Programmed assembly of multi-layered protein/nanoparticle-carbon nanotube conjugates†

Mei Li,[a] Erik Dujardin[b] and Stephen Mann*[a]

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Biomolecular interactions are used to programme the assembly of multi-layer, multi-functional CNT-based conjugates.

The conjugation of carbon nanotubes (CNT) with biomolecules and nanoparticles is an emerging field of research that has important potential applications in bionanotechnology.[1] For example, the metallic/semiconductive properties of CNTs have been exploited to produce functional, technologically relevant devices such as transistors, actuators and sensors. In the latter, derivatization of the CNT sidewall with appropriate biomolecules significantly improves the binding specificity[2] for detection of target molecules such as glucose,[3,4] DNA,[5] streptavidin,[6] and antibodies.[5] As CNT–biomolecule conjugates can be obtained using highly specific chemical interactions, this method offers a promising approach to reversibly solubilize CNTs with respect to other carbonaceous materials[7–9] or selectively disperse different types of CNT (*e.g.* semiconductive polymorphs) from bulk mixtures in CNT purification and separation techniques.[7,10–12] Moreover, as CNT–biomolecule conjugates combine a biocompatible outer coating with a rigid, needle-shaped nanotube core,[13,14] they have been used to transport CNT-adsorbed proteins across cell membranes for controlled cytoplasmic release and biological activation.[15–17] Similarly, mammalian cells have been transfected with exogenous DNA by immobilizing plasmid DNA on nickel-containing CNTs and transporting the conjugate into the cells by applying an external magnetic field.[18]

Although there are numerous reports on CNT–biomolecule conjugates, these are almost exclusively limited to constructs consisting of a single layer of biomolecules connected directly to the CNT wall by covalent or hydrophobic interactions, or *via* a linker molecule. As applications in sensing and transfection are now established in principle, multi-component forms of these conjugates should significantly enhance the potential of bio-functionalized CNTs, for use for example in parallel multi-analyte detection or simultaneous endocytosis of several proteins. Recently, electrostatic matching has been used to coat CNTs with layers of oppositely charged polyelectrolytes,[19] and here we describe a similar layer-by-layer procedure but based on programmed biomolecular assembly to produce a multi-component, multi-layered CNT-based conjugate. The conjugate consists of a multi-walled CNT core coated with four functionalised layers that successively comprise protein-encapsulated iron oxide nanoparticles (biotinylated ferritin (*b*Fn)), the tetravalent biotin-binding protein, streptavidin (SA), 24-base three-stranded biotin-terminated oligonucleotide duplexes, and oligonucleotide-coupled Au nanoparticles (Fig. 1). We demonstrate that the core/multi-shell architecture can be constructed stepwise by specific recognition processes involving biotin–SA binding or DNA duplexation, and that these interactions can be exploited for reversible assembly/disassembly of the Au nanoparticle layer.

Addition of *b*Fn to a dispersion of multi-walled CNTs in buffer at 70 °C for 5 min followed by rapid cooling on ice produced biotinylated ferritin-conjugated CNTs (*b*Fn–[CNT]) that exhibited increased hydrophilicity and solubility in aqueous solutions.‡ Unstained TEM images showed extensive surface decoration along the entire length of the CNTs with discrete 5 nm-sized electron dense nanoparticles (Fig. 2a, b). The particles were randomly arranged across the CNT surface, and EDX analysis and electron diffraction confirmed that they consisted of poorly crystallized hydrated iron oxide. Significantly, the surface-adsorbed

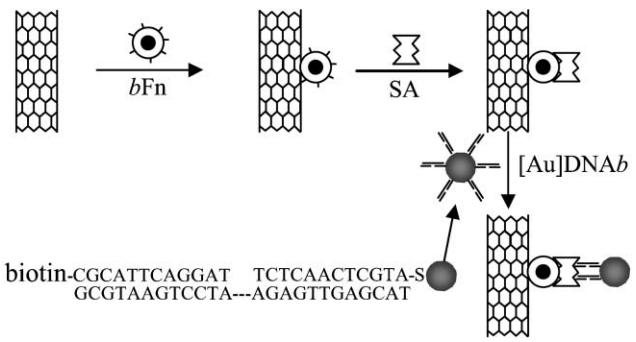

Fig. 1 Schematic showing stepwise construction of multi-layered protein/nanoparticle CNT conjugates. Top left to right; adsorption of *b*Fn onto multiwalled CNTs by hydrophobic interactions followed by SA binding to exposed biotin residues. Top to bottom right: addition of biotin-terminated oligonucleotide-functionalized Au nanoparticles ([Au]–DNA*b*) to produce multi-layered CNT-based conjugate. Prior to addition, the Au nanoparticles were capped with a mercapto-terminated 12-base single strand oligonucleotide (*ss*A) followed by duplexation with a 24-base single strand oligonucleotide (*ss*C), half of which was complementary to the *ss*A sequence, and then binding of a 5′-biotin-terminated 12-base single strand oligonucleotide (*ss*B). As *ss*B was complementary to the uncoupled single strand of partially duplexed *ss*C associated with the surface-bound *ss*A oligonucleotide, addition of *ss*B produced Au nanoparticles with surface-attached DNA double helices comprising exposed biotinylated residues.

[a]*Centre for Organized Matter Chemistry, School of Chemistry, University of Bristol, Bristol, UK BS8 1TS. E-mail: s.mann@bristol.ac.uk*
[b]*NanoSciences group, CEMES-CNRS UPR 8011, 29 rue J. Marvig, 31055 Toulouse Cedex 4, France*

† Electronic supplementary information (ESI) available: Preparation methods for *b*Fn; UV-vis absorption spectra of Au nanoparticle sol and Au nanoparticles in [Au]–DNA*b*–SA–*b*Fn–[CNT]; TEM image showing disassembly of Au nanoparticles after addition of excess biotin to a dispersion of [Au]–DNA*b*–SA–*b*Fn–[CNT] conjugates. See http://dx.doi.org/10.1039/b509109h

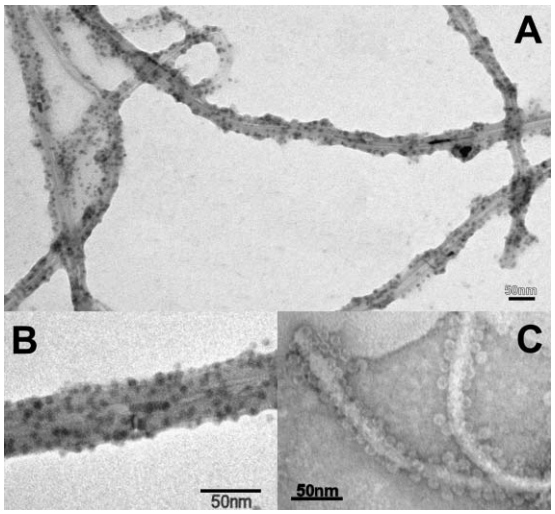

Fig. 2 TEM micrographs, (a, b) unstained image showing electron dense iron oxide nanoparticle cores associated with *b*Fn molecules attached on the surface of CNTs, and (c) negatively stained image showing intact polypeptide shell of CNT-attached ferritin. Scale bars = 50 nm.

nanoparticles were spatially separated, which together with data obtained from uranyl acetate-stained TEM samples (Fig. 2c), indicated that the iron oxide nanoparticles were enclosed within a 4 nm-thick polypeptide shell, confirming that the quaternary structure of *b*Fn remained intact after adsorption to the CNT surface.

Experiments undertaken at room temperature, or involving heating to 70 °C followed by slow cooling to room temperature, did not produce *b*Fn–[CNT] nanoconjugates. This was consistent with previous observations that demonstrated that whereas proteins such as SA and HupR were strongly adsorbed onto multi-walled CNTs at room temperature and in buffer, no significant interactions with Fn or *b*Fn were observed.[21] Our results indicated that attractive hydrophobic interactions between *b*Fn and the CNT surface were enhanced by heating to 70 °C, and that rapid cooling of the surface-adsorbed protein molecules produced irreversible attachment. This suggested that changes in the structure of the protein molecules associated with the heating and adsorption steps remained in place after rapid cooling and that the equilibrium conformation was not re-established even when the samples were stored in buffer for several months or weeks in the fridge or at room temperature, respectively. However, as shown by the stained TEM images, the extent of protein denaturation was not sufficient to significantly affect the quaternary structure of the protein, or as described below, the ability of CNT-coated *b*Fn molecules to non-covalently bind SA in aqueous solution.

Addition of SA to a suspension of the *b*Fn–[CNT] nanoconjugates at room temperature produced protein-coated nanotubes consisting of an outer layer of bound SA molecules.§ The SA–*b*Fn–[CNT] conjugates were hydrophilic and readily dispersed in aqueous buffer, and TEM investigations showed no loss of *b*Fn from the CNT surface. Moreover, a substantial number of the conjugated SA molecules were oriented such that biotin-terminated DNA-functionalized Au nanoparticles ([Au]–DNA*b*) could be subsequently coupled to residual binding sites exposed on the nanotube surface to generate a [Au]–DNA*b*–SA–*b*Fn–[CNT] hybrid nanostructure.¶ As shown in Fig. 3, TEM images confirmed that the DNA-modified Au nanoparticles were specifically assembled on the protein-functionalized CNT surface. Iron oxide cores of conjugated *b*Fn molecules were also observed in association with the Au nanoparticles, although the former were of much lower contrast. EDX analysis confirmed the presence of Au (9.6 keV) and Fe (6.4 keV), as well as S (2.3 keV) from the conjugated proteins and mercapto-terminated DNA. Significantly, the assembled Au nanoparticles were spatially separated across the surface of the protein-modified CNTs, and images of particles viewed along the projected sides of the nanotubes confirmed that the Au nanoparticles were not in direct physical contact with the CNT surface. The TEM images were consistent with corresponding optical spectra, which showed no shift in the 524 nm Au plasmon resonance band for the conjugated [Au]–DNA*b*–SA–*b*Fn–[CNT] nanostructures (ESI, S1†), and strongly suggested the presence of a multi-layered shell architecture of biomolecule-linked Au and iron oxide nanoparticles around the highly anisotropic CNT core.

Disassembly of the Au nanoparticles from the CNT surface was achieved by reversible denaturation of the 24-base-pair DNA duplex into single-stranded oligomers.‖ Unwinding of the DNA duplex was undertaken by heating the [Au]–DNA*b*–SA–*b*Fn–[CNT] nanostructures to 70 °C. TEM investigations of samples extracted from the mixtures at above the DNA melting temperature showed few Au nanoparticles associated with the CNTs, although the streptavidin–*b*Fn conjugates remained strongly bound (Fig. 4a). Significantly, when the temperature was decreased to room temperature, reassembly of the Au nanoparticles on the surface of the CNTs was observed (Fig. 4b). Alternatively, reversible disassociation of the Au nanoparticles was achieved by addition of excess free biotin to shift the streptavidin-binding equilibrium in favour of uncoupled biotin-terminated DNA-functionalized nanoparticles (ESI, S2†).

In summary, biomolecular interactions have been used to programme the assembly of multi-layer, multi-functional

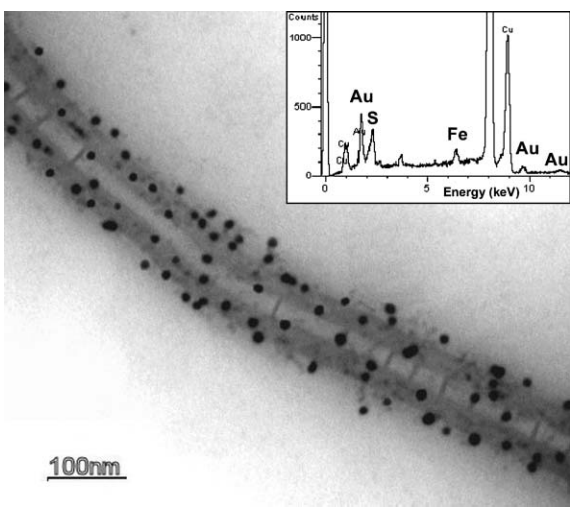

Fig. 3 Unstained TEM image of a [Au]–DNA*b*–SA–*b*Fn–[CNT] hybrid nanoconjugate showing single CNT with assembled Au and ferritin nanoparticles. Inset, EDXA spectrum corresponding to the TEM image showing the presence of Au, Fe and S (background peaks for Cu are also observed).

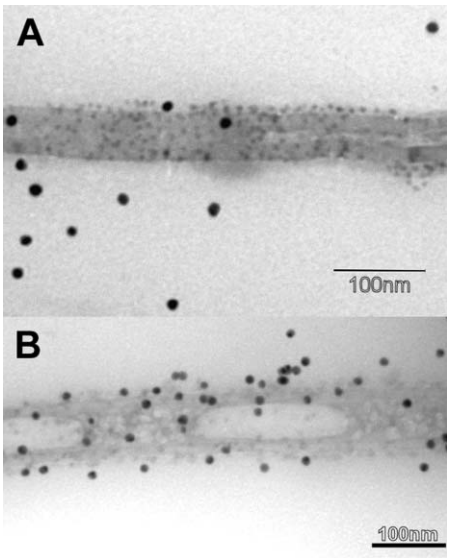

Fig. 4 TEM image of [Au]–DNA*b*–SA–*b*Fn–[CNT] conjugates showing (a) disassembly of Au nanoparticles after heating a dispersion to 70 °C; note the ferritin nanoparticles remain firmly attached. (b) Reassembly of [Au]–DNA*b* nanopaticles on SA–*b*Fn–[CNT] conjugates after cooling to room temperature.

CNT-based conjugates. The procedure results in the layered assembly of two different types of bio-functionalized inorganic nanoparticles that exhibit properties, such as superparamagnetism (ferritin), and electrical conductivity and surface plasmon resonance (Au), which in association with the reversible assembly/disassembly of the [Au]–DNA*b* building block, could be attractive functions in a wide range of potential bio-sensing environments. The interlinking of streptavidin molecules between the ferritin and Au nanoparticles could also provide additional functional centres, which might be exploited for example in the binding of small molecule biotinylated analytes to the CNT-based layered conjugate. Finally, ferritin can be routinely demineralized and reconstituted with a range of inorganic nanoparticles, notable the ferromagnetic oxide, magnetite (Fe_3O_4),[24] suggesting that magnetic, catalytic or photoactive building units could also be incorporated into multi-layered protein/nanoparticle CNT conjugated architectures by stepwise programmed assembly.

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Notes and references

‡ Biotinylated ferritin (*b*Fn) was synthesized according to standard procedures,[20] and coupled to CNTs by rapid cooling of heated reaction mixtures (ESI†).

§ 50 μL of SA (1 mg mL^{-1}) was added to a suspension of *b*Fn–[CNT] and incubated at room temperature for 4 h. Excess SA was removed by centrifugation and redispersion as described above.

¶ Au nanoparticles (mean diameter = 11 nm) were synthesized by citrate reduction of aqueous $HAuCl_4$ according to established procedures,[22] and capped with a 3′-mercapto-terminated 12-base single strand oligonucleotide (*ss*A; 5′-TCTCAACTCGTA-propyl-mercapto-3′, Eurogentec Ltd) as described previously.[23] 12.1 μL of a 24-base single strand oligonucleotide (4.1 μM) (*ss*C; 5′-TACGAGTTGAGAATCCTGAATGCG-3′, Eurogentec Ltd) was added to 100 μL of the *ss*A-capped Au nanoparticle colloidal solution containing 6×10^{15} particles L^{-1} in phosphate buffer. Subsequently, 5 μL of a 10 μM solution of a 5′-biotin-terminated 12-base single strand oligonucleotide (*ss*B; 5′-biotin-propyl-CGCATTCAGGAT-3′, Eurogentec Ltd) was added at a *ss*A–Au : *ss*B : *ss*C molar ratio of 1 : 50 : 50, and the dispersion incubated at room temperature for 2 h and then stored at 4 °C overnight. 100 μL of a suspension of [Au]–DNA*b* was added to the modified CNTs and the mixture left at room temperature for 4 h, and then at 4 °C overnight. The conjugated material ([Au]–DNA*b*–SA–*b*Fn–[CNT]) was separated from non-associated Au nanoparticles by centrifugation at 8 krpm for 5 min, followed by washing twice with HEPES buffer. The samples were stored in 500 μL HEPES buffer at 4 °C.

‖ Experiments to investigate reversible assembly were undertaken using two methods. Firstly, dispersions of conjugated [Au]–DNA*b*–SA–*b*Fn–[CNT] were heated at 70 °C for 5 min and then dried onto TEM grids without cooling, or after cooling back to room temperature. Secondly, biotin (10 μL, 1 mg mL^{-1}) was added to 500 μL of a dispersion of [Au]–DNA*b*–SA–*b*Fn–[CNT] to give a 20-fold molar excess of biotin over streptavidin. The samples were incubated at room temperature for 2 h and then air-dried onto TEM grids.

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