

Synthesis, characterization of reduced graphene oxide using –Malus pumila and –Dellinea indica extracts and study of its antimicrobial properties

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Research Article

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Abstract

In this work, reduced graphene oxide is prepared using *Malus pumila* and *Dellinea indica* plant's aqueous extracts, via sonication method. After ultrasonication the brown colored graphene oxide (GO) gets converted into black colored reduced graphene oxide (rGO) which indicates the successful reduction of graphene oxide. Characterization techniques like Fourier transform infrared spectroscopy (FTIR), Field Emission Scanning electron microscopy (FESEM), Raman spectroscopy and X-ray diffraction spectroscopy were used to confirm the reduction process. FTIR confirms the presence of bioactive molecules in the phyto extracts and microorganisms which are used to reduce the graphene oxide. Raman analysis confirms the reduction of GO by observed increase in the I_d/I_g ratio. The presence of oxygen containing groups and its removal were characterized through structural analysis. The field emission scanning electron microscopy performed to confirm the conversion of graphene oxide into reduced graphene oxide. The synthesized rGO were tested for antimicrobial activities and were found to have efficient antimicrobial activity.

Introduction

Technology has revolved ever since knowing the unique properties of graphene after its invention in 2004. Graphene is the first material isolated in two dimensions (2D). [1] [2] [3] Graphene belongs to the class of carbon materials where 2D materials made up of conjugated sp^2 carbons are arranged in a honeycomb pattern. Graphene is a monolayer of sp^2 hybridized carbon atoms in honeycomb pattern. It has unique two dimensional structure and electronic, mechanical and optical properties. [4][5] Graphene a flexible material has excellent thermal conductivity and high electrical mobility at room temperature. These properties of graphene make it a useful material in the fields of electronics, energy storage as well as biological applications. [1][6] Currently most common approach for large scale production of graphene is through chemical reduction of graphene oxide. A number of approaches are available including chemical, electrochemical and thermal reduction [4] [7]. There are basically three approaches to synthesize graphene: direct growth by chemical vapor deposition, mechanical exfoliation of graphite, exfoliation of graphite oxide followed by its reduction. These approaches may be causing harm to the environment. [7][8]. Therefore it is advisable to use methods which are environment friendly. Chemical vapor deposition generates graphene film in a large area. Being the high temperature process it increases cost and makes it unsuitable for direct fabrication of graphene. Mechanical cleavage is laborious and cannot produce large graphene films. The third method of exfoliation of graphite oxide followed by its reduction is the efficient one. But the quality of rGO depends on the reducing agents and other experimental conditions. Various reductants used for the process are sodium borohydride, hydrazine hydrate, dimethyl hydrazine, hydroquinone, hydroiodic acid etc. Some of these reagents are toxic and chemical reduction may require from few hours to few days [7][9]

In this study, we have synthesized reduced graphene oxide by using chemical, biological and microbial pathways. Along with the method of chemical reduction using hydrazine hydrate, two microbial species and extracts of two different fruits were used for the reduction purpose. The microbial species used are

Escherichia coli, *Pseudomonas aerogenos* and *Staphylococcus aureus* and the fruits of the plants selected were Apple fruit extract and Elephant Apple fruit extract.

Escherichia coli abbreviated as E coli are the bacteria found in the environment, and in the intestines of healthy people and animals. E coli are a large and diverse group of bacteria. It is a Gram-negative, anaerobic, coliform bacteria. Most strains of E coli are not harmful.

Pseudomonas aerogenosa is a type of bacteria that is found commonly in the environment. Out of the many different types of *Pseudomonas*, the one that most infectious in human beings is called *Pseudomonas aeruginosa*, which can cause infections in the blood, lungs (pneumonia), or other parts of the body after surgery.

Apple (*Pyrus malus*) fruit extract

Pyrus malus is a species of apple. The fruit is a tremendous source of polyphenol antioxidants.

Elephant Apple fruits extract

Dillenia indica commonly known as Elephant Apple is a small to medium sized tree growing upto 15m tall. Its characteristic round fruits are large greenish yellow, with multiple seeds and the fruits are edible. The fruit pulp is sour and it is used in Indian cuisines. The extracts of fruits of above two plants is used for the reduction of graphene oxide.

Graphite oxide has a layered structure similar to that of graphite, but between the two planes of carbon atoms in graphite oxide are the oxygen-containing groups, which increases the interlayer distance and also makes the atomic-thick layers hydrophilic. Ultra sonication is carried for the exfoliation of these sheets.

When these exfoliated sheets contain only one or a few layers of carbon atoms like graphene, then these sheets are named graphene oxide (GO). So, GO is a single-atomic-layered material made up of carbon, hydrogen, and oxygen elements obtained by the oxidation of graphite. It is dispersible in water and easy to process. Most importantly, the GO can be reduced to graphene-like sheets by deletion of the oxygen-containing groups and with the recovery of a conjugated structure. The reduced GO (rGO) sheets are usually considered one kind of chemically derived graphene and are known as rGO. Other names given to rGO are functionalized graphene, chemically modified graphene, chemically converted graphene, or reduced graphene.

The main important advantages of reduced graphene are: (i) it can be prepared using cheap raw material graphite and by using cost-effective chemical methods with a high yield and (ii) it is highly hydrophilic and forms stable aqueous dispersions.

The graphene sheet consists of only trigonally bonded sp² carbon atoms and is perfectly flat. The heavily oxygenated GO sheets consist partially of tetrahedral bonded sp³ carbon atoms, which are

displaced slightly above or below the graphene plane. Graphene is a monolayer of sp^2 hybridized carbon atoms arranged in a honey comb lattice.

There are three different types of methods for the synthesis of the graphene which are:

- a. growth by chemical vapor deposition,
- b. mechanical exfoliation of graphite and
- c. exfoliation of graphite oxide followed by its reduction.

Reduction of GO is a much more efficient, low-cost method for bulk production of graphene sheets. GO is obtained from natural graphite powder by modified Hummers method. GO can be reduced to graphene either by chemical methods (like exposure to pure hydrazine or sodium borohydride), or by thermal treatment and by various other methods. Each method has its own benefits. In this study we have discussed few methods and properties exhibited by the materials synthesized by these methods.

Numerous physical and chemical routes are available for the synthesis of graphene. The first successful approach to get single-layer graphene sheets was mechanical cleavage of graphite [1]; however, it is difficult to control the number of layers for peeled-off fragments, and it gives low yield so not useful for large-scale production. Epitaxial growth of graphene layers requires high temperatures and an ultrahigh vacuum environment, which is expensive and thus it is less used and limits the widespread application of graphene. Chemical vapor deposition (CVD) produces large area films of 1 to ~ 12 graphene layers [19][20][21][22], but a step is added to remove the Ni particles in order to obtain graphene sheets. Li et al [23] have reported the CVD-growth of predominantly single-layer graphene with lateral sizes up to centimeters on copper foils using methane; however, a high temperature (1000°C) and vacuum were required for the graphene synthesis. Graphene oxide (GO) is a water soluble nanomaterial. It is hydrophilic and molecules of water can easily intercalate between the graphite layers [24][25][26]. Three methods are used for the synthesis of graphite oxide: Staudenmaier [27], Brodie [28] [29], Hummers-Offeman/ (Hummers Method) [30] [31]. All these methods involve the oxidation of graphite, but differ in the type of oxidizing agents, mineral acids used, time of preparation and the type of washing and drying processes. Graphite oxide synthesized from Hummers method has higher interlayer distance and C/O atomic ratio than the remaining two methods and thus it is widely used. Generally GO is insulator in nature; but it is made conductive by exposing it to reducing agents like hydrazine hydrate, hydroiodic acid or Sodium Boro Hydrate, through high temperature treatment or by UV assisted photo catalysis and thermal treatment (low- temperature annealing reduction of graphite oxide). Hydrazine reduced GO shows remarkable electrical properties. The chemical reduction of graphite oxide is one of the accepted procedures to make reduced graphene oxide films in large scale. Here we made an attempt to synthesize reduced graphene oxide films by oxidation of graphite to form graphite oxide followed by exfoliation and reduction of GO.[34][35].

Materials and Methods

Graphite powder, sodium nitrate, sulphuric acid, potassium permanganate, hydrochloric acid were purchased from Sigma-Aldrich. The other chemicals like Sodium borohydride etc. were of analytical grade. Whatman filter paper no 41 were used as per the requirement. Demonized water was used wherever required.

Preparation of Microbial Broth was done by the following method

1. Add 13g of nutrient broth powder (CM0001B) in 1L of distilled water.
2. Mix and dissolve them completely.
3. Pour them into the final containers (e.g. conical flask)
4. Sterilize by autoclaving at 121°C for 15 minutes.

Preparation of Phytoextract: From Apple and elephant apple fruit

The fruit of the plant was thoroughly washed with tap water and then by deionized water. It is peeled off with the peeler and then sliced into small pieces before grinding into the paste. 100 gm of fruit juice was used for the reduction purpose.

Preparation of Graphene oxide

The preparation process of rGO consists of three steps: i) Oxidation or intercalation (ii) exfoliation and (iii) reduction. Firstly Graphite oxide was prepared from the graphite powder by modified Hummer's method. A 2g of graphite powder mixed thoroughly with sodium nitrate is put into concentrated H_2SO_4 (46ml). KMnO_4 (6 g) is added gradually with stirring and cooling in an ice bath, so that the temperature of the mixture was maintained below 20°C. The mixture again stirred at 35°C for 45 min, and distilled water (75 ml) is added slowly which causes violent effervescence and temperature is increased to 85-90°C. After one hour, the reaction is terminated by the addition of distilled water (100 ml) and 30% H_2O_2 solution (30 ml), after which the color of the suspension changes to bright yellow. The suspension is then washed with 1:10 HCl solution (300 ml) for removal of metal ions. Then it is filtered with Whatman filter paper using vacuum filter pump. The paste collected from the filter paper is then dried at 60°.

Chemical method of synthesis of Reduced Graphene Oxide

In the preparation of reduced graphene oxide, first graphite oxide (100 mg) is added with distilled water in a 250 ml conical flask; this gave an inhomogeneous yellow-brown dispersion. This dispersion was sonicated using a ultrasonication bath cleaner (150 W). Hydrazine hydrate (1 ml) was then added and the solution was heated in the oil bath at 90°C for eight hours over which the reduced GO gradually precipitated into a black solid. This product was isolated by filtration and dried at room temperature. The resultant product is the reduced graphite oxide [26]. Finally for exfoliation, reduced graphite oxide was taken in the concentration 3.5mg/ml and the suspension was subjected to ultrasonication for 30 min.

Biological method of synthesis of Reduced Graphene Oxide

1gm of graphene oxide is mixed with 100 ml deionized water and ultrasonicated for one hour. 100ml of fruit extract is added to it and ultrasonication is continued for 12 hours. After 12 hours it is filtered and dried in oven at 60°C.

Results and Discussion

FT-IR spectra were recorded using spectrophotometer. FT-IR spectroscopy was performed to analyze the presence of various functional groups. FT-IR spectra of graphene oxide exhibits the presence of oxygen functionalities in graphene oxide. The peaks for these oxygen functionalities are weakened in the IR spectra of reduced graphene oxide.

Raman Spectra

To verify the presence of graphene oxide and ensure reduction of graphene oxide Raman spectroscopy is done. It is one of the most reliable technique and potent tool to differentiate between GO and rGO. Raman spectrum is useful to know the defects in graphene layers and their electronic structures. Raman analysis of graphene materials is based on formation and intensity of D and G bands. Generally D band is formed because of breathing mode k point photons of A_{1g} symmetry and G band is formed based on first order scattering of E_{2g} photon of sp^2 carbon atoms. Raman spectrum of chemically synthesized GO and rGO are shown in the diagram. For graphene oxide G and D bands are observed at 1599 and 1355 cm^{-1} respectively. After the reduction by the plant extract and microbes D band is observed at 1333.04, 1336.402 and 1343.50 cm^{-1} respectively whereas G band appeared at 1572, 1573 and 1575 cm^{-1} respectively.

The crystalline structure of the synthesized GO and rGO are confirmed using powder XRD. Graphite flakes show a characteristic peak of 26.6 whereas graphite on chemical oxidation peak is shifted to 10.34 which is attributed to (001) plane and revealed that graphite is completely oxidized to graphene oxide.

Further after sonication with plant extracts and microbes graphene oxide is reduced to reduced graphene oxide, it gives broad peaks at 26.8, 25.5 and 27.35 respectively for treatment with *E coli*, *S aureus* and apple extract respectively. This proves efficient reduction of graphene oxide by biological method.

FE-SEM spectral images

FE-SEM is used to know the surface morphology of the substance. In the given images we can observe the presence of layers which indicates that exfoliation has occurred during oxidation process and also

during ultrasonication for reduction process. GO sheets are different than graphite by presence of vast oxygen containing groups over the surface thus reducing the electrical conductivity. Chemically synthesized GO is found to be much thicker because many layers are stacked one above the other. rGO images show that reduced graphene oxide sheets are well-exfoliated and thickness much reduced than GO. This proves that biologically active molecules present in the plant extract play important role in elimination of oxygen functionalities from surfaces.

Antimicrobial properties (Kirby Bauer Method)

Antimicrobial activity test was performed by the commonly used Agar diffusion method which is designed to determine the smallest amount of the antibiotic required to inhibit the growth of the microorganisms. In a typical procedure a sterile cotton swab dipped into a culture of Test organism suspension was taken. The entire agar surface of each plate was inoculated first in horizontal and then in vertical direction to ensure the even distribution of the microorganism over the agar surface using the swab. The agar surface was dried for 5 min. These Mueller–Hinton agar plates were taken and aseptically (4-mm) holes were punched using a presterilised cork borer in the agar. Using a wax pencil, mark the underside of the Petri dish to label the wells. With the help of micropipette test solution was added in the well. The procedure was repeated for all wells and incubation of all plates was carried at 37°C for 24-48 hrs. in an incubator.[36]

1. Examine all the plates for the clear zone of inhibition surrounding the discs/ well.
2. Measure the diameter of zone of inhibition in mm using a ruler on the underside of the plate. Record the zone size. (K R Aneja.)

Following strains of microbial species were used.

- | | |
|--|--|
| 1. Ec: - <i>Escherichia coli</i> (NCIM2065) | 2. Pm: - <i>Proteus mirabilis</i> (NCIM2388) |
| 2. Sa: - <i>Staphylococcus aureus</i> (NCIM2178) | 4. Bs: - <i>Bacillus subtilis</i> (NCIM2063) |
| 5. Ca:- <i>Candida albicans</i> (NCIM 310) | 6. An: - <i>Aspergillus niger</i> (ATCC504) |

Conclusion

In the present study, graphene oxide (GO) was successfully synthesized from graphite flakes using modified Hummers method and its further reduction is carried out biologically using plant extracts of the fruits of the plants apple and elephant apple and microorganisms to obtain reduced graphene oxide (rGO). This biological reduction method exemplified to be an eco-friendly, cost-effective, and sustainable alternative to traditional chemical reduction methods. Characterization studies confirmed the successful synthesis of graphene oxide and its reduction. FT-IR analysis revealed the significant decrease in percentage of oxygen-containing functional groups in the product, indicating its effective deoxygenation.

Raman spectroscopy confirmed the structural transformation from GO to rGO through characteristic shifts in D and G bands and Id/ Ig ratio 2D and 2G peaks, whereas XRD analysis demonstrated structure after biological reduction. FE-SEM images showed well-exfoliated, thin, and wrinkled graphene sheets, confirming effective ex-foliation, reduction and improved morphology of rGO. The antimicrobial studies proved that both GO and biologically reduced graphene oxide exhibit inhibitory activity for various bacterial and fungal strains including *Escherichia coli*, *Proteus mirabilis*, *Staphylococcus aureus*, *Bacillus subtilis*, *Candida albicans*, and *Aspergillus niger*. Among the synthesized materials, biologically reduced graphene oxide samples showed comparatively enhanced antimicrobial activity, indicating that biological reduction improves the functional properties of graphene-based materials. The antimicrobial effect is found to be concentration dependent and is may be due to membrane disruption, oxidative stress, or surface interaction of graphene sheets with microbial cells.

To summarize, the study establishes that biological synthesis of reduced graphene oxide using plant extracts and microorganisms is an efficient, environmentally benign approach. The synthesized rGO exhibit promising structural, morphological, and antimicrobial properties, indicating its potential applications in biomedical materials, antimicrobial coatings, water purification, other nanotechnology-based applications and sensors. Further investigations on large-scale synthesis will enhance its applicability in advanced electronics fields.

Declarations

Conflict of Interests:

On behalf of all authors, the corresponding author states that there is no any financial and non-financial conflict of interest.

Author Contribution

Dr S Waghmode made substantial contribution for conceptualization and interpretation and revised manuscripts. Dr S Gadade assisted in the characterization of materials synthesized, its interpretation and draft writing. Dr N Patil assisted in antimicrobial activities of all synthesized materials and its interpretation and draft writing. Dr D Butala carried all experimental work, analysis; drafted the work , approved the version to be published. All authors contributed towards submission of the article.

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Data Availability

No datasets were generated or analyzed during the current study. The data related with study is provided in the supporting materials or can be made available on requests.

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Tables

Tables 1 to 5 are available in the supplementary files section

Figures

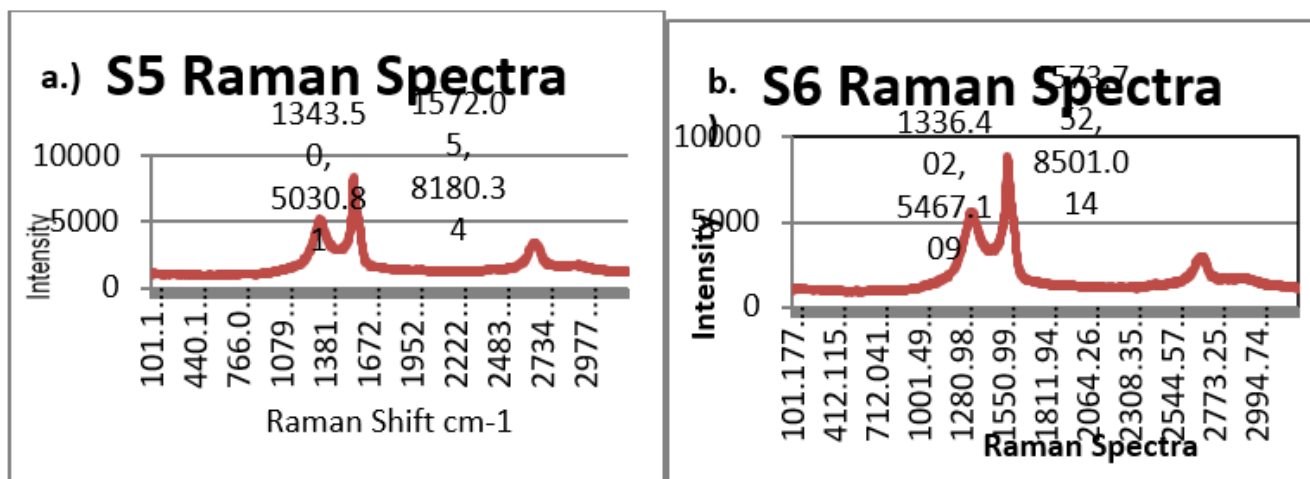


Figure 1

Raman Spectra of (a) reduced graphene oxide prepared by reduction using phytoextract (b) Reduced graphene oxide prepared by microorganisms

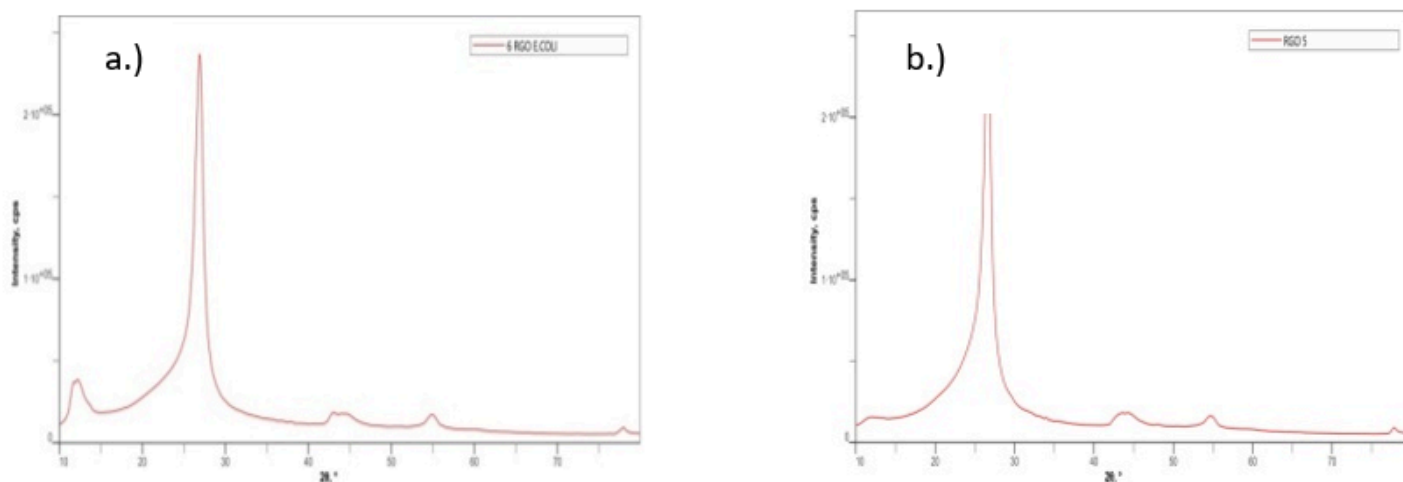


Figure 2

XRD spectrum of reduced graphene oxide prepared by (a) phytoextract (b) microorganisms

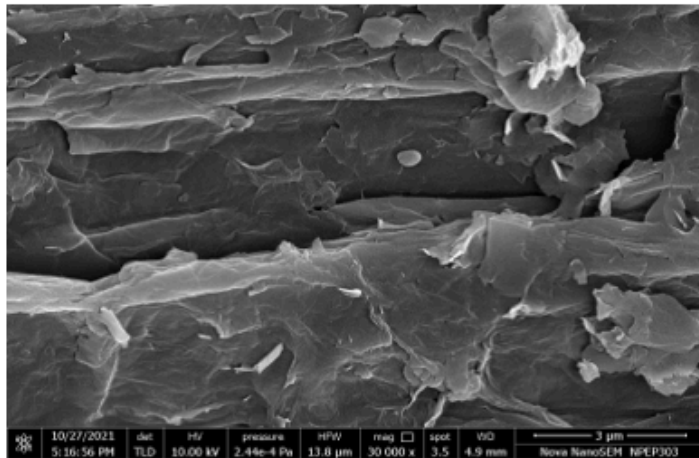
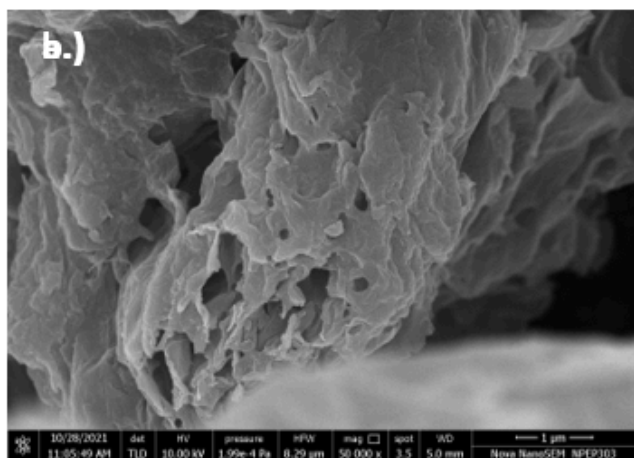


Figure 3

FE-SEM of reduced graphene oxide produced by (a) phytoextract (b) microorganisms

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