Protein-Affinity of Single-Walled Carbon Nanotubes in Water

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The interactions of single-walled carbon nanotubes (SWNTs) with ferritin protein in water were investigated. It was found that SWNTs are naturally protein-affinitive in an aqueous ferritin solution, resulting in significant ferritin−SWNT conjugation and the solubilization of the nanotubes. The conjugation is further enhanced and stabilized in the presence of a coupling agent for amidation to promote the formation of covalent linkages. The natural protein-affinity of SWNTs may be alleviated or even eliminated by the functionalization of the nanotubes with hydrophilic polymers or better with oligomeric poly(ethylene glycol) moieties.

Introduction

Potential biological and biomedical applications of carbon nanotubes have promoted a number of recent investigations, including especially those on their aqueous solubility and their conjugation with biocompatible oligomers and polymers,[1−4] carbohydrates,[5−8] amino acids,[9] peptides,[10,11] nucleic acids,[12] and proteins.[13,14] Of particular interest are the interactions of carbon nanotubes with proteins (or enzymes), as related to the development of nanoscale biosensors and biocatalytic devices.[15−18] In fact, proteins such as bovine serum albumin (BSA) are often used as biological models and as linkers between synthetic materials and more fragile biological species such as antibodies.[19]

Various approaches have been explored for the conjugation of proteins with nanotubes. For example, Sun and co-workers reported on the functionalization of both single-(SWNTs) and multiple-walled (MWNTs) carbon nanotubes with BSA protein under carbodiimide activated amidation reaction conditions.[13a] The results from the characterization using atomic force microscopy (AFM) showed that the protein species are intimately associated with individual thin bundles of SWNTs and MWNTs. The BSA−nanotube conjugates thus prepared are comparable with those obtained from a different method, in which functionalized carbon nanotubes bearing ester linkages were used as starting materials in ester-to-amide transformation reactions with BSA protein.[13b] Separately, protein−nanotube conjugation through nonspecific interactions has been examined by several research groups.[14,15,20−22] Balavoine et al. reported that streptavidin could form ordered helical structures on the MWNT surface.[21] Dai and co-workers found no meaningful attachment of ferritin protein to SWNTs and thus used a bifunctional molecule as linker in the preparation of ferritin−SWNT conjugates.[20] However, Azamian et al. reported strong interactions of ferritin with SWNTs under their experimental conditions, with the protein covering the nanotube surface without the presence of coupling reagents.[14] More recently, Boussaad and co-workers used changes in the conductivity of semiconducting SWNTs on a solid substrate to monitor the natural adsorption process of cytochrome *c* onto the nanotube surface.[22] These investigations have contributed to the understanding of the nonspecific interactions between nanotubes and proteins, but they have also revealed the complexity of the issue. Even in the attachment of proteins to carbon nanotubes the extent to which the protein species are adsorbed on the nanotube surface remains to be evaluated.

The work reported here was centered on the SWNT−protein interactions in water with the consideration that most biological systems are associated with an aqueous environment. The results show that there is natural affinity of purified SWNTs toward ferritin in aqueous solution, with significant adsorption of the protein species on the nanotube surface and that the ferritin−SWNT conjugation is enhanced and stabilized in the presence of a carbodiimide coupling agent for amidation reactions. The results also show that the nanotube surface may be protected from protein adsorption via the functionalization of the nanotube with hydrophilic polymers or better with oligomeric poly(ethylene glycol), a benchmark protein-resistant material.

Experimental Section

Materials. Ferritin (Type I from horse spleen, $M_W \sim 440\,000$) samples of 77 mg/mL (Lot 21K7074) and 91 mg/mL (Lot 72K7071) in 0.15 M NaCl solution were purchased from Sigma. Diamine-terminated oligomeric poly(ethylene glycol) (PEG_{1500N}, $M_W \sim 1500$), poly(vinyl alcohol) (PVA, $M_W \sim 70\,000-100\,000$), and poly(propionylethylenimine) (PPEI, $M_W \sim 200\,000$) were obtained from Aldrich. The PPEI polymer was partially hydrolyzed, as reported previously,[3] to yield poly(propionylethylenimine-*co*-ethylenimine) (PPEI-EI). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC, 98+%) was purchased from Alfa Aesar. Dialysis membrane tubing made of polyvinylidene fluoride (cutoff molecular weight ~ 1 million) was supplied by Spectrum Laboratories.

The SWNT sample was produced in Prof. A. M. Rao's laboratory (Physics Department, Clemson University) by using the arc-discharge method.[23] The sample was purified via refluxing in aqueous HNO_3 (14%) for 48 h, followed by vigorous centrifuging, repeated washing with deionized water, and drying in a vacuum oven.[3,4] The estimated SWNT content in the purified sample was >90%. Water-soluble functionalized

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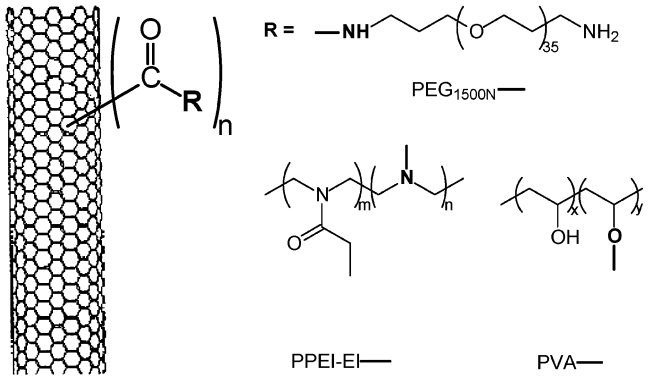
SCHEME 1



SWNT samples PEG_{1500N}−SWNT,[2b] PVA−SWNT,[4] and PPEI-EI−SWNT[3d] (Scheme 1) were prepared as previously reported. The nanotube contents in these samples were generally on the order of ∼10% (wt/wt).

Measurements. Transmission electron microscopy (TEM) analyses were conducted on a Hitachi HF-2000 TEM system and a Hitachi HD-2000 TEM/STEM system, both equipped with CCD cameras for digital imaging.

Ferritin−Nanotube Interactions. In a typical experiment for the natural adsorption, a purified SWNT sample (12.4 mg) was dispersed in KH_2PO_4 buffer solution (pH = 6−7, 4 mL) via sonication in a bath sonicator (Cole Palmer B3-R, 55 kHz) for 1 h. To the dispersion was added the as-purchased ferritin solution (1 mL). The mixture was stirred for 24 h, followed by centrifuging at ∼3000*g* to obtain a homogeneous supernatant (stored at 4 °C) for characterization.

The same experiment was carried out in the presence of EDAC (10 mg/mL, 0.5 M) in the mixture, yielding similarly homogeneous supernatant (stored at 4 °C) for characterization.

For an examination of interactions between ferritin and the functionalized SWNTs, the water-soluble sample PPEI-EI−SWNT was dissolved in water to form a homogeneous solution. To the aqueous solution (∼1 mg/mL nanotube equivalent) was added the as-purchased ferritin solution (∼0.1 mL). The mixture was shaken continuously for 12 h. Upon centrifuging to remove residual particulates, the homogeneous solution (stored at 4 °C) was used for characterization. The same experimental conditions were used for the other water-soluble functionalized SWNT samples PVA-SWNT and PEG_{1500N}−SWNT.

Results and Discussion

The ferritin from horse spleen typically contains a shell of 24 protein subunits (apoferritin) with diameters up to 12−13 nm. The encapsulated iron core (6−7 nm) is in the form of a hydrated Fe(III) oxy-hydroxide particle, which can readily be identified under TEM (Figure 1).

There is significant adsorption of ferritin species on the nanotube surface in the aqueous dispersion containing purified SWNTs and the protein. The ferritin−SWNT conjugation results in the solubilization of a portion of the nanotube sample, turning the brownish red solution of ferritin into a dark-colored solution. The solubilized portion is ∼25% of the starting sample, estimated on the basis of the residual nanotube weight. The significant adsorption of ferritin on the nanotube surface is rather evident in TEM images, as shown in Figure 1 and Figure 2. The ferritin−SWNT conjugates are also well dispersed (Figure 1).

Similarly strong natural protein affinity of SWNTs has been observed and documented in the literature.[14,15,20−22] For example,

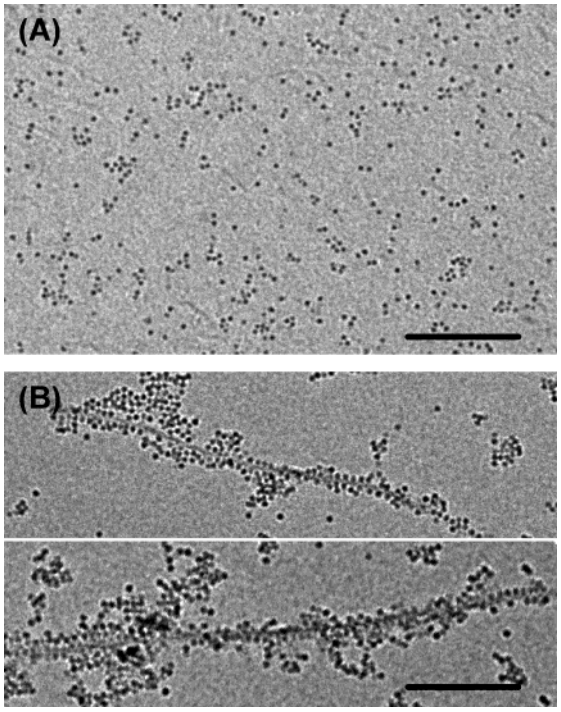


Figure 1. TEM images of the specimens from the ferritin solutions (A) without and (B) with purified SWNTs (200 nm for both scale bars).

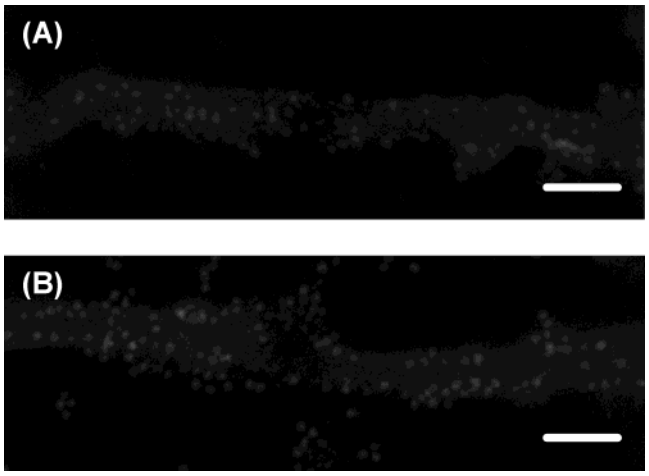


Figure 2. TEM images (dark field) of the ferritin−SWNT conjugates obtained (A) without and (B) with EDAC at higher magnification (50 nm for both scale bars).

Azamian et al. reported on the adsorption of both positively charged (cytochrome *c*, pI 10.8) and negatively charged (ferritin, pI 4.6) proteins onto SWNTs at physiological pH.[14] Somewhat surprising was the result of Dai and co-workers from their negative control experiment, in which no meaningful natural adsorption of ferritin was observed.[20] The inconsistency between experiments in different laboratories suggests that the protein−nanotube conjugation via nonspecific interactions is probably complicated, depending on experimental conditions such as the source and history of the nanotube sample.[14,15,24] Mechanistically, several possible origins have been considered for the nonspecific interactions, including electrostatic interactions, hydrophobic interactions, hydrogen bonding, and others associated with the very high surface area of the nanotube.[25] The complex local features such as defects on the nanotube may also play a significant role. However, particularly noteworthy is the increasing amount of experimental evidence for the amino-affinity of SWNTs, namely that molecules bearing amino

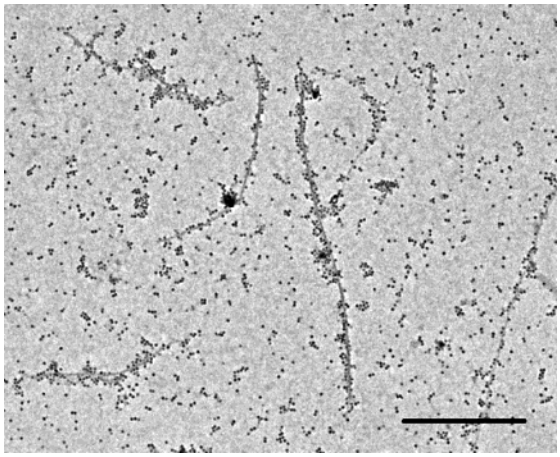


Figure 3. A TEM image of the ferritin−SWNT conjugates obtained with EDAC in the reaction mixture (scale bar = 400 nm).

moieties are affinitive to the nanotube surface, drastically changing the electrical conductance of especially semiconducting SWNTs.[26] Since ferritin and other proteins contain multiple amino units,[27] amino-based nonspecific interactions may contribute significantly to the observed protein−SWNT conjugation.

The presence of the activation agent EDAC in the reaction mixture for the experimental condition favorable to amidation apparently promotes the ferritin−SWNT conjugation through covalent linkages. It has been shown that BSA protein can be covalently conjugated with carbon nanotubes via the amidation of nanotube-bound carboxylic acids.[13a,3c] Similarly, the EDAC-activated coupling of ferritin with SWNTs also results in a dark-colored homogeneous solution. The ferritin−SWNT conjugates thus obtained are also well-dispersed according to TEM analysis (Figure 3). In fact, the amidation reaction condition is more effective in the nanotube solubilization, resulting in the solubilization of ∼40% of the starting SWNT sample (versus ∼25% in the natural adsorption via nonspecific interactions discussed above). As compared in Figure 2, there are also relatively more ferritin species on the nanotube surface in the sample obtained in the presence of EDAC. It is likely, however, that even in the presence of EDAC the ferritin−SWNT conjugation is contributed by adsorption via nonspecific interactions. This is supported by the results concerning the dialysis of the conjugate solutions.

The homogeneous solutions of the ferritin−SWNT conjugates obtained with and without EDAC were both dialyzed in large pore-size membrane tubing (cutoff molecular weight ∼ 1 million) against fresh deionized water for 24 h. As shown in Figure 4, for both samples there are less ferritin species on the nanotube surface in comparison with those before the dialysis (Figure 2), though the decrease in the sample obtained with EDAC is not as pronounced as that without EDAC. In fact, the conjugate solution obtained without EDAC became less stable after the dialysis, exhibiting some precipitation in a few days. The precipitated SWNTs, with fewer adsorbed ferritin species, could be solubilized again in a repeat of the natural adsorption experiment. The results suggest not only contributions of nonspecific interactions in both conjugate samples but also the dependence of the natural adsorption on the overall protein concentration in the solution. In both the nonspecific interactions and the covalent functionalization, the adsorption of ferritin on the nanotube surface is probably a dominating process. In the presence of EDAC in the functionalization, the formation of amide linkages with the nanotube-bound carboxylic acids locks some of the adsorbed ferritin species in place, which remain stable under the dialysis condition.

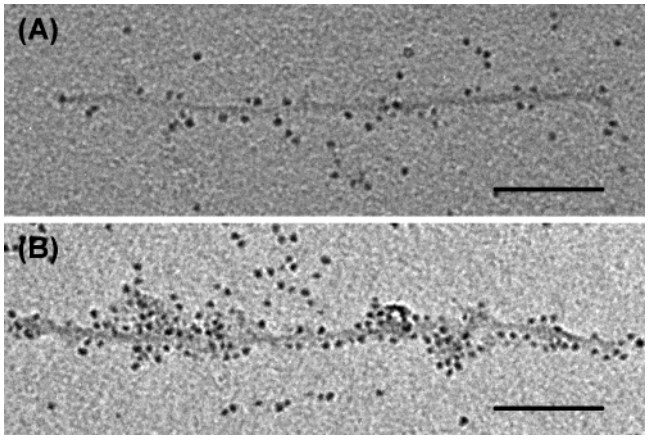


Figure 4. TEM images of the specimens from the ferritin−SWNT conjugate solutions obtained (A) without and (B) with EDAC after dialysis (100 nm for both scale bars).

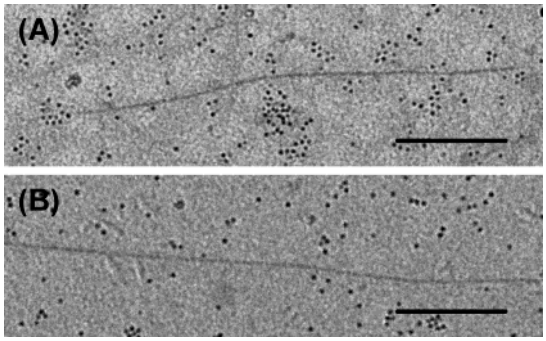


Figure 5. TEM images of the specimens from aqueous solutions of (A) ferritin and PPEI-EI−SWNT and (B) ferritin and PVA−SWNT (200 nm for both scale bars).

The protein-affinity of carbon nanotubes was also evaluated from a different angle by using water-soluble functionalized SWNTs. For PPEI-EI−SWNT, as an example, recent characterization by high-resolution TEM coupled with electron energy loss spectroscopy (EELS) has shown that the soluble sample contains individual and thin bundles of SWNTs covered with PPEI-EI polymer.[3d] Because of the surface modification by the water-soluble polymer, these SWNTs are expected to have a hydrophilic surface. The protein-affinity of PPEI-EI−SWNT is considerably less than that of the purified SWNT sample discussed above. As shown in Figure 5, ferritin species largely avoid the PPEI-EI-functionalized SWNTs. Similarly, the PVA-functionalized SWNTs are also expected to have a PVA-covered surface. These functionalized nanotubes are largely avoided by ferritin species as well (Figure 5). The PPEI-EI and PVA polymers apparently protect the nanotubes to make the modified nanotube surface protein-resistant.

In fact, the introduction of hydrophilic surface functional groups represents a signature strategy in the protection of the surface from protein adsorption.[25,28] Many factors such as steric repulsion, hydration, and solvent structuring at the surface have been investigated extensively in the creation of a protein-resistant surface. Experimentally, poly(ethylene glycol) (PEG) moieties have been identified as being strongly protein-resistant on various surfaces.[28] Thus, the oligomeric PEG-functionalized SWNTs (PEG_{1500N}−SWNT) were used in this study to evaluate the interactions with (or resistance toward) ferritin. Since PEG_{1500N}−SWNT is soluble in water,[2b] the experiment was carried out in an homogeneous aqueous solution. As shown in Figure 6, there is hardly any conjugation of the nanotube with ferritin, in sharp contrast to the results shown in Figure 2. The

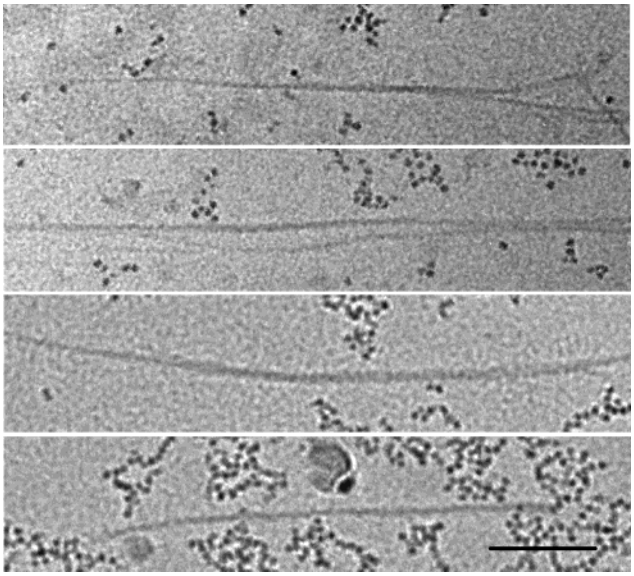


Figure 6. TEM images (different regions on the same grid) of the specimen from the aqueous solution of ferritin and PEG_{1500N}−SWNT (scale bar = 100 nm).

PEG-modified nanotube surface is indeed strongly resistant toward the adsorption of ferritin. Similar protein-resistant effects of PEG-modified nanotubes were observed by Dai and co-workers in a very different experimental configuration.[15] While the oligomeric PEG_{1500N} chains are much shorter than those of the PPEI-EI ($M_W \sim 200\,000$) and PVA ($M_W \sim 70\,000$−$100\,000$) polymers, the protein-resistance of the PEG_{1500N}−SWNT sample is at least as strong as those of the PPEI-EI−SWNT and PVA−SWNT samples (Figure 5 and Figure 6).

The amide linkage between the oligomeric PEG and nanotube in PEG_{1500N}−SWNT is necessary to maintain the sample stability in interactions with protein species. A similar PEG-functionalized SWNT sample based on ester linkages (PEG_{1500}−SWNT)[13b] is susceptible to ester-to-amide transformation reactions with pendant amino functionalities on the protein surface, despite the protein−resistant properties of the sample. In fact, the PEG_{1500}−SWNT sample was used in the reaction with BSA protein in a small pore-size dialysis tubing (for the permeation of oligomeric PEG molecules and the retention of protein species) for the preparation of BSA−SWNT conjugates under ambient conditions.[13b] The thermodynamically favorable ester-to-amide transformation obviously overcomes the strong protein-resistance of PEG moieties on the SWNT surface.

In summary, SWNTs are naturally protein-affinitive in an aqueous ferritin solution, resulting in significant ferritin−SWNT conjugation and the solubilization of the nanotubes. The conjugation is further enhanced and stabilized in the presence of a coupling agent for amidation to promote the formation of covalent linkages. The natural protein-affinity of SWNTs may be alleviated or even eliminated by the covalent functionalization of the nanotubes with hydrophilic polymers or better with oligomeric PEG moieties, which is consistent with the fact that PEG is considered as a benchmark material for protein-resistant surfaces. The protein-resistant properties of these functionalized SWNTs make them attractive candidates for biocompatibility evaluation. The tunable protein-affinity of SWNTs in aqueous environments is valuable to their potential biological and biomedical applications.

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