boehm and Scholz, 1965]. Initially the quality of reduced GO was lower than pure graphene and also the defects present were high. Later on synthesis of GO was optimized and reduction of which gave highly conductive graphene flakes closely resembling pure graphene [Eigler et al., 2013]. Some of the methods that produces graphene from GO easily are rapid thermal treatment of GO [Schniepp et al., 2006] and light scribing

of GO film by LASER [Kady et al., 2012]. Graphene oxide finds use as a potential agent for water purification. GO films are tested as reverse osmosis membrane. GO films can be rendered permeable to water but at the same time impervious to other materials. Features of GO films that aid in RO filtration are high strength, negligible thickness and permeability. Thus, GO films also can be used as excellent molecular sieves [Joshi et al., 2014].

Graphene Oxide as we are primarily concerned has various uses in biotechnological and biomedical field. One major advantage of GO's electronic and mechanical properties is that it can easily be molded into biosensors. Biosensing refers to detection of a bioactive compound using detectors and electronically analyze the quality and quantity of the bioactive agent [Kuila et al., 2011]. GO based biosensors have been used to detect single-stranded DNA, proteins etc. in fluorescence based approach [Lu et al., 2009]. GO is used as the material of construction for Fluorescence Resonance Energy Transfer (FRET) biosensor [Lu et al., 2009]. GO based materials have been used to form matrices for LDI-MS, especially for ssDNA and proteins due to efficient transfer of UV energy between matrices and analytes [Tang et al., 2010]. Another major application of GO is the delivery agents. GO sheets modified with Polyethyleneimine (PEI) have used as gene delivery agents with high efficiency of transfection [Chen et al., 2011]. Chitosan bound GO was used to deliver pDNA and an anti-cancer drug camptothecin in HeLa cell lines [Bao et al., 2011]. Some drug molecules have pH dependent solubility in solution. In such cases too, GO conjugated with the drug has proven to be a good nanocarrier for drug delivery. GO conjugated with Doxorubicin (DOX) was used to target HeLa cell lines, where release of drug is a pH dependent process [Zhang et al., 2010]. Ibuprofen and 5FU were also delivered like this in another experiment [Rana et al., 2011]. Polyethyleneglycol (PEG) conjugated nano-GO was also used to target DOX in cancer cell lines [Yang et al. 2010]. In addition to being delivery agents it helps in induction of photothermal stress inside cancer cells resulting in cancer cell death by apoptotic and necrotic mechanisms [Markovic et al., 2011].

Graphene oxide and reduced GO both were analyzed for their effects on bacterial growth. These were found to inhibit bacterial growth [Hu et al., 2010], which was attributed to development of oxidative stress due to damage to the plasma membrane [Liu et al., 2011]. Paradoxically, some other experiments have yielded that GO films actually enhance the bacterial growth [Ruiz et al., 2011]. GO based scaffolds can also be used for tissue engineering applications. GO surfaces also acted as differentiating factors of hMSC's. Depending upon surface functionalisation differentiation was directed towards either

osteogenic or adipogenic lineages [Lee et al., 2011]. Thus, we find that graphene oxide has multitude of applications in biotechnology and biomedical fields and with continuing research more applications are expected to emerge.

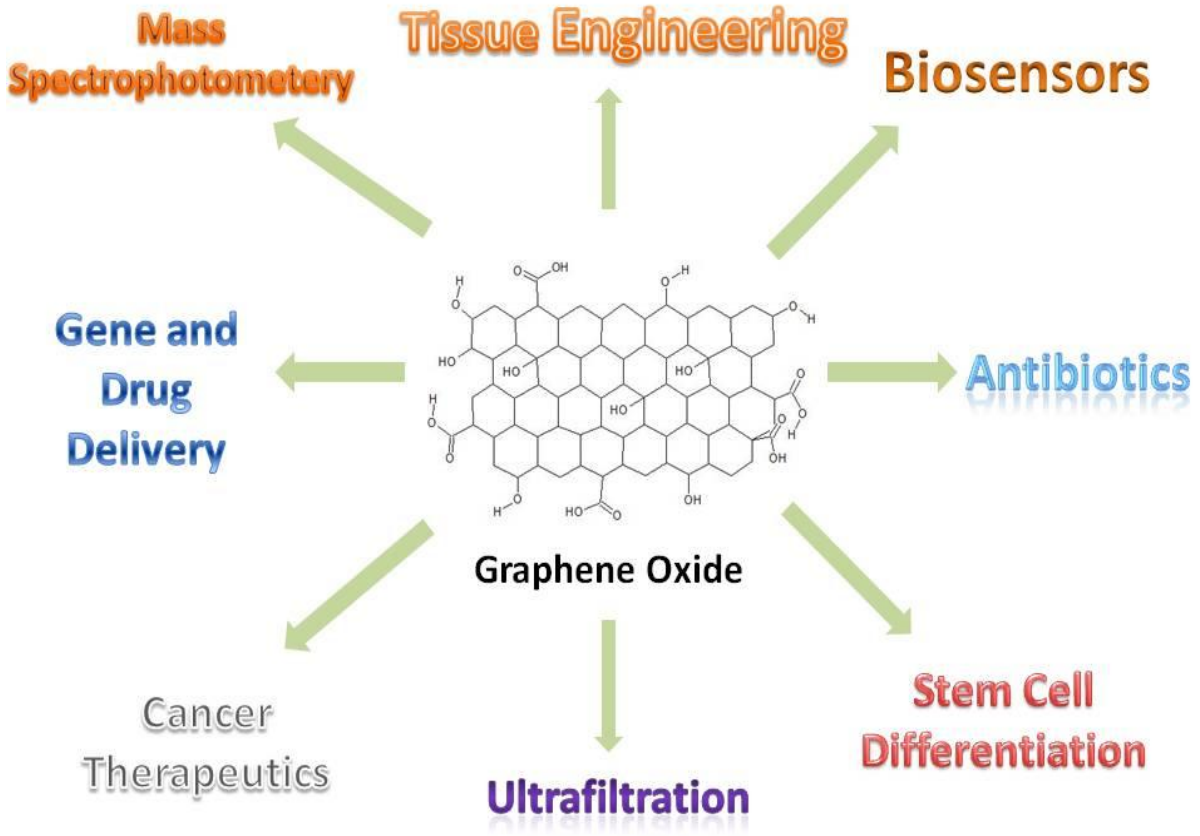


Figure 1: Various biomedical applications of graphene oxide (GO).

Graphite as such can be imagined as stacked form of millions of graphene layer. Hence, it is only logical that graphite is used to exfoliate graphene/ graphene oxide sheets. Although in course of experiments we find that this exfoliation is not completely efficient. Some amount of untreated graphite always is present in the final solution. This has to be removed by various purification methods, which in turn results in higher cost of production of graphene oxide. Moreover a common problem plaguing nanotechnology presently is large scale synthesis of nanoparticles. Whenever small scale synthesis methods are scaled-up to synthesize nanoparticles we find that synthesized particles are of heterogenous size and shapes, aggregation, uneven accumulation of nanoparticles etc. are bothersome issues that are being faced during industrial scale synthesis of nanoparticles. These issues also arise during synthesis of graphene oxide (GO).

Through the means of this research work, we want to develop further the synthesis protocol for the production of graphene oxide. We aim to optimize the basic protocol in a lab stage, for which we thought of scaling up the process of GO synthesis in a low yield range in order to observe and analyze the minute changes that occur in the shape and sizes of GO nanoparticles. These observations will help us understand the dynamics of scaling up the synthesis of graphene oxide at an industrial scale plant. Our optimization also would help in maximizing the yield of final graphene oxide content in the purified solution. We have extensively modified the purification protocol for this purpose leading to a cheaper and time saving purification methodology with lesser steps. We also aim to utilize graphene oxide as biomaterial towards biomedical applications. In accordance with that aim we wanted to analyze the hemocompatibility and anti-bacterial effect of the GO samples produced by our modified protocol. Thus, we were able to analyze the effects of process parameters on the yield of graphene oxide and also confirmed the biocompatibility of GO in order for it to be utilized as a suitable biomaterial for various biomedical applications.

Chapter 2

REVIEW OF LITERATURE

Since the initial discovery of graphene in 2004 by A.K. Giem and K.S. Novoselov, interest has only risen in application of graphene's properties in various fields. Graphene is the first of the one or few atom thick sheet. Graphene has made it possible to study quantum-relativistic effects on nanoscale at room temperature; it is possible due to unusual electronic properties of graphene. Its charge carriers have properties like that of relativistic particles and hence fits into Dirac's equation and not into Schrodinger's as small scale particles are expected to be. It also is an ideal candidate to study Quantum-Electrodynamics (QED). Modified graphene's semiconductor like properties makes it an ideal choice as replacement for silicon in microchips and it is likely that computing speeds will reach completely different level as compared to what's possible with silicon (Giem and Novoselov, 2007).

Graphene has been shown to possess excellent electronic properties due to which it is believed to revolutionize the field of electronics. Charge carrier confinement of graphene is excellent and when it is combined with high carrier mobility makes graphene an ideal choice of material for highly scaled electronics. In spite of such excellent properties, graphene based transistors aren't feasible for near future as they lack of band gap and perform poorly in real time circuits for now. There have been attempts to utilize these properties instead of considering them as hindrances. Some examples that utilize these properties of graphene include ambipolar electronics, IR detectors, graphene-silicon systems etc. (Palacios, 2011).

Optical information connections are the foundation of today's telecom base. The combination of electronic and optic segments on one chip is a standout amongst the most appealing courses to further enhance the system's performance. They show the fabrication of photodetectors based on Chemical Vapor Deposition (CVD) generated graphene on silicon optical waveguides. The devices are shown to operate bias-free in the C-band at 1550 nm and show an extrinsic −3 dB bandwidth of 41 GHz. They exhibit that these detectors work at data rates up to 50 GBit/s with excellent signal integrity. Thus the application of graphene in data transfer through optic fibers is proved (Schall et al., 2014).

Exhaustive review of graphene and graphene oxide based biosensors was done. Large surface area and conductivity of graphene enables it to behave like an "electron wire" between redox

regions on protein surface and the electrodes this result in a rapid current flow which helps in accurate detection and quantification of Biomolecules. Some of the Biomolecules that were detected by graphene and graphene oxide based biosensors are glucose, Cyt-c, NADH, Hb, cholesterol, hydrogen peroxide, ion sensors, gas sensors and DNA sensors. Field effect transistors based on graphene also were studied. Common advantages of these biosensors include low working potential, high sensitivity, low detection limits and long term stability (Kuila et al., 2011).

Nanomaterials exhibit exciting biological properties which can be utilized for biomedical applications. Some of them are small sizes, large surface area to volume ratio, ability to interact with cells and tissues. Rightly graphene derived nanomaterials have been termed as "2D Wonder Materials" due to their excellent properties which have been exploited in many fields. Recent trends show that these can also be applied in biomedical field especially in drug delivery and tissue engineering. They review the recent advancements in applications of graphene and GO in drug delivery and tissue engineering applications (Goenka et al., 2014).

Graphene-derived substances like GO should be analysed vigorously for their deleterious effects on health and environment. Graphene possesses anti-bacterial activity. The authors compared graphite, GO, graphite oxide and reduced GO for their effect on the growth of *E. coli*. GO has the maximum anti-bacterial effect followed by rGO, Gt and GtO. SEM and DLS results show that GO flakes are the smallest in size as compared to other four materials. SEM results also show that bacterial cells are lysed as a result of contact with GO sheets. Superoxide anion mediated ROS response was absent but glutathione was oxidised, conductors were able to oxidise glutathione more efficiently than insulators. Synergistic effect of membrane rupture and oxidative stress results cell lysis. Antibacterial mechanisms of the compounds were elucidated as deposition on GO sheets followed by rupture through contact and glutathione oxidation induced cell lysis. Thus size, conductivity and functional groups can be modified to reduce the impact on health and environment (Liu et al., 2011).

There exists a non-consensus about the antibacterial effect of GO nanoparticles and their biocompatibility in the scientific literature. Researchers studied antibacterial properties of GO and their effect on mammalian cells. At low concentrations, GO resulted in increasing the growth of bacteria. They proliferated at faster rate and attained higher optical densities as compared to the cultures without graphene oxide. SEM analysis showed formation of bio-films around GO flakes. Aggregation of cells and extracellular matrix occurred near GO rich areas. Increasing the concentration of GO resulted in increase in the rate of bacterial growth proving that GO is an enhancer. GO sheets weren't able to produce bacterial dead zones thus indicating their lack of antibacterial effect. Bacteriostatic activity was also found to be lacking in GO. Functionalisation with silver resulted in formation of dead zones. Mammalian cells also showed similar results with increased attachment and proliferation. Thus, they demonstrate that GO doesn't possess antibacterial activity and also isn't cytotoxic for mammalian cell lines, instead it behaves as a growth enhancer (Ruiz et al., 2011).

Mesenchymal stem cells (MSC) possess ability to differentiate towards many lineages, controlling this is an important aspect of tissue engineering wherein cells are seeded onto scaffolds for their rapid growth and development. Effect of graphene and GO in directing uncommitted stem cells towards a specific lineage is probed. Noncovalent binding of osteogenic inducers on graphene results in differentiation towards osteogenic lineage. Binding abilities of graphene and graphene oxide with different growth factors is an important factor directing the differentiation of MSC's. Unlike graphene oxide, graphene is unable to direct the MSC's towards adipogenesis because insulin, an important adipogenic growth factor gets denatured post binding to graphene sheets, Functionalisation of GO protects insulin from denaturation. Thus GO acts as an excellent differentiation agent for MSC's (Lee et al., 2011).

Since its initial synthesis in 2004, graphene and graphene derived materials have become the most vigorously researched upon topic in the field of material science. This has resulted in many scientific papers elucidating the properties of graphene. One of the fields in material science that has been influenced a lot by graphene is electrochemical energy storage devices. Future effect of graphene on this aspect seems little uncertain. The paper discusses

applications of graphene as active material and also as inactive component in energy storage devices such as lithium-ion batteries, electrochemical capacitors to upcoming magnesium-ion batteries. They address the issues and list out the advantages of graphene based system application in energy storage systems (Raccichini et al., 2015).

The research group analyzed the elastic properties and intrinsic breaking strength of monolayer graphene membranes. They achieved it by inducing grooves in the layer on a nano scale using atomic force microscope. The force displacement pattern is according to non-linear stress-strain response with elastic stiffness of about 340 Nm^{-1} (2^{nd} order). The breaking stress was estimated at 42 Nm^{-1}. This denotes high intrinsic strength of the sheets. These numbers correspond to intrinsic strength of 130 gigapascals for bulk graphite. Thus, they show that monolayer graphene is one of the strongest materials available in the world. They also show that atomically ideal nanomaterials can be tested for deformations further than linear regime of stress-strain plot (Changgu et al., 2008).

In 1884, Edwin Abbot mathematical novel "Flatland", a square narrates its life story as a member of two dimensional worlds. Scientists have long been attracted by unique properties of 2D materials such as semiconductor chips. It comes as no surprise that when scientists discovered graphene; they immediately got down to study its properties. As the carbon flatland, Graphene presents itself with lots of intriguing properties such conductivity, zero band-gap etc. To synthesize it wasn't easy since not many methods could distinguish between monolayer and many-layer graphene. Graphene's symmetry results in a relativistic behavior by its electrons where they move at speeds close to light and fulfill Dirac's equation. This tells us that electrons in graphene can break though any potential barrier. Graphene is very soft solid and a true 2D material since ripples in the surface are few angstroms in height and many nanometers in length. This flatland only promises more and more applications with each passing day (Giem and MacDonald, 2007).

After the discovery of monolayer graphene, rat race began to synthesize it using the cheapest method possible. One of the most important methods for production and preparation of

graphene is Chemical Vapor Deposition (CVD). The authors have reviewed the growth of graphene on various metal substrates by chemical vapor deposition. They also study applications of chemically grown graphene such conductors in organic photovoltaic cells and Field Effect Transistors (FET). Usage of polycrystalline Ni as a substrate resulted in formation of monolayer and few-layered graphene. This is attributed to presence of grain boundaries on Ni films. They significantly increased the ratio of monolayered graphene in the product by using single crystalline Ni surfaces, which possess smooth surfaces without grain boundaries. Another metal that yields high quantity of monolayered graphene is Cu. This is due to the low solubility of carbon in Cu. Since the growth of graphene on Cu substrate is a surface reaction, only single layer graphene is synthesized. Whereas in Ni substrate more than one layers form graphene by carbon segregation and precipitation. Polymethyl Methacrylate (PMMA) was used to transfer the graphene layer from the metal substrates. Chemically deposited graphene presents many unique properties especially electronic properties that might be applied in various industries. Like few layer graphene can act as conductive electrodes for photovoltaic cells. Also graphene with large surface area can also be grown on Cu surfaces and such graphene possess excellent electronic properties which can be applied in Field Effect Transistors (FET) (Zhang et al., 2013).

Quasi-free standing monolayer graphene is obtained by intercalation of hydrogen atoms. This is done using Silicon carbide (SiC) as a substrate. The hydrogen moves across the initial graphene layer and the topmost layer of the SiC. The Si atoms in this top layer are bound to hydrogen because of hydrogen bonding resulting in formation of a buffer layer. Once the all of the Si atoms are bonded to hydrogen, bound monolayer graphene becomes a quasi-free standing structure with linear π-bands. Subsequently, epitaxial monolayer graphene becomes a bilayer due to self folding and surface disturbances. The bonding of hydrogen to top layer of Si in SiC is stable in air and can be disturbed by heating it to temperatures near $900^{O}C$ (Reidl et al., 2009).

The enquiry into the properties of graphene has resulted in plethora of new innovative discoveries. Graphene was hailed as the new wonder material after silicon. Hence, obviously many methods of synthesis of graphene emerged. One of the most promising of those

methods is reduction of graphene oxide to chemically reduced graphene. One major issue with this method is graphene oxide during synthesis incorporates in its structure some permanent defects. This results in breakage of honeycomb lattice and formation of planar twists in GO. Thus the graphene produced as such cannot be used for many applications. Wet chemistry synthesis approach to graphene is used by the research group. This approach chemically converts graphene oxide synthesized via a new method and having almost zero defects in the planar structure to graphene. Thus the graphene produces via wet chemical synthesis is free of structural defects and has an intact honeycomb lattice (Eigler et al., 2013).

The study was aimed at analyzing the atomic weight of various types of graphite from the different ores around the world for e.g. graphite of New Brunswick, Greenland, Travancore, Ceylon etc. First demonstration of synthesis Graphitic oxide was also a part of the experiment, where in finely ground graphite powder was allowed to react with 1:4 ratios of HNO_3 and H_2SO_4 along with chlorate of potash. When the graphite was allowed to react so, it resulted in a compound which increased in weight from the original graphite and disintegrated upon heating. Thus, by measuring the elemental composition of graphite by combustion and acid hydrolysis atomic weight of graphite is calculated accurately (Brodie, 1859).

Brodie's method of graphitic oxide synthesis involved high concentration of harmful substances and gases. The present study aimed at reducing the cost, time consumed and evolution of toxic gases during synthesis of graphitic oxide. Earlier methods also involved constant threat of explosion due to chlorine gas. The group hence allowed graphite to react with sodium nitrate, conc. sulphuric acid and potassium permanganate in a non-aqueous medium. This method led to formation of graphitic oxide in less than 2 hrs and at temperatures well below 50^oC. They also found the criteria for efficient oxidation of graphite to form graphitic oxide; ideally the ratio of carbon to the oxygen atoms in the final compound should be from 2.1 to 2.9. One more way to judge the oxidation efficiency is by color, if the GO is yellow in aqueous suspension, then it means the GO is adequately oxidized. Instead if it's green to black it denotes poor oxidation. Elemental composition of the graphitic oxide was also conducted (Hummers and Offeman, 1958).

Nanofilms of graphene oxide hold much promise in the field of optoelectronics and energy storage. GO has commonly been prepared by Hummers method or a modification of it, which utilizes strong oxidizing agents such as nitric acid and sulphuric acid. These methods are termed as top-down approaches. They are easy but has obvious disadvantage of burns and explosion. Also width of the obtained GO nanoflakes is small. They elucidate a bottom-up synthesis approach for GO with modifying the thickness in a large range. The method also results in GO nanoflakes with increased yet stable lateral sizes. They synthesize GO nanoflakes using Glucose as a single reactant in hydrothermal process. The process is touted to be scalable, easy, economical, labor intensive and cheap method for GO synthesis. The properties of these GO nanosheets are similar to that produced by top-down approaches. They also constructed a photodetector with GO to demonstrate its electric properties (Tang et al., 2012).

When the possibility of growing graphene oxide via bottom-up synthesis was proven, many new compounds were tested for formation of graphene oxide post physico-chemical treatment. Here, the researchers reveal a bottom-up synthesis method for graphene quantum dots and graphene oxide nanosheets. This has been achieved by modifying the carbonization of citric acid and dissolving the end-products in sodium hydroxide solution. Quantum dots are about 15nm in length and 2nm in thickness. They exhibit powerful photoluminescent yield and this PL activity is independent of excitation wavelength. Contradicting them, the GO nanosheets are hundreds of nanometers in length and about a nanometer in thickness. They also show photoluminescent yield which is much weaker as compared to quantum dots, and this activity is dependent on excitation wavelength unlike that of quantum dots (Dong et. al., 2012).

One of the primary methods of reducing graphene oxide is by combustion. Although while burning graphene oxide we find that the process is very slow. This anomaly has been attributed to the heat loss by evaporation of water absorbed between the crystals of graphene oxide. The graphene oxide crystals generate heat when slowly decomposed just under the point of combustion (threshold temperature at which material begins to burn). Hence, on slow

rate of heating the sample temperature is higher than ambient temperature at any given point of time. The temperature of combustion is also affected by the method by which graphene oxide was prepared. Also, the impurities reduce the point of combustion significantly (Boehm and Scholz, 1965).

Functionalized graphene refers to the surface modifications of graphene sheets with various functional groups. Here, the researchers present a way of synthesizing functionalized graphene monolayer sheets by thermal exfoliation of graphene oxide. The method results in formation of graphene sheets possessing many trenches and troughs. This is due to the fact that these sites participated in the redox processes during oxidation of graphite. The surface features analyzed by AFM agree well with molecular modeling predictions. Even though GO is an insulator, graphene sheets functionalized by thermal exfoliation is very conducting. Functionalized graphene sheets have shown excellent properties as a filler agent in nanocomposites. These graphene sheets may one day help in development of conducting polymers and ultracapacitors (Schniepp et. al., 2006).

A novel method for synthesis of graphene oxide is elucidated. Most commonly used method till now is Hummers method. The researchers find that increasing the concentration of potassium permanganate while not using sodium nitrate at all and conducting the reaction in a liquid base of 9:1 ratio of sulfuric acid to ortho-phosphoric acid results in a highly efficient oxidation of graphite flakes. They find that yield of graphene oxide is higher than both of the conventional Hummers method and the modified Hummers method (where they just increase the amount of potassium permanganate). Although the GO synthesized here is highly oxidized, yet on reduction with hydrazine it yields graphene with similar electrical properties as that of graphene obtained by reduction of GO produced by Hummers method and the modified Hummers method. They use an extended filtration and wash process to purify the graphene oxide obtained. The yield of GO is approximately 5g against initial graphite concentration of 3g. As compared to the conventional Hummers method, this new synthesis method doesn't evolve harmful toxic gases (case in point, nitrous oxides) and the reaction takes place at moderate temperature thus reducing the risk of explosions. This method is important for industrial level synthesis of GO (Marcano et. al., 2010).

Chapter 3

MATERIAL & METHODS

Materials

Graphite (Sigma Aldrich #332461), Sulphuric Acid (Merck), Potassium Permanganate (Himedia), Deionised Water (Millipore), Ortho-Phosphoric Acid (Himedia), Hydrogen Peroxide (Merck), Hydrochloric Acid (Thrmo Fisher), Ethanol (Merck), Sodium Chloride (Merck), Luria-Bertani Broth (Himedia). Chemicals were of analytical grade and were used for experiments as they were obtained.

Instruments: Ultraviolet-Visible Spectrophotometer (Perkin-Elmer Lambda 35), Ultra Centrifuge (Thermo Scientific Multifuge X1R), Field Emission Scanning Electron Microscope (Nova NanoSEM 450), X-Ray Diffraction Analyzer (Regaku Ultima 4), Raman Spectroscope (Horiba JY, France), Ultrasonicator (Hielscher UP100H), Vortex Spinner, Magnetic Heater-Stirrer (Genei), Weighing Balance (Presica XR 205SM-DR), Autoclave, Laminar Air Flow Hood, Shaking Incubator, Microplate Reader (Bio-Rad).

Methods

Synthesis and Yield of GO: Graphene oxide was synthesized by modifying Marcano's Synthesis method. The modifications to the protocol given by Marcano *et al.* include reducing the initial quantity of graphite flakes, decreasing the number of filtration steps and duration of heat treatment. 50mg of graphite flakes were mixed with 300mg of $KMnO_4$, to this mixture 6ml of H_2SO_4 and 0.67ml of H_3PO_4 was added, it was then left for stirring at 50^oC for 15 hours. After that mixture was allowed to cool down and 8ml of dI H_2O-Ice was added to it. After stirring for sometime 1ml of H_2O_2 was added to stop the reaction. Mixture was then washed with deionised water once followed by 5% HCl and again twice with dI water. The precipitate was resuspended in dI water and dried to obtain GO flakes. These nanoflakes were used to create stock solutions which were sonicated to disperse the GO in suspension. The same procedure was repeated for synthesis of GO samples with 100, 200, 300, 400 and 500mg of initial graphite concentration, which were subsequently identified as Graphene Oxide (GO) Sample-1 (50mg), Sample-2 (100mg), Sample-3 (200mg), Sample-4 (300mg), Sample-5 (400mg), Sample-6 (500mg). The Yield (in mg) of the produced GO

nanoparticles was measured by measuring the total mass of GO flakes obtained per unit mass of graphite utilised as is depicted in the following formula.

$$\text{Yield} = \frac{\text{Weight of GO obtained}}{\text{Weight of Graphite used}} \quad (1)$$

Characterization of GO: UV-Visible absorption spectra of all the GO samples were obtained from UV-Visible spectrophotometer (Perkin-Elmer Lambda 35). The samples were analyzed in full spectrum scan in the range 200-800nm at the concentration of 0.05mg/ml. Size and shape of the GO nanoparticles were studied by FESEM imaging where samples were observed under Nova NanoSEM 450 device, for this analyzes samples were pulse sonicated (at 90% Frequency and 0.5 Hz Amplitude) for efficient dispersion, 10µl of 1mg/ml of GO solution was pipetted out and dried on a small glass slide in a dessicator. Crystal nature of GO nanoparticles was observed by XRD analysis. Powdered GO samples were mounted on the sample holder (pre-cleaned with propanone) and were scanned in the range of 5^0-40^0 at a scan rate of 10^0 min^{-1}. Raman Spectroscopy was done to confirm formation of GO nanoparticles. For analysis 200µl of 0.5mg/ml solution of GO samples were taken in a small cylindrical cuvette. Raman spectra of the samples were taken at LASER wavelength of 488nm.

Hemolysis Assay: Human blood was drawn using a sterile syringe and poured into heparinised tubes. Serum was removed from the blood by repeated centrifugation at 1000 rpm for 10min until clear solution remained as supernatant. Settled RBC's were collected in Autoclaved Saline solution (0.9% NaCl). This RBC solution was then diluted to 0.8% (v/v). GO samples were added in various concentrations (5, 10, 20, 40, 80 and 100µg/ml) to 4ml of diluted RBC solution in different tubes. Solution was incubated for 1hr for hemolysis. After 1 hr. solution was centrifuged at 1000 rpm for 5 min. and the absorbance of the supernatant was observed at 404nm.

Effect on Bacterial Growth: 100µl of freshly prepared cultures of bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) were inoculated in test tubes containing 5ml of Luria-Bertani broth media having various concentrations of GO Sample 3 and 6 (concentrations used were

50, 100, 200, 300µg/ml). It was then set in a shaking incubator at 37^{o}C at 120rpm. After 16 and 24 hours, 300µl of media solution was taken from tubes into 96 well titer plate and their absorbance was measured at 600nm in a microplate reader. This showed the relative growth of the bacteria co-cultured with GO samples.

Chapter 4

RESULTS & DISCUSSION

GO samples were synthesized according to the modified Marcano's method as described above (Fig 2). The yields of these samples were measured and the yield was found to be maximum for GO Sample-3 it then slightly decreased only to increase later, thus we can say that the quantity of graphite present as the initial doesn't linearly relate to the final yield of purified graphene oxide (GO), still the yield obtained is good for all the samples (Fig 3).

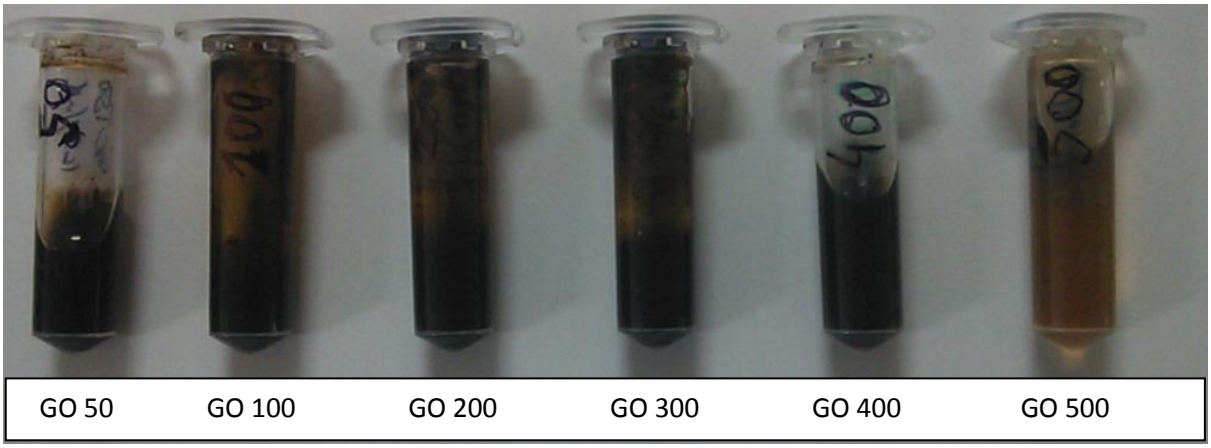


Figure 2: GO samples synthesized by modified Marcano's method after purification (nos. denote the initial quantity of graphite (in mg) used in synthesis).

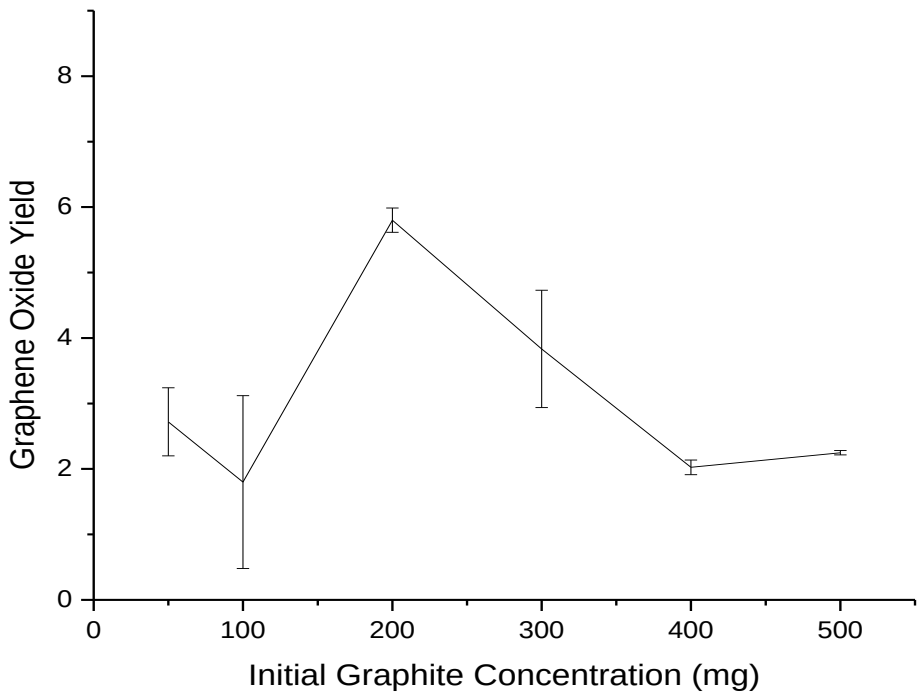


Figure 3: Plot showing variation in GO Yield according to change in Initial graphite concentration.

The suspension of GO nanoparticles is yellow in color, this indicates successful oxidation of graphite into GO as reported by Hummers *et.al.* UV-Visible spectrum scan was carried out on all the samples, we found that the samples absorbed wavelengths in the range of 229-231nm. It corresponds to π- π* transition of electrons in C=C (alkyne) bonds. The samples also show a small shoulder peak ~300nm which can be attributed to electronic transitions in C=O (carbonyl) bonds, which indicates successful oxidation of GO samples (Fig 4). The consistency of UV absorption peaks prove the stability of synthesis protocol for GO. The results are in agreement with those reported earlier in literature.

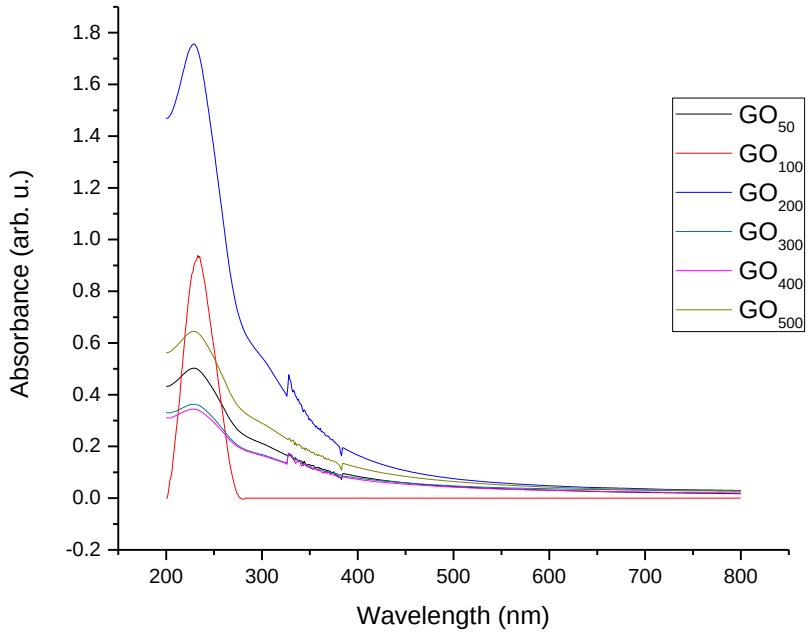


Figure 4: UV-Vis spectrum of 0.05mg/ml GO solutions in wavelength range 200-800nm.

In order to analyze the shape and size of the samples FESEM Imaging was done. Sizes of the obtained GO nanoparticles ranged from 82-700nm. This can be associated with sonication. Samples which were sonicated for longer duration resulted in smaller sizes (<100nm). As we scaled up the process from low to high graphite concentration, we found that GO nanoparticles became more and more spherical in nature. Surprisingly less amount of initial graphite also led to more aggregation in the sample which may have been caused by improper exfoliation of GO nanoparticles from graphite (Fig 5 a-f).

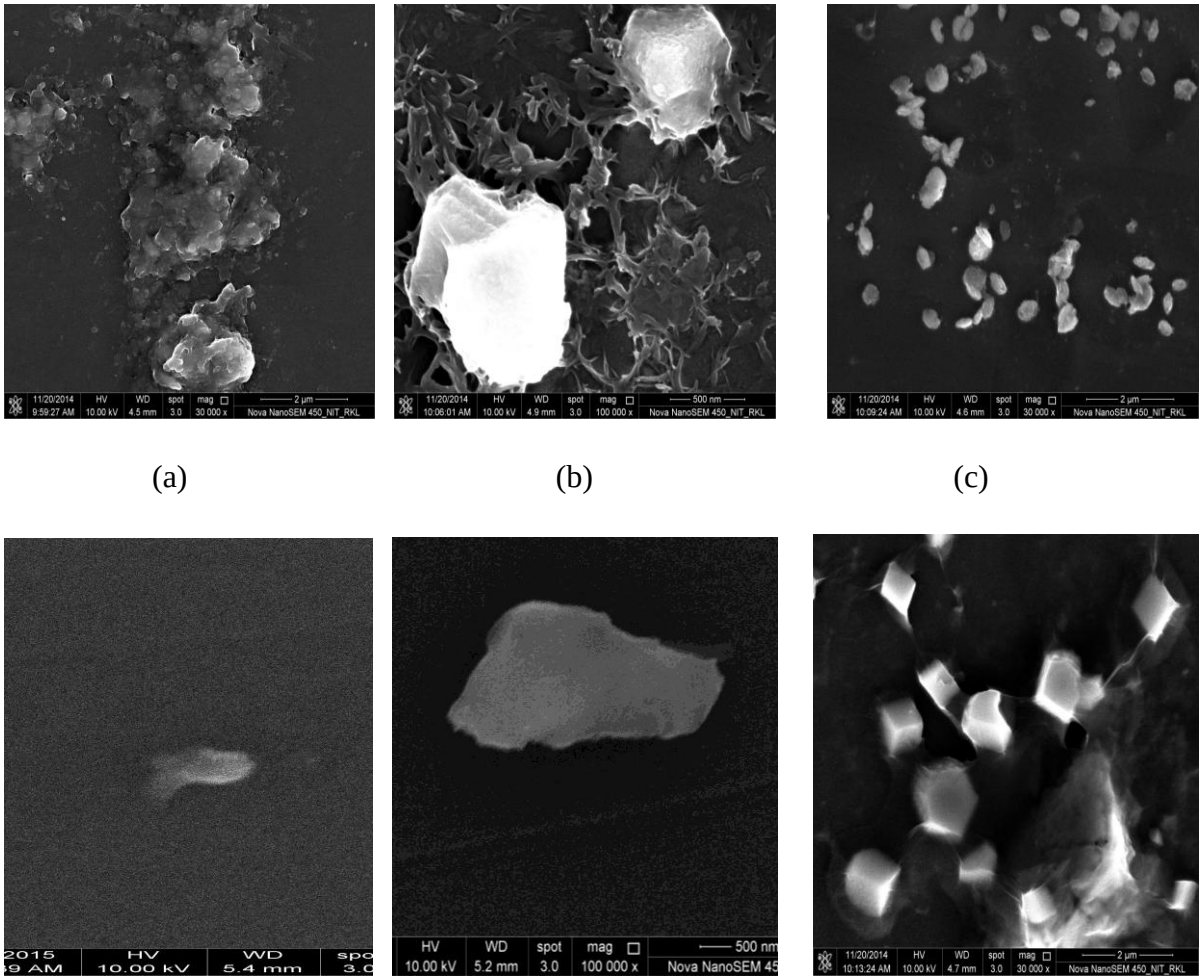


Figure 5: FESEM image of various GO samples synthesized using different concentration of graphite (a) GO_{50} synthesised using 50mg graphite (avg. size=aggregates), (b) GO_{100} using 100mg graphite (avg. size=632nm), (c) GO_{200} using 200mg (avg. size=383nm), (d) GO_{300} using 300mg graphite (avg. size=91nm), (e) GO_{400} using 400mg graphite (avg. size =543nm), (f) GO_{500} using 500mg graphite (avg. size=794nm).

X-Ray Diffraction analysis was carried to analyze the lattice structures of GO crystals. The peak reflection of samples at 2θ equal to ~10^{o} are in the range of 0.8-1nm which about thrice of graphite. This increase is due to presence of oxygen functionalities in between the graphene oxide layers as is introduced during synthesis. Our XRD peaks also agree with various reported literature, thus confirming synthesis of graphene oxide (Fig 6).

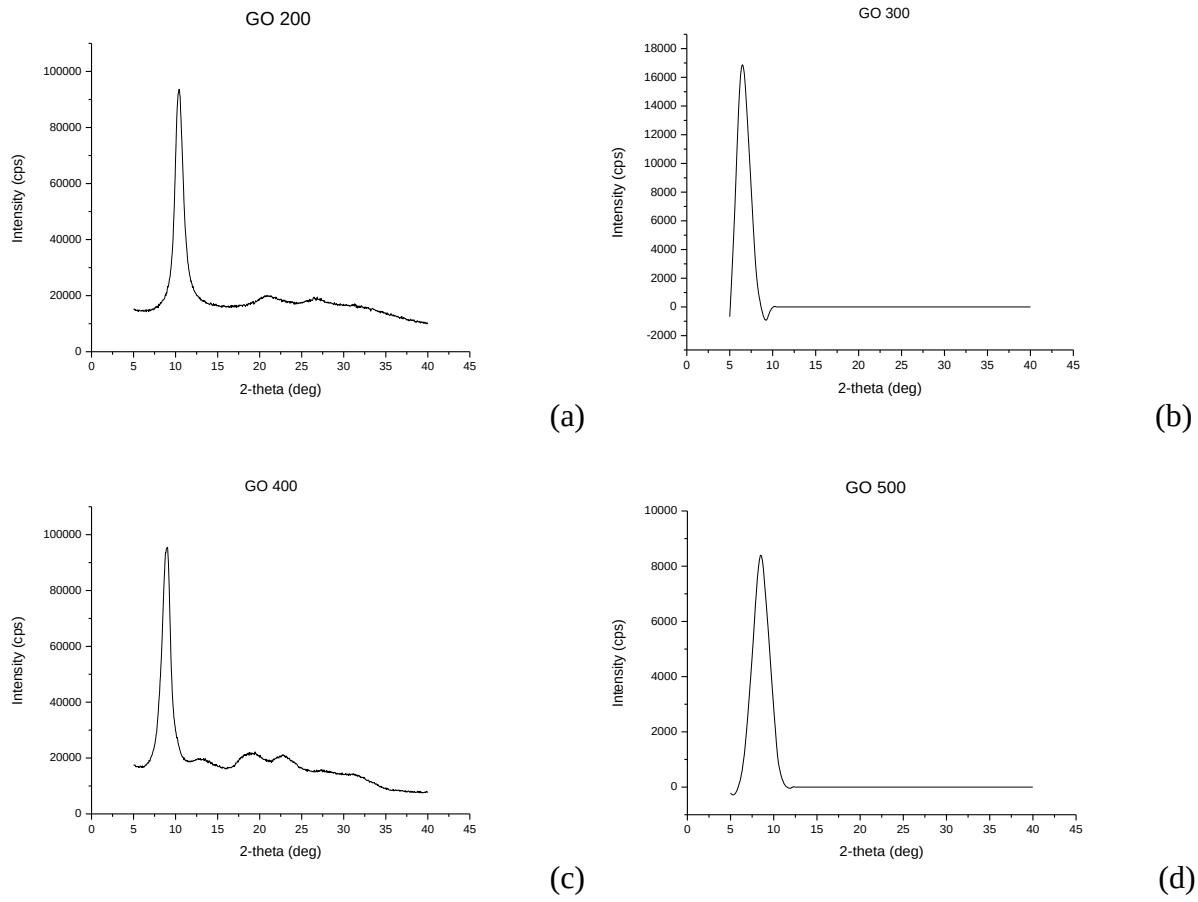


Figure 6: XRD pattern and characteristic peaks of GO, (a) GO_{200}, (b) GO_{300}, (c) GO_{400}, (d) GO_{500}.

Raman spectral analysis was done on the GO samples in order to study their structure. In agreement with existing literature we obtain two peaks of the sample. One peak is at ~1350 cm^{-1} and another at ~1590 cm^{-1}. The former peak is G-Band and latter is D-Band. G-Band relates to carbon atoms and D-Bands correspond to the surface distortions present in the graphene oxide. Strong D-band indicates high degree of lattice distortions of GO surface, which confirms with the pseudoplanar nature of graphene oxide (Fig 7).

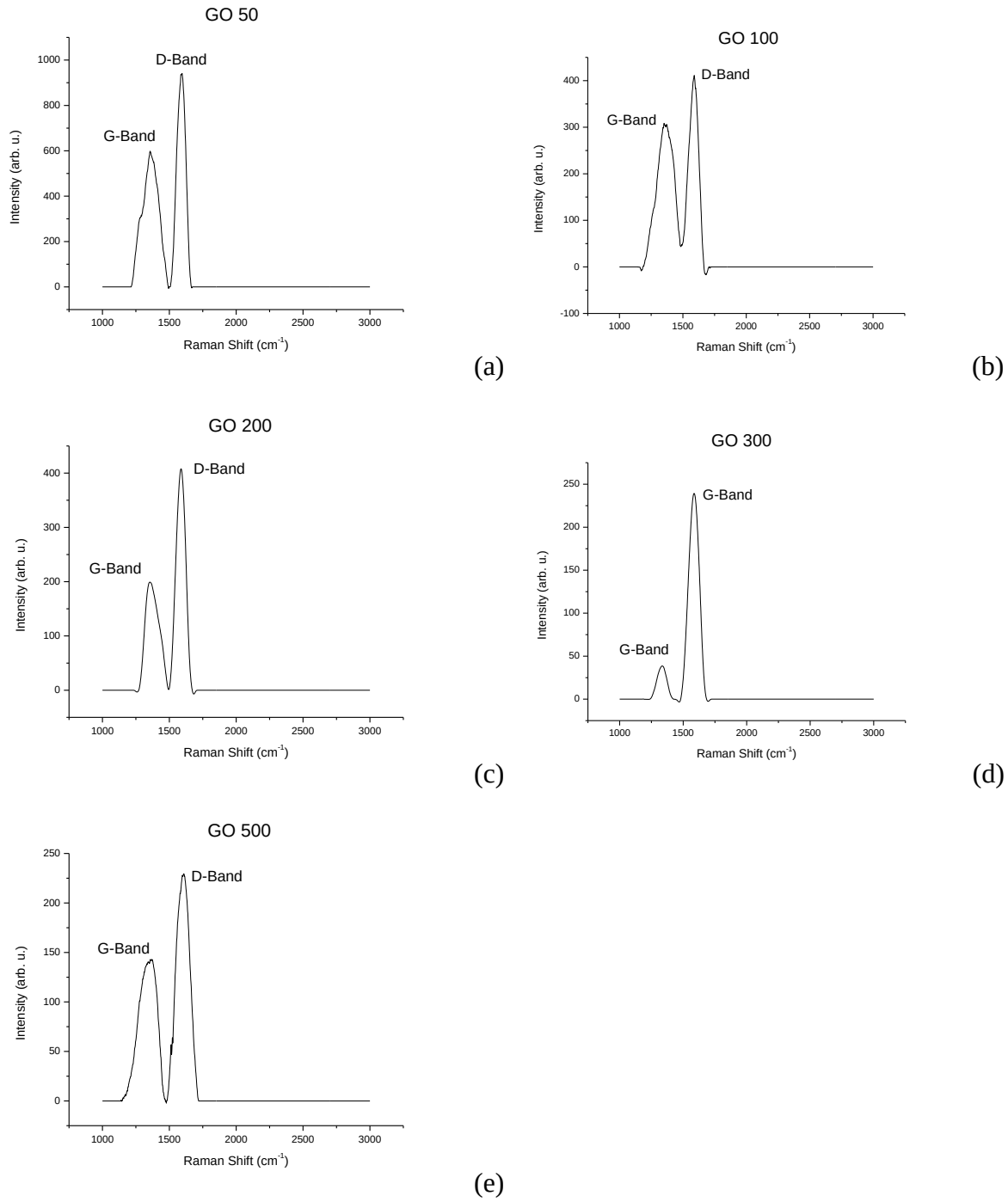
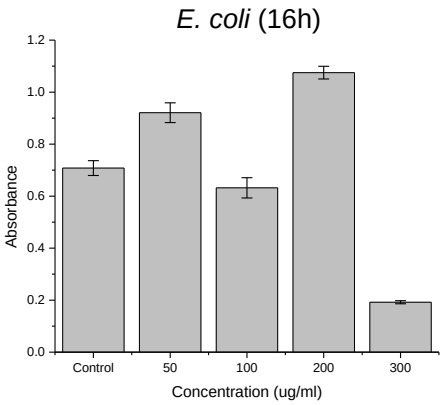


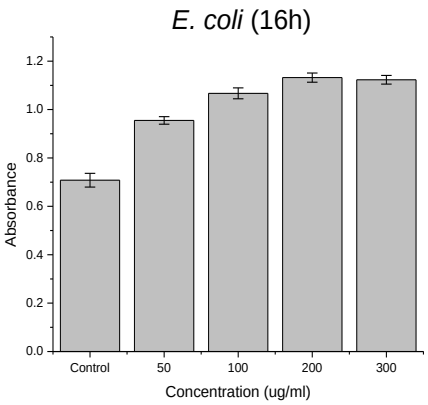
Figure 7: Raman spectrum of GO samples at 488nm LASER, (a-d) GO_{50} to GO_{300} and (e) GO_{500}.

Cytotoxicity of the produced GO samples was analyzed by culturing bacteria in medium (Luria-Bertani broth medium) containing graphene oxide. For this purpose we selected two Gram-negative bacteria *E. coli* and *P. aeruginosa*. The bacteria were allowed to grow in the medium for a period of 24 hrs, during which their growth was monitored alongside a control group without GO in their medium in order to realize the effect of GO on their growth. We

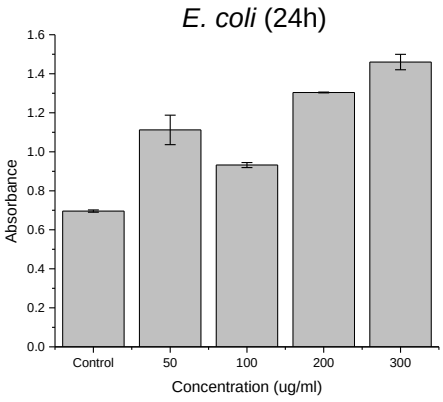
discovered that GO in growth medium uniformly enhanced the growth of both *E. coli* and *P. aeruginosa*. This contradicts research works of some groups many of which has GO conjugated with other growth inhibitory substances like Ag^{+} ions, lysozymes, lipid peroxidases etc. but agrees with few other groups too leading to a paradoxical conclusion. Groups which report anti-bacterial activity of GO attribute the effect to the sharp nature of GO nanoflakes and hence we attribute enhancement effect of our GO samples to the fact that they have slightly spherical shape primarily devoid of sharp edges, thus our GO samples were unable to induce shear stress on the bacterial cell membrane and also there is no generation of Reactive Oxygen Species (ROS) in response from the bacteria. Enhancement of the bacterial growth occurs presumably due to adaptation of bacteria to grow on the GO flakes (acting as substrate) where the bacterial cells are adsorbed and in turn GO gets reduced due to metabolizing bacteria.



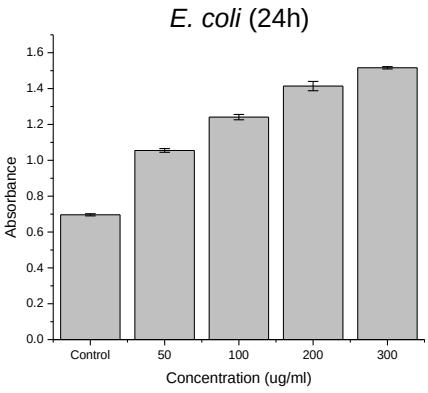
(a)



(b)



(c)



(d)

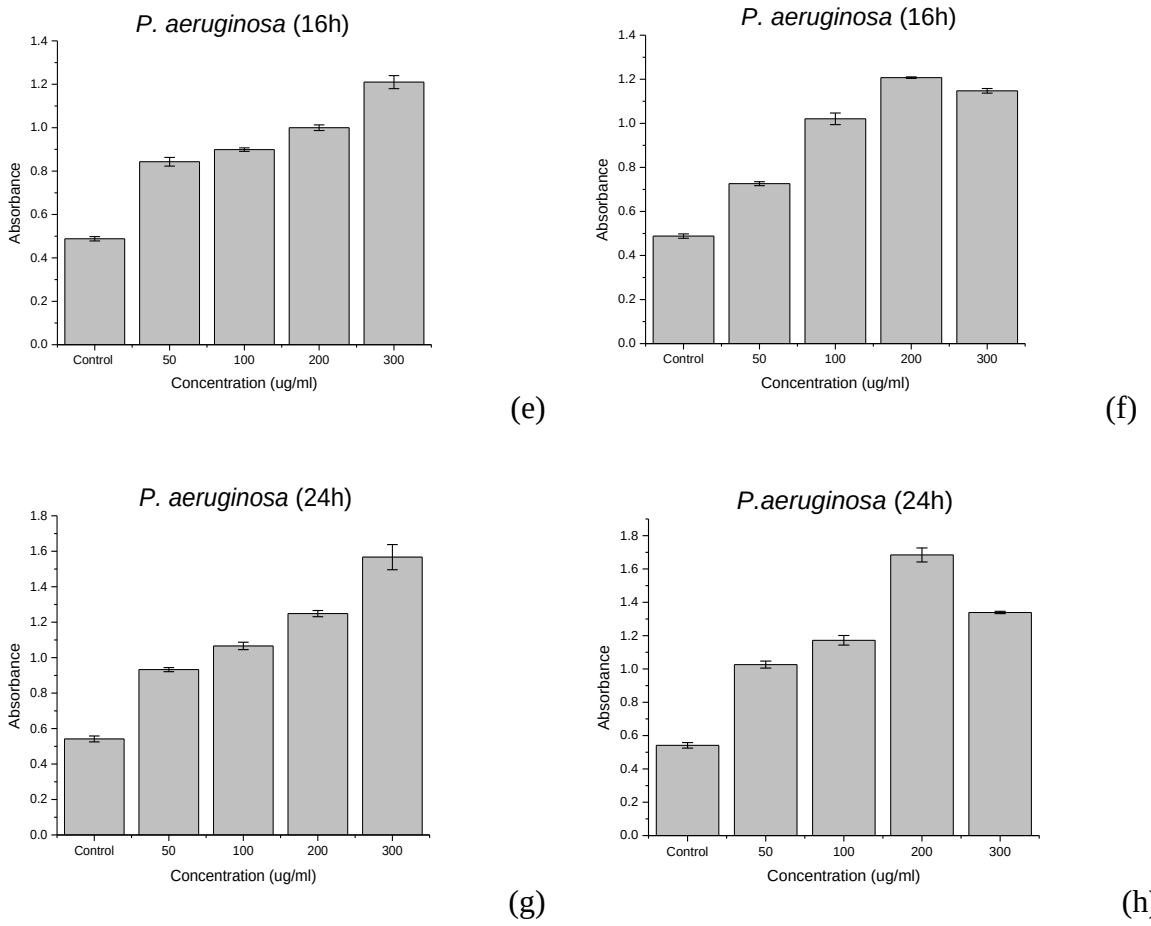


Figure 8: Plots showing the effect of various GO samples on the growth of *E. coli* and *P. aeruginosa*. (a) and (c) shows effect of GO_{200} on *E.coli*, (b) and (d) shows effect of GO_{500} on *E. coli*. (e) and (g) shows effect of GO_{200} on *P. aeruginosa*, (f) and (h) shows effect of GO_{500} on *P. aeruginosa.*

Hemolysis assay of produced GO samples was conducted in order to analyze the hemocompatibility of the samples. It is a major factor that determines the use of a substance as a biomedical agent for drug delivery or tissue engineering. The extent of hemolysis was measured by measuring the absorbance values of hemoglobin released in supernatant post hemolysis. Value of absorbance of supernatant which was hemolysed by Triton X at 404nm is 0.2624. Hence, in comparison hemolysis caused by our graphene oxide samples is very less (Fig 9 (a-f)). This effect can be attributed to the spherical geometry of GO nanoparticles as confirmed by the FESEM imaging due to which the smooth edges of the GO nanoparticles are unable to pierce through the plasma membrane of erythrocytes. In accordance with the

previously reported literature we too find that aggregated GO nanoparticles (GO Sample-1) result in least hemolysis as compared to monodisperse nanoparticles (GO Sample-6).

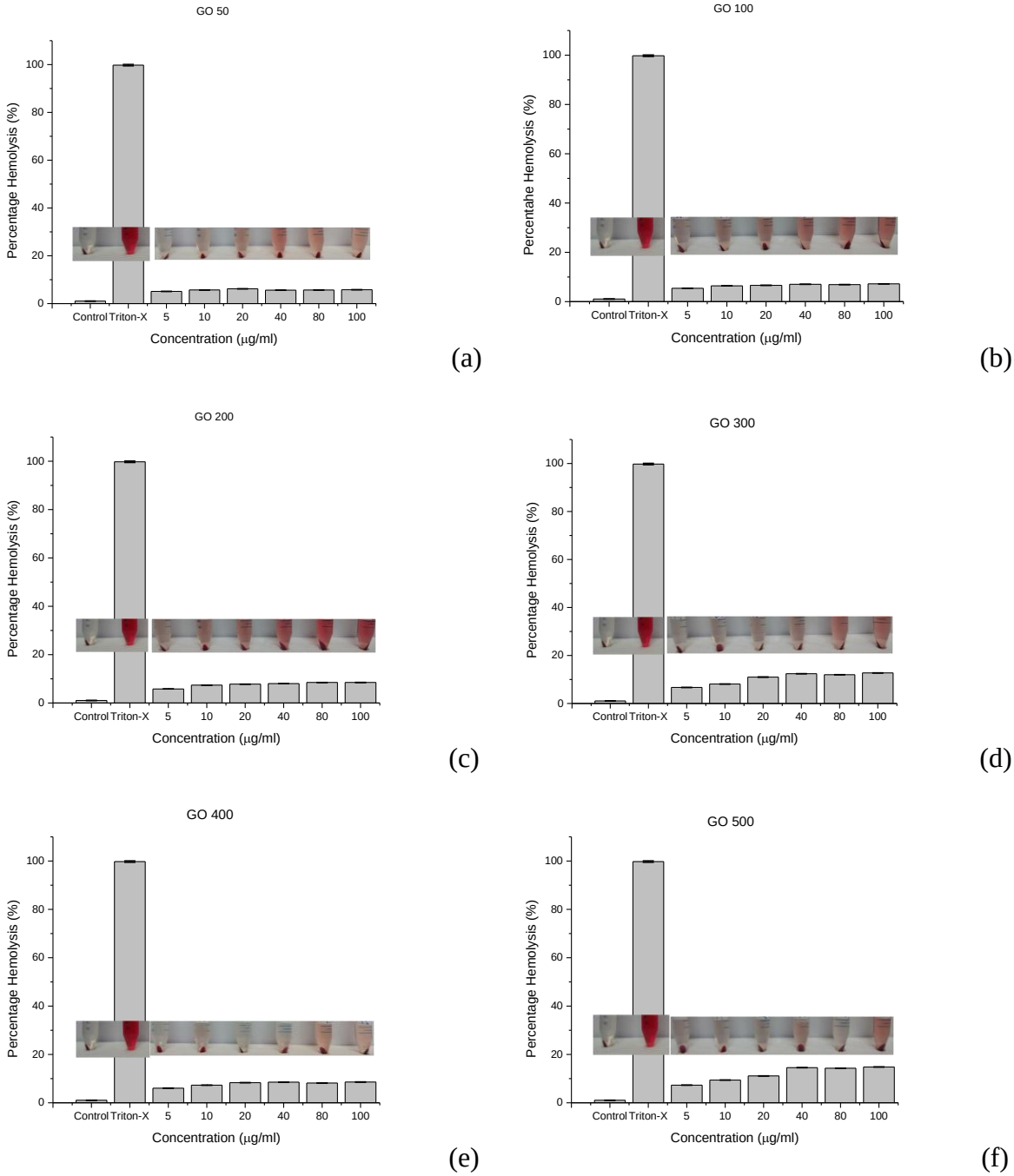


Figure 9: Plots showing percentage hemolysis (as compared to 1% Triton X solution) of various GO Samples.

Chapter 5

CONCLUSION

We successfully optimized the Marcano's synthesis protocol in order to achieve maximum yield in low-reactant lab scale synthesis using graphite as the limiting reactant. We characterized the produced graphene oxide samples with various methods (UV-Vis spectrophotometry, FESEM Imaging, XRD and Raman Spectroscopy techniques) to confirm good quality synthesis of purified graphene oxide. We also optimised the yield of this modified protocol, where we got maximum yield against initial graphite concentration of 200mg. We analysed the samples for their hemocompatibility and their effect on growth of bacteria (*E. coli* and *P. aeruginosa*). We observed that our GO samples lysed erythrocytes to much lesser extent as compared to the standard detergent (Triton-X). This result confirms a high degree of hemocompatibility of our GO samples, which is an essential prerequisite for any material to be utilized for biomedical applications. The synthesized samples of graphene oxide caused enhancement of the growth of both the bacteria studied. This can be attributed to adsorption of bacterial cells on the surface of GO nanoflakes. This may lead to better drug delivery system for medical research. Further characterizations can be performed in order to probe the samples for applications in various fields like mechanical sciences, electronics etc. Various other tests can be done to assess the biocompatibility of GO samples. Reduction of GO to graphene is one another major application that could be looked into using this protocol in the near future.

Chapter 7

REFERENCES

Bao H. Q.; Pan Y. Z.; Ping Y.; Sahoo N. G.; Wu T.; Li L.; Li J.; Gan L. H. “Chitosan functionalized graphene oxide as a nanocarrier for drug and gene delivery” *Small* 2011, 7, 1569–1578.

Brodie, B. C. “On the Atomic Weight of Graphite” Philosophical Transactions of the Royal Society of London 1859, 149: 249.

Changgu Lee; Xiaoding Wei; Jeffrey W. Kysar; James Hone. “Measurement of the Elastic Properties and Intrinsic Strength of Monolayer Graphene” Science 321, 2008, (385): 385–388.

Chen B.; Liu M.; Zhang L. M.; Huang J.; Yao J. L.; Zhang Z. J. “Polyethylenimine functionalized graphene oxide as an efficient gene delivery vector” *Journal of Mater Chem.* 2011, 21, 7736–7741.

Daniel Schall; Daniel Neumaier; Muhammad Mohsin; Bartos Chmielak; Jens Bolten; Caroline Porschatis; Andreas Prinzen; Christopher Matheisen; Wolfgang Kuebart; Bernhard Junginger; Wolfgang Templ; Anna Lena Giesecke; Heinrich Kurz. “50 GBit/s Photodetectors Based on Wafer-Scale Graphene for Integrated Silicon Photonic Communication Systems”, *ACS Photonics*, 2014, 1 (9), pp 781–784.

Daniela C. Marcano; Dmitry V. Kosynkin; Jacob M. Berlin; Alexander Sinitskii; Zhengzong Sun; Alexander Slesarev; Lawrence B. Alemany; Wei Lu; James M. Tour. “Improved Synthesis of Graphene Oxide” ACS Nano, 2010, 4, 4806-4814.

Eigler, S.; Enzelberger-Heim, M.; Grimm, S.; Hofmann, P.; Kroener, W.; Geworski, A.; Dotzer, C.; Röckert, M.; Xiao, J.; Papp, C.; Lytken, O.; Steinrück, H.P.; Müller, P.; Hirsch, A. "Wet Chemical Synthesis of Graphene" Advanced Materials 25, 2013, (26): 3583–3587.

El-Kady M. F.; Strong V.; Dubin S.; Kaner R. B. "Laser Scribing of High-Performance and Flexible Graphene-Based Electrochemical Capacitors" Science, 2012, 335(6074): 1326–1330.

Geim, A. K.; MacDonald, A. H. "Graphene: Exploring carbon flatland" Physics Today 60 2007, (8): 35–41.

Hu W. B.; Peng C.; Luo W. J.; Lv M.; Li X.; Li D.; Huang Q.; Fan C. "Graphene-based antibacterial paper" *ACS Nano* 2010, 4, 4317–4323.

Hummers, W. S.; Offeman, R. E. "Preparation of Graphitic Oxide" Journal of the American Chemical Society 80, 1958, (6): 1339.

Ji Chen; Bowen Yao; Chun Li; Gaoquan Shi. "An improved Hummers method for eco-friendly synthesis of graphene oxide" *CARBON* 64, 2013, 225 –229.

Joshi R. K.; Carbone P.; Wang F. C.; Kravets V. G.; Su Y.; Grigorieva I. V.; Wu H. A.; Geim A. K.; Nair R. R. "Precise and Ultrafast Molecular Sieving Through Graphene Oxide Membranes" Science, 2014, 343 (6172): 752–4.

Lee W. C.; Lim C. H.; Shi H.; Tang L. A.; Wang Y.; Lim C. T.; Loh K. P. "Origin of enhanced stem cell growth and differentiation on graphene and graphene oxide" *ACS Nano* 2011, 5, 7334–7341.

Liu S. B.; Zeng T. H.; Hofmann M.; Burcombe E.; Wei J.; Jiang R.; Kong J.; Chen Y. "Antibacterial activity of graphite, graphite oxide, graphene oxide, and reduced graphene oxide: membrane and oxidative stress" *ACS Nano* 2011, 5, 6971–6980.

Lu C. H.; Yang H. H.; Zhu C. L.; Chen X.; Chen G. N. "A graphene platform for sensing Biomolecules" *Angew. Chem., Int. Ed.* 2009, 48, 4785–4787.

Manash R. Das; Rupak K. Sarma; Ratul Saikia; Vinayak S. Kale; Manjusha V. Shelke; Pinaki Sengupta. "Synthesis of silver nanoparticles in an aqueous suspension of graphene oxide sheets and its antimicrobial activity" *Colloids and Surfaces B: Biointerfaces* 83, 2011, 16–22

Markovic Z. M.; Harhaji-Trajkovic L. M.; Todorovic-Markovic B. M.; Kepic D. P.; Arsikin K. M.; Jovanovic S. P.; Pantovic A. C.; Dramicanin M. D.; Trajkovic V. S. "*In- vitro* comparison of the photothermal anticancer activity of graphene nanoparticles and carbon nanotubes" *Biomaterials* 2011, 32, 1121–1129.

Novoselov, K. S.; Geim, A. K.; Morozov, S. V.; Jiang, D.; Zhang, Y.; Dubonos, S. V.; Grigorieva, I. V.; Firsov, A. A. "Electric Field Effect in Atomically Thin Carbon Films" *Science* 2004, 306, 666–669.

Rana V. K.; Choi M. C.; Kong J. Y.; Kim G. Y.; Kim M. J.; Kim S. H.; Mishra S.; Singh R. P.; Ha C. S. "Synthesis and drug-delivery behavior of chitosan-functionalized graphene oxide hybrid nanosheets" *Macromol. Mater. Eng.* 2011, 296, 131–140.

Riedl, C.; Coletti, C.; Iwasaki, T.; Zakharov, A.A.; Starke, U. “Quasi-Free-Standing Epitaxial Graphene on SiC Obtained by Hydrogen Intercalation” Physical Review Letters 103, 2009, (24): 246804.

Rinaldo Raccichini; Alberto Varzi; Stefano Passerini; Bruno Scrosati. “The role of graphene for electrochemical energy storage” *Nature Materials* 2015, 14, 271–279.

Ruiz O. N.; Fernando K. A.; Wang B.; Brown N. A.; Luo P. G.; McNamara N. D.; Vangsness M.; Sun Y. P.; Bunker C. E. “Graphene oxide: a nonspecific enhancer of cellular growth” *ACS Nano* 2011, 5, 8100–8107.

Schniepp H. C.; Li J. L.; McAllister M. J.; Sai H.; Herrera-Alonso M.; Adamson D. H.; Aksay I. A. “Functionalized single graphene sheets derived from splitting graphite oxide” The Journal of Physical Chemistry. B, 2006, 110(17), 8535–9.

Sorokin P. B.; Chernozatonskii L. A. “Graphene-based semiconductor nanostructures” *Phys. Usp.* 2013, 56, 105-122.

Sumit Goenka; Vinayak Sant; Shilpa Sant. “Graphene-based nanomaterials for drug delivery and tissue engineering” Journal of Controlled Release, Volume 173, 10 January 2014, Pages 75–88.

Tang L. A.; Wang J.; Loh K. P. “Graphene-based SELDI probe with ultrahigh extraction and sensitivity for DNA oligomer” *J. Am. Chem. Soc*. 2010, 132, 10976–10977.

Tang, L.; Li, X.; Ji, R.; Teng, K. S.; Tai, G.; Ye, J.; Wei, C.; Lau, S. P. "Bottom-up synthesis of large-scale graphene oxide nanosheets" Journal of Materials Chemistry 22, 2012, (12): 5676.

Tapas Kuila; Saswata Bose; Partha Khanra; Ananta Kumar Mishra; Nam Hoon Kim; Joong Hee Lee. "Recent advances in graphene-based biosensors" *Biosensors and Bioelectronics*, Volume 26, Issue 12, 15 August 2011, Pages 4637–4648.

Tomás Palacios. "Graphene electronics: Thinking outside the silicon box", *Nature nanotechnology* 6, 2011, 464–465.

Yang K.; Zhang S.; Zhang G. X.; Sun X.; Lee S. T.; Liu Z. "Graphene in mice: ultrahigh *in-vivo* tumor uptake and efficient photothermal therapy" *Nano Lett.* 2010, 10, 3318–3323.

Yi Huang; Hang Li; Lei Wang; Yali Qiao; Chuanbing Tang; Chanil Jung; Yeomin Yoon; Shiguang Li; Miao Yu. "Ultrafiltration Membranes with Structure-Optimized Graphene-Oxide Coatings for Antifouling Oil/Water Separation" *Advanced Materials Interfaces*, Volume 2, Issue 7, January 21, 2015.

Yi Zhang; Luyao Zhang; Chongwu Zhou. "Review of Chemical Vapor Deposition of Graphene and Related Applications" *Acc. Chem. Res.*, 2013, 46 (10), pp 2329–2339.

Yongqiang Dong; Jingwei Shao; Congqiang Chen; Hao Li; Ruixue Wang; Yuwu Ch; Xiaomei Lin; Guonan Chen. “Blue luminescent graphene quantum dots and graphene oxide prepared by tuning the carbonization degree of citric acid” *Carbon*, Volume 50, Issue 12, October 2012, Pages 4738–4743.

Zhang L. M.; Xia J.; Zhao Q.; Liu L.; Zhang Z. “Functional graphene oxide as a nanocarrier for controlled loading and targeted delivery of mixed anticancer drugs” *Small* 2010, 6, 537–544.