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Characterization of Multienzyme-Antibody-Carbon Nanotube Bioconjugates for Immunosensors

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Abstract

Characterization studies of a multi-enzyme-antibody-carbon nanotube bioconjugate designed for the amplification of electrochemical immunosensing are described. Secondary antibodies for prostate specific antigen (PSA) were covalently linked to highly carboxylated multi-walled carbon nanotube (CNT) along with multiple horseradish peroxidase (HRP) enzyme labels. These bioconjugates provide ultra-sensitive amperometric detection of PSA on a single-wall carbon nanotube forest sandwich immunosensor platform. A single layer of HRP on the surface of the CNT was suggested by images from atomic force microscopy (AFM) and transmission electron microscopy (TEM). HRP on the bioconjugate surface was visualized by confocal microscopy using *in-situ* HRP-catalyzed polymerization yielding a fluorescent product, and HRP activity was estimated in a conventional assay. Binding of quantum-dot labeled PSA to antibodies on the bioconjugate was used for visualization by TEM. Combining TEM and enzyme activity results gave estimates of ~82 HRPs and 30 ± 15 secondary antibodies per 100 nm of antibody-HRP-CNT bioconjugate.

Keywords

Nanotube Bioconjugate; Protein Immunosensor; Atomic Force Microscopy; Electron Microscopy; Confocal Microscopy; Quantum Dots

1. INTRODUCTION

Accurate measurement of protein biomarkers in human serum and tissue is important for early disease detection and diagnosis, signaling pathway studies, and drug development.^{1,2} However, eventual development of rapid, point of care measurement of disease biomarker proteins using miniaturized arrays will require highly sensitive, versatile detection approaches. Novel nanomaterials conjugated with biomolecules for molecular recognition and multi-label sensing have been employed to provide ultra-sensitive protein detection that may prove to be useful for array applications.^{3–6} For example, multiple enzyme labels assembled using layer-by-layer (LBL) electrostatic assembly on multiwall carbon nanotubes (CNTs) have been used to provide detection of 5 aM for DNA and 67 aM for proteins.⁷

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We recently fabricated multi-label CNT bioconjugates for electrochemical immunosensors. CNTs were covalently conjugated to secondary antibodies (Ab₂) for prostate specific antigen (PSA) along with multiple horseradish peroxidase (HRP) labels.⁸ These Ab₂-CNT-HRP nanoparticles were used with single-wall carbon nanotube (SWNT) forest platforms⁹ in sandwich immunoassays for prostate cancer biomarker PSA in human serum and tissue lysates (Scheme 1). Capture antibodies (Ab₁) were attached to the SWNT forests to bind PSA in the sample. The Ab₂-CNT-HRP bioconjugates were then added to bind to the antigen on the sensor surface, and PSA was detected by the amperometric signal generated from enzyme-catalyzed electrochemical reduction of hydrogen peroxide by the HRP. This amplification procedure provided detection of PSA in undiluted calf serum at a detection limit of ~4 pg mL⁻¹ (100 aM) PSA.⁸ Quantitative detection of PSA in lysates of approximately 1000 prostate cancer cells was also demonstrated.

Characterization of nanotube bioconjugates used for multi-label detection (as in the above example) is important for the future progress of this approach. In particular, particle size, enzyme activity, and number of labels and antibodies per nanotube length are important parameters to optimize in order to fine tune sensitivity, detection limit, and linear range of the analytical method.

In this paper, we report on the characterization of Ab₂-CNT-HRP bioconjugates using atomic force (AFM) and transmission electron microscopy (TEM), amperometry, enzyme activity, and enzyme-catalyzed polymerization of a fluorescent product. Amperometry monitored the specific binding of the Ab₂-CNT-HRP bioconjugate with immobilized antigens on the sensor surface. TEM and AFM images confirmed the increased diameter of the CNT bioconjugates compared to bare CNTs. Raman Spectroscopy imaging confirmed that the tubular shapes observed by other microscopic methods were indeed shortened carbon nanotubes. Successful binding of HRP on the sidewall of functionalized CNT was demonstrated by *in-situ* HRP catalyzed polymerization and confocal fluorescent microscopy.

2. EXPERIMENTAL DETAILS

2.1. Chemicals and Materials

Horseradish peroxidase (HRP, MW = 47,000), lyophilized 99% bovine serum albumin (BSA), 4-Hydroxy-phenylacetic acid (HPA) and Tween-20 were purchased from Sigma/Aldrich. Tracer secondary anti-PSA antibody (Clone number CHYH2) with and without HRP conjugation, and PSA (MW 34,000) standard were obtained from Anogen/Yes Biotech Lab, Ltd., antibodies are specific to *t*-PSA. Multi-walled carbon nanotubes were purchased from Helix Inc. Immunoreagents were dissolved in pH 7.0 phosphate saline (PBS) buffer (0.01 M in phosphate, 0.14 M NaCl, 2.7 mM KCl) unless otherwise noted. 1-(3-(dimethylamino) propyl)-3-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide (NHSS) were dissolved in water immediately before use.

2.2. Preparation of the Ab₂-CNT-HRP Bioconjugates

We used covalent binding via amidization to obtain stable nanotube bioconjugates. Carboxylic acid groups were first converted to active esters via diimide-activation using EDC and NHSS. Excess EDC and NHSS were then removed and the active esters reacted with the amine groups on proteins. This two-step process avoids intermolecular crosslinking between proteins which can lead to bundling of the Ab₂-CNT-HRP. The procedure was describe in detail previously,⁸ and involves simultaneous loading of HRP and anti-PSA antibody onto functionalized multiwall CNTs via EDC/NHSS coupling. Excessive and non-specifically adsorbed reagents were removed by several washing/centrifugation steps. The essential features are summarized below

1. MWCNTs were functionalized and shortened with 6 hours' sonication in 3:1 H_2SO_4 and HNO_3 , followed by washing with water to pH neutral, then dried in vacuum.
2. 1.5 mg of the MWCNTs was dispersed in 2 mL pH 7 PBS buffer and sonicated 10 min.
3. The CNT dispersion was then mixed with 1 mL of fresh 400 mM EDC and 100 mM NHSS in pH 6.0 MES buffer and vortexed at room temperature for 15 min.
4. The resulting solution was centrifuged at 15000 RPM for 5 min. and the supernatant was discarded, and this step was repeated.
5. Anti-PSA antibody(Ab_2) at 0.005 mg mL^{-1} and HRP at 1 mg mL^{-1} were added to the above mixture and stirred for 2 hrs.
6. The reaction mixture was centrifuged at 15000 RPM at 4°C for 10 min., and supernatant removed.
7. 1 mL PBS buffer was added to the solid remaining in the vial, and this was centrifuged at 15000 RPM at 4°C for 10 min., supernatant was discarded, and this step was repeated for 4 more times to remove free HRP and Ab_2 .
8. 1 mL 0.05% Tween-20/PBS was added to the conjugate collected from step 7, and this was vortexed and stored at 4°C . This mixture was diluted 10 $\times$ with PBS/0.05% Tween-20 immediately before use to give an Ab_2 -CNT-HRP dispersion with $11 \text{ pmol HRP mL}^{-1}$.

The PSA-quantum dot bioconjugate probe for TEM studies was synthesized by covalently linking to amine-derivatized PEG coated quantum dots (Invitrogen Qdot 655 Nanocrystals) using EDC chemistry as described above with the PSA/Q-dot molar ratio 1.2 to 1.

2.3. Instrumentation and Characterization Procedures

A Veeco Nanoscope IV Multimode Atomic Force Microscope (AFM), a Philips EM 420 Transmission Electron Microscope (TEM) operating at 100 kV, and a Renishaw Raman Microscope 2000 using a 1.58 eV excitation laser focused on a $<1 \mu\text{m}$ spot by 10×100 objectives were used for the characterization of the Ab_2 -CNT-HRP. A CHI 660 electrochemical workstation was used for amperometry at ambient temperature ($22 \pm 2^\circ\text{C}$) in a three-electrode cell. Rotating disk amperometry at 3000 RPM was done at -0.3 V versus SCE and -0.2 V versus SCE for the mediated and unmediated systems respectively.

The previously reported procedure was used to prepare SWNT immunosensors.⁸ Briefly, 30 μL of freshly prepared 400 mM EDC and 100 mM NHSS in water were placed onto prefabricated SWNT forest sensors, then washed off after 10 mins. Then, 10 μL of 0.1 ng mL^{-1} anti-PSA was placed on the sensor surface and incubated for 3 hr. The sensor was then washed with 0.05% Tween-20 and PBS buffer. PSA antigen was diluted in pH 7.0 PBS buffer containing 0.05% Tween-20. Washing steps used here and below were the same as described previously.⁸ When singly-labeled HRP- Ab_2 was used, the sensor was incubated with 10 μL 4 pmol mL^{-1} anti-PSA-HRP in 2% BSA and 0.05% Tween-20. Using the bioconjugates, 10 μL of 10 $\times$ diluted Ab_2 -CNT-HRP bioconjugate was placed on the sensor surface. Sensors were then incubated for 1.25 hours at 37°C . This was followed by a second washing step and subsequent rotating disc amperometry using a CHI electrochemistry workstation.

HRP catalyzed polymerization was used for the detection of enzyme activity by fluorescence microscopy. A reported procedure was followed.¹⁰ A 0.05 M solution of hydroxyphenylacetic acid (HPA) substrate at pH of 6.5 was used to dilute the Ab_2 -CNT-HRP bioconjugate sample

10-fold. While stirring, H_2O_2 was added to a final concentration of 0.06 M and the reaction was allowed to proceed overnight. A 10 μL aliquot of the bioconjugate was placed on a glass slide for confocal fluorescence microscopy.

Enzyme activity of the $\text{Ab}_2\text{-CNT-HRP}$ bioconjugate was measured quantitatively using 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as a substrate. HRP was activated by hydrogen peroxide to oxidize ABTS to form a soluble end product with a characteristic absorbance at 405 nm.¹¹ 10 mg ABTS (Sigma) was dissolved in 100 mL of 0.05 M phosphate-citrate buffer at pH 5.0. Immediately before use 25 μL of 30% hydrogen peroxide was added to the buffer solution. The $\text{Ab}_2\text{-CNT-HRP}$ bioconjugate dispersion was injected and absorbance readings were taken every 6 seconds using a Cary 50 spectrophotometer.

Transmission Electron Microscopy (TEM) was done using a Philips EM300 Transmission Electron Microscope operating at 80 kV. Grids were 10–20 nm evaporated carbon films, and were irradiated with UV light for 1 hr to increase hydrophilicity. 3 μL of sample dispersion was placed on a grid and allowed to adsorb for approximately 1 min. The grid was then washed with several drops of pure water and allowed to stand for 1 min. The water in the sample was then wicked with filter paper and allowed to dry for 15 min. Photographic negatives were scanned using the Epson Expression 1680 scanner, SilverFast Ai, and were processed using Adobe Photoshop CS software.

3. RESULTS

3.1. Single versus Multi-Label Detection of PSA

We previously reported results using SWNT forest immunosensors for the detection of PSA in calf serum, human serum and cancer tissue lysates.⁸ This immunoassay was previously shown to be resistant to interferences from the large peripheral protein content of serum samples. Figure 1 illustrates comparison between single and multi-label immunodetection in buffer solutions which do not contain peripheral proteins. These data show a representative comparison of steady state amperometric results using conventional single label $\text{Ab}_2\text{-HRP}$ with the multilabel $\text{Ab}_2\text{-HRP-CNT}$ for PSA in buffer solutions. These data illustrate the advantages in sensitivity and detection limit obtained from the multi-label detection scheme and are similar to results for PSA in calf serum.

Using the singly labeled $\text{Ab}_2\text{-HRP}$, these calibration data are expressed by a sensitivity of 5.9 nA/ng mL^{-1} and limit of detection (LD, as non-specific binding control with no PSA plus $3\times$ the avg. noise level) of 0.4 ng mL^{-1} . Using the multi-label $\text{Ab}_2\text{-HRP-CNT}$, the sensitivity increased to 8430 nA/ng mL^{-1} , ~ 1400 times larger than with $\text{Ab}_2\text{-HRP}$. The LD of this multi-label assay in buffer was ~ 10 pg mL^{-1} in 10 μL sample, or 250 aM. Thus, the multi-label approach improved the LD 40-fold.

3.2. AFM, Resonance Raman, and TEM Imaging

Diluted dispersions of the $\text{Ab}_2\text{-HRP-CNT}$ bioconjugate and control nanotubes were placed on the surface of a freshly cleaved piece of mica and washed with water. Tapping mode atomic force microscopy (AFM) images were acquired on $5\times 5\ \mu\text{m}$ sections of the mica with the aim of obtaining images of isolated nanotubes (Fig. 2). The heights of AFM images of oxidized multiwall CNT before bioconjugation were 25 ± 5 nm on average. After bioconjugation with Ab_2 and HRP, the images revealed an average 10 nm increase in the diameter of the nanotubes for an average diameter of 35 ± 5 nm. This increase is consistent with the average thickness of a monolayer of HRP, which has molecular dimensions of $4.0 \times 6.7 \times 11.7$ nm,¹² and confirms earlier results suggesting roughly a monolayer of HRP on the multiwall CNT on a smaller

sample size.⁸ Although some protein aggregates may be present, our results suggest that the removal of excessive EDC/NHSS before protein binding described in the experimental section minimizes the creation of very large protein aggregates.

To confirm that the tubular structures in AFM were CNTs, Raman spectroscopy (RS) imaging was done on the same sample that was analyzed by AFM. RS spectral maps were obtained on $10 \times 10 \mu\text{m}$ area of the mica sample with resolution of $0.4 \mu\text{m}$. The characteristic RS graphene G-band of the carbon nanotubes in Ab₂-HRP-CNT on mica is shown in Figure 2(a). The profile of G-band intensity acquired from RS mapping in Figure 2(b) shows sub-micrometer long features that mirror the topologies observed by AFM, albeit with poorer resolution. These results suggest that tubular shapes observed by AFM originated from CNTs covered with proteins, i.e., that they are indeed the Ab₂-HRP-CNT bioconjugates.

Transmission electron microscopy (TEM) was also used to confirm the presence of protein layers on the surface of the Ab₂-HRP-CNT bioconjugate (Fig. 4). $5 \mu\text{L}$ of the sample dispersions were deposited on lacy carbon grids, and TEM measurements at $230,000\times$ magnification were made. The bare carboxylated nanotubes had an average diameter of $24 \pm 2 \text{ nm}$ (Fig. 4(a)). Compared to bare nanotubes, after protein binding, the average diameter increased to $32 \pm 2 \text{ nm}$ (Fig. 4(b)), an average increase in diameter of 8.6 nm was consistent with the 10 nm increase from AFM. Both AFM and TEM gave results consistent with the successful binding of roughly a monolayer of HRP on the sidewalls of the carboxylated nanotubes.

3.3. Enzyme Activity of Ab₂-HRP-CNT

Enzyme activity of the bioconjugate was imaged by using an enzyme reaction giving a fluorescent product, as well as by using a conventional optical assay for HRP activity. In the fluorescence imaging, an *in-situ* HRP-catalyzed polymerization was done (see experimental). HRP coated on the side walls of the CNT catalyzed the polymerization of the 4-oxyphenol substrate at pH 6.5 and formed a fluorescent polymeric layer along the sidewalls of the CNT bioconjugates, as illustrated by fluorescent images (Fig 5(a)). With HRP immobilized on the sidewalls, fluorescent polymer product grows along the line of the CNTs and forms a nearly uniform fluorescent layer. As a result, tubular fluorescent shapes can be seen clearly in Figure 5(a). The control is a confocal microscopic image of HRP-CNT-Ab₂ without the hydrogen peroxide added to drive the enzyme reaction (Fig. 5(b)). Here, many fewer fluorescent images can be observed. These are most likely due to weak fluorescence from tryptophan residues in HRP.¹³

The enzyme activity of HRP on Ab₂-HRP-CNT bioconjugates was assayed by using ABTS as substrate and hydrogen peroxide to activate the HRP. This provided a linear increase in absorbance of the product at 405 nm (Fig. 6), and the slope of this plot was used to estimate¹¹ an HRP activity of 21 units per mL of undiluted Ab₂-HRP-CNT bioconjugate. This corresponds to approximately $84 \mu\text{g mL}^{-1}$ of HRP. Thermal gravimetric analysis of the bioconjugate showed that there were $297 \mu\text{g mL}^{-1}$ of the CNTs in the bioconjugate samples.⁸ Using a model of multiwall CNTs with an average diameter of 25 nm (from AFM and TEM) and a length of $1 \mu\text{m}$ gave approximately 1.4×10^{12} nanotubes per mL of dispersion. From this concentration and the activity data, we estimated that there are 82 active HRP labels per 100 nm length of the CNTs.

3.4. TEM Detection of Ab₂ on Ab₂-HRP-CNT

PSA labeled with Qdots were used to visualize bound antibodies on the HRP-CNT-Ab₂ bioconjugate using TEM. The Qdots are relatively monodisperse with a core size of $5\text{--}10 \text{ nm}$ and an outer PEG layer of $\sim 20 \text{ nm}$. Qdot-PSA pre-equilibrated with HRP-CNT-Ab₂ bind to the Ab₂ on the bioconjugates, and provide a way to visualize and count Ab₂ from TEM images.

TEM micrographs of HRP-CNT-Ab₂ before and after reaction with Qdot-PSA and washing are shown in Figure 7. Micrographs of unreacted HRP-CNT-Ab₂ (Figs. 7(B and C)) and HRP-CNT show minimal dark spots. These controls (Figs. 7(B–D)) suggest that without the presence of Ab₂ on the CNT bioconjugate or in absence of treatment with Qdot-PSA (i.e., HRP-CNT), features characteristic of Qdots were not observed. However, for HRP-CNT-Ab₂ treated with Qdot-PSA, small cylindrical spots are clearly visible on the TEM images, and cannot be washed off, suggesting that these features represent Qdot-PSA bound to Ab₂ (Figs. 7(E–F)). Furthermore, Qdots alone (Fig. 7(A)) and those that are on HRP-CNT-Ab₂ (Figs. 7(E and F)) have the characteristic dimensions and shapes of the rod-like Qdots. The Qdots on the bioconjugates are a little smaller than when they are alone on a carbon TEM grid, probably because on the CNTs they are not all oriented on the same axis or plane in the bioconjugate samples. When PSA is linked non-dimensionally biased onto the Qdots, PSA binds to Ab₂ attached all around the CNT walls, giving rise to a range of orientations of each bound Qdot-PSA and resulting in different apparent sizes in the 2-dimensional TEM images.

Based on counting the Qdots bound to HRP-CNT-Ab₂ in the TEMs, we estimate that ~30 PSA molecules ($n = 8$, RSD 55%) bind per 100 nm, suggesting 30 ± 16 Ab₂ per 100 nm length of the HRP-CNT-Ab₂ bioconjugates.

4. DISCUSSION

Combined results from AFM, TEM, RS and *in-situ* HRP catalyzed polymerization (Figs. 2 through 7) confirmed the successful formation of the HRP-CNT-Ab₂ bioconjugates. The Two-step covalent coupling utilizing EDC and NHSS onto the oxidized CNTs successfully loaded an active thin layer of HRP enzymes and Ab₂ onto oxidized multiwall CNTs. Results from enzyme activity (Fig. 6) and TEM (Fig. 7) revealed 82 HRPs and 30 ± 16 Ab₂ per 100 nm length of multiwall CNT. However, Figures 7(D, E) suggests that the Ab₂ may be rather unevenly distributed along the nanotubes.

Compared to the use of a conventional single HRP-Ab₂ label, the HRP-CNT-Ab₂ bioconjugate amplified amperometric signals for PSA in buffer to achieve sensitivity ~1400-fold greater in mediated immunoelectrochemical assays (Fig. 1) on a SWNT forest platform. Similar results were reported earlier for PSA in calf serum.⁸ This ultra sensitive multi-enzyme bioconjugate immunoassay approach is general and could easily be adapted to other protein biomarkers. Additional signal amplification might be achieved by increasing the number of enzyme labels on the bioconjugate, and better linear range might be achieved by optimizing the HRP/Ab₂ ratio and CNT length. The techniques reported here can be used in general to characterize multi-enzyme labeled nanoparticles for immunoassays to help achieve this optimization. The next step is to establish relationships between characterization results and the bioanalytical performance of the bioconjugate, which are unclear at present.

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Fig. 1.

Amperometry for PSA detection in pH 7.4 buffer on SWNT immunosensors. Capture antibodies were linked to SWNT immunosensor surfaces, which were then equilibrated with PSA in 10 μ L buffer for 1.25 hr then with 2% BSA and 0.05% Tween-20 for 1.25 hr. Sensors were then equilibrated with (A) conventional Ab₂-HRP and (B) multi-label Ab₂-HRP-CNT. Amperometric current was measured at -0.3 V versus SCE and 3000 rpm after placing sensors in buffer containing 1 mM hydroquinone mediator, then injecting H₂O₂ to 0.4 mM. Controls shown on right: (a) full SWNT immunosensor omitting addition of PSA (NSB control), (b) immunosensor built on bare PG surface at 40 ng mL⁻¹ PSA for A or 300 pg mL⁻¹ for B, (c) immunosensor built on Nafion-iron oxide-coated PG surface at 40 ng mL⁻¹ PSA for A or 300 pg mL⁻¹ for B, (d) immunosensor built on Nafion-PG surface at 40 ng mL⁻¹ PSA, in A only.

Fig. 2. Tapping mode AFM images of multiwall CNTs isolated on smooth, freshly-cleaved mica surfaces (A) carboxylated, but not derivatized with protein; and (B) carboxylated and derivatized as Ab₂-HRP-CNT bioconjugates.

Fig. 3. (a) Raman spectra of Ab₂-HRP-CNT on mica with large D-band and G-band indicated by arrow (red), and mica alone (black); (b) Raman mapping of G-band intensity, scale on right.

Fig. 4. Transmission electron microscopy (TEM) at 100 kV using a Philips EM 420 TEM (a) bare MWCNT, (b) Ab₂-HRP-CNT bioconjugate.

Fig. 5. Confocal fluorescence microscope images of (A) 10× diluted Ab₂-HRP-CNT and 0.05 M 4-hydroxyphenylacetic acid in pH 6.5 phosphate buffer and 20 mM H₂O₂. (B) 10× diluted Ab₂-HRP-MWCNT and 0.05 M 4-hydroxyphenylacetic acid in pH 6.5 phosphate buffer.

Fig. 6. Results of enzyme activity assay of HRP-CNT-Ab₂ bioconjugate activated by H₂O₂ with ABTS as substrate to give colored product with absorbance at 405 nm.

Fig. 7. TEM images of bioconjugates and controls used to visualize the number of anti-PSA antibodies (Ab_2) on the bioconjugate using PSA labeled with quantum dots (Q-dots). (A) Q-dots (Invitrogen Qdot 655 nanocrystals) shown to illustrate size and dispersity; (B) and (C) HRP-CNT- Ab_2 bioconjugate controls deposited on grid from water, no Qdots; (D) Bioconjugate without Ab_2 mixed with Q-dot labeled PSA. (E) and (F) micrographs of HRP-CNT- Ab_2 bioconjugates mixed with Q-dot labeled PSA.

Scheme 1.

Illustration of detection principles using multi-label Ab_2 -HRP-CNT bioconjugates on a SWNT forest immunosensor platform. On the left is a cartoon of a SWNT immunosensor that has been equilibrated with PSA antigen, along with the biomaterials above it used for fabrication. The picture on the right shows the immunosensor after treating with Ab_2 -HRP-CNT to provide numerous enzyme labels per binding event. The final detection step involves immersing the immunosensor after secondary antibody attachment into a buffer containing mediator in an electrochemical cell, applying voltage, and injecting hydrogen peroxide.