

# Template-Synthesized Protein Nanotubes

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## ABSTRACT

A layer-by-layer deposition strategy for preparing protein nanotubes within the pores of a nanopore alumina template membrane is described. This method entails alternately exposing the template membrane to a solution of the desired protein and then to a solution of glutaraldehyde, which acts as cross-linking agent to hold the protein layers together. The number of layers of protein that make up the nanotube walls can be controlled at will by varying the number of alternate protein/glutaraldehyde cycles. After the desired number of layers have been deposited on the pore walls, the alumina template can be dissolved to liberate the protein nanotubes. We show here that glucose oxidase nanotubes prepared in this way catalyze glucose oxidation and that hemoglobin nanotubes retain their heme electroactivity. Furthermore, for the glucose oxidase nanotubes, the enzymatic activity increases with the nanotube wall thickness.

**Introduction.** There is considerable interest in protein-functionalized nanoparticles, with potential applications ranging from biosensors,<sup>1–3</sup> enzymatic bioreactors,<sup>4</sup> disease treatment,<sup>5</sup> and membranes for bioseparations.<sup>6</sup> Protein-functionalized nanotubes have been of particular recent interest;<sup>7,8</sup> for example, we have functionalized template-synthesized nanotubes with antibodies<sup>6</sup> and enzymes,<sup>9</sup> and there have been numerous reports of protein-functionalized fullerene nanotubes.<sup>10,11</sup> In all of these examples, the nanotube itself acts as a carrier for the attached protein molecules, and since the protein is attached to the surfaces of the tube, most of the mass or volume of the system is the nanotube, not the protein. In some cases it might be advantageous to have nanotubes composed entirely of the protein itself, rather than the protein being a minor component of the system. We describe here a method for preparing such protein nanotubes.

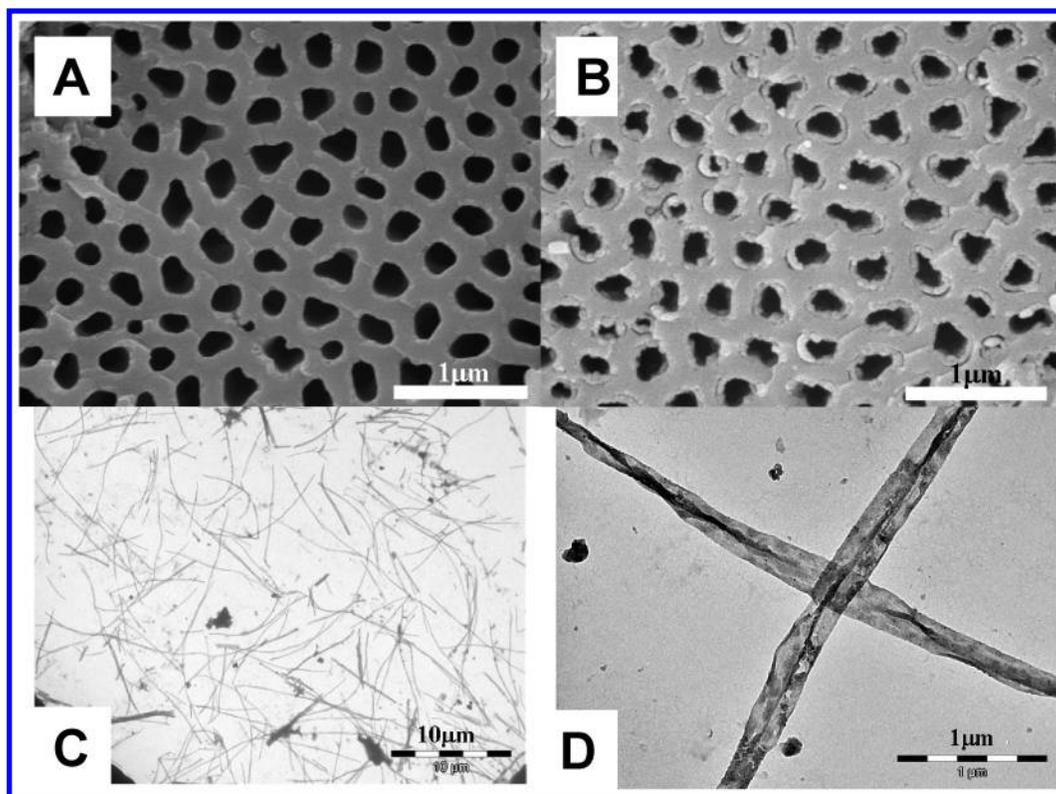
The synthetic strategy developed builds on our work on layer-by-layer nanotube template synthesis.<sup>12</sup> We showed that Mallouk's alternating  $\alpha,\omega$ -diorganophosphonate/Zr(IV) chemistry<sup>13</sup> can be used for preparing nanotubes within the pores of nanopore alumina template membranes.<sup>12</sup> This method entails alternate emersion of the alumina template into a solution of the  $\alpha,\omega$ -diorganophosphonate and then into a solution of ZrO<sup>2+</sup>. In the first deposition step, one end of the diorganophosphonate adsorbs to the alumina pore wall, and the other end is available for chelating the Zr(IV) layer, which can in turn be used to attach the next layer of diorganophosphonate.

A similar alternate-immersion strategy was developed to make the protein nanotubes. The nanopore alumina template

is immersed first into a solution of 3-amino propylphosphonic acid (APA), resulting in attachment (again via the phosphonate) of a monolayer of this molecule to the pore walls. The amino groups are then reacted with an excess quantity of the protein-immobilization agent glutaraldehyde (GA),<sup>14,15</sup> leaving unreacted aldehyde groups on the pore walls. The membrane is then exposed to a solution of the desired protein, which reacts via free amino sites with the aldehyde groups on the pore wall. The membrane is then alternately exposed to GA and then the protein solution again to layer-by-layer deposit the protein nanotubes. A similar method has been used to deposit multilayer glucose oxidase films on flat substrates.<sup>16</sup> After the desired number of protein/GA layers have been deposited on the pore walls, the alumina template can be dissolved to liberate the protein nanotubes. We show here that glucose oxidase nanotubes prepared in this way catalyze glucose oxidation and that hemoglobin nanotubes retain the heme electroactivity.

**Experimental Section.** The alumina template membrane used was a commercially available filter that was 60  $\mu\text{m}$  thick, had nominally 200 nm-diameter pores, and a pore density of  $\sim 10^9$  pores  $\text{cm}^{-2}$  (Figure 1A). Pieces of this membrane with area  $\sim 1$   $\text{cm}^2$  were used for these studies. As discussed previously,<sup>12</sup> we want the nanotube-forming material, in this case the protein, to deposit along the pore walls but not on the faces of the template membrane. Face (or membrane-surface) deposition is unwanted because this will ultimately block the pores. For the diorganophosphonate/Zr(IV) nanotubes,<sup>12</sup> deposition at the membrane faces was prevented by sputtering a thin ( $\sim 5$  nm) film of Au on both faces. Au is a good surface-blocking material because, unlike alumina, the phosphonate group does not adsorb strongly to Au.

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**Figure 1.** SEM images of the surface of the alumina template before (A) and after (B) deposition of 6-layer GOD nanotubes. (C, D) TEM images of the liberated nanotubes taken at two different levels of magnification.

Protein nanotube synthesis also begins with deposition of a phosphonate, APA, so deposition of this material on the membrane faces was again prevented by sputtering both faces of the membrane with Au. After deposition of APA along the pore walls, the membrane is then immersed into the GA solution (*vide infra*). Since there is no APA on the membrane faces, GA does not deposit on the faces either. However, in the next step the membrane is immersed into the protein solution, and it is impossible to prevent nonspecific adsorption of the protein to the Au surface films. However, we have found that the quantity of protein deposited at the membrane faces via this nonspecific mechanism is much less than if the membrane faces were not sputtered with Au. As a result, with the Au-sputtered membrane, the pores at the membrane surface do not get blocked with protein, and protein nanotubes are deposited along the pore wall.

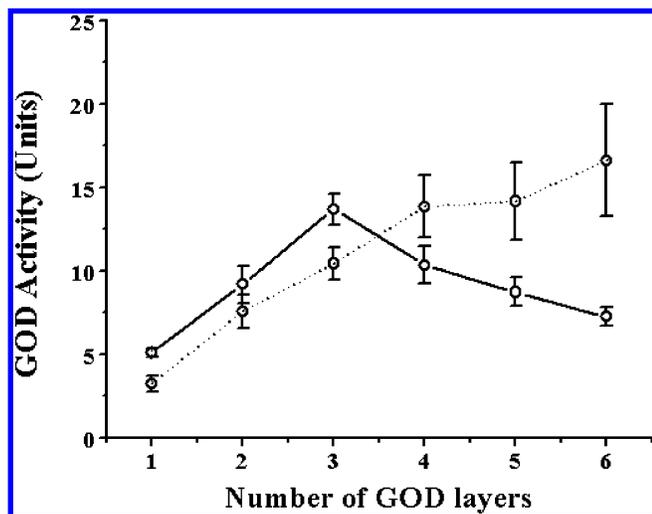
Details of the synthetic procedure can be found with the Supporting Information. Briefly, a 5 mM APA solution (pH = 5.8) was used to deposit the APA monolayer. A 2.5% solution of glutaraldehyde (GA) was used to deposit the first GA layer. The protein solutions (glucose oxidase or hemoglobin) had concentrations of 10 mg of protein per mL, and the protein layers were deposited by vacuum filtration of the protein solution through the membrane. To deposit the second GA layer the membrane was immersed into a 0.025% GA solution. The protein nanotubes were then built up layer-by-layer by alternate vacuum filtration of the protein solution through the membrane followed by immersion of the membrane in the 0.025% GA solution. After deposition of the desired number of protein/GA layers, the nanotubes were liberated by dissolution from the template membrane. This

was accomplished by immersion of the membrane for 24 h into a 5% phosphoric acid solution at 0 °C. The liberated nanotubes were collected by vacuum filtration and stored dry at 4 °C until use.

It is essential to show that the liberated nanotubes retain their biochemically activity. For the glucose oxidase (GOD) nanotubes this was accomplished by assaying their activity for glucose oxidation using the well-known aminophenazone/ peroxidase method.<sup>17</sup> The details of this colorimetric assay for GOD activity are presented in the Supporting Information. This method provides the GOD activity in units, where one unit corresponds to the production of one micromole of H<sub>2</sub>O<sub>2</sub> per min at 37 °C. The GOD activity of the liberated nanotubes was compared to the GOD activity of an identical quantity of nanotubes that was left embedded within the template membrane. For the latter assay, the GOD-nanotube-containing template membrane was simply immersed into the assay solution.

To show that the liberated Hb nanotubes are not denatured, we attached the tubes to an indium/tin oxide (ITO) electrode and used cyclic voltammetry to show that they retained their heme electroactivity. The details of the attachment procedure can be found with the Supporting Information. Cyclic voltammetry was performed on an EG&G 273 potentiostat in phosphate buffer solution (pH = 7.0), with the ITO as the working electrode, a platinum foil as the counter electrode, and a Ag/AgCl reference electrode.

**Results and Discussion.** *Glucose Oxidase (GOD) Nanotubes.* We designate the nanotubes by the number of alternate protein/GA immersion cycles used to build up the walls of the tube; i.e., 1-layer, 2-layer, etc., nanotubes. Figures 1 A

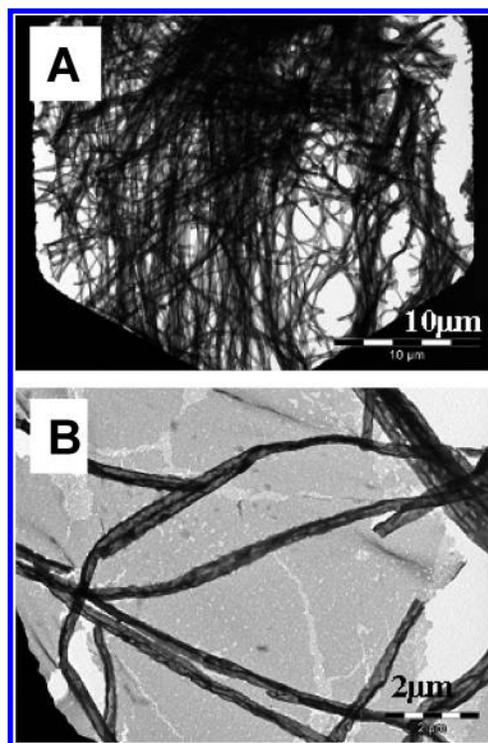


**Figure 2.** Plots of GOD activity vs layer number for GOD nanotubes. Dashed line = nanotubes liberated from the template membrane. Solid line = membrane-embedded nanotubes. The data represent averages of measurements on three identically prepared nanotube samples.

and B show scanning electron microscopic (SEM) images of the surfaces of alumina template membranes before and after deposition of 6-layer GOD nanotubes within the pores. Evidence for nanotube deposition can be seen by the decrease in the average pore diameter after deposition. An approximate measure of the wall thickness of the nanotubes can be obtained from such images; wall thickness of  $\sim 15$  nm and  $\sim 30$  nm were obtained for 3-layer and 6-layer nanotubes, respectively. These thicknesses are in qualitative agreement with what might be anticipated from the dimensions of the GOD molecule,  $5 \text{ nm} \times 5 \text{ nm} \times 7 \text{ nm}$ .<sup>15</sup>

More direct evidence for nanotube formation can be obtained by dissolving the membrane and imaging the liberated nanotubes using transmission electron microscopic (TEM). The lower magnification TEM image (Figure 1C) shows that tubes with lengths equivalent to the membrane thickness can be obtained but that many of the tubes are broken during dissolution of the membrane and filtration to collect the nanotubes. The higher magnification image (Figure 1D) shows the outside diameter and portions of the hollow inner core of the GOD nanotubes.

A critical issue for these studies is whether the phosphoric acid solution used to dissolve the template membrane and liberate the nanotubes denatures the protein making up the tubes. To explore this issue quantitatively, we compared the GOD activity for GOD nanotubes left embedded within the pores of the membrane (no exposure to phosphoric acid) with the GOD activity for an identical quantity of GOD nanotubes that had been liberated from the membrane (Figure 2). The GOD activities for the liberated 1-, 2-, and 3-layer nanotubes are slightly less than the activities for the corresponding membrane-embedded nanotubes, but the difference is similar in magnitude to the error in the measurement. For the membrane-embedded nanotubes, there is no further increase in GOD activity for the 4-, 5-, and 6-layer tubes (Figure 2). Indeed, the embedded nanotubes show a slight, but real, decrease in activity on going from the 3-layer to 6-layer

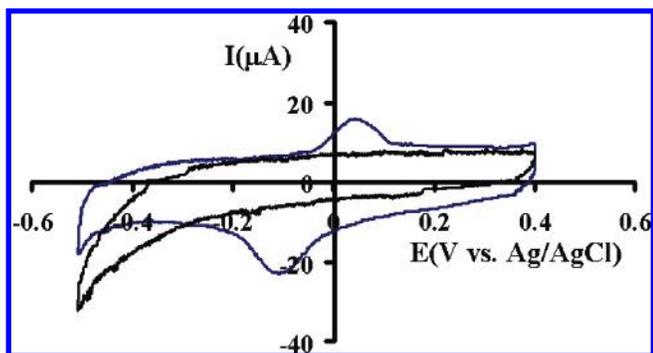


**Figure 3.** TEM images of the liberated 6-layer Hb nanotubes.

tubes. In contrast, the liberated nanotubes show a significant increase in activity on going from the 3-layer to 6-layer tubes.

When the nanotubes are left embedded within the pores of the template membrane, the glucose to be oxidized must diffuse from bulk solution to the membrane surface, then down the hollow core of the tube, and finally into the tube walls to access the GOD making up the tubes. (That diffusion of glucose through the tube walls occurs was proven by studies of activity vs film thickness for layer-by-layer-deposited GOD films on flat surfaces.<sup>18,19</sup>) The  $\text{H}_2\text{O}_2$  produced by the enzymatic reaction must then diffuse out of the tube wall and into the hollow core of the nanotube. The  $\text{H}_2\text{O}_2$  (or its reaction products) must then diffuse to one of the membrane faces and out into bulk solution. Hence, there are significant barriers to mass transport with the membrane-embedded nanotubes. Furthermore, the flux down the length of the hollow core of the nanotube will decrease with increasing layer number because the wall thickness increases, and hence the inside tube radius decreases, with layer number.

The barriers to mass-transport for the liberated nanotubes are not as severe. First, because the liberated tubes are dispersed throughout the assay solution, the diffusional distance to bring glucose to (and products away from) a tube is less than for the membrane-embedded tubes. Second, while glucose can diffuse down the length of the hollow core of the nanotube, it can also access the GOD by diffusion from solution through the outer surfaces of the tube. This is not possible for a membrane-embedded nanotube because the outer surface of the tube is in contact with the pore wall. Because glucose was easier access to the GOD in the liberated nanotubes, the mass-transfer resistance is lower for the liberated tubes. This diminution of the mass-transport is



**Figure 4.** Cyclic voltammograms for the ITO electrode before (black curve) and after (blue curve) attachment of the Hb nanotubes. Scan rate = 60 mV s<sup>-1</sup>.

undoubtedly responsible for the higher enzymatic activities for the liberated 4-, 5-, and 6-layer nanotubes (Figure 2). Put another way, the decrease in mass-transfer resistance for the liberated nanotubes means that a larger fraction of the GOD making up the tube wall is available for enzymatic reaction.

**Hemoglobin (Hb) Nanotubes.** Figure 3 shows TEM images of liberated 6-layer Hb nanotubes. Again, the lower magnification image (Figure 3A) shows that tubes with lengths equivalent to the membranes thickness can be obtained but that many of the tubes are broken during dissolution of the membrane and filtration to collect the nanotubes. The higher magnification image (Figure 3B) shows the outside diameter and portions of the hollow inner core of the GOD nanotubes.

There has been considerable interest in the redox properties of Hb. Typically a mediator or promoter is required in order to observe the heme electrochemistry;<sup>20</sup> however, there are numerous reports of direct electron transfer to Hb immobilized within various types of organic<sup>21</sup> and inorganic<sup>22,23</sup> matrices. When the protein is denatured, the heme redox activity is lost.<sup>24–26</sup> Figure 4 shows cyclic voltammograms for the ITO electrode before and after attachment of the liberated Hb nanotubes. The immobilized Hb nanotubes show heme electroactivity with a formal potential (approx. -40 mV vs Ag/AgCl) similar to that observed for Hb immobilized in a silica matrix at a glassy carbon electrode.<sup>22</sup>

**Conclusions.** We have described a simple method to make nanotubes composed of proteins. Because of the versatility of the reagent glutaraldehyde, this method should be applicable to nearly any protein. The wall thickness of the protein nanotubes can be controlled at will, and for the GOD nanotubes, the enzymatic activity increases with wall thickness.

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**Supporting Information Available:** Experimental details describing nanotube synthesis, liberation of nanotubes, GOD activity assay, and Hb nanotube immobilization. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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