In Vivo Delivery of Nitric Oxide-Sensing, Single-Walled Carbon Nanotubes

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In Vivo Delivery of Nitric Oxide-Sensing, Single-Walled Carbon Nanotubes

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Abstract

Detection of nitric oxide (NO) in vivo by single walled carbon nanotubes (SWNT) is based on the fluorescent properties of SWNT and the ability of NO to quench the fluorescence signal. Alterations of the signal can be utilized to detect a small molecule in vivo that has not previously been possible by other assay techniques. The protocols described here explain the techniques used to prepare NO-detecting SWNTs and to administer them to mice by both intravenous and subcutaneous routes. These techniques can also be utilized with other SWNT sensors as well as non-SWNT sensors.

Keywords

in vivo; single walled carbon nanotubes; nitric oxide; sensor; intravenous; subcutaneous; hydrogel

INTRODUCTION

We describe here a procedure for the in vivo detection of NO through the use of SWNT. Detection of this important reactive nitrogen species (RNS) is accomplished through the quenching of the SWNT’s inherent fluorescence. This procedure utilizes a SWNT wrapped with a specific DNA sequence, creating an NO sensor (Kim et al., 2009; Zhang et al., 2011) but can readily be altered to create sensors for other analytes by changing the SWNT wrapping (Heller et al., 2009) and also can be utilized with many variations of nanoparticles. The basic principle is to create a biocompatible sensor that allows relatively non-invasive delivery and various time courses for signal detection: long-term detection through hydrogel implantation and short-term detection through intravenous injection.

SWNT have a number of advantages for use as biological sensors. Key properties are their photostability (Wray et al., 1988), inherent fluorescence and failure to photobleach, allowing for long term (multiple month) sample collection (Iverson et al., 2013). Since mice are typically kept under a constant 12 hr light/dark cycle, use of a fluorescent tag that

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photobleaches when exposed to light would be difficult or impossible to use for long term \textit{in vivo} studies. Another benefit of SWNT sensors is that they fluoresce in the near-infrared region, an area where interference by blood and water is minimal (Wray et al., 1988). The specific SWNT described here are unique because they detect nitric oxide (NO), a small biological signaling molecule that has, until now, been measurable only through secondary means, such as nitrate and nitrite concentrations in body fluids and tissue samples. No previous \textit{in vivo} NO measurement technique has been applicable over this long time scale.

\section*{STRATEGIC PLANNING}

Before placing an \textit{in vivo} sensor there are many factors that must be considered. The first and most important issue to consider is where the analyte of interest is located within the body. For example, if the analyte of interest is only found within the lungs then a subcutaneous hydrogel obviously would not be an efficient delivery technique, whereas an intravenous injection could provide the information desired. Planning the delivery method to fit the required data is essential. Another important parameter to consider is which species and strain of experimental animal will be used. In this paper we describe a mouse model but other animal species could readily be used with these sensors and these techniques. An important factor to keep in mind is that the excitation and emission light must penetrate the tissue for a successful fluorescence reading of the sensor; thus, use in an animal with pigmented skin would be difficult.

\textit{NOTE}: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

\section*{BASIC PROTOCOL 1: FABRICATION OF NITRIC OXIDE SENSORS THROUGH DNA WRAPPING OF SINGLE WALLED CARBON NANOTUBES}

The design of a carbon nanotube probe for a specific analyte is a lengthy process of trial and error, but once a probe has been proven to react specifically with one analyte it can be synthesized and used repeatedly without the extensive characterization that is initially necessary. For the detection of NO, an (AAAT)$_7$ wrapped single wall carbon nanotube has proven successful. The following procedure, shown diagrammatically in Figure 1, is very versatile and can be utilized to wrap any DNA sequence around single wall carbon nanotubes; however, the characterization steps to ensure specificity are not detailed here (see Iverson et al., 2013) and would need to be performed on other DNA sequences to determine their specificity.

In this procedure solid carbon nanotubes are used. Since all of the health implications of exposure to carbon nanotubes are not known, it is recommended that a nanoenclosure be utilized for any manipulation using them; once they are in suspension they do not appear to aerosolize in the manner that is observed in the solid state.

In this procedure, tip sonication is used to mix the carbon nanotube and DNA solution, a procedure that heats the solution to a sufficiently high temperature to sterilize it.
Nonetheless, the use of sterile materials is recommended throughout. It should also be noted that the tip sonication process can lead to melting of the microcentrifuge tube if it comes into direct contact with the tip, so care must be taken when aligning the instrument.

Towards the end of the process two centrifugation steps are performed to remove any non-wrapped SWNT. If the wrapping procedure is unsuccessful, most of the sample will form a pellet during this step. If the carbon nanotubes come out of suspension, the sample loses its dark color, and the remaining solute should be discarded and the procedure repeated. If this problem persists, it is possible that the tip sonication process is not thoroughly mixing the sample and a longer time or higher power should be evaluated.

**Materials List**

- SWNT (SouthWest NanoTechnologies SG65i, tube diameter 0.77 +/− 0.02 nm, high aspect ratio of > 1,000, carbon content of > 95% by weight, > 40% (6,5) chirality SWNT and > 95% of SWNT are semiconducting)
- 5′ thiol-modified d(AAAT)₇ (Integrated DNA Technologies)
- Sterile normal saline (0.90% w/v NaCl in water)
- Static gun
- Nanoenclosure (XPert Nano Enclosure by Labconco)
- Ultrasonicator/Tip sonicator (Qsonica 1/8″ microtip probe)
- Bath sonicator
- UV/Vis

**Wrapping carbon nanotubes with DNA**

1. Dissolve the DNA sample by adding 200 μL sterile saline to 2 mg of DNA; this can be done in advance and aliquots (1 mL aliquots are convenient) can be stored for up to 6 months at −20 °C until needed. If using a stored, frozen sample remove aliquot from −20 °C and allow it to thaw at room temperature.

2. Weigh out 1 mg of carbon nanotubes in a nanoenclosure and place it into a microcentrifuge tube. Use a static gun on both the stock container of carbon nanotubes and the microcentrifuge tube to prevent adhesion of the solid carbon nanotubes to the plastic walls of the containers.

3. Add 800 μL of saline to the carbon nanotubes while the container is still in the nanoenclosure. Mix the solution well until all/most of the SWNT is in solution (monitor visually).

4. In a chemical hood, add 200 μL of DNA solution to the SWNT solution. To mix the solution, place the microcentrifuge tube in a bath sonicator for 10 minutes followed by two 20-minute cycles with a tip sonicator set at 4 W. For tip sonication it is important to keep the sample cool, so an ice bath surrounding the microcentrifuge tube is essential. Replace melted ice after the first 20 minutes and clamp the tube in place before starting each of the 20-minute runs. The extreme
heat of the tip sonication process is sufficient to sterilize the solution and the sample should be handled under sterile conditions from this point onward.

5. After thorough mixing with the sonicator, transfer the microcentrifuge tube to a centrifuge and spin at 16,100 rcf (or 16,100 g) for 90 minutes. After 90 minutes transfer the top 80% of the sample to a sterile microcentrifuge tube and discard the pellet. To maintain sterility, this should be done in a biosafety cabinet. Centrifuge the sample again for 90 minutes at 16,100 rcf, transferring the top 80% of the sample to a sterile microcentrifuge tube, again in a biosafety cabinet, and discard the pellet.

6. The final step is to determine the concentration of the SWNT solution by measuring absorbance at 632 nm, dividing by the extinction coefficient (0.036 for this example) and multiplying by the dilution factor. This relationship expresses SWNT concentration in mg L\(^{-1}\) in the solution.

BASIC PROTOCOL 2: HYDROGEL ENCAPSULATION, DELIVERY AND DETECTION OF DNA WRAPPED SINGLE WALLED CARBON NANOTUBES

Carbon nanotube sensor synthesized as described above can be encapsulated within a biocompatible gel for administration to the animal. Encapsulation within a gel promotes stabilization and long-term measurement capabilities. Subcutaneous delivery of a gel to an animal involves a small incision, requiring a sterile environment, sterile instruments and recovery time. Mice are relatively resistant to superficial infections, and the procedure should be done under conditions minimizing pain, as prescribed by AALAC approved methods. After the hydrogel has been placed the animal can be returned to its cage without food or water restrictions and imaging of the implant can begin as soon as the incision has healed. Embedding the carbon nanotube sensors within the alginate hydrogel creates a biocompatible, stable, consistent sensor that can be assayed as frequently as required without trauma to the animal. When this method was used for subcutaneous placement in mice, the sensors were shown to remain intact and to retain their fluorescence for over 300 days.

The following protocol describes procedures for encapsulation, delivery and detection of an NO carbon nanotube sensor, but can be applied to any nanoparticle of appropriate size, hydrophilicity and charge; see Figure 2 for an overview of the encapsulation process. After extensive characterization (Iverson et al., 2014) we found that increasing the concentration of the carbon nanotube in the hydrogel does not always increase the fluorescence signal; a concentration of 10 mg L\(^{-1}\) provides the maximal fluorescence and is therefore the concentration used.

Materials

- PRONOVA SLM 20 alginate (NovaMatrix)
- Sterile SWNT solution (30 mg L\(^{-1}\))
- Sterile normal saline (0.90% w/v NaCl in water)
Dialysis microcentrifuge tube (Slide-A-Lyzer MINI Dialysis Devices, 2K MWCO)

Sterile BaCl\(_2\) (0.1 M in water)

Sterile surgical instruments (forceps, surgical scissors, 4-0 – 5-0 polypropylene or nylon sutures, sterile drape, needle driver, betadine scrub, 70% isopropyl alcohol)

Razor

Depilatory agent (Nair bikini cream sensitive formula)

Imaging instrument (Maestro CRi™)

Isoflurane and isoflurane delivery instrument

**Prepare alginate and carbon nanotube gels**

1. Dissolve 250 mg alginate in 8.3 mL sterile saline to create a 3% (w/v) solution. Use a sterile syringe to add the liquid to the alginate container to maintain sterility. Place solution on shaker until all solid has dissolved (monitored visually, usually 24–72 hours).

2. Aliquot 666.7 μL of 3% alginate solution, using a sterile syringe, and 333.3 μL of sterile SWNT solution into a sterile microcentrifuge tube. Mixture will be viscous and will need extensive mixing with a mini-vortex and pipetting up and down.

3. Add 200 μL well mixed sample to the top portion of the dialysis microcentrifuge tube. Add BaCl\(_2\) to the bottom of the dialysis microcentrifuge tube setup and place the sample containing portion on top. Make sure that the BaCl\(_2\) solution is touching the dialysis membrane. Close the lid and leave gel at room temperature overnight.

4. After hydrogel has solidified remove it from the dialysis microcentrifuge tube and place it in excess sterile saline. Wash the gel at least three times with sterile saline and keep it in a saline bath until it is placed into the animal (if gel dries out its properties will be permanently altered).

**Surgically place hydrogel subcutaneously**

5. Prepare mouse for surgical placement of the hydrogel by anesthetizing the animal with 1–3% isoflurane gas, shaving the area around the proposed incision site and then applying a depilatory agent. The depilatory agent may need to be applied multiple times to remove all of the hair, but should not be left on the animal for extended periods of time; to prevent irritation, perform multiple applications. Clean the incision area with betadine and ethanol and cover the mouse with a sterile drape with a small hole in the center to allow access to the surgical field.

6. Using sterile forceps and scissors create a small incision in the skin of the mouse, then place the scissors in the closed position into the incision between the skin and muscle layers. Open the scissors to perform a blunt dissection of the...
skin away from the muscular area. This portion of the procedure should not result in cutting of any tissue or bleeding.

7 Place the alginate hydrogel in the pocket created beneath the skin and close the wound with two to three stitches. Due to the rigidity of the alginate gel there will be a visible lump under the skin, but when the incision has healed the mouse should not be bothered by this implant.

8 Allow the mouse to awaken under a heat lamp to prevent chilling, and observe the animal to insure that the implantation has been successful.

Long term imaging of carbon nanotubes

9 To image the animal, first anesthetize with 1–3% isoflurane gas and if needed apply depilatory cream to the area of interest (wait until the incision has fully healed before applying depilatory cream).

10 Place animal in the imaging box, making sure that it continues to receive anesthesia for the entire imaging procedure. For reproducible long-term images it is important to place the animal in the same position and to flatten the area of interest as much as possible.

11 After image acquisition remove the anesthesia and allow animal to awaken, usually within a few minutes. For a short time afterwards place it under a heating lamp to ensure recovery.

BASIC PROTOCOL 3: PEGYLATION, INTRAVENOUS DELIVERY AND DETECTION OF DNA WRAPPED SINGLE WALLED CARBON NANOTUBES

If a short term in vivo sensor is required, it is possible to avoid the surgical steps described above and administer the SWNT sensor intravenously instead. This option involves less trauma to the animal, takes less time to perform and requires fewer instruments. Unfortunately, DNA-wrapped SWNT prepared as above comes out of suspension when injected intravenously and causes blockage when injected into the mouse tail vein. This complication is readily circumvented by PEGylation of the SWNT. The following procedure can be used to PEGylate and inject many different nanoparticles, but only detection of nitric oxide in mouse liver is described here. Extensive evaluation has revealed that high concentrations of SWNT accumulate within the liver 30 minutes after SWNT injection, so that is the time frame used in our experiments. We also found that while an extremely high concentration of SWNT can cause vessel occlusion, a 50 mg L\(^{-1}\) solution consistently provided tail vein clearance and was used in our experiments.

Materials List

SWNT (SouthWest NanoTechnologies SG65i, tube diameter 0.77 +/- 0.02 nm, high aspect ratio of > 1,000, carbon content of > 95% by weight, > 40% (6,5) chirality SWNT and > 95% of SWNT are semiconducting)

TCEP stock solution (0.5 M)
5’ thiol-modified d(AAAT)\textsubscript{7} (Integrated DNA Technologies)
Nanopure water
Methoxy PEG (5 kDa) maleimide
PBS (phosphate buffered saline)
Depilatory agent (Nair bikini cream sensitive formula)
Imaging instrument (Maestro CR\textsuperscript{i}™)
Isoflurane and isoflurane delivery instrument

**Prepare PEGylated SWNT sensor**

1. Dissolve the DNA sample by adding 10 μL sterile saline to 1 mg of DNA, this can be done in advance and aliquots (1 mL aliquots are convenient) can be stored for up to 6 months at −20 °C until needed. If using a stored, frozen sample then remove aliquot from −20 °C and allow it to thaw at room temperature.

2. Aliquot 10 μL of TCEP stock solution, 4.49 μL of 5’ thiol-modified d(AAAT)\textsubscript{7} and 485.5 μL of nanopure water into a small beaker. Stir the solution for one hour to break the disulfide bonds on the DNA strands.

3. Dissolve Methoxy PEG (5 kDa) maleimide at a concentration of 100 mg mL\textsuperscript{−1} in PBS. Combine 600 μL of DNA solution and 600 μL of PEG solution in a microcentrifuge tube and mix for at least 20 minutes (can leave overnight if desired). Then wrap SWNT with PEGylated DNA using the procedure described in Basic Protocol 1.

**Inject and detect PEGylated SWNT sensor**

4. Prepare mouse for procedure by anesthetizing with up to 3% isoflurane gas, shaving the region of interest (in this case the abdomen, for visualization of the liver) and applying depilatory cream. It is possible that the depilatory agent will need to be applied multiple times to remove all of the hair. As noted earlier, the depilatory cream left on the animal for extended periods of time is a skin irritant. Instead, multiple applications of the cream should be used.

5. Dilute PEGylated SWNT sensor to 50 mg L\textsuperscript{−1} with sterile saline. Using a 0.3 cc 29 gauge 0.5 inch insulin syringe, inject 200 μL of PEGylated SWNT solution in either the right or left tail vein. Due to the dark color of the SWNT solution it is visible as it travels up the tail vein.

6. After 30 minutes, with the mouse anesthetized the entire time to attain consistent results between animals, image the mouse’s abdomen (liver) to determine SWNT presence and/or quenching. The Maestro CR\textsuperscript{i}™ instrument was used for initial studies, but other image acquisition instruments may be used. Optimization of laser power to acquire the greatest signal without burning the animal is necessary. Optimization of exposure time will also be needed. For our...
studies a 561 nm laser was used, emission wavelength of 950–1050 nm in 10 nm steps was gathered for 20 seconds at each wavelength. The Maestro CrI™ instrument contains a data processing program or the Matlab code for our data analysis method can be downloaded (Iverson et al., 2013).

After image acquisition remove the anesthesia from the animal and place it under a heating lamp to help with the discomfort from the anesthesia and depilatory agent.

**REAGENTS AND SOLUTIONS**

**TCEP stock solution (0.5M)**

Mix 0.25 g TCEP (Tris(2-carboxyethyl)phosphine hydrochloride from Sigma Aldrich) and 2 mL sterile PBS in clean, sterile vial

**COMMENTARY**

**Background Information**

Single walled carbon nanotubes are ideal in vivo sensors due to their photostability and emission wavelength in the tissue transparency window, where interference from blood and water is minimal (Wray et al., 1988). The ability of SWNT to detect single molecules (Heller et al., 2009), specifically NO (Kim et al., 2009; Zhang et al., 2011), makes SWNT the only long term NO in vivo sensors (Iverson et al., 2013) available to researchers.

**Critical Parameters**

An important aspect to consider when planning in vivo carbon nanotube studies is the fluorescence intensity of the carbon nanotubes. It is important to excite the sample at the optimal wavelength (E₁₁ or E₂₂) in order to produce as large a baseline signal as possible. This will minimize the signal-to-noise ratio and ensure accuracy of instrument readings.

Another important factor for in vivo studies is the strain of animal to be used. Since the excitation and emission signal needs to be transmitted through the skin it is important to use albino or hairless mouse strains.

Finally, it is important to consider what fluorescence detection instrument will be used for the in vivo measurements. If the excitation signal power is too high it can burn the skin of the animal, causing pain and skewing data. The instrument must allow for the animal to be restrained or sedated, depending on the length of the signal detection period, and the instrument should permit repeat of studies to ensure reproducibility of results.

**Troubleshooting**

**Carbon nanotubes precipitate during wrapping**—It is possible that the carbon nanotubes will not be properly wrapped by the DNA, either with or without PEG, and they will therefore not stay suspended in solution (since carbon nanotubes are hydrophobic). If this happens, it is possible that the sonication process was not long enough or at a high enough power. To solve the problem, first try to increase the time of the tip sonication step...
(with a new batch of SWNT and DNA) and if that is not successful a higher power for the tip sonicator should be tried.

**In vivo biocompatibility or animal distress**—With the second protocol, the implantation of the hydrogel, sample and instrument sterilization is essential. Cleanliness of the sample preparation and surgical area is of critical importance; ensure that instruments are clean and sterile and that sterile technique is used to prepare the sample and place the gel. Care needs to be taken that an overdose of anesthetic is not administered to the animal. For hydrogel placement, it is essential that the animal be completely anesthetized and does not react to pain (the toe pinch test) but for shaving, tail vein injection and imaging procedures the animal can be less heavily sedated. Carefully observe the animal’s breathing pattern during the entire time that it is under isoflurane anesthesia, and if breathing slows or stops the gas should be immediately removed and the animal allowed to recover in fresh air. If the animal stops breathing and its heart stops, resuscitation can be attempted by chest compression and relaxation.

An additional point of concern for the biological compatibility of the hydrogel is the BaCl2 cross-linking agent, which is highly toxic and must be fully rinsed from the gel before implantation.

If injection of PEGylated solution into the mouse’s tail vein results in total vessel blockage the animal experiences pain after the removal of the anesthesia and may have to be euthanized. Vessel blockage can occur for multiple reasons, the most likely being that PEGylation of the DNA was not successful or that the solution injected contained aggregates of SWNT. PEGylation of the DNA sample can be ensured by gel electrophoresis before wrapping the SWNT. To avoid the injection of SWNT aggregates, an extra centrifugation step (as described at the end of the wrapping protocol) can be performed.

**Insufficient or irregular signal transmission**—If there is difficulty detecting a fluorescence signal from the animal once the sensors have been placed, two main possibilities should be assessed; first, the power of the excitation signal, and second the presence of inflammation at the site of the sensor. Checking the excitation signal at the site of the sample can be done relatively easily by using a tissue phantom; in the case of muscle tissue chicken breast works well. Place a section of tissue phantom in the imaging instrument with a carbon nanotube sensor at the same concentration and depth as that in the mouse, then test for signal detection. If the signal is still not acquired by the instrument, the carbon nanotube sensor should be placed at a shallower depth or at a higher concentration in the phantom tissue. Continue this until the signal detection has been optimized and use these parameters in the animal study.

The second aspect to check when a fluorescence signal is not detected in the animal is the possibility of inflammation. Examine the area for a reaction to the sensor or depilatory agent once or twice a day. If the area is inflamed stop applying the depilatory agent until the tissue looks healthy and try imaging the sensor again. Using the depilatory agent for a shorter time may allow for the removal of the hair without inflammation of the surrounding tissue. Any inflammation induced by the placement of the sensor should decrease spontaneously and
disappear within a week. If sensor-based inflammation persists it is likely that the sensor was not sterile when placed and stricter sterility guidelines should be followed for future samples.

**Anticipated Results**

The first protocol will result in the creation of a carbon nanotube sensor, see figure 3, that can be immediately used for *in vitro* studies or utilized for the *in vivo* studies described in the second protocol. The second protocol will also produce a hydrogel that can be used *in vitro* if desired as well as the *in vivo* long-term studies described. This data can be used to follow NO production or disease progression through the lifetime of the animal or over desired time intervals. The third protocol provides real-time assessment of NO production without the necessity for surgical manipulations.

**Time Considerations**

The SWNT wrapping procedure either with the PEGylated or non-PEGylated DNA can easily be completed in one day, but if necessary the sample can sit overnight after tip sonication and before the centrifugation step. The creation of the alginate gel takes two days, but again the gel can be made ahead of time and allowed to sit in the BaCl$_2$ bath until it is rinsed and prepared for placement. To place a gel or perform a tail vein injection in one mouse takes an hour or less, but as with any animal work additional time may be required.

**Acknowledgments**

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**References**


Figure 1. Schematic of process for SWNT wrapping by DNA

Single walled carbon nanotubes are hydrophobic and wrapping with DNA or other polymers must be performed to create a SWNT suspension in saline. By simply mixing the SWNT and DNA with vigorous sonication steps the SWNT becomes wrapped by the DNA, the centrifugation steps remove the non-wrapped SWNT, creating the final solution.
Once the SWNT has been wrapped with DNA it can be encapsulated within an alginate hydrogel and placed subcutaneously in a mouse. The slow and even addition of the cross-linking agent, in this case BaCl\textsubscript{2}, is an essential step in the creation of a uniform gel, this can be accomplished in multiple ways but the easiest procedure is depicted here.

Figure 2. Schematic of SWNT encapsulation by the alginate hydrogel

Once the SWNT has been wrapped with DNA it can be encapsulated within an alginate hydrogel and placed subcutaneously in a mouse. The slow and even addition of the cross-linking agent, in this case BaCl\textsubscript{2}, is an essential step in the creation of a uniform gel, this can be accomplished in multiple ways but the easiest procedure is depicted here.
Figure 3. Schematic of SWNT wrapping and NO detection by the alginate hydrogel encapsulated SWNT and PEGylated SWNT
a) Schematic of the DNA wrapped SWNT, both with and without an associated PEG chain.
b) NO detection by subcutaneously implanted alginate encapsulated SWNT is shown by the quenching of the fluorescence signal (pseudo colored in green) and then the return of the signal 4 days later after the implantation associated inflammation has subsided. c) NO detection of healthy ‘control mice’ or inflamed ‘RcsX treated’ mice by PEGylated SWNT 30 min after tail vein injection, in situ images were taken while the mouse was under anesthesia, the mouse was then immediately sacrificed and its liver was excised. The fluorescence in the excised liver samples shows that the SWNT was present in the livers of both mice while the in situ images show the presence or lack of NO by the quenching or lack of quenching respectively.