Liquid Exfoliation of Layered Transition Metal Dichalcogenides for Biological Applications

Emily P. Nguyen,[1,2] Torben Daeneke,[1] Serge Zhuiykov,[3] and Kourosh Kalantar-zadeh[1]

[1]School of Electrical and Computer Engineering, RMIT University, Melbourne, Victoria, Australia
[2]Division of Materials Science and Engineering, CSIRO, Clayton, Victoria, Australia
[3]Department of Applied Analytical and Physical Chemistry, Faculty of Bioscience Engineering, Ghent University Global Campus, Incheon, South Korea

Known to possess distinctive properties that differ greatly from their bulk form, layered two-dimensional materials have been extensively studied and incorporated into many versatile applications ranging from optoelectronics to sensors. For biomedical research, two-dimensional transition metal dichalcogenides (2D TMDs) have garnered much interest as they have been shown to exhibit relatively low toxicity, high stability in aqueous environments, and the ability to adhere to biological materials such as proteins. These materials are promising candidates, demonstrating potential applications in biosensing, cell imaging, diagnostics, and therapeutics. Preparation and exfoliation of 2D TMDs play an important part in these various applications as their properties are heavily dependent on the number of layers and lateral size. Described in this article are protocols for the liquid exfoliation of 2D TMDs from their bulk materials. Additional protocols are also provided for functionalizing or modifying the surface of the exfoliated 2D TMDs. © 2016 by John Wiley & Sons, Inc.

Keywords: exfoliation • functionalization • ion intercalation • liquid phase exfoliation • transition metal dichalcogenides

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INTRODUCTION

This protocol article outlines the main two methods used for the liquid exfoliation of transition metal dichalcogenides (TMDs) for biological applications and experiments: exfoliation by ionic intercalation (Basic Protocol 1 and Alternate Protocol) and ultrasonication-assisted exfoliation (Basic Protocol 2). However, not all of these processed two-dimensional (2D) materials are readily biocompatible (Chng and Pumera, 2015), and surface modification and functionalization are sometimes required (Liu et al., 2014; Guan et al., 2015; Presolski and Pumera, 2015). As such, protocols are also provided for changing suspension solvents (Support Protocols 1 and 2) and for surface modifications and functionalization techniques (Support Protocols 3 and 4).

The use of 2D TMDs in biomedical and biological applications has increased in recent years. The properties TMDs possess, when exfoliated down to their 2D form, differ greatly from their bulk counterparts (Balendhran et al., 2013). 2D TMDs have unique

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electrical, optical, and chemical properties that make these materials suitable for a range of applications (Duan et al., 2015).

For biomedical applications, the most explored and researched morphologies for diverse nanosystems are spherical nanoparticles (Ashley et al., 2011). This is due to ease of preparation to achieve the morphology and a more fundamental understanding of such quasi-zero-dimensional objects. However, previous studies have shown that mesoporous nanoparticles were more favorable for drug delivery due to the larger surface area than simple nanoparticles (Chen et al., 2015). Thus, it is expected that structures that have large surface areas would be more appealing for biomedical applications. The exfoliation of the bulk stratified material down to single- or multi-layered sheets allows for just that: it increases the amount of accessible surface area of the material.

For large quantity production of 2D TMDs, chemical exfoliation methods are often used as they offer high yields (Coleman et al., 2011; Nicolosi et al., 2013), and some of their crucial structural and compositional parameters are easy to control. Chemical exfoliation of layered bulk TMDs in liquids/solvents is one of the most developed strategies for making 2D TMD sheets for biomedical applications, as well as catalysis and electrochemical storage (Chen et al., 2015).

For the chemical exfoliation of TMDs, two major approaches are usually taken. The first is a process generally based on "ion intercalation" (Basic Protocol 1 and Alternate Protocol). As TMDs are able to accommodate significant quantities of chemical species into the space between the layers (interlayer spacing; Kalantar-zadeh et al., 2015), this method aims to introduce charged ions into the interlayer space of a layered bulk crystal to aid in expansion of the spacing. This results in the disintegration of bulk crystal structure while still maintaining the individual sheets. Various chemical reactions and processes can then be used in assisting the exfoliation afterwards.

The other chemical exfoliation method is liquid phase exfoliation using high-intensity ultrasound waves, also known as sonication (Basic Protocol 2). This method is one of the simplest routes where sonication is used to shear the layered bulk material into single- or multi-layered sheets (Cunningham et al., 2012; Kalantar-zadeh and Ou, 2016). This exfoliation technique is usually performed in a specifically selected solvent or aqueous surfactant, and then the exfoliated 2D material is separated and collected via centrifugation. This method of chemical exfoliation is often favorable for bioprocesses as it does not involve any chemical reactions and generally achieves moderate to high yields (Yao et al., 2013; Huo et al., 2015). Additionally, it has been shown that the exfoliated TMDs can be separated into different lateral dimensions by varying the speed of the centrifugation (O'Neill et al., 2012).

Chemical pretreatment or functionalization of the surface, prior to or during exfoliation, can also aid in the liquid phase exfoliation of TMDs as it may make the material more compatible with the solvent. Such modifications can alter the surface energy and adhesion factors, which can eventually facilitate a more efficient exfoliation process. Additional chemical surface treatments can prevent re-aggregation of the 2D sheets and provide stability in incompatible solvents and environments (Zhou et al., 2014).

Functionalization of the TMD surface after exfoliation can also be just as beneficial as pretreatment. For example, as with many other nanosystems for biological applications, biocompatibility and stability are important factors to consider. Although it has been demonstrated that 2D TMD nanosheets can exhibit relatively high biocompatibility, it has also been reported that these exfoliated sheets may not be stable in some physiological conditions, and thus surface modifications are required (Chen et al., 2015; Kalantar-zadeh et al., 2015). Depending on the type of surface modification,

functionalization can potentially allow the exfoliated TMDs to have high dispersity, stability, improved biocompatibility, and site specificity (Wang et al., 2014).

Surface modification and functionalization can easily be achieved through physical adsorption or covalent chemical bonding to the surface (and at exposed edges and corners) with certain agents and reagents (Chen et al., 2015; Nguyen et al., 2015b). Surface modification agents can range from polymers and surfactants to small organic molecules and proteins. For instance, a common polymer used for functionalization of 2D TMDs is polyethylene glycol, a polymeric agent used in various fields including medicine (Liu et al., 2014; Chen et al., 2015). In addition to improving the biocompatibility and stability of 2D TMDs, surface modification and functionalization can also broaden the potential for a range of new and improved applications. Presently, 2D TMDs (as exfoliated and/or surface-modified) are being explored in a variety of biomedical applications including drug delivery, biosensing, diagnostic therapies, and imaging (Chen et al., 2015; Kalantar-zadeh et al., 2015).

ELECTROCHEMICAL METHOD FOR INTERCALATION EXFOLIATION OF TRANSITION METAL DICHALCOGENIDES

BASIC PROTOCOL 1

One of the most common ion intercalation approaches involves the intercalation of lithium ions (Li^+) into the interlayer spacing. The intercalated bulk material is then immersed into water, where the Li^+ ions react to form LiOH and H_2 gas and exfoliate the bulk material into mono- or multi-layer sheets (Nicolosi et al., 2013).

There are two methods to exfoliate TMDs by intercalation: electrochemically (as described here; Fig. 1), with an anode and a cathode setup, and chemically (see Alternate Protocol) using n-butyl lithium. When using these methods, surface modification and functionalization during the exfoliation procedure can be a challenging task and has not been commonly reported.

The electrochemical approach to exfoliate TMDs is fairly rapid and facile; however, it has been reported that this method often leads to defects on the surface and edges of

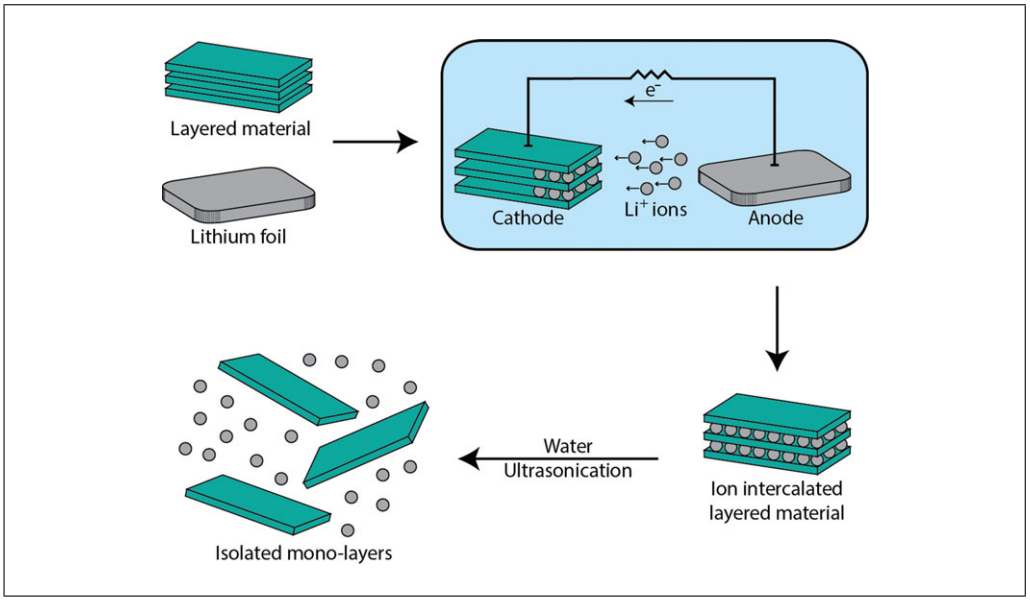

Figure 1 Setup of a typical electrochemical ion intercalation exfoliation of two-dimensional transition metal dichalcogenides (2D TMDs). This exfoliation method actively intercalates the Li^+ ions into the layered material by using a current source. Upon exposure to water molecules, the intercalated ions interact with water to produce hydrogen gas that separates the planes. Other conductive materials can be used for the anode.

the exfoliated 2D TMD and also introduces phase impurities which are able to alter the physical and chemical properties of the exfoliated 2D TMD (Nguyen et al., 2015a).

Materials

Bulk TMD powder (e.g., Sigma Aldrich)
Acetylene black
Polyvinylidene fluoride (PVDF)
N-methyl-pyrrolidone (NMP)
Copper foil
Ethyl carbonate
Dimethyl carbonate
Lithium hexafluorophosphate ($LiPF_6$)
Lithium (Li) foil
Argon gas supply
Acetone
Ethanol
Solvent appropriate for the product of interest

Galvanostatic power supply
Glove box with argon gas inlet (required if Li foil is used as the anode)
Glass sample vial
Bath or probe sonicator
Centrifuge tubes
Centrifuge

1. To make the cathode, mix together bulk TMD powder, acetylene black, and PVDF in a mass ratio of 80:10:10, and disperse in a small amount of NMP to make a slurry. Uniformly coat a square of copper foil with the slurry and allow to dry overnight under vacuum.
2. To make the electrolyte solution, prepare a 1:1 volume ratio solution of ethyl carbonate and dimethyl carbonate. Into this solution, dissolve enough $LiPF_6$ to make a 1 M solution.
3. Zero the current on the power supply and assemble the test cell, with Li foil as the anode, in the glove box. Purge the glove box with argon gas.
4. Apply a suitable DC voltage (minimum of 5 V) to the setup for 15 min.

   A good way to monitor the effectiveness of the intercalation is by watching the amount of gas bubbles on the cathode. Apply more voltage as necessary.
5. Carefully remove the cathode, and wash with acetone or ethanol to remove any excess $LiPF_6$.
6. Place the cathode in a glass sample vial with an appropriate volume of distilled water, and bath sonicate or probe sonicate the sample.

   The solution should turn opaque within a short amount of time (about 5 to 10 min), and copious amounts of gas should be produced.
7. Remove the copper foil and transfer the suspension to a centrifuge tube. Centrifuge 90 min at $>2500 \times g$, room temperature.
8. Wash the sediment by removing the supernatant and redispersing the sediment in an appropriate solvent. Repeat the washing step a few more times as required.

   The exfoliated material is now ready for use and should be stored in a closed container (i.e., a sample jar or vial).

CHEMICAL INTERCALATION TO EXFOLIATE TRANSITION METAL DICHALCOGENIDES

If an electrochemical setup is not possible, the TMD material can be intercalated chemically; however, this procedure will require a longer preparation time (up to 2 days). Figure 2 depicts the procedure for chemical intercalation to exfoliate TMDs.

Materials

Argon gas supply
Bulk TMD powder (e.g., Sigma Aldrich)
1.6 M n-butyl lithium in hexanes
Hexane
Solvent appropriate for the product of interest

Glove box with argon gas inlet
20- or 40-ml sample vials
Vacuum filtration apparatus
Bath sonicator
Centrifuge tubes
Centrifuge

CAUTION: The reagents used in this process (especially n-butyl lithium) are quite dangerous and pose health hazards. Appropriate protective equipment is recommended, and precaution and safety practices should be exercised.

1. In a glove box purged with argon gas, place bulk TMD powder in a sample vial and add n-butyl lithium (1 g TMD powder per 1 ml n-butyl lithium). Stir for 48 hr.

   This step intercalates the lithium ions into the bulk powder.

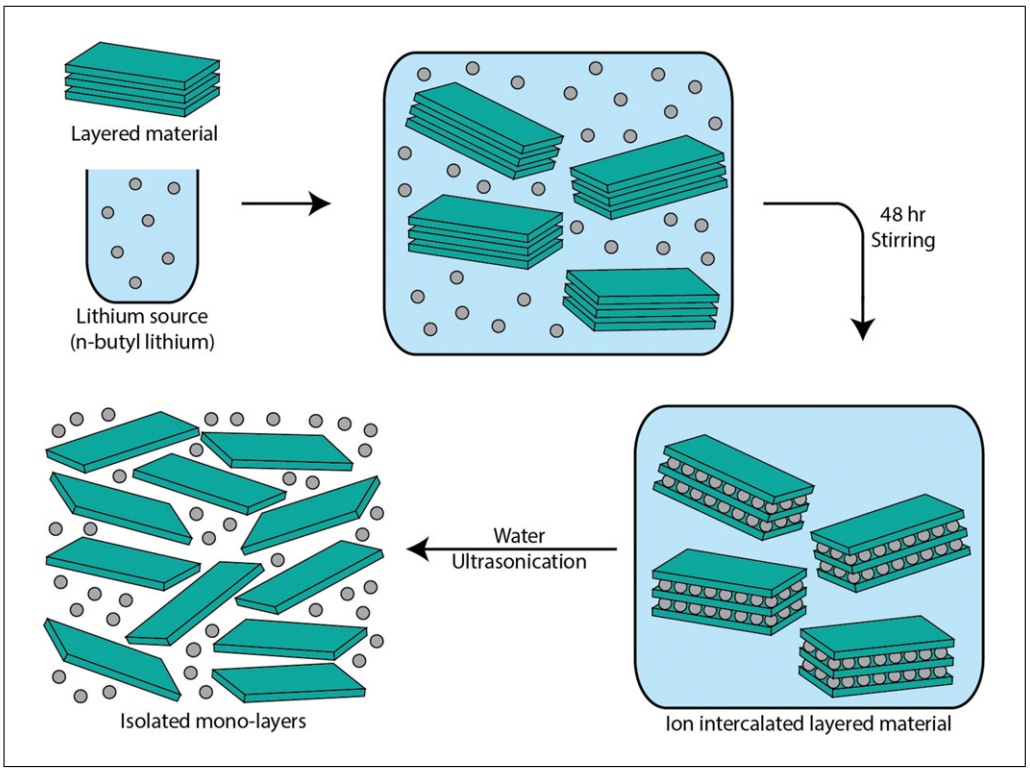

Figure 2 Setup of chemical ion intercalation exfoliation. The Li^+ ions intercalate themselves into the layered material over time. Exposure to water molecules exfoliates the bulk material.

2. Working quickly to avoid de-intercalation, collect the bulk powder by vacuum filtration, and wash the powder with hexane to remove excess n-butyl lithium. Repeat this washing step a few more times, and then allow the bulk powder to dry in the glove box under argon gas.
3. Transfer the intercalated bulk powder into a sample vial and seal tightly. Remove the sample vial from the glove box and put into the bath sonicator.
4. While sonicating, add distilled water to the sample vial (generally six to seven times the volume of n-butyl lithium used).

   Large amounts of gas should be produced, and the solution should become opaque.
5. Sonicate further for about 1 hr or until gas is no longer produced.
6. Transfer the suspension into a centrifuge tube, and centrifuge 90 min at $>2500 \times g$, room temperature.
7. Wash the sediment by removing the supernatant and redispersing the sediment in an appropriate solvent. Repeat the washing step a few more times as required.

BASIC PROTOCOL 2

SONICATION-ASSISTED LIQUID EXFOLIATION OF TRANSITION METAL DICHALCOGENIDES

Unlike the intercalation methods to exfoliate TMDs, sonication-assisted liquid phase exfoliation (Fig. 3) offers the advantages of requiring no chemical reactions, the potential and ease of tailoring this method to meet the requirements of intended experiments, and the ability to functionalize the 2D material simultaneously while exfoliating. For this protocol, either a bath sonicator or a probe sonicator can be used as the ultrasonic source.

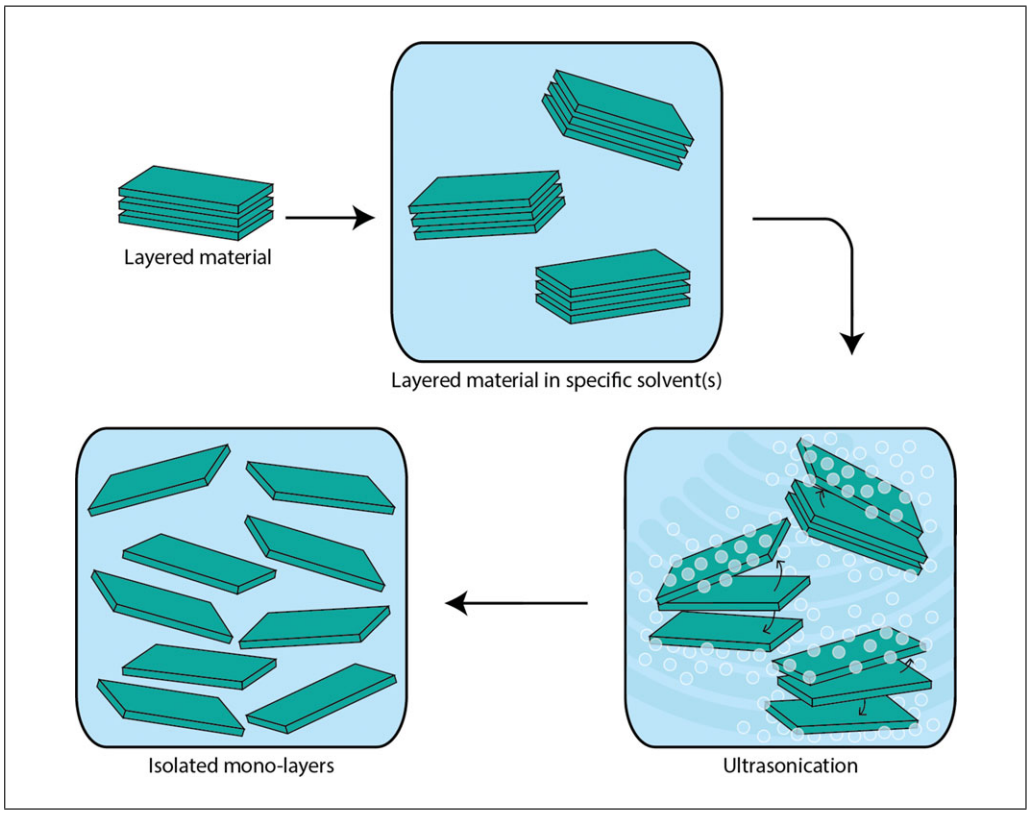

Figure 3 Setup of ultrasonication-assisted liquid exfoliation of layered materials. The shearing force from the ultrasonication cleaves off surface layers of the stratified bulk material.

Materials

Bulk TMD powder (e.g., Sigma Aldrich)
Solvent(s) of choice

Glass sample vials
Bath or probe sonicator
Centrifuge tubes
Centrifuge

1. Weigh out the bulk TMD powder and pour out solvent as required.

   Usually not a great amount of starting materials is required, and most report to measuring out the bulk powder in milligrams and the solvents in milliliters.

   Appropriate solvents will depend on the TMD powder being exfoliated. Studies have shown that solvents with similar surface energies to that of the TMD work best (Coleman et al., 2011).

2. Place the powder and solvent into a vessel compatible with the sonicator, usually a glass sample vial. Lightly shake the vial to mix the powder and the solvent.

3. Place the sample vial into the sonicator. Sonicate the solution for 30 to 90 min at 100 to 200 W and 20 kHz.

   Sonication time and amplitude can vary depending on the exfoliation level desired as well as machine make and model.

   If using a probe sonicator, stirring of the solution during sonication is also optional.

4. Once sonication is complete, allow the sample to cool, and transfer the solution into a centrifuge tube.

5. Centrifuge the sample at a desired speed and time.

   Usually, to separate the exfoliated material from the excess bulk powder, centrifugation is performed at a minimum of 2500 × g at room temperature. However, there have been reports that by careful selection of the centrifugation speed and sediment recycling it is possible to obtain specifically sized exfoliated sheets (O'Neill et al., 2012).

CHANGING SUSPENSION SOLVENTS BY EVAPORATION

SUPPORT PROTOCOL 1

Sometimes the resulting suspension of exfoliated 2D materials is in a solvent that may have resulted in high yields, but is not compatible with the intended use for the experiment. In this case changing suspension solvents may be required. Some solvents are easy to remove by evaporation; however, applying high temperatures to the 2D TMD suspensions may cause aggregation of the 2D material. It is best to evaporate the solvent at lower temperatures such as at 40°C.

Materials

Exfoliated TMD suspension
Intended suspension solvent

Glass petri dish
Hot plate
Vacuum oven (optional)
Fume cupboard
Sample vial
Bath sonicator

1. Pour the suspension into a glass petri dish (to expose the maximum surface area).
2. Place the petri dish on a hot plate at a low temperature (maximum 40°C) or into a vacuum oven (at a very low temperature such as 25°C) if the solvent has a high boiling point (i.e., n-methyl-pyrrolidone). If the solvent has a low enough boiling point that can be easily evaporated off (i.e., ethanol), then leave the petri dish in a fume cupboard to allow the solvent to evaporate by itself.
3. Once the solvent has fully evaporated, add the intended suspension solvent to resuspend the 2D material and collect in a new sample vial. Sonicate using a bath sonicator, as required, if the film adheres too strongly onto the petri dish.

After evaporation, the exfoliated TMDs should remain on the petri dish as a colored film.

SUPPORT PROTOCOL 2

CENTRIFUGATION TO SEPARATE 2D MATERIAL FROM SOLVENT

It is possible to use a centrifuge to separate 2D material from the solvent. However, as 2D TMDs are small and lightweight, a fast centrifuge speed is required, with a minimum speed of 13,500 × *g*.

Materials

Exfoliated TMD suspension
Intended suspension solvent

Centrifuge tubes
High-speed centrifuge (>13,500 × *g*)
Bath sonicator

1. Pour the exfoliated TMDs into the centrifuge tubes. Use a water balance if necessary.
2. Centrifuge at a minimum of 13,500 × *g*, room temperature.

Centrifugation times vary depending on the model; however, 90 min is usually sufficient.

3. Remove the supernatant and redisperse the sediment with the intended solvent. Sonicate using a bath sonicator to redisperse the sediment, if required.
4. Repeat steps 2 and 3 a few more times to ensure no or minimal residue of the original solvent is present.

SUPPORT PROTOCOL 3

POSTEXFOLIATION FUNCTIONALIZATION OR SURFACE MODIFICATION OF 2D TRANSITION METAL DICHALCOGENIDES

It is possible to use exfoliated 2D material without further functionalization or surface modification. However, for experiments that require or investigate the use of functionalization agents and/or surface chemistries, there are numerous methods to add molecules to the surface of 2D TMDs. The most common way to functionalize the surface of the 2D materials is to add the modification agent(s) after the exfoliation procedure, as described here.

Materials

Exfoliated TMD suspension
Surface modification/functionalization agent

Sample vial
Magnetic plate with stir bars that fit into the sample vial
Bath sonicator
Dialysis tubing
Centrifuge

1. Decant off a small volume of the exfoliated TMDs and place in a sample vial.
2. Add in a small amount of the surface modification agent in an appropriate ratio (i.e., 1:1 or in excess).

   The addition of the surface agent can be done either dropwise or by mixing directly into the 2D TMD suspension. Specific procedures may need to be applied depending on the agent used.

3. Stir the mixture using a magnetic stir bar overnight, or sonicate for short periods of time taking care to ensure the bath sonicator does not heat up the mixture.

   This will ensure thorough mixing and functionalization of the surface of the 2D TMD.

4. To remove the excess functionalization agents, either dialyze the mixture with dialysis tubing or centrifuge at a high speed (13,500 × *g*, room temperature) with a few cycles of washing.

   If using dialysis tubing, ensure that the pores are smaller than the exfoliated TMDs.

FUNCTIONALIZATION OR SURFACE MODIFICATION OF 2D TRANSITION METAL DICHALCOGENIDES SIMULTANEOUSLY DURING SONICATION

SUPPORT PROTOCOL 4

It is possible to functionalize the TMD while exfoliating. This is usually performed in a bath sonicator at low energy. A probe sonicator is not recommended as the probe becomes too hot and may cause the functionalization agent to change in nature (e.g., denature if it is a protein). This method has been shown to be successful with a variety of small biological molecules such as bovine serum albumin, glutathione, and glycine as well as organic molecules such as citric acid, benzoic acid, and aniline (Guan et al., 2015). Note that this protocol can only be performed with sonication-assisted liquid phase exfoliation (Basic Protocol 2).

Materials

Surface modification/functionalization agent
Bulk TMD powder (e.g., Sigma Aldrich)
Solvent appropriate for the product of interest

Centrifuge tube
Bath sonicator
Centrifuge

1. Prepare an appropriate volume of solution of the surface modification agent in a centrifuge tube, and add a small amount (i.e., a few milligrams) of the bulk powder.
2. Bath sonicate the mixture for about 30 to 90 min, in 15 to 20 min intervals to ensure the water bath does not get too hot. Change the bath water periodically to ensure temperature control.
3. Centrifuge 90 min at 2500 × *g*, room temperature, to pellet/remove the excess bulk powder.
4. Collect the supernatant and centrifuge 90 min at a minimum of 13,500 × *g*, room temperature, to collect the functionalized 2D material at the bottom of the centrifuge tube.
5. Redisperse the sediment in any compatible solvent, and wash the 2D material several times.

COMMENTARY

Background Information

Transition metal dichalcogenides (TMDs) have a general chemical formula of MX_2, where M refers to transition metals (Mo, W, Nb, Zr, etc.) and X refers to chalcogen atoms (S, Se, and Te). There can be up to 40 different types of TMD crystal phases depending on the combination of chalcogen and transition metal (Wang et al., 2012; Nicolosi et al., 2013). A single plane of TMD comprises of a layer of M atoms sandwiched between layers of X atoms. These planes are then held together by weak van der Waals forces and therefore can be exfoliated into single layer sheets (Fig. 4).

The term planar refers to the relatively large lateral dimensions of the exfoliated material, consisting of "measureable" lateral dimensions in the x and y directions. The "lack" of dimension in the z direction allows the material to have dramatic changes in the physical and chemical properties of the material with respect to its bulk (Chhowalla et al., 2015). The reduction of the number of layers dramatically influences the electronic energy states. These exfoliated 2D TMDs can be metallic, semi-metallic, or semi-conducting depending on the level of doping found in the lattice structure as well as coordination and oxidation state of the metal atoms (Cunningham et al., 2012; Nicolosi et al., 2013).

There are reported techniques to exfoliate 2D TMDs from the stratified bulk, ranging from mechanical to chemical exfoliation methods, with each method having its advantages and disadvantages (Tan and Zhang, 2015). The mechanical exfoliation processes of stratified TMDs produce relatively pristine and well-crystallized 2D sheets onto a substrate. 2D TMDs produced using these techniques are more commonly used for fundamental condensed matter physics and intrinsic characteristic studies as the electronic grade of the material is high. However, this method results in low yield and random landing of the flakes on the substrates and, as such, cannot be used for many biomedical applications where high amounts are required (Chen et al., 2015).

Critical Parameters

Solvent selection

The most integral part of sonication-assisted liquid phase exfoliation of TMDs is choosing a suitable solvent for the sonication stage. Physical and chemical properties of the selected solvent—such as boiling point, surface tension, and energy and solubility parameters—will have an effect on the resulting 2D TMD. Research and modelling have shown that the most effective solvents for the exfoliation of TMDs are those that are similar in surface energy to the bulk material (Nicolosi et al., 2013; Tang and Zhou, 2013). Solvents such as N-methyl-2-pyrrolidone (NMP) and isopropyl alcohol have been demonstrated to be the most effective (Coleman et al., 2011).

Ball milling/grinding

For sonication-assisted exfoliation, it has been shown that ball milling the bulk powder prior to sonication can increase the concentration (Yao et al., 2013). Grinding the powder with a mortar and pestle is also an option to increase the overall concentration, although the resultant concentration will be less than that if the powder was ball milled. Ball milling and/or grinding will ultimately introduce surface defects to the resulting 2D materials.

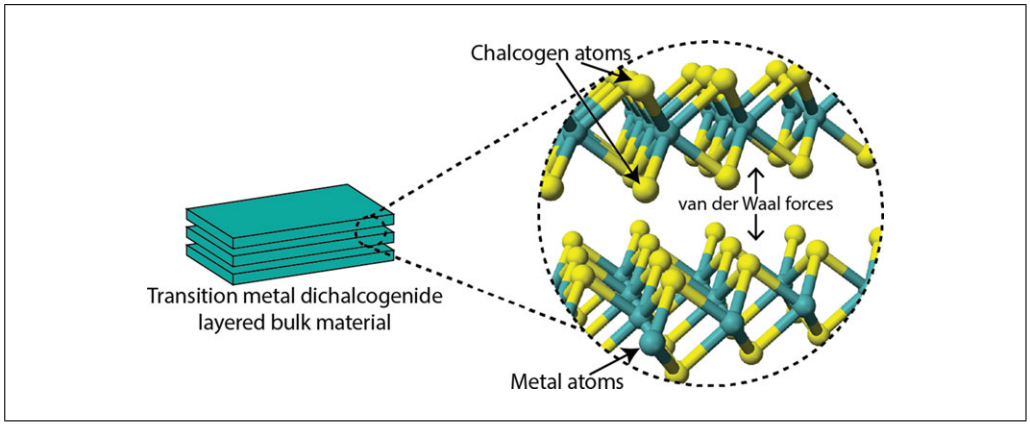

Figure 4 In the bulk form, mono-layered transitional metal dichalcogenides (TMDs) are held together by weak van der Waals forces.

Solvent blends and two-solvent synthesis

It is also possible to conduct a two-solvent exfoliation process where the grinding or ball milling stage is carried out in a solvent different to that in the sonication stage or where both stages are conducted with mixed solvents, such as 1:1 NMP and ethanol (Carey et al., 2015; Nguyen et al., 2015a). It has been reported that liquid exfoliation of TMDs in solvent blends led to slightly higher yields than those with just one solvent (Zhou et al., 2011).

Troubleshooting

The most common problems encountered with the exfoliation protocols are usually low concentration yields and aggregation of the exfoliated TMD over time. This may be due to many factors, but the main reason is often the choice of poor or incompatible solvent. Selecting a different solvent or experimenting with other solvents and/or solvent blends may be an answer. Moreover, ball milling or grinding can also aid in increasing the yield.

Anticipated Results

Basic Protocols 1 and 2 will produce suspensions of exfoliated TMDs. Depending on the TMD exfoliated, the suspension will be colored and may or may not also be pearlescent. Suspensions that are almost colorless and transparent are indicative of low concentrations. These suspensions are ready for the intended experiment; however, it is important to characterize the material according to size, shape, and layer. There are numerous simple techniques that can be used for characterizing the exfoliated materials, including atomic force microscopy, electron microscopy, and optical and vibrational spectroscopies (Wang et al., 2012; Tang and Zhou, 2013; Chhowalla et al., 2015).

Time Considerations

If all necessary solvents, materials, and reagents are ready, these protocols can be conducted and completed within a day. However, to be able to optimize and validate these steps, it may take up to a working week to exfoliate these materials and to functionalize them.

Acknowledgements

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