Immobilization of Small Proteins in Carbon Nanotubes: High-resolution Transmission Electron Microscopy Study and Catalytic Activity

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The morphologies of the small proteins Zn_2Cd_5-metallothionein, cytochrome c_3 and β-lactamase I immobilized inside carbon nanotubes have been studied by high-resolution transmission electron microscopy (HRTEM); single protein molecules and their associated forms were clearly observed inside the central cavity and a significant amount remained catalytically active indicating that no drastic conformational change had taken place.

The recently discovered carbon nanotubes[1,2] are prepared on a gram scale by the arc vapourisation of carbon. The caps which are present on the ends of the initially formed tubules may be removed[3,4] by treatment with oxidising acids such as nitric acid. In a typical sample, the internal diameters of the central tubes vary from 30 to 60 Å, and the openings at the ends of the tubes may be smaller in cross section than the interior diameter of the tube. Solutions of metal salts readily pass into the interior of the opened tubes, leading,[3] after heating, to their being filled with metal oxide crystals, *e.g.* of NiO, Fe_3O_4 and UO_2. Hydrogen reduction converts the oxides to pure metal crystals, *e.g.* Ni, Pd, Pt, Fe, Ru and Co. The treatment with oxidising acids not only opens the tube ends but also creates acidic sites which may be located both on the outer and inner carbon layers and especially at the opened ends. It appears that the majority of the acid sites are[4,5] carboxylic and phenolic acid groups. The attachment and immobilization of enzymes and other proteins on modified glassy carbon materials with retention of activity has[6] been described. We were interested, therefore, in determining whether proteins could be similarly immobilised on carbon nanotubes and especially whether suitably small proteins could be introduced into the protective interior of the tubes.

Samples of opened nanotubes were prepared[6] by refluxing the closed tubes with nitric acid for 24 h, followed by thoroughly washing with distilled water and drying *in vacuo* at 180 °C for 12 h. A substantial proportion of nanoparticles are also present in the resulting nanotube samples as observed in TEM micrographs. In a typical experiment the opened nanotubes were suspended in an aqueous protein solution for 24 h and volatile water was then removed under reduced pressure at room temperature over a period of at least 24 h. The resulting protein–nanotube material was transferred to a 400 mesh copper grid and examined by HRTEM (JEOL 2000FX), using an accelerating voltage of 200 kV. Optimum conditions were employed in order to reduce damage and consequent contamination of the sample with amorphous material.

Sample **A** was prepared using a purified sample of the cysteine-rich protein, Zn–Cd metallothionein (rabbit liver, metallothionein/II with Zn : Cd molar ratio of 2 : 5, M_r = 7000) (0.55 mg) in 2 ml of distilled water (39.3 μmol l^{-1}) and mixed with 2.0 mg of an opened nanotube sample. The micrographs showed that most of the nanotubes have internal cross section of 3–5 nm and the ends of > 90% of the tubes were open. All of the opened tubes contained distinct darker grey areas in the inner tubes. Careful energy-dispersive X-ray (EDX) analysis of selected protruding tubes containing the internal grey areas showed the presence of Cd, Zn and S over the grey areas and there was no visible external amorphous material. Therefore, we infer that the grey internal patches consist of protein-based material. These are observed along the entire length of the tubes and, interestingly, they occur at fairly regular separations of 15–25 nm [Fig. 1(*a*–*c*)]. In a blank experiment the grey areas did not appear when the tubes were treated with aqueous buffer solution not containing the protein. Fig. 1(*a*–*c*) shows a nanotube of sample **A** under higher magnification. The edges of all the grey areas appear as a smooth concave meniscus. This suggests the protein can strongly attach to the internal tube walls. We measured the dimensions of 60 grey patches of protein material inside the nanotubes and found that their average diameters matched those of the inner tube (3.0–5.0 nm) and the lengths varied from 3.1 to 15.0 nm. Two lengths predominated, namely 3.7 ± 0.5 and 6.8 ± 0.5 nm, whilst higher multiples of these lengths, *ca.* 9.5, 12.5 and 15.5 nm were occasionally observed. The crystal structure of the metallothionein/II indicates a tetragonal unit cell of dimensions 3.1 × 3.1 × 12 nm^3 accommodating[7,8] eight molecules. The molecules are packed in pairs and arranged parallel to each other and into two groups of four along the *z*-axis with a solvent channel bisecting the two groups. It seems reasonable that the smallest grey areas consist of protein dimers and those of length 6.8 ± 0.5 nm are tetramers. Dimers of metallothionein/II in solution are thought[8] to be the precursors of the crystal. The HRTEM studies also show the presence of grey areas on the outside of the tubes and on the surfaces of the nanoparticles. However, their morphology could not be clearly imaged.

The sample **B** was prepared by treatment of opened nanotubes (2.7 mg) with a solution of purified cytochrome c_3 (*Desulfovibrio vulgaris*, M_r = 14000) (25 μmol l^{-1}, using 0.7 mg of cytochrome c_3 in 2 ml of distilled water). The HRTEM

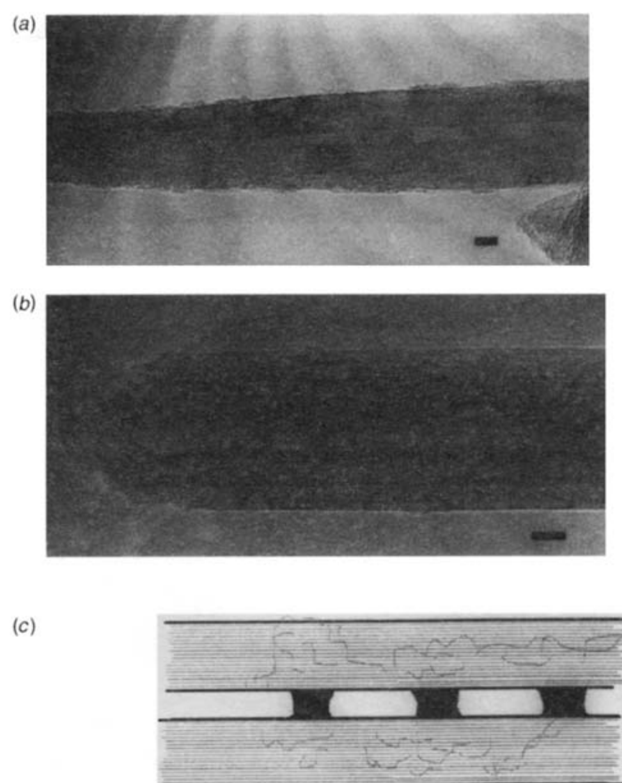

Fig. 1 (*a*) and (*b*) Micrographs showing concave meniscus of internal metallothionein patches. External metallothionein patches are also present. The scale bar is 5 nm. (*c*) Sketch of details in (*b*).

photographs of sample **B** are given in Fig. 2(*a*) and (*b*). They show many grey areas of amorphous material on the exterior surfaces of the nanotube. However, there are also some clearly visible grey patches inside the tube cavities and careful EDX analyses over these showed the presence of iron. These grey areas are quasi-spherical and of dimensions smaller than that of the host inner tube. The diameters of the smallest grey patches in the tube are about 3.3 nm: of similar size to cytochrome c_3 (a spherical protein[9] with a 3.0 nm diameter and having 4 haem units). It seems probable that the quasi-spherical grey areas are due to single protein molecules. Some larger grey areas inside the tubes of about 13.5 and 17 nm in length may be attributed to protein aggregates.

Sample **C** was prepared by treatment of opened tubes (20 mg) with the purified enzyme, β-lactamase I (*Bacillus cereus* 569H/9, M_r = 29 000) (0.11 mg) dissolved in 1 ml of distilled water (3.8 μmol l^{-1}). A HRTEM examination of sample **C** showed the presence of grey amorphous material, on both the interior and exterior surfaces of the tubes, see graphical abstract. The β-lactamase I is[10] small enough (3.3 × 3.8 × 4.9 nm^3) to fit into the inner tube cavity and we presume that the grey areas consist of aggregates of this protein.

A sample of purified flavocytochrome b_2 tetramer (*Saccharomyces cerevisiae*, M_r = 230 000) (0.22 mg) in 1 ml of distilled water (0.94 μmol l^{-1}) was tested for redox activity, prior to the addition of tubes, to confirm that the active tetrameric structure had been retained. Sample **D** was prepared with the addition of 20 mg of opened nanotubes to this solution. Examination of sample **D** by HRTEM showed the presence of grey areas on the nanotube external surface only: no grey areas were observed inside the tubes. The absence of these, in the case of this protein, is not surprising since the ellipsoidal flavocytochrome b_2 tetramer[11] is too large (10 nm in diameter, 6 nm thick) to enter the tube cavity.

We have carried out experiments on samples **B**, **C** and **D** designed to establish the stability of attachment of the proteins to the carbon surfaces. Typically, sample **B** in an Eppendorf tube was shaken with water, with buffered aqueous solutions of different pH or with aqueous sodium chloride (2 mol l^{-1}), each for 12 h. Spectroscopic analysis of the supernatant solutions showed, for samples **B** and **C**, that no protein has been extracted, and for sample **D** that $>90\%$ of cytochrome b_2 remained immobilized. Thus, the proteins are firmly attached to the carbon surfaces and it seems likely that the acidic sites known to be present contribute to the protein binding.

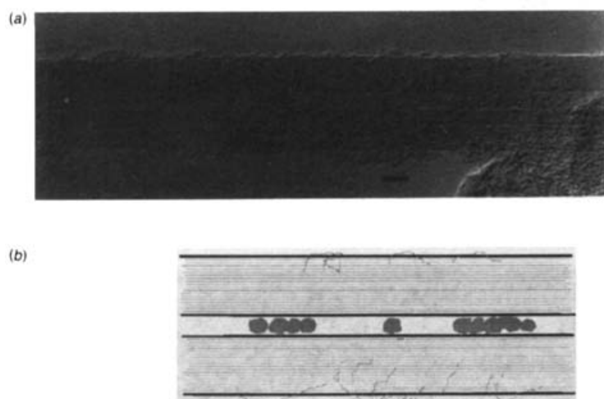

Fig. 2 (*a*) Micrograph of a cytochrome c_3 molecule (3.1 nm) and aggregates inside a nanotube. The protein can also be seen on the outside surface of the nanotubes. The scale bar is 5 nm. (*b*) Sketch of details in (*a*).

β-Lactamase I displays[12] extremely fast turnover rates. We have carried out activity studies on the hydrolysis of phenoxymethylpenicillin (penicillin V) by nanotube-immobilized β-lactamase I. The immobilised β-lactamase sample was treated with an excess of penicillin V (1 mmol l^{-1}) so that the initial rate of hydrolysis was proportional only to the total concentration of protein used. In the absence of immobilised protein no hydrolysis of substrate was observed. The initial rate of hydrolysis was determined by analysis of progressive curves for the initial 5 min of the reaction obtained by monitoring absorbance changes at 232 nm. Solutions of β-lactamase used for the preparation were calibrated for activity and gave an average turnover number of 1042 s^{-1}, in agreement with literature value.[12] The immobilized β- lactamase sample showed moderate activity (16.4% compared to standard solutions). The decrease in activity may be associated with the various effects of immobilisation[13,14] but at least some of the proteins retain their active site structure. On washing the immobilized sample with distilled water and then adding the same substrate concentration, activity was unchanged. These data do not distinguish between activity of the protein on interior and exterior carbon surfaces.

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