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Corking Nitrogen-Doped Carbon Nanotube Cups with Gold Nanoparticles for Biodegradable Drug Delivery Applications

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Abstract

Carbon nanomaterials have been proposed as effective drug delivery devices; however their perceived biopersistence and toxicological profile may hinder their applications in medical therapeutics. Nitrogen doping of carbon nanotubes results in a unique “stacked-cup” structure, with cups held together through van der Waals forces. Disrupting these weak interactions yields individual and short-stacked nanocups which can be subsequently corked with gold nanoparticles resulting in sealed containers for delivery of cargo. Peroxidase-catalyzed reactions can effectively uncork these containers, followed by complete degradation of the graphitic capsule, resulting in effective release of therapeutic cargo while minimizing harmful side effects. The protocols reported herein describe the synthesis of stacked nitrogen-doped carbon nanotube cups followed by effective separation into individual cups and gold nanoparticle cork formation resulting in loaded and sealed containers.

Keywords

Drug-delivery; chemical vapor deposition; nitrogen-doped carbon nanotubes; graphitic exfoliation; citrate reduction; enzymatic biodegradation

INTRODUCTION

Advancements in drug delivery have been made through the implementation of newly developed nanomaterials including, drug-polymer conjugates, polymeric nanoparticles, solid lipid nanoparticles, dendrimers, liposomes, micelles, gold nanoparticles (GNPs), and carbon nanomaterials (Muthu et al., 2014). While these nanomaterials have been tested as drug delivery devices, several issues still remain including non-specific delivery of therapeutic cargo, inflammation due to accumulation of drug delivery devices, and short circulation time. Carbon nanomaterials exhibit many unique properties making them ideal candidates for drug delivery including large surface area, the ability to encapsulate designated cargo, in addition to the ability for external functionalization of cargo and biocompatibility agents. Graphitic carbon nanomaterials exist in several different allotropes, depending on how many

Conflicts of Interest

The authors report no competing financial interest

of their dimensions are below 100 nm, including zero dimensional (0D) fullerenes, 1D carbon nanotubes, 2D graphene, and graphene quantum dots with all dimensions <10 nm.

While carbon nanotubes (CNTs) have been seen as an attractive material for drug delivery devices, they have been shown to cause oxidative stress both *in vitro* and *in vivo* resulting in inflammation and cell damage in the lungs and liver. Pristine CNTs directly induce the formation of reactive oxygen species (ROS) resulting in oxidative damage ultimately leading to cell damage and apoptosis (Muthu et al., 2013). The high aspect ratio of CNTs, similar to that of asbestos, causes DNA damage and inflammation; however, these side effects are shown to decrease with shorter CNTs as multi-walled carbon nanotubes (MWCNTs) with lengths less than 200 nm have negligible side effects *in vivo* (Yamashita et al., 2010). Further functionalization of pristine CNTs, primarily with polyethylene glycol (PEG), improves not only solubility in aqueous solution, thus increasing circulation time, but also diminishes the harmful side effects resulting from the formation of ROS. Additionally, functionalizing CNTs with nitrogen functional groups facilitates their degradation by the body, after delivery of designated cargo, resulting in less cytotoxic behavior (Yang et al., 2012).

Nitrogen can be substituted into CNTs as many different functionalities including pyridinic nitrogen, pyrrolic nitrogen, graphitic nitrogen, and oxidized nitrogen; these functionalities can be further altered or converted through chemical reaction to achieve desired nitrogen species (Dommele et al., 2008). The incorporation of nitrogen atoms into the graphitic lattice of CNTs creates additional strain to the CNT structure resulting in the formation of “stacked-cups” termed herein as nitrogen-doped carbon nanotube cups (NCNCs) (Figure 1A) (Allen et al., 2008). The biocompatibility of NCNCs can be improved upon oxidation with concentrated mineral acids, this process leaves the inherent stacked cup structure intact (Figure 1B) and further aids in the separation procedure. Adjacent cups within this stacked-cups structure are held together through van der Waals forces; disrupting these weak intermolecular forces results in individual and short-stacked segments of NCNCs (Figure 1C). NCNC separation has been achieved through different mechanical and chemical processes including grinding with mortar and pestle, KCl intercalation (Tang et al., 2014), and oxidation and subsequent probe-tip ultrasonication.

Upon separation it has been shown that nitrogen functional groups are found to be abundant on the opening of individual NCNCs (Florea et al., 2012), providing an active site for functionalization in order to effectively cork NCNCs (Figure 1D). Probe-tip sonication alters inherent nitrogen functionalities into dangling amine groups, due to the intense energy output, which have been shown to coordinate strongly to gold clusters (Zhao et al., 2015). This phenomenon was exploited in order to seed GNPs on the open end of NCNCs, which further grow, through sodium citrate reduction of chloroauric acid, into a solid GNP cork completely sealing NCNCs. Loading of specific molecules or cargo into the corked NCNCs can be achieved by incorporating the desired cargo into the corking reaction which has been confirmed through surface enhanced Raman scattering of the cargo with the GNP cork. A schematic diagram for the synthesis of corked NCNCs is shown in Figure 1E.

In order for GNP corked NCNCs to be an effective drug delivery system, a mechanism for cork detachment and release of cargo is necessary. Previous studies have shown that carbon nanomaterials are biologically degraded by peroxidases such as horseradish peroxidase (HRP), myeloperoxidase (MPO), and eosinophil peroxidase (EPO) both *in vitro* and *in vivo* (Kotchey et al., 2013). All three of these enzymes contain a ferriprotoporphyrin IX heme group which acts as the active site for CNT degradation (Figure 2A). When the enzymes are in their resting state the heme group exists in its ferric, Fe(III), form; upon the addition of hydrogen peroxide the heme center is converted into an oxo-ferryl iron ($\text{Fe}^{4+}=\text{O}$) which can be further reduced back to the ferric state by the peroxidase cycle. It has been shown that CNTs with oxygen functionalities degrade more rapidly through incubation with these enzymes than pristine CNTs. The improvement in CNT degradation upon oxidation is due to not only better solubility in aqueous solutions but also due to the electrostatic interactions of carboxyl groups orienting CNTs close to the heme active site. MPO is an enzyme that is naturally found in neutrophils which are the most abundant white blood cells in the body, in addition to being involved in normal inflammatory responses. MPO is further able to oxidize halogens at high rates producing hypohalous acids which further improve CNT degradation.

Since MPO has shown excellent degradation of oxidized CNTs, and is biologically relevant providing opportunities for practical applications, it has been used to degrade separated NCNCs as shown in Figure 2B. After 20 days of incubation with MPO, H_2O_2 , and NaCl, NCNCs were found to be degraded as shown by lighter solution color as compared to NCNCs only incubated with H_2O_2 which was further confirmed by TEM imaging and Raman spectroscopy. MPO was then further applied to degrade GNP corked NCNCs effectively detaching the GNP cork, releasing the loaded cargo, and completely degrading the graphitic carbon capsule negating harmful side effects. Our previous report has shown that GNP corked NCNCs, upon incubation with MPO, H_2O_2 , and NaCl, can be effectively uncorked within 5 days followed by complete degradation of the graphitic capsule after 20 days (Zhao et al., 2015). TEM of GNP corked NCNCs after 5 days of incubation with MPO (Figure 2C) shows the GNP being completely detached from the graphitic NCNC. Additionally, the detachment of the GNP decreases the Raman intensity of the graphitic D and G bands as there is no more surface enhancement from GNP coordination (Figure 2D). In the first protocol NCNCs will be synthesized through chemical vapor deposition (CVD) which will then be utilized in the second protocol to isolate individual cups from their stacked configuration and subsequently cork with GNPs. Several characterization techniques will be shown in order to prove the loading of cargo and complete sealing of the NCNC cavity as a result of GNP formation.

BASIC PROTOCOL 1

Synthesis of Stacked NCNCs through Chemical Vapor Deposition (CVD)

This protocol outlines the procedure for synthesizing NCNCs through CVD from a mixture of ferrocene, xylenes, and acetonitrile in a hydrogen and argon environment at elevated temperatures. During this synthesis, the metal ions in ferrocene are reduced upon interaction with hydrogen gas, continually forming iron nanoparticles which act as catalysts initiating carbon nanotube growth. Xylenes act as the carbon source and acetonitrile acts as the

nitrogen source, at the elevated temperatures these molecules are broken down into individual carbon and nitrogen atoms. The surface of the iron nanoparticles is highly active in the formation of iron carbides creating a graphitic envelope on the particle. The iron nanoparticle effectively moves as the carbon nanotube is formed from the surface; due to the strain incorporated into the graphitic lattice by the doped nitrogen atoms, the carbon nanotube eventually pulls away from the iron nanoparticle exposing a fresh catalyst surface allowing for growth of the next graphitic envelope. This growth mechanism results in the unique stacked cup structure of nitrogen-doped carbon nanotubes (Dommele et al., 2008).

Materials

Lindberg Blue M 3 zone furnace
Kimwipes (Kimtech Science)
Acetone
5 foot glass rod
Quartz tube ($\frac{1}{2}$ inch diameter, 4 feet long)
Quartz crossplate ($\frac{1}{2}$ inch $\times$ 1 foot)
3 thermal blocks (3 inch diameter with $\frac{1}{2}$ inch bored hole in the center and 3 inches thick)
Kera wool (or other insulating material)
Stainless steel end caps, one with solution injector
2 O-rings ($\frac{1}{2}$ inch diameter)
2 stainless steel washers ($\frac{1}{2}$ inch diameter)
2 mass flow controllers
Pure H₂ gas cylinder
Pure Ar gas tank cylinder
2 bubblers
Microbalance (Mettler Toledo)
Ferrocene (98% purity)
Xylenes (98.5% purity)
Acetonitrile (99.5% purity)
Syringe pump
25 mL air tight syringe
Respirator
Double-sided razor blade

Assembling the Chemical Vapor Deposition Apparatus

- 1 Wrap a kimwipe around the glass rod, wet with acetone, and run through the inside of the quartz tube to clean. Additionally, wipe down the quartz crossplate with a kimwipe wet with acetone to clean. Place the quartz crossplate 1/3 of the way into the quartz tube.
- 2 Place the thermal blocks on the quartz tube. One thermal block should be placed at either end of the quartz tube and the third thermal block should be placed directly in front of the quartz crossplate.
- 3 Clean the stainless steel endcaps, washers, o-rings, and injector with acetone wet kimwipes. Seal both ends with the end caps, washers, and o-rings with the injector being placed on the side closest to the quartz crossplate, the injector used in our lab can be seen inset Figure 3A.
- 4 Place the quartz tube into the Lindberg Blue furnace so that the edge of the crossplate is at the boundary between the second and third zones. The thermal block in the middle should then be set at the edge of the crossplate resting entirely in the third zone. Kera wool is wrapped around this thermal block to make a tight seal isolating the first and second zones from the third zone.
- 5 The end cap without the injector should have a rubber hose running to a bubbler.
- 6 The end cap with the solution injector should have three outlets on it, one of which leads to a bubbler (which should be filled with more water than the bubbler on the other end of the tube), one which should be connected to the incoming gas flow, and one that will be used to inject the catalyst precursor solution. Figure 3A shows a picture of the CVD apparatus used in our laboratory.
- 7 Once the tube is sealed tight turn on the Ar gas at a flow of 127 standard cubic centimeters per minute (sccm) and spray a dilute soap solution on all connections to make sure there are no gas leaks (gas leaks are indicated by growing soap bubbles forming at connections). If there are no gas leaks, turn on the H₂ gas at a flow of 38 sccm.
- 8 At this point the furnace can be turned on and preheating can begin. Set the third zone (one with the injector) to 250 °C and the first and second zones to 800 °C. The third zone is for evaporation of the solvent while the first and second zones are responsible for the deposition of the synthesized carbon nanotubes.

Nitrogen-Doped Carbon Nanotube Cup Growth by CVD Synthesis

- 9 Weigh out 37.5 mg of ferrocene. Tare a vial on the microbalance and add the 37.5 mg of ferrocene. Add 0.5 g of acetonitrile to the vial using a Pasteur pipet. Add xylenes to the vial using a Pasteur pipet until the total mass of the solution is 5 g. This results in a solution with 0.75 wt % ferrocene, 10 wt % xylenes, and 89.25 wt % xylenes. Larger volumes of the solution can be made for multiple runs if these ratios are scaled up appropriately.

- 10 Plastic tubing should be connected to the end of the 25 mL air tight syringe. Draw in all 5 g of catalyst precursor into the syringe with some additional air. Then hold the syringe upright so the air bubble is at the injector and push out the air and continue until the solution fills the plastic tubing.
- 11 Set the syringe into the syringe pump and start pumping at 9 mL/hr until the solution starts moving and comes to the end of the plastic tubing. Then place the rubber tubing (which should have a metal adapter at the end) into the CVD injector.
- 12 Run the syringe at 9 mL/hr for roughly 6 minutes and 30 seconds (which may change depending on the length of your specific injector). When a brown smoke is seen at the end of the quartz tube (away from the injector) the flow rate of the syringe pump should be reduced to 1 mL/hr. The CVD run should last 1 hour after the solution flow rate is reduced.

Collecting Synthesized Nitrogen-Doped Carbon Nanotube Cups

- 13 After 1 hour has passed, turn off the temperature control unit as well as the H₂ flow to the CVD apparatus. Allow the Ar to continue to flow to help cool the quartz tube and push out H₂ gas in addition to any additional fumes created during the CVD synthesis.
- 14 After 15 minutes open the CVD furnace and allow the furnace to cool to room temperature. At this point the Ar gas may also be turned off.
- 15 Put on the respirator as to prevent breathing in any carbon nanotubes. Detach the inlets and outlets from the quartz tube and remove from the furnace.
- 16 Remove the end caps and wipe them down with kimwipes wet with acetone to remove any waste material. Use the glass rod to push the quartz crossplate out of the quartz tube. Be careful as this will be hotter than the quartz tube.
- 17 Clean the inside of the quartz tube in the same fashion as before the experiment. Be careful as there will be a large amount of carbon nanotubes on the sidewalls of the quartz tube.
- 18 Wipe down the double-sided razor blade with a kimwipe wet with ethanol. There should be a thick black mat of deposited NCNCs on the quartz crossplate closest to where the injector was. Use the double-sided razor blade to scrape off the NCNCs onto a piece of weigh paper and transfer the NCNCs to a vial.
- 19 After the CVD synthesis is complete the tube needs to be cleaned. Place the crossplate back into the quartz tube, do not put the stainless steel endcaps or the thermal blocks on the quartz tube. Place the tube in the furnace and heat at 1000 °C for 30 minutes.

BASIC PROTOCOL 2

Separation of Stacked NCNCs by Probe-Tip Sonication and Subsequent Cargo Loading and Gold Nanoparticle Corking

This protocol effectively separates as-synthesized stacked NCNCs into individual and short-stacked NCNCs through oxidation and subsequent probe-tip ultrasonication. The typical CVD synthesis performed in Basic Protocol 1 yields around 10–15 mg of NCNCs and multiple syntheses are conducted in order to collect enough material for practical applications. Alternatively, commercial nitrogen-doped carbon nanotubes, exhibiting the similar “stacked-cup” morphology (NanoTechLabs, Item # NTL-12116) can be utilized in this separation procedure. Acid oxidation of NCNCs facilitates their separation and further provides functionalities for complete suspension in aqueous environments. Upon separation by probe-tip sonication, individual NCNCs are then corked with gold nanoparticles through sodium citrate reduction of chloroauric acid. The resulting corked NCNCs can be identified through transmission electron microscopy (TEM) in addition to a strong surface enhanced Raman signal due to coordination of the GNP cork with the NCNC. Introducing desired cargo into the corking reaction mixture results in the uptake of the cargo into the cup-like cavity of individual NCNCs which are then trapped upon the completion of the GNP cork. The loading of cargo into the NCNCs can be shown by the enhancing of their Raman peaks due to charge transfer with the GNP cork much like that shown for the D and G bands of the graphitic carbon capsules.

Materials

Nitrogen-doped carbon nanotube cups (synthesized in Basic Protocol 1)

Microbalance (Mettler Toledo)

Sulfuric Acid (H_2SO_4 , 95.0 to 98.0 w/w%)

Nitric Acid (HNO_3 , 70%)

Round bottom flask (250 mL)

Kimwipes (Kimtech Science)

Three-prong clamp

Bath Sonicator (Branson 5510)

Nanopure Water (Barnstead Nanopure filtration unit)

Filtration apparatus

Polytetrafluoroethylene (PTFE) 0.2 μm filter (EMD Millipore, hydrophobic)

Hydrochloric acid (HCl , 0.01 M in nanopure water)

Sodium hydroxide (NaOH , 0.01 M in nanopure water)

100 mL beaker

Probe-tip sonicator (Qsonica Q500 equipped with 1/2 inch titanium probe tip, 20 kHz max intensity)

Chloroauric acid (HAuCl_4 , 1 mg/mL in nanopure water)

Sodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$, 1 wt% in nanopure water)

Magnetic stir bar (10 $\times$ 3 mm)

Vial and cap (21 × 70 mm, 4 dram)

Hot and stir plate with oil bath

Oxidize nitrogen-doped carbon nanotube cups (NCNCs)

- 1 Weigh out 10 mg of NCNCs on an analytical microbalance.
- 2 Transfer the NCNCs to a 250 mL round-bottom flask containing 40 mL of 3:1 v/v H₂SO₄/HNO₃ acid solution. Cover the opening of the round bottom flask with a kimwipe and secure with a rubber band to prevent the entry of any water or loose particles.
- 3 Place the round bottom flask into the center of the bath sonicator so that the water level of the bath is at the same level as the acid solution inside the flask. Hold the flask in position with a three-prong clamp as shown in Figure 3B.
- 4 Turn on the bath sonicator. The NCNCs should begin to evenly disperse throughout the acid solution, if any NCNCs get stuck on the sidewalls of the flask, gently swirl the flask to suspend these NCNCs into the acid solution.
- 5 Allow the solution to sonicate for 4 hours checking occasionally to ensure all NCNCs remain in the acid solution.
- 6 Once sonication time is complete, remove the round bottom flask and immerse it in a beaker of ice water. Inside a ventilated fume hood, slowly add ~100 mL of nanopure water to the round bottom flask in order to dilute the acid solution for filtration. Dark fumes will be produced and heat will be generated as the water is added.
- 7 Assemble a filtration apparatus in order to wash off the excess acid and collect the oxidized NCNCs. Use a 0.2 µm PTFE membrane.
- 8 Once the acid solution has been filtered, wash the NCNCs on the PTFE membrane with 500 mL of nanopure water, 250 mL of 0.01 M NaOH, 250 mL of 0.01 M HCl, and 500 mL of nanopure water.
- 9 After the wash is complete, scrape the NCNCs off of the membrane using a small spatula and collect for probe-tip sonication separation. As some NCNCs remain in the grooves of the membrane brief sonication of the membrane in a small volume of nanopure water will remove the rest of the oxidized NCNCs.

Separate the oxidized NCNCs through probe-tip ultrasonication

- 10 Suspend roughly 5 mg of oxidized NCNCs in 75 mL of nanopure water in a 100 mL beaker. Small narrow beakers are best for the probe tip separation.
- 11 Place the tip of the probe-tip sonicator ~1/3 of the way into the solution of oxidized NCNCs and hold the beaker in place with a three-prong clamp.
- 12 With the amount of energy produced by the tip, the solution heats up quickly. In order to keep it cool, make an ice bath in a larger beaker and surround the

beaker with the oxidized NCNCs as shown in Figure 3C. This ice bath will need to be changed every hour to keep the solution close to room temperature.

- 13 The probe-tip sonicator should be set to 60% of its total intensity (12 kHz) and cycled with on and off times. This can either be done with 30 seconds on and 30 seconds off or 45 seconds on and 15 seconds off. This ensures that the tip does not overheat in the sonication process.
- 14 Run the tip-sonicator for 8 hours of total on time, if doing 30 seconds on and 30 seconds off this would be 16 hours of total time.
- 15 After the 8 hours of sonication time is complete take the beaker of separated NCNCs and cover with parafilm. Leave the beaker to sit out for two days to allow the TiO₂ particles shed by the probe-tip to sediment out of solution. Then collect the top dark solution which will contain the oxidized and separated NCNCs.

Cork NCNCs with gold nanoparticles through sodium citrate reduction of chloroauric acid

- 16 Prepare an oil bath to be held at 70°C, the hot plate used should also be able to spin a magnetic stir bar.
- 17 Make 2 mL of a 0.01 mg/mL NCNCs solution (in nanopure water) in a 4 dram vial. Slowly add 100 µL of 1 mg/mL HAuCl₄. Add a small stir bar (11 mm × 3 mm) and stir the solution at 1000 RPM for 20 minutes. The desired cargo should be added to the solution at this stage to allow enough time for incorporation into the NCNC cavities.
- 18 After 20 minutes, add 60 µL of 1 wt% sodium citrate dropwise to allow for thorough mixing. Allow the reaction to proceed for 2 hours. As the reaction progresses the solution should develop a red hue to it indicating the formation of gold nanoparticles.
- 19 After the reaction time is finished, do a brief sonication in order to break up any aggregates.
- 20 In order to collect the corked NCNCs, centrifuge the sample at 3400 RPM for 30 minutes, removing the supernatant, and keeping the pellet. The supernatant, containing free gold nanoparticles, will have a red color while the pellet, containing GNP corked NCNCs, will be purple in color. These colors are very different from the black solution of separated NCNCs as shown in Figure 4A.
- 21 The GNP corked NCNC pellet should then be washed with nanopure water in order to remove any unreacted reagents. Add roughly 5 mL of nanopure water and resuspend the pellet followed by another 30 minutes of centrifugation, remove the supernatant and keep the GNP-NCNC pellet. This wash should be repeated 3 to 5 times. The resulting GNP-NCNC pellet can then be suspended in nanopure water.
- 22 TEM images will confirm the corking of NCNCs with GNPs. In addition this coordination enhanced the D and G Raman bands from the graphitic carbon

lattice of the NCNCs which can be used in order to track the GNP-NCNC throughout any experiments. This enhancement is not seen however when free GNPs are mixed with NCNCs as shown in Figure 4B. Furthermore, the Raman signature of any loaded cargo is also enhanced through charge transfer with the GNP cork as shown in Figure 4C.

COMMENTARY

Background Information

NCNCs have been synthesized through a wide range of CVD procedures altering the carbon source, nitrogen source, and metal catalyst. Changing these parameters results in different structures in addition to different nitrogen functionalities. The synthesis used in these protocols results in well-defined stacked cups structure; however analogous syntheses could also be employed by altering the precursor composition and synthesis temperature to provide starting material with desired properties (Dommele et al., 2008). The metal catalyst employed in nanotube growth has also been altered and may result in unique properties in the separation and corking reactions (Tang et al., 2013), while other reports have achieved carbon nanotube growth through metal-free methods (Yu et al., 2010).

Corking NCNCs was first achieved through functionalization of the open end nitrogen functionalities with 3-mercapto-propionic acid which was able to form strong gold-sulfur bonds with commercial GNPs (Zhao et al., 2012). While corking was achieved through this reaction, the efficiency was relatively low due to a distribution for the diameter of synthesized NCNCs and the use of a single size commercial GNP. The sodium citrate reduction of chloroauric acid further improved the corking efficiency, however 100% efficiency is still not achievable. The least efficient corking reactions that occur are around 30% which is dependent on the distribution of nitrogen functionalities around the open end of the cup in addition to how many of those functionalities have been converted to amine groups through probe-tip sonication. Because of this problem with efficiency, multiple runs are required to achieve enough sample for practical applications.

Additionally, the uncorking mechanism is limited to some extent due to the specific reaction pathway for degradation of NCNCs. While different cell types have been proven to detach GNP corks followed by complete degradation of the NCNC container, without peroxidase catalyzed pathways degradation and uncorking, as it is understood at this point, is not achievable. Therefore, GNP corked NCNCs would not be appropriate for applications that cannot exploit the peroxidase cycles responsible for the demonstrated uncorking phenomenon. Analogous enzymes may potentially be able to degrade NCNCs, however this has not been shown in trials to date. However, for applications that do utilize these peroxidase degradation pathways the delivery of the cargo is specific for cells types with these enzymes allowing for targeted delivery minimizing premature and nonspecific delivery of cargo. We have shown the effective loading of corked NCNCs with two specific molecules; Rhodamine 123 and paclitaxel (Zhao et al., 2015). While this technique has been proven effective, the implementation of different cargo could have an unforeseen effect on the corking mechanism. As such corrections to this procedure will need to be made in order to achieve the most efficient loading and corking necessary for different molecules.

Critical Parameters

CVD synthesis of NCNCs is very sensitive to small changes in the apparatus setup. For instance, the exact location of the end of the solution injector in the quartz tube can directly correlate to the yield of nitrogen-doped carbon nanotubes. The desire for the stacked-cup structure creates an additional amount of complexity to the apparatus setup. To this end, when starting CVD synthesis several trial runs may need to be performed to optimize the specific apparatus used; once optimized, keep all further runs consistent to prevent unnecessary variations between samples. To manage the gas flow for CVD synthesis, each flow controller needs to be calibrated for their respective gasses. To do this, turn the flow controller on to a specific percentage and measure the rate of gas into a graduated cylinder filled with water upside in a beaker of water. Measure the flow in a given period of time and convert to standard cubic centimeters per minute (sccm) to determine the percentages needed to achieve the desired flow rates. Be careful as hydrogen gas can be flammable; perform these measurements in the fume hood. Additionally, the bubbler away from the injector should be bubbling while the bubbler at the end with the injector should not bubble. If this is not the case, remove some water from the bubbler away from the injector or add water to the bubbler at the injector. This ensures that the gasses and evaporated solvent are flowing through the tube and not backed up preventing proper synthesis and deposition of NCNCs.

The oxidation process breaks down the NCNCs so roughly half of the original 10 mg is lost. Furthermore, the tip sonication also breaks down material and can reduce the overall yield of the separated NCNCs especially with extended sonication times. To this end, it is necessary to ensure an efficient separation, by keeping the sonicated solution cool in addition to making sure the NCNCs are at a very low concentration, in order to minimize the necessary sonication time. Therefore, it is necessary to consider the required amount of GNP cored NCNCs before starting the protocols. An oxidation and probe-tip sonication can yield roughly 100 mL of a 0.1 mg/mL solution of separated NCNCs, when performed efficiently, which can be used for several corking procedures. However, since each corking procedure utilizes only 2 mL of 0.01 mg/mL separated NCNCs and efficiencies can be as low as 30% a single run can result in only 20 µg of GNP cored NCNCs maximum. To this end, roughly 50 corking procedures would have to be done in order to achieve 1 mg of GNP cored NCNCs. If pressed for time, pulse centrifugation can be performed in order to remove the TiO₂ particles after probe tip sonication. Centrifuge the solution at 3400 RPM for 10 seconds and keep the top layer leaving behind the TiO₂ particles. Do this until no more residual TiO₂ remains which can be confirmed through X-ray photoelectron spectroscopy or energy-dispersive X-ray spectroscopy.

Troubleshooting (overcoming or avoiding common problems)

The problems associated with the corking of NCNCs are directly related to the efficiency of oxidation and probe-tip sonication. One issue that occurs with oxidation of NCNCs is the aggregation of NCNC forming clumps which prevents the oxidation of those NCNCs. To achieve the greatest efficiency of oxidation, assure no clumps of NCNCs are formed in the solution; this can be done by increasing the amount of H₂SO₄/HNO₃ acid solution used for oxidation allowing better dispersion of the NCNCs. Poor oxidation will result in inefficient

separation, if this problem is encountered longer oxidation times, up to 6 hours, can be performed to improve overall oxidation. However, this will also break down more NCNCs resulting in shorter stacks and a greater loss of NCNCs during the filtration process.

The tip sonication can also encounter problems not associated with the level of oxidation in the sample. If the NCNC solution is too concentrated, the separation efficiency will be diminished. Ensure that the concentration of NCNCs in the separation solution is below 1 mg/mL. Temperature control in the tip sonication is incredibly important; if the temperature increases too much, evaporation will occur thus decreasing separation efficiency.

Additionally, the high temperatures could alter the functionalities of NCNCs or compromise their structure. Make sure to check the ice bath frequently and replace it if all the ice has melted. If some of the water does evaporate from the solution, add more nanopure water to improve the efficiency of NCNC separation.

The GNP corking reaction is straightforward, however several small problems can lead to inefficient corking. First, the temperature should not drop below 70°C as the efficiency is dramatically decreased at lower temperatures. Ensure that the stir bar is directly in the middle of the vial and creating a vortex for even mixing; non uniform mixing can have a negative effect on the corking mechanism. Often times the GNP corking suspension is climbing the vial walls and aggregates form around the top of the solution in an apparent coffee ring around the vial. These aggregates contain NCNCs that are not being corked, and as such the vial should be shaken in order to get those aggregates back into the solution. Check the vial every 15 minutes in order to ensure no aggregates are forming outside of the solution.

Anticipated Results

The CVD synthesis described in Protocol 1 results in NCNCs with a nitrogen content from 1–2 atomic % as determined by X-ray photoelectron spectroscopy (XPS) as shown in Figure 5A. Additionally, 10–15 mg as-synthesized NCNC fibers will be producing ranging from 10 – 50 nm in diameter and be several microns in length on average. After completing the acid oxidation process, an oxygen atomic percentage near 15% is achieved depending on the efficiency of the oxidation (Figure 5A). Furthermore, NCNC fibers are shortened substantially with many segments being under one micron in length. The separation process can vary depending on the concentration of the NCNCs and the average temperature of the separation solution, on average roughly 100 mL of 0.1 mg/mL separated NCNCs will be produced with an average length around 250 nm. This sample can be used for several corking procedures producing similar GNP corked NCNCs from different corking reactions. It is possible that the resulting separation solution may have some longer NCNC segments which would be undesirable for practical applications. To this end, filtration through a 0.2 μm PTFE membrane will allow for a more uniform dispersion of separated NCNCs resulting in nearly identical GNP corked NCNC samples from different reactions.

The nitrogen content will also decrease after oxidation and increase following probe-tip separation as determined by XPS analysis (Figure 5A). During the oxidation, chemical reactions occur preferentially at the nitrogen functionalities resulting in the overall decrease in nitrogen content. During probe-tip separation amorphous carbon is broken down in

addition to isolating individual cups exposing more nitrogen to the outer chemical environment thus explaining the increase in nitrogen content. Additionally, due to the high energy of probe-tip sonication, nitrogen functionalities are preferentially converted into dangling amine bonds as shown in the high-resolution XPS spectra in Figure 5B and 5C. This is essentially ideal as previous computational results have indicated that dangling amine bonds have the highest affinity for gold clusters (Zhao et al., 2015), thus resulting in the best GNP capping efficiency.

Time Considerations

CVD synthesis takes about two hours to perform. The oxidation of NCNCs and filtration of the sample takes roughly 6 hours with the possibility of being longer depending on the efficiency of the vacuum filtration system. Probe-tip separation of the NCNCs takes 13 hours, including time to change the ice, if done with 45 s on time and 15 s off time. This procedure can be split into two days if necessary, simply cover the beaker and let it sit out overnight when not doing tip sonication. The GNP capping procedure takes 5 hours including the time to wash the sample.

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Figure 1. Transmission electron microscopy (TEM) images of A) as-synthesized, stacked nitrogen-doped carbon nanotube cups (NCNCs); B) oxidized NCNCs; C) separated NCNCs; D) gold nanoparticle corked NCNCs; E) schematic diagram for the preparation of corked NCNCs.

Figure 2.

A) The catalytic peroxidase cycle for HRP, MPO, and EPO, compound I is reduced directly to the resting state *via* conversion of halide to hypohalite; B) photograph of (1) NCNCs incubated with hMPO, H_2O_2 , and NaCl for 20 days, and (2) NCNCs treated only with H_2O_2 for 20 days; C) TEM image of GNP corked NCNCs after incubation with hMPO, H_2O_2 , and NaCl for 5 days; D) Raman spectra of GNP corked NCNCs at increasing incubation times with hMPO, H_2O_2 , and NaCl. Panel A reproduced with permission from (Kotchey et al., 2013), panels B, C, and D reproduced with permission from (Zhao et al., 2015).

Figure 3.

A) Image of the chemical vapor deposition apparatus, the inset shows the custom made solution injector; B) image of the acid oxidation process in the bath sonicator; C) image of the probe tip sonicator set up for separating adjacent NCNCs.

Figure 4.

A) Images of (1) separated NCNCs, (2) supernatant of corking reaction (free GNPs), (3) pellet of corking reaction (GNP cored NCNCs); B) Raman spectra of separated NCNCs (black), NCNCs mixed with GNPs (blue), and NCNCs corked with GNPs (red); C) Raman spectra of free Rh123, (1) GNP cored NCNCs loaded with Rh123, and (2) Rh123 mixed with pre-corked NCNCs. All panels reproduced with permission from (Zhao et al., 2015).

Figure 5.

A) Survey XPS of stacked NCNCs (black), oxidized NCNCs (red), and separated NCNCs (blue); high resolution N1s XPS of B) oxidized NCNCs and C) separated NCNCs and their respective deconvolutions; N1 (pyridinic), N2 (pyrrolic/amine), N3 (graphitic), and N5 (oxidized nitrogen). Panels B and C reproduced with permission from (Dong et al., 2015)