Preparation, characterization and surface functionalization of nano Graphene Oxide using lysozyme: Evaluation of antibacterial activity against Escherichia coli.

Dissertation submitted in partial fulfillment

of the requirements of the degree of

Master of Technology

in

Biotechnology

by

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(Roll Number: 214BM2032)

based on research carried out

under the supervision of

Prof. Subhankar Paul

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This is to certify that the work presented in the dissertation entitled *Preparation, characterization and surface functionalization of nano Graphene Oxide using lysozyme: Evaluation of antibacterial activity against Escherichia coli* submitted by *Bathuri Venkata chaithanya*, Roll Number 214BM2032, is a record of original research carried out by him under my supervision and guidance in partial fulfillment of the requirements of the degree of *Master of Technology* in *Department of Biotechnology and Medical Engineering*. Neither this dissertation nor any part of it has been submitted earlier for any degree or diploma to any institute or university in India or abroad.

_____

Prof. Subhankar Paul

Supervisor

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ABBREVIATIONS

GOns	Graphene oxide nanosheets
LYZ	Lysozyme
mg	Milligram
µg	Microgram
nm	Nanometer
E. coli	Escherichia coli
XRD	X-ray Diffraction
FESEM	Field Emission Scanning Electron Microscope
ROS	Reactive oxygen species
fGOns	Functionalized Graphene oxide nanosheets
DSC	Differential Scanning Calorimetry
TGA	Thermo Gravimetric Analysis

Abstract

A simple and environment friendly method controlled pyrolysis of citric acid and modified Hummer's method had used for the synthesis of Graphene Oxide nanosheets (GOns). Here, the GOns are functionalized with hen egg white lysozyme protein by the process of chemical cross-linking results in formation of fGOns. The synthesized GOns was characterized using various standard biophysical techniques such as X-ray diffraction crystallography, UV-Vis spectroscopy, fluorescence spectroscopy, FESEM imaging, DLS particle size and zeta-potential analysis, DSC and TGA analysis. The XRD crystallography results showed a peak at $2\Theta = 9.86°$ for pyrolysis synthesized GOns and $2\Theta = 10.54°$ for modified Hummer's synthesized GOns. The UV-Vis spectra showed a broad peak between 230-235nm for the GOns synthesized by two different methods. FESEM images shows the sharp edge shape of the GOns whereas, fGOns acquired spherical shape. The particle size distribution and zeta potential of GOns was measured. The fGOns was characterized by fluorescence spectroscopy as there is a decrease in fluorescence intensity for fGOns when compared to Lysozyme protein fluorescence confirms the binding of LYZ on GOns surface. The UV-Vis absorption spectra showed a peak shift around 225nm for the fGOns. Further, the effect of GOns and fGOns towards the growth kinetics of E.coli was evaluated by observing the E.coli growth profile and estimation of total intracellular protein concentration. Results showed that pyrolysis synthesized GOns and fGOns exhibiting bacterial growth inhibition during log phase of growth profile. Whereas, modified hummer's synthesized GOns showed the enhanced growth of E.coli.

Keywords: Pyrolysis, functionalized GOns, desolvation, chemical cross-linking, biophysical techniques.

CHAPTER 1

INTRODUCTION

1. Introduction

Graphene oxide is a thin single layered carbon sheet that was decorated with several oygen containing functional groups such as carboxylic groups at the edges, epoxide groups arranged on the basal plane has attracted biological researchers for its remarkable biological properties. GO is the thinnest and strongest compound among the carbon based nanomaterials. Graphene oxide surfaces acts like partially hydrophobic but the most of the surface has hydrophilic regions capable of hydrogen bonding and ion complexing, and contains negative charge on the edges due to the presence of many carboxylic groups (Sanchez *et al*., 2011).

Among the family of graphene like nanomaterials, graphene oxide has shown promising applications in biological research area. Graphene Oxide is highly oxidised form of chemically modified graphene, produced by oxidation of graphite followed by sonication or other dispersion methods to produce monolayer nanosheets in aqueous suspensions (Sanchez *et al.,* 2011). Graphene Oxide is usually synthesised by chemically exfoliating graphite, here, we are preparing graphene oxide (GO) by carbonization of organic compound i.e., citric acid is subjected to thermal treatment (pyrolysis) (Mahanta and Paul, 2015).

Currently much of the research focussing on using graphene and graphene like materials such as graphene oxide towards antibacterial activity, anticancer drug carriers, etc., Here, we are focussing on the study of antibacterial activity of functionalised graphene oxide. The antibacterial activity of Graphene Oxide mechanism is due to the induction of membrane and oxidative stress by the sharp edge effect of graphene oxide nanosheets (GOns) which destabilizes the bacterial membrane integrity on its interaction (Liu *et al.,* 2011).

Graphene oxide has excellent electrical, mechanical, thermal and optical properties. Due to this GO has been widely used for its applications in biological sensors, enzyme immobilization, electronics etc. The bulk synthesis of GO was produced by hummer's method which involves direct oxidation of graphite to form GOns. It is a lucrative method as this method productivity is high in large scale, at a very minimal cost. The traditional Hummer's method uses sodium nitrate because of which toxic gases like NO_4 and N_2O_4 were evolved. The improved Hummer's process

eliminates the use of sodium nitrate for the GOns synthesis makes the process more simple and environment friendly without affecting the GOns productivity. (Chen et al., 2013).

GO is usually synthesized by chemically exfoliating graphite. GO can be synthesized either by top down and bottom-up approach. Top down methods uses high-resolution electron beam lithography for carving graphite, Chemical oxidation of graphite, electrochemical synthesis etc. Low yield was the major drawback of top-down methods. The bottom-up approaches involves the thermal treatment of organic compound precursors. (Dong et al., 2012). GO inhibits the growth of the significant amount of cells when incubated with the cells. The cytotoxicity was greatly reduced when GO was functionalized with FBS and incubated in the medium with 10% FBS. (Cedervall et al., 2007).

Biomolecules such as proteins, nucleic acids and lipids have intrinsic properties for the self-assembly into the functional biological unit, known as the cell. The Cellular activities like amyloid fibril formation, antigen-antibody interactions, chromatin assembly, and bilayer phospholipid membrane self-assembly are excellent examples of molecular self-assembly.

Biological moderation of GOns surfaces enhances its biocompatibility and solubility. Hence this is drawing researcher's attention for its remarkable surface characteristics prone to the surface modifications (Allen et al., 2009). Though GOns exhibits its antibacterial potential against both gram+^{ve} and gram-^{ve} bacterial strains because of the difference in cell membrane properties GOns exhibiting more antibacterial activity towards gram-ve bacteria. (Akhavan et al., 2010).

A recent investigation has shown that incubation of GO with *E.coli* and P. aeruginosa can cause reduction in the growth about 50% and 60% respectively. The results are evident as the growth inhibition zones was observed for the *E.coli* and *S.aureus* when GO paper used (Bao et al, 2011). When GO was used for disk diffusion assay the GO dipped filter disks placed in the center of a nutrient media plate whose surface was already spreaded with bacteria, no zone of growth inhibition was observed (Das et al., 2011).

Single layered GO sheets are not permeable to many molecules. The GOns have ability to wrap the bacteria making it biologically isolating from the nutrient medium. Thereby, making the wrapped cells starve and not allowing to proliferate. The cell viability loss was observed by the

colony counting test. One third of the wrapped bacteria with GOns can be reactivated after 48hr of inactivation within the GOns. (Liu et al., 2012).

GO is having toxicity to human fibroblast cells which is dose dependent and when the dose of GO is increased upto 50 μg/mL it causes cytotoxicity. On the other side, other investigation shows good biocompatibility of GO where GO enter inside the A549 cells and showed no toxicity unconcerned of the size or dosage of GO. (Ryoo et al., 2010 & Chang et al., 2011). Whenever proteins binds to planer surfaces it induces some changes in the secondary structure but due to large curvature of nanoparticle the secondary structure of protein is retained. It is reported that in any conjugation process there is always some kind of perturbation in the protein structure to an extent. ZnO and Au NPs are biocompatible as well as inert hence they are mostly used nanoparticle. Au NPs are used in drug delivery for DNA and protein detection. When lysozyme is adsorbed onto silica NPs surface there is a change in the conformation of secondary structure and tertiary structure. (Vertegel et al., 2004; Wu and Narsimhan et al., 2008; Bhattacharya et al., 2007).

CHAPTER 2

REVIEW OF LITERATURE

2. Literature Review

2.1 Synthesis of GOns

GO was first synthesized in 1859 by the oxidation of Ceylon graphite using strong oxidizing agents by Brodie. Then onwards different methods like Staudenmaier, Hofmann, Tour etc are proposed for the synthesis of GO. Though all the methods principle follows same the oxidation of graphite but the use of different chemicals for oxidation differs. All these methods use hazardous chemicals and time consuming. Hummer's method of GO synthesis is fast and easy alternative process for the bulk GO preparation (Hummer's et al., 1958). GO can be directly synthesized by the pyrolysis of organic compounds which is a simple and environmental friendly and time saving method (Dong et al., 2012).

2.2 Interaction of GOns with proteins

Graphene oxide surface was abundant in containing oxygen functional groups such as carboxyl groups at the edges of the GO, epoxide groups and hydroxyl groups at the both sides of basal planes. The abundance of the functional groups makes the GO surface reactive for the biomolecules like proteins to interact. The large flat surface provides reactive groups at the both sides makes it a remarkable compound for the loading of proteins on its surface. The protein molecules can be immobilized on the GO surface without using any cross-linkers or conformational changes occurring in the proteins. The adsorption of protein molecules on the GO surfaces is majorly due to the hydrophobic interactions between them. Besides the direct adsorption of protein molecules on the GO surface the covalent binding of protein molecules using cross-linkers like glutaraldehyde were used to achieve more stable binding of the proteins with the GO surface proposed by Su et al., 2003. Xu et al proposed a protocol in which trypsin was immobilized by preparing polylysine (PL) and PEG modified GO as substrate. Here PL-PEG modified GO was used as receptors for the immobilization of trypsin.

The enzyme adsorption on the GOns surfaces was due to the synergetic effect of the interactions between the protein and GO functional groups. The study on interaction between the chymotrypsin and GOns showed that there's is a significant reduction in its activity. This is due to the coexistence of large molecular interaction forces between the GO and enzyme. (De et al., 2011).

The preparation and further crosslinking of human serum albumin protein nanoparticles showing new directions for drug delivery. The key parameter for the protein nanoparticles is its particle size, for its in vivo applications. Preparing the protein nanoparticles with uniform size is a challenging task. The controllable synthesis of protein nanoparticles with uniform particles size distribution was achieved by the desolvation process of proteins. During its synthesis many parameters like pH of the solution, rate of desolvating agent addition, amount of the protein. But the major factor which determines the particle size was the pH. The nanoparticles agglomeration was prevented by varying the pH values outside the isoelectric pH and low salt concentration. Particle size was the major determining factor for the proteins to adsorb in biological fluids. The emulsion methods for the preparation of BSA (bovine serum albumin) protein nanoparticles in the nano sub-sized groups of 200nm range was reported by Muller et al., (1996).

The major disadvantage of the emulsion method is the use of surfactants for the stabilization of emulsion and the use of organic solvents for the separation of oily residues. Overcoming these an alternative approach has been proposed by Lin et al., 1993 that is the desolvation process. This method is surfactant free and the protein nanoparticles of diameter nearly 100nm can be prepared. The method involves the drop wise addition of desolvating agent acetone to the aqueous HAS protein solution and further crosslinking was done using glutaraldehyde. The size range of 90-250 nm HSA nanoparticles were synthesized by controlling the amount and rate of desolvating agent added. The nanoparticles size was reduced by increasing the pH values at the pI=5.3, this is due to increasing ionization of HSA aqueous solution. The efficiency of the desolvation process in preparing nano spherical particles was characterized by mainly the rate of addition of desolvating agent and the amount of crosslinking agent used. The other crucial parameter which determines the particles size is pH of the HSA solution. These HSA nanoparticles are able to taken up by the cells through the process of opsonisation. The surface charge of the nanoparticles synthesized by the desolvation technique is found to be -44mv which defines the good stability of the particles. The very stable solutions of HSA nanoparticles can be used for efficient drug delivery systems. The cellular uptake of the nanoparticles to investigate the drug delivery mechanism the primary human blood derived macrophages was used. It was observed that the nanoparticles of size range

of 100-200nm were taken up into the intracellular compartments of macrophages reported by the Langer et al., 2003.

2.3 Antibacterial activity of GOns

The antibacterial activity of carbon based nanoparticles such as Graphite (Gt), Graphene oxide (GO), reduced Graphene oxide (rGO) at the same concentrations of 100 µg/ml on E. coli was evaluated. Here E.coli cells were incubated with same concentrations and the rate of death was determined by the counting of colonies. The results showed that the percentage of bacterial inactivation of GO sample is six fold higher than the graphite and two fold higher than the rGO proves the antibacterial action of the GO. (Liu et al., 2011).

Recent research showed the antibacterial potential of different carbon based nanomaterials on the growth of P. aeruginosa. At the GO and rGO concentrations of 75 µg/ml the growth of the P. aeruginosa displayed the growth inhibition in the exponential phase. The 90% of the growth reduction was observed after 15 hours of incubation in the presence of GO and rGO. Whereas, comparing to rGO the GO growth curve showed higher inhibition trend. (Gurunathan et al., 2012).

Achieving the minimum extent of toxicity of nanomaterials for its application in nanomedicine is always a challenging task for the researchers. GO at the higher concentrations exhibits toxicity both in vitro and in vivo. (Feng et al., 2011 & Yang et al., 2012). Whereas, functionalization of GO does not exhibit such toxicity towards animals. This is due to the change in the surface chemistry of GO with the biomolecules. The functionalized nGO-PEG compounds had showed higher affinity towards serum proteins and induces complement C3 cleavage less extent than unfunctionalized GO. This shows the capability of attenuation of the complement C3 activation of functionalized GO which was evident for the increased biocompatibility when compared with the free GO. (Tan et al., 2013).

The antibacterial activity of GOns was also explained by the lateral dimension dependent activity of the GOns. The large GOns are capable of trapping around the E.coli surfaces completely when compared to the smaller GOns. The results shows that cell loss viability increased for the GOns size range 0.010 to 0.127 µm but there is no significant increase in cell viability loss even though there is an increase in number of large GO sheets. (Liu et al., 2012).

CHAPTER 3
OBJECTIVES

3. Objectives

- To prepare Graphene Oxide nanosheets (GOns) using controlled pyrolysis of citric acid and modified Hummer's method.
- Characterization of synthesized GOns.
- Surface functionalization of GOns with hen egg white Lysozyme using chemical cross-linking.
- Evaluation of the effect of GOns and fGOns towards the growth kinetics of E.*coli*.

CHAPTER 4
MATERIALS AND METHODS

4. Material

Citric acid was purchased from Loba chemie Pvt Ltd., Mumbai, India. Graphite flakes and crosslinker glutaraldehyde were purchased from Sigma- Aldrich, India. The chemicals used in all experiments are of the pure and analytical grade. The proteins BSA and Lysozyme was purchased from Hi-Media laboratories Pvt Ltd., Mumbai, India. The E.coli strain DH5α stock culture was obtained from Life sciences department, NIT Rourkela. All glassware's were purchased from borosil, India.

4.1 Preparation of Graphene Oxide nanosheets (GOns) by controlled pyrolysis of citric acid

GOns was prepared by pyrolysis of citric acid. 2gm of citric acid was taken in a test tube and heated to 200°c in a furnace. When citric acid is subjected to high temperatures, the powder turns into a pale yellow liquid. Within 5-10 min whole powder turns into liquid. After sometime it turns to pale orange liquid and finally a black mass is obtained which is GO. Now this black mass is dissolved in 50ml of NaOH (10mg/ml) solution. Then GO solution pH was adjusted to 7.0. Finally, the obtained solution was sonicated for 30min using probe ultra sonicator.

4.2 Preparation of GO nanosheets (GOns) by modified Hummer's method:

Hummer's method is generally the easy and inexpensive method that is followed for large scale synthesis of Graphene oxide. This method follows the oxidation of graphite to GO by using sodium nitrate and potassium permanganate. Graphite flakes (0.5g) and sodium nitrate (0.25g) were mixed in 12ml of sulphuric acid under stirring condition for 90min at room temperature. Then, potassium permanganate (1.5g) was added slowly to the mixture under vigorous stirring. In order to avoid explosion, temperature of the beaker is maintained less than 15^0c by placing it in ice bath. Then the beaker is kept at 35^0c. After 1hr the solution turns to pasty brownish colour by evolving gases. Then the mixture was diluted with 25ml of deionized water under vigorous stirring. Then the temperature was increased to 95˚c which resulted in evolution of gas. This should be maintained for 15 min, then the colour changed to brown. This was further diluted with 40ml deionized water and stirred continuously. The reaction was terminated finally by adding 5ml of hydrogen peroxide

(30% v/v). It resulted in change of colour from brown to light yellowish colour. To remove the metal ions from mixture it was further washed with 10% (v/v) HCl for 4 times by centrifugation. Further washed 8 times with deionized water till the pH of solution reaches neutral. The prepared mixture was further sonicated nearly 20 min at 0.5 cycle and 80% amplitude for exfoliation of graphene layers to monolayered GO.

4.3 Preparation of functionalized GOns:

Functionalized GOns was prepared following the protocol described by Mahanta and Paul, 2015. Two ml of 5 mg/ml aqueous solution of lysozyme (Lyz) was stirred at 500 rpm using a magnetic stirrer at room temperature for 10 min followed by addition of 5 ml ethanol (desolvating agent) slowly drop by drop. After desolvation, GOns was immediately added at the same concentrations of protein following the addition of glutaraldehyde to 0.1% and stirred continuously (800 rpm) at room temperature for 12 hr. The prepared functionalized GOns was purified by 3 times of centrifugation at 10,000rpm for 30 min. Finally, the pellet was re-suspended in 2 ml of milliQ water.

4.4 Characterization techniques

The GO nanosheets (GOns) were characterized in a Perkin-Elmer UV-VIS spectrophotometer, Lambda35 conducted at a scan range 200-700nm. The UV-VIS absorption spectra of the GO sample was recorded and plotted in the Excel. Fluorescence emission spectra was done in Perkin-Elmer LS55 fluorescence spectroscopy at the scan rate 300nm/min. X-ray diffraction technique was done to know the crystalline properties of GO. The GO samples were scanned in the range 5°-30° at the rate of 5°/min. FESEM was used to characterize the shape and morphology of the GOns. The average size distribution of particles and surface charge zeta potential was measured using Zeta sizer instrument.

4.5 Antibacterial activity studies

4.5.1 Preparation of fresh E.coli culture

The E.coli stock culture was revived on LB agar plates and a single colony of E.coli was picked and inoculated in LB broth and was allowed to grow until O.D reaches 0.5 at 37˚C and 140 rpm in an orbital shaking incubator.

4.5.2 Growth profile of E.coli in presence of GOns and fGOns

The antibacterial potential of GOns &fGOns were evaluated by observing the growth profile of E.coli, in presence of GOns and fGOns. The prepared fresh E.coli inoculum was inoculated into four different flasks with fresh LB broth. One flask is labelled as E.coli control, the other is for lysozyme, GOns and fGOns separately. GOns with concentration of 300µg/mL was added to the LB media. Similarly fGOns of same concentration was added to the LB media to observe the growth pattern of E.coli in presence of GOns and fGOns. All the culture flasks were incubated at 37˚C in an orbital shaking incubator for 34 hours. At every 2 hour interval for 34 hours absorbance was measured at 600nm with the help of UV-Vis spectrophotometer. Absorbance readings were noted at regular said time intervals for all the samples.

4.5.3 Total intra-cellular protein concentration determination by Bradford assay

Measuring total intra-cellular protein concentration is an indirect observation of the growth of bacterial cells. Fresh E.coli inoculum was inoculated into the fresh media containing GOns and fGOns at concentration of 300 µg/ml in two different flasks. One flask containing only inoculum is used as E.coli culture control. All the culture flasks were incubated at 37˚C in an orbital shaking incubator for 24h. 2ml of culture was withdrawn from the flasks at time intervals of 4, 6, 8, 12, 17, 24h. Then the collected samples boiled for 1min in the water-bath and then centrifuged to collect pellet which contains total cells. The pellet was dissolved in 1ml of 1X TE buffer and further boiled for 1 min in a water bath for cell lysis. Further, all the tubes were centrifuged for 15min at 5000rpm to collect supernatant which contains total intra cellular protein. The total intra cellular protein concentration was measured following the standard Bradford assay protocol. The absorbance

readings were taken at 595nm and the corresponding unknown protein concentration was measured by plotting against BSA standard curve using MS-Excel.

4.6 Disk Diffusion Assay

The antibacterial potential can be studied by disk diffusion assay. Nutrient agar plates were prepared upon with circular Whatmann's filter disks were placed on the agar. The filter discs were dipped in 300µg/mL and 500µg/mL of GOns and allowed to dry for 2 min. Then carefully the disks were transferred onto the bacteria spread agar plates and pressed gently to ensure stable attachment of disks on the surface. The agar plates were inverted and kept at 37˚C and 140rpm for overnight to observe zone of inhibition around disks.

CHAPTER 5

RESULTS AND DISCUSSION

5. Results

5.1 Preparation of Graphene oxide:

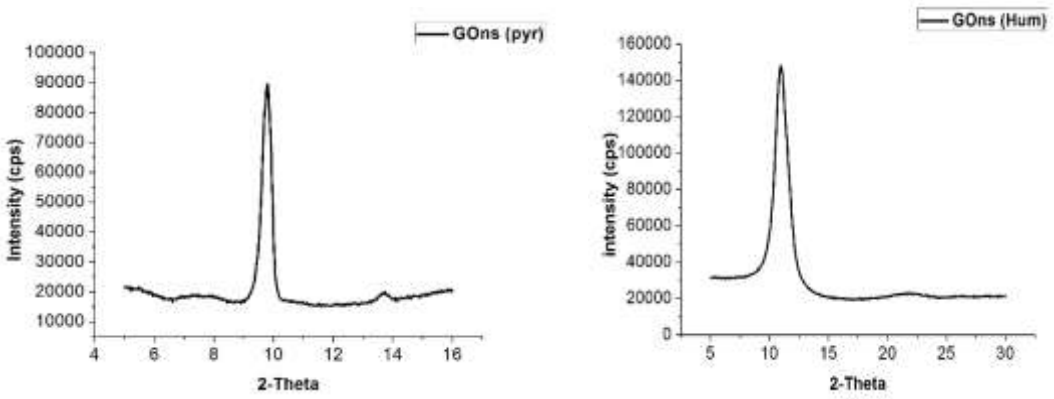
Graphene oxide was synthesized by following the controlled pyrolysis method of synthesis and improved Hummer's method of synthesis as mentioned in the Materials and Method section.

(a) **(b)**

Figure 1: Photographic image of GOns synthesized by: a) modified hummer's method b) controlled pyrolysis of citric acid.

5.2 X-Ray Diffraction Patterns of GOns

XRD analysis is used to find the crystalline properties of the GO. GOns synthesized by controlled pyrolysis of citric acid gives a characteristic peak around $2\Theta = 9.86°$ and the interlayer distance was found to be 0.895 nm. GOns synthesized by modified Hummer's method gives a characteristic peak around $2\Theta = 10.54°$ which is very less compared to graphite peak, this indicates oxidation of graphite powder to GO. The interlayer spacing between GOns was found to be 0.834 nm.

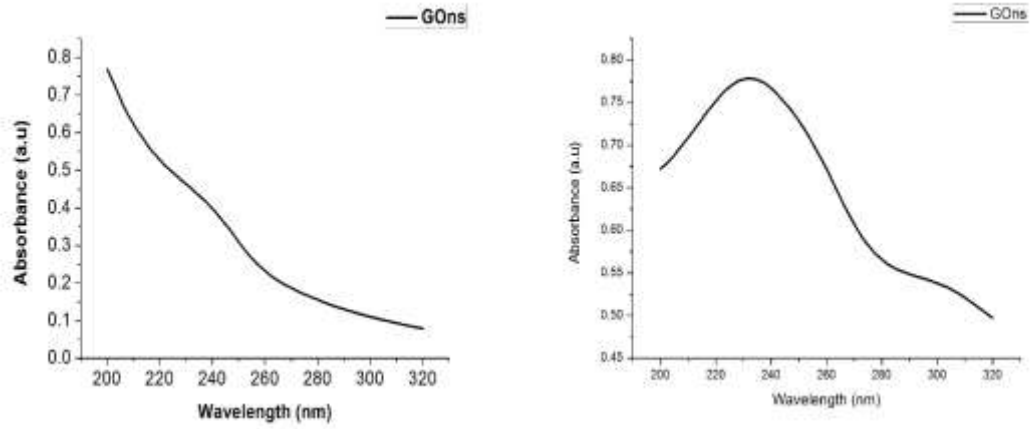


(a) (b)

Figure 2: XRD pattern of GOns synthesized by: (a) controlled pyrolysis method (b) modified Hummer's method.

5.3 UV-Vis Spectral analysis of different GOns

This figure shows the UV-visible absorption spectrum of the suspended GO nanosheets. UV-Vis absorption spectra of GO shows the broadening of peak around 230-235nm. The absorption peak at about 230nm suggest the π- π* transitions of aromatic C-C bond.



(a) (b)

Figure 3: UV-VIS spectra scanned at the range of 200-320nm of GOns synthesized by: (a) controlled pyrolysis method (b) modified Hummer's method.

5.4 DLS size distribution and zeta potential analysis of GOns

Dynamic light scattering (DLS) instruments were used to measure the average particle size distribution and zeta potential measurement which gives the surface charge of the particles. The average particle size distribution of GOns synthesized by controlled pyrolysis method was 125nm whereas, the GOns synthesized by modified Hummer's method particles size was 380nm. The zeta potential measurements of GOns synthesized by controlled pyrolysis method was -54.7 (mv) whereas, the GOns synthesized by modified Hummer's method was -32.3 (mv).

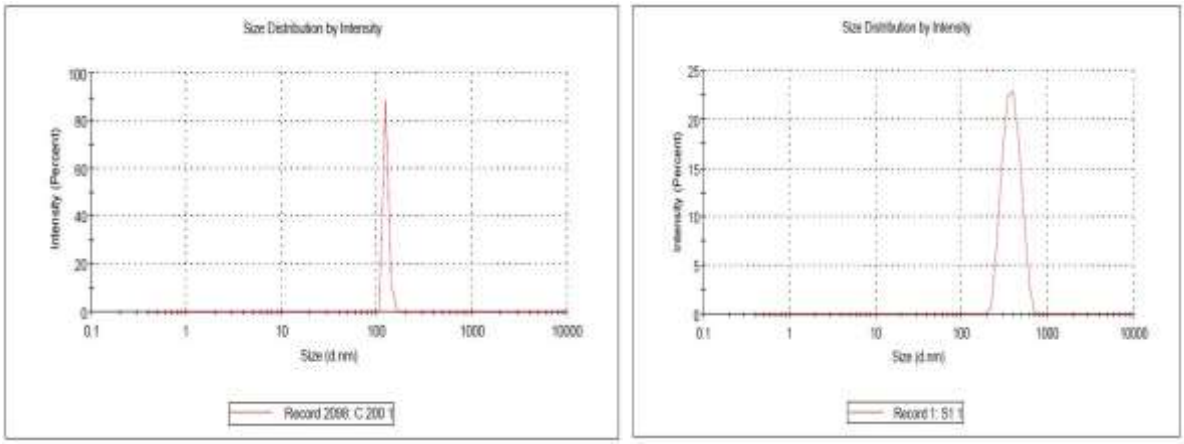
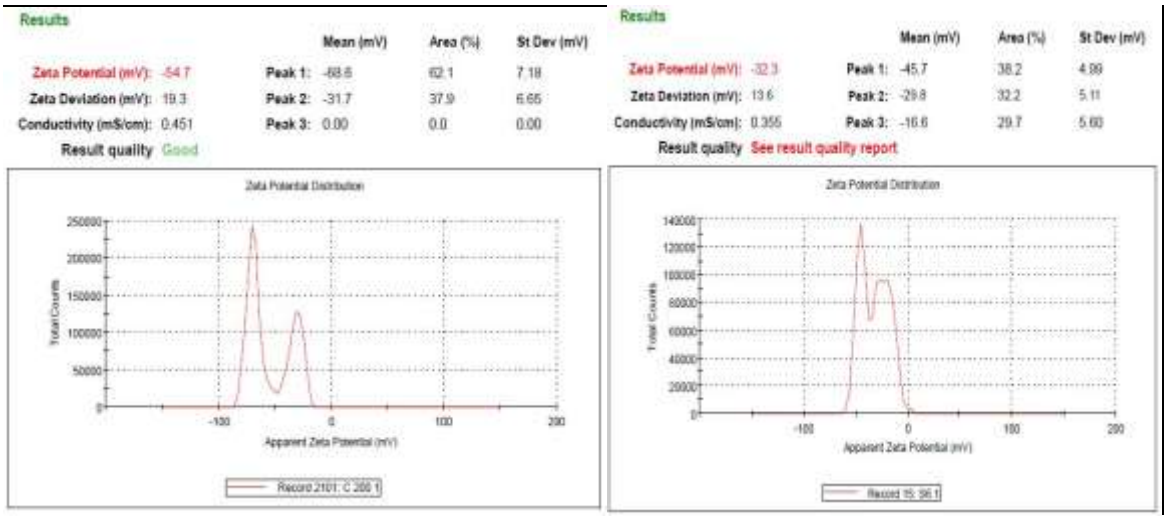


Figure 4: Particle size distribution of GOns synthesized by: a) controlled pyrolysis method b) modified Hummer's method.

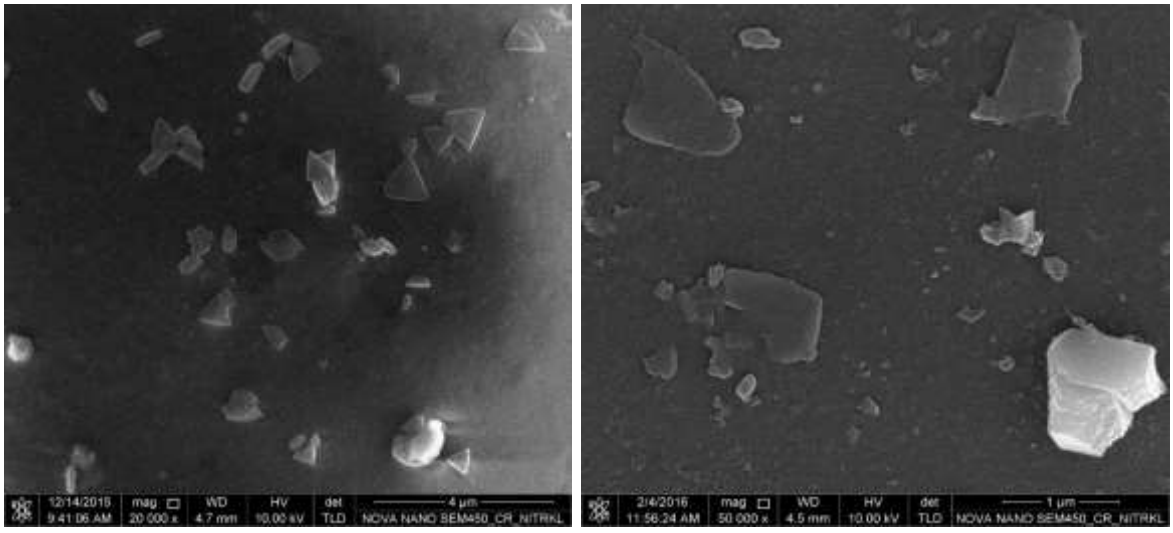


(a) (b)

Figure 5: Zeta potential measurement of GOns synthesized by: a) controlled pyrolysis method b) modified Hummer's method.

5.5 FESEM images of GOns

The morphology and size of GOns synthesized by the two methods were observed under FESEM. The thin nanosheets were of average size 200nm was observed for pyrolysis synthesized GOns whereas, average size of 100nm GOns was observed for improved hummer's method of synthesis.



(a) (b)

Figure 6: FESEM images of GOns synthesized by: a) controlled pyrolysis method b) modified Hummer's method.

5.6 Thermal stability analysis of GOns

5.6.1 TGA analysis

TGA had done to measure the change in the mass percentage of a sample with the temperature. TGA determines the thermal and oxidative abilities of the pyrolysis synthesized GOns. Here, the TGA results showed that the GOns maximum change in mass was observed at the temperature between 150°-200°C.

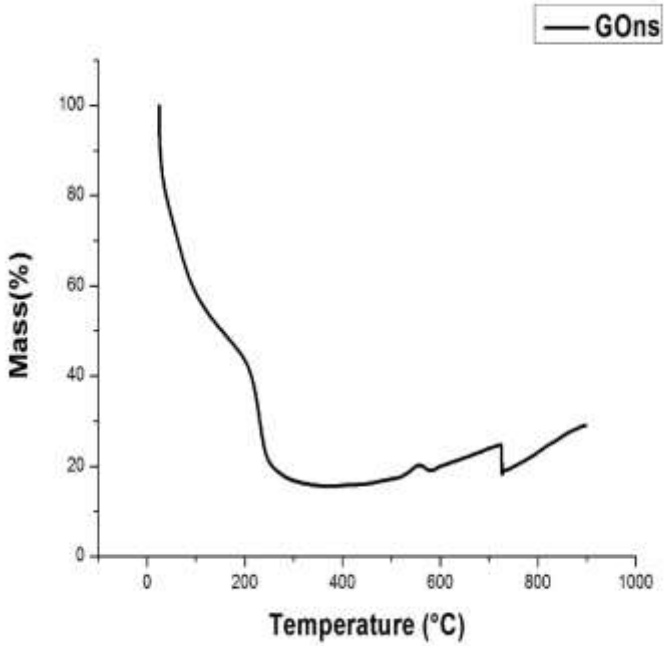


Figure 7: TGA curve of pyrolysis synthesized GOns

5.6.2 DSC analysis

DSC measures the amount of heat need to flow to maintain the temperatures of both sample and reference when a phase transition occurs. The results showed that the phase change in pyrolysis synthesized GOns was occurred at the temperatures of 200°-250°C and 700°-750°C.

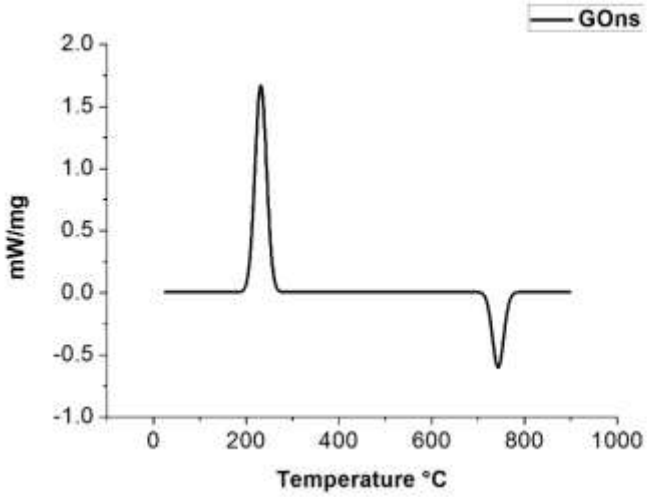


Figure 8: DSC curve of pyrolysis synthesized GOns.

5.7 Characterization of protein functionalized GOns (fGOns)

5.7.1 UV-Vis spectroscopic analysis of fGOns

The broad characteristic peak at 280nm was observed for Lysozyme. For the fGOns a broad peak was observed at 225nm indicates the peak shift. The shift in the peak of lysozyme confirms the formation of fGOns. The peak shift occurs due to the binding of protein onto the surface of GOns results in conformational change of protein.

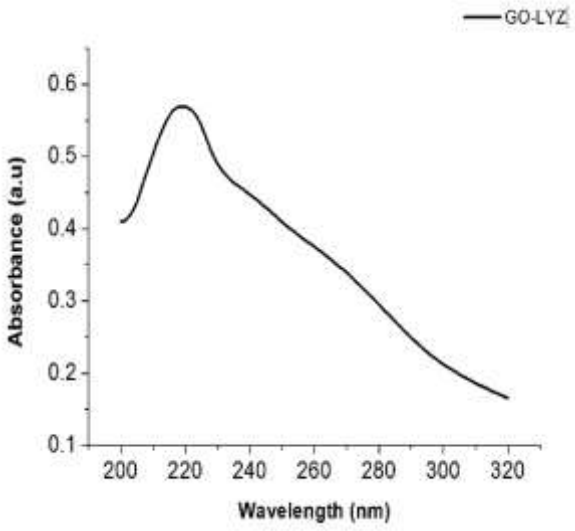


Figure 9: UV-VIS absorption spectra of fGOns at the scan range 200-320nm.

5.7.2 FESEM images of fGOns

The morphology of fGOns was observed by FESEM imaging. The prepared fGOns sample was spotted onto the glass slide and the respective images were taken using FESEM. The images were taken at the scale of 500nm at 10000x magnification. The average size of the fGOns was approximately 250nm. The morphology of the fGOns appears as smooth spherical particles differs from GOns which appears as thin sharp edged particles.

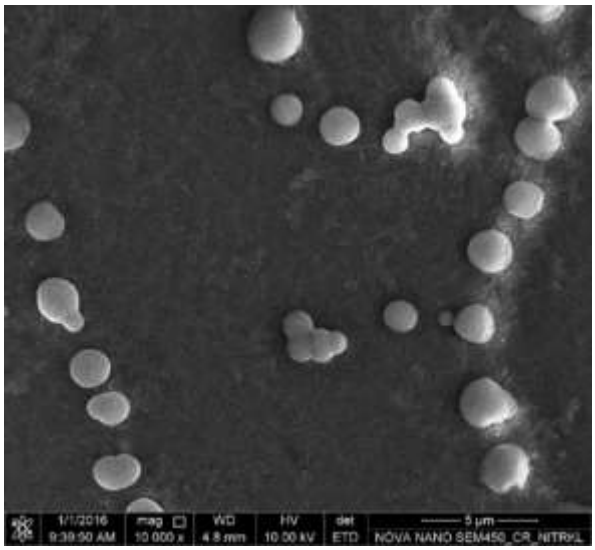


Figure 10: FESEM image of fGOns.

5.8 Fluorescence emission spectra measurements of fGOns

5.8.1 Trp fluorescence of fGOns

The fluorescence emission spectra of lysozyme and GOns was monitored within the spectral scan range region between 310 and 380 nm. The slit width for the excitation and emission were used at 10nm. The Trp fluorescence emission spectra were recorded using excitation maxima of 280nm. It was observed that tryptophan fluorescence intensity was reduced in the presence of GOns. Such a decrease in the Trp fluorescence perhaps may be due to the translocation of the Trp residues on the surface of the protein to an environment having more polar residues which results in quenching of the fluorescence. The decrease in the fluorescence was due to the adsorption of Lysozyme with GOns which results in conformational changes in the protein. Moreover, GOns have been reported to be a good quencher of fluorescence.

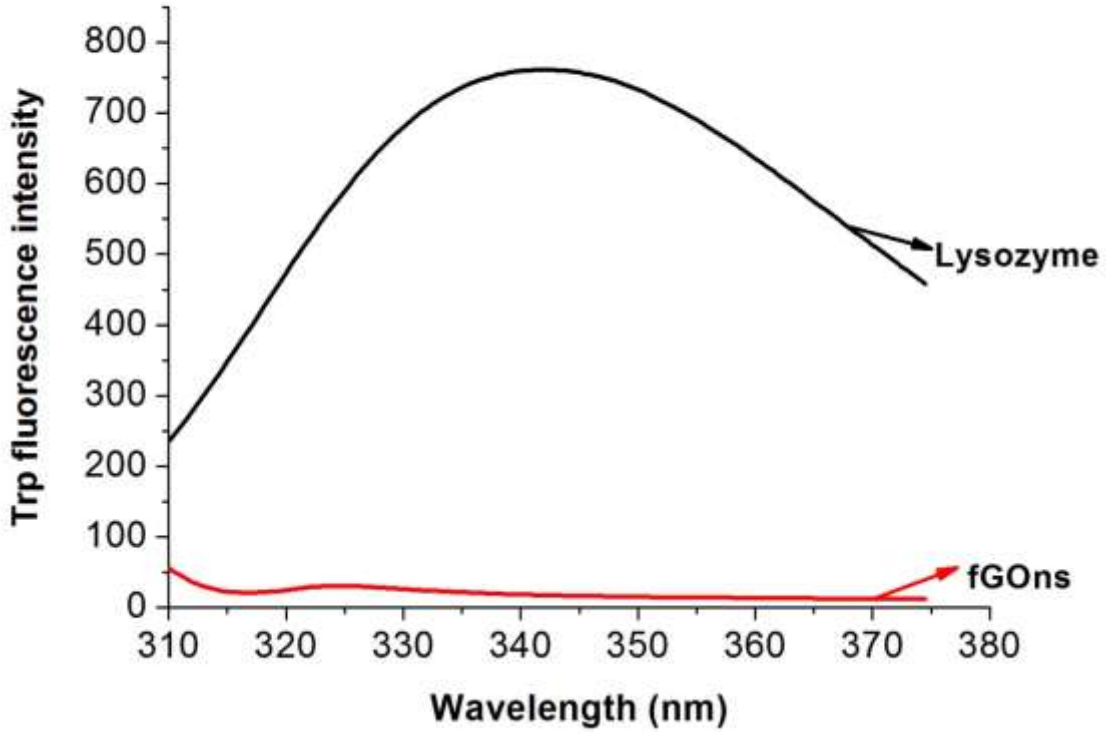


Figure 11: Trp fluorescence emission spectra of Lysozyme and fGOns recorded at the range 310-375nm.

5.8.2 ANS fluorescence of fGOns

ANS fluorescent properties will change as it binds to the hydrophobic regions of the protein. To understand the surface hydrophobic character of fGOns, extrinsic fluorescence using 1-anilino napthalene-8-sulfonic acid (ANS) was also performed. The figure shows that protein-bound ANS fluorescence intensity was reduced for fGOns compared to free LYZ. The reduced intensity of fluorescence for fGOns indicates that the binding of lysozyme protein on GOns surface.

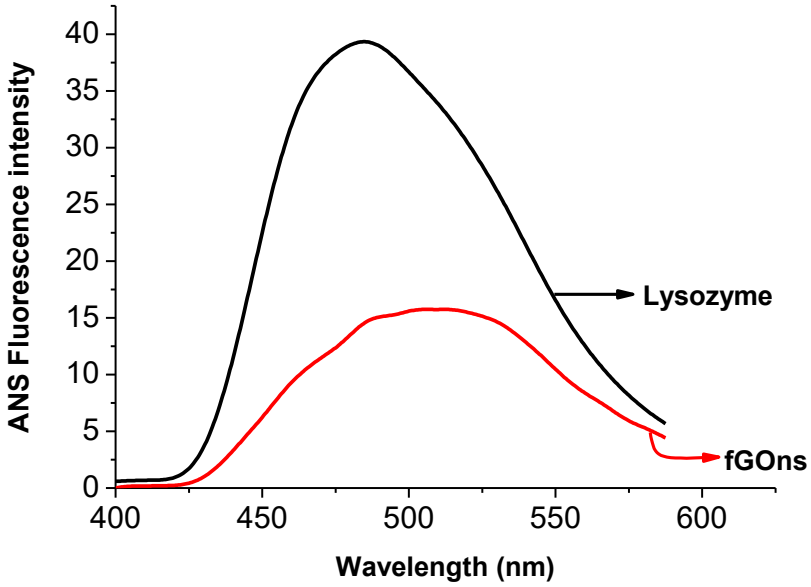


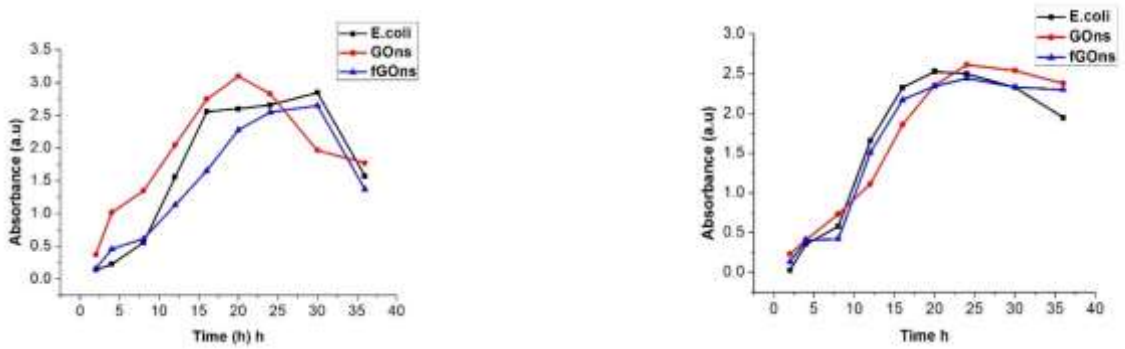
Figure 12: ANS Fluorescence spectra of lysozyme and fGOns recorded at the range 400-580nm.

5.9 Effect of GOns and fGOns on E.coli growth

5.9.1 Time vs Absorbance growth profile of E.coli

At 300 µg/ml of GOns and 300 µg/ml of fGOns prepared by controlled pyrolysis method the E.coli growth profile was reduced compared to control. The absorbance readings were taken at the wavelength of 600nm for every 2 hours total of 34 hours. The reduction of growth can be due to the action of test samples. There is significant inhibition of E.coli growth in the presence of GOns and fGOns as evident from the log phase of the growth profile. But fGOns exhibiting less inhibition when compared to free GOns during log phase growth profile.

At 300 µg/ml of GOns and 300 µg/ml of fGOns synthesized using modified Hummer's method the E.coli growth was enhanced for GOns when compared to control. Whereas, fGOns exhibiting growth inhibition during log phase. The absorbance readings were for the bacterial culture taken at the wavelength of 600nm at time intervals 2, 4, 8, 12, 16, 20, 24 h.



(a) **(b)**

Figure 13: E.coli Growth profile of GOns and fGOns at the time interval of 2-36 h and concentrations of 300 µg/ml: a) pyrolysis GOns b) modified Hummer's GOns.

5.9.2 Determination of total intracellular protein concentration by Bradford assay

At the concentration of 300 µg/ml of GOns and 300 µg/ml of fGOns prepared by pyrolysis of citric acid, the E.coli intracellular concentration was measured at different time intervals of culture time and then compared with control. As observed in the above section there is significant inhibition of E.coli growth in the presence of GOns and fGOns during the log phase, the total intracellular protein concentration was not significantly increasing because of the cell lysis. But fGOns shows slightly more significant increase in protein concentration indicates less inhibition of growth during log phase when compared to free GOns.

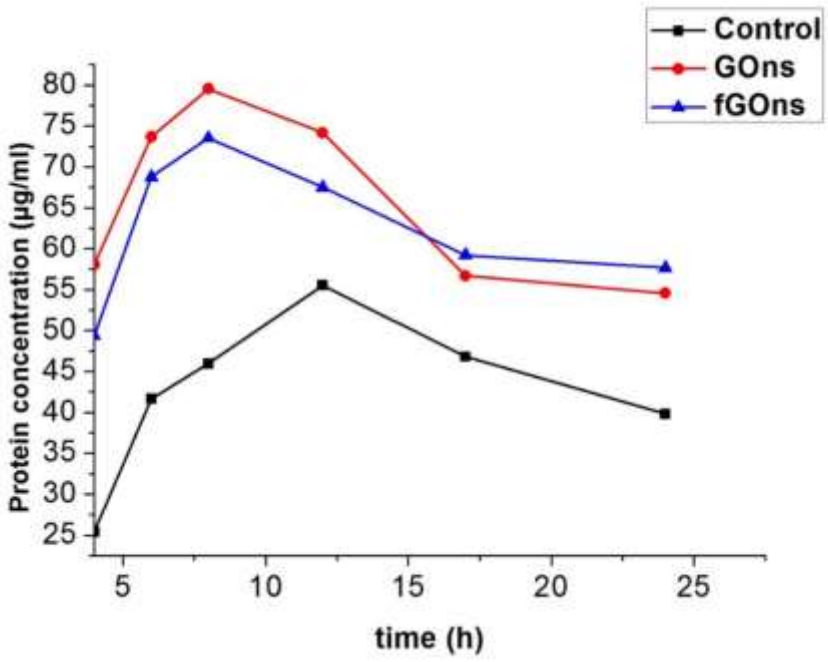


Figure 14: Time vs E.coli protein concentration profile of GOns and fGOns synthesized by controlled pyrolysis at the time interval of 4-24 h.

The GOns and fGOns synthesized by modified Hummer's method at the concentrations of 300 µg/ml was added to the E.coli culture. The total intracellular protein concentration during the E.coli culture time at different time intervals 4, 6, 8, 12, 17, 24h was measured by collecting samples. The Bradford assay standard procedure was followed for protein concentration estimation as mentioned in the section 4.5.3. As the observation from the fig.15 the GOns showed increasing total protein concentration with time shows that GOns enhancing the growth of bacterial cells when compared to control. The fGOns protein concentration profile shows that there is growth inhibition as the protein concentration levels are not significantly increasing when compared to control after 6h.

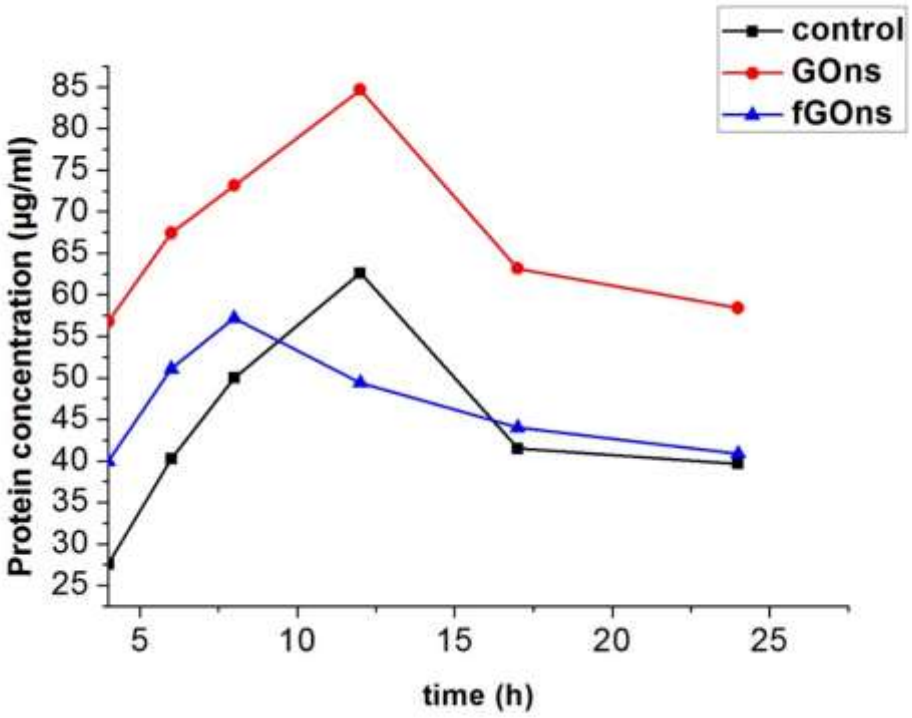


Figure 15: Time vs E.coli protein concentration profile of GOns and fGOns synthesized by modified hummer's method. .

5.10 Disk diffusion assay of GOns:

The filter disks dipped in the concentrations of 500 µg/ml GOns are placed on the surface of agar after drying for 2min. The plates were inverted and kept in incubator at the temperature of 37°c for the overnight. No zone of inhibition was found. It might be the reason that GOns is non-diffusing in agar gel due to its shape.

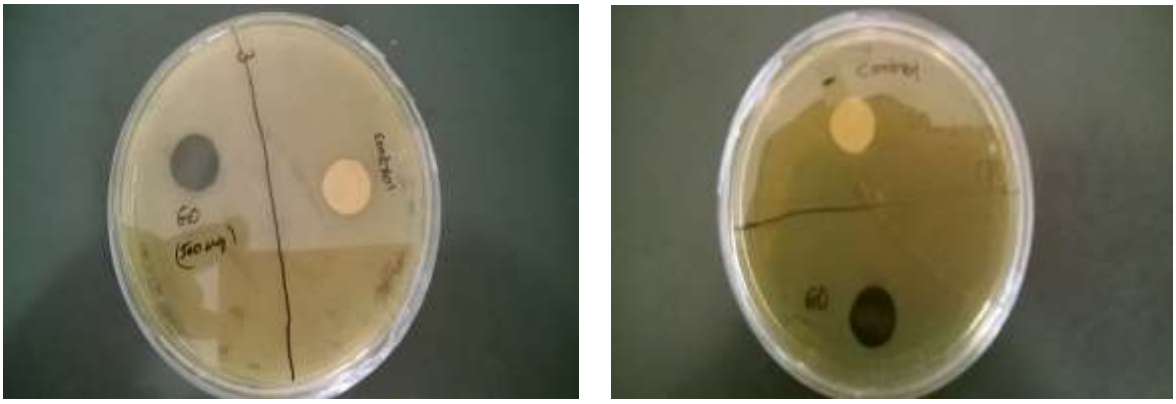


Figure 16: (a) pyrolysis synthesized GOns spotted disks on E.coli spreaded agar plates (b) hummer's synthesized GOns spotted disks on E.coli spreaded agar plates.

CHAPTER 6

CONCLUSION

6. Conclusion

Among various carbon based nanomaterials, GO has drawn much attention due to its outstanding physico-chemical and biological characteristics. Here the GOns was synthesized using an environment friendly and simple approach controlled pyrolysis of citric acid and modified hummer's method. The biophysical techniques like X-ray crystallography, UV-Vis spectroscopy techniques confirms the synthesis of GOns. DSC and TGA curves analyses the GOns thermal stability. Whereas, UV-Vis spectroscopy, tryptophan fluorescence and ANS fluorescence confirms the formation of fGOns. FESEM imaging analysis has done to observe the morphology and size of the GOns and fGOns. The average particle size distribution and zeta potential measurement has done using Zeta sizer instrument. The antibacterial activity of GOns and fGOns towards a gram negative bacterium E.coli was compared. The growth profile inhibition results shows that GOns and fGOns synthesized by pyrolysis method exhibiting bacterial growth inhibition whereas most of the bacterial growth inhibition was observed during log phase only. fGOns exhibiting less bacterial growth inhibition when compared to free GOns. Hummer's method GOns growth profile results showed the enhanced growth of bacterial cells.

CHAPTER 7

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