

Retention of biological activity and near-infrared absorbance upon adsorption of horseradish peroxidase on single-walled carbon nanotubes

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Received 16 January 2007, in final form 18 March 2007

Published 8 May 2007

Online at stacks.iop.org/Nano/18/235601

Abstract

The objective of this study is to demonstrate the adsorption of horseradish peroxidase (HRP) on single-walled carbon nanotubes (SWNTs) using the sodium cholate suspension–dialysis method and to determine the effect of HRP adsorption on the biological activity of HRP and the UV–vis–NIR spectra of the SNWTs. The results indicate that this method results in a stable SWNT–protein suspension with complete retention of enzymatic activity of adsorbed HRP and also retention of a substantial fraction of the NIR absorption at 980 nm. The loading of protein on the SWNTs is high, and the overall yield of preparing the SWNT–protein suspension is also high. This process is promising for preparing SWNT–protein suspensions for biological applications where maintaining protein activity and SWNT absorption are important.

 Supplementary data are available from stacks.iop.org/Nano/18/235601

1. Introduction

Single-walled carbon nanotubes (SWNTs) are under active development for applications in various biological systems, including the delivery of biological cargoes into cells, biosensor development, bioelectrochemistry and biomedical devices [1–6]. One way to use SWNTs in biological systems is by attachment to proteins so that a SWNT–protein complex can be delivered to a specific site. Although protein attachment by covalent functionalization may be useful in some applications, this method has resulted in the elimination of important features in the UV–vis–NIR absorption spectra of the SWNTs [7, 8]. While adsorption of biomolecules to SWNTs does not provide as strong a linkage as using covalent functionalization, this method has been found to preserve the UV–vis–NIR absorption of the SWNTs, a property that could be exploited for the selective destruction of cancer cells [9].

It is particularly important to preserve the optical absorption and photoluminescence of SWNTs in the range of NIR, since biological systems exhibit a significantly deep penetrability but very low absorption of NIR photons in the range 700–1100 nm.

In preparing SWNTs for use in biological applications, it is desirable to obtain aqueous suspensions of SWNT–protein complexes, in which the nanotubes should remain well dispersed and preferably individually separated from each other. However, in many cases, even when a pseudo-stable suspension is formed, the nanotubes remain in large aggregates and lose most of their unique properties, such as optical absorption and photoluminescence. To solve this problem, Graff *et al* [10] have used a novel method in which the SWNTs are first completely suspended in a solution with a low concentration of the sodium cholate, a bile salt which acts as a surfactant. Subsequently, the protein to be adsorbed is added to the suspension and the sodium cholate is removed by dialysis. In that study, the protein concanavalin-A was adsorbed to form a stable solution-phase complex with the

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SWNTs; however, neither the conyavalin-A activity nor the UV-vis-NIR absorption spectra was determined. Other methods that have been used to adsorb proteins on SWNTs are the organic solvent displacement method [11] and the aqueous sonication method [12]; however, these methods gave problems with complete dispersion of SWNTs and with the maintenance of protein activity or structure.

The objective, therefore, of this study is to demonstrate the adsorption of a protein on SWNTs using the sodium cholate suspension-dialysis method developed by Graff *et al* and to determine the effect of protein adsorption on the biological activity of the protein and the UV-vis-NIR spectra of the suspended SWNTs. The protein chosen for this study is horseradish peroxidase (HRP), a monomeric enzyme with a molecular weight of 40 kDa. The biological activity of HRP can be quantified using an enzyme activity assay. CoMoCAT nanotubes were used in this study since they are enriched in the (6, 5) nanotubes type that exhibits a sharp absorption as well as a fluorescence band at around 980 nm [13].

2. Materials and methods

A saturated suspension of single-wall carbon nanotubes (SWNTs) was made by first adding 3 mg of pristine nanotubes (CoMoCAT sample supplied by SouthWest Nanotechnologies) to 7 ml of a 2 wt% aqueous solution of sodium cholate (Sigma-Aldrich). CoMoCAT nanotubes have an average diameter of 0.81 nm [13]. To disperse the SWNTs, the mixture was horn sonicated for 1 h using a homogenizer (22% amplitude, Cole-Parmer model CPX750) resulting in a dark black liquid. This suspension of SWNTs was then centrifuged at 30 100g for 1 h.

HRP was adsorbed on SWNTs using the following procedure at 4°C: sodium phosphate was added to the SWNT suspension to give a concentration of 20 mM. To this solution 20 mg of HRP (Worthington Biochemical) was added, and dialysis using a 10 kDa dialysis membrane (Spectrum Laboratories) was carried out with 2 l of sodium phosphate buffer solution at pH 7.4 for 12 h to remove sodium cholate. The resulting solution was transferred to a 100 kDa dialysis membrane (Spectrum Laboratories) and dialyzed against 2 l sodium phosphate buffer at pH 7.4 to remove unadsorbed protein, with a change of the 2 l of buffer at 2, 4, 16 and 24 h from the start of dialysis. The final dialysis was carried out for 4 h. The final suspension was centrifuged at 29 600g for 1 h and the supernatant was retained. A control experiment was also performed in which no HRP was added and dialysis to remove sodium cholate with a 10 kDa membrane was carried out for 48 h.

The enzymatic activity of HRP in free solution and adsorbed on SWNTs was measured using an assay for peroxidase [14]. HRP catalyzes 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in the presence of hydrogen peroxide to form an oxidized ABTS. For this assay, 2.9 ml of 9.1 mM ABTS solution, 0.05 ml of enzyme solution and 0.100 ml of 0.3% (w/w) of H₂O₂ solution are mixed. For a blank, 0.05 ml of 40 mM potassium phosphate buffer with 0.25% (w/v) of bovine serum albumin (BSA) at pH 6.8 is used instead of the enzyme solution. Absorbance at 405 nm is measured for 2 min. A unit (U) of HRP activity is defined as



Figure 1. Comparison of SWNT suspensions. Left: no HRP protein was added and the suspension of SWNTs in sodium cholate was dialyzed for two days using a 10 kDa membrane. Right: SWNTs suspended in sodium cholate with HRP added were dialyzed, first with a 10 kDa membrane and then with a 100 kDa membrane. The SWNT-protein suspension was held at 4°C for ten days.

the enzyme activity that causes 1 μ mol of ABTS to be oxidized per minute at the assay conditions.

The total protein concentration was determined using the Bradford assay. This assay uses a Coomassie blue dye reagent (from Bio-Rad).

The absorption of light as a function of wavelength was measured using a Bruker Equinox 55 FTIR/FTNIR/FTVis spectrometer; 60 scans at 30 cm⁻¹ were averaged on each spectrum in order to achieve a high signal-to-noise ratio.

Atomic force microscopy (AFM) images of HRP and sodium cholate adsorbed onto SWNTs were obtained using a Digital Instruments NanoScope III AFM instrument in the tapping mode. MikroMasch Ultrasharp NSC15 silicon nitride cantilevers were used with a backside aluminium coating and typical resonant frequencies of 325 kHz and force constants of 40 N m⁻¹. All images were captured at 512 samples per line for clearer pictures. Samples were diluted 50 times with deionized water before 20 μ l was cast on a freshly cleaved mica surface by slow spin coating at 500 rpm. Then, samples were dried under vacuum for 5 h at room temperature to remove water before imaging.

3. Results and discussion

A stable SWNT-protein suspension was obtained after the final centrifugation of the preparation process. This suspension remained stable after holding for 10 days at 4°C (figure 1). After the dialysis with the 10 kDa membrane to remove the sodium cholate, it was determined from a methylene chloride extraction of the final dialysis solution that 98% of the sodium cholate was removed, leaving a sodium cholate concentration of 0.04 wt% in the SWNT-protein suspension.

In one control experiment starting with the pristine SWNT suspension in 2 wt% sodium cholate, no HRP was added and the dialysis with a 10 kDa membrane was carried out for 48 h. No aggregation of SWNTs was observed after 12 and 24 h; but after 48 h, the SWNTs were aggregated in black clumps and settled out of suspension (figure 1), which indicates that the adsorption of sodium cholate is reversible. This finding

Table 1. Enzyme activity and total protein results.

Sample	Enzyme activity, (U/mg) protein	Protein concentration (mg l ⁻¹)	Enzyme activity/enzyme activity of native protein
SWNT–protein suspension after centrifugation			
Day 0	197	345	0.98
Day 5, held at room temperature and centrifuged again	0	16	0
Final dialysis solution (2 l) using 100 kDa membrane	0	0	—

is consistent with previous work showing that the percentage of SWNTs suspended falls as the surfactant concentration falls [15]. In another control experiment, a portion of SWNT–protein suspension after the final centrifugation was held at room temperature. After five days, the sample was observed to have amounts of black SWNT precipitate.

The enzyme activity and total protein measurements for the SWNT–protein suspension that was obtained by this procedure after centrifugation are shown in table 1. A control experiment for the measurement of enzyme activity on SWNTs suspended only in sodium cholate gave zero activity. The results in table 1 indicate that the native HRP activity was almost entirely retained. The enzyme activity and protein concentration in the 2 l dialysis solution after the final dialysis with the 100 kDa membrane were both found to be zero (see table 1); this indicates that HRP was adsorbed on the SWNTs, since HRP with 40 kDa molecular weight can easily pass through the 100 kDa membrane. After five days of holding the SWNT–protein suspension at room temperature, the sample was centrifuged and the supernatant was found to have no enzyme activity and a protein concentration of only 16 mg l⁻¹ (table 1), indicating denaturation of HRP that led to precipitation of the SWNT–protein complex.

The UV–vis–NIR absorption spectra (normalized) of the SWNT–protein suspension before and after the final centrifugation are compared to that for the pristine SWNT suspension in figure 2. Non-normalized UV–vis–NIR absorption spectra data and Raman spectra data are given in supplementary data (available at stacks.iop.org/Nano/18/235601). In the 300–400 nm wavelength range of the spectra, the absorbance was significantly greater for both of the samples with SWNT–protein than for SWNTs alone, and the SWNT–protein after centrifuging had significantly higher absorbance in this same range than the SWNT–protein before centrifuging. This latter observation indicates that the SWNT–protein before centrifuging contained SWNTs with varying amounts of protein adsorbed per SWNT, and that centrifuging removed the SWNTs with lower amounts of protein adsorbed. The NIR absorption spectra of all three samples exhibit a strong absorption band at approximately 980 nm, which is typical for CoMoCAT samples and ascribed to the S11 transition of (6, 5) nanotubes [16]. The peak intensities were similar for the pristine SWNTs and the SWNT–protein pre-centrifugation samples, with a slight redshift for the SWNT–protein samples. The slight, but consistent, redshift can be interpreted in terms of a SWNT–protein interaction stronger than that with the surfactant. Similar shifts with respect to surfactant SWNT suspensions have been previously observed for SWNTs with adsorbed DNA [17].

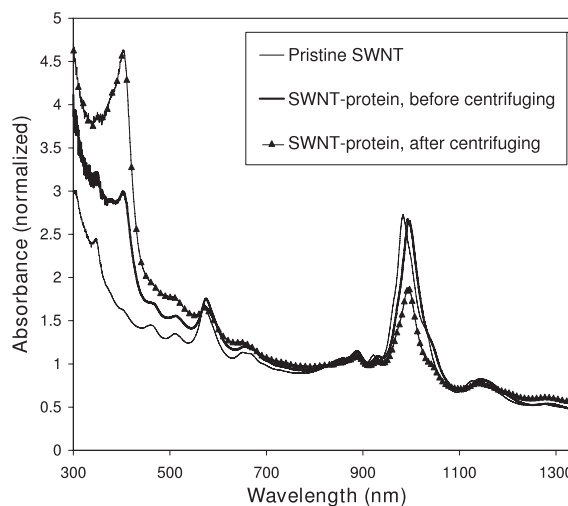


Figure 2. UV–vis–NIR absorption spectra of the pristine SWNTs suspended in sodium cholate, SWNT–protein complex before the final centrifugation and SWNT–protein complex after the final centrifugation.

The concentration of the SWNT–protein suspension before and after centrifugation is compared to that for the pristine SWNT suspension in table 2. These concentrations were determined from the absorbance at 800 nm, a valley in the absorption spectrum where the absorbance is minimally influenced by the specific S11 transitions of the SWNT or by the adsorbed protein. This data indicates that a 10% loss of SWNTs occurred in the preparation process up to the final centrifugation and that 11% was lost in the final centrifugation. The overall SWNT yield of 79% is very good. In addition, the protein and SWNT concentration data in tables 1 and 2 for the SWNT–protein suspension after centrifugation indicate that the loading of the protein was 2250 μg protein/mg SWNTs (= 56.2 nmol protein/mg SWNTs).

AFM images of sodium cholate and HRP adsorbed onto SWNTs are shown in figure 3. The arrows indicate the adsorption of sodium cholate (figure 3(a)) and HRP (figure 3(b)). The height of the images of HRP (3.8–6.0 nm) is considerably higher than those of sodium cholate (1.0–1.5 nm) and is consistent with the size of proteins that are about the same size as HRP. For example, β -lactoglobulin (molecular weight 35 kDa) has a diameter of 5.4 nm as determined from diffusion measurements [18].

It is interesting to compare these results to those obtained by Karajanagi *et al* [11] and Matsuura *et al* [12]

Table 2. SWNT concentrations, as measured by the absorbance at 800 nm.

Compound	SWNT concentration, mg l ⁻¹	Yield, %
Pristine SWNTs	194	100.0
SWNT–protein, pre-centrifugation	174	89.6
SWNT–protein, post-centrifugation ^a	153	78.9

^a From table 1 data, protein weight/SWNT weight = 2250 $\mu\text{g mg}^{-1}$ (= 56.2 nmol protein/mg SWNTs).

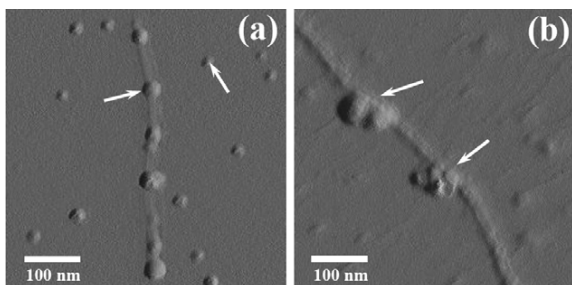


Figure 3. AFM images showing SWNT with sodium cholate and HRP protein. (a) SWNT in sodium cholate after sonication and centrifugation. Arrows indicate solid sodium cholate with a height of 1.0–1.5 nm (does not include 0.8 nm SWNT height). (b) SWNT–protein after dialysis and centrifugation. Arrows indicate protein associated with SWNT. The height is 3.8–6.0 nm (does not include 0.8 nm SWNT height).

for the adsorption of proteins on SWNTs using different methods to accomplish the adsorption. Karajanagi *et al* first dispersed the SWNTs in dimethylformamide (DMF) and then gradually replaced the DMF with an aqueous phosphate buffer. Soybean peroxidase or α -chymotrypsin was adsorbed on the SWNTs in the aqueous buffer and the suspension was centrifuged at 8000 rpm in a microcentrifuge (which should be at approximately 6000g). Like HRP, soybean peroxidase or α -chymotrypsin are monomeric enzymes. For soybean peroxidase, up to a maximum of only 28% of the original specific enzyme activity was retained and the maximum loading was 575 $\mu\text{g protein/mg SWNTs}$ (= 12.0 nmol protein/mg SWNTs), or four times lower on a weight basis than we obtained using HRP (2250 $\mu\text{g protein/mg SWNTs}$). Using atomic force microscopy (AFM), the SWNTs with soybean peroxidase adsorbed appeared as bundles of 3–4 SWNTs. For α -chymotrypsin, no greater than 1% of the native specific enzyme activity was retained and the maximum loading was 673 $\mu\text{g protein/mg SWNTs}$ (=26.9 nmol protein/mg SWNTs), or three times lower on a weight basis than for HRP in our study.

Matsuura *et al* [12] studied the adsorption of four proteins (papain, pepsin, egg white lysozyme and human serum albumin) on SWNTs. Protein adsorption was accomplished by adding bundled SWNTs to an aqueous protein solution and then sonicating to disperse the SWNTs. Sonication resulted in the mixture reaching 60–70 °C. After sonication, the suspension was centrifuged at 16000g to remove insoluble materials. This procedure resulted in dispersion of the

SWNTs when either lysozyme or albumin was used, but no dispersion using either papain or pepsin. After the removal of unadsorbed protein using precipitation of the SWNTs by the addition of sodium chloride or ammonium sulfate salt and then resuspension of the SWNTs, there was a significant change in the circular dichroism (CD) spectra for both lysozyme and albumin adsorbed on SWNTs compared to the CD spectra for the native proteins, indicating that the adsorbed protein was partially unfolded. Absorbance measurements of the SWNTs with adsorbed proteins gave peaks that were redshifted and lower in intensity compared to SWNTs suspended in sodium cholate solution—findings that we observed also.

These comparisons of the present work with those of Karajanagi *et al* and Matsuura *et al* indicate that the sodium cholate suspension–dialysis method may be superior to the solvent displacement method and the aqueous sonication method for adsorption of protein on SWNTs with respect to the retention of biological activity. The sodium cholate suspension–dialysis method may also be superior to the solvent removal method for maximizing protein loading. However, it is possible that the differences in biological activity and protein loading for HRP compared to the proteins studied by Karajanagi *et al* and Matsuura *et al* may be due to differences in the characteristics of the proteins.

4. Conclusions

The results indicate that the sodium cholate suspension–dialysis method gives a stable SWNT–protein suspension with complete retention of enzymatic activity of adsorbed HRP and also retention of a substantial fraction of the NIR absorption at 980 nm of the SWNTs. The loading of protein on the SWNTs is high and the overall yield of preparing the SWNT–protein suspension is also high. This process, therefore, is promising for the preparation of SWNT–protein suspensions for biological applications where maintaining protein activity and SWNT absorption are important.

Acknowledgments

We gratefully acknowledge the financial support from the Department of Energy–Basic Energy Sciences (Grants DE-FG03-02ER15345 and DE-FG02-06ER64239).

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