Horseradish peroxidase-driven fluorescent labeling of nanotubes with quantum dots

Vladimir V. Didenko1,2 and David S. Baskin2,3

1Baylor College of Medicine, Houston, 2Michael E. DeBakey Veterans Affairs Medical Center, Houston, and 3Methodist Neurological Institute, Houston, TX, USA

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We describe the first enzyme-driven technique for fluorescent labeling of single-walled carbon nanotubes (SWNTs). The labeling was performed via enzymatic biotinylation of nanotubes in the tyramide-horseradish peroxidase (HRP) reaction. Both direct and indirect fluorescent labeling of SWNTs was achieved using either biotinyl tyramide or fluorescently tagged tyramides. Biotinylated SWNTs later reacted with streptavidin-conjugated fluorophores. Linking semiconductor nanocrystals, quantum dots (Q-dots), to the surface of nanotubes resulted in their fluorescent visualization, whereas conventional fluorophores bound to SWNTs directly or through biotin-streptavidin linkage, were completely quenched. Enzymatic biotinylation permits fluorescent visualization of carbon nanotubes, which could be useful for a number of biomedical applications. In addition, other organic molecules such as proteins, antibodies, or DNA can be conjugated to biotinylated SWNTs using this approach.

INTRODUCTION

Biochemical approaches for coupling different molecules to the surface of single-walled carbon nanotubes (SWNTs) would expand the circle of researchers working on the life science applications of these nanostructures and would allow nanotechnology experimentation in biomedical research laboratories. Of particular importance is fluorescent labeling of SWNTs. Fluorescent SWNTs are essential for the development of nanophotonics, quantum computing, and probes for biomedical research. In spite of their nanometer-scale diameters, fluorescent SWNTs can be directly observed by optical microscopes, which will enable the construction of nanosize devices via optically controlled assembly. SWNTs can also make unique composite fluorophores because, due to their length, they permit the attachment of multiple fluorescent dyes to a single nanotube.

Two major obstacles need to be surmounted for successful enzyme-driven preparation of fluorescent nanotubes. First, enzymatic approaches to label SWNTs have to be developed. Secondly, fluorescence quenching of the nanotube-coupled fluorophores should be prevented, as it is known that the fluorophores directly attached to SWNTs are quenched via energy transfer (1,2).

Here we describe the first procedure for enzymatic fluorescent labeling of SWNTs. The procedure is based on peroxidase-driven biotinylation of SWNTs with subsequent reaction with streptavidin-conjugated fluorophores. The fluorescence quenching property of SWNTs is bypassed by using colloidal semiconductor nanocrystals, quantum dots (Q-dots), instead of conventional fluorophores.

MATERIALS AND METHODS

Fluorescence Labeling of SWNTs

Biotinylation of SWNTs or direct ligation of fluorescent tyramides to the surface of nanotubes was performed using purified and filtered preparation of SWNTs stabilized in 1% sodium dodecyl sulfate (SDS) (HiPco tubes received from Dr. R.E. Smalley, Rice University, Houston, TX, USA) containing predominantly individual SWNTs and SWNT ropes (3). The reaction was initiated in 50 mM Tris-HCl, pH 8.0, containing 3 μg/mL SWNTs; 200 ng/μL horseradish peroxidase (HRP), 0.01% hydrogen peroxide, 0.2% SDS, and a 1:50 dilution of biotinyl tyramide reagent (from the TSA™ Biotin System; Perkin-Elmer Life Sciences, Boston, MA, USA) or a 1:50 dilution of Alexa-Fluor®-tagged tyramides (Molecular Probes, Eugene, OR, USA) Alexa Fluor-594 (red fluorescence), Alexa Fluor-488 (green fluorescence), and Alexa Fluor-350 (blue fluorescence) for 1 h at 37°C. Although SDS concentration in the labeling mixture fell below 1%, we did not see any precipitation of the functionalized nanotubes, even after 1-month storage at 4°C. After the reaction, the biotin-tagged SWNTs were combined with streptavidin-fluorescein isothiocyanate (FITC) or streptavidin-Q-dots by mixing at a 1:1 ratio (v/v).

Q-dots (Adirondack Green, COOH-terminated semiconductor nanocrystals) were purchased (Evident Technologies Troy, NY, USA) and then conjugated to streptavidin in our laboratory using the EviTags™ Protein Labeling Kit (Evident Technologies) exactly as recommended by the manufacturer.

Gel Electrophoresis of Labeled SWNTs

SWNTs labeled with fluorescent dyes were separated from unreacted conjugates using 0.3% agarose gel electrophoresis. Small aliquots of labeling mixture containing various preparations of fluorescently labeled SWNTs were loaded in 15% Ficoll® on a 0.3% agarose gel in 1× Tris-borate-EDTA (TBE), and run for 30 min to 1 h at 100 V. They were observed using an FBTIV-88 transilluminator (Fisher Scientific, Hampton, NH, USA). Above the 312 nm emission peak, transilluminator lamps provide sufficient spectral output for the visualization of fluorophores excited in green and red parts of spectrum (see Figures 2 and 3), which is routinely used for the evaluation of fluorescent molecular probes (probes.invitrogen.com/handbook). Gel images were captured on a PC1015 Canon digital photo camera (Canon U.S.A., Lake Success, NY, USA) equipped with a 4-megapixel charge-coupled device (CCD) and processed in Adobe® Photoshop® 7.0.
Imaging of Labeled SWNTs

The fluorescence of labeled SWNTs deposited on a glass slide was assessed, and images were captured using an Olympus IX70 fluorescent microscope (Leeds Instruments, Irving, TX, USA) and a MicroMax digital video camera (Princeton Instruments, Trenton, NJ, USA). A bandpass filter set (Chroma Technology, Rockingham, VT, USA) was used (FITC and Alexa Fluor-488 excitation D490/40, emission 520/10; Alexa Fluor-594 excitation D560/40, emission 620/30; Alexa Fluor-350 excitation D360/40, emission 460/20). Composite images were created in MetaMorph® (Molecular Devices, Sunnyvale, CA, USA). Adirondack Green Q-dots can be excited at a broad range of wavelengths, so Q-dot-labeled SWNTs deposited on a glass slide were imaged using FITC-Alexa Fluor-488 excitation and emission filters.

Transmission electron microscopy (TEM) of SWNTs-Q-dot complexes was performed on a Hitachi H-7500 Transmission Electron Microscope equipped with Gatan 2K × 2K CCD (Hitachi, Tokyo, Japan) using Formvar-coated copper grids (Electron Microscopy Services, Hatfield, PA, USA).

RESULTS AND DISCUSSION

The functionalization of SWNTs (biotinylation or direct attachment of fluorophores to their surface) was accomplished by a new application of the classical tyramide-HRP reaction (4). In the reaction, HRP activates tyramide-biotin (or tyramide-fluorophore) conjugates, generating active tyramide radicals. The addition of SWNTs to this reaction mixture results in their biotinylation or direct linkage of fluorophores via attachment of tagged tyramides. A proposed mechanism is presented in Figure 1, based on Reference 4.

We found that solutions containing SWNTs with attached fluorophores could be separated from unreacted conjugates using low-concentration (0.3%) agarose gel electrophoresis (Figures 2 and 3). During electrophoresis, the fluorescently labeled SWNTs moved toward the positive electrode, but were trapped at the anode side of loading wells because they could not penetrate deeply into the gel (e.g., Figure 2, lane 1). The negative charge of SWNTs could be attributed to the anionic SDS molecules still remaining on their surface as well as to the attached anionic tyramide conjugates. In all cases, conjugates that were not attached to SWNTs moved toward the anode (Figures 2 and 3). Therefore, the layer of agarose on the anode side of loading wells because they could not penetrate deeply into the gel (e.g., Figure 2, lane 1). The negative charge of SWNTs could be attributed to the anionic SDS molecules still remaining on their surface as well as to the attached anionic tyramide conjugates. In all cases, conjugates that were not attached to SWNTs moved toward the anode (Figures 2 and 3). Therefore, the layer of agarose on the anode side of loading wells served as a molecular sieve, retaining SWNTs but allowing unreacted tyramides and streptavidin conjugates to pass through. This convenient property allowed for the analysis of various labeled SWNT preparations because any signal in the well would be due exclusively to fluorophores attached to SWNTs.

Using 0.3% gel electrophoresis, we found that streptavidin-FITC conjugates and Alexa Fluor tyramides were completely quenched upon attachment to nanotubes (Figure 2, lane 3 and Figure 3, lanes 2–4). Fluorescent signal from SWNTs was observed only when labeled with streptavidin Q-dots (Lane 1 of Figures 2 and 3). Verification of nanotube labeling was done by TEM. TEM images of SWNT-Q-dot complexes showed individual nanotubes covered with Q-dots (Figure 4B). Dense clusters of Q-dots were positioned on the nanotubes in a “beads-on-a-string” pattern (depicted in Figure 4A), so that the sides of individual Q-dots were in direct contact.

The size of the Q-dots is directly related to their fluorescence spectrum. The TEM-measured “hard-sphere” diameters of green-fluorescing Q-dots in our experiments were approximately 6 nm, which is consistent with reported sizes of 3 nm (blue fluorescence) and 10 nm (red fluorescence) (www.qdots.com/live/render/content.asp?id=83). A “soft-sphere” diameter associated with the organic polymer coating was seen as a darker circle around the core and was approximately twice as big with an additional nanometer added due to the streptavidin covering. Streptavidin molecules covering Q-dots were also observable in the higher power TEM images (Figure 4B).

Figure 1. Enzymatic functionalization of SWNTs by biotinyl tyramide radicals created by horseradish peroxidase. SWNTs, single-walled carbon nanotubes.
Fluorescently labeled SWNTs were observed under an optical microscope. A suspension of SWNTs labeled with Q-dots was deposited onto a glass slide by 10 s centrifugation at 100× g and examined using an Olympus IX70 fluorescent microscope under 490 nm excitation and 520 nm emission light, using an oil immersion objective. Strongly fluorescent SWNT ropes (Figure 5, A and D) and individual SWNTs (Figure 6A) were seen. SWNTs labeled with FITC and Alexa Fluor-488 were not observable under these conditions. Similarly, Alexa Fluor-350- and Alexa Fluor-594-labeled SWNTs were not fluorescent when their appropriate excitation/emission filters were used (data not shown).

The combined width of fluorescing individual SWNT-Q-dot complexes measured by TEM was in a 15–30 nm range, which is below the optical detection limit. Thus, individual Q-dot-labeled SWNTs were not visible by light microscopy (Figure 6B). However, fluorescing objects with sizes below the optical resolution limit and potentially a single fluorescent molecule can be observed because each fluorophore can be thought of as a light source. Therefore, when we examined Q-dot-labeled SWNTs deposited on the glass slide, we detected multiple fluorescent nanotubes visualized as strings of brightly fluorescent Q-dots (Figures 5 and 6).

Our results indicate that the chemical fluorophores Alexa Fluor-594 (red fluorescence), Alexa Fluor-488 (green fluorescence), Alexa Fluor-350 (blue fluorescence), and FITC are quenched by SWNTs when attached to their surface. This effect was observed both for tyramide-linked fluorophores (Alexa Fluor-488, Alexa Fluor-350, and FITC) and directly attached tyramide fluorophores. The combined results suggest that the quenching effect is not limited to Q-dots but also applies to other fluorophores when they are attached to SWNTs. This finding has significant implications for the use of SWNTs in biological applications, as it may limit the sensitivity and selectivity of fluorescence-based detection methods.

**Figure 2.** Gel electrophoresis of biotinylated SWNTs conjugated with streptavidin-Q-dots and streptavidin-FITC. (Lane 1) Biotinylated SWNTs reacted with Q-dots conjugates with streptavidin; (lane 2) Q-dot conjugates with streptavidin; (lane 3) biotinylated SWNTs reacted with FITC conjugates with streptavidin; (lane 4) FITC conjugates with streptavidin. Nanotubes do not enter the gel, and a band of fluorescent nanotubes labeled with Q-dots is formed when they become trapped in the wall of the loading well (lane 1). By contrast, Q-dot- and FITC-streptavidin conjugates that are not attached to SWNTs enter the gel (all lanes). FITC-labeled SWNTs do not fluoresce (lane 3). SWNTs, single-walled carbon nanotubes; FITC, fluorescein isothiocyanate; Q-dots, quantum dots.

**Figure 3.** Gel electrophoresis of SWNTs with directly attached Alexa Fluor tyramides and biotinylated SWNTs conjugated with streptavidin-Q-dots. (Lane 1) Biotinylated SWNTs reacted with Q-dot-streptavidin conjugates; (lane 2) SWNTs reacted with Alexa Fluor-350 tyramide (blue fluorescence); (lane 3) SWNTs reacted with Alexa Fluor-594 tyramide (red fluorescence); and (lane 4) SWNTs reacted with Alexa Fluor-488 tyramide (green fluorescence). Note the absence of fluorescence in the wells in cases of direct tyramide labeling (lanes 2–4); by contrast, Q-dot-labeled SWNTs in lane 1 are visible as a fluorescent band in the wall of the loading well. Unreacted Q-dots as well as blue, red, and green tyramide monomers and dimers move toward the anode and are visible as smears in the gel. SWNTs, single-walled carbon nanotubes; Q-dots, quantum dots.

**Figure 4.** Visualization of biotinylated carbon nanotube with attached Q-dot-streptavidin conjugates. (A) Illustration of streptavidin-conjugated Q-dots attached to a biotinylated SWNT. The significant difference in size between the Q-dots and the nanotubes prevents fluorescence quenching. (B) Electron micrograph of an individual biotinylated nanotube (diameter is approximately 0.7 nm) with attached Q-dots (diameter is approximately 6 nm). Scale bar = 20 nm. Denser areas of labeling are separated by a short unlabeled stretch. Above are magnified TEM images of individual Q-dots. Note streptavidin molecules coating Q-dots. Scale bars = 10 nm. Q-dots, quantum dots; SWNTs, single-walled carbon nanotubes; TEM, transmission electron microscopy.
dyes) and for a biotin-streptavidin linkage (FITC). This correlates with the results of others showing that a variety of fluorescent molecules such as Alexa Fluor-488, BODIPY® FL, 3,3′-dipentyloxacarbocyanine iodide, 3,3′-ethyloxacarbocyanine iodide (5), pyrene (6), and naphthalimide chromophore (1) are efficiently quenched by SWNTs. This phenomenon was observed with fluorophores covalently and noncovalently bound to the nanotubes’ surface (1,5). However, in several cases, the noncovalent or indirect attachment of a fluorescent dye to SWNTs results in a less efficient deactivation of the photoexcited chromophore. For example, the incomplete quenching of

Figure 5. Q-dot-labeled SWNT ropes deposited on a glass slide. (A) Fluorescent microscopy (excitation, 490 nm; emission, 520 nm); (B) light microscopy; (C) combined light and fluorescent microscopy; and (D) magnified fluorescent images. Structures below the optical resolution limit can be observed. Note the clusters of fluorescence along SWNTs. Scale bars = 2.5 μm. Q-dots, quantum dots; SWNTs, single-walled carbon nanotubes.
Cy3 linked to SWNTs by noncovalent adsorption of Cy3-labeled DNA was recently described; this may have occurred because the fluorophore was positioned at the end of the 15-mer-long oligonucleotide string and did not have full contact with the SWNT surface. Other experiments demonstrated that when FITC is indirectly linked to nanotubes, its fluorescence is maintained. In this case, the dye did not have direct contact with the nanotube surface and was positioned at the end of a 25-mer oligonucleotide, which was subsequently hybridized to a complimentary oligonucleotide linked to SWNTs.

In another study, we have described the preservation of fluorescence of the same Alexa Fluor dyes as used in the present experiments when they were attached to a polymer string wrapped around individual nanotubes. The protection presumably occurred due to the insulation effect of the polymer separating SWNTs and fluorophores.

In this study, we observed quenching of chemical fluorophores but not Q-dots, although all were connected to SWNTs surface via the same tyramide linkers. This indicated that tyramide linkers are not responsible for the preservation of Q-dot fluorescence.

Diameters of Q-dots are at least an order of magnitude bigger than that of conventional fluorophores and also exceed SWNT diameters by about the same degree. This minimizes interactions between the nanotube surface and the outer parts of Q-dots, and could explain why the Q-dots’ fluorescence was not quenched, unlike that of the other fluorophores we tested.

Isolated unlabeled SWNTs can in principle be visualized by photoluminescence; however, this option is inconvenient in biological settings because of the low-signal intensity compared with fluorescent labeling. Indeed CdSe Q-dots, such as those used in our experiments, have a quantum yield of about $5 \times 10^{-1}$, which is 1000 times higher than the photoluminescence quantum yield of unlabeled SWNTs (approximately $5 \times 10^{-4}$).

Figure 6. Q-dot-labeled individual SWNT deposited on a glass slide. (A) Fluorescent microscopy (excitation, 490 nm; emission, 520 nm); (B) light microscopy; (C) combined light and fluorescent microscopy. Note that structures below optical resolution limit can be observed only on fluorescent but not in light microscopy images. Note the clusters of fluorescence along the SWNT. Scale bar = 2 μm. Q-dots, quantum dots; SWNTs, single-walled carbon nanotubes.

[Figure 6 caption: Q-dot-labeled individual SWNT deposited on a glass slide. (A) Fluorescent microscopy (excitation, 490 nm; emission, 520 nm); (B) light microscopy; (C) combined light and fluorescent microscopy. Note that structures below optical resolution limit can be observed only on fluorescent but not in light microscopy images. Note the clusters of fluorescence along the SWNT. Scale bar = 2 μm. Q-dots, quantum dots; SWNTs, single-walled carbon nanotubes.]
HRP-driven labeling of SWNTs is the first enzyme-based nanotube functionalization technique. Moreover, the utilization of Q-dots in the HRP reaction for the first time accomplishes fluorescent visualization of SWNTs using a purely biochemical approach. Nonbiochemical attachment of Q-dots to SWNTs has been previously described and performed via electrostatic aggregation of Q-dots on the surface of nanotubes (12). The aggregation occurred via interaction between zinc ions in the Q-dots shells and oxygen atoms in the sulfonate group of SDS molecules used to stabilize SWNTs in solution (12). Although the study showed that the reaction resulted in the generation of fluorescent adducts, the reaction is not applicable for biomedical research because it relies on SDS concentrations of approximately 1%, which are toxic to many biological organisms. Enzymatic modification and fluorescent labeling of nanotubes as described here does not rely on SDS and is suitable for life science applications. In addition, the strength of the Q-dots’ attachment to nanotubes is also significantly stronger compared with electrostatic interactions.

The highly luminous fluorescent probes based on carbon nanotubes carrying multiple Q-dots would make possible the labeling of individual molecular events and single protein or DNA molecules. Although the possible toxicity of cadmium-containing Q-dots should be considered in live-cell labeling, no toxic response was detected when these Q-dots were tested in vivo in mice and pigs (13). In addition, Q-dots based on indium gallium phosphide are nontoxic and can be used instead.

We conclude that enzyme-driven biotinylation of carbon nanotubes makes possible their fluorescent visualization by Q-dots. Moreover, the reaction is not limited to the Q-dot application and permits linking of a variety of biological molecules to nanotubes. Thus, biotinylated SWNTs can be used in a variety of applications with DNA and proteins. The procedure can be performed in a biological laboratory setting, bridging fields of biomedicine and nanomaterials.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES


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Address correspondence to Vladimir V. Didenko, 2002 Holcombe Blvd., Bldg. 109, Room 204, Houston, TX 77030, USA. e-mail: vdidenko@bcm.tmc.edu

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