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Additional Information

Production of ready-to-use few-layer graphene in aqueous suspensions

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Graphene has promising physical and chemical properties such as high strength and flexibility, coupled with high electrical and thermal conductivities. It is therefore being incorporated into polymer-based composites for use in electronics and photonics applications. A main constraint related to the graphene development is that, being of a strongly hydrophobic nature, almost all dispersions (usually required for its handling and processing toward the desired application) are prepared in poisonous organic solvents such as *N*-methyl pyrrolidone or *N,N*-dimethyl formamide. Here, we describe how to prepare exfoliated graphite using a ball mill. The graphene produced is three to four layers thick and ~500 nm in diameter on average, as measured by electron microscopy and Raman spectroscopy; can be stored in the form of light solid; and is easily dispersed in aqueous media. Our methodology consists of four main steps: (i) the mechanochemical intercalation of organic molecules (melamine) into graphite, followed by suspension in water; (ii) the washing of suspended graphene to eliminate most of the melamine; (iii) the isolation of stable graphene sheets; and (iv) freeze-drying to obtain graphene powder. This process takes 6-7 or 9-10 d for aqueous suspensions and dry powders, respectively. The product has well-defined properties and can be used for many science and technology applications, including toxicology impact assessment and the production of innovative medical devices.

INTRODUCTION

Graphene is a 2D, one-atom-thick material entirely made of carbon atoms arranged in a honeycomb lattice. Because of their distinctive mechanical, optical, and electronic properties, graphene and its derivatives are ideal candidates for a variety of applications[1]. However, a reliable and scalable graphene production method still stands as a bottleneck for its broad use. Where perfect monolayer graphene can be obtained by epitaxial growth or CVD (chemical vapor deposition) methods, the as-obtained materials are limited to specific high-performance applications in the fields of electronics or optoelectronics, because the amounts obtained are very small (i.e., high-area monolayers at several orders of magnitude below the milligram scale)[2]. The price is also an issue, as CVD graphene is affordable in tiny amounts, which can satisfy the requirements of most of optoelectronic applications, but is prohibitively expensive when required in bulk (as a guideline, the price could be ≥$56,000/m²; see e.g., https://www.graphene-supermarket.com/CVD-Graphene-on-Metals). The nanocomposite material industry and nanomedicine require larger quantities of graphene, and have therefore used chemical derivatives of graphene (namely, its oxide, graphene oxide (GO), and its reduced counterpart, reduced graphene oxide (rGO)) instead, because these derivatives can be produced in huge amounts in any laboratory, as they require very common chemical reagents and affordable labware. There are, therefore, detailed methods for preparing and functionalizing GO and rGO[3], but few procedures are available for non-oxidized graphene. Although GO and rGO (and their derivatives) are very interesting materials, their properties are different from those of pristine graphene, and similar methods to exploit this material would be beneficial.

A cost-effective approach for producing suitably large amounts of graphene is the mechanical exfoliation of graphite[4–6]. This can be performed in liquid media with the help of ultrasound[7–9] or high-shear forces[4], or under other conditions, such as with supercritical fluids or by dry ball-milling[5,6]. These methods mainly provide few-layer graphene materials of reasonable sizes, acceptable levels of defects, and good structural characteristics that enable their exploitation in composite or medical applications. So far, the mechanical exfoliation of graphite has led to the efficient separation and stabilization of graphene sheets in water, if water-soluble dispersant species (i.e., surfactants such as 1-pyrenesulfonic acid and SDS) are used in the exfoliation process[4,10]. However, many surfactants may cause toxicity problems if they are not thoroughly removed. Therefore, after having obtained the aqueous dispersion, it is desirable to remove as much of the dispersants as possible, leaving the minimum quantity indispensable to maintaining the stability of graphene in suspension, in its exfoliated state and with its structural integrity.

To address this, we developed a facile and inexpensive ball-milling process to exfoliate graphite into few-layer graphene (≤5 layers), using melamine as the exfoliating agent[11]. The mechanism of exfoliation and stabilization of graphene with melamine has been studied in detail and also modeled using density functional theory methods[12,13]. Briefly, it was found that melamine has strong π–π interactions with the basal planes of graphene layers while assembling into extended 2D networks during the exfoliation in the ball mill, and favors the anchoring of water molecules during the redispersion process. This is an easily scalable and non-oxidative methodology, which allows the researcher to produce stable suspensions of three-to-four-layer graphene[14] in polar solvents, including water, paving the way for biomedical applications such as hydrogel nanocomposites[15], production of cast films for studying cellular interactions in neuroscience[16], and ecotoxicological studies[17]. Although some potential applications require perfectly formed graphene monolayers[2], there are many other applications in which three-to-four-layer graphene would work just as well[18].

This protocol is suitable for applications involving bulk quantities of aqueous graphene, such as polymer composites, inks, or biomedicinal investigations. For instance, this mechanically exfoliated graphene has been found to be very active against myeloid leukemia, whereas graphene oxide is completely inefficient[14]. In other potential applications of this aqueous few-layer graphene in the biological/biomedical field, we have ascertained that it has low cytotoxicity to skin cells[19], revealed the direct relationship between environmental impact and its surface area[17], and achieved the fabrication of nanocomposite hydrogels in which graphene provided an improved on-demand drug delivery with an electrical stimulus[15].

The presence of melamine in these graphene samples could raise safety concerns[20], so this protocol also describes how to efficiently remove and detect minimal amounts of melamine through a variety of techniques[21]. To use few-layer graphene in our research, our laboratory has developed a simple, reliable, and sensitive colorimetric visualization of melamine in graphene dispersions through the use of gold nanoparticles (AuNPs)[21]. The resulting aggregates of AuNPs with melamine show color changes, which can be monitored using UV–Vis spectroscopy, in order to quantify the amount of melamine in graphene dispersions. The original absorbance of AuNPs at 520 nm decreases, whereas a new band at 640 nm appears, which is characteristic of melamine–AuNP aggregates. The ratio of A_{640}/A_{520} for different concentrations of melamine is plotted against this concentration, exhibiting Lambert–Beer behavior ($y = 0.18x$), with excellent linearity ($r^2 = 0.99$). This methodology is used to control the amount of removed melamine during the washing process and to quantify the melamine, which could remain in the graphene dispersions.

Furthermore, the biological assays performed so far show that melamine traces do not cause any kind of toxicity issues nor any other adverse reaction[14,16,19]. These assays include *in vitro* culture of hippocampal neuronal cells and subsequent electrophysiological studies[16], the *ex vivo* interaction of primary human monocytes coming from leukemia patients[14] and the *in vitro* culture of skin keratinocytes at different exposure times and concentrations[19].

Eventually, the lyophilization of these water suspensions gives rise to a light solid, which can be easily dispersed in either water or culture media. We have also made sure that the redispersed solid retains the properties of the original few-layer graphene[22]. In particular, both Raman spectroscopy and transmission electron microscopy (TEM) are key techniques we used to confirm the same graphene size, thickness, and defects.

Typical shortcomings of graphene prepared by graphite exfoliation are low yields, the need for high-boiling-point solvents, and great difficulty in removing the dispersant species used to promote the exfoliation[8]. In our approach, we are able to combine the proper experimental techniques and solid-state chemistry to obtain few-layer graphene in good yield (~30 mass%) in the form of a fine powder, dispersible in water with only traces of dispersant. This is of particular interest, as most similar methods report lower yields (e.g., graphite exfoliation with urea, which is reported with a yield of 2.4%[23]) or exhibit larger amounts of residual dispersant (typically 10–20 wt%), even after thorough washing[24,25]. Graphene produced as described in articles that report higher yields is either not water-dispersible (such as that exfoliated by pyrene[26]) or poorly water dispersible (such as that mechanochemically exfoliated with elemental sulfur[27]). In cases in which the graphene produced is water-dispersible, it usually contains massive amounts of dispersants[28]. The advantages of using our protocol are that the product is a solid graphene powder produced in good yield, with high water (or cell culture media) dispersibility, and that the original structural and physicochemical features of graphene are retained.

The possibility of having a few-layer graphene as a solid, dispersible in water, culture media and, in principle, in any organic solvent, opens new horizons in the technology of graphene:

(i) This solid can be safely stored and shipped;

(ii) This solid can be dispersed in aqueous media or any organic solvent;

(iii) This solid can be used for studies in materials science (e.g., inks, composites);

(iv) The safety issues of this new nanoform of carbon, which have raised substantial concern, can be addressed under the proper preparative conditions, without the use of additional potentially toxic detergents[29].

In summary, this methodology is simple and reproducible, and has already been used for biological applications, including toxicological studies[14–19].

Overview of the procedure

The procedure is divided into four main sections, detailed in the following paragraphs.

Melamine intercalation and dispersion in water (Steps 1–16). Graphite powder and melamine are forced into contact by the use of a planetary ball mill, which provides sufficient energy to overcome the π-stacking energy of the sp^2 planes, and allows aromatic compounds to act as the exfoliating agent[12]. Among the many compounds that could be used for this purpose, melamine was determined to be the most efficient, because of its ability to form extended 2D networks[11,12]. The process is carried out on a dry basis and under an air atmosphere.

The resulting heterogeneous solid mixture is removed from the ball mill using water and mild ultrasonication.

The experimental conditions described below for the ballmilling process (e.g., graphite/melamine ratio, sizes of the jar and balls, times and revolutions) are designed for lab-scale production. This process is scalable when the characteristics of the different technical variables are taken into account, although it is important to underline that planetary mills are currently available only for lab and pilot scales (they are provided with jars of up to a maximum capacity of 1 liter)[30]. It is widely accepted in mechanochemistry that the specific (i.e., per unit mass of sample) energy entry is a critical parameter that must be taken into account to determine the optimal conditions[26,30]. Therefore all the instrumental settings should be scaled with a view to keeping such specific energy constant when scaling from the lab-scale optimum to the larger scale.

Melamine removal (Steps 17–26). Most of the melamine is removed by washing with water at 70 °C. Among the many methods that could be applied to such purpose, we have chosen dialysis, for its simplicity, for its cost-effectiveness, and for being the least dependent on the operator. In addition, the maintenance of the colloidal stability of aqueous few-layer graphene during the washing process is critical for a successful production, so the

dialysis is helpful in keeping graphene in the dispersion state[25] and is compatible with the application of mild sonication pulses, which would be otherwise difficult. The extent to which melamine is removed by dialysis is qualitatively monitored by the naked eye through the addition of a AuNP solution to aliquots extracted from the washing waters. As will be shown below, AuNPs are an invaluable analytical tool to detect (and even quantify) melamine in this protocol. A rapid, nondestructive, and visual monitoring is enabled by the color change of AuNPs from magenta (naked) to dark blue (complexed with melamine).

Separation and isolation of stable few-layer graphene sheets in suspension (Steps 27–33). After eliminating the majority of the melamine, leaving only the minimum that is indispensable to keeping the graphene in suspension, it is necessary to separate the stable few-layer graphene sheets in suspension from the accompanying nonexfoliated graphite. For this, we prefer natural sedimentation, through which most graphite leftovers deposit at the bottom. Then a critical extraction step is

performed in order to isolate the desired material. The concentration of graphene is determined by UV–Vis absorption at 660 nm with a weight extinction coefficient of 15.0184 ml/mg per cm (see Anticipated Results).

Drying of the aqueous graphene suspension and redispersion of the powder (Steps 34–39). Depending on the specific needs and the envisioned fate of the aqueous graphene, one might want to remove water without compacting the graphene sheets. Vacuum-filtering or thermal evaporation usually causes the material to pack during the drying process; this occurs by capillary forces, often irreversibly, and impairs the subsequent processability of the material. It has been known for years that freeze–drying, a useful technique for efficiently drying carbon nanomaterials, avoids these drawbacks and preserves the structure of the dried entities[31]. The quantitative determination of the remaining melamine in the resulting graphene powder is performed by elemental analysis and thermogravimetry. The powder can be redispersed in a variety of solvents using mild ultrasound.

MATERIALS
REAGENTS
Preparation of few-layer graphene through the ball-milling process
- Graphite powder (Bay Carbon, SP-1 grade)
- Melamine (Sigma-Aldrich, CAS no. 108-78-1) **! CAUTION** For safety reasons, a lab coat, gloves, and a face mask should be worn during the manipulation of powders.
- Ultrapure water (18.2 MΛ·cm; Milli-Q system from Millipore)

Preparation of AuNPs
- Gold (III) chloride hydrate (HAuCl₄ · H₂O; Sigma-Aldrich, CAS no. 27988-77-8) **! CAUTION** HAuCl₄ · H₂O can cause skin corrosion and serious eye damage. For safety reasons, a lab coat, gloves, an eye shield, and a face mask should be worn during its manipulation.
- Trisodium citrate dihydrate (Sigma-Aldrich, CAS no. 6132-04-3)
  ! CAUTION For safety reasons, a lab coat, gloves, and a face mask should be worn during the manipulation of trisodium citrate dihydrate.

Characterization
- Silicon wafers (P/boron <100>, resistivity: 0.01–0.02; WRS Materials)
- Lacey carbon film on 300-mesh nickel grids (Electron Microscopy Sciences, cat. no. LC300-Ni)

EQUIPMENT
- Planetary mill (Retsch, model no. PM100), provided with a 25-ml steel jar and ten stainless-steel balls of 1-cm diameter
- Freeze-drying system (Telstar Lyoquest)
- UV–Vis–NIR spectrophotometer (Cary, model no. 5000)
- Thermogravimetric instrument (TA Instruments, model no. TGA Q50), provided with Pt pans
- Raman microspectrometer (Renishaw, InVia model)
- High-resolution transmission electron microscope (JEOL, model no. 2100)
- 1-cm quartz cuvettes (Agilent, part no. 6610000800)
- X-ray photoelectron spectrometer (VG Escalab, model no. 200R)
- Elemental analyzer (LECO CHNS-932, model no. 601-800-500)
- Ultrasonic cleaning bath without heating (Selecta, model no. 3000513; set to 50/60-Hz frequency and 360 W of nominal power)
- Ultrapure water system (Purelab UHQ (ELGA))
- Omnipore hydrophilic Teflon membranes (0.2-μm pore size; Millipore, cat. no. JGWP02500)
- Thermogravimetric analysis instrument (TA Instruments, model no. TGA Q50)
- 10-ml syringe
- Long needle
- Spectra/Por1 dialysis membrane (MWCO = 6–8 kDa, 20.4-mm diameter, 32-mm nominal flat width; Spectrum Laboratories)

REAGENT SETUP
Ultrapure water Immediately before use, prepare ultrapure water by purifying previously deionized water using the Purelab UHQ (ELGA) device according to the standard protocol.
AuNP synthesis Prepare AuNPs according to the procedure described in the literature[32]. Briefly, AuNPs were prepared by reduction of gold(III) chloride trihydrate with trisodium citrate. In a typical procedure, add 25 ml of trisodium citrate (38.8 mM) to a boiling solution of gold (III) chloride trihydrate (500 ml, 0.5 mM) and stir the solution for 15 min until a magenta suspension is obtained. The concentration of the AuNP suspension is determined using UV–Vis spectroscopy, as has been reported in the literature[33].
! CAUTION HAuCl₄ · H₂O can cause skin corrosion and serious eye damage. For safety reasons, a lab coat, gloves, an eye shield, and a face mask should be worn during its manipulation. **· CRITICAL** Owing to the colloidal nature of AuNPs, they can be highly unstable if proper requirements of storage and use are not met. AuNPs for graphene characterization should be freshly prepared as far as possible. However, storing a stock is possible if it is always kept in a refrigerator at 4 °C, and contained in a dark bottle or other means of isolation from light and external impurities. Do not use AuNPs if their magenta color is not vivid or if they become turbid or blue. The operator must periodically obtain a UV–Vis spectrum of stock AuNPs and compare it with the one obtained the day they were synthesized. Any deviation from the first spectrum is sufficient reason to discard these AuNPs and to prepare them anew.

EQUIPMENT SETUP
Raman spectroscopy Raman characterization is performed using an InVia Renishaw microspectrometer. Raman samples are prepared from stable dispersions of graphene by drop-casting over silicon oxide surfaces (small pieces of silicon wafers (WRS Materials)), and left to evaporate in ambient conditions. Raman spectra are acquired with the 532-nm laser focused under the ×100 objective and at an incident power of 1% (keeping the energy density <~1 mW/μm²), for 10 s of exposure time, in the range from 1,100 to 3,000 per cm.
TEM To perform TEM analyses, stable dispersions of graphene are diluted, drop-cast on carbon-coated microscopy grids, and dried under vacuum. From among the different grids that can be used for this purpose, we have chosen Lacey carbon film nickel grids (3.00 mm, 200 mesh), as these provided us with the best results. The samples are studied by high-resolution TEM using a JEOL 2100 at an accelerating voltage of 100 kV.
UV–Vis spectroscopy UV–Vis spectra are recorded in 1-cm quartz cuvettes (Agilent, part no. 6610000800) on a Cary 5000 UV–Vis–NIR spectrophotometer. Dual-beam mode and baseline correction are used throughout the measurements to scan the samples.

Thermogravimetric analysis The thermogravimetric analyses are performed with a TGA Q50 (TA Instruments) at 10 °C per min under a nitrogen atmosphere, from 100 to 800 °C, to sample aliquots of 0.5–1 mg contained inside a Pt pan. The thermogravimetric program was preceded by an isotherm at 100 °C for 20 min in order to remove possible traces of moisture.

PROCEDURE

Intercalation of melamine molecules within graphitic planes ● TIMING 40 min

1| Combine 7.5 mg of graphite and 22.5 mg of melamine in a stainless-steel grinding jar (25 ml) containing ten stainless-steel balls (1-cm diameter).

! CAUTION For safety reasons, a lab coat, gloves, and a face mask should be worn during the manipulation of powders.

2| Close and place the grinding bowl in the planetary mill and tighten it with the clamping device.

! CAUTION Ensure that the weight of the jar, filled with balls and a sample, is compensated for by setting the counterweight (700 g for a 25-ml jar with ten 1-cm balls and 30 mg of sample), and the clamping device is totally locked.

3| Program the planetary mill according to the following milling conditions:

Revolutions per min	Time	Atmosphere	Interval operation
100	30 min	Air	Spinning reversal every 3 min

4| Once the milling has ended, the inner walls of the jar and the surface of the milling balls will be covered with a solid consisting of a heterogeneous mixture of exfoliated graphite, nonexfoliated graphite, and melamine. Take the jar out of the planetary mill. You then must release the mixture from the balls (Steps 5–9) and from the jar (Steps 10–15). These steps can be performed simultaneously; or in either order (**Fig. 1**). Sonication pulses should be ~10 s each time. Keep the water level inside the bath always up to the mark indicated by the manufacturer, and ensure that the temperature in the sonication bath does not exceed 30 °C.

· **CRITICAL STEP** Use metal tweezers for the manipulation of balls.

Release of the solid from the milling balls ● TIMING 5 min

5| Carefully transfer the balls to an Erlenmeyer flask (25-ml or 50-ml capacity), one by one with the metal tweezers, and add 10 ml of Milli-Q water.

6| Sonicate for 10 s in a bath (50/60 Hz, 360 W) and gently shake the flask by hand, using circular movements to release the attached solid from the balls.

7| Using metal tweezers, remove the balls, again carefully and one by one, then sonicate the suspension for 10 s to keep the solid in suspension and thus prevent the unstable colloid from collapsing.

8| Transfer the 10-ml suspension to the prefinal dispersion container.

9| Sonicate again for 10 s to keep the solid in suspension.

Release of the solid from the jar ● TIMING 4 h 15 min

**10|** Add 10 ml of Milli-Q water to the jar.

11| Sonicate the jar for 10 s, with gentle shaking by hand, to release the attached solid from the walls.

12| Transfer the 10-ml suspension to an Erlenmeyer flask (20-ml or 50-ml capacity).

13| Sonicate the Erlenmeyer flask for 10 s, again to ensure the suspension of the solid in water.

14| Transfer the suspension to the prefinal dispersion container.
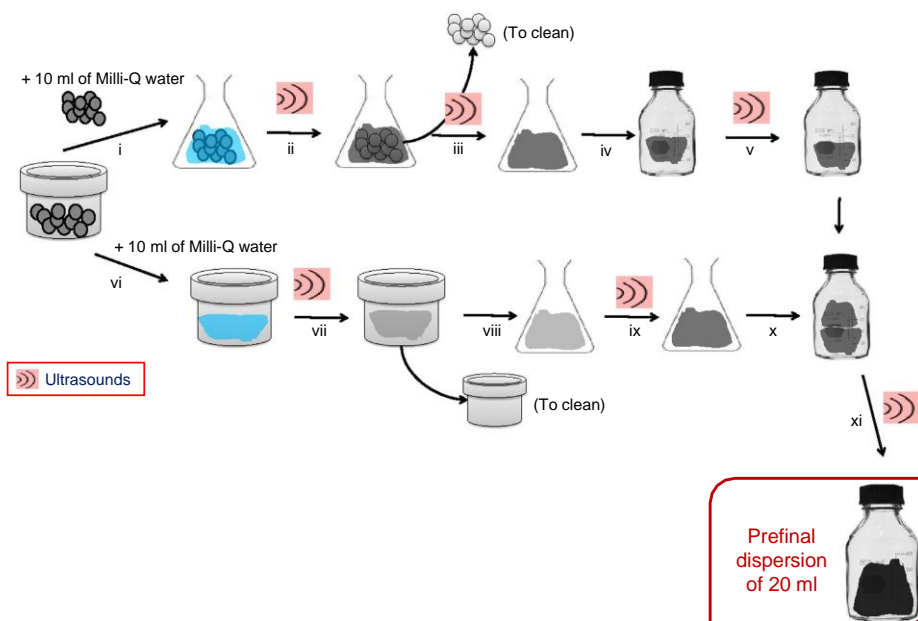
Figure 1 | Roadmap for releasing the solid mixture from the milling container by sonication in ultrapure water. (i) Transference of the balls to a flask holding 10 ml of Milli-Q water; (ii) sonication to release the attached solid from the balls; (iii) removal of the balls and sonication of the suspension; (iv) transference of the 10-ml suspension to the prefinal dispersion container; (v) sonication of the prefinal dispersion container; (vi) addition of 10 ml of Milli-Q water to the jar; (vii) sonication of the jar to release the attached solid from the walls; (viii) transfer of the 10-ml suspension to a flask; (ix) sonication of the flask; (x) transference of the suspension to the prefinal dispersion container; (xi) sonication of the prefinal dispersion container (now filled with 20 ml).

15| Sonicate the prefinal dispersion container (filled with 20 ml) for 10 s to keep the solid in suspension.
· **CRITICAL STEP** Keep the temperature in the sonication bath <30 °C, and ensure that there are no sudden temperature variations, checking the bath water periodically with a household thermometer. The aqueous suspension of the milled solid mixture, before removing the melamine excess, is an unstable colloid and thus sensitive to temperature.
! CAUTION Prolonged manipulation of steel balls inside glassware might result in glass breakage. Perform the manipulations gently and do not perform abrupt movements.

16| Repeat Steps 1–15 six times in order to obtain two containers of 60 ml of prefinal dispersion.
· **CRITICAL STEP** The prefinal dispersion container should be sonicated upon each addition of a new dispersion in order to ensure the homogenization of the sample and the suspension state.

Removal of the melamine surplus ● **TIMING 36–48 h**
· **CRITICAL** Dialyze each 60-ml container of prefinal dispersion separately.

17| Sonicate the prefinal dispersion container (60 ml) for 30 s.
· **CRITICAL STEP** Keep the temperature in the sonication bath <30 °C. Do not prolong the sonication for more than 10 s in each pulse.

18| Hydrate the dialysis sack membrane using ultrapure water for at least 30 min before being filled, in order to make it easier to handle.

19| Transfer the prefinal dispersion (60 ml) to the dialysis sack via a 10-ml syringe with a long needle.
· **CRITICAL STEP** Special care must be taken to avoid piercing the sack with the needle during its filling.
· **CRITICAL STEP** The dialysis sack should be closed with handmade knots and not with regular closure clamps to avoid the leakage of the sample and to facilitate further shaking.

20| Shake the dialysis sack by hand, holding it by the knots and using a horizontal motion for a few seconds, and place it (bent in a symmetrical 'U' shape) in an Erlenmeyer flask filled with 500 ml of Milli-Q water. See **Figure 2a,b**.

21| Sonicate the Erlenmeyer flask with the dialysis sack inside for no more than 1 min, through different pulses, to ensure the homogenization of the sample inside the membrane.

· **CRITICAL STEP** Keep the temperature in the sonication bath <30 °C. Do not prolong the sonication for more than 10 s in each pulse. When sonicating the dialysis sack, the position of the Erlenmeyer flask in the ultrasonic bath may affect the dispersion of the sample. Ensure that the waves are transmitted across the sack.

22| Place the Erlenmeyer flask with the dialysis sack containing the dispersion on a hot plate at 70 °C with slow stirring for at least 2 h (**Fig. 2a,b**).
· **CRITICAL STEP** Ensure that the temperature of the hot plate is well controlled and also that the magnetic stirring bar does not hit the dialysis sack.
? TROUBLESHOOTING

Figure 2 | Images of the melamine washing process. (**a,b**) Dialysis sack placement and handling (Step 20). (**c**) Incorporation of the dialyzed suspension into a proper container for the settlement stage (Step 26).

23| After the elapsed time of dialysis, check for the presence of melamine in the washing waters by analysis with AuNPs[21]. Place 2 ml of water washings and 1 ml of AuNPs in a vial and mix them by pipetting once. Check the color of the mixture: magenta color indicates no melamine; purple or blue colors indicate that melamine is still being washed away from the sample (see Anticipated Results).

24| If there is still melamine in the water, replace the dialysis waters with fresh Milli-Q water and repeat Steps 20-23.

25| Repeat until a magenta color appears twice in a row in the analysis of water washings by AuNPs. Note that the dialysis usually implies one cycle overnight. In a typical experiment, no melamine is usually found after five cycles of dialysis (see Anticipated Results).

26| Once the dialyses are completed, transfer the dispersions from the dialysis sack (60 ml each) to a stabilization container (final volume of 120 ml), as shown in **Figure 2c**.

Separation of few-layer graphene from nonexfoliated graphite ● **TIMING** 5 d
27| Keep the dispersion in the stabilization container (final volume of 120 ml) at room temperature (20-25 °C) for 5 d. During this time, the nonexfoliated graphite precipitates and the exfoliated graphene remains in suspension.
· **CRITICAL STEP** Make sure not to move the container in any way while resting for the 5 d, as this will interfere with the sedimentation process.

Isolation of stable few-layer graphene sheets in suspension ● **TIMING** 3-27 h
28| The few-layer graphene dispersion should be isolated from the nonexfoliated graphite by manual extraction. The aqueous medium will contain the unwanted graphite in the bottom sediments and in some floating particles on the surface of the liquid. The liquid bulk will have a gradient of few-layer graphene, with a higher concentration at the bottom and a lower concentration at the surface. Thus, the extraction must be performed from bottom to top, avoiding the graphite-rich ends (**Fig. 3**). Start the extraction process by inclining the stabilization container at 45°.
? TROUBLESHOOTING

29| Extract the dispersion from the lowest part of the liquid with a pipette without touching either the bottom of the container or the graphite 'cloud' floating on the surface of the dispersion, and transfer it stepwise to the final container. Sonicate the dispersion for 10 s to homogenize.
· **CRITICAL STEP** Keep the temperature in the sonication bath <30 °C and do not prolong the sonication for more than 10 s in a single pulse. It is important to avoid both the graphite floating on the surface of the liquid and the sediment on the bottom, as the nonexfoliated graphite concentrates in those places. The pipetting must be done slowly and carefully in order not to disturb the state of equilibrium between the precipitate and the dispersion. **Supplementary Video 1** illustrates the extraction procedure.
? TROUBLESHOOTING

30| Quantify the melamine in the graphene dispersion. To do this, add 1 ml of AuNPs to 2 ml of the few-layer graphene aqueous suspension. Determine the absorbance at 500-650 nm (see Anticipated Results). In a typical experiment, the melamine concentration will be ≤1 p.p.m., for a nominal graphene concentration of 0.1 mg/ml.
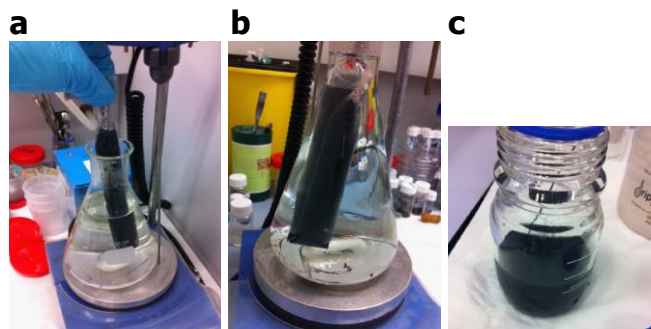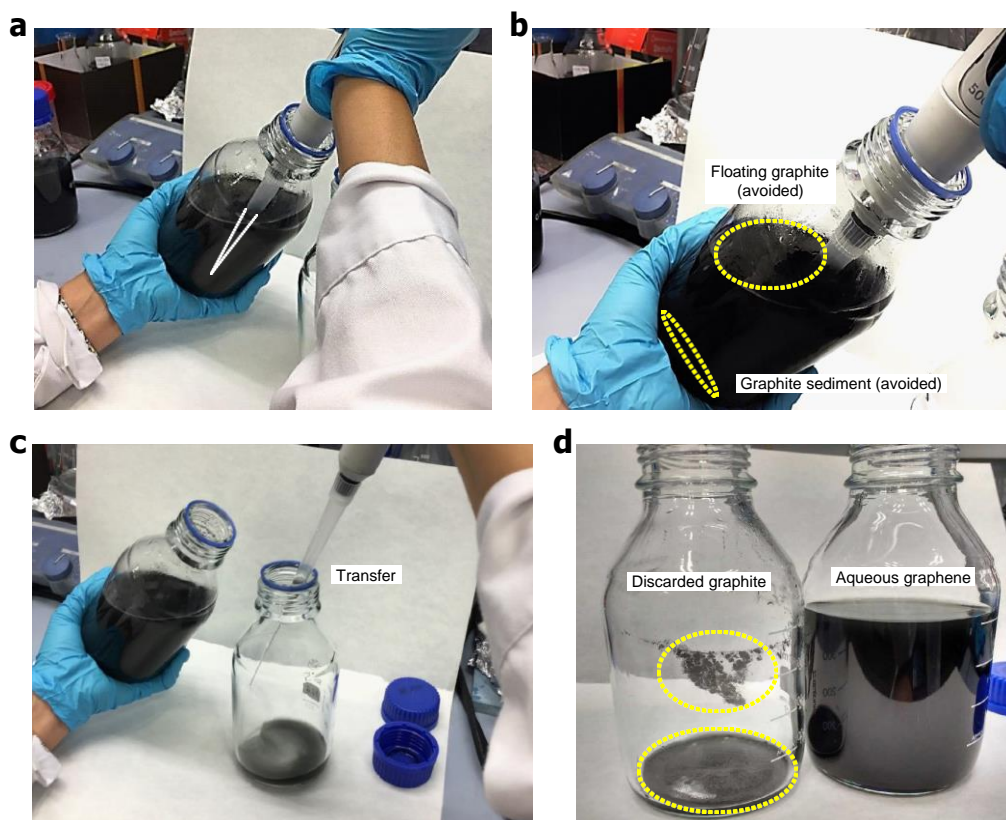
Figure 3 | Photographs of the process of extracting stable graphene sheets in water after the sedimentation process. (**a**) Insertion of the micropipette tip close to the lowest part of the liquid. (**b**) Magnification of the previous image, in which it is shown that graphite on the surface and the bottom is avoided. (**c**) Illustration of the transference process. (**d**) Finalization of the extraction. Dotted white lines provide visual aid for locating the micropipette tip, whereas dotted yellow circles denote the presence of graphite.

· **CRITICAL STEP** The UV-Vis measurement should be performed immediately after the addition of the AuNPs, in order to avoid spontaneous aggregation of nanoparticles.

31| Determine the concentration of few-layer graphene in the dispersion by UV-Vis spectroscopy at 660 nm; the extinction coefficient was empirically determined to be 15.0184 ml/mg per cm. In a typical experiment, the concentration of few-layer graphene is ~0.1 mg/ml (see Anticipated Results).
? TROUBLESHOOTING

32| Verify the concentration of few-layer graphene in suspension by weight using a Teflon filter membrane in common vacuum-filtering equipment. To do this, vacuum-filter a known volume of graphene aqueous dispersion (typically 10 ml) and bring the membrane to complete dryness. By weighing the membrane before and after filtering, and knowing the filtered volume, a graphene concentration in water (in mg/ml) is obtained (see Anticipated Results).
? TROUBLESHOOTING

33| Determine the ratio between the graphene and melamine concentrations. This gives a quick indication of how successful the removal of melamine was. Graphene is sufficiently clean if the ratio [melamine (p.p.m.)]/[graphene (mg/ml)] is below 0.01.
? TROUBLESHOOTING
■ **PAUSE POINT** The few-layer graphene in aqueous medium can be stored for 1 month at 20–25 °C without changes in properties. Closed glass or plastic bottles are suitable containers, with no special precautions against light or moisture needed. However, sudden temperature changes must be avoided.

Drying of the aqueous graphene suspension ● **TIMING** 72-96 h
34| Fill transparent plastic containers (**Fig. 4**) with ~60–80 ml of the few-layer graphene dispersion each.

35| Freeze the dispersion by immersing the sealed container in liquid nitrogen. Take as much time as necessary to ensure the full freezing of the whole sample.

! CAUTION Take appropriate safety precautions when working with liquid nitrogen, such as wearing a fully fastened lab coat, specific thermal gloves, and safety goggles.

· CRITICAL STEP Avoid the direct contact of liquid nitrogen with the dispersion, as this could be a source of graphene contamination from external impurities. Use the plastic lid of the container, but do not tighten completely, to allow the pressure to equalize.

36| Cover the containers with a piece of aluminum foil with five equidistant 1-mm-sized holes and seal them with two elastic bands.

37| Place the containers in the freeze-drying system and program it for 72 h at –80 ˚C under vacuum (0.005 mbar). The resulting powder is shown in **Figure 4**.
? TROUBLESHOOTING

and carefully tapping it from the bottom. Aluminum foil serves as a good surface for collecting and transferring these powders to their final container. As the typical mass concentration of the aqueous few-layer graphene is ~0.1 mg/ml (see Anticipated Results), it is possible to estimate how much solid will be collected after the freeze–drying step, keeping in mind that there will be slight deviations because of unavoidable losses.

! CAUTION For safety reasons, a lab coat, gloves, and a face mask should be worn during the manipulation of powders.

· CRITICAL STEP Perform the manipulation of few-layer graphene powder away from airstreams, as it is very light and fluffy.

■ PAUSE POINT The graphene powder can be stored in any kind of container (as long as it is sealed) and at any range of room temperatures. No specific protection from air, light or moisture is necessary. Glass vials, however, are preferred over plastic for the storage, because electrostatic interactions can cause the powder to fly off of plastic surfaces.

Redispersion of the graphene powder ● TIMING 1-5 min

39| This graphene powder can be easily redispersed in a wide variety of polar solvents, including water. To do this, add the liquid medium carefully on top of the corresponding amount of powder graphene, followed by manual or vortex agitation for a few seconds, and bath ultrasonication (while manually shaking) for up to 1 min in short pulses (no longer than 10 s each). This process may be equally performed in other polar organic solvents (such as DMF or DMSO) and other aqueous media (such as cell culture medium). There is a detailed video in the supporting information of ref. 22 showing how to redisperse this graphene powder (http://www.rsc.org/suppdata/c6/nr/c6nr03246j/c6nr03246j2.avi).

· CRITICAL STEP As graphene powder is very light and fluffy, pouring liquids on top of it might result in the loss of material. Adding the liquid slowly and dropwise is advised.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
22	The sample precipitates during dialysis	The colloidal stability of the medium has collapsed. This can happen as a result of sudden temperature changes or unintended hitting	Ensure that the temperature is fixed at 70 ˚C and that the magnetic bar does not hit the sack. Sonicate the system for few seconds and control stirring

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
28 and 29	The state of equilibrium between the precipitate and the dispersion is altered during the isolation	The dispersion has been accidentally shaken during the extraction step	Sonicate the dispersion, let it settle down for 24 h and repeat the isolation of graphene (Steps 28 and 29)
31 and 32	The concentration of the graphene as determined by weighing is < 0.1 and does not equal the concentration of graphene as determined by UV-Vis	Part of the precipitated bulk graphite has been accidentally taken up with the graphene during Steps 28 and 29	Let the dispersion settle down for 24 h and repeat the isolation of graphene (Steps 28 and 29)
33	Melamine/graphene mass ratio is ≥0.01	Dialysis was not completed	Repeat washing (Steps 17–26) and isolation (Steps 28 and 29)
	The aqueous graphene dispersion ends up partly precipitated within 1 month of storage time	The colloidal stability has collapsed, probably due to inappropriate storage conditions (e.g., sudden temperature changes, shuttling or shaking motions)	Sonicate the dispersion, let it settle down for 24 h, and use it if no major precipitation occurs. Otherwise, dispose of it
37	The lyophilization was not completed	The timing of the lyophilization depends on the volume to be dried	Keep running the freeze-drying device until the powder can be clearly seen through the walls of the plastic container. The typical sublimation rate of an ice front is ~1 mm/h

● TIMING

Steps 1–16, intercalation of melamine molecules within graphitic planes and water dispersion: 5 h

Steps 17–26, removal of the melamine surplus: 36–48 h

Step 27, separation of few-layer graphene from nonexfoliated graphite: 5 d

Steps 28–33, isolation of the stable few-layer graphene sheets in suspension: 3–27 h

Steps 34–38, drying of the aqueous graphene suspension: 72–96 h

Step 28, redispersion of graphene powder: 1–5 min

ANTICIPATED RESULTS
Few-layer graphene in aqueous environment

The newly formed few-layer graphene product must be washed to remove the excess melamine. To check that the melamine is being removed, and to determine when to stop washing the product, the washes are analyzed using AuNPs. If there is no melamine, the solution is magenta; if melamine is present, the solution turns blue (Steps 23–25, **Fig. 5a**). Most of the melamine is removed between the second and the third fraction, both showing a blue color when exposed to nanoparticles (Steps 23–25). The fourth and the fifth fractions show magenta color as melamine is <1 p.p.m. (**Fig. 5a**).

UV-Vis spectroscopy is not only used to quantify residual melamine (Step 30, **Fig. 5b**) but also to determine the graphene mass concentration (Step 31). We typically obtain concentration values at safe levels of melamine (<1 p.p.m., for a graphene concentration of ≥0.1 mg/ml, Step 33).
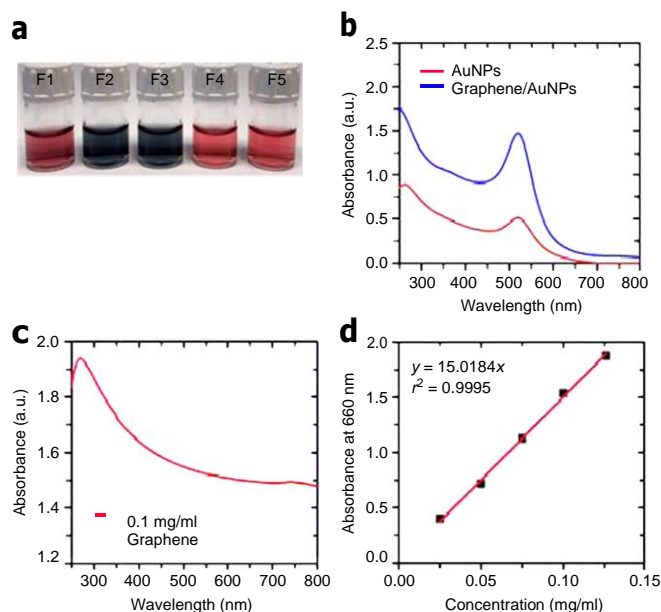

Figure 5 | Visual and spectroscopic characterization of the aqueous few-layer graphene. (**a**) Qualitative analysis of the concentration of melamine into fractions of washing water. (**b**) Representative UV-Vis absorption spectrum of AuNPs and few-layer graphene/AuNPs, with <1 p.p.m. of melamine content[21]. (**c**) Representative UV-Vis absorption spectrum of 0.1 mg/ml few-layer graphene in water[21]. (**d**) Calibration plot of few-layer graphene in water[21]. a.u., arbitrary units.

Although it is important to minimize the amount of residual melamine, because it is potentially toxic in some applications, it is essential that a very small amount remain after washing to enable successful redispersion and stabilization of the few-layer graphene in water. A recently published theoretical study has revealed that these almost negligible amounts of melamine act as anchoring points for water molecules, substantially increasing the number of stabilizing hydrogen bonds without altering the graphene–melamine interactions (mainly because of π-π stacking)[13]. The overall combination of such weak forces eventually leads to a stable graphene suspension in aqueous media.

The mass concentration of graphene prepared in Step 33 is calculated by UV-Vis spectroscopy (**Fig. 5c,d**; Step 31). The absorbance at 660 nm, divided by cell length, is plotted against the concentration exhibiting Lambert–Beer behavior (y = 15.0184x; r^2 = 0.9995). We typically obtain concentration values of ~0.1 mg/ml few-layer graphene in Milli-Q water.

To verify the concentration values obtained by UV-Vis, the solid residue coming from a known volume of graphene dispersion is weighed (Step 32). For this, 10 ml of few-layer graphene dispersion is filtered through a tared membrane filter, dried under vacuum for 2 h and weighed again. The concentration is calculated according to the following equation:

$$[graphene(mg/mL)] = \frac{(Final\ weight - tared\ filter\ weight)(mg)}{10(mL)}$$

Few-layer graphene in powder form

Once characterized in the aqueous environment, graphene dispersion can be lyophilized in a freeze–dryer device, resulting in low-density black powder (Steps 34–38). The characterization of the powder is conducted by elemental analysis, X-ray photoelectron spectroscopy (XPS) and thermogravimetric analysis[22]. Elemental analysis gives average values of 91.03 ±0.51 wt% C, 0.74 ± 0.04 wt% H, 0.52 ± 0.02 wt% N, and 7.71 ± 0.48 wt% O. We can estimate, from these data, traces of melamine are <0.8 wt% in the solid powder. TGA in an inert atmosphere usually displays a weight loss of <10%, which corresponds to the melamine content and a low concentration of oxygen groups, created during the ball-milling treatment (**Fig. 6a**).

Further proof of the nonoxidative nature of the milling treatment can be obtained using XPS (**Fig. 6b,c**). The C1s peak can be deconvoluted into three different components. The most intense peak at 284.8 eV is assigned to sp[2] C-atoms of the graphene sheets, which is a signature of graphitic carbon. The component at 286.2 eV has usually been attributed to C–O bonds (together with C-N) and the components at 288 eV to C=O species[22]. It is clear that the spectrum of powdered graphene, after mechanochemical treatment, displays a similar pattern, revealing that the intrinsic structure of graphite remains largely intact after treatment, and that no major oxidation occurs during milling (**Fig. 6b**). However, the N1s spectrum (**Fig. 6c**) exhibits a similar intensity of the components at 399.6 and 400.5 eV to C=N and C–N species, respectively, because of the presence of melamine[11,22].

The powder can be redispersed in water or any other polar organic solvent at a desired concentration[22], enabling concentrated graphene suspensions (up to 0.3 mg/ml in water with acceptable stability) to deposit this material onto different surfaces that can be further characterized by Raman spectroscopy and TEM, as shown in **Figure 7**.

Raman spectroscopy provides essential information that can be used to characterize graphene[2,34]. Raman spectra of a representative aliquot from few-layer graphene show the two most intense peaks of graphene (**Fig. 7a**), the G peak at around 1,580 per cm and the 2D band at around 2,700 per cm. D appears as a shoulder at around 1,600 per cm on the G band, which is characteristic of few-layer graphene. In addition, $I(2D)/I(G)$ usually falls below 1, confirming the samples to be few-layer graphene[35]. This few-layer graphene typically displays full width at half maximum (FWHM) of the 2D band ranging from 63 to 65 per cm[22]. When graphene is affected by defects, the Raman feature at 1,345 per cm (D band) appears in the spectrum.

The evolution of the intensity ratio between the G and the D bands, $I(D)/I(G)$, has recently been used to provide a method to quantify the density of defects in graphene. $I(D)/I(G)$ values ranging from 0.2 to 0.5, which is the case of this kind of sample[22], are attributed to defects located at the edges of our submicrometer flakes[36], revealing that the structure of graphene basal planes is not damaged after all the processing steps.

The average number of layers can be calculated from the intensities of the 2D bands of both graphene and its parent graphite by a formula proposed by J. N. Coleman and co-workers[28].

$$N_G = 10^{0.84M+0.45M^2}$$
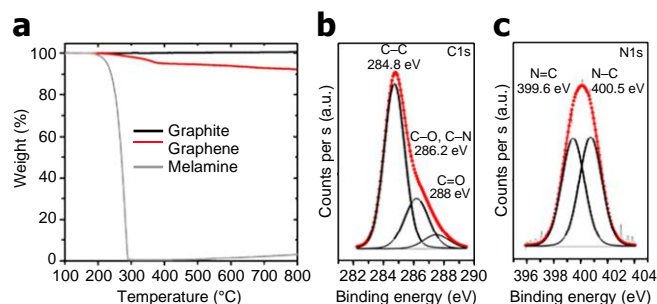
Figure 6 | Chemical characterization of graphene in powder form. (**a**) TGA plots of graphite, melamine and few-layer graphene powder. (**b,c**) C1s (**b**) and N1s (**c**) XPS of few-layer graphene powder. Adapted with permission from ref. 22, Royal Society of Chemistry.

Where M corresponds to

$$M = \frac{I_{2D\text{-ene}}(w = w_{p(\text{-ite})})/\ I_{2D\text{-ene}}(w = w_{s(\text{-ite})})}{I_{2D\text{-ite}}(w = w_{p(\text{-ite})})/\ I_{2D\text{-ene}}(w = w_{s(\text{-ite})})}$$

In this equation, I_{2D} is the intensity of the $2D$ band in Raman spectra for graphene (-ene) and graphite (-ite), taken at the specific position ($\grave{u}$), in which the first shoulder of graphite appears ($\grave{u}_s$, at about 2,675 per cm), and in which the second shoulder of graphite appears ($\grave{u}_p$, at about 2,720 per cm). All the data used in these calculations must be taken from the same graphite sample. We have applied this formula in previous studies[14], revealing that few-layer graphene produced through this protocol contains an average of three to four layers when the solid powder is redispersed in water, or approximately four layers when redispersed in cell culture media.

TEM analysis is used to determine the lateral sizes of graphene samples (**Fig. 7b**). The lateral dimension distribution is determined using Fiji-win32 software (https://imagej.net/Fiji/Downloads). Graphene samples prepared using this protocol have a wide size distribution (~200–2,000 nm), but their major population (~80%) lies in between 400 and 800 nm. **Figures 7c,d** show representative few-layer graphene flakes.
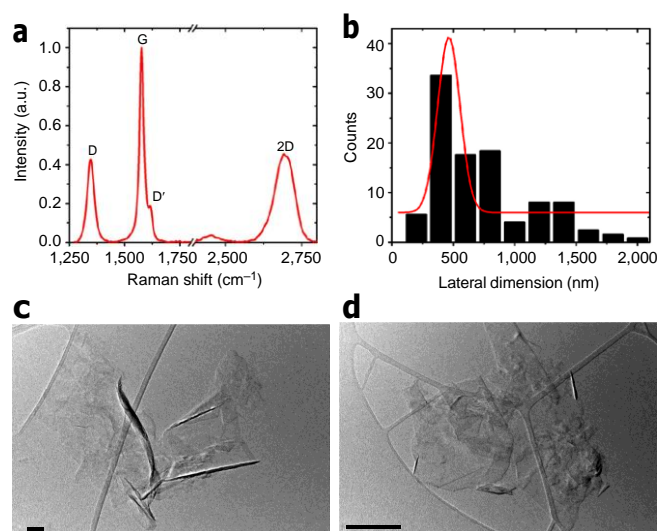
Figure 7 | Structural and morphological characterization of graphene flakes. (**a**) Representative Raman spectrum of few-layer graphene. (**b**) Lateral size distribution of few-layer graphene from TEM images, in which the counts represent the frequency of occurrence as percentages. (**c,d**) Representative TEM photos of few-layer graphene. Scale bars, 200 nm (**c**); 500 nm (**d**). a, b adapted with permission from ref. 22, Royal Society of Chemistry.

Note: Any Supplementary Information and Source Data files are available from the authors.

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