Noncovalent Sidewall Functionalization of Single-Walled Carbon Nanotubes for Protein Immobilization

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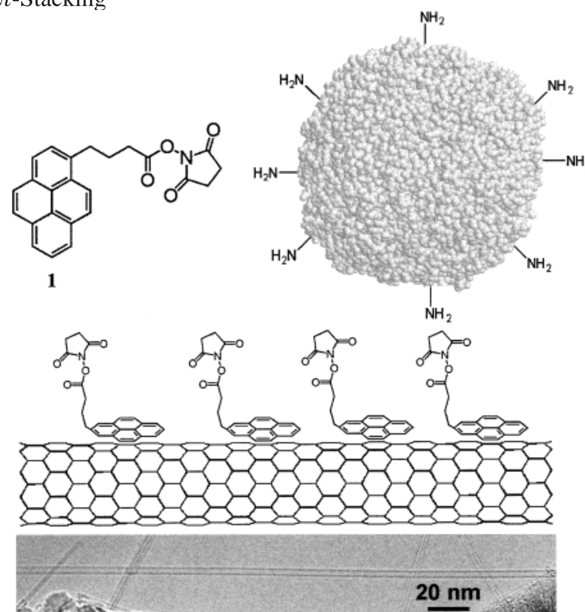
Single-walled carbon nanotubes (SWNTs) are molecular wires that exhibit interesting structural, mechanical, electrical, and electromechanical properties.[1−3] A SWNT is unique among solid-state materials in that every atom is on the surface. Surface chemistry could therefore be critical to the physical properties of SWNTs and their applications.[3−10] SWNT sidewall functionalization is important to soluble nanotubes,[4−6] self-assembly on surfaces, and chemical sensors.[8−10] For these purposes, it is imperative to functionalize the sidewalls of SWNTs in noncovalent ways to preserve the sp^2 nanotube structure and thus their electronic characteristics.

Immobilization of biomolecules on carbon nanotubes has been pursued in the past, motivated by the prospects of using nanotubes as new types of biosensor materials.[11−15] The electronic properties of nanotubes coupled with the specific recognition properties of the immobilized biosystems would indeed make for an ideal miniaturized sensor. A prerequisite for research in this area is the development of chemical methods to immobilize biological molecules onto carbon nanotubes in a reliable manner. Thus far, only limited work has been carried out with multiwalled carbon nanotubes (MWNTs).[11−15] Metallothionein proteins were trapped inside and placed onto the outer surfaces of open-ended MWNTs.[11−14] Streptavidin was found to adsorb on MWNTs presumably via hydrophobic interactions between the nanotubes and hydrophobic domains of the proteins.[15] DNA molecules adsorbed on MWNTs via nonspecific interactions were also observed.[12−14]

In this communication, we report a simple and general approach to noncovalent functionalization of the sidewalls of single-walled carbon nanotubes, and subsequent immobilization of various biological molecules onto nanotubes with a high degree of control and specificity. The noncovalent functionalization involves a bifunctional molecule, 1-pyrenebutanoic acid, succinimidyl ester (**1**) (Molecular Probes, Inc., USA), irreversibly adsorbed onto the inherently hydrophobic surfaces of SWNTs in an organic solvent dimethylformamide (DMF) or methanol. The pyrenyl group, being highly aromatic in nature, is known to interact strongly with the basal plane of graphite via π-stacking,[16,17] and also found here to strongly interact with the sidewalls of SWNTs in a similar manner, thus providing a fixation point for **1** on the nanotubes. The anchored molecules of **1** on SWNTs are highly stable against desorption in aqueous solutions. This leads to the functionalization of SWNTs with succinimidyl ester groups that are highly reactive to nucleophilic substitution by primary and secondary amines that exist in abundance on the surface of most proteins (Scheme 1). The mechanism of protein immobilization on nanotubes, then, involves the nucleophilic substitution of *N*-hydroxysuccinimide by an amine group on the protein, resulting in the formation of an amide bond. This technique enables the immobilization of a wide range of biomolecules on the sidewalls of SWNTs with high specificity and efficiency, as demonstrated here with ferritin, streptavidin, and biotinyl-3,6-dioxaoctanediamine (biotin-PEO-amine).

Scheme 1. 1-Pyrenebutanoic Acid, Succinimidyl Ester **1** Irreversibly Adsorbing onto the Sidewall of a SWNT via π-Stacking[a]



[a] Amine groups on a protein react with the anchored succinimidyl ester to form amide bonds for protein immobilization. Lower panel: A TEM image of an as-grown SWNT on a gold TEM grid.

Our samples contained suspended SWNTs synthesized directly on meshed gold grids in a manner described previously.[18] In brief, gold grids for transmission electron microscopy (TEM) were dipped in a liquid catalyst comprised of a triblock copolymer P-123, $AlCl_3$, $FeCl_3$, and MoO_2Cl_2 dissolved in ethanol and *sec*-butanol.[19] The catalyst-coated grids were calcined at 500 °C for 18 h, followed by chemical vapor deposition of methane at 900 °C for 10 min. This led to the synthesis of abundant individual and bundled SWNTs suspended over the holes of the gold grids. A TEM image of a typical SWNT is shown in Scheme 1.

For noncovalent functionalization of suspended SWNTs, a grid sample was incubated in a 1-pyrenebutanoic acid, succinimidyl ester solution (6 mM of **1** in DMF or 1 mM in methanol) for 1 h at room temperature, after which the sample was rinsed three

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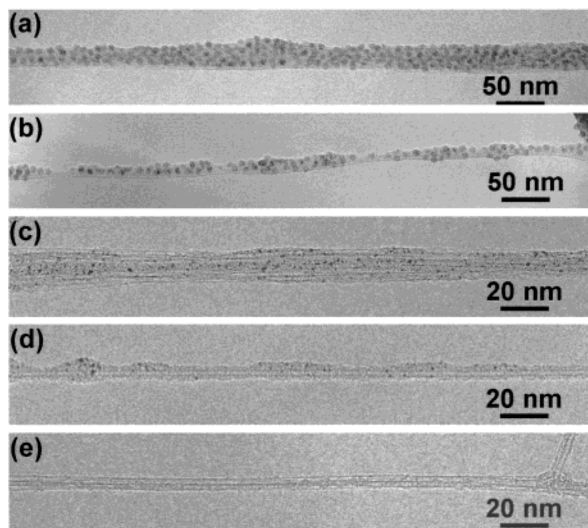

Figure 1. (a) A TEM image of a bundle of SWNTs functionalized by **1** followed by ferritin immobilization. The round dark spots are the (∼4 nm) iron cores of ferritin on the bundle. (b) A TEM image of ferritin immobilized onto an individual SWNT. (c) A TEM image of streptavidin-Au conjugates immobilized onto a bundle of SWNTs. The dark spots represent the 1.4 nm Au particles bound to streptavidin molecules. (d) A TEM image of streptavidin-Au conjugates immobilized on an individual SWNT. (e) A TEM image showing the absence of protein immobilization on as-grown nanotubes without functionalization by **1**.

times in pure DMF or methanol to wash away excess reagent. For protein immobilization, the SWNTs functionalized by **1** were incubated in an aqueous solution of protein for 18 h at room temperature, rinsed thoroughly in pure water for 6 h, and then dried. Proteins immobilized on the suspended SWNTs were characterized by TEM imaging and X-ray photoemission spectroscopy (XPS, see Supporting Information for XPS characterization). Ferritin from horse spleen (Sigma, USA) diluted to a concentration of 5 mg/mL in a 7.5 mM NaCl solution and streptavidin labeled by 1.4 nm gold nanoparticles (Nanoprobes, USA) diluted to 8 μg/mL in phosphate buffered saline (1.5 mM NaCl) at pH 7.4 were chosen for immobilization on SWNTs for their high contrast in TEM visualization.

We observe successful immobilization of both ferritin and streptavidin onto SWNTs functionalized by **1** (Scheme 1) in the TEM. Figure 1a shows a typical example of ferritin molecules densely immobilized on a SWNT bundle. The ∼4 nm iron cores of each ferritin are clearly resolved. Figure 1b shows ferritin molecules attached to an individual SWNT. Again, the ferritin cores are easily visible, as well as the apoproteins that appear amorphous surrounding the cores. Typically, we observe that the immobilized proteins appear dense on SWNT bundles, while gaps tend to exist between proteins on single tubes. This can be rationalized by taking into consideration the increased surface area and favored surface topography of bundled SWNTs for protein binding. The concave regions formed between neighboring individual SWNTs on the bundle surface allow ferritin molecules (outer diameter ∼10 nm) to be anchored by **1** on both of the neighboring tubes, thus holding the protein more effectively to the bundle than to an individual SWNT.

Streptavidin labeled by Au nanoparticles are immobilized in a similar fashion (Scheme 1) onto bundles and individual SWNTs as shown in Figure 1c,d. Here, the black dots represent not the streptavidin molecules themselves, but the covalently attached 1.4 nm Au particles. As in ferritin immobilization, the SWNT bundle exhibits a larger apparent density of immobilized streptavidin (Figure 1c) than the individual SWNT (Figure 1d).

To demonstrate the expansive range of biomolecules that could be immobilized on SWNTs, we employed the same approach to immobilize a much smaller molecule, biotin-PEO-amine (Pierce Chemical, USA) onto SWNTs. This was carried out by incubating SWNTs functionalized by **1** in an aqueous solution of biotin-PEO-amine (20 mg/mL) for 18 h to covalently link the biotin group to **1** via the same amide-forming reaction mechanism described above. The sample was then exposed to a solution of streptavidin-Au conjugate to achieve streptavidin−biotin coupling. Subsequent TEM confirmed that streptavidin does indeed bind strongly to the immobilized biotin groups on the SWNT, with images that revealed streptavidin-Au structures immobilized on SWNTs similar to those shown in Figure 1c,d.

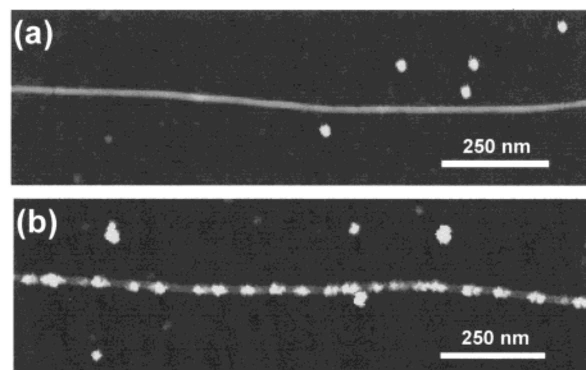

Figure 2. (a) An AFM image showing a SWNT bundle (diameter = 4.5 nm) free of adsorbed ferritin after incubation in a ferritin solution. (b) An image showing ferritin molecules (apparent heights ∼10 nm) adsorbed on a SWNT bundle (diameter = 2.5 nm) functionalized by **1** and incubated in a ferritin solution.

To confirm the functionalization of SWNTs by **1** and thus the protein immobilization mechanism outlined in Scheme 1, we carried out control experiments in which as-grown SWNT grid samples were incubated in aqueous solutions of proteins only, without any prior treatment with **1**. In this case, we observed nanotubes free of adsorbed protein on their sidewalls (Figure 1e).

Further experiments carried out with nanotubes on substrates revealed that our approach to SWNT functionalization and protein immobilization is efficient and specific. SWNTs grown by laser ablation were deposited onto a SiO_2 substrate from suspension in 1,2-dichloroethane (1 mg of SWNT/10 mL of solvent). The substrate was incubated in **1** (6 mM, in DMF) for 1 h, rinsed thoroughly in pure DMF, exposed to a dilute ferritin solution (10 μg/mL in a 15 μM NaCl solution) for 18 h, and finally rinsed in pure water for 3 h. Atomic force microscopy (AFM) revealed SWNTs dotted with immobilized ferritin molecules along their lengths (Figure 2b). For a control, SWNTs on a substrate were exposed to the solution of ferritin only, without treatment by **1**. The nanotubes were found to be free of adsorbed ferritin (Figure 2a). These results show that the functionalization and protein immobilization reactions are selective to the nanotubes and not to the substrate.

In summary, we have developed a controlled and nanotube-specific method for immobilizing proteins and small biomolecules onto noncovalently functionalized SWNTs. The versatility of this simple approach can be extended beyond biological molecules to many systems such as small molecules with desired properties, polymerizable molecules, and inorganic nanoparticles. The method may also hold the key to forming stable suspensions of functionalized SWNTs in solutions and open up the possibility of self-assembly of nanotubes with unperturbed sp^2 structures and electronic properties.

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Supporting Information Available: XPS characterization of functionalized SWNT with immobilized proteins (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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