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Controlling the Graphene–Bio Interface: Dispersions in Animal Sera for Enhanced Stability and Reduced Toxicity

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Abstract

Liquid phase exfoliation of graphite in six different animal sera and evaluation of its toxicity are reported here. Previously, we reported the exfoliation of graphene using proteins and here we extend this approach complex animal fluids. The kitchen blender with high turbulence flow gave high quality and maximum exfoliation efficiency in all sera tested, when compared to shear and ultrasonication methods. Raman spectra and electron microscopy confirmed the formation of 3–4 layer, submicron size graphene, independent of the serum used. Graphene prepared in serum was directly transferred to cell culture media without post treatments. Contrary to many reports, nanotoxicity study of this fully dispersed graphene to human embryonic kidney cells, human lung cancer cells and nematodes (*C. elegans*) showed no acute toxicity for up to 7 days at various doses (50–500 µg/mL). But prolonged exposure at higher doses (300–500 µg/mL, 10–15 days) showed cytotoxicity to cells (~95% death) and reproductive toxicity to *C. elegans* (5–10% reduction in brood size). The origin of toxicity was found to be due to the highly fragmented smaller graphene sheets (<200 nm), while the larger sheets were non-toxic (50–300 µg/mL dose). In contrast, graphene produced with sodium cholate as the mediator has been found to be cytotoxic to these cells at these dosages. We demonstrated the toxicity of liquid phase exfoliated graphene is attributed to highly fragmented fractions or non-biocompatible exfoliating agents. Thus, low toxicity graphene/serum suspensions are produced by a facile method in biological media, this approach may accelerate the much-anticipated development of graphene for biological applications.

Graphical abstract

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Supporting Information

Includes properties of graphene produced at all conditions, more TEM images and method for TEM and Raman studies.

The Supporting Information is available free of charge on the ACS Publications website.

Author Contributions

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Introduction

Direct exfoliation of graphite to few layer, high quality graphene in animal sera, and its biological toxicity are reported here. Because of unique mechanical and physiochemical properties, graphene is considered as the material of the 21st century.¹ Rapid development of graphene research focuses on the optoelectronic applications of graphene for foldable, lightweight and ultrafast electronic devices.² High surface area, light weight, high mechanical strength and strong fluorescence quenching properties of graphene is attractive for biological and health care applications, such as biosensors, drug delivery, and bioelectronics.³ However, good colloidal stability and low toxicity of graphene is vital for the implementation of graphene materials for biological applications, and current studies address this issue.

Production of graphene under biologically relevant conditions is challenging because of the high dispersion forces among graphene sheets in water.⁴ Graphene oxide (GO) with oxygen functional groups introduced on graphene basal plane by oxidative methods (e.g., Hummers method)⁵ lowers these dispersive forces and solubilizes it in water.⁶ GO is often used as a model system to demonstrate the biological applications of graphene derivatives.³ For biological applications, the graphene oxide samples are usually modified using polymers (for example, PEG),⁷ proteins,⁸ DNA,⁹ or other biomolecules.¹⁰⁻¹¹ However, the presence of metal impurities, oxidative functional groups and extensive heterogeneity in its functional groups⁵ limits the use of GO for systematic biological studies. Therefore, low-defect graphene with minimum chemical processing by a green, sustainable, and scalable method in media that are compatible with various biological protocols is urgently required, but not available, until now.

Direct exfoliation of graphite to graphene in water is achieved by using small organic molecules,¹² amphiphilic stabilizers such as surfactants,¹³ proteins,^{8,14,15} and carbohydrates.¹⁶ The reagent used for this process (exfoliating agent) should stabilize graphene in water, but it should not be toxic to most biological systems, if the preparations are to be used for biological applications. Biomolecules such as serum proteins are better candidates as exfoliation agents due to their wide spread compatibility with numerous biological systems. Previously our group and others reported protein-based exfoliation of graphite in a shear reactor with encouraging results.⁸ Herein, our focus is on the preparation of graphene suspensions in animal sera for cell and animal model studies. The data show that the

graphene loaded sera are excellent media for this purpose, when compared to graphene produced in surfactants (Figure 1).

Preparation of graphene in serum is attractive for the biological applications of graphene, not graphene oxide. Serum is a complex mixture of proteins, peptides, amino acids, salts, sugars, and many other small molecules,¹⁷ in which exfoliation of graphite is feasible because of its protein content.⁸ However, the presence of carbohydrates, sugars, and other small molecules could affect the exfoliation efficiency, physical characteristics such as size, the extent and type of defects as well as the number of layers present and its colloidal stability. Previously, melamine exfoliated graphene showed good colloidal stability in cell culture media for 24 h.¹⁸ Graphene in animal serum could improve the stability and biocompatibility of the suspensions to cell culture media or body fluids, and serum could be a good platform to study nanotoxicity of graphene with specific cell lines or animals *in vivo*.^{19,20} Graphene produced in animal sera is expected to have a strong impact on graphene biotechnology, because many animal model studies can be carried out with good compatibility. For example, if one would like to do a mice model study with a graphene sample, mice serum can be used to prepare the graphene and administer to the animal, without having to deal with the compatibility issues from the media used for the graphene preparation.

Graphene produced in serum (~7 mg/mL) was used to study the toxicity of graphene to human cells and nematodes at a wide range of doses (Figure 1). Human Embryonic Kidney 293T (HEK-293) cell line is a good model system for toxicity studies because of its robustness and high transfection efficiency,²¹ and it has already been used to study cytotoxicity of other carbon materials.²² Then, the toxicity studies have been extended to a living organism *C. elegans* (nematodes), which served as a good model system because of the availability of its complete genome data, transparent body, and variable cellular activities.²³ A wide range of dose dependent toxicity studies of graphene up to 500 $\mu\text{g mL}^{-1}$ was feasible in the current studies, because high concentrations of graphene could be produced directly in cell media. In contrast, dilution with the culture media decreases the dose range of graphene produced by other methods. Our study involving the scalable production of graphene with high colloidal stability in sera and its toxicity data might help the graphene research to develop other graphene-based products for biological applications.

Experimental

Materials

Graphite (~150 μm) and Sera (Bovine, Chicken, Human, Horse, Porcine and Rabbit) was purchased from Sigma Aldrich (St. Louis, MO). High shear disperser (stator/rotor diameter = 18/12.7 mm) with speed control was purchased from IKA Inc. BSA was obtained from Equitech-Bio Inc. (Kerrville, TX). Oster® Kitchen 10 speed blender got from a local shop.

Exfoliation of graphite in serum

Fluid dynamics in a kitchen blender was used to prepare graphene in serum. Typically, graphite (100 mg/mL) and bovine serum (2-15%, by volume) was taken in the blender jar and the pH was adjusted to 7.4 using dilute HCl or NaOH. In case of other sera,

concentration of 10%) was used for exfoliation. The total volume of the mixture was adjusted to 200 mL, prior to the blending at 17 000 rpm using a 6-arm blender blade. Similarly, IKA high shear disperser (T25) at 17 krpm was used to exfoliate graphite in animal sera in 200 mL volume. Ultrasonication experiments were performed in 20 mL volume (10% serum, 100 mg/mL graphite), in a Branson bath sonicator (9.5 L) for 10 -15 h, at ~25 °C. Samples (1 mL) were taken out from the mixture to monitor progress. The rate of exfoliation (mg h^{-1}) was calculated using the kinetic traces, after removing the unexfoliated graphite by centrifugation (1500 rpm for 45 minutes). Graphene concentration was measured using its reported extinction coefficient at 660 nm,⁸ and the exfoliation efficiency ($\text{mg mL}^{-1} \text{h}^{-1}$) determined for each batch, in triplicates.

Graphene that is more suitable for biological assays was prepared under completely sterile conditions to avoid contamination. Blender jar, motor and other assemblies were kept under UV irradiation in a biosafety hood for 12 h. Blender blade, graphite and MilliQ water were autoclaved to ensure sterility. Exfoliation, separation and transfer were also carried out in a biosafety hood.

***In vitro* toxicity of graphene to HEK-293T and H1299 cells**

The *in vitro* compatibility of serum- and cholate-exfoliated graphene sheets were investigated by co-incubating them with human embryonic kidney HEK-293T cells, which are one of the most widely characterized human cells lines available, and H1299 human non-small cell lung carcinoma cell line, which are commonly used for studying nanoparticle-induced lung toxicity.²⁴ The standard growth conditions of 37 °C, 5% CO₂ and 95% relative humidity were maintained throughout the study.

Briefly, 0.4×10^5 HEK-293T or H1299 cells were seeded in each well of 24 well plates in 500 μL of complete growth media which consisted of DMEMTM (Dulbecco's Modified Eagle Medium) supplemented with 9% fetal bovine serum and 2% non-essential amino acids. The cells were allowed to adhere to the bottom of the wells of 24-well plate overnight and then exposed to a range of concentrations of graphene dispersions at 25% initial cell confluency. The compatibility of incubated material with the cells was measured (in triplicates) by using the following procedures.

Intracellular metabolism—The intracellular metabolism of HEK-293T or H1299 cells co-incubated with as-prepared serum or cholate-exfoliated graphene and size-fractionated serum-exfoliated graphene were measured by quantifying the intracellular (total) dehydrogenase activity using CCK-8 kit as reported previously.²⁵

Phase-contrast optical microscopy—The overall extracellular morphology of a cell is an important indicator of its health.²⁶ The morphologies of the metabolically active cells co-incubated with graphene dispersions were monitored from short-term (1 – 24 hours) up to 16 days.

In vivo* toxicity on *Caenorhabditis elegans

Caenorhabditis elegans a free living microscopic soil nematode was used as whole animal model for toxicity screening of the graphene dispersions. The wild-type N2 (ancestral) strain of *C. elegans* was obtained from Caenorhabditis Genetics Centre in Minneapolis, MN and were fed with OP50 strain of *E. coli* seeded on nematode growth medium (NGM) agar plates. *E. coli* OP50 was grown on Luria Bertani agar under incubation at 37°C. Development of *C. elegans* from LI stage to the adult hermaphrodite form is reached within 3 days at 20°C. Prior to each experiment, the worms were age synchronized and adults were lysed by treatment with 500 µL of 1 M sodium hydroxide and 200 µL of 5.65% sodium hypochlorite to release the eggs. The eggs were allowed to hatch on NGM with *E. coli* OP50.

The worms were tested for survivability on acute (48 h) and prolonged exposure (10 days) and activity with graphene at concentrations ranging from 50 to 500 µg/mL in exfoliants bovine serum and sodium cholate. Briefly, a 12-well assay was performed where adult *C. elegans* on NGM were exposed to varying concentrations of graphene for 48 h (acute toxicity assay). Toxic effects on prolonged exposure were assessed by maintaining the seeded plates for a period of 10 days. In order to control for proper food availability and avoid starvation induced stress, worms in the chronic toxicity assay were transferred to fresh agar with OP50 and graphene every 2 days.

At each sampling time, the worms were observed and enumerated using the binocular dissecting microscope. Sample size of 20 was used for each nanomaterial concentration and time (duplicate wells of 20 worms each) and the entire study was repeated three times. Reproductive toxicity was assessed by placing one nematode in young adult stage from a synchronized culture in each well of 12 well plate under the same culture conditions and graphene test concentrations as described previously. The nematodes were maintained for 3 days (72 h) and the number of offspring at all stages beyond the eggs were counted. For each test concentration and control, the number of progeny from four wells were obtained for each test replicate and the test was repeated three times.^[26]

Results

Preparation of high quality graphene in biological media by a simple, scalable, green approach in a kitchen blender was demonstrated here (Figure 1). The efficiencies of graphene exfoliation in six animal sera by three different methods (kitchen blender, ultrasonication, and shear reactor) were compared and our results are described below.

Synthesis of Graphene in Serum

Graphite exfoliation was optimized first in a kitchen blender using bovine serum as a model system. The exfoliation efficiency ($\text{mg mL}^{-1} \text{ h}^{-1}$) of graphene was quantified by varying bovine serum concentration from 1 to 10% (by volume), at constant graphite concentration (100 mg mL^{-1}). Graphene produced at regular intervals of blending at a constant blade speed of 17 krpm was estimated by measuring the absorbance of graphene suspensions at 660 nm (after removing the unexfoliated graphite) as a function of exfoliation time. From

these data, the rate of exfoliation (mg h^{-1}) and exfoliation efficiency ($\text{mg mL}^{-1} \text{h}^{-1}$) have been determined.⁸ The efficiency increased from 2.5 to $\sim 4.5 \text{ mg mL}^{-1} \text{h}^{-1}$ (Figure 2A) with increasing serum concentration (1 to 5%), but no further increase was observed above 5% serum. At the maximum serum concentration that we tested (10%), 1 g graphene was produced in an h in 200 mL sample at pH 7.4. Next, we examined the rates and efficiencies of graphene production in a kitchen blender with human, chicken, horse, porcine, and rabbit sera, under similar conditions.

Figure 2B shows the feasibility of making graphene in any serum with exfoliation efficiencies ranging from 2.5 - 5.0 $\text{mg mL}^{-1} \text{h}^{-1}$ (10% serum, kitchen blender). The efficiencies are compared with our previous study which used bovine serum albumin (BSA, 3.0 mg/mL , 4.0 $\text{mg mL}^{-1} \text{h}^{-1}$)⁸ and 10% serum contains similar levels of total protein in serum. Thus, the comparable levels of graphene efficiencies suggest that serum proteins may be the primary active species responsible for the observed exfoliation efficiencies in these sera. However, the efficiency could vary based on the origin, age, fat content etc. of the serum. The versatility of our approach is that graphene can be prepared in all these sera, and possibly others, as required by the experimental model of interest. Encouraged by these findings, we checked the feasibility of making graphene in other sera in a high shear force disperser as well as by ultrasonication under similar conditions.

The efficiency of graphite exfoliation in the kitchen blender in sera was compared with exfoliation by ultrasonication,²⁷ which is often utilized in the literature, and by a shear (rotor/stator) reactor²⁸ (Figure 2.B, SI Table SI) to determine the preferred means of exfoliation.²⁹ In all sera, kitchen blender (17 krpm, 700 W) was the most effective with efficiencies averaging $\sim 4.5 \text{ mg mL}^{-1} \text{h}^{-1}$, followed by shear reactor (17 krpm, 300 W) ($\sim 2.0 \text{ mg mL}^{-1} \text{h}^{-1}$), while ultrasonication (Bath 9.5 L, 50-60 Hz, 470 W) gave a poor efficiency of $0.02 \text{ mg mL}^{-1} \text{h}^{-1}$. Ultrasonication, therefore, was unable to achieve high enough graphene concentrations. The wattages of these devices under the settings used are within 300-700 W range and when the exfoliation efficiencies are normalized with wattage of the source, the observed efficiency for shear reactor $0.0067 \text{ mg mL}^{-1} \text{h}^{-1} \text{W}^{-1}$ is comparable to that obtained with the kitchen blender, $0.0063 \text{ mg mL}^{-1} \text{h}^{-1} \text{W}^{-1}$ but that of the ultrasonication has been much smaller, $0.000043 \text{ mg mL}^{-1} \text{h}^{-1} \text{W}^{-1}$. Exfoliation experiment were performed using sonication and high shear at 10% serum concentration based on the assumption that the dependence of protein concentration follow the same trend as in kitchen blender (Figure 2B). In principle, the rate of exfoliation is a function of initial concentration of protein and graphite but the efficiency depends on the mechanical force applied. Next, we examined the colloidal stability of graphene produced in bovine serum for practical applications in more detail.

Stabilities of graphene dispersions at three different temperatures were evaluated for storage and shipping conditions (10% bovine serum, 2 mg/mL , pH 7.4). The absorbance at 660 nm of the suspensions were monitored for samples stored at $\sim 4^\circ \text{C}$ (fridge), -4°C (freezer) and 37°C (water bath) over extended periods of time (Figure SI). The dispersions stored in the freezer at -4°C showed no decrease in their concentrations even after 30 days ($\sim 97\%$ retention (SI Figure SI), whereas a small decrease was noted for samples stored in the fridge at 4°C ($\sim 88\%$ retention) over the same time period. Graphene stored at 37°C , however,

showed considerable decrease in concentration after a month (~38% retention), but was stable for 10 days (~96%) without significant loss. Thus, storage at lower temperatures is benign, which is convenient for practical applications such as bioassays and biosensing where stability is required. This study also suggests that graphene dispersions can be transported under freezing or cold temperatures on these timescales. The loss in stability at room temperature could be due to protein degradation in the sera owing to the protease activity, thus allowing the graphene sheets to agglomerate and eventually precipitate out (Figure SI). Next, we evaluated the structural properties of graphene by Raman spectroscopy and TEM studies.

Raman Spectral Characterization

Raman spectroscopy is widely used to evaluate the size, number of layers, and the type of defects in the graphene samples.³⁰ Air-dried graphene samples on a glass slide were used to collect Raman spectra by 514 nm laser excitation, and a large number of spectra (~100) from different locations of the sample have been collected and averaged (SI Figure S3). Peak positions/intensities of the samples, in comparison with graphite, showed major changes in D, D' and 2D bands (Figure 3A). The prominent change was an increase in relative intensity of the D band (I_D at ~1350 cm^{-1}), which is related to the presence of edge defects (SI Figure S3), with respect to that of the G band (I_G) after exfoliation.³⁰ Thus, increase in I_D/I_G ratio represents increased abundance of defect sites, accompanied by micromechanical cleavage and/or oxidation during exfoliation (presence of sp^3 carbons).

The increase in I_D/I_G intensity ratio was independent of the serum type used, but depended on the method of exfoliation (Figure 3B). For example, graphene prepared in bovine serum showed I_D/I_G ratio of 0.32 ± 0.02 , when exfoliated with the kitchen blender. But sample exfoliate by a shear reactor had a reduced value of 0.23 ± 0.03 , while sample made by sonication had a much higher value of 0.41 ± 0.06 . Thus, sonication caused the most defects, while the shear reactor, at shear rates below turbulence, gave the best quality graphene. This trend in values of I_D/I_G ratio (sonication > blender > shear reactor) was the same among all serum-mediated samples. Next, we quantitated Raman data to characterize the size, defect type, and average number of layers of graphene in the flakes.

The 2D peak (~2700 cm^{-1}), which does not require defects for its activation, showed a shift to lower frequencies, increase in intensity, and change in peak shape after exfoliation (Figure 3A). Since the 2D peak shape has a single Lorentzian in single layer graphene, the spectrum splits depending on the number of layers (N).³⁰ The changes in the I_{2D}/I_G values for all the samples are given in SI Table S2. Again, the changes were independent of the serum type but they differed based on the exfoliation method. Irrespective of the serum used, higher I_{2D}/I_G ratio was observed for exfoliation in a kitchen blender (average of ~0.65), followed by sonication (~0.58) and shear (~0.55). These changes were reflected in the analysis of number of layers of graphene, as following.

Statistical analysis of size, number of layers and the defect type of the graphene was carried out using the Raman data.²⁸ The flake size was found to be 0.4-1 μm in length with 2-6 layers in each flake, on average, and these parameters were found to be independent of the type of serum used in the three methods described here (Table 1). But minor changes were

noted. Samples exfoliated with the kitchen blender showed a size range from 0.5 to 1 μm and 3.5-4.4 numbers of layers, whereas the shear reactor resulted in much larger sheets (1.1 – 1.6 μm) but had more number of layers (5.8 – 6.9) per flake. This is probably because of the low turbulence in the shear reactor at the speed (17 krpm, $\sim 3 \times 10^4 \text{ s}^{-1}$ shear rate) when compared to that provided by the kitchen blender ($\sim 10^{-6} \text{ s}^{-1}$)³¹. Thus, the method of exfoliation is a governing factor, rather than the type of serum used, for flake size control over a limited range (0.4-1 μm in length) over the range of 2-6 layers per flake.

Microscopy studies

The above flake sizes were further confirmed by TEM analysis of the graphene samples (20 $\mu\text{g/mL}$) coated on a Cu-grid where we counted roughly ~ 50 different regions on the sample grid. The largest length of each sheet and the area (in nm^2) were measured using ImageJ software, after calibrating the image pixels to the known length of the scale bar. The image analysis confirmed successful exfoliation of graphite to submicron size graphene sheets (Figure 4A and B). TEM data analysis of ~ 200 sheets from one experiment showed a mean of 0.5 ($\pm 0.2 \mu\text{m}$) μm size (bovine serum/kitchen blender) consistent with the Raman data analysis. Similar TEM analysis performed for human serum samples, which showed mean size of 0.5 ($\pm 0.3 \mu\text{m}$) (SI Figure S2). Interestingly, TEM images showed considerable level of smaller sheets (below 200 nm) in the suspension, which was not indicated by the Raman analysis (SI Figure S3).

To further confirm the presence of smaller sheets in our graphene, the samples were centrifuged for 90 minutes at 8000 rpm in a desktop centrifuge to remove the larger sheets.³¹ The supernatant was found to contain much small sheets, as analyzed by Raman spectroscopy and TEM. This was further validated by the quantification of their proportion in the total sample by absorbance measurements. In general, kitchen blender gave a larger extent of smaller particles ($\sim 15\%$), followed by lower amounts by the shear reactor ($\sim 8\%$), and even lesser amounts by sonication ($\sim 3\%$). Kitchen blender produced more turbulence than the shear reactor, enhancing the fragmentation pathway, and, as a result, a higher proportion of small particle fraction was generated. The higher defects seen in the Raman spectra (Figure 3B) of graphene prepared by the kitchen blender could be due to the greater presence of smaller fraction in this sample. Raman analysis of the separated fraction of the larger particles showed graphite like spectra because of aggregation during centrifugation. Ultrasonication gave a lower fraction of smaller particles, which suggested low fragmentation but the greater number of defects in these samples must have been originated ($\sim 0.5 \mu\text{m}$. Table 1) from oxidative damage and/or edge defects.

Average flake area is also important, rather than the average length (longest dimension), but measurement of flake area from TEM images is difficult because of different shapes, aggregation and curling that often occurs on the TEM grid during solvent evaporation. Using an image processing software (ImageJ), areas of a large number of sheets were measured. As prepared graphene (lateral size $\sim 500 \text{ nm}$), showed mean size of $\sim 36000 \text{ nm}^2$ (about 200 sheets were measured) while the fraction of smaller graphene sheets (lateral size $< 200 \text{ nm}$) separated from the larger sheets were also analyzed which gave average size of 1700 nm^2 (SI Figure S4. D and E). Smaller values noted from area measurements could be possibly

due to the presence of elongated sheets, aggregation and curling of flakes as the solvent is evaporated from the TEM grid. The large standard deviations noted from the area measurements indicate that the flakes are more heterogeneously distributed on the TEM grid than in the liquid suspension.

Raman analysis of the small size graphene fraction was carried out for bovine serum/kitchen blender system, which showed I_D/I_G ratio of ~ 1 , representing high degree of defects (SI Figure S4). High intensity of 2D band also noted, which suggested fewer number of layers per flake, which is advantageous for some applications.³⁰ TEM analysis of this sample showed that the upper size limit of the smaller fraction was 200 nm (SI Figure S4), but a statistical analysis was not carried out because very small sizes were quite difficult to measure. Later, we found that particle size plays a major role in the cytotoxicity of graphene (vide infra). Overall, we acquired multiple TEM images and hundreds of Raman spectra on a number of samples under a variety of conditions and these confirmed the consistent formation of few layer, submicron size graphene, comparable to graphene dispersions exfoliated by other methods. The variability in these parameters is also indicated in Table 1 and Figure 4B, which is in acceptable range, when Compared to previously reported values.^{14,15,16}

The graphene is further characterized by protein content analysis and by circular dichroism (CD) studies. The amount of protein bound to graphene in serum (bovine) was estimated using the well-established Bradford assay, using BSA as the standard.³² After removing the free and loosely bound protein by repeated centrifugation and washing of the flakes (twice), the composition of graphene suspension was found to be 2.4 (± 0.3) mg/mL of protein per 3.6 mg/mL graphene (SI Figure S5. A) ($\sim 40\%$ protein and $\sim 60\%$ graphene by weight). The zeta potential of this graphene dispersion was -25 mV (at pH 7.0), and the negative charge could be due to ionizable groups from the protein bound to the graphene flakes.

The CD spectra of the graphene suspensions (SI Figure S5. B), after removal of the loosely bound protein as above, indicated considerable loss of the protein secondary structure and most of the bound protein is essentially denatured. This is likely due to the binding of the hydrophobic residues of the proteins to the hydrophobic graphene surface, causing denaturation. Even so, our graphene suspensions of large flakes of 500 nm in lateral dimensions were not toxic to cells or nematodes, as demonstrated below.

***In vitro* cytotoxicity of serum exfoliated graphene**

Cytotoxicity of our graphene produced in sera was tested using human embryonic kidney 293T (HEK-293T) cells as a model system. One advantage of our graphene preparations is that we could match the type of serum most suited for a particular cell line. This was not the case up until now as most graphene dispersions were either in organic solvents or in non-sera. The graphene/serum toxicity to HEK-293T cells was evaluated by scoring intracellular dehydrogenase activity measured by standard methods,³³ and these results have been verified by light microscopy throughout the study. Initially, certain dose (~ 100 $\mu\text{g/mL}$) of graphene in bovine serum (10%) was co-incubated with the cells, which were started with normal confluency. Prior to this work, most of the cytotoxicity studies reported in the literature were carried out for only up to 48 h,³⁴ and for comparison with these reported

data, we examined the metabolic rate at 48 h time point. To our surprise, no cell toxicity was noted at this time point. So, we decided to monitor the cells for extended periods of time, up until the control cells (unexposed to graphene) were still healthy (Figure 5). By extending the time of exposure and increasing the dose, toxic effect of graphene was noted, but only after exposure for a week. The effects of graphene on the intracellular metabolism as well as the overall cell morphology was analyzed in immortalized HEK-293T cells at 25% initial confluency in comparison to untreated control (pristine cells). As prepared graphene (average size 500 nm) did not affect the intracellular metabolism, cell division, and cell morphology of the cells for the first seven days, as indicated above, up to a maximum dose of 300 $\mu\text{g/mL}$. Higher doses were not possible due to dilution with cell media. At the highest concentration examined (300 $\mu\text{g/mL}$), intercellular metabolism was reduced to about 30% on the 8th day of exposure (Figure 5. A) and at the lowest concentration of graphene tested (50 $\mu\text{g/mL}$), graphene had no toxic effect at this time point or after 10 days, but controls cells started dying after 10 days. Thus, our graphene was not toxic to these cells at 50 $\mu\text{g/mL}$ and toxicity increased with dose, only after 7 days of incubation. These findings are first of their kind and further testing was necessary before generalizing these results.

We also examined another cell line to test if HEK-293T cells are exceptional or unique for graphene toxicity. Human lung carcinoma cells (HI299) were exposed to graphene in bovine serum at increasing doses for extended periods of time, because cancer cells like HI299 are immortal, and divide indefinitely. Due to the adaptive nature of cancer cells, very long exposure times were needed to measure changes in metabolic rates (10-15 days). Again, there was no detectable toxicity until the 8th day, as in the case of HEK-293T cells but on day 11, the metabolic rate was reduced to 30% at the highest dose of 300 $\mu\text{g/mL}$, whereas cells exposed to a low dose of 50 $\mu\text{g/mL}$ were intact until day 12 (Figure 5.A). But most of the cells were dead during days 13-15, on a dose dependent manner. The comparative data with two cell lines clearly demonstrates the long-term toxic effects of graphene to human cells, irrespective of their nature. Next, we investigated the influence of flake size on cellular toxicity.

The graphene in bovine serum had an average size of ~500 nm and it is unlikely to enter the cells. Also, toxicity of nanomaterials was shown to depend on the particle size, as well as charge and specific functional groups on the surface.³⁵ The latter two factors are similar in serum coated graphene, but the potential toxicity of the presence of very small particles is desired to be tested. To investigate the influence of the size of our graphene, we fractionated the as-prepared graphene into larger (200 – 1000 nm) and smaller (< 200 nm length, SI Figure S4A) size particles, co-incubated them separately with HEK-293T as well as HI299 cells (< 200 nm length, SI Figure S6). Some cells turned darker, suggesting graphene uptake. Figure 5.B 5.C and 5.D show that the metabolic data are superimposable with that of as prepared graphene (~500 nm) but the doses were much lower (10 to 60 $\mu\text{g/mL}$). Clearly, cell toxicity was mostly due to smaller graphene flakes, independent of the cell lines used. The graphene sheets are represented here in terms of average length but area of a 2D sheet is perhaps more relevant than length for some applications. Area measurements of flakes by TEM, however, have been much smaller than the length measurements and these could be inaccurate because of different shapes, aggregation and curling of the flakes, as noted above.

The fraction containing smaller sheets (200 nm) showed higher cytotoxicity than the fraction containing larger size (500 nm).

The size-dependent toxicity highlights the size effects, that were not known before for unoxidized graphene. To account for the role of protein coating on graphene in toxicity, we attempted to do cytotoxicity of surfactant exfoliated graphene (1% sodium cholate, kitchen blender),³² but the surfactant alone was too toxic and cells did not survive (SI Figure S7). This attempt, however, indicates the need for preparing benign graphene samples for biological applications, larger size for non-toxicity applications and smaller sizes to induce toxicity. Thus, toxicity of graphene could be enhanced or reduced by controlling the flake size, one of the many factors that might contribute to toxicity. Encouraged by these data, we extended our toxicity studies to a multicellular organism, nematodes (*C. elegans*) *in vivo*.

In vivo* toxicity screening with *C. elegans

The wild type N2 (ancestral) strain of nematodes has been chosen for toxicity studies as these are good model systems in terms of survivability, activity, and reproductive toxicity. Survivability after acute and prolonged exposure with graphene at concentrations ranging from 50 to 500 µg/mL were tested on age-synchronized adult *C. elegans*, grown on nematode growth medium (NGM). In the acute toxicity studies, twenty nematodes were exposed to graphene prepared in bovine serum for 48 h. Following exposure to graphene, the health of the worms was observed at 24 and 48 h. Toxic effects on prolonged exposure were assessed by maintaining the seeded plates for a period of 10 days.³⁶

Effect of graphene exfoliated in bovine serum and sodium cholate at different doses were studied with appropriate controls (10% bovine serum and 1% sodium cholate) and compared with nematodes not exposed to graphene or controls. In the acute toxicity assay, results indicated that graphene on concentrations from 50 - 500 µg/mL, irrespective of the exfoliating agent used, did not cause mortality in the worms, demonstrating the absence of acute toxicity. These results are comparable to the control samples, where all the worms were alive at the end of the 48 h period (SI Figure S8), as in the cell toxicity studies. On prolonged exposure to graphene, a dose-dependent increase in toxicity was observed by the tenth day of exposure.

In the initial periods of exposure, all the twenty worms exposed to graphene were alive and the viability of nematodes was similar to the control samples across all concentrations tested. By the tenth day of exposure, the survival rates for the high concentrations tested (500 µg/mL and 250 µg/mL) were reduced to 58% (8 live worms) and 86% (17 live worms) respectively, compared to the control (20 live worms; Figure 6.A and 6.B). Despite the reduced survivability at higher concentrations, the activity in terms of feeding and locomotion behavior was found to be similar to those of untreated control worms.

For a deeper assessment of graphene toxicity on nematodes, we analyzed the reproductive potential when exposed to the material at concentrations varying from 50 to 500 µg/mL. A brood size assay as in Figure 6.C showed that the worms exposed at highest concentration (500 µg/ml graphene in serum and sodium cholate) only exhibited small reduction in the brood size (136 and 140 respectively) when compared to the control (150 number). At lower

concentrations including 50 and 100 $\mu\text{g/mL}$, identical results with no significant deviation from the untreated control samples were observed. This study shows that graphene is safe up to 50 $\mu\text{g/mL}$, but at higher dosages, the toxicity depends on the exposure time.

Discussion

Liquid-phase exfoliation of graphite in animal serum produced stable dispersions of few layer graphene with less than a micron average size. High yield and colloidal stability in biological media was exploited to test the cytotoxicity of graphene to human cells and *C. elegans* under completely dispersive environments.

Post-modification or dispersing agents (e.g., surfactants) are required to disperse carbon nanomaterials in biological media, but certain surfactants could be lethal to biological systems. *In situ* biofunctionalization or preparation of graphene in biological media such as serum or cell media is a novel approach to overcome this problem. Graphene is an interesting system to explore this approach, where individual sheets can be exfoliated from graphite, by overcoming the dispersive interactions. Proteins with even moderate affinity for graphene and sufficient surface charge stabilize graphene in aqueous conditions (Zeta potential is about -25 ± 4 mV at pH 7).⁸ The stability of protein adsorbed 2D sheets are attributed to the combination of repulsive electrostatic interactions and the steric hindrance of 3D protein molecules. Such protein induced stabilization of double layer hydroxides and phosphonates were demonstrated under physiological conditions and variable ionic strengths.^{37,38,39} In the present approach protein was incorporated to the layers at the synthetic step.

The micromechanical force needed to delaminate graphite crystal can be provided by ultrasonication or high shear. A comparison of the efficiency of these two forces has not been done previously, and here we have compared three different methods of exfoliation in terms of exfoliation efficiency, flake size, and number of layers of graphene (Figure 2B and Table 1). Of the three methods tested, ultrasonication was slower for exfoliation and resulted in relatively graphene/graphite particles (~ 0.5 μm length and ~ 5 number of layers per flake), with a higher abundance of defects ($I_D/I_G = 0.4$) than shear methods. Sonication was shown to induce oxidative damage to the sheets, particularly when parent graphite has defects.⁴⁰ But the exfoliation assisted by shear in kitchen blender or the shear reactor showed lower defects ($I_D/I_G = 0.3$ and 0.2 , respectively), from the same parent graphite, which indicates that sonication may induce minor damage to the basal plane unlike the other two methods. In the case of ultrasonication, where high energy micro-jets are produced, which can penetrate through the graphite sheets to expand the layers. Thus, much smaller sheets may be produced, with a high number of edge and vacancy defects.

In shear exfoliation, normal force parallel to the graphite layer induces the delamination of the graphene, where the collisions between the graphite platelets, accompanied by the turbulence in the medium, cause fragmentation of the sheets. It would be easier to delaminate smaller graphite platelets because of weaker van der Waals forces between small surface area sheets (van der Waal's force is proportional to the contact area). This mechanism confirms the size difference in graphene made by using kitchen blender and the

shear reactor. Kitchen blender produces higher turbulence in comparison to the shear reactor, so relatively smaller sheets were formed (average 0.6 μm) compared to that of shear reactor, and it produced graphene with an average size of 1.2 μm . This also reflects in the number of layers where small graphene sheets are well delaminated (average 4 layers) in the kitchen blender while the shear reactor had flakes with an average of 6 layers at same blade speed (17 krpm). So, the data suggest that turbulence in the exfoliation medium dramatically improves the exfoliation efficiency by increasing the number of collisions between the graphite platelets, which enhances the fragmentation pathway.

Defining the toxicity of graphene and its derivatives is an ongoing challenge, where inconsistent results were found in the literature.³⁴ The primary reason behind this varying observation is the purity and dispersibility of graphene.³⁴ Graphene oxide and reduced graphene oxide have toxic effects on cells and higher organisms, implying the role of defects in toxicity.¹⁸ However, only few studies are available for the toxicity of unoxidized graphene, due to its poor dispersive nature in biological media. Graphene (few layer) grown by chemical vapor deposition (CVD) shows severe toxicity to neural cells due to the generation of high reactive oxygen species (ROS) at 100 $\mu\text{g/mL}$ dose for 24 h exposure.⁴¹ In contrast to these data, we found no toxicity of graphene in serum (~500 nm) even at a higher dose (300 $\mu\text{g/mL}$) for an exposure time of 7 days to human cell lines. Our graphene, with good colloidal stability in serum (Zeta potential about -25 ± 4 mV) is less toxic, required longer exposure times, at higher doses than graphene produced by CVD. No foreign material other than serum was used for the preparation of graphene by our method, which clearly rules out the possibility of toxicity from impurities in the media. Toxicity of graphene depends on the method of preparation and flake size, with larger flakes being less toxic.

Size dependent cytotoxicity of graphene oxide derivatives are established previously.⁴²⁻⁴³ Reduced graphene oxide sheets below 100 nm lateral size induced cell death at 1-10 $\mu\text{g/mL}$ doses in 1 h exposure, whereas the micron size sheets showed similar toxicity at 10 times higher doses after 96 h exposure.^{42,43} This supports our findings that the larger sheets are benign to the cells due to lower uptake compared to smaller sheets. In contrast, the unoxidized graphene nanosheets (>200 nm) prepared in this report did not show cytotoxicity over a week at 60 $\mu\text{g/mL}$ dose, suggests that the intrinsic cytotoxicity comes from oxidative edges and defects. Reduced graphene oxide with sharp edges showed severe cell membrane damage in bacteria compared to regular GO sheets.⁴⁴ In this study we did not observed loss of membrane integrity of the cells in optical images (Figure S8). An in-depth mechanistic study required in the future to unravel the observed toxicity of unoxidized graphene nanosheets.

The reduced toxicity of our graphene/serum dispersions could be due to the serum proteins adsorbed on the sheets, passivating the high-energy surface of graphene. This protein layer could lower the toxicity by decreasing the hydrophobicity of graphene surface, making it more soluble in water and imparting a net negative charge. But protease degradation of this protein coating could occur inside the cell and eventually expose the hydrophobic regions of graphene sheets.⁴⁵ This might be the reason for the delayed toxicity of graphene in our studies, where the toxicity has been noticed after the seventh day. HI299 is a cancer cell line known for high protease activity,⁴⁶ and yet this cell had reduced toxicity when compared to

noncancerous HEK cells, suggesting alternate possibilities for graphene toxicity. To explore another possibility, we attempted to study the cytotoxicity of graphene exfoliated in a non-protein medium, sodium cholate. Unfortunately, sodium cholate was found to be toxic to these cell lines even at 10 nM concentrations. So, *C. elegans*, another model system was used to study graphene toxicity, where the organism was not susceptible to sodium cholate.

Significant toxicity of aqueous solutions of graphene and graphene oxide have been reported on *C. elegans*.⁴¹ However, this is the first study to evaluate the toxic potential of unoxidized graphene which is fully dispersed in bovine serum and sodium cholate with *C. elegans*. Ingestion of graphene flakes in concentrations ranging from 50-100 µg/mL did not result in any significant reduction in population number and survival rate till the end of a 10-day study. This is in accordance with previous findings that the toxicity of pristine graphene is dose dependent in *C. elegans*⁴⁸ but the stability of these materials in biological media was not taken into account. The exposure of large graphite platelets (3-60 layers and 1-10 µm size), not well-dispersed in solution, consistently showed no detectable toxicity, measured by longevity and reproductive capacity (250 µg/mL dose).⁴⁹ Surprisingly, loading of the worms with large amounts of graphene did not adversely influence their survival for several days.

Oxidative stress may be responsible for the reduced survivability of nematodes exposed to higher doses of graphene (Figure 6A and B), following a 10-day exposure. The worms were actively feeding, motile and responsive to stimuli, after the accumulation of graphene in their guts, clearly indicating the absence of lethal toxicity at lower doses. Reproductive toxicity studies based on brood size of *C. elegans* found that worms exposed to graphite nanoplatelets with graphene up to a concentration of 250 µg/mL showed no deviation from the untreated controls for progeny production.⁴⁹ Results obtained in the present study, where the reduction in the number of eggs produced was demonstrated only in nematodes exposed to a higher dose of 500 µg/mL of graphene. The accumulation of graphene particles in secondary organs like reproductive system and resultant oxidative damage may be responsible for the reduction in the reproductive performance.⁴⁷ Furthermore, the small reduction in brood size could be due to the defective development of vulva necessary for egg laying in *C. elegans*, as reported on exposure to silica nanoparticles, which in turn reduced the number of progeny.⁵⁰ In addition, our studies reveal that there was no significant difference in toxicity associated with the two exfoliating agents, serum and sodium cholate.

At doses ranging from 20-250 µg/mL graphene exfoliated with either of the two exfoliating agents, serum or sodium cholate, did not result in any lethality or reproductive toxicity in *C. elegans*. The formation of serum protein layer on the graphene (corona/armor) may be a protective factor in the toxic effects of the pristine graphene.¹⁷ We showed above that 2.4 (±0.3) mg/mL of protein was bound to 3.6 mg/mL of graphene (SI Figure S5.A), and that the bound protein had little or no secondary structure as indicated by extensive loss in the CD spectrum of the graphene suspension (SI Figure S5.B).

Studies by others showed that protein coating masks the toxicity of graphene oxide in cells.¹⁷ In another context, protein corona around graphene oxide reduced the toxicity to a certain extent, but not permanently, possibly due to the degradation of protein corona by protease activity inside the cell.⁴⁵ This hypothesis was also tested here using nematodes by

comparing the toxicity of graphene exfoliated in serum and in non-proteinaceous material, sodium cholate. With or without protein corona, graphene had similar effect on nematode activities (Figure 6). Thus, protein corona around graphene may not be a permanent solution to graphene toxicity, but may mask the sharp edges or corners in the layers. Possibly the proteases in the cells or organisms would cleave the protecting protein layers and eventually the graphene surface may be exposed to the system. Thus, monitoring the toxicity of nanomaterials to maximum possible periods of exposure is essential for accurate results. The method established here using serum as inexpensive and benign exfoliating agent is a stepping stone in controlling or taming graphene biotechnology.

Conclusions

An approach to prepare nano layered materials directly in cell culture conditions is illustrated in this report. Based on our previous understanding of protein mediated graphite exfoliation, we designed graphene production in sera, which is a protein rich cell culture component. Synthesis of graphene in six different animal sera and its implementation in material toxicity studying showed enhanced colloidal stability and decreased nanotoxicity. Several inconsistencies in nanotoxicity of graphene is noted in the past decade, primarily due to the aggregation of graphene in biological media and also due to the formation of protein coatings on the flakes. Graphene in sera reported here is well characterized and well-defined by microscopy, spectroscopy and toxicity studies which is suitable for biological applications. The cytotoxicity studies using two different human cell lines show that well dispersed graphene is toxic at higher doses ($>200 \mu\text{g/mL}$) after prolonged exposure (7 to 10 days), which was mainly due to flakes that are below 200 nm in size. The toxicity studies extended to *C. elegans* supports the cell toxicity studies where no acute toxic effect on nematodes was observed, although the entire digestive track of the worms was accumulated with graphene. Comparative study of graphene exfoliated using surfactant and proteins proved that protein coating is not a solution to prolonged nanotoxicity. Here, we demonstrated that nanomaterials can be prepared in biological media, without further purification or sterilization prior to use. We were able to address some of the reasons for variations in nanotoxicity results of graphene reported in the literature and provide control in modulating its toxicity by using proper stabilizing agents and by controlling the flake size.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Ferrari AC, Bonaccorso F, Fal'ko V, Novoselov KS, Roche S, Boggild P, Borini S, Koppens FHL, Palermo V, Pugno N, Garrido JA, Sordan R, Bianco A, Ballerini L, Prato M, Lidorilcis E, Kivioja J, Marinelli C, Ryhanen T, Morpurgo A, et al. Science and technology roadmap for graphene, related

- two-dimensional crystals, and hybrid systems. *Nanoscale*. 2015; 7:4598–4810. [PubMed: 25707682]
2. Novoselov KS, Falko VI, Colombo L, Gellert PR, Schwab MG, Kim K. A roadmap for graphene. *Nature*. 2012; 490:192–200. [PubMed: 23060189]
 3. Chung C, Kim YK, Shin D, Ryoo SR, Hong BH, Min DH. Biomedical Applications of Graphene and Graphene Oxide. *Acc Chem Res*. 2013; 46:2211–2224. [PubMed: 23480658]
 4. Li L, Bedrov D, Smith GD. Water-Induced Interactions between Carbon Nanoparticles. *J Phys Chem*. 2006; 5110:10509–10513.
 5. Hummers WS, Offeman RE. Preparation of Graphitic Oxide. *J Am Chem Soc*. 1958; 80:1339–1339.
 6. Si Y, Samulski ET. Synthesis of Water Soluble Graphene. *Nano Lett*. 2008; 8:1679–1682. [PubMed: 18498200]
 7. Chen J, Liu H, Zhao C, Qin G, Xi G, Li T, Wang X, Chen T. One-step reduction and PEGylation of graphene oxide for photothermally controlled drug delivery. *Biomaterials*. 2014; 35:4986–4995. [PubMed: 24656608]
 8. Pattammattel A, Kumar CV. Kitchen Chemistry 101: Multigram Production of High Quality Biographene in a Blender with Edible Proteins. *Adv Funct Mater*. 2015; 25:7088–7098.
 9. Wang Y, Li Z, Wang J, Li J, Lin Y. Graphene and graphene oxide: biofunctionalization and applications in biotechnology. *Trends Biotechnol*. 2011; 29:205–212. [PubMed: 21397350]
 10. Ge Y, Wang J, Shi Z, Yin J. Gelatin-assisted fabrication of water-dispersible graphene and its inorganic analogues. *J Mater Chem*. 2012; 22:17619–17624.
 11. Uysal Unalan I, Wan C, Trabattoni S, Piergiovanni L, Farris S. Polysaccharide-assisted rapid exfoliation of graphite platelets into high quality water-dispersible graphene sheets. *RSC Adv*. 2015; 5:26482–26490.
 12. León V, Rodríguez AM, Prieto P, Prato M, Vázquez E. Exfoliation of Graphite with Triazine Derivatives under Ball-Milling Conditions: Preparation of Few-Layer Graphene via Selective Noncovalent Interactions. *ACS Nano*. 2014; 8(1):563–571. [PubMed: 24380430]
 13. Lotya M, King PJ, Khan U, De S, Coleman JN. High-Concentration, Surfactant-Stabilized Graphene Dispersions. *ACS Nano*. 2010; 4:3155–3162. [PubMed: 20455583]
 14. Ahadian S, Estili M, Surya VJ, Ramon-Azcon J, Liang X, Shiku H, Ramalingam M, Matsue T, Sakka Y, Bae H, Nakajima K, Kawazoe Y, Khademhosseini A. Facile and green production of aqueous graphene dispersions for biomedical applications. *Nanoscale*. 2015; 7:6436–6443. [PubMed: 25779762]
 15. Gravagnuolo AM, Morales-Narváez E, Longobardi S, da Silva ET, Giardina P, Merkoçi A. In Situ Production of Biofunctionalized Few-Layer Defect-Free Microsheets of Graphene. *Adv Funct Mater*. 2015; 25:2771–2779.
 16. Yi M, Shen Z. A review on mechanical exfoliation for the scalable production of graphene. *J Mater Chem A*. 2015; 3:11700–11715.
 17. Moore DH. Species differences in serum protein patterns. *J Biol Chem*. 1945; 161:21–32. [PubMed: 21005711]
 18. León V, González-Domínguez J, Fierro J, Prato M, Vázquez E. Production and stability of mechanochemically exfoliated graphene in water and culture media. *Nanoscale*. 2016; 5(30): 14548–14555.
 19. Hu W, Peng C, Lv M, Li X, Zhang Y, Chen N, Fan C, Huang Q. Protein Corona-Mediated Mitigation of Cytotoxicity of Graphene Oxide. *ACS Nano*. 2011; 5:3693–3700. [PubMed: 21500856]
 20. Yang K, Li Y, Tan X, Peng R, Liu Z. Behavior and Toxicity of Graphene and Its Functionalized Derivatives in Biological Systems. *Small*. 2013; 9:1492–1503. [PubMed: 22987582]
 21. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a Human Cell Line Transformed by DNA from Human Adenovirus Type 5. *J Gen Virol*. 1977; 36:59–72.
 22. Jiang C, Jia J, Zhai S. Mechanistic Understanding of Toxicity from Nanocatalysts. *Int J Mol Sci*. 2014; 15:13967. [PubMed: 25119861]

23. Nass, R., Hamza, I. Current Protocols in Toxicology. John Wiley & Sons, Inc; 2001. The Nematode *C. elegans* as an Animal Model to Explore Toxicology In Vivo: Solid and Axenic Growth Culture Conditions and Compound Exposure Parameters.
24. Tedja R, Marquis C, Lim M, Amal R. Biological impacts of TiO₂ on human lung cell lines A549 and H1299: particle size distribution effects. *J Nanopart Res.* 2011; 13:3801–3813.
25. Yoshimatsu K, Koide H, Hoshino Y, Shea KJ. Preparation of abiotic polymer nanoparticles for sequestration and neutralization of a target peptide toxin. *Nat Protocols.* 2015; 10:595–604. [PubMed: 25790112]
26. Benjamin, EM., Bourdeau, P., Somers, E., Richardson, GM., Hickman, JR. Short-term toxicity tests for non-genotoxic effects. *J Appl Toxicol*; SCOPE 41, IPCS Joint Symposia 8, SGOMSEC 4 Wiley; Chichester (1990). 1991. p. 156-156.
27. Ciesielski A, Samori P. Graphene via sonication assisted liquid-phase exfoliation. *Chem Soc Rev.* 2014; 43:381–398. [PubMed: 24002478]
28. Paton KR, Varrla E, Backes C, Smith RJ, Khan U, O'Neill A, Boland C, Lotya M, Istrate OM, King P, Higgins T, Barwich S, May P, Puczkarski P, Ahmed I, Moebius M, Pettersson H, Long E, Coelho J, O'Brien, et al. Scalable production of large quantities of defect-free few-layer graphene by shear exfoliation in liquids. *Nat Mater.* 2014; 13:624–630. [PubMed: 24747780]
29. Yi M, Shen Z. Kitchen blender for producing high-quality few-layer graphene. *Carbon.* 2014; 78:622–626.
30. Ferrari AC, Basko DM. Raman spectroscopy as a versatile tool for studying the properties of graphene. *Nat Nano.* 2013; 8:235–246.
31. Varrla E, Paton KR, Backes C, Harvey A, Smith RJ, McCauley J, Coleman JN. Turbulence-assisted shear exfoliation of graphene using household detergent and a kitchen blender. *Nanoscale.* 2014; 6:11810–11819. [PubMed: 25164103]
32. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry.* 1976; 72:248–254. [PubMed: 942051]
33. Love SA, Maurer-Jones MA, Thompson JW, Lin Y-S, Haynes CL. Assessing Nanoparticle Toxicity. *Annu Rev Anal Chem.* 2012; 5:181–205.
34. Bianco A. Graphene: Safe or Toxic? The Two Faces of the Medal. *Angew Chem Int Ed.* 2013; 52:4986–4997.
35. Sharifi S, Behzadi S, Laurent S, Laird Forrest M, Stroeve P, Mahmoudi M. Toxicity of nanomaterials. *Chem Soc Rev.* 2012; 41:2323–2343. [PubMed: 22170510]
36. Stiernagle, T. Maintenance of *C. elegans*, WormBook, The *C. elegans*. 2006. Research Community. http://www.wormbook.org/chapters/www_strainmaintain/strainmaintain.html
37. Vasti C, Bedoya DA, Rojas R, Giacomelli CE. Effect of the protein corona on the colloidal stability and reactivity of LDH-based nanocarriers. *Journal of Materials Chemistry B.* 2016; 4:2008–2016.
38. Pavlovic M, Li L, Dits F, Gu Z, Adok-Sipiczki M, Szilagyi I. Aggregation of layered double hydroxide nanoparticles in the presence of heparin: towards highly stable delivery systems. *RSC Advances.* 2016; 6:16159–16167.
39. Deshapriya IK, Kim CS, Novak MJ, Kumar CV. Biofunctionalization of α -Zirconium Phosphate Nanosheets: Toward Rational Control of Enzyme Loading, Affinities, Activities and Structure Retention. *ACS Applied Materials & Interfaces.* 2014; 6:9643–9653. [PubMed: 24853777]
40. Skaltsas T, Ke X, Bittencourt C, Tagmatarchis N. Ultrasonication Induces Oxygenated Species and Defects onto Exfoliated Graphene. *J Phys Chem C.* 2013; 117:23272–23278.
41. Zhang Y, Ali SF, Dervishi E, Xu Y, Li Z, Casciano D, Biris AS. Cytotoxicity Effects of Graphene and Single-Wall Carbon Nanotubes in Neural Phaeochromocytoma-Derived PC12 Cells. *ACS Nano.* 2010; 4:3181–3186. [PubMed: 20481456]
42. Akhavan O, Ghaderi E, Emamy H, Akhavan F. Genotoxicity of graphene nanoribbons in human mesenchymal stem cells. *Carbon.* 2013; 54:419–431.
43. Akhavan O, Ghaderi E, Akhavan A. Size-dependent genotoxicity of graphene nanoplatelets in human stem cells. *Biomaterials.* 2012; 33:8017–8025. [PubMed: 22863381]
44. Akhavan O, Ghaderi E. Toxicity of Graphene and Graphene Oxide Nanowalls Against Bacteria. *ACS Nano.* 2010; 4:5731–5736. [PubMed: 20925398]

45. Ma Z, Bai J, Jiang X. Monitoring of the Enzymatic Degradation of Protein Corona and Evaluating the Accompanying Cytotoxicity of Nanoparticles. *ACS Appl Mater Interfaces*. 2015; 7:17614–17622. [PubMed: 26200209]
46. Ginestra A, La Placa MD, Saladino F, Cassarà D, Nagase H, Vittorelli ML. The amount and proteolytic content of vesicles shed by human cancer cell lines correlates with their in vitro invasiveness. *Anticancer Res*. 1998; 18:3433–3437. [PubMed: 9858920]
47. Jung SK, Qu X, Aleman-Meza B, Wang T, Riepe C, Liu Z, Li Q, Zhong W. Multi-endpoint, High-Throughput Study of Nano material Toxicity in *Caenorhabditis elegans*. *Environ Sci Technol*. 2015; 49:2477–2485. [PubMed: 25611253]
48. Chatterjee N, Yang JS, Park K, Oh SM, Park J, Choi J. Screening of toxic potential of graphene family nanomaterials using in vitro and alternative in vivo toxicity testing systems. *Environ Health Toxicol*. 2015; 30:e2015007. [PubMed: 26602558]
49. Zanni E, De Beilis G, Bracciale MP, Broggi A, Santarelli ML, Sarto MS, Palleschi C, Uccelletti D. Graphite Nanoplatelets and *Caenorhabditis elegans*: Insights from an in Vivo Model. *Nano Lett*. 2012; 12:2740–2744. [PubMed: 22612766]
50. Wu Q, Yin L, Li X, Tang M, Zhang T, Wang D. Contributions of altered permeability of intestinal barrier and defecation behavior to toxicity formation from graphene oxide in nematode *Caenorhabditis elegans*. *Nanoscale*. 2013; 5:9934–9943. [PubMed: 23986404]

ABBREVIATIONS

BSA	Bovine Serum Albumin
GO	graphene oxide
HEK cells	Human Embryonic Kidney cells

Figure 1.

Graphene was prepared in six different animal sera (Bovine, Chicken, Horse, Human, Porcine and Rabbit serum) in a kitchen blender and the exfoliation efficiency ($\text{mg mL}^{-1} \text{h}^{-1}$) was compared with those by ultrasonication and a shear reactor. Nanotoxicity of low-defect, graphene in bovine serum was analyzed against two mammalian cell lines and in *C. elegans*.

Figure 2.

A. Dependence of bovine serum concentration on the efficiency of graphite exfoliation in a kitchen blender at 17 krpm blade speed. **B.** Dependence of graphite exfoliation efficiency on the type of serum and the method of exfoliation by a kitchen blender (17 krpm), shear reactor (17 krpm) and ultrasonication.

Figure 3.

A. Raman spectrum of graphene (solid line) is compared with graphite (dotted) shows increase in intensity of D band (~1345 cm⁻¹), D' band (~1625 cm⁻¹), and peak shift (about 30 cm⁻¹) of 2D band (~2695 cm⁻¹). **B.** Changes in ID/IG ratio in different sera using different modes of exfoliation.

Figure 4.

A. TEM images of exfoliated graphene in bovine serum **B.** Statistical distribution of graphene length analyzed from several TEM images and ~200 flakes.

Figure 5.

A. Cytotoxicity exhibited by as-prepared bovine serum-exfoliated graphene (~500 nm) with HEK-293T cells **B.** Cytotoxicity exhibited by smaller size (<200 nm) graphene with HEK-293T cells. **C.** Cytotoxicity exhibited by as-prepared bovine serum-exfoliated graphene (~500 nm) with H1299 cells. **D.** Cytotoxicity exhibited by smaller size (<200 nm) graphene sheets with H1299 cells. The cytotoxicity differences between HEK-293T cells and H1299 cells could be a cell-line specific phenomenon. All the co-incubation trials were performed in duplicates and the data was analyzed using unpaired student's *t*-test (* $p < 0.05$).

Figure 6.

A. Toxicity of graphene prepared in bovine serum to *C. elegans* at higher doses ($>50 \mu\text{g/mL}$ per 20 worms). **B.** Toxicity of graphene exfoliated in sodium cholate showed similar trend as graphene in bovine serum. **C.** Reproductive toxicity of graphene to nematode shows changes in brood size, depending on the dose, irrespective of the exfoliating media. All the data sets are statistically validated using student's t-test ($*p < 0.05$).

Length and number of layers of graphene produced in animal serum by different modes of exfoliation

Table 1

Serum Type	Kitchen Blender		Shear (rotor/stator)		Ultrasonication	
	Number of Layers	Length (µm)	Number of Layers	Length (µm)	Number of Layers	Length (µm)
BSA ⁸	3.6 ±0.4	0.50 ±0.10	6.3 ±1.9	1.7 ±0.5	5.6 ±0.6	0.45 ±0.03
Bovine	3.7 ±0.2	0.71 ±0.06	6.9 ±1.4	1.4 ±0.7	5.3 ±0.3	0.53 ±0.10
Chicken	4.4 ±0.5	0.73 ±0.13	5.7 ±1.2	1.2 ±0.4	3.6 ±0.3	0.41 ±0.01
Horse	4.0 ±1.0	0.54 ±0.08	6.3 ±1.8	1.6 ±0.6	5.0 ±0.6	0.52 ±0.02
Human	3.6 ±0.4	0.58 ±0.06	5.9 ±1.7	1.1 ±0.4	4.3 ±0.5	0.45 ±0.01
Porcine	3.7 ±0.1	1.05 ±0.01	5.8 ±0.8	1.5 ±0.5	4.7 ±0.4	0.48 ±0.03
Rabbit	3.6 ±0.6	0.60 ±0.07	5.8 ±1.6	1.5 ±0.3	4.5 ±0.3	0.48 ±0.03