Nanobiohybrids

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Controlled Hybrid Nanostructures through Protein-Mediated Noncovalent Functionalization of Carbon Nanotubes**

Katri Kurppa, Hua Jiang, Géza R. Szilvay, Albert G. Nasibulin, Esko I. Kauppinen, and Markus B. Linder*

Metallic nanoparticles (NPs) exhibit interesting electronic and optical properties, especially as organized assemblies.[1–3] Controlled positioning of NPs in nanoarchitectures is essential as the interdistances of neighboring NPs are critical for their collective properties.[4] Also single-walled carbon nanotubes (SWNTs) have many attractive properties for use in nanotechnology.[5] Hierarchically controlled functionalization of the nanotube sidewalls with different NPs is a relevant goal that can lead to new ways of exploiting these properties.[6] Creation of such hybrid structures with control over particle positioning presents a challenging task.[7–11]

NP arrangement in arrays is presently carried out mainly by laborious top-down methods.[12] A more feasible approach to large-scale production of defined nanostructures is bottom-up self-assembly. For this purpose, nature's pool of biomolecules provides us with a diverse toolbox.[3,13–15] Organized arrays of NPs have been created by DNA self-assembly.[1] However, control in structures that demand higher hierarchy is poor with flexible, unordered molecules as templates. More-precise structures can be achieved by using proteins, which self-assemble by interactions based on three-dimensional molecular recognition.[16] Proteins also often have a rigid, defined structure that is in a size scale that is compatible with many nanostructures. The dimensions achieved by protein self-assembly are in principle much smaller than those in DNA-based architectures. Proteins can also be precisely modified by genetic engineering to yield even more specificity in nanostructure design.

Hydrophobins are small, amphiphilic proteins found in filamentous fungi.[17] Their natural function is to adhere to surfaces and function as surfactants. Herein we show that the functional property of a hydrophobin called HFBI can be utilized for controlled functionalization of carbon nanotubes. These features allowed the formation of new protein–nanotube composite structures in which the nanotubes are embedded in protein films. The protein–carbon nanotube interaction was further used to create hybrid structures of carbon nanotubes and regularly spaced gold nanoparticles (AuNPs), named the nano^3 hybrid.

We studied the solubilization and functionalization phenomena of SWNTs by using two different SWNTs. Initial solubilization studies were carried out with more-abundant commercially available SWNTs produced by arc discharge. For functionalization studies, we used as-produced SWNTs (see the Supporting Information).[18,19] Two different hydrophobin proteins were used, the naturally occurring (wild type) HFBI, a class II hydrophobin from *Trichoderma reesei*, and its genetically engineered variant named NCysHFBI.[20] The structure of HFBI is amphiphilic due to a patch of aliphatic hydrophobic side chains as shown in Figure 1 a.[21] This feature causes HFBI to be very surface active and to bind to hydrophobic surfaces such as graphite.[22] At the air–water interface, HFBI forms highly elastic films that are one

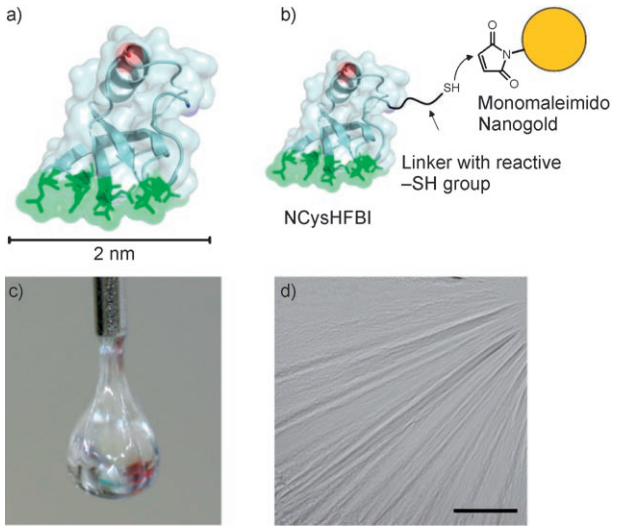

Figure 1. a) HFBI has a compact structure with a large hydrophobic patch (shown in green, side view) as part of an otherwise polar surface. b) A schematic representation of the conjugation of monomaleimido nanogold NPs to NCysHFBI. c) Self-assembly of HFBI leads to the formation of elastic monomolecular films at the surface containing the protein, for example water drops. d) Stretch marks seen on a microscope image of the drop surface illustrate the elasticity and coherence of the film. The scale bar is 50 μm.

[*] K. Kurppa, Dr. H. Jiang, G. R. Szilvay, Prof. E. I. Kauppinen, Dr. M. B. Linder
VTT Biotechnology
VTT Technical Research Centre of Finland
Tietotie 2, P.O. Box 1000, 02044 Espoo (Finland)
Fax: (+358) 20-722-7071
E-mail: katri.kurppa@vtt.fi
Dr. A. G. Nasibulin, Prof. E. I. Kauppinen
Nano Materials Group
Laboratory of Physics and Centre for New Materials
Helsinki University of Technology
Biologinkuja 7, P.O. Box 1000, 02044 Espoo (Finland)

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molecule thick and in which the protein molecules have self-assembled in a distinct, highly ordered hexagonal pattern.[22,23] The structure of HFBI is cross-linked by intramolecular disulphide bonds making it very rigid, compact, and stable. NCysHFBI was modified from the wild-type HFBI by linking a stretch of 13 amino acids to its amino terminus (Figure 1b). At the end of the added stretch is a Cys amino acid residue, which has a free reactive -SH group. The -SH groups of different molecules form intermolecular disulphide bonds, resulting in covalently linked dimers $(NCysHFBI)_2$. The intermolecular disulphide can selectively be reduced by dithiotreitol to yield monomeric NCysHFBI with a free -SH group for further conjugation reactions.

The protein–SWNT samples were prepared by adding the aqueous protein solution to a known amount of SWNTs, followed by ultrasonication and centrifugation. HFBI proved to be an extremely capable solubilizing agent for SWNTs. For comparison of the solubilization capability, we performed the same procedures with a formerly reported example, bovine serum albumin (BSA).[24] Previous reports show that as much as 10 mg mL^{-1} (0.15 mM) BSA is needed to solubilize 50 μg mL^{-1} SWNTs.[24] By using HFBI, efficient solubilization of 200 μg mL^{-1} SWNTs was achieved with a concentration as low as 0.25 mg mL^{-1} (0.03 mM). Use of the same concentration of BSA yielded negligible solubilization. The dimeric $(NCysHFBI)_2$ was as efficient as wild-type HFBI. The transmittance values calculated from measured absorbance at 550 nm were 28% and 96% for HFBI-SWNT and BSA-SWNT solutions, respectively (Figure 2). Centrifuged solutions of the hydrophobin–SWNT conjugates were stable for months at room temperature and could be diluted and otherwise handled in a normal manner.

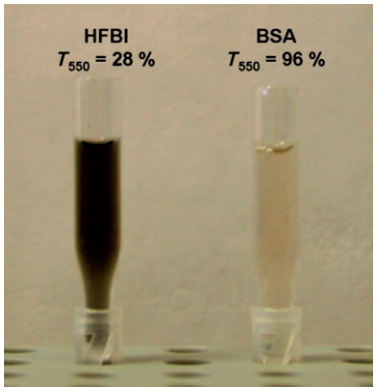

Figure 2. Solutions of SWNTs solubilized by HFBI (left) and BSA (right). The color and transmittance, T_{550}, of the centrifuged supernatants represent the amounts of SWNTs in the solution.

Optical characterization of hydrophobin–SWNT conjugates by UV/Vis spectroscopy displays Van Hove peaks that are characteristic of solubilized SWNTs (Figure 3a).[25,26] Broad peaks in the spectra most likely imply the prevalence of bundled carbon nanotubes in the solution.[25] This is possibly a consequence of the rigid, relatively large structure of the protein that prohibits the peeling of individual tubes from the tight bundles formed in the synthesis reactor. No large differences were observed in the spectra of HFBI–SWNT or $(NCysHFBI)_2$–SWNT conjugates. CD spectroscopy measurements showed identical spectra for HFBI, HFBI—SWNT, and NCysHFBI–SWNT (Figure 3b). This shows that neither the interaction between HFBI and SWNTs nor the ultrasonication treatment cause changes in the protein structure.

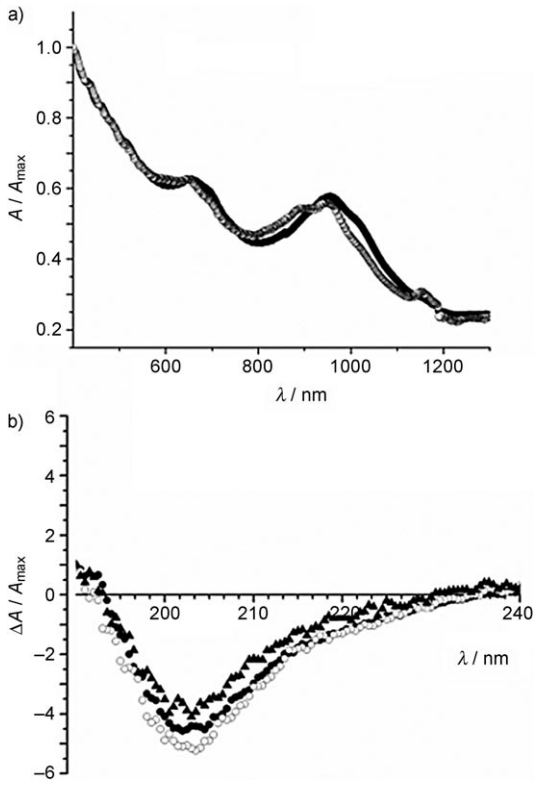

Figure 3. a) UV/Vis spectra of NCysHFBI–SWNT and HFBI–SWNT. b) The CD spectra of HFBI–SWNT, NCysHFBI–SWNT, and HFBI are identical. ● = HFBI–SWNT, ○ = NCysHFBI–SWNT, ▲ = HFBI.

We analyzed the HFBI- and $(NCysHFBI)_2$-functionalized SWNTs by TEM (Figure 4). HFBI–SWNT and $(NCysHFBI)_2$–SWNT samples were prepared by drop-casting the sample solution on TEM grids covered with a holey carbon film. The hydrophobins showed the interesting property of forming thin films spanning the entire carbon film of the grid. SWNTs were embedded in this protein film as individual tubes or bundles. As the protein film also covered the holes of the carbon film, SWNTs were also seen as only supported by the protein film. (Figure 4a and b) Imaging was conducted without staining or protection of the samples. Beam damage was evident after prolonged imaging of a single area of the protein film, causing the formation of rapidly enlarging holes in the protein film. Films prepared from the dimeric $(NCysHFBI)_2$ were more stable in this respect than wild-type HFBI films. This could be due to the additional covalent disulphide bond between NCysHFBI monomers that rigidifies the structure.

The interaction between NCysHFBI and SWNTs was used to create novel hybrid nanostructures of carbon nanotubes, AuNPs, and protein molecules. The single reactive -SH

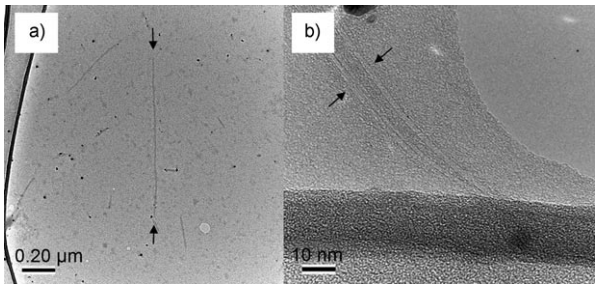

Figure 4. TEM images imaged at 80 kV. a) The $(NCysHFBI)_2$ film suspended over the holey carbon film with SWNTs embedded within. The two arrows indicate the ends of one of the SWNTs. The dark spots are catalyst particles. The edge of the holey carbon film is seen on the left side of the image. b) SWNTs (in the area between the arrows) in the protein film. A hole in the $(NCysHFBI)_2$ film is seen in the upper right corner and the edge of the holey carbon film as a darker area at the lower part of the image.

group of NCysHFBI allows stoichiometric conjugation of other functional groups to only one site of the protein. We used a commercially available monomaleimido nanogold labeling reagent (Nanoprobes, Inc.) to label the single -SH group of the NCysHFBI molecule with a single AuNP. The AuNPs are discrete gold particles of 1.4 nm in diameter and consist of 55–75 gold atoms. A schematic representation of the AuNP–NCysHFBI conjugation is presented in Figure 1b. The AuNP–NCysHFBI conjugate was purified by chromatography and then used to solubilize SWNTs. After sonication and centrifugation, the pelleted hybrid structures of NCysHFBI–AuNPs and SWNTs, named nano^3 hybrids, were resuspended into a solution of unmodified NCysHFBI to ensure a protein excess, which was needed for film formation.

Transmission electron microscopy showed that NCysHFBI–AuNPs bound exclusively along the carbon nanotubes (Figure 5a). Only a few stray gold particles were visible in the surrounding NCysHFBI film. Interestingly, the AuNPs were evenly spaced along the SWNTs. Measuring 58 interparticle distances from several images and samples, the average distance between gold particles was found to be (2.6 ± 0.4) nm (Figure 5b). A schematic representation of the nano^3 hybrid is shown in Figure 5c. To verify that the positioning of AuNPs was due to the protein conjugation, a control experiment was made. When SWNTs were solubilized in a mixture of nonconjugated NCysHFBI and free AuNPs, the TEM images showed a random distribution of AuNPs in the protein film (see the Supporting Information and Figure 1). Solutions of Au-functionalized NCysHFBI and SWNTs suspended in a solution of excess NCysHFBI were stored up to a week at $+4\,^{\circ}C$. Even then, only some individual gold particles were observed in the protein film surrounding the functionalized SWNTs. This finding is well descriptive of the high affinity and specificity of the HFBI–SWNT interaction.

Our results demonstrate the self-assembly of three different nanoscale building blocks to form a new type of hybrid material. Functionalization of SWNTs with AuNP–NCysHFBI resulted in novel hybrid nanostructures in which a one-dimensional regular array of AuNPs is bound to the

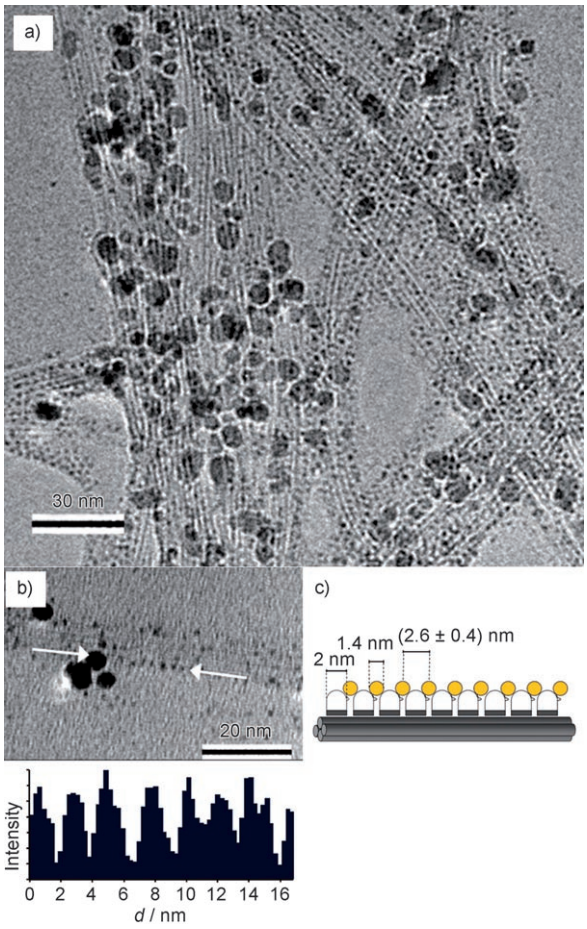

Figure 5. TEM images imaged at 200 kV (a) or 80 kV (b). a) Nano^3 hybrids embedded in a $(NCysHFBI)_2$ film. b) An example of a stretch of aligned NPs that was used for calculating particle distances. The intensity profile shown underneath the TEM image was calculated from the row of AuNPs between the arrow heads. c) A schematic view of the nano^3 hybrid displaying the diameters of the protein (2 nm) and the AuNPs (1.4 nm) and the interdistances of the AuNPs ((2.6 ± 0.4) nm).

carbon nanotube sidewalls. These nano^3 hybrid structures as well as the HFBI–SWNT composite films are prime examples of the structural control that can be achieved with self-assembling proteins. Because HFBI is structurally rigid and was engineered to bind only one AuNP per HFBI molecule, the spacing of the AuNPs on the SWNTs followed the size of the protein, distributing the NPs evenly at a distance of 2.6 nm. Owing to the relatively long 13 amino acid linker connecting the AuNP and the protein, the specific location of the NP with respect to the SWNT cannot be exactly defined. The regular arrangement of AuNPs, however, suggests that the interaction between the HFBI moiety and the SWNT surface is spatially oriented and occurs through a specific location on the protein, presumably the hydrophobic patch.

In conclusion, we have shown efficient solubilization and functionalization of SWNTs by HFBI and an engineered variant NCysHFBI. This interaction allowed the formation of novel protein–SWNT composite films as well as hybrid nanostructures of carbon nanotubes and AuNPs, the

nano[3] hybrids. In these hybrid structures, AuNPs were organized on carbon nanotube sidewalls in 1D arrays with a spacing of 2.6 nm, which implies underlying protein organization. Our results differ from previous examples of biomolecule-mediated positioning of NPs in that more structural levels were achieved by combining different types of materials. We show that rational use of proteins can lead to integration of different nanoscale objects in an ordered and hierarchical manner. This opens a route for combining their optical and electronic functions in new ways and can form a valuable addition to the toolbox of biomolecules that are finding use in nanotechnology.

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