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Preparation and Characterization of Carbon Nanotube-Protein Conjugates

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Abstract

This chapter describes methods of immobilizing proteins on carbon nanotubes, using two different routes—physical adsorption and covalent attachment. We also provide an overview on how such conjugates can be characterized with the help of various techniques, such as Raman, Fourier transform infrared (FT-IR), circular dichroism (CD), and fluorescence spectroscopies, in addition to the standard enzyme kinetic analyses of activity and stability. Both the attachment routes—covalent and noncovalent—could be used to prepare protein conjugates that retained a significant fraction of their native structure and function; furthermore, the protein conjugates were operationally stable, reusable, and functional even under harsh denaturing conditions. These studies therefore corroborate the use of these immobilization methods to engineer functional carbon nanotube-protein hybrids that are highly active and stable.

Key terms

enzyme immobilization
carbon nanotubes
physical adsorption
covalent attachment
nanotube solubilization

1.1 Introduction

Nanomaterials, such as carbon nanotubes (CNTs) offer a unique combination of electrical, mechanical, thermal, and optical properties [1] that make them promising materials for various applications ranging from sensing [2] and diagnostics to biotransformations and the cellular delivery of peptides and proteins [3, 4]. For instance, Barone et al. [5] have developed carbon nanotube-glucose oxidase conjugates that can act as glucose sensors. Recently, Dai and coworkers [2] demonstrated the recognition of monoclonal antibodies by a recombinant human antigen immobilized onto carbon nanotubes. Carbon nanotubes have also been used for both biomolecule delivery and targeted therapy. Pantarotto et al. [3] demonstrated that carbon nanotubes functionalized with peptides can penetrate cell membranes of human and murine fibroblasts, and serve as carriers for biomolecule delivery. Dai and coworkers [4] observed internalization of nanotube-protein conjugates in nonadherent human cancer cells as well as adherent cell lines. Kam et al. [6] demonstrated that functionalized CNTs could be used to selectively target cancer cells and destroy them by irradiating CNTs with near-infrared (NIR) light. These studies represent a fraction of the exciting opportunities at the interface of nanotechnology and biotechnology. It is, however, important to interface carbon nanotubes with biomolecules, such as proteins, to realize some of these applications. As a result, various methods of functionalization have been developed recently to functionalize CNTs with proteins. In this chapter, we describe three methods of preparing carbon nanotube-protein conjugates, each of them possessing distinct structural, mechanical, and functional characteristics.

Noncovalent attachment is probably the simplest technique for attaching proteins onto carbon nanotubes. The adsorption of proteins onto CNTs is hypothesized to be a result of the attractive hydrophobic interactions between carbon nanotubes and proteins [7]. This method has been found to preserve a significant fraction of the native structural and functional properties of several proteins as well as the physicochemical properties of nanotubes [2, 8–10]. The resulting formulations prevail in the form of aggregates, which can be easily separated from other solution components. However, the limited solubility of these conjugates in water limits their attractiveness for many applications in biotechnology [11, 12]. Nevertheless, such conjugates have been used for biosensing, diagnostics and preparing antifouling nanocomposites films [13].

To overcome the aforementioned limitation of water solubility, Karajanagi et al. have described a simple method that uses proteins to solubilize single-walled carbon nanotubes (SWNTs) in water [14]. Efficient solubilization of SWNTs has previously been achieved using surfactants [15, 16], polymers [17, 18], single stranded DNA [19], peptides [20], and polysaccharides [12, 21]. The direct solubilization of SWNTs using a variety of proteins differing in size and structure is a simple and scalable alternative that enables the generation of individual nanotube solutions. Moreover, proteins are rich in structure and function and have numerous reactive groups, such as hydroxyls, amines, thiols, carboxylic acids, and others, which can be used as orthogonal reactive handles for further functionalization of SWNTs.

Finally, Asuri et al. have developed an alternative method of preparing water-soluble conjugates of carbon nanotubes with a broad range of proteins [22]. CNTs can be acid oxidized to produce hydrophilic carboxylic acid and hydroxyl groups along their sidewalls [23, 24], thereby leading to water solubility. Proteins can then be covalently attached to oxidized water-soluble CNTs using carbodiimide activation of the carboxylic

acid groups. These water-soluble conjugates not only display low diffusional resistance [25] and high activity with stable protein attachment [26], but also have added advantages of high stability and reusability, thereby overcoming the traditional limitations of water-soluble proteins. Though the covalent immobilization of proteins onto CNTs leads to stable protein attachment, the chemical modification of the CNTs surface may compromise the desirable electronic properties of CNTs. Such water-soluble CNT-protein conjugates may find application in fields other than biosensing, for example, biotransformations, biomaterials, medicine, and self-assembled materials.

It is, therefore, clear that many methods have been explored to prepare functional nanotube-protein conjugates. Each of these methods possesses its own unique set of advantages and disadvantages, and the best choice of the method depends on the desired end application of the hybrid conjugates.

1.2 Materials

Raw and purified HIPCO single-walled carbon nanotubes (SWNTs) (1–1.5 nm diameter, ca. 10 μm length, <35 wt% ash content) were purchased from Unidym (Houston, TX). Multiwalled carbon nanotubes (MWNTs) (10–20 nm diameter, 5–20 μm length, 95 wt% purity) were purchased from Nanolab, Inc. (Newton, MA). Enzymes—soybean peroxidase (SBP), horseradish peroxidase (HRP) and *Mucor javanicus* lipase (MJL)—were purchased from Sigma-Aldrich (St. Louis, MO) as salt-free, dry powders. Bicinchoninic acid (BCA) assay kit for determining solution phase protein concentrations was purchased from Pierce Biotechnology, Inc. (Rockford, IL). Guanidine hydrochloride (GdnHCl), sodium dodecylbenzene sulfonate (NaDDBS), and all other chemicals were obtained from Sigma. Bovine serum albumin (BSA) was purchased from Fisher Scientific International, Inc. (Hampton, NH). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). All enzymes and chemicals were used as received without any further purification.

1.3 Methods

1.3.1 Physical Adsorption of Proteins on Carbon Nanotubes

Attachment of proteins to carbon nanotubes via physical adsorption represents a facile method of preparing nanotube-protein conjugates, wherein an aqueous dispersion of SWNTs is mixed with a protein solution to achieve adsorption. The unadsorbed protein is washed off and nanotube-protein conjugates are then resuspended in aqueous solution. The detailed procedure is described below:

1. Sonicate a fixed amount of raw SWNTs in dimethylformamide (DMF) at a concentration of 1 mg/mL for 30 minutes using a bath sonicator (Model 50T, VWR International, West Chester, PA) with rated power of 45W to obtain a uniform dispersion in solution.
2. Dispense 1 mL of the resulting SWNT dispersion into an Eppendorf microcentrifuge tube, and centrifuge the solution at 8,000 rpm for 1 minute. Remove the supernatant and resuspend the settled SWNTs in an aqueous buffer (50 mM phosphate buffer, pH

7.0) by vortexing the solution. Repeat this wash procedure at least five more times to remove any residual organic solvent. This gradual change from organic phase to an aqueous phase renders unfunctionalized SWNTs more dispersed in buffer. Finally, disperse 1 mg SWNTs in 500 μL aqueous buffer (15 minutes).

NOTE: The selection of buffer for preparing conjugates is dependent upon the choice of protein and the retention of its native function in that buffer.

3. Prepare a fresh solution of protein in the aqueous buffer. Add the aqueous dispersion of SWNTs (500 μL, 2 mg/mL) to the protein solution (500 μL), and shake the mixture on a platform shaker for 2 hours at 200 rpm and room temperature (2 hours 15 minutes).

   NOTE: In the case of thermally unstable proteins or enzymes undergoing autolysis, such as trypsin, shaking should be carried out at 4°C to prevent deactivation during incubation.

4. After incubation, centrifuge the SWNT dispersion at about 8,000 rpm for 1 minute to settle the SWNT-protein conjugates. Carefully decant the supernatant (ca. 800 μL) without loss of any conjugates. Typically, perform six such washes, with fresh buffer added each time to remove unbound protein (20 minutes).

   NOTE: While collecting the supernatant, tilt the microcentrifuge tube and gently pipette out approximately 800 μL supernatant from close to the tube walls without disturbing the settled SWNTs, so that the supernatant does not contain SWNTs, which can interfere with BCA assay for protein content determination in supernatants. A swinging bucket microcentrifuge is ideal for this step, as the SWNTs settle at the bottom and not on the walls of the tube after centrifugation. Do not resuspend the SWNT-protein conjugates by vortexing, as this can lead to desorption of the protein from SWNT surface. Resuspend the conjugates by gently inverting and tapping the microcentrifuge tube containing the conjugates.

5. Analyze the supernatants for protein content using the BCA assay (for protein concentration range of 20–2,000 μg/mL) or the micro-BCA assay (for protein concentration range of 0.5–20 μg/mL). Determine the amount of protein attached onto the SWNTs by mass balance. Use the SWNT-protein dispersion for further analysis and characterization. (1 hour 15 minutes) The process flowchart is shown in Figure 1.1.

The total time to carry out the procedure is approximately 5 hours.

1.3.2 Protein Assisted Solubilization of Carbon Nanotubes

For preparation of protein solubilized carbon nanotubes, an aqueous dispersion of nanotubes is dispensed in a concentrated protein solution and exposed to ultrasonication for a predetermined time period. The supernatant, collected after sequential steps of ultracentrifugation of the CNT-protein dispersion, contain solubilized carbon nanotubes that are stable at room temperature and show no signs of aggregation. The detailed procedure is described below:

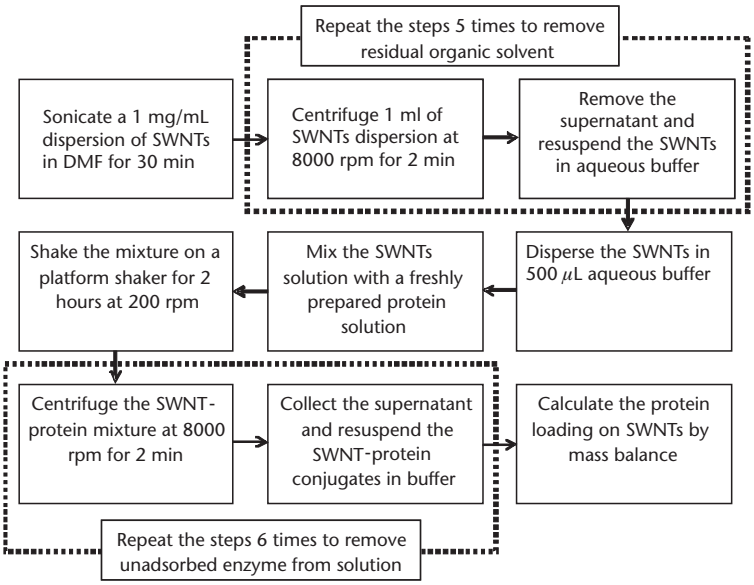

Figure 1.1 Process flowchart for the physical adsorption of proteins onto SWNTs.

1. Disperse purified SWNTs in DMF at a concentration of 1 mg/mL by sonication, and replace the organic phase gradually with an aqueous phase through repeated washing with milliQ water (as stated in section 1.3.1) (45 minutes).
2. Disperse 200 μg of SWNTs in 4-mL protein solution (10 mg/mL) and sonicate the dispersion of SWNTs for 2 hours using a bath sonicator (Model 50T, VWR International, West Chester, PA) with rated power of 45W (2 hours).
3. Ultracentrifuge the dispersed solution at 123,000g for 30 minutes.
4. Carefully collect 60% of the supernatant and ultracentrifuge at 185,000g for 30 minutes.
5. Collect 75% of the supernatant that contains protein adsorbed SWNTs. Use this solution for further analysis and characterization.

The total time to carry out the procedure is approximately 4 hours.

NOTE: The sonication efficiency and, hence, the quality of the dispersion varies with the volume of the solution sonicated. For best dispersions, use 4 mL volume for sonication.

1.3.3 Covalent Attachment of Proteins onto Carbon Nanotubes

For covalently attaching proteins onto carbon nanotubes, the carbon nanotubes are first functionalized with carboxylic acid groups by acid treatment. The carboxylic acid groups are then "activated" to form succinimide esters using carbodiimide chemistry [23]. These activated carboxylic groups react with amine groups on proteins enabling the covalent attachment of proteins onto carbon nanotubes. A detailed description of the procedure is given below:

1. Sonicate a fixed amount of multiwalled carbon nanotubes (MWNTs) in a mixture of concentrated sulfuric acid and nitric acid (3:1, v/v; 400 mL/100 mg carbon nanotubes) using a bath sonicator with a rated power of 45W for 3 hours. Periodically replace the contents of the bath with ice cold water to ensure that the MWNT suspension does not get heated up during sonication (3 hours).

   NOTE: Acid oxidation not only leads to the functionalization of MWNTs with carboxylic acid groups, but also causes cutting of MWNTs. Longer sonication times result in finer oxidized MWNTs.

2. Add the nanotube-containing acid solution (400 mL) to an ice-cold solution of milliQ water (3,600 mL) gradually with constant swirling. Allow 10 to 15 minutes for dissipation of the heat generated on diluting the acid mixture.
3. Filter the solution through a 0.22-μm polycarbonate filter membrane (Isopore membrane, Millipore) in batches of approximately 200 mL to remove the acid. After each filtration, disperse the nanotube film or bucky paper (a mass of carbon nanotubes tangled with each other to form a film or mat) thus formed in 50-mL milliQ water by ultrasonication in the bath sonicator for approximately 10 minutes, until the nanotubes are dispersed entirely in solution. Dilute this suspension with 150-mL milliQ water (40 minutes).
4. Repeat the ultrasonication/filtration step at least three times until water-soluble MWNTs are obtained and the pH of the filtrate becomes neutral (2 hours).

   NOTE: These oxidized and "cut" nanotubes can be stored in aqueous solution (1 mg/mL) at room temperature.

5. After the final filtration, disperse the oxidized nanotubes (2 mg/mL) in MES (2-(*N*-Morpholino) ethanesulfonic acid) buffer (50 mM, pH 6.2), and add an equal volume of 400-mM *N*-hydroxysuccinimide (NHS) in MES buffer (5 minutes).
6. Sonicate the mixture for 30 minutes in a bath sonicator.
7. Add *N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) (20 mM in MES buffer) to the nanotube solution to initiate the coupling of NHS to the carboxylic groups on the oxidized nanotubes, and stir the mixture at 400 rpm for 30 minutes at room temperature.
8. Filter the activated nanotube solution through a polycarbonate filter membrane (0.22 μm) and rinse thoroughly with MES buffer to remove excess EDC and NHS (20 minutes).
9. Transfer the nanotube film immediately into a freshly prepared protein solution (2 mg/mL, 10 mM phosphate buffer, pH 8.0), and sonicate for few seconds to disperse the nanotubes in solution.

   NOTE: Do not allow the nanotube film to dry out completely on the filter membrane as it may lead to hydrolysis of the active ester and hence decreased attachment of the protein.

10. Shake the mixture on an orbital shaker at 200 rpm for 3 hours and at room temperature to allow the attachment of proteins to the nanotubes.

NOTE: Proteins such as proteases or thermally unstable proteins require that this step be carried out at 4°C to prevent protein deactivation.

11. Filter the nanotube-protein suspension and wash it three times with milliQ water (5-mL/mg nanotubes) and once with 1% Tween-20 (5-mL/mg nanotube) to remove any nonspecifically bound protein (2 hours).
12. Allow flocculates of nanotube-protein conjugates, if any, to settle overnight and use the supernatant for further experiments.
13. Quantify the amount of immobilized protein by elemental analysis of the oxidized nanotubes and the nanotube-protein conjugates. The schematic of the process of protein functionalization on nanotubes is shown in Figure 1.2.

The total time to carry out the procedure is approximately 11 hours.

1.4 Data Acquisition, Anticipated Results, and Interpretation of Data

We have employed various techniques, such as Raman, FT-IR, CD, and fluorescence spectroscopies in addition to the standard enzyme kinetic analyses of activity and stability, to understand how the attachment onto CNTs influences protein structure and function. The choice of technique depends on the method used for protein attachment and the resulting characteristics of the formulation (e.g., protein loading and dispersibility). In this section, we discuss these characterization techniques and include our own data of experiments to enable the reader to evaluate the CNT-protein conjugates prepared by the previously described methods of protein attachment onto carbon nanotubes.

1.4.1 Characterization of Proteins Physically Adsorbed onto Carbon Nanotubes

We have used enzymes as probes of protein structure and function. To measure the retention of enzyme activity upon attachment, it is necessary to quantify the amount of enzyme physically adsorbed onto carbon nanotubes. Measuring enzyme activity and detecting the change in its secondary structure by FT-IR spectroscopy before and after

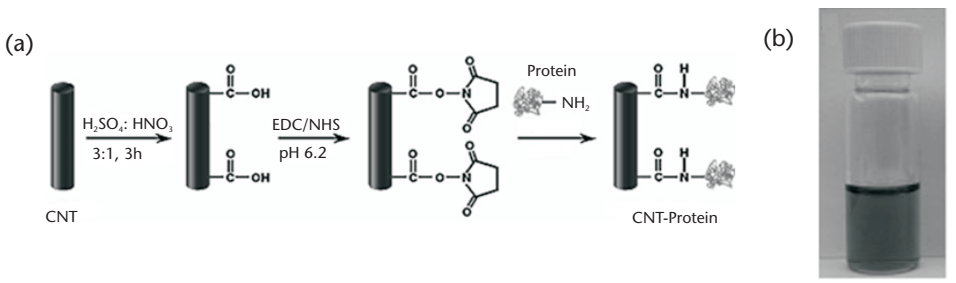

Figure 1.2 CNT-protein composites. (a) Schematic of protein functionalization of carbon nanotubes. (b) Photograph of water-soluble MWNTs. (Adapted from Asuri et al. [22].)

adsorption is useful for studying the influence of the hydrophobic nanoscale environment of carbon nanotubes on protein structure and function. Also, enzyme activity measurement can be used to determine the stability of enzymes adsorbed onto nanotubes under harsh conditions.

1.4.1.1 Measurement of Loading of Proteins on Carbon Nanotubes by the BCA Assay

The Pierce BCA Protein Assay uses a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. It involves the reduction of Cu^{2+} to Cu^{1+} ions by proteins to form a water soluble complex with BCA that strongly absorbs at 562 nm. Using this assay, the loading of proteins on carbon nanotubes is calculated as follows:

$$\text{Amount of protein loaded per mg SWNT} = C_i \cdot V_i - \sum_n C_j \cdot V_j \tag{1.1}$$

where,

C_i = Initial concentration of protein before exposing it to SWNTs;

V_i = Initial volume of protein solution added to SWNT dispersion;

C_j = Concentration of supernatant in *j*th wash;

V_j = Volume of supernatant in *j*th wash;

n = Number of washes performed.

Representative data for the loading of SBP on SWNTs is shown in Figure 1.3(a). The adsorption of SBP followed a pseudosaturation behavior, with a maximum loading of 575 μg SBP/mg SWNTs (Figure 1.3(a)). We observed that adsorbed SBP has a strong affinity for the SWNTs, with almost complete adsorption observed within the first minute (data not shown). Protein adsorption was irreversible at lower loadings. For example, at a loading of 250-μg protein/mg SWNT, essentially no protein desorption was observed (Figure 1.3(b)). The AFM images of SWNT-SBP conjugates are shown in Figure 1.3(c) and (d). The globular structures seen on the wire like SWNTs represent SBP molecules. Line scans reveal that a region on the SWNTs that does not contain SBP has a height of 3.7 nm, while a region containing SBP has a height of 9.6 nm, the difference (5.9 nm) being the height of adsorbed SBP molecules.

1.4.1.2 Retention of Protein Activity Upon Physical Adsorption

Adsorption onto CNTs can influence the structure, function, and stability of proteins. Since the catalytic activity of proteins relies on the retention of their native structure, measurement of catalytic activity can be used to evaluate the influence of the nanoscale environment of a CNT on protein properties. Using enzymes as highly sensitive probes of protein function, we studied the strong influence of the CNT surface on protein function and stability in harsh environments.

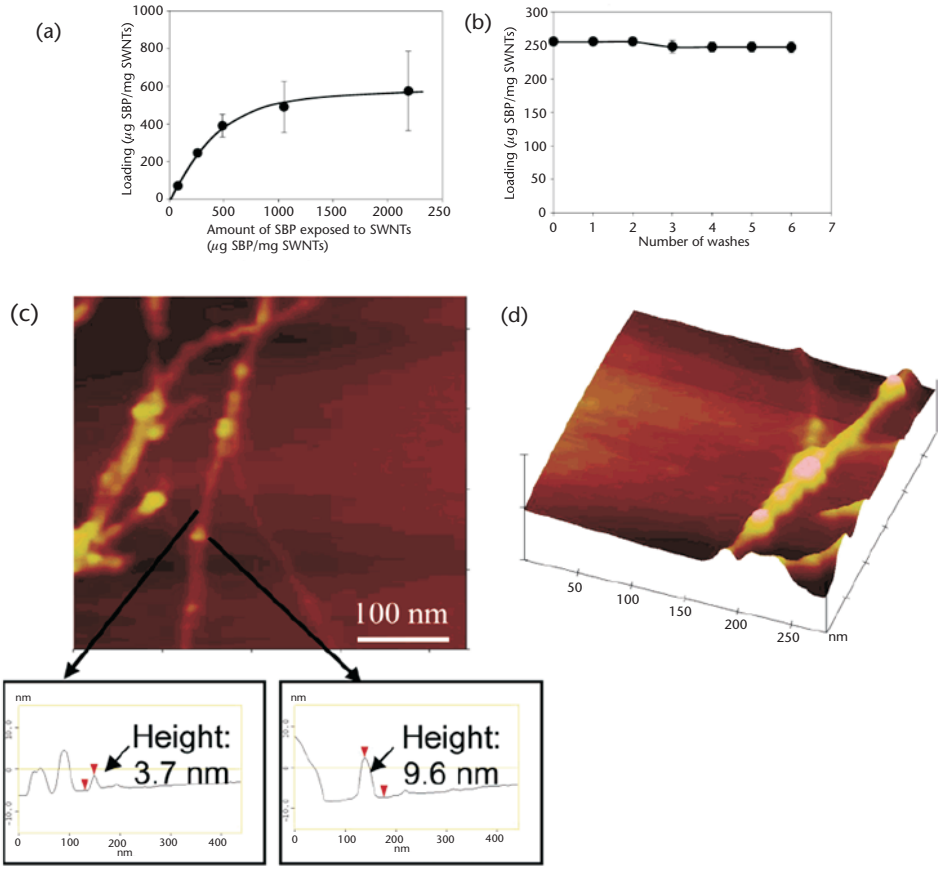

Figure 1.3 Loading of SBP on SWNTs: (a) Protein loading as a function of amount of SBP exposed to SWNTs. (b) Protein loading as a function of washing with fresh buffer. (c) AFM images of SBP adsorbed onto SWNTs. (d) Surface plot of height image for SBP adsorbed onto SWNTs revealing SBP molecules on the SWNTs. (Reprinted with permission from Karajanagi et al. . Copyright (2004) American Chemical Society [7].)

Determination of Protein Activity Upon Physical Adsorption

The structure, function, and spatial orientation of proteins attached onto carbon nanotubes strongly depends on the interactions of the nanotube surface with proteins. Since the catalytic activity and exquisite selectivity of proteins requires the near complete retention of native structure, measurement of enzyme activity can be used to evaluate the influence of the hydrophobic nanoscale environment of nanotubes on enzyme structure and function. To that end, comparison of the activity of native and immobilized enzyme can provide insight into the influence of carbon nanotubes on the retention or loss of native-like enzyme properties.

As an example, the activity of native SBP was measured using *p*-cresol as the substrate [27]. SBP catalyzes the oxidation of *p*-cresol in the presence of H_2O_2 to form fluorescent oligo- and polyphenol products. The initial reaction rates were measured by tracking the increase in fluorescence of the reaction mixture at excitation and emission wavelengths of 325 nm and 402 nm, respectively, using an HTS 7000 Plus Bio Assay Reader (Perkin-Elmer, Wellesley, MA). For a typical solution-phase assay, 0.15-μg/mL SBP was used with 20-mM *p*-cresol and 0.125-mM H_2O_2 in a volume of 200 μL. To measure the

activity of SBP adsorbed onto SWNTs, a well-mixed dispersion of SWNT-SBP at a concentration of 1.0 mg/mL was prepared in aqueous buffer. For a typical experiment, 0.2–2.5 μg of SWNTs were used on the basis of the loading of SBP. It was found that SBP retained significant specific activity at all loadings (Figure 1.4) ranging from 18 to 280 μg SBP/ mg SWNTs. The specific activity of SBP was strongly dependent on the loading; up to 28% of native solution activity was obtained at 50% of maximal surface coverage, and this value dropped to ca. 10% at 3% of maximal surface coverage (Figure 1.4). The increase in specific activity of adsorbed SBP with an increase in the surface coverage on SWNTs may be due to a higher retention of native structure at higher surface loadings.

Protein Stability under Harsh Conditions

Physical adsorption of proteins to carbon nanotubes enhances the stability of proteins in strongly denaturing environments where native proteins undergo substantial deactivation. To determine protein stability at elevated temperatures (Figure 1.5(a)), the nanotube-protein conjugates were subjected to these temperature conditions for different periods of time and cooled in an ice bath. Initial enzymatic reaction rates were then determined at room temperature. To determine rate constants in approximately 100% methanol (Figure 1.5(b)), the initial rates were measured in methanol as a function of incubation time. The deactivation constant was determined from the slope of a straight-line fit through the plot of $\log_e$ (% activity retained) versus time.

1.4.1.3 Determination of Protein Secondary Structure Using Fourier Transform Infrared (FT-IR) Spectroscopy

FT-IR spectroscopy is an established tool for the structural characterization of proteins [29]. The secondary structure of a protein can be quantitatively determined from a spectrum by considering the amide I region, between 1,600 and 1,700 cm^{-1}. This region, which consists mainly of the C-O stretching vibration of the backbone peptide bonds in proteins, was used to obtain the α helix and β sheet contents of the protein [30, 31]. We

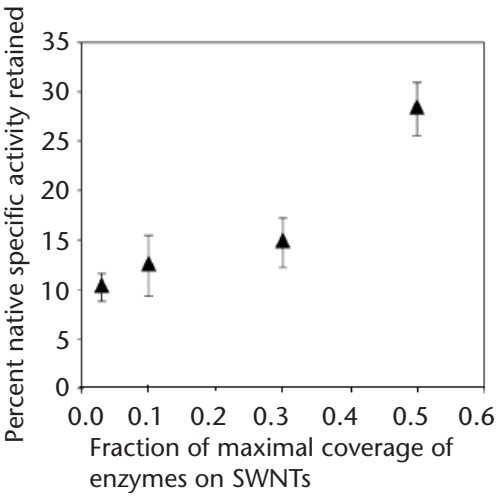

Figure 1.4 Enzymatic activity retained as a function of the surface coverage of SBP adsorbed on SWNTs (▲). (Reprinted with permission from Karajanagi et al. [7]. Copyright (2004) American Chemical Society.)

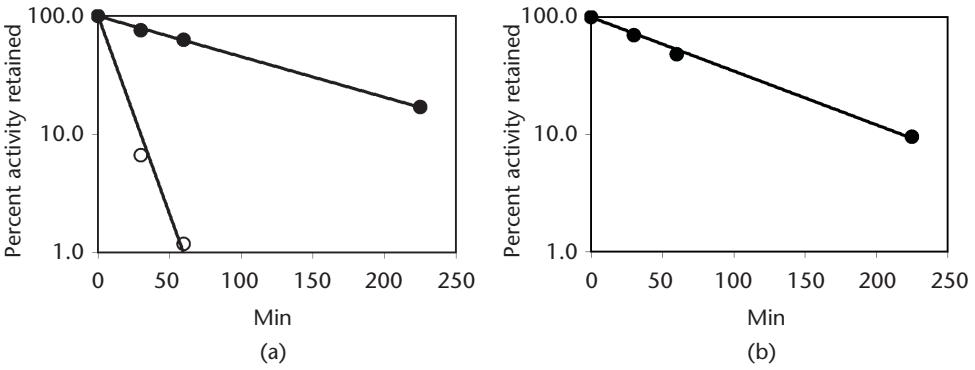

Figure 1.5 Time-dependent deactivation of native SBP (○) and SBP on SWNTs (●) (a) at 95°C and (b) in 100% methanol. The activities are normalized relative to the initial activity (activity at $t = 0$ min). Figure 1.5(b) does not contain % activity data for native SBP as it shows no activity in 100% methanol. (Reprinted with permission from Asuri et al. [28]. Copyright (2006) American Chemical Society.)

used FT-IR spectroscopy to compare the secondary structure of proteins before and after their adsorption onto carbon nanotubes. The differences in secondary structure between the soluble and adsorbed states are represented by the simple sum of magnitudes of changes in α helix and β sheet contents. For example, SBP showed a total change in α helical and β sheet content of 13% (Table 1.1), which suggests that SBP retains much of its native structure and activity upon absorption onto SWNTs.

1.4.2 Characterization of Protein-Solubilized Carbon Nanotubes

The aggregation state of the protein solubilized carbon nanotube dispersions can be characterized by ultraviolet-visible (UV-Vis) and Raman spectroscopy. These methods can be used effectively to distinguish between solubilized and nonsolubilized carbon nanotubes. We describe the use of these two techniques to characterize the solubilized CNTs.

1.4.2.1 Characterization of Carbon Nanotube Dispersions Using UV-Vis Spectroscopy

The UV-Vis absorption spectra of dispersions of SWNTs are known to be sensitive to their aggregation state [15]. The UV-Vis spectrum for SWNTs in water in the absence of a dispersing agent was essentially featureless, which indicates the presence of aggregates of SWNTs (data not shown). In contrast, the UV-Vis spectra for solutions of SWNTs

Table 1.1 Secondary Structure Percentages of SBP in Solution and Absorbed onto SWNTs, as Determined by FT-IR Spectroscopy Calculated from the Amide I Spectra

Sample	% α Helix	% β Sheet
Native solution of SBP	36.1 ± 1.2	25.1 ± 2.5
SBP absorbed onto SWNTs	27.9 ± 4.1	20.6 ± 6.9

(Adapted from Karajanagi et al. [7])

obtained using the proteins BSA and MJL exhibited sharp and well-resolved peaks (Figure 1.6). These sharp van Hove peaks are a characteristic of aqueous solutions containing debundled, individually dispersed SWNTs. The UV-Vis spectrum for SWNTs dispersed in water using NaDDBS also shows similar sharp features (Figure 1.6). We note that the spectra for SWNT-BSA and SWNT-MJL show peaks in the region beyond 900 nm that are red-shifted by approximately 10 to 15 nm with respect to those for SWNT-NaDDBS. This shift may be attributed to the greater accessibility of water to the SWNT surface for SWNT-BSA and SWNT-MJL than for SWNT-NaDDBS. It has been shown [32] that proteins can form a more porous layer on the SWNT surface than surfactants, thereby permitting water and other small molecules to associate with the surface.

1.4.2.2 Raman Spectroscopy to Probe Aggregation State of SWNTs

Raman spectroscopy is a versatile tool, which enables us to probe the aggregation state of SWNTs in solutions. In the case of protein-solubilized SWNT dispersions, the radial breathing mode (150–350 cm^{-1}) and tangential mode observations can be used as indicators of the quality of nanotube dispersion. To obtain the Raman spectra of the solubilized SWNT-BSA conjugates, 10 μg of the conjugates were placed on a cleaned silicon substrate and samples were analyzed using a laser excitation at 785 nm at a power of 10 mW, with a 50x lens. (Spectra were recorded from 0–3000 cm^{-1} for 4 minutes). The wavenumber calibration was carried out using the 521-cm^{-1} line of silicon substrate as a reference. The relative intensities of Raman peaks in the region between 230 and 270 cm^{-1} were found to be good indicators of the nanotube dispersion because of the change in Raman spectrum depending on their dispersed state. Specifically, in the aggregated state, (10,2) nanotubes are in resonance and (10,5) nanotubes are off resonance, while when SWNTs are dispersed, (10,5) nanotubes are in resonance and (10,2) nanotubes are off resonance. Accordingly, we see a peak at 267 cm^{-1} for SWNT aggregates, whereas solubilized SWNT-BSA conjugates show no peak at 267 cm^{-1} but a prominent peak at 234 cm^{-1} (Figure 1.7(a)). Furthermore, the peak corresponding to the tangential mode (centered at 1,591 cm^{-1}) for soluble SWNT-BSA was narrower than that for the aggregated

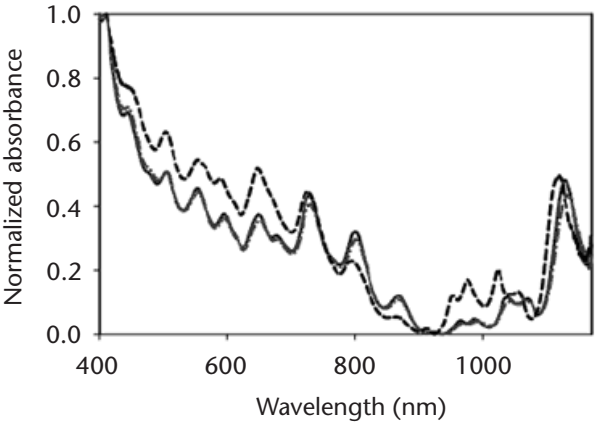

Figure 1.6 UV-Vis absorption spectra of SWNTs dispersed in water using NaDDBS (dashed line), BSA (solid line), and MJL (dash-dot line) normalized at 410 nm. (Reprinted with permission from Karajanagi et al. [14]. Copyright 2006 American Chemical Society.)

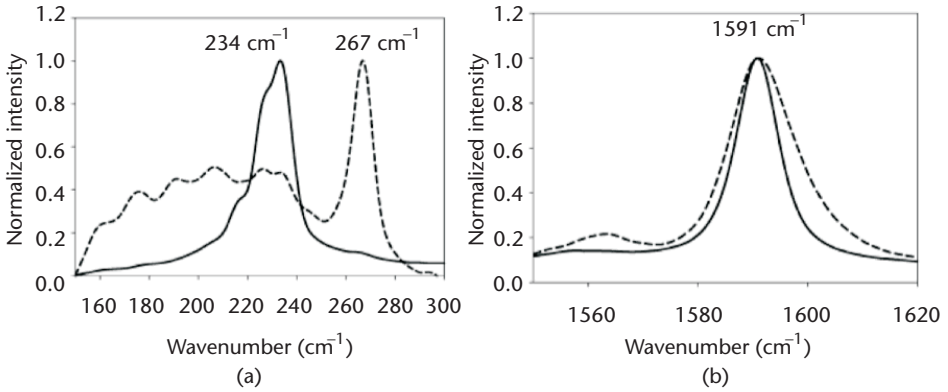

Figure 1.7 Raman spectroscopic analysis of SWNT-BSA conjugates (solid line) and SWNTs (dashed line) in (a) Radial breathing mode, (b) Tangential mode at 785-nm excitation. (Reprinted with permission from Karajanagi et al. [14]. Copyright 2006 American Chemical Society.)

SWNTs, with a decrease in the full width at a half-maximum of approximately 5 cm^{-1} (Figure 1.7(b)), which is in agreement with similar observations for solutions containing individually dispersed SWNTs [20, 33, 34].

1.4.3 Characterization of Covalently Attached Carbon Nanotube-Protein Conjugates

We determined the retention of protein structure and function upon covalent attachment to carbon nanotubes using Hammett analysis of protein activity as well as spectroscopic techniques, such as CD and fluorescence spectroscopies. Structural analysis by CD or fluorescence spectroscopy is not possible for conjugates prepared by physical adsorption of proteins onto bundles of nanotubes or for covalent MWNT-protein conjugates because of interference from the carbon nanotubes. On the other hand, because of the higher solubility and higher protein loading obtained in case of covalent attachment of proteins to oxidized SWNTs, CD, and fluorescence measurement-based structural studies are possible. The activity measurements of the nanotube-protein conjugates indicated that the conjugates demonstrated not only enhanced stability in harsh conditions, but also operational and storage stability.

1.4.3.1 Hammett Analysis for Protein Structure-Activity Relationship

It is often important to study the structural perturbations of the protein to further probe the effects of immobilization. However, analyses of proteins on MWNTs, such as CD and FT-IR spectroscopy, are hindered by the strong absorbance and intrinsic fluorescence of nanotubes. Hammett analysis, on the other hand, is a well-established kinetic technique to probe an enzyme's transition state structure [27]. In the case of SBP catalysis, the Hammett coefficient ρ provides a measure of the sensitivity of SBP's catalytic efficiency to the electronic nature of substituents on phenolic substrates (electron-donating or electron-withdrawing), as reflected in the values of their substituent electronic parameter σ. Positive values of σ represent electron withdrawal by the substituent from the aromatic ring, whereas negative values indicate electron release to the ring. Deviation in ρ values for SBP bound to a support from that for the native enzyme in aqueous buffer

would indicate that the active site structure of the enzyme is perturbed by adsorption onto the support.

$$\log\left(\frac{V_{max}}{K_M}\right) = \sigma \cdot \rho + constant \tag{1.2}$$

To that end, we determined the Hammett coefficient, ρ, based on a modified form of the Hammett equation for SBP (1.2), using a series of phenolic substrates, *p*-OC_2H_5, *p*-CH_3, *p*-CH_2OH, and *p*-Cl with different values of the electronic parameter (σ) varying from -0.24 to +0.23 [27].[1] The standard kinetic parameters—maximum reaction rate (V_{max}) and Michaelis constant (K_M)—were determined for the different substrates using nonlinear Michaelis-Menten fits. Figure 1.8 depicts the Hammett analysis for native SBP and MWNT-SBP in aqueous buffer. Interestingly, the Hammett coefficients for native and immobilized SBP are essentially identical. The comparable values of ρ indicate that the differences in the active site structure for native and immobilized SBP are minimal; therefore, the mechanism of catalysis for MWNT-SBP is similar to that for native SBP. Thus, the high retention of catalytic activity for the MWNT-SBP conjugates is consistent with the enzyme retaining its intrinsic active site structure throughout the attachment process.

1.4.3.2 Determination of Protein Secondary Structure Using Circular Dichroism (CD) Spectroscopy

CD spectroscopy is used for studying the conformational stability of a protein under harsh conditions—thermal stability, pH stability, and stability against chemical denaturants. CD measures the difference in absorbance of a sample between left-hand polarized light and right-hand polarized light; these differences arise because of

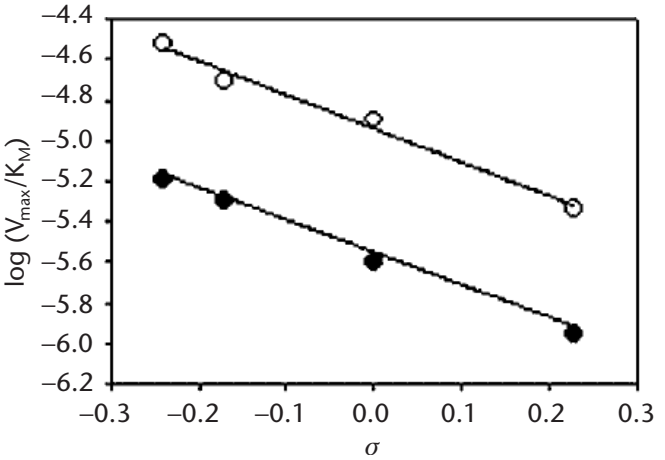

Figure 1.8 Influence of the substituent electronic parameter, σ, on the catalytic efficiency of native SBP (○) and MWNT-SBP conjugates (●) in aqueous buffer. Slope of the lines gives the Hammett coefficient in each case: ρ for native SBP = –1.6 ± 0.1; ρ for MWNT-SBP = –1.5 ± 0.2. (Adapted from Asuri et al. [22].)

[1] The values of the electronic parameter (σ) for *p*-OC_2H_5, *p*-CH_3,*p*-CH_2OH, and *p*-Cl are –0.24, –0.17, 0.00, and 0.23 respectively.

structural asymmetry in a molecule. Secondary protein structure is usually comprised of α helices and β sheets, each producing a characteristic spectrum in the far-UV range (190–250 nm). α helices produce a spectrum with valleys around 208 and 222 nm, while β sheets show a single valley around 215 nm. As proteins lose their native structure and become less ordered, the absence of regular structure is reflected in zero CD intensity. Thus, by measuring the far-UV CD spectrum of a protein before and after attachment onto nanotubes, one can get an idea of how the structure of the protein has been altered. Using data processing software that can analyze a CD spectrum and determine the relative content of α helix and β sheet, we determined that HRP attached to SWNTs retained 68% of its native α helix content (Figure 1.9).

We used CD spectroscopy to monitor the change in the secondary structure of HRP upon exposure to varying concentrations of GdnHCl denaturant and high temperatures. The secondary structure of HRP and SWNT-HRP conjugates were thus monitored by CD, using a protein concentration of 0.05 mg/mL, in the presence or absence of denaturant. After equilibrating the samples with GdnHCl for 24 hours, CD spectra were measured (Figure 1.10(b)). The concentration of GdnHCl required to denature the protein by 50% in the sample (C_m) increased from 1.6 to 2.4M as a result of conjugation. For thermal denaturation, the temperature was slowly raised (0.5°C/min) from 20°C to 99°C while spectra were taken (Figure 1.10(a)). The temperature required to unfold the protein by 50% in the sample (T_m) increased from 79°C to 92°C for the SWNT-HRP conjugate. Characterization by CD spectroscopy therefore revealed a substantial increase in protein stability under stronger denaturing conditions and higher temperatures when covalently attached to SWNTs.

1.4.3.3 Characterization of Protein Tertiary Structure Using Tryptophan Fluorescence

Proteins contain three aromatic amino acid residues (tryptophan, tyrosine, and phenylalanine), which contribute to their intrinsic fluorescence. In particular, the polarity and charge densities surrounding tryptophan residues influence both the fluorescence intensity and maximal emission fluorescence wavelength (λ_{max}). As the protein denatures, losing its tertiary structure, the environment around buried tryptophan resi-

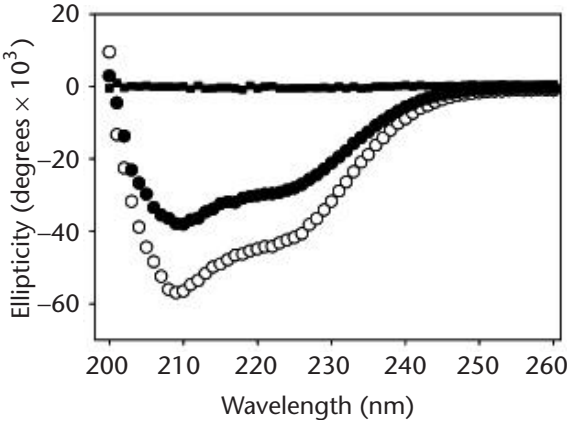

Figure 1.9 Far-UV CD spectra of native HRP (○), SWNT-HRP (●), and bare SWNTs (■). (Reprinted with permission from Asuri et al. [35]. Copyright 2007 American Chemical Society.)

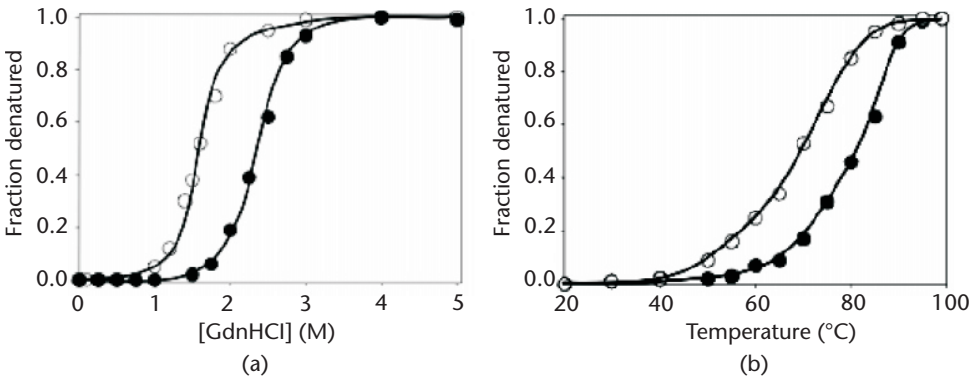

Figure 1.10 Fraction of HRP denatured determined by monitoring the CD signal at 222 nm of native HRP (○) and SWNT-HRP (●) as a function of (a) GdnHCl concentration and (b) solution temperature. (Reprinted with permission from Asuri et al. [35]. Copyright 2007 American Chemical Society.)

dues changes drastically, eventually leading to their exposure to solution. Thus, upon protein denaturation, the fluorescence intensities and tryptophan emission wavelength tend toward those of free tryptophan in solution; structural changes can thus be inferred from alteration of the tryptophan's microenvironment.

As an example, the protein HRP contains one buried tryptophan residue at position 117 [36]. When GdnHCl was used as the denaturant (Figure 1.11), the fluorescence intensities and λ_{max} values for both native HRP and SWNT-HRP conjugate were lower than those for free L-tryptophanamide when excited at 283 nm at lower Gdn HCI concentrations [27, 36]; however, at higher GdnHCl concentrations, both the values approached those of L-tryptophanamide, indicating that HRP's tryptophan residue was now more accessible to the solvent due to protein denaturation. The SWNT-HRP conjugates showed a more gradual increase toward the values of L-tryptophanamide than native HRP, indicating that they are more stable under denaturing conditions than native HRP.

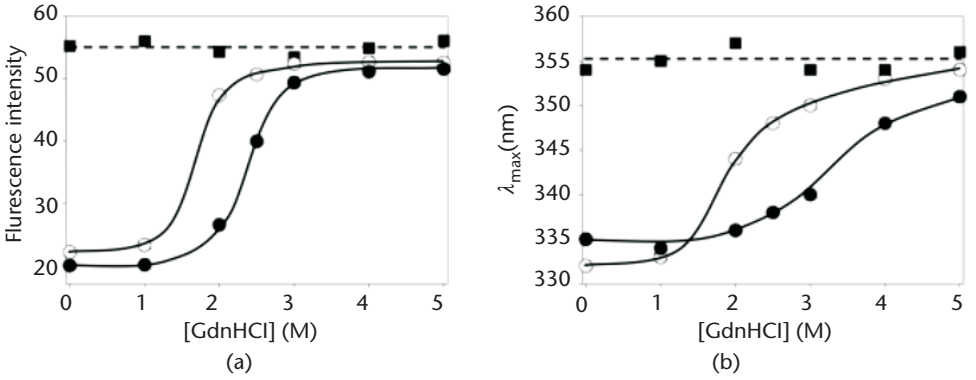

Figure 1.11 (a) Fluorescence intensity (excitation at 283 nm and emission at λ_{max}) and (b) λ_{max} of L-tryptophanamide (■), native HRP (○), and SWNT-HRP (●) as a function of GdnHCl concentration. (Reprinted with permission from Asuri et al. [35]. Copyright 2007 American Chemical Society.)

1.4.3.4 Thermostabilization of Proteins Via Covalent Attachment onto Carbon Nanotubes

Exposure of proteins to high temperatures can lead to irreversible unfolding and deactivation, posing a critical limitation to their commercial use. We have found that covalent attachment of proteins onto MWNTs leads to thermostabilization of the protein. There is a certain optimal temperature (T_{opt}) at which the protein's catalytic activity is at its maximum, beyond which the protein unfolds and gets deactivated irreversibly. The thermostabilization of proteins upon immobilization causes an elevation in T_{opt} values. While the T_{opt} for native SBP was found to be approximately 75°C, the MWNT-SBP conjugates displayed a T_{opt} of approximately 90°C (Figure 1.12), which is close to the native melting temperature of SBP (T_m = 90.5°C) [37]. This enhanced stability of MWNT-SBP leads to a 2.5-fold increase in the maximal initial reaction rate at 90°C as compared to that of native SBP at 75°C, thus rendering the protein formulation well suited for applications where harsh conditions are required.

1.4.3.5 Operational and Storage Stability of Carbon Nanotube-Enzyme Conjugates

Two other issues concerning the commercial use of native enzymes in biocatalysis are difficulty of enzyme reuse and loss of enzymatic activity on prolonged storage. While macroscopic supports provide ease of separation and reusability of immobilized enzymes, stabilization provided by such supports is significantly less compared to nanoscale supports [28]. On the other hand, use of inherently long oxidized MWNTs for attaching enzymes not only stabilizes enzymes under different reaction and storage conditions but also allows easy recovery of conjugates from reaction mixture through filtration. A mat-like film forms after filtering the reaction mixture through the filter membrane; this film can be redispersed in an aqueous buffer by minimal sonication. For example, SBP, which was covalently attached to MWNT, retained about 70% of its initial activity even after being reused over 100 times (Figure 1.13(a)). Additionally, such conjugates were found to be stable for an extended period. Even after 30 days, the MWNT-SBP conjugates retained ca. 70% of their initial activity (Figure 1.13(b)). On the other hand, native SBP retained only about 30% of its initial activity. These observations indeed suggest that

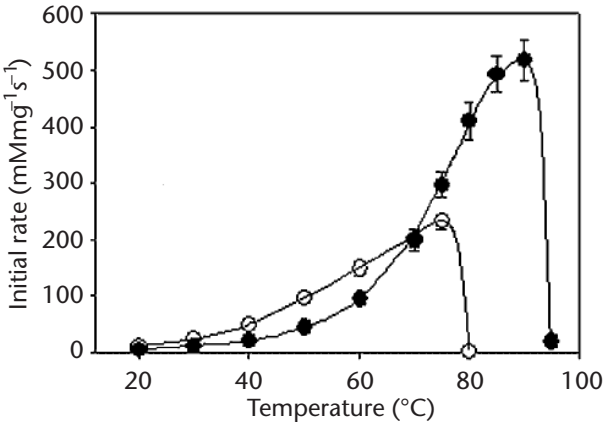

Figure 1.12 Influence of temperature on the kinetics of native SBP (○) and MWNT-SBP (●) in aqueous buffer. (Adapted from Asuri et al. [22].)

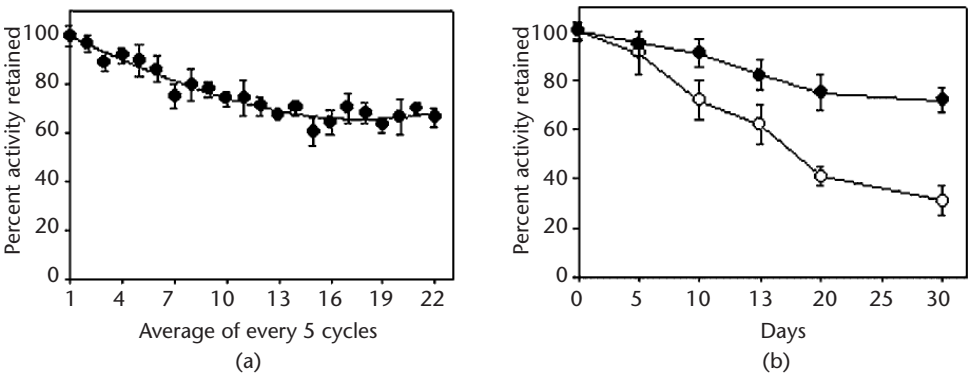

Figure 1.13 Operational and storage stability of MWNT-SBP. (a) Reusability of MWNT-SBP conjugates. (b) Retention of enzymatic activity in aqueous buffer at room temperature—native SBP (○) and MWNT-SBP (●). (Adapted from Asuri et al. [22].)

nanoscale supports, such as MWNTs, make the enzyme formulation reusable and storage compatible.

1.5 Discussion and Commentary

As discussed in the previous sections, characterization of CNT-protein conjugates exhibits the subtle differences observed due to these different methods of protein immobilization on CNTs. Biofunctionalization of CNTs and characterization of resultant hybrid material has been carried out for various reasons. First, it is of great interest to study how different biomolecules interact with carbon nanotubes compared to conventional micro or macroscale supports. For this study, biomolecules were interfaced with nanotubes through physical adsorption or covalent attachment. Second, use of CNTs, as drug delivery vehicles or for construction of self-assembled nanoscaled superstructures, requires them to be water soluble. The amphiphilic nature of biomolecules can be exploited in solubilizing CNTs. Additionally, these biofunctionalized CNTs can be used in a wide range of applications, which include biosensing, bioelectrochemistry, biomedicine, and intracellular delivery of peptides and proteins. In the course of preparing such nanobiocomposite materials and realizing their potential applications, we have critically optimized our protocols to overcome some of the problems that can occur with these techniques. In this section, we discuss some precautions to take while preparing nanotube-protein conjugates.

CNTs, being in the form of clumpy or fluffy black powder, should be handled using personal protective equipment and in safety hoods with adequate ventilation. If inhaled, remove to fresh air. If breathing difficulties persist, get medical attention. In case of contact, immediately flush eyes or skin with plenty of water for at least 15 minutes. If irritation develops or persists, get medical attention. During physical adsorption of proteins, we have observed that protein adsorption occurs in the initial 5 to 10 minutes of mixing CNTs and protein solutions. Therefore, before mixing these solutions, it is necessary to achieve a uniform dispersion of CNTs through effective sonication. As an additional precaution, it is advisable to thaw the protein-containing vial after removing

it from storage temperature of 4°C or –20°C. This is to ensure that the protein powder does not pick up unwanted moisture on exposure to air.

As a common safety practice, it is recommended to handle acids in fume hoods while carrying out acid oxidation of CNTs. During the sonication step, intermittent swirling of nanotube-acid mixture would maintain well-mixed reaction conditions. The rise in temperature due to exothermic acid oxidation and sonication leads to heating up of water bath, which could cause excessive oxidation of CNTs and hence formation of fine, non-recoverable particles. Therefore, periodic replacement of water in the bath with ice-cold water is necessary for maintaining desired operating conditions. Filtration of CNTs after acid oxidation leads to formation of a densely packed nanotube mat. A simplistic approach to get uniform nanotube suspension would be to disperse this nanotube film in enough volumes of milliQ water by sonication. After ester functionalization and filtration of oxidized CNTs, ensure that the filtered CNTs film does not dry out completely; disperse the film immediately in protein solution. The pH of the buffer used for carrying out EDC-NHS chemistry was found to govern the extent of ester functionalization onto carbon nanotubes. While a pH range of 4 to 7 is suitable, lower pH conditions results in higher functionalization and hence higher protein attachment.

There are a few notable differences between the three methods of nanotube-protein preparation, and the choice of one over the other is governed by the properties of conjugates desired and their end application. Some of the differences between physical adsorption of proteins onto CNTs, protein solubilization of CNTs, and covalent attachment methods of proteins onto CNTs are listed in Table 1.2.

Troubleshooting Table

Problem	Explanation	Potential Solution
Low catalytic activity of proteins upon covalent attachment.	Low protein attachment onto oxidized CNTs in EDC-NHS reaction steps.	Prolonged exposure of active ester to air can lead to its hydrolysis. Add protein solution immediately after nanotube activation.
Presence of CNT aggregates after acid oxidation and filtration steps.	Inefficient oxidation of CNTs.	Increase acid treatment duration.

1.6 Applications Notes

The methods of noncovalent and covalent functionalization of carbon nanotubes with proteins have been used in numerous applications two of which are highlighted in this

Table 1.2 General Comparison Between the Three Methods of Protein Attachment onto CNTs

Physical Adsorption of Proteins onto CNTs	Protein Solubilization of CNTs	Covalent Attachment of Proteins onto CNTs
Ease of attachment	Ease of attachment	Cumbersome with many steps involved
Facile method of attachment preserves native structural and functional properties of both CNTs and proteins	Ultrasonication can lead to protein denaturation	Chemical modification of CNTs can compromise its native electronic and mechanical properties
Leaching of proteins upon agitation and storage	Leaching of proteins upon agitation and storage	No leaching effect observed
Conjugates present in aggregate form	Conjugates are water-soluble	Conjugates are water-soluble
Conjugates can be separated from solution by centrifugation	Conjugates can be separated from solution by filtration	Conjugates can be separated from solution by filtration

section. As our first example, we consider the role of nanobiocomposites in carrying out biotransformation in biphasic medium wherein phase transfer biocatalysis involves the mass transfer of water-insoluble substrates from organic to aqueous phase. Therefore, interfacial adsorption of enzymes is desired to carry out biotransformations at the aqueous-organic interface. We have demonstrated that SWNTs along with the attached protein can be directed to aqueous-organic interfaces with the aid of surfactants [38]. SWNTs as a protein support not only provide high intrinsic surface area but also overcome any intraparticle diffusional limitations that restrict use of enzymes in biphasic system. We showed that physical adsorption increased specific enzyme activity by three orders of magnitude as compared to native enzymes in aqueous phase, with enhanced stability at high temperatures. Thus, the nanotube-mediated interfacial assembly of enzymes can be very advantageous in directing greater amounts of enzymes from the bulk aqueous phase to the interface and in increasing the stability of enzymes against inactivation.

The procedure of covalent attachment of proteins onto carbon nanotubes has been successfully employed to produce highly active and stable DNAzyme-carbon nanotube hybrids. Certain small single-stranded DNA fragments possess catalytic activity (e.g., endonuclease-type activity) and are known as DNAzymes [39]. Yim et al. covalently attached streptavidin to acid-treated MWNTs using EDC-NHS chemistry, followed by the binding of biotinylated DNAzyme to yield MWNT-DNAzyme conjugates that were soluble in aqueous buffer [40]. The MWNT-DNAzyme conjugates followed Michaelis-Menten kinetics under the conditions where substrate concentration is higher than that of DNAzyme (Figure 1.14). Additionally, this hybridization led to a formulation providing very high turnover numbers, without the need for substrate-DNAzyme hybridization between each catalytic event. Conjugating such DNAzymes with nanomaterials can be of potential use in the development of biosensors to detect metal ions and nucleic acids as well as in designing strategies for directing nanoparticle assembly [41, 42].

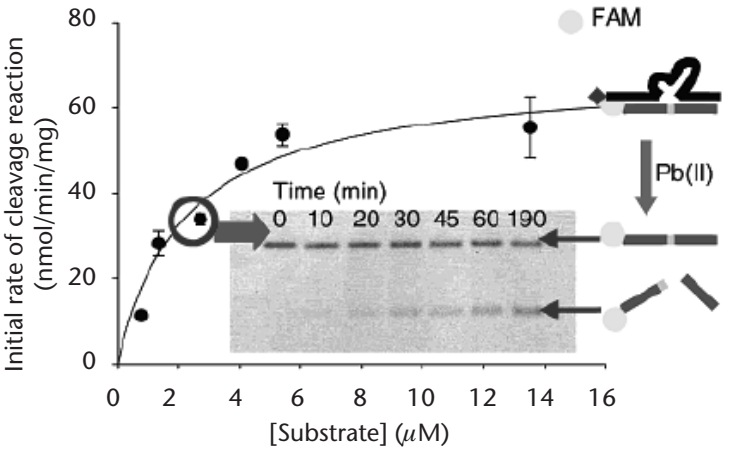

Figure 1.14 Catalytic activity of MWNT-DNAzyme conjugates. The line represents a nonlinear fit of the Michaelis-Menten expression to the data. Inset shows analysis of extent of conversion of fluorescently labeled substrate DNA by polyacrylamide gel electrophoresis (PAGE), with upper band representing uncleaved DNA and lower band representing cleaved fragments. (Reprinted with permission from Yim et al. [40]. Copyright 2005 American Chemical Society.)

1.7 Summary Points

Physical adsorption of proteins onto CNTs is a simple and effective method for preparing nanotube-protein conjugates without any modification of electronic and mechanical properties of CNTs.

Protein assisted solubilization of CNTs can be important for biomedical applications, such as biomedical devices, cellular delivery; besides, the wide variety of functional groups on adsorbed proteins can act as orthogonal reactive handles for the functionalization of CNTs.

Water-soluble CNT-protein conjugates, prepared by acid oxidation of CNTs and covalent attachment of proteins, possess high enzyme activity, high stability and reusability, and low diffusional resistance; these conjugates can find application in biomaterials, biotransformations, medicine and self-assembled materials.

Acknowledgments

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References

[1] Ajayan, P. M., "Nanotubes from carbon," *Chemical Reviews* Vol. 99, No. 7 1999, pp. 1787–1799.

[2] Chen, R. J., Bangsaruntip, S., Drouvalakis, K. A., Kam, N. W. S., Shim, M., Li, Y. M., Kim, W., Utz, P. J., and Dai, H. J., "Noncovalent functionalization of carbon nanotubes for highly specific electronic biosensors," *Proceedings of the National Academy of Sciences of the United States of America* Vol. 100, No. 9 2003, pp. 4984–4989.

[3] Pantarotto, D., Briand, J. P., Prato, M., and Bianco, A., "Translocation of bioactive peptides across cell membranes by carbon nanotubes," *Chemical Communications*, No. 1 2004, pp. 16–17.

[4] Kam, N. W. S., Jessop, T. C., Wender, P. A., and Dai, H. J., "Nanotube molecular transporters: Internalization of carbon nanotube-protein conjugates into mammalian cells," *Journal of the American Chemical Society* Vol. 126, No. 22 2004, pp. 6850–6851.

[5] Barone, P. W., Parker, R. S., and Strano, M. S., "In vivo fluorescence detection of glucose using a single-walled carbon nanotube optical sensor: Design, fluorophore properties, advantages, and disadvantages," *Analytical Chemistry* Vol. 77, No. 23 2005, pp. 7556–7562.

[6] Kam, N. W. S., O'Connell, M., Wisdom, J. A., and Dai, H. J., "Carbon nanotubes as multifunctional biological transporters and near-infrared agents for selective cancer cell destruction," *Proceedings of the National Academy of Sciences of the United States of America* Vol. 102, No. 33 2005, pp. 11600–11605.

[7] Karajanagi, S. S., Vertegel, A. A., Kane, R. S., and Dordick, J. S., "Structure and function of enzymes adsorbed onto single-walled carbon nanotubes," *Langmuir* Vol. 20, No. 26 2004, pp. 11594–11599.
The authors investigate the structure and function of proteins immobilized onto SWNTs to develop a better understanding of SWNT-protein interactions.

[8] Panhuis, M. I. H., Salvador-Morales, C., Franklin, E., Chambers, G., Fonseca, A., Nagy, J. B., Blau, W. J., and Minett, A. I., "Characterization of an interaction between functionalized carbon nanotubes and an enzyme," *Journal of Nanoscience and Nanotechnology* Vol. 3, No. 3 2003, pp. 209–213.

[9] Carrillo, A., Swartz, J. A., Gamba, J. M., Kane, R. S., Chakrapani, N., Wei, B. Q., and Ajayan, P. M., "Noncovalent functionalization of graphite and carbon nanotubes with polymer multilayers and gold nanoparticles," *Nano Letters* Vol. 3, No. 10 2003, pp. 1437–1440.

[10] Shim, M., Kam, N. W. S., Chen, R. J., Li, Y. M., and Dai, H. J., "Functionalization of carbon nanotubes for biocompatibility and biomolecular recognition," *Nano Letters* Vol. 2, No. 4 2002, pp. 285–288.

[11] Pompeo, F., and Resasco, D. E., "Water solubilization of single-walled carbon nanotubes by functionalization with glucosarnine," *Nano Letters* Vol. 2, No. 4 2002, pp. 369–373.

[12] Star, A., Steuerman, D. W., Heath, J. R., and Stoddart, J. F., "Starched carbon nanotubes," *Angewandte Chemie-International Edition* Vol. 41, No. 14 2002, pp. 2508.

[13] Asuri, P., Karajanagi, S. S., Kane, R. S., and Dordick, J. S., "Polymer-nanotube-enzyme composites as active antifouling films," *Small* Vol. 3, No. 1 2007, pp. 50–53.

[14] Karajanagi, S. S., Yang, H. C., Asuri, P., Sellitto, E., Dordick, J. S., and Kane, R. S., "Protein-assisted solubilization of single-walled carbon nanotubes," *Langmuir* Vol. 22, No. 4 2006, pp. 1392–1395.

The authors report a simple method that uses proteins to solubilize SWNTs in water.

[15] O'Connell, M. J., Bachilo, S. M., Huffman, C. B., Moore, V. C., Strano, M. S., Haroz, E. H., Rialon, K. L., Boul, P. J., Noon, W. H., Kittrell, C., Ma, J. P., Hauge, R. H., Weisman, R. B., and Smalley, R. E., "Band gap fluorescence from individual single-walled carbon nanotubes," *Science* Vol. 297, No. 5581 2002, pp. 593–596.

[16] Islam, M. F., Rojas, E., Bergey, D. M., Johnson, A. T., and Yodh, A. G., "High weight fraction surfactant solubilization of single-wall carbon nanotubes in water," *Nano Letters* Vol. 3, No. 2 2003, pp. 269–273.

[17] O'Connell, M. J., Boul, P., Ericson, L. M., Huffman, C., Wang, Y. H., Haroz, E., Kuper, C., Tour, J., Ausman, K. D., and Smalley, R. E., "Reversible water-solubilization of single-walled carbon nanotubes by polymer wrapping," *Chemical Physics Letters* Vol. 342, No. 3–4 2001, pp. 265–271.

[18] Huang, W. J., Fernando, S., Allard, L. F., and Sun, Y. P., "Solubilization of single-walled carbon nanotubes with diamine-terminated oligomeric poly(ethylene glycol) in different functionalization reactions," *Nano Letters* Vol. 3, No. 4 2003, pp. 565–568.

[19] Zheng, M., Jagota, A., Semke, E. D., Diner, B. A., McLean, R. S., Lustig, S. R., Richardson, R. E., and Tassi, N. G., "DNA-assisted dispersion and separation of carbon nanotubes," *Nature Materials* Vol. 2, No. 5 2003, pp. 338–342.

[20] Dieckmann, G. R., Dalton, A. B., Johnson, P. A., Razal, J., Chen, J., Giordano, G. M., Munoz, E., Musselman, I. H., Baughman, R. H., and Draper, R. K., "Controlled assembly of carbon nanotubes by designed amphiphilic peptide helices," *Journal of the American Chemical Society* Vol. 125, No. 7 2003, pp. 1770–1777.

[21] Numata, M., Asai, M., Kaneko, K., Bae, A. H., Hasegawa, T., Sakurai, K., and Shinkai, S., "Inclusion of cut and as-grown single-walled carbon nanotubes in the helical superstructure of schizophyllan and curdlan (ss-1,3-glucans)," *Journal of the American Chemical Society* Vol. 127, No. 16 2005, pp. 5875–5884.

[22] Asuri, P., Karajanagi, S. S., Sellitto, E., Kim, D. Y., Kane, R. S., and Dordick, J. S., "Water-soluble carbon nanotube-enzyme conjugates as functional biocatalytic formulations," *Biotechnology and Bioengineering* Vol. 95, No. 5 2006, pp. 804–811.

The authors present a method of preparing water-soluble carbon nanotube-enzyme conjugates, which retain high activity and are stable at high temperatures.

[23] Jiang, K. Y., Schadler, L. S., Siegel, R. W., Zhang, X. J., Zhang, H. F., and Terrones, M., "Protein immobilization on carbon nanotubes via a two-step process of diimide-activated amidation," *Journal of Materials Chemistry* Vol. 14, No. 1 2004, pp. 37–39.

[24] Vix-Guterl, C., Couzi, M., Dentzer, J., Trinquecoste, M., and Delhaes, P., "Surface characterizations of carbon multiwall nanotubes: Comparison between surface active sites and Raman Spectroscopy," *Journal of Physical Chemistry B* Vol. 108, No. 50 2004, pp. 19361–19367.

[25] Wang, P., "Nanoscale biocatalyst systems," *Current Opinion in Biotechnology* Vol. 17, No. 6 2006, pp. 574–579.

[26] Huang, W. J., Taylor, S., Fu, K. F., Lin, Y., Zhang, D. H., Hanks, T. W., Rao, A. M., and Sun, Y. P., "Attaching proteins to carbon nanotubes via diimide-activated amidation," *Nano Letters* Vol. 2, No. 4 2002, pp. 311–314.

[27] Ryu, K., and Dordick, J. S., "How Do Organic-Solvents Affect Peroxidase Structure and Function," *Biochemistry* Vol. 31, No. 9 1992, pp. 2588–2598.

[28] Asuri, P., Karajanagi, S. S., Yang, H. C., Yim, T. J., Kane, R. S., and Dordick, J. S., "Increasing protein stability through control of the nanoscale environment," *Langmuir* Vol. 22, No. 13 2006, pp. 5833–5836.

The authors report that carbon nanotubes may be used to enhance protein stability in harsh environments.

[29] Jackson, M., and Mantsch, H. H., "The use and misuse of FTIR Spectroscopy in the determination of protein-structure," *Critical Reviews in Biochemistry and Molecular Biology* Vol. 30, No. 2 1995, pp. 95–120.

[30] Dong, A., Huang, P., and Caughey, W. S., "Protein secondary structures in water from 2nd-derivative amide-I infrared spectra," *Biochemistry* Vol. 29, No. 13 1990, pp. 3303–3308.

[31] Vedantham, G., Sparks, H. G., Sane, S. U., Tzannis, S., and Przybycien, T. M., "A holistic approach for protein secondary structure estimation from infrared spectra in H_2O solutions," *Analytical Biochemistry* Vol. 285, No. 1 2000, pp. 33–49.

[32] Barone, P. W., Baik, S., Heller, D. A., and Strano, M. S., "Near-infrared optical sensors based on single-walled carbon nanotubes," *Nature Materials* Vol. 4, No. 1 2005, pp. 86–U16.

[33] Dalton, A. B., Stephan, C., Coleman, J. N., McCarthy, B., Ajayan, P. M., Lefrant, S., Bernier, P., Blau, W. J., and Byrne, H. J., "Selective interaction of a semiconjugated organic polymer with single-wall nanotubes," *Journal of Physical Chemistry B* Vol. 104, No. 43 2000, pp. 10012–10016.

[34] Rao, A. M., Chen, J., Richter, E., Schlecht, U., Eklund, P. C., Haddon, R. C., Venkateswaran, U. D., Kwon, Y. K., and Tomanek, D., "Effect of van der Waals interactions on the Raman modes in single walled carbon nanotubes," *Physical Review Letters* Vol. 86, No. 17 2001, pp. 3895–3898.

[35] Asuri, P., Bale, S. S., Pangule, R. C., Shah, D. A., Kane, R. S., and Dordick, J. S., "Structure, function, and stability of enzymes covalently attached to single-walled carbon nanotubes," *Langmuir* Vol. 23, No. 24 2007, pp. 12318–12321.

The authors report that enzymes covalently attached to SWNTs retain a high fraction of their native structure and function, with enhanced stability in harsh environments.

[36] Welinder, K. G., "Amino-Acid Sequence Studies of Horseradish-Peroxidase .4. Amino and Carboxyl Termini, Cyanogen-Bromide and Tryptic Fragments, the Complete Sequence, and Some Structural Characteristics of Horseradish Peroxidase-C," *European Journal of Biochemistry* Vol. 96, No. 3 1979, pp. 483–502.

[37] McEldoon, J. P., and Dordick, J. S., "Unusual thermal stability of soybean peroxidase," *Biotechnology Progress* Vol. 12, No. 4 1996, pp. 555–558.

[38] Asuri, P., Karajanagi, S. S., Dordick, J. S., and Kane, R. S., "Directed assembly of carbon nanotubes at liquid-liquid interfaces: Nanoscale conveyors for interfacial biocatalysis," *Journal of the American Chemical Society* Vol. 128, No. 4 2006, pp. 1046–1047.

The authors demonstrate that SWNT-enzyme conjugates can be directed to aqueous-organic interfaces and used for interfacial biocatalysis.

[39] Li, Y. F., and Breaker, R. R., "Deoxyribozymes: new players in the ancient game of biocatalysis," *Current Opinion in Structural Biology* Vol. 9, No. 3 1999, pp. 315–323.

[40] Yim, T. J., Liu, J. W., Lu, Y., Kane, R. S., and Dordick, J. S., "Highly active and stable DNAzyme - Carbon nanotube hybrids," *Journal of the American Chemical Society* Vol. 127, No. 35 2005, pp. 12200–12201.

[41] Liu, J. W., and Lu, Y., "A colorimetric lead biosensor using DNAzyme-directed assembly of gold nanoparticles," *Journal of the American Chemical Society* Vol. 125, No. 22 2003, pp. 6642–6643.

[42] Sando, S., Sasaki, T., Kanatani, K., and Aoyama, Y., "Amplified nucleic acid sensing using programmed self-cleaving DNAzyme," *Journal of the American Chemical Society* Vol. 125, No. 51 2003, pp. 15720–15721.