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| Citation       | Parviz, Dorsa and Michael Strano. “Endotoxin-Free Preparation of Graphene Oxide and Graphene-Based Materials for Biological Applications.” Current Protocols 10, 4 (December 2018): e51 © 2018 John Wiley & Sons, Inc. |
|----------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| As Published   | http://dx.doi.org/10.1002/cpch.51                                                                                                                                                                      |
| Publisher      | Wiley                                                                                                                                                                                             |
| Version        | Author’s final manuscript                                                                                                                                                                          |
| Citable link   | https://hdl.handle.net/1721.1/125976                                                                                                                                                                |
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Endotoxin-Free Preparation of Graphene Oxide and Graphene-based Materials for Biological Applications

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Abstract

Due to their two-dimensional structure and unique properties, graphene and its derivatives have been extensively studied for their potential applications in various fields ranging from electronics to composites. Particularly, their high surface area, electrical conductivity, mechanical strength, dispersability in aqueous phase, and possibility of surface modification make them promising candidates for biomedical applications including biosensing, drug delivery, tissue engineering, cell imaging, and therapeutics. The functioning of graphene nanosheets in these applications is dependent on their structure and properties, which are mainly determined by their preparation and processing methods. Exfoliation techniques are the most common methods for preparation of graphene nanosheets for biomedical applications due to their high yield and scalability. Further modification of these methods is necessary to produce biocompatible and toxin-free graphene that can be safely incorporated into biological media. Here, we describe protocols for chemical and mechanical exfoliation of graphite to produce endotoxin-free and highly stable graphene oxide and graphene dispersions. Additional protocols are provided for proper pre- and post-processing of nanosheets and endotoxin measurement techniques.

Keywords
Graphene; graphene oxide; exfoliation; liquid-phase exfoliation; oxidation; endotoxin; colloidal stability

INTRODUCTION

This protocol outlines two main methods for preparation of endotoxin-free (a) graphene oxide, and (b) graphene dispersions for biological and medical applications. Basic Protocol 1 and Alternate Protocol describe two different modified hummer’s methods for high-yield synthesis and exfoliation of graphene oxide (GO) from bulk graphite. It is feasible to maximize the oxidation of graphite and the production yield by pre-oxidation of graphite before using it in main synthesis process (Supporting Protocol 1). Basic Protocol 2 describes the direct liquid-phase exfoliation of graphene from graphite in aqueous systems using biocompatible stabilizers to achieve higher yield and dispersion stability. All these protocol are modified to prevent endotoxin-contamination in various steps of sample preparation.
Thus, proper measurement of endotoxin level in samples is a significant to assure that this goal is achieved (Supporting Protocol 2). Moreover, the GO and graphene nanosheets produced using these methods have a broad size distribution. Post-processing of the dispersions using centrifugation method is required to narrow down the size distribution and enhance the colloidal stability of the product (Supporting Protocol 3). The graphene dispersion contain excessive unbound stabilizer molecules, which are not desirable in many application, hence, Supporting Protocol 4 provides guidelines for removal of the excessive stabilizer.

Since the exfoliation of a single-layer graphene nanosheet in 2004, graphene and its derivatives have been vastly studied for their potential biological and biomedical applications. The high electrical conductivity and surface area of pristine graphene (non-functionalized form of graphene) are the origins of the increased interest in the graphene-based biosensors (Chung et al. 2013). These properties have motivated researchers to investigate the potential application of graphene in cell growth, cell differentiation, and tissue engineering (Bitounis et al. 2013). On the other hand, graphene oxide (the functionalized counterpart of graphene) has a different surface chemistry that offers hydrophilicity, biocompatibility, surface reactivity, and fluorescence quenching (Dreyer et al. 2010). Attempts have been made to use GO in drug delivery, gene delivery, in vivo and in vitro biosensing, bioimaging, and theraneustic applications (Reina et al. 2017; Chung et al. 2013).

Various production methods yield graphene-based materials of different chemistry and properties. However, the scalability, high yield, and possibility of tailoring the surface chemistry of nanosheets in exfoliation methods make them more appealing for the biomedical and biological applications (Niu et al. 2016; Parviz et al. 2016). Exfoliation refers to the peeling of nanosheets from layered the bulk graphite. Due to the intersheet strong van der Waals (vdW) forces, external energy is required for the efficient exfoliation process. This external energy may be provided in mechanical, thermal, chemical, or electrochemical form in either liquid or solid phase (Tao et al. 2017; Stankovich et al. 2007). The mechanical and chemical exfoliations in the liquid phase are the most popular routes due to their simplicity and higher yield of product.

The chemical exfoliation is also known as the intercalation-oxidation-exfoliation route and relies on the covalent functionalization of bulk graphite and exfoliation of the resultant graphite oxide to achieve colloidal dispersions of single layer graphene oxide. The yield of this method is quite high since at the optimal reaction conditions most of the graphite oxidizes to yield GO. Various reaction pathways have been suggested for graphite oxidation among which the Hummer’s method and its modified versions are the fastest and most popular ones (Hummers and Offeman 1958; Xu et al. 2011). In Hummers method, sulfuric acid, sodium nitrate, and potassium permanganate are used as intercalating and oxidizing agents. However, the byproducts of this method are toxic and the reaction is extremely exothermic. In the modified Hummer’s methods the potassium permanganate/graphite ratio is increased to compensate for the removal of the sodium nitrate from the synthesis process (Gilje et al. 2007; Marcano et al. 2010). Moreover, some pre-oxidation steps have been proposed to achieve maximum oxidation and yield in the final product (Kovtyukhova et al. 2017; Reina et al. 2017; Chung et al. 2013).
Graphene oxide produced through this chemical exfoliation route contains hydrophilic functional groups and thus, it is compatible with and dispersible in biological media.

In the mechanical exfoliation route, the pristine graphene nanosheets are directly exfoliated from bulk graphite by application of mechanical force in the liquid medium using ultrasonication or shear mixing (Hernandez et al. 2008). However, the reaggregation of exfoliated nanosheets due to the strong intersheet vdW forces is the main challenge of this method. Particularly, the difference in the surface energy of graphene and water makes it really difficult to exfoliate these nanosheets in aqueous systems. Electrostatic or steric stabilization of the nanosheets during exfoliation in presence of surfactants and polymers is the most common strategy to prepare aqueous dispersions of graphene (Guardia et al. 2011; Lotya et al. 2009; Wajid et al. 2012). This stabilization approach relies on non-covalent functionalization of graphene and thus, preserves the pristine crystalline structure of the nanosheets.

The graphene nanosheets prepared for application within or at the interface of biological media need to meet some other requirements such as initial sterility and colloidal stability. These parameters can affect the toxicity of graphene nanosheets in biological environment (Ma et al. 2014). For example, exposure to air in the synthesis environment may result in an endotoxin-contamination of the nanosheets (Vallhov et al. 2006). Also, failure to prepare stable and free of aggregates dispersions may cause severe and immediate aggregation of nanosheets upon exposure to biological media (Liu et al. 2012). Additionally, for applications in biological media, it is important to select a biocompatible stabilizing surfactant or polymer. Therefore, it is important to modify the production and post-processing methods such that no extrinsic toxicity is introduced to the final products prior to exposure to the biological media.

Lipopolysaccharide, also known as endotoxin, is the most studied pyrogen that exist in the outer membrane of gram negative Bacteria. It is a common source of toxicity in biomaterials, as they activate and/or stimulate the host to release inflammatory cytokines. Exposure to high doses of endotoxins (>0.5EU/mL according to FDA standards) can lead to organ failure. Endotoxins cannot be removed using usual sterilization protocols. With advances in graphene-based technologies for biological applications, it is important to develop protocols for production of graphene nanosheets in an endotoxin-free manner. Proper measurement of the endotoxin level in the final graphene product is a significant part of developing such protocols (Ding and Ho 2001). Conventionally, the endotoxin measurement is performed using Limulus amebocyte lysate (LAL) assay for quality control of graphene-based samples (Mukherjee et al. 2016). However, this widely used assay suffers from multiple drawbacks including the limited sources of limulus, inconsistent sensitivity, lack of specificity, and the interference of the nanomaterials with the assay at the detection wavelength. The recombinant Factor C (rFC) assay is a more recent alternative to the LAL assay (Ding and Ho 2001). Recombinant Factor C has been produced by genetic engineering of the Factor C, the endotoxin-sensitive protein of the LAL and it is capable of binding to endotoxin. The rFC assay provides a highly sensitive, specific, and simple method for in vitro measurement of the endotoxin level in the samples.

*Cur Protoc Chem Biol. Author manuscript; available in PMC 2019 December 01.*
The purification and removal of aggregated nanosheets and graphitic residues is another essential step for preparation of well-dispersed graphene nanosheets for biological applications. The interactions of these nanosheets in biological media strongly depend on their size distribution. On the other hand, the polydispersity of the graphene nanosheets produced via exfoliation methods is one of the major issues that directly affect their stability over time (Bonaccorso et al. 2016). If the larger aggregated nanosheets are not removed from the final products, they can accelerate the aggregation process of initially stable nanosheets over time and upon the change of the dispersion media. Proper centrifugation methods are useful in removal of the large aggregated materials, as well as narrowing the size distribution of the nanosheets.

**BASIC PROTOCOL 1: SYNTHESIS AND EXFOLIATION OF GRAPHENE OXIDE USING IMPROVED HUMMERS’ METHOD**

The graphene oxide dispersion preparation includes three main steps: (i) oxidation of graphite (ii) purification of graphite oxide, and (iii) exfoliation of graphene oxide (Figure 1). We recommend that these three steps be complemented by (iv) an additional centrifugation step in order to remove the non-exfoliated and larger graphitic materials.

The oxidation of graphite is commonly performed by one of the modified Hummer’s methods (Toh et al. 2014). Most of these methods require several synthesis steps and precise control of the synthesis temperature. A recent modification of Hummer’s method, introduced by Marcano et al., offers a simpler and less hazardous protocol while maximizing the production yield (Marcano et al. 2010). This method replaces the usage of sodium nitrate with higher acid/graphite ratio and addition of phosphoric acid to synthesis process. Hence, toxic gases such as NO$_2$ do not evolve during the oxidation step. Simultaneous acid intercalation and oxidation occurs in the presence of sulfuric acid, phosphoric acid, and potassium permanganate, which omits the need for precise temperature control during the addition of oxidizing agent to the graphite and acid solution.

The purification process is essential for removal of the non-oxidized graphitic materials, residual metal ions, and neutralization. Multiple cycles of acid and ethanol wash followed by neutralization using water is performed to obtain a neutral pH in the product. The neutralization is required to enhance the hydrolysis of the functional groups and thus, prevent aggregation of the GO nanosheets.

The final step is the exfoliation of the graphite oxide into single-layer graphene oxide by sonication. This step can be performed on freshly synthesized graphite oxide kept in the solution, or dried powder of the graphite oxide collected from the purification step.

Lastly, it must be emphasized that in all the above-mentioned steps, precursors, intermediates, and products must be protected from endotoxin contamination. The main contamination sources include air, water, glassware and plastic used in the synthesis, and contact with skin (Mukherjee et al. 2016). Precursors, intermediates and products may be exposed to these sources of contamination at various steps of the synthesis. Thus, general rules must be followed in this protocol to obtain endotoxin-free GO nanosheets. These rules
are presented in Figure 2. Usage of endotoxin-free water and non-pyrogenic plastics, wearing gloves in all the synthesis steps, and cleaning the glassware in a base bath prior to usage are the main measurements that must be taken to avoid endotoxin-contamination. Additionally, the purification and exfoliation steps must be performed in a laminar flow hood to prevent contamination by air. The synthesis step, however, may be performed in a normal lab hood since the harsh acidic condition of this step minimizes the contamination possibility. These general rules also apply to the Alternate Protocol and the Supporting Protocol 1.

Note: Use endotoxin-free water in all the oxidation, purification, and dispersion preparation steps. All the plastic involved in experiments must be non-pyrogenic. All the glassware must be cleaned in a base and kept in a laminar flow hood prior to usage in the oxidation, purification, and dispersion steps. Wear gloves in all steps of the sample preparation. Rinse your gloves with 70% ethanol prior to experiments and dry them immediately. The oxidation steps can be carried in a clean fume hood. The purification and dispersion preparation steps must be carried in a sterile laminar flow hood.

**Materials**

- Graphite
- Sulfuric acid
- Phosphoric acid
- Potassium permanganate
- Hydrogen peroxide
- Hydrochloric acid
- 70% ethanol solution
- Sodium bicarbonate
- Endotoxin-free water

**Equipment**

- Glass vials, beakers, & sample containers
- Erlenmeyer flask (2L)
- Graduated cylinder
- Disposable plastic spatula
- Magnetic stirrer & glass rod
- Metal U.S. standard testing sieve (200 μm)
- Cover glass
Instruments

- Laminar flow hood
- Hotplate with magnetic stirrer and thermocouple
- Lyophilizer (i.e., freeze-dryer)
- Bath or tip Sonicator
- Centrifuge
- Centrifuge tubes

Oxidation of Graphite—Note: This step can be carried in normal hood.

1. Measure 450 ml of the endotoxin-free water in a container with lid and keep it in the freezer to form endotoxin-free ice.

2. Set the hot plate at 55 °C and wait until the temperature stabilizes.

3. Weigh 3 g of the graphite powder and place it in a 2000-mL glass Erlenmeyer flask.

4. Weigh 18 g of the potassium permanganate and add it to the graphite powder.

5. Lightly shake the flask to mix the powders in it.

6. Measure 360 mL of sulfuric acid ($H_2SO_4$) using graduating cylinder and glass funnel and pour it into a 1000-mL glass beaker.

7. Measure 40 mL of phosphoric acid ($H_3PO_4$) using graduating cylinder and glass funnel and add it to the to sulfuric acid in the glass beaker. Stir the acids manually with a glass rod for few minutes to properly mix them.

8. Add the acid mixture to the graphite/potassium permanganate mixture very quickly. This mixture has a dark green color.

   (Caution: If the acid is added slowly, the ratio of the potassium permanganate/acid in the container will exceed the desired value and a highly exothermic reaction will be initiated. The heat generated by this highly exothermic reaction can cause fire).

9. Place the reaction flask on the pre-heated hot plate at 55 °C. Place a magnetic stirrer in the solution and adjust the stirring rate. Allow the reaction to go on for 12 hrs.

10. After 12 hours, turn off the heater and let the reaction mixture to cool down to room temperature. The mixture has a brown-purple shade.

11. Take the endotoxin-free ice out of the freezer and place it in a 2000-mL glass beaker.

12. Pour the reaction mixture onto the endotoxin-free ice in the beaker. Initially, the sample turns deep purple and upon addition of the entire sample, it will turn dark brown.
13. Measure 5 ml of hydrogen peroxide ($H_2O_2$) and **gradually** add it to the solution. At this point, the solution must have a yellowish-orange color.

14. Leave the reaction flask in the hood overnight with a glass cover on top so that the solid content settles.

**Purification of Graphite oxide**—Note: This step must be carried in a sterile laminar flow hood.

1. Pour excessive amount of sodium bicarbonate in a 4-L glass beaker and designate it as acidic waste container.

   (All acidic waste and supplies with acid on them must be neutralized in the designated acidic waste container with excess sodium bicarbonate before disposal).

2. Pour the acidic supernatant of the sample in the acidic waste container.

3. Rinse the metal U.S. Standard testing sieve of desired mesh size (<200 μm) with ethanol and endotoxin-free water, respectively, and dry it with immediately.

4. Sift the sample through the metal U.S. Standard testing sieve and collect the sieved sample in a 2000-mL Erlenmeyer flask.

5. Prepare 2000 mL of 10% hydrochloric acid (HCl) solution by adding 500 mL of 37% HCl to 1500 mL of endotoxin-free water.

6. Wash the sieved sample with endotoxin-free water and 10% HCl solution in succession. For each wash add 1000 mL of the washing solvent to the sample, stir the sample for a day, allow the solid content to settle while the container is covered, dispose the acidic supernatant in the acidic waste container, and collect the sediment for the next wash step, respectively. Repeat this process for two to three times to assure the removal of residual metal ions.

7. Wash the sample collected from the prior step (acid-wash) with endotoxin-free water and ethanol in succession. For each wash, add 500 mL of the washing solvent to the sediment collected in prior step to, stir the sample for a 5 hours, allow the solid content to settle while the container is covered, dispose the acidic supernatant in the acidic waste container, and collect the sediment for the next wash step, respectively. Repeat this process for two to three times to assure the removal of residual organic and amorphous compounds.

8. Wash the sample collected from the prior step (ethanol-wash) with endotoxin-free water until the pH of the samples become neutral (>5.5). For each wash, add 500 mL of endotoxin-free water to the sediment collected in prior step, stir the sample for a few hours, collect the sample either through settling or centrifugation, and dispose the supernatant.

   (Note: as the pH of the sample increases, the sample takes longer to settle and sediment in the container; centrifugation can accelerate the wash process. For centrifugation, pour the samples into centrifugation
tubes and balance with endotoxin-free water, if necessary. However, it must be cautioned that multiple cycles of centrifugations risk of exposure to the endotoxins).

9. Collect the sample into a clean glass container with lid and add 200 ml of endotoxin-free water to the sample to keep it wet and prevent aggregation.

10. Pour the sample in sample vials and freeze-dry the sample using a lyophilizer to collect the graphite oxide powder as the final product of this step.

(Note: This step may be skipped if the graphite oxide is going to be immediately processed for exfoliation and dispersion preparation (see step 3).

(Note: when prepared for non-biological applications, at this step the sample is collected through filtration and dried at room-temperature. However, the filtration process increases the risk of endotoxin contamination, and drying process causes aggregation. Therefore, we strongly recommend avoid any filtration and/or room-temperature drying at the final step of purification).

**Exfoliation of Graphene Oxide** — Note: This step must be carried in a sterile laminar flow hood.

1. Weigh appropriate amount of graphite oxide powder and add it to desired volume of endotoxin-free water in a glass beaker.

   (Concentration as high as 10 mg/mL can be used for sonication step, however, dispersions with concentrations higher than 1 mg/mL are not stable over time and require immediate dilution after sonication and centrifugation).

   (Note: If the sonication is performed immediately after purification step 2.9, freeze-drying process can be skipped. To prepare the dispersion sample, shake the graphite oxide solution (from step 2.9) manually to mix the sample. Then, dilute 10 mL of the sample by adding 90 mL of endotoxin-free water in a glass beaker.

2. Prepare a 1L ice bath for temperature control during the sonication and place it in the sonicator sound-closure box.

3. Place the sample beaker in the ice bath and center it.

4. Rinse the sonicator tip with endotoxin-free water and ethanol several times.

5. Lower the sonicator tip and insert it into the sample until it touches the bottom of the beaker.

6. Center the sonicator tip in the beaker and raise it to half of the height of the sample.
7. Adjust the sonicator settings and sonicate the sample for 30–60 min at 20 kHz and 100–200 W.

(The sonication amplitude and time can be tuned to obtain the desired level of exfoliation. The larger sonication power and longer sonication times result in reduction of the nanosheet size, therefore we do not recommend sonication at powers higher than 200 W and longer than 60 min).

8. When the sonication is over, collect the dispersed sample in non-pyrogenic conical centrifuge tubes.

9. Repeat steps 3.1–6.8 until the entire original sample is sonicated.

10. Centrifuge the sonicated samples at room temperature (22 °C) for 15–60 min at 2500–3500 g-force.

(The centrifugation time and g-force can be tuned to achieve narrower or broader nanosheet size distribution.)

11. Collect the supernatants in a glass vial with lid.

12. Optional step: adjust the pH of GO dispersion to 8 or slightly higher by addition of ammonium hydroxide.

(Note: GO becomes more acidic over time and this change in the pH results in very slow aggregation over time. If the dispersions are to be stored for a long time before usage, the adjustment of pH to more basic environment slows down the aggregation of nanosheets.)

13. Vacuum-filter 20 mL of the final dispersion over a PTFE membrane (0.2 μm pore size) to measure the final concentration of GO in the dispersion.

(If the concentration is higher than 1 mg/mL and the samples are subject to long-term storage, dilute the sample using water to slow the aggregation process and maintain colloidal stability and particle size distribution.)

Post-Synthesis Clean Up and Equipment maintenance

1. Be aware of the “acidic waste” container, before it becomes full dispose the waste and fill it with fresh Sodium bicarbonate.

2. Remember to neutralize all the glassware that is contaminated with acid with sodium bicarbonate before cleaning them with water.

3. Clean the sonicator tip and all other non-disposable tools after usage with DI water, ethanol, and DI water, respectively and keep them in the laminar flow hood.

4. Make sure that graphene oxide mixtures and dispersions are covered all the time to prevent any change in the pH and consequent aggregation.
5. Be aware of the sonicator tip aging. Immediately replace the worn-out tip with a new one.

ALTERNATE PROTOCOL 1: SYNTHESIS AND EXFOLIATION OF GRAPHENE OXIDE USING MODIFIED HUMMER’S METHOD

Graphene nanosheets may be prepared using a more conventional modified Hummer’s method (Gilje et al. 2007). This method still avoids the usage of the sodium nitrate, and the potassium permanganate/graphite ratio is similar to the improved hummers’ method introduced in Basic Protocol 1. However, the acid intercalation and oxidation are performed sequentially and the oxidation reaction is longer than the original Hummers’ method. The product of this method may be less oxidized than the GO produced via Basic Protocol 1 and thus, has slightly lower yield. Moreover, this method requires precise temperature control during the oxidation step. If the temperature is not accurately adjusted as instructed, the exothermic reaction of the oxidizing agent with the graphite/acid solution will be hazardous. Incomplete oxidation of graphite is another consequence of inaccurate temperature adjustment. However, this method is more similar to the original Hummers’ method and thus more commonly used.

Note: The purification and exfoliation steps are similar to the ones described in the Basic Protocol 1. Thus, we only describe the protocol for oxidation step here. The general rules applied to the Basic Protocol 1 for protection against endotoxin contamination must be followed in this protocol as well.

Materials

Graphite
Sulfuric acid
Phosphoric acid
Potassium permanganate
Hydrogen peroxide
Hydrochloric acid
Ethanol
Sodium bicarbonate
Endotoxin-free water

Equipment

Glass vials, beakers, & sample containers
Erlenmeyer flask (2L)
Graduated cylinder
Glass thermometer
Disposable plastic spatula
Magnetic stirrer & glass rod
Metal U.S. standard testing sieve (200 μm)
Cover glass
Centrifuge tubes

**Instruments**

Laminar flow hood
Hotplate with magnetic stirrer
Lyophilizer
Bath or tip Sonicator
Centrifuge

**Oxidation**

1. Measure 230 mL of sulfuric acid and keep it in the refrigerator overnight.
2. Prepare an ice bath using sodium chloride salt and wait till the temperature reaches −5°C.
3. Pour 230 mL of sulfuric acid in a 1000-mL Erlenmeyer flask.
4. Put a magnetic stirrer in the flask and place it in the ice bath. Use a glass thermometer to monitor the acid temperature.
5. When the acid temperature reaches 5°C, add 5 g of graphite powder to the flask. Let the sample stir for 30 min so that the graphite and acid mix well.
6. Weigh 30 g of potassium permanganate.
7. **Gradually** add the potassium permanganate to the reaction mixture while monitoring the temperature constantly. The reaction mixture temperature must be kept below 10°C.
   
   (Note: The addition of potassium permanganate to the reaction mixture is extremely exothermic. Temperature control is required to prevent the hazard of setting the reaction mixture on fire. Slow addition of the potassium permanganate is the key to the temperature control.)
8. After addition of all the potassium permanganate wait for the temperature to stabilize. Then remove the flask from the ice bath and stir the reaction mixture at room temperature for two hours. After this time, the reaction mixture becomes a viscous and dark green in color.
9. Put the flask back into the ice bath and measure 450 mL of endotoxin-free water.
10. Add the water to the reaction mixture dropwise while monitoring the temperature of mixture constantly. The temperature must be kept between 45°C and 50 °C.
(Note: the addition of water to concentrated acid is very hazardous, thus maintain a very low rate of water addition throughout this step.)

11. Remove the flask from the ice bath and stir the reaction mixture for one to two hours at room temperature.

12. Transfer the sample to a larger glass container and add another 1500 mL of endotoxin-free water to it. At this stage, the sample must have a brown color.

13. **Gradually** add 20 mL of 30% hydrogen peroxide (H2O2) to the solution. The solution should turn yellowish-orange upon addition of all the H2O2. Darker brown color indicates less oxidation and/or exfoliation level.

14. Leave the reaction container in the hood overnight with a glass cover on top so that the solid content settles.

15. Follow by the purification and dispersion steps described in the Basic Protocol 1.

**BASIC PROTOCOL 2: DIRECT LIQUID-PHASE EXFOLIATION OF GRAPHENE FROM GRAPHITE IN AQUEOUS SYSTEMS**

Graphene may be directly exfoliated from bulk graphite via sonication in presence of solvent (Figure 3). For aqueous dispersions, a stabilizer (i.e., surfactant or polymer) must be used to facilitate the exfoliation, increase the yield of production, stabilize the nanosheets in the dispersion, and prevent reaggregation. The choice of the stabilizer, stabilizer concentration, and stabilizer/graphite ratio directly affects the graphene yield and colloidal stability in the final dispersions (Parviz et al. 2012; Tao et al. 2017). Moreover, for usage in biological applications, the stabilizer must be biocompatible.

To avoid endotoxin contamination, measurement must be taken to minimize the exposure from air, water, skin, and sample preparation environment. Similar to previous protocol, endotoxin-free water and non-pyrogenic plastics must be used in all steps of the experiments; glassware must be cleaned in a base bath prior to usage in the experiments. All the exfoliation and centrifugation steps must be performed in a fume hood and any contact with the skin must be avoided by wearing gloves. These rules are summarized in Figure 4 and also apply to Supporting Protocol 2, 3, and 4.

**Note:** Use endotoxin-free water in all the exfoliation and centrifugation steps. All the plastic involved in the experiments must be non-pyrogenic. All the glassware must be cleaned in a base and kept in a laminar flow hood prior to usage in the experiments. Wear gloves in all steps of the sample preparation. Rinse your gloves with 70% ethanol prior to experiments and dry them immediately. All the sample preparation steps including sonication and preparation for centrifugation must be carried in a sterile laminar flow hood.

**Materials**

- Expanded graphite
- Stabilizer (surfactant, polymer of choice)
Endotoxin-free water
Cleaning ethanol

**Equipment**

Glass vials, beakers, and sample containers
Disposable plastic spatula
Pipettes and pipette tips
Centrifuge tubes

**Instruments**

Laminar flow hood
Tip Sonicator
Sonicator tip
Centrifuge

**Liquid-Phase Exfoliation**

1. Weigh appropriate amount of stabilizer that yields the desired concentration and place it in a glass sonication container.
   
   (Note: The higher stabilizer concentration improves the exfoliation yield and stability of the dispersions, however, excessive amount of stabilizers are not recommended).

   (Note: the sonication container must be chosen to achieve the best sonication efficiency and energy delivery to the sample; it is recommended that sample fills up to 75%−85% of the height of the sonication container.

2. Add appropriate volume of endotoxin-free water, place a magnetic stirrer in the beaker, and stir the sample until the stabilizer is completely dissolved.

3. Add appropriate amount of expanded graphite to the stabilizer solution in order to obtain a desired ratio of graphite/stabilizer.

   (Note: usually the graphite concentration must exceed the stabilizer concentration to obtain higher yield, however, addition of too much graphite increases the viscosity and prevents efficient exfoliation. A good ratio of graphite/stabilizer is usually between 3 and 10, depending on the choice of stabilizer).

4. Lightly shake the beaker until the graphite is completely mixed in.

5. Prepare ice bath for temperature control during the sonication and place it in the sonicator sound-closure box.

6. Place the sample beaker in the ice bath and center it.
7. Rinse the sonicator tip with endotoxin-free water and ethanol several times.
   (Note: sonicator tip should be consistent with the volume being
   sonicated for efficient exfoliation, according to the equipment
   specification).

8. Lower the sonicator tip and insert it into the sample until it touches the bottom of
   the beaker.

9. Center the sonicator tip in the beaker and raise it to half of the height of the
   sample.

10. Adjust the sonicator settings and sonicate the sample for 30–60min at 20 kHz
    and 100–200 W.
    (Note: The sonication amplitude and time can be tuned to obtain the
    desired level of exfoliation. The larger sonication power and longer
    sonication times result in reduction of the nanosheet size, therefore we
    do not recommend sonication at powers higher than 200 W and longer
    than 60 min).

11. When the sonication is over, collect the dispersed sample in non-pyrogenic
    conical centrifuge tubes with lid.

12. Balance the sample for centrifugation according to the centrifuge specifications.
    Make sure to use endotoxin-free water in this step.

13. Centrifuge the sonicated samples at room temperature (22 °C) for 2–4 hours at
    2500–3500 g-force.
    (Note: The centrifugation time and g-force can be tuned to achieve
    narrower or broader nanosheet size distribution. Also, the lower
    centrifugation duration and g-force corresponds to lower colloidal
    stability of the dispersions)

14. Collect the supernatants in a glass vial with lid.

15. Collect the supernatants in a glass vial with lid and dispose the sediment.

**SUPPORT PROTOCOL 1: PRE-OXIDATION OF GRAPHITE FOR MAXIMIZED
OXIDATION AND YIELD**

The extent of graphite oxidation in Modified Hummer’s method depends on the accessibility
of the individual graphene layers to the oxidizing agent. The acid intercalation increases the
interlayer spacing in the bulk graphite and enhances the extent of oxidation for individual
nanosheets. A pre-oxidation step using anhydrous acids and oxidizing agents assists in
increasing the interlayer spacing in the bulk graphite and makes individual graphene layers
more accessible during the main oxidation step (Kovtyukhova et al. 1999). The GO obtained
from pre-oxidation and oxidation of graphite possesses slightly higher oxygen to carbon
ratio compared to those obtained from the Alternate Protocol 1 alone. Since this step does
not significantly change the properties of final GO, it is optional to couple it with the Alternate Protocol 1.

Note: All the general rules applied to the Basic Protocol 1 must be followed in this protocol to avoid endotoxin contamination.

Materials

Graphite
Sulfuric acid
Potassium persulfate
Phosphorus pentoxide
Ethanol
Sodium bicarbonate
Endotoxin-free water

Equipment

Glass vials, beakers, & sample containers
Graduated cylinder
Disposable plastic spatula
Magnetic stirrer & glass rod
PTFE Hydrophilic filtration membrane (0.45 μm)
Cover glass
Oil bath
Petri dish with cover

Instruments

Oven
Hotplate with magnetic stirrer
Glass Thermometer
Vacuum filtration unit
Desiccator and desiccants

Pre-oxidation of graphite

1. Add excessive amount of Sodium bicarbonate to a 4-L glass beaker, label the beaker as “acidic waste”, and leave it in the hood.

   (The beaker should always contain excessive amount of sodium bicarbonate to neutralize the acidic disposal).
2. Measure 10 g of the graphite, put it in a glass Petri dish with a cover on top and place it in an oven at 150 °C for 24 hours.
3. Set the oil bath on the hotplate at 90 °C and wait for temperature to stabilize.
4. Pour 50 mL of sulfuric acid (H_2SO_4) into a 250mL beaker and add a magnetic stirrer to the container.
5. Place the beaker in the oil bath and start stirring the sample.
6. Weigh 10 g of potassium persulfate (K_2S_2O_8).
7. Gradually add the potassium persulfate (K_2S_2O_8) until the solution becomes clear.
8. Weigh 10 g of phosphorus pentoxide (P_2O_5).
9. Quickly add the phosphorus pentoxide (P_2O_5) to the solution.
   (Phosphorus pentoxide immediately absorbs the atmospheric moisture and becomes sticky, so this step should be performed quickly. The minimal absorbed moisture leads to fume formation upon addition to the solution that is normal).
10. Wait until the solution becomes completely clear.
11. Gradually add 10 g of the previously dried graphite to the solution.
12. Place a cover glass on top of the beaker.
13. Allow the reaction to go on for 6 hours. Make sure that the temperature remains at 90 °C during this step.
14. After 6 hrs, remove the beaker from the oil bath and leave it in the hood overnight with a cover glass on top, so the solid content settles.
15. Pour the acidic supernatant in the prepared acidic waste container. Pour the acid slowly to prevent foam formation upon acid-base reaction.
16. Prepare an ice bath.
17. Place the solution beaker in the ice bath.
18. Slowly, add 200 ml of endotoxin-free water to the solution.
19. Prepare the vacuum filtration unit and filter the content of the beaker.
20. Wash the filtered pre-oxidized graphite with endotoxin-free water until the pH of the filtrate water reaches 5.5–6. Whenever the receiving flask becomes full, dispose its content in the “acidic waste” container.
   (The washing process takes a while and usually requires 3.5–4 liter of endotoxin-free water).
21. After the pH of the filtrate water reaches ~ 6, wash the sample with 200 ml of endotoxin-free water and ethanol in succession for two to three times.
22. Collect the filtered pre-oxidized graphite in a petri dish and cover it with the lid.
23. Fill the desiccator with desiccants.
24. Place the sample-containing petri dish in the desiccant and seal the desiccator lid.
25. Leave the sample in the desiccator to dry at room temperature for 3–4 days.

**SUPPORT PROTOCOL 2: MEASUREMENT OF ENDOTOXIN LEVEL IN GRAPHENE OXIDE AND GRAPHENE DISPERSIONS**

Recombinant Factor C assay uses the LPS receptor of the LAL in combination with a fluorogenic substrate to quantify the endotoxin level in the samples (Ding and Ho 2001). The presence of trace amounts of endotoxin in the sample and their binding onto the rFC active sites, enzymatically activates the rFC and leads to hydrolysis and coagulation of the substrate. The fluorescence measurements at proper emission and excitation wavelengths can reveal the presence of the endotoxin in the samples. Spiking the samples with increasing concentration of the endotoxin standard and comparing the fluorescence with those of the non-spiked samples assures the lack of interference of the sample and the assay at the detection wavelengths.

*Note: All the general rules applied to the Basic Protocol 2 must be followed in this protocol to avoid endotoxin contamination.*

**Materials**
- Graphene and graphene oxide dispersions
- Recombinant Factor C Assay kit (e.g. Endozyme)
- Sodium hydroxide
- Hydrochloric acid
- Endotoxin-free water

**Equipment**
- Pipettes and pipette tips
- Glass and plastic sample vials
- 96-well microplates

**Instruments**
- Laminar flow hood
- Vortexer
- Incubator (optional)
Endotoxin measurement using rFC assay

1. Reconstitute the endotoxin standard provided in the assay kit according to the kit instructions.

2. Prepare dilutions of the endotoxin standard at concentration recommended in the kit instruction.

3. Dilute the nanosheet dispersions to ~ 1 μg/mL using endotoxin-free water.

4. Prepare 0.1, 0.01, and 0.001 N solutions of sodium hydroxide and hydrochloric acid using endotoxin-free water.

5. Adjust the pH of the diluted dispersion samples to 7 (± 0.1) by addition of appropriate amount of the sodium hydroxide or hydrochloric acid solutions.

6. Place the endotoxin standard dilutions and dispersion samples in the microplate according to the volume recommended in the kit instructions.

7. Add another batch of dispersion samples in the microplate and spike them with the endotoxin standards according to the kit instructions.

8. Prepare appropriate amount of assay reagents.

9. Add appropriate amount of assay reagent to each well according to the kit instructions.

10. Place the microplate in the reader, wait one minute for the samples to stabilize and adjust the temperature.

11. Read the fluorescence signal at time zero at proper excitation and emission wavelengths according to the kit instructions.

12. Incubate the samples at 37 °C for 90 min (alternatively the microplate can be kept in the room temperature for 90 min).

13. Read the fluorescence signals after 90 min at proper excitation and emission wavelengths according to the kit instructions.

14. Analyze the results according to the kit instructions to determine the endotoxin level in the samples.

SUPPORT PROTOCOL 3: SORTING OF GRAPHENE AND GRAPHENE OXIDE NANOSHEETS USING ITERATIVE CENTRIFUGATION

The polydispersity of nanosheets in the products of liquid-phase exfoliation is an inherent and yet, undesirable property of these materials (Bonaccorso et al. 2016). Many applications such as drug delivery require control over the size of nanosheets (Ma et al. 2014). Iterative centrifugation is a method for size sorting with larger throughput compared to other methods such a density gradient centrifugation (Backes et al. 2016). Using iterative centrifugation with increasing g-force in each cycle, the nanosheets can be separated based on their density and size.
Note: All the general rules applied to the Basic Protocol 2 must be followed in this protocol to avoid endotoxin contamination.

Materials

Graphene and graphene oxide dispersions
Endotoxin free water
Stabilizer (surfactant, polymer)

Equipment

Centrifugation tubes

Instruments

Centrifuge
1. Pour graphene dispersion into centrifuge tubes and balance with endotoxin-free water if necessary.
2. Centrifuge at 1000 g-force for two hour.
3. Collect the sediment into fresh stabilizer solution of similar concentration and volume as the original dispersion. Store them as the dispersions with largest lateral size and/or thickness of nanosheets.
4. Centrifuge the supernatant at 1500 g-force.
5. Collect the sediment into fresh stabilizer solution and centrifuge the supernatant at 2000 g-force.
6. Continue the centrifugation cycles by sequentially increasing the g-force. Collect the sediments of each step as the product with narrower size distribution.

(Note: the g-force and centrifugation time intervals can be tuned to obtain narrower or broader nanosheet size distribution.)

Similar method can be used to sort graphene oxide dispersions, the only difference is that the sediments of each centrifugation cycle must be collected into fresh endotoxin-free water instead of stabilizer solution.

SUPPORT PROTOCOL 4: REMOVAL OF EXCESSIVE STABILIZER FROM GRAPHENE DISPERSIONS

The entire stabilizer present in the solvent does not adsorb on the nanosheets surface. A fraction of the stabilizer is present in the dispersion as unbound molecules. Presence of these unbound molecules (especially when polymers are used) is undesirable for many biological applications. Thus, this extra unbound stabilizer may be removed from the nanosheets dispersion using multiple centrifugation cycles.
Materials

Graphene dispersions
Endotoxin-free water

Equipment

Centrifuge tubes
Dialysis tube with clips

Instruments

Centrifuge
Bath sonicator

1. Pour the graphene dispersion in centrifuge tubes with lid and balance with endotoxin-free water if necessary.
2. Centrifuge the sample at 15000 g-force.
3. Decant away the supernatant.
4. Redisperse the sediment into equal volume of the endotoxin-free water.
5. Repeat the centrifugation cycle at least 4–5 times to make sure that the excessive, unbound stabilizer is completely removed.
6. Optional: Mildly sonicate the final dispersion of graphene in a bath sonicator for 30–60 min to assure the colloidal stability of the sample.

Alternatively, the excessive surfactant may be removed through dialysis with pure water. Multiple dialysis cycles (each of 48 hours) may be performed to fully remove the excessive stabilizer molecules. Make sure the dialysis tube pore size is smaller than the average nanosheet size. Also, Cover the dialysis container all the time to prevent endotoxin contamination from air.

COMMENTARY

Background Information

Graphene is the most recent addition to the carbon family consisting of graphite, diamond, carbon nanotubes (CNT) and fullerenes (Geim and Novoselov 2007). It is a freestanding atomic layer of sp²-hybridized carbon atoms arranged into a honeycomb lattice. Each carbon atom is connected to three other carbon atoms with covalent σ bonds and shares a delocalized double bond with adjacent atoms through its π orbital. Single layers of graphene are held together through strong vdW forces in the bulk graphite. A single-layer pristine graphene may be a few microns in lateral size, which is terminated at the edges by...
sp³ hybridized carbon atoms. As expected, pristine graphene is a hydrophobic material and prone to agglomeration in water and many other solvents.

Graphene oxide is an important graphene derivative that has been widely used as a precursor for production of graphene nanosheets for different applications (Stankovich et al. 2006; Schniepp et al. 2006; Gilje et al. 2007) Graphene oxide refers to a highly oxidized single layer of graphene in which the basal plane and the edges are heavily functionalized with oxygen-containing groups including epoxide, carboxyl, and carbonyl groups (Dreyer et al. 2010). Due to the high polarity of the oxygen-containing groups, GO is easily dispersible in water and many other solvents. The solution-processability of GO is important for preparation of bulk graphene products. Also, the functional groups are reactive sites that can be targeted in various chemical reactions for modification of GO structure into a graphene derivative with tunable properties (Georgakilas et al. 2012).

Since the interlayer vdW attractive forces in graphite are weak, they can be dominated by an external force, resulting in separation of adjacent layers. Also, the interlayer attractive forces can be diminished by intercalation of various atoms and molecules into the graphite structure and increasing the interlayer spacing (Fu et al. 2011). Exfoliation may be carried out in solid or liquid phase using various sources of external energy. Maintaining the exfoliated state of the graphene sheets requires an energy barrier that prevents the reaggregation of the sheets, especially in a medium, which prompts the Brownian motion of the sheets, i.e., liquid phase. Such an energy barrier can be provided by covalent functionalization (e.g., oxidation of graphite into graphite oxide) or by non-covalent functionalization using various types of ionic and non-ionic surfactants, polymers, and small aromatic molecules (Parviz et al. 2016). Another stabilization approach is the exfoliation in organic solvent with matching surface energy with graphene, followed by drying/centrifugation and redispersion in aqueous systems (Hernandez et al. 2008). However, the residual toxic organic solvents in the products make this stabilization approach less appealing for biological and biomedical applications.

Critical Parameters

The most important parameters in GO synthesis using modified Hummer’s methods are the potassium permanganate/graphite and acid/graphite ratio. The potassium permanganate/graphite ratio may be varied from 1 to 6 to tune the oxidation level of GO. Also, various acid/graphite ratios have been reported in the literature ranging from 46 to 135 (Toh et al. 2014). If ratios other than the ones mentioned in the protocols are being used, extreme temperature control must be provided using and ice bath. If the amount of potassium permanganate exceeds the acid content for longer than a few second with no temperature control, extremely exothermic reaction occurs and the graphite will catch fire. Furthermore, neutralization of the final graphite oxide before exfoliation step is important to achieve higher exfoliation yield and colloidal stability over time, as it affects the surface charge of the nanosheets and their aggregation over time.

The most important factors in preparation of graphene aqueous dispersions are the choice of stabilizer, concentration of stabilizer, sonication time, and sonication power. The ionic surfactants and small aromatic molecules have been reported as the most effective stabilizers.
with higher graphene yields (Guardia et al. 2014). However, not all these stabilizers are biocompatible and thus, cannot be used in preparation of graphene for biological and medical applications. Sodium cholate, polyvinylpyrrolidone (PVP), and polyethylene glycol (PEG) are among the biocompatible stabilizers with high graphene yield in the product (0.5–1 mg/mL). The concentration of the stabilizers directly affects the concentration and colloidal stability of the final graphene dispersion. The dependence of the final dispersion on the stabilizer concentration is mostly stabilizer-specific. However, it has been reported that the initial increase of the stabilizer concentration improves the yield of graphene, but after a specific concentration, it may not affect the graphene yield anymore. Also, the higher ratio of initial graphite to stabilizer (as long as the sample is not too viscous for sonication step) results in higher exfoliation yield. Additionally, increasing the sonication time and power drastically increases the graphene yield. However, some researchers have reported the decrease in the lateral size of the graphene nanosheets upon longer sonication times.

For both graphene and graphene oxide dispersions, a proper centrifugation step and removal of all the residual graphitic sheets is vital for the colloidal stability of the nanosheets. Presence of larger nanosheets in the dispersion accelerates the aggregation of nanosheets in the dispersion and reduces the colloidal stability over time.

**Troubleshooting**

It has been indicated that over time graphene oxide becomes acidic in aqueous dispersions (Dimiev, Alemany, and Tour 2013). This may cause slow aggregation of GO nanosheets. Increasing the pH of the dispersion after exfoliation assists with the long-term stability of the nanosheets. Also, storing the dispersions at concentrations below 1 mg/mL reduces the possibility of acidification and aggregation.

The main issues that may be encountered in graphene dispersion preparation are the low yield and stability of the dispersions. Altering the type of the stabilizer, increasing the stabilizer concentration, increasing the stabilizer/graphite ratio, and increasing the sonication time can potentially improve the yield of the product. However, if stabilizer is above its critical micelle concentration (CMC), temperature fluctuations may cause nucleation and aggregation of excessive stabilizer and the nanosheets bound to them. Removal of the excessive stabilizer according to the Supporting Protocol 4, and precise temperature control of the storage environment can resolve this issue. Finally, applying proper centrifugal force and removal of the larger graphitic aggregates can enhance the colloidal stability of nanosheets.

Glassware and samples containers are the main endotoxin contamination source for graphene samples that are prepared by endotoxin-free water. If endotoxin contamination above the FDA-approved level is observed in the samples, extreme cleaning of the glassware, and keeping them from air exposure can potentially help to resolve this issue.

**Anticipated Results**

Basic Protocol 1 and Alternate Protocol 1 will produce graphene oxide dispersions. Dispersions of higher concentration will have darker brown color and as the concentration decreases, the dispersions become more transparent and will be golden-brown. Basic...
Protocol 2 will produce graphene dispersions with the stabilizer of choice. These dispersions will be black in color. Depending on the choice of stabilizer and its concentration, the dispersions may have gray shade.

The samples will consist of nanosheets with various lateral size and thickness and proper characterization methods can be used to evaluate their size distribution and chemical properties such as type of functional groups and the carbon/oxygen ratio.

**Time Considerations**

If all the materials, equipment and instruments are ready, the synthesis, purification and exfoliation of one batch of graphene oxide according to Basic Protocol 1 can be performed within 7–10 days. For basic Protocol 2, if all the required materials and instruments are ready, the protocol can be carried in one day.

**ACKNOWLEDGEMENTS**

Research reported in this publication was supported by the HSPH Center for Nanotechnology and Nanotoxicology and National Institute of Environmental Health Sciences of the National Institutes of Health (under award number, NIH grant # U24ES026946) as part of the Nanotechnology Health Implications Research (NHIR) Consortium. The engineered nanomaterials used in the research presented in this publication have been synthesized and characterized by the Engineered Nanomaterials Resource and Coordination Core of the NHIR consortium. We acknowledge Prof. Philip Demokritou, Dr. Georgios Pyrgiotakis, and Dr. Glen Deloid for their inputs and help with the materials characterization and endotoxin measurements. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Figure 1.
Schematic of graphene oxide preparation process using modified Hummers’ method. In this method, acid intercalates into the interlayer spacing of the graphite and facilitates the oxidation of the graphene surface in presence of the oxidizing agent. The expansion of the oxidized layers yields graphite oxide. Extensive purification removes the non-oxidized graphite, residual metal ions and organic compounds. Final mechanical exfoliation of graphite oxide produces graphene oxide (GO) monolayers.
Figure 2.
Requirements for production of endotoxin-free graphene oxide dispersions. The endotoxin from various sources can contaminate the sample at different steps of production. Thus, it is important to take appropriate measurements in each step to minimize the possibility of endotoxin-contamination.
Figure 3.
Schematic of direct liquid-phase exfoliation process for production of graphene aqueous dispersions. The exfoliation of graphene via external mechanical force (e.g., sonication) must be accompanied by stabilization of exfoliated nanosheets to prevent reaggregation.
Figure 4.
Requirements for production of endotoxin-free graphene/stabilizer aqueous dispersions. The endotoxin from various sources can contaminate the sample at every step of production. Thus, it is important to take appropriate measurements in each step to minimize the possibility of endotoxin-contamination.