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ARTICLE *in* BIOMACROMOLECULES · OCTOBER 2006

Impact Factor: 5.75 · DOI: 10.1021/bm060412l · Source: PubMed

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Fabrication of Protein Nanotubes Based on Layer-by-Layer Assembly

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Received April 28, 2006; Revised Manuscript Received July 9, 2006

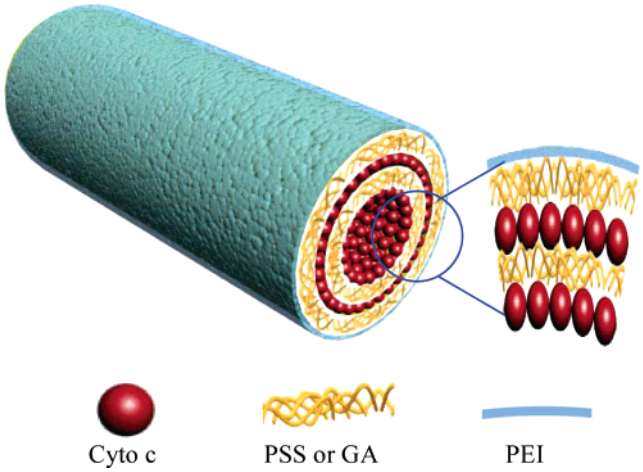
Nanotubes of cytochrome C (cyto-c) with glutaraldehyde (GA) or PSS based on the layer-by-layer (LbL) assembly through covalent binding and electrostatic adsorption have been fabricated. The combination of the template method and the LbL method for fabrication of nanotubes exhibits low cost, simplicity, and versatility. The tubular morphology of the assembled glutaraldehyde and cytochrome C film was demonstrated by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) measurements.The components of the tubes were determined by energy-dispersive X- ray spectra (EDAX). It is found that the assembled tubes keep the proteins' biochemical activity and electronic activity by cyclic voltammograms. The measurements of ultraviolet spectra and circular dichroism (CD) on the assembled nanotubes confirmed the cyto-c existence in the tubes.

Introduction

Nanotubular structures have attracted extensive research interest due to their unique physicochemical and electronic properties and their potential applications.[1−5] In the past decades, carbon nanotubes as well as other kinds of tubes composed of inorganic compounds, organic polymers, or metals have been synthesized and characterizated.[6−10] Different components of nanotubes have been considered for application in different fields; for instance, carbon nanotubes are good for application of nanoelectronics. Silica nanotubes are potentially used as biocatalysts, fuel cells, sensors, and separation systems.[11−15] A common route to prepare nanotubes of different composition has been developed via a membrane template. Such a method is becoming an effective way to synthesize one-dimensional nanomaterials.[16] Highly ordered nanotube arrays can be obtained by using template membranes with their highly ordered pores.[17] In most cases, sizes, shapes, and other structural properties of the assembled system can be controlled as well by the template method. The layer-by-layer assembly based on the alternate adsorption of oppositely charged species has generated much interest and has been developed into a general approach to fabricating ultrathin films on solid surfaces.[18] Combination of the LbL technique with the template method is recognized as a premier way for the preparation of designed nanotubes, largely due to its low cost, simplicity, and versatility.[9,19] The LbL technique permits the coating of substrates of various shapes and sizes with uniform layers of varying composition.[9,20,21]

Protein-based nanotubes have potential applications as biosensors and enzymatic bioreactors and for disease treatment and bioseparations.[22−25] LbL assembly has been extended to build up multilayer films of proteins or other biomacromolecules and is employed for the design and construction of biodevices. Cytochrome c (cyto-c) is a well-characterized redox protein that has received considerable attention in recent years.[26,27] Cytochrome c takes part in the cell respiration process and is a very important electron transfer agent during bio-oxidation.[27] The direct electron transfer reaction of cyto-c has been the most widely investigated among all redox proteins.

Scheme 1. Schematic Representation of Cyto-c/GA or Cyto-c/PSS Nanotubes with LbL Assembly on the Inner Wall of the Alumina Template Based on Covalent Bonding or Charge Interaction



The aim of the present work is to fabricate cyto-c nanotubes through the layer-by-layer assembly method in the pores of a template membrane. The synthesis procedure is based on our previous work on electrostatic adsorption by polyelectrolyte and chemical reaction via covalent bonds.[28,29] Briefly, the protein can be bonded with glutaraldehyde (GA) after they adsorb in high density on the pore walls of an alumina template to fabricate layer-by-layer films. Also, since cyto-c is an amphoteric protein (pH value at isoelectric point is 10.4),[30] it has a net positively charged surface at pH below its isoelectric point, while it is opposite above its isoelectric point. Thus at pH 7.0, the cyto-c has a net positively charged surface, while poly(sodium styrenesulfonate) (PSS) possesses negative charges. They can form a multilayer by LbL assembly through oppositely charged adsorption. In the present work (Scheme 1), we take PSS and poly(ethylenimine) (PEI) as the first two layers inside the template pores. In the following, we deposited cyto-c and

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10.1021/bm060412l CCC: $33.50
Published on Web 08/22/2006

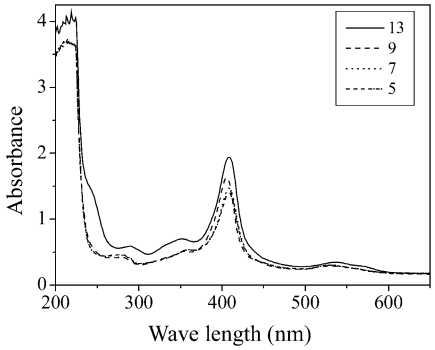

Figure 1. UV spectra of cyto-c solution at different pH values.

GA, respectively, on the polymer film. The final obtained (cyto-c/GA)$_5$ nanotubes exhibit a well-defined and reversible electrochemical response at underlying electrodes in a tubular structure with GA and PSS. The composite of protein cyto-c is not changed within the tubes. In all of these examples, the nanotube itself acts as a carrier for the attached protein molecules, as reported early by Martin;[31] it may become advantageous to have nanotubes composed entirely of the protein itself instead of a minor component of protein in the system.

Result and Discussion

The stability of the cyto-c system under drastic pH condition changes was measured by ultraviolet (UV) absorption, which clarified the structural stability of cyto-c with the pH modification. Figure 1 shows the UV spectra of the cyto-c buffer solution from pH 5 to 13. The UV spectrograph of the cyto-c solution shows that the absorption spectrum at 280 nm results from the side chain groups of some amino acid. In the far-UV range, around 220 nm shows the peptide bond absorbance of the protein. The UV absorption experiments demonstrate that the composite of protein cyto-c is not changed within the pH range. At pH > 10.4, the UV spectra exhibit a red shift, indicating that the protein secondary structure might be alternated at higher base condition.

The (cyto-c/GA)$_5$ nanotube formation can be evidenced by imaging the liberated nanotubes using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The SEM image in Figure 2A shows the typical tubular structure of the obtained samples, and some of them have lengths that are almost identical to the template thickness. Figure 2B displays the hollow inner core of the (cyto-c/GA)$_5$ nanotubes, and the obtained tubes look flexible. It should be noted that the dimension of the obtained tubes from SEM and TEM images is larger than the expected one due to the different collapse degree caused under different vacuum conditions. The TEM image in Figure 2C provides the information on the outside diameter and portion of the hollow inner core of the (cyto-c/GA)$_5$ nanotubes. The wall thickness of the (cyto-c/GA)$_5$ nanotubes is about 30 nm, so each bilayer is about 6 nm. The energy-dispersive X-ray (EDAX) analysis in Figure 2D gives the main elements of the obtained tubes with carbon, nitrogen, oxygen, and phosphate (except hydrogen), indicating that the expected composites from cyto-c and GA are existing in the tubes. Due to the low density of sulfur, the EDAX analysis does not give its contribution in the protein tubes.

The SEM image in Figure 3A shows that the (cyto-c/PSS)$_5$ nanotubes are also successfully fabricated by a similar method. The walls of the nanotubes are uniform with smooth surfaces. The TEM image in Figure 3B further demonstrates the tubular

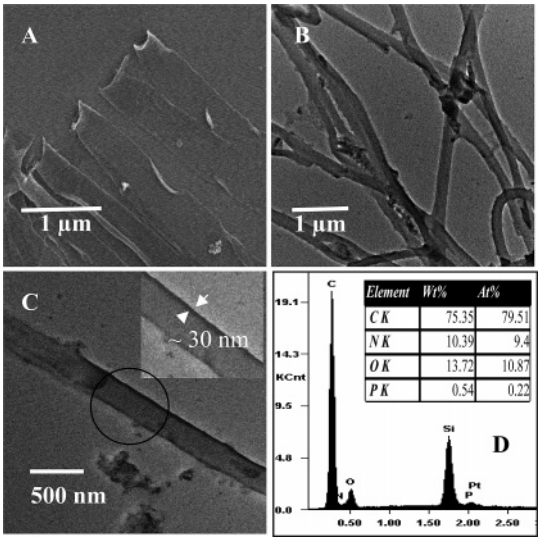

Figure 2. (A) SEM image of assembled (cyto-c/GA)$_5$ tubes. (B) TEM image of the (cyto-c/GA)$_5$ tubes. (C) TEM image of a selected (cyto-c/GA)$_5$ tube, with the insert image showing the wall thickness of the tube. (D) The energy-dispersive X-ray spectra (EDAX) analysis of assembled (cyto-c/GA)$_5$ tubes.

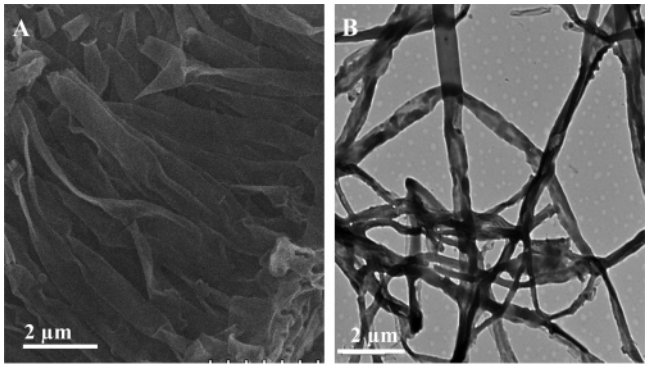

Figure 3. (A) SEM images of (cyto-c/PSS)$_5$ nanotubes. (B) TEM image of (cyto-c/PSS)$_5$ nanotube.

structure and shows the outside diameter and portion of hollow inner core of cyto-c nanotubes.

The bioactivity analysis will provide the electric characteristics of the assembled (cyto-c/GA)$_5$ and (cyto-c/PSS)$_5$ tubes. If the cyto-c is denatured during assembly, the heme redox activity will be lost. In view of this, we have performed measurements of the electrocatalytic properties of the tubes. Figure 4A,B shows the cyclic voltammograms of (cyto-c/GA)$_5$ and (cyto-c/PSS)$_5$ tubes, respectively, for the glassy carbon electrode after attachment of the liberated nanotubes. Direct electrochemistry of the heme proteins in both cases is observed. The measurement results from the immobilized (cyto-c/GA)$_5$ and (cyto-c/PSS)$_5$ tubes demonstrate the heme electro activity with a formal potential similar to each other, which means that the assembled cyto-c tubes with GA or PSS keep the bioactivity and induced property of the protein.

Figure 5A shows the UV spectra of pure cyto-c aqueous solution and (cyto-c/GA)$_5$ and (cyto-c/PSS)$_5$ tubes in water. The featured absorption bands at 408 nm from the three curves represent the contribution of cyto-c, and the absorption peak at 230 nm of (cyto-c/GA)$_5$ is from GA as well as its aggregation. The peak at 220 nm proves the existence of PSS in the (cyto-c/PSS)$_5$ nanotubes. The UV spectra basically determined the composition of the assembled proteins tubes.

To prove that the structure of cyto-c will not be significantly changed at the selected pH range, we carried out circular

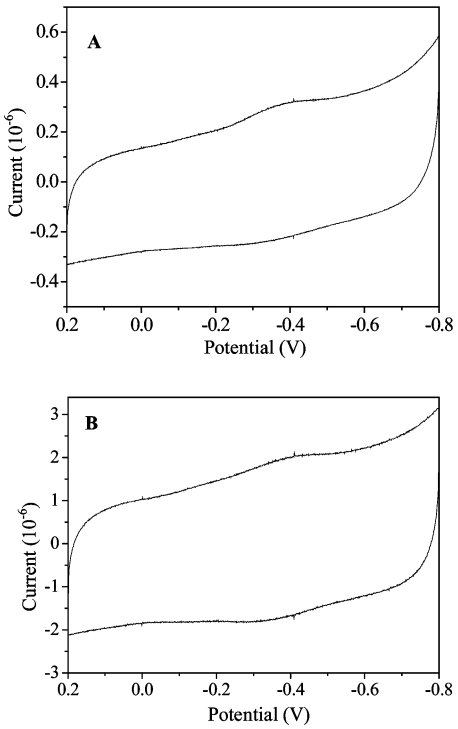

Figure 4. Cyclic voltammograms for the electrode after attachment of the (cyto-c/GA)$_5$ (A) and (cyto-c/PSS)$_5$ (B) nanotubes.

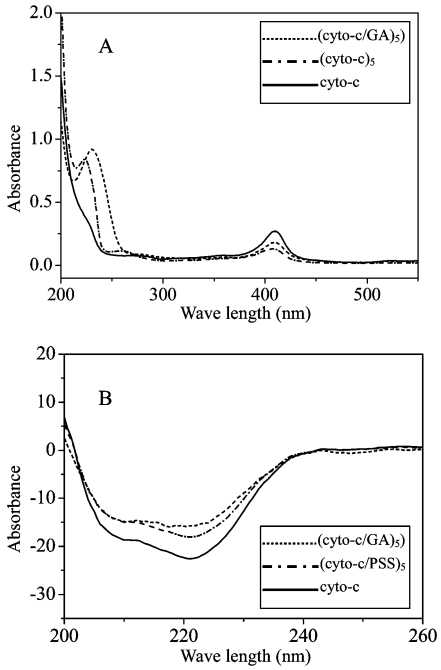

Figure 5. UV (A) and CD (B) spectra of (cyto-c/GA)$_5$ nanotube, (cyto-c/PSS)$_5$ nanotubes, and pure cyto-c water solution.

dichroism (CD) measurements. The circular dichroism (CD) spectra in Figure 5B show the featured adsorption of cyto-c in (cyto-c/GA)$_5$ and (cyto-c/PSS)$_5$ tubes. Comparing to the CD spectra of pure cyto-c in aqueous solution, we do not find the obvious variation of the α-helix conformation corresponding to the two minimum peaks in the above different pH condition for the assembled (cyto-c/GA)$_5$ and (cyto-c/PSS)$_5$ tubes. It indicates the lack of change of the cyto-c structure under the assembled condition after fabrication into (cyto-c/GA)$_5$ nanotubes.

Conclusion

In conclusion, cytochrome c tubes were successfully synthesized by means of the layer-by-layer method through chemical reaction with GA and electronic absorption with PSS on the inner wall of template pores. The assembled nanotubes have a uniform size and bendable shape. We also proved that after fabrication the tubes possess the electronic and bioactive properties of proteins, which may result in tubes having potential applications as carriers of biocatalysts.

Experimental Section

Materials. The ordered porous alumina film is a product from Whatman Co. (which was sold as having 0.2 μm pores); the measured inner pore diameter is ca. 300 nm, and the thickness of the film is about 60 μm. The cyto-c was obtained from Sigma. The PSS was purchased from Aldrich. All chemicals were used as received without further purification. The water used through out the experiment is purified with a Milli-Q system from Millipore Co.

Synthesis of (cyto-c/PSS)$_5$ Nanotubes. The protein nanotubes are synthesized on the basis of the methods reported previously.[9,31] Briefly, the alumina template membrane was first sputtered by a thin Au film (∼5 nm) to prevent the protein from remaining at the surface of template. The Au-coated alumina membrane was first immersed in PEI (1 mg/mL, 0.1 M NaCl) solution, sonicated for 1 min, and incubated for 1 h. Then, the PEI-coated alumina template was immersed in negatively charged PSS solution, $C = 1$ mg/mL (pH 7, containing 0.1 M NaH_2PO_4 and 0.1 M KBr). It will take about 1 h for for adsorption. Since PEI is positively charged, PSS will absorb on the PEI surface. The same procedure is suitable for cyto-c adsorption, at $C = 1$ mg/mL, pH 7, containing 0.1 M NaH_2PO_4 and 0.1 M KBr solution. After 5 times of "immersing-and-drying" cycles, a multilayer of (cyto-c/PSS)$_5$ forming on the inner walls of the template pores is expected. Fabrication of (cyto-c/GA)$_5$ nanotubes was carried out by a similar procedure to that discussed above. The GA aqueous solution (0.025%) was sonicated for 15 min. The (cyto-c/PSS)$_5$ and (cyto-c/GA)$_5$ multilayer-film-containing template was immersed into 5% phosphoric acid solution for about 3 h. This procedure will cause the Au film to delaminate from the alumina, and then, it was carefully wiped away. The film template was immersed into fresh 5% phosphoric acid solution for 24 h at 0 °C until the alumina was dissolved thoroughly. A short period of ultrasonic treatment is permitted, and then after the removal of the template, the (cyto-c/PSS)$_5$ and (cyto-c/GA)$_5$ nanotubes are liberated into the solution. A great amount of water was used to wash out the samples in order to remove the residual salt.

Characterizations. The glassy carbon electrode was first coated by PEI (1 mg, 0.1 M NaCl solution) for 1 h, rinsed by water, and dried by N_2 stream. Then, it was immersed into 0.25% GA solution for 30 min and washed by water. Afterward, the electrode was immersed into (cyto-c/PSS)$_5$ and (cyto-c/GA)$_5$ solution for 6 h and dried by N_2 for the detection of cyclic voltammogram. All the immersion procedures were performed at 4 °C.

The scanning electron microscopy (SEM) micrographs were acquired on an S-4300 (HITACHI, Japan), and the transmission electron microscopy (TEM) images were obtained by 200-CX (JEM, Japan). Cyclic voltammograms were recorded on a CHI660B instrument (CHENHUA, China). The UV−vis spectra were carried out on a HITACHI U-3010 UV−vis spectrometer, and CD spectra were measured on a JASCO J-810 spectropolarimeter.

Acknowledgment. This work was financially supported by the National Nature Science Foundation of China (nos. 20574077 and 20403022), the Chinese Academy of Sciences, as well as the collaborative project of the German Max Planck Society.

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BM060412L