

Fabrication of bovine serum albumin nanotubes through template assisted layer by layer assembly

by

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ABSTRACT

One-dimensional nanostructures have offered unique advantages in many fields. Protein based nanotubes, in particular, are desirable for biomedical applications due to their ease of functionalization and intrinsic biocompatibility.

Template-assisted methods are widely used to fabricate cylindrical nanostructures like carbon nanotubes, metal nanowires, polymer nanorods, etc. In the fabrication of protein nanostructures, the layer by layer (LbL) technique has long been applied to deposit protein multilayers on planar and spherical substrates. The success in each area led to the conclusion that the combination of these two techniques will potentially bring us the capability of fabricating protein nanotubes in a more controllable fashion.

In this work, protein nanotubes have been successfully deposited inside nanoscopic pores by sequential filtration of bovine serum albumin (BSA) solution at pH 3.8 and pH 7.0 through the channels in the anodic aluminum oxide (AAO) template. The morphologies of the obtained nanostructures have been examined using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Also, a simple analysis from UV/Vis spectroscopy has shown that the solutions used in our experiment will not significantly damage the bioactivity of BSA. Our future work will focus on strengthening the mechanical stability of the protein nanotubes and controlling their morphology more precisely.

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CHAPTER 1: INTRODUCTION

The rapid development in materials science has brought the huge advance in the applications of highly functionalized nanomaterials as well as innovations in the fabrication techniques for these materials. Out of all the assembling methods, template assisted methods have proven to be extremely useful for the fabrication of one-dimensional nanomaterials. Layer by layer (LbL) technique has been widely used in the controlled growth of two-dimensional nanostructures that are deposited on a planar or spherical substrate. The successful adoption of LbL technique in templates with nanochannels is rapidly emerging as an enabling fabrication approach to synthesis cylindrical nanomaterials. This work was inspired by several pioneering works that have combined the template assisted fabrication and LbL assemblies to produce nanostructures. The **objective** of this work is to explore the possibility of fabricating single component protein nanotubes by applying the LbL method, i.e. alternately depositing oppositely charged bovine serum albumin (BSA) molecules, within the nanochannels provided by anodic aluminum oxide (AAO) templates.

A literature review of the procedures, process variables, and applications of both template assisted nanofabrication and LbL methods is presented in Chapter 2. The results of this endeavor are presented in Chapter 3. The effect of the pH of the solutions used in our experiment is discussed in terms of the bioactivity of BSA molecules based on the results from UV/Vis spectroscopy. The morphologies of the obtained nanostructures have been examined using scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

Additionally, we have tried to reinforce the mechanical stability of the protein nanotubes by the introduction of a supporting double layer that offers covalent bonds between the aluminum oxide surface and the protein layers. Finally, we have used home-made AAO templates, of which the thickness and the

pore size can be easily controlled, to improve the controllability of the dimensions of the obtained nanotubes. Those preliminary results are discussed in Chapter 4.

Finally, the conclusions of this project and prospect of future work are discussed in Chapter 5.

CHAPTER 2: BACKGROUND

2.1 Template assisted fabrication

The rapid growth of microscopy in the last couple of decades has enabled people to visualize smaller and finer structures of matters. Along with the tremendous success scientists have experienced in the synthesis of nanoscopic materials of different kinds and shapes, two very useful approaches, namely self assembly and template assembly have emerged.

The self-assembly of nanoparticles is conceptually simple; particles of a controlled size are obtained when a disordered system forms an organized structure as a consequence of specific interactions among the components, without outside direction [1]. However, self assembly methods often depend upon the unique chemistries and functionalities of the building blocks, and the morphologies and dimensions of the self-assembled nanostructures rely significantly on the properties of the original bulk materials [2-4].

Template assisted fabrication, on the other hand, is relatively free of this concern because it can utilize an external force to confine the process of the reaction. Moreover, with the help from the supporting substrates, template assisted fabrication can be used to synthesize uniform nanoparticles with different geometries, which can be desirable for a lot of applications.

2.1.1 AAO template

The templates that are commonly used for template assisted fabrication are track-etched membranes and anodic aluminum oxide (AAO) templates, both of which have open-through nanochannels across their thicknesses. Track-etched membranes are formed by nuclear track etching of polycarbonate [5] or polyester [6] thin films. These membranes have some disadvantages which include the low pore density

and random pore distribution over the surface. In comparison, templates made of porous alumina can have a much higher porosity as well as a perfect periodicity under controllable conditions.

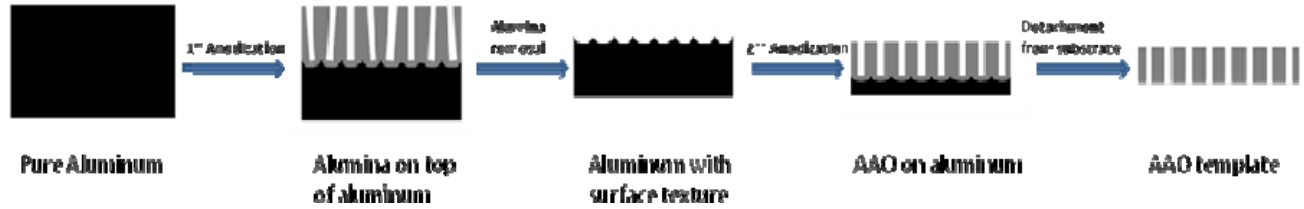


Fig.1 Schematic diagram of two-step anodization process

Figure 1 shows a schematic diagram of the two-step anodization process used to fabricate AAO templates. Essentially, the well-aligned nanopores are the product of the mechanical stress associated with the volume expansion that occurs during the conversion of aluminum to aluminum oxide [7]. By varying parameters like solution type [8], anodization voltage [9] and time in the electrochemical setup, we can obtain long-range ordered templates with tunable pore size, inter-pore spacing and thickness. These properties are very important for controlling the dimensions and related performance of the nanostructures produced from these templates.

2.1.2 Template assisted fabrication of 1-D nanomaterials

Nanostructures of different material chemistries have been synthesized using different template assisted approaches for various kinds of applications. For example, nanostructures have been fabricated using metals, alloys and binary compounds that are useful in the areas of magnetic and electronic devices by the means of electrodeposition. Specifically, Fe [10], Co [11], and Ni [12] nanowires have been synthesized in AAO templates using alternating current (AC) deposition from aqueous electrolytes of FeSO_4 , CoSO_4 and NiSO_4 respectively. The nanowires obtained from this conventional method are in the form of hexagonally arranged cylinders standing perpendicular to the film plane. From the data of hysteresis loops, these nanowires could be very useful for magnetic recording media [13]. Deposition using a direct current (DC) power source is also applicable in the synthesis of one dimensional nanostructures. A consistent

effort has been made on the electrodeposition of free standing Cu nanorod arrays on Cu foil via a template assisted method. The obtained nanostructure would be a high-performance current collector in Li ion batteries [14].

AAO templates can also serve as a matrix for the growth of carbon nanotubes. A demonstration of this was first published by Li *et al* [15]. Starting from electrodeposition of a small amount of Co as a catalyst at the bottom of the nanochannels, the template was then heated in a chemical vapor deposition (CVD) furnace at 600°C for 4-5 hours under CO flow. Then it was replaced by an acetylene flow for 2 hours at 650°C. Finally, the sample was annealed in nitrogen for 12 hours. Figure 2 is a hexagonally patterned array of carbon nanotubes synthesized using this method. Since the pore size of AAO template is proportional to the applied voltage, a more complicated Y-shaped or double Y-shaped nanotube can be fabricated by reducing the voltage during anodization [16].

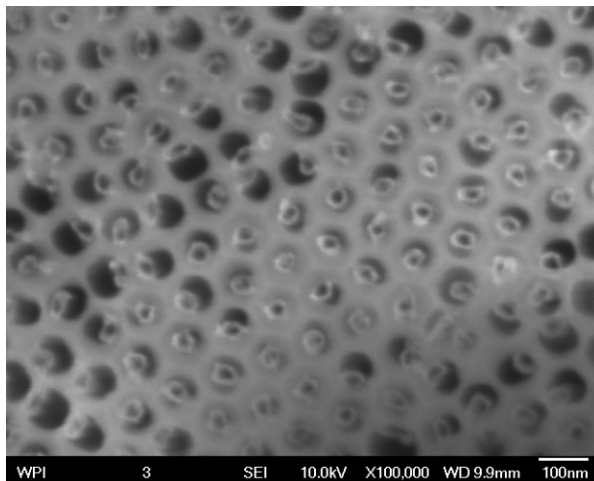


Fig.2 Hexagonal array of carbon nanotubes in AAO template

(Unpublished data from Huanan Duan, Nanomaterials and Nanomanufacturing Lab, WPI)

With respect to polymeric nanostructures, the nano-confinement from AAO template is more often used with a combination of wetting phenomena. By simply contacting polymer melts or solutions to the surface of AAO templates, cylindrical nanostructures can be formed within a very short period of time. Depending on the morphologies (tubes or rods), two mechanisms have been proposed: 1. If the polymer melts (or solutions) have a surface energy lower than that of the inner-surface of the AAO pores, they can rapidly wet the walls of nanochannels by forming a precursor film. After solidification, polymers adsorbed on the walls will become nanotubes; 2. If polymer melts have a surface energy higher than that of the inner surface of AAO nanochannels, conventional capillary wetting would happen and only nanorods are formed [17, 18].

To investigate the effects of the nanoscaled confinement on the as-made polymer nanostructures, Russell's group at UMass Amherst has template-wetted AAO with a diblock copolymer of styrene and butadiene (PS-b-PBD) with different morphologies. It was found that with asymmetric copolymers, cylindrical micro-domains align along the pore axis due to the preferential wetting from the PBD block; in the case of lamellar PS-b-PBD, concentric cylinders formed in the pore and the number of cylinders depended on the ratio of pore diameter [19]. They have also fabricated nanorods with Rayleigh instability induced periodic encapsulated holes, which were believed to have potential applications as delivery devices [20].

2.2 Layer by layer assembly

Fabrication of nanostructured single or multi component films with control over their nanoscopic internal orientation and organization is important step towards the creation of highly functionalized devices. For about 60 years, the pursuit of manufacturing molecularly controlled thin films has been dominated by the Langmuir-Blodgett (LB) method [21, 22], in which monolayers are formed on an aqueous surface and then transferred onto a solid substrate. Although the true nano-manipulation from the LB films has

brought significant advancement especially in biological areas, the LB technique requires special equipment and has severe limitations in the film quality and stability.

In 1991, a new species of thin films based on the layer by layer (LbL) assembly technique were first introduced by G. Decher [23]. As shown in Figure 3, his experiment started by creating a positively charged surface. Then the negatively charged sodium salt of poly(styrene sulfonate) (PSS) and positively charged molecules of poly(allylamine hydrochloride) (PAH) were sequentially deposited from respective solutions via electrostatic interaction. Between the adsorption of oppositely charged polyions, rinsing was applied to wash off the loosely bound molecules. Repeating these basic steps could lead to polyelectrolyte thin films with desirable thicknesses [24].

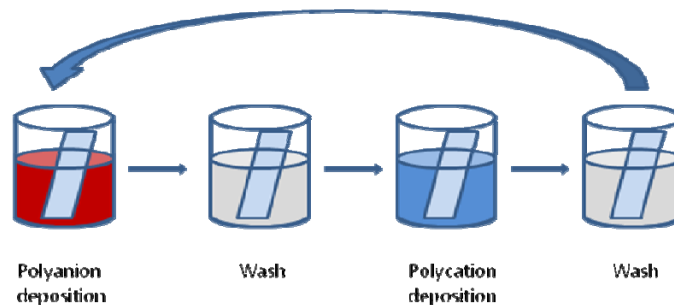


Fig.3 Schematic diagram of film deposition using a solution dipping method

The LbL assembly has advantages over the LB technique in many ways. LbL thin films can be conveniently fabricated, are inexpensive in nature, and can be obtained from the deposition of a wide variety of materials. Another important quality of LbL assembly is the high degree of control over the thickness of films, which can be grown either linearly or non-linearly with incremental thickness that is sensitive to the fabrication parameters.

2.2.1 Assembling processes

The fabrication of multilayers is not limited to electrostatic forces between charged species, and can also be assembled in an LbL fashion via almost any other type of interactions.

By means of LbL method, Sun *et al* have fabricated multilayers comprising of diazo-resin (DAR) and poly(acrylic acid) (PAA) through covalent bonds. Upon UV radiation, a fraction of the carboxylate groups in PAA can react with diazonium groups at the interfaces of the adjacent single layer in the LbL film. In this way, partly covalently attached multilayered assemblies can be formed [25]. Using covalent chemistry, Kohli *et al* have also demonstrated the growth of multilayers from copolymerization of isocyanate-containing monomers and amine-containing monomers [26].

In the category of secondary interactions, hydrogen bonds can be applied to associate components in LbL assemblies. Wang *et al* have proposed a new approach for the construction of LbL thin films based on the alternate assembling of poly(4-vinylpyridine) and poly(acrylic acid), the interaction between which was identified as hydrogen bonding by IR spectroscopy [27]. Furthermore, it has also been reported that even dispersion forces can be used to assemble the multilayers [28].

With respect to the equipment necessary for LbL assembly, most of the research is conducted using a simple setup of solution dipping shown in Figure 3, either manually or automatically [29]. However, LbL assemblies are not limited to solution dipping.

The application of sprayed layers was introduced by Schlenoff. By sequential spraying of poly(styrene sulfonate) and poly(diallyldimethylammonium) solutions, a highly uniform polyelectrolyte multilayer was rapidly obtained over a large area. The obtained film was virtually identical in membrane properties to those prepared by solution dipping [30]. Another way of fabricating multilayer thin films is spin coating, which was used in Pompeo's work on lipid multilayers [31] and Nohria's work on polypyrrole nanoassembly [32].

Compared to solution dipping, only a small amount of the polyelectrolyte solutions are needed for both techniques to coat large areas of substrates. Those developments have extended the controllability of the LbL assemblies since more parameters can be incorporated to the fabrication processes.

2.2.2 Film materials

Another unique advantage for the LbL method is the huge selection of materials that is available for multilayers, since interactions among the building blocks can be either general or specific. While the early study of LbL thin films were focused on several model polymer pairs like PAA/PAH, PSS/PAH etc., polymers with more functionalities, small molecules, inorganic particles, protein or even DNA have been investigated in the multilayer deposition.

For example, Loh *et al* have fabricated a multifunctional thin film by alternately dipping a glass substrate into 1.0 wt% PVA solution and carbon nanotube-PSS dispersion. By varying the carbon nanotube concentration and altering the types of polyelectrolytes, it can be used as both a strain and pH sensor [33].

Multicomponent LbL films can have more oriented internal structures if they are assembled with more specific attractions. Using LbL technique, Ansell *et al* have synthesized a cobalt-diisocyanide/metal-bisphosphonate hybrid superstructure based on covalent bonding. The obtained thin film was expected to have nonlinear optical properties [34].

2.2.3 Effects of process parameters

From the thousands of investigations into a wide variety of materials that have been deposited into multilayer thin films, two factors have been determined to be most influential in deciding the internal structures, ionic strength and solution pH, especially for LbL assemblies with electrostatic interaction [35-37].

As described earlier, polyelectrolytes are assembled by the alternate adsorption of oppositely charged species onto previous layers. Therefore charge overcompensation for each deposited layer is indispensable during the consecutive growth of multilayers [38]. Intrinsically, charged groups on polyelectrolyte, whether in the dipping solution or in the bulk film, are compensated by the polyelectrolyte with opposite charges. However, if salt is introduced into the system, the original equilibrium will be disrupted because extrinsic charge compensation can now happen between polyelectrolyte and salt ions. In a polyelectrolyte solution with higher ionic strength, the salt ions partially neutralize the strong charge that used to repel the same charge species [39]. Consequently, instead of spreading into relatively linear chains, free molecules in the solution will deposit on the previous layers in a rather coiled conformation, resulting in a thicker increment for each layer [35]. Also, since the salt ions are sufficiently small, they can penetrate into a certain depth of the adsorbed multilayer, and loosen its internal structures [36].

With regard to weak polyelectrolytes, which have a dissociation constant in the range of 2-10 and are not fully charged in solution, the morphology of multilayers can not only be manipulated by ionic strength, but more dominantly by simply changing the pH environment.

Using LbL assemblies of PAA/PAH, Shiratori *et al* have investigated how the average incremental layer thickness of an adsorbed PAA and PAH layer can be varied according to the solution pH. In their experiment, both PAA and PAH solutions were at the same pH values from 2-10. A dramatic increase in thickness was observed over a very narrow pH range, which arose from the sensitiveness of weak polyelectrolytes towards solution pH. In the region of pH 6.0-7.5, both PAA and PAH were charged sufficiently to overcome chain coiling entropy, whereas in the region of pH 4.5-6.0, polyelectrolyte chains were adsorbed in a more “loopy” conformation. In the same work, a more comprehensive matrix can be found showing the relationship of dipping solution pH and thickness growing step [37].

Similar to the effect of salt ions on the structures of cross-linked strong polyelectrolytes, acid-base equilibria can disrupt the internal organization of weak polyelectrolytes. The degree of this disruption is much higher for the reason that coiled conformation of weak polyelectrolytes makes them more penetrable and compared to salt ions, protons are easier to diffuse in and out therefore swell or de-swell the multilayers [40].

A more interesting example utilizing the pH-responsive behaviors of weak polyelectrolytes is the fabrication of a spinodal decomposed microporous thin film. By exposing the multilayer to acidic solution of pH 2.4 for a short period of 15 seconds, polyelectrolyte pairs of pH 3.5/7.5 PAA/PAH multilayer would be broken as carboxylate groups are protonated by acid, and re-organized into a more energetically favorable arrangement. Consequently, the multilayer film undergoes a dramatic increase in thickness and phase separates into a microporous structure [41].

2.2.4 Applications of LbL assembled multilayers

As we have stated previously, the LbL method does not require expensive and complicated equipment and can accommodate various kinds of materials. Therefore, new applications have been proposed continuously, based on the unique physical or chemical properties of this molecularly blended nanocomposite. Following are a very limited number of representative developments in the recent years.

Kotov and his coworkers have fabricated a photoactive multilayer thin film from Te nanowires and light-sensitive polyelectrolytes. The obtained film showed an optical gating effect and this “light-on-light-off” cycle was demonstrated to be very stable for more than 100 repetitions given ambient environment [42].

Using sodium montmorillonite clay nanoparticles as physical barriers, Jang *et al* have deposited clay-PAA bilayers on a polyethylene terephthalate film via LbL technique. The resulting transparent film had an unprecedented low oxygen transmission rate of less than 0.005 cc/m²/day/atm, which made it a good candidate for food packaging [43].

Meanwhile, huge potential of the LbL assemblies have also been demonstrated in diverse areas of chemical and biological fields. Particularly, they can be ideally suitable for the fabrication of stimuli-responsive systems due to the soft and flexible nature of the incorporated polyelectrolyte molecules.

One of the most important applications, for example, is that by carefully selecting the components in the multilayers, controlled release can be achieved from LbL films deposited on both flat membranes and spherical surfaces. From alternately depositing of poly(β -amino ester) and a series of model therapeutic polysaccharides, a hydrolytically degradable LbL thin film for controlled drug releasing has been fabricated by Hammond and her coworkers. The films have exhibited pH-dependent, pseudo-first-order releasing profile as they degrade [44].

Smart capsules have also been fabricated by LbL deposition of multilayers on spherical particles. In the work by Lvov *et al*, PAA and PSS were alternately assembled on melamine-formaldehyde microparticles for four repetitions, followed by decomposition of the core at pH 1. The obtained capsule was non-permeable to large molecules in water, but was able to open its surface to load protein in a water/ethanol 1:1 mixture [45].

LbL assemblies are extremely tolerant to the chemistries and morphologies of the deposition substrates. As a matter of fact, with the combination of patterning of the template, multilayers can be engineered to perform more complicated tasks. An amazing example can be found in Ling *et al*'s work. In this work, a 3D multimaterial nanostructure was constructed by supramolecular LbL assembly on a patterned substrate created by nanoimprint lithography. The manipulation in different levels will potentially functionalize the system into a multi-tasking sensor with differentiable sensitivity in each level [46].

2.3 Layer by layer assembly in nano-porous template

From the consistent effort people have made world-wide, the template-assisted method and LbL technique have become two of the most successful and mature methods for the fabrication of nanomaterials, but have shown their advantages in different areas: while it is easier to use template-assisted method to fabricate 1-D nanomaterials, LbL technique is more often used in the manufacturing of planar or 2-D nano-assemblies. Therefore, it is natural to think: what if LbL technique is applied inside the nanopores of those templates?

To find an answer to this question, Koutyukhova *et al* have attempted to LbL synthesize multilayer TiO₂/PSS and ZnO/PSS tubules on the pore walls of a nano-porous polycarbonate membrane by successive immersion of the membrane in aqueous solution of each component. After electrochemical or chemical plating of Au rods inside the tubules, the template was dissolved and the resulting nanostructure showed a surprisingly strong current-voltage hysteresis and was potentially interesting in the field of switching devices [47].

More efforts have been made in the pursuit of LbL fabrication of protein-based nanotubes because of their ease of functionalization and good biocompatibility that are particularly desirable for biomedical applications.

In 2004, Martin and his co-workers published a method in which a 1.25mM solution of 1, 10-decanediylbis(phosphoric acid) (DBPA) and a 5.0mM solution of ZrOCl₂·2.8H₂O were alternately used to deposit nanotubes made of DBPA/Zr bilayers in the nanochannels of AAO template [48]. Following this pioneering work, Li *et al* successfully fabricated LbL nanotubular assemblies of Cytochrome c (Cyto-c)/Glutaraldehyde (GA) and Cyto-c/PSS, via covalent bonding and electrostatic interaction respectively. The obtained nanotubes have shown uniform length and thickness in agreement with the dimensions of AAO template. Circular dichroism (CD) measurements suggested that there was no obvious variation of bioactivity from the processing pH values compared to pure Cyto-c in aqueous solution [49].

A further step in functionalizing this kind of nanotubes has been made by Lu *et al* in the study on artificial hemoprotein nanotubes. In the same fashion, a model protein of human serum albumin (HSA) and hemoprotein (FeP) were engineered into nanotubes. The oxygen binding affinity of the nanotubes was two-fold lower than that of monomeric HSA-FeP, which possibly owed to the low association rate constant across the multilayer of nanotubes [50].

These promising results have all demonstrated that by combining LbL technique and template assisted fabrication, it is possible to have better control of the dimensions of the obtained nanotubes, in which the length and the diameter of the nanotubes can be varied by the thickness and pore size of the template, and LbL method controls the wall thickness as well as internal structures in a higher resolution.

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CHAPTER 3: JOURNAL MANUSCRIPT

Fabrication of bovine serum albumin nanotubes through template assisted layer by layer assembly

Dawei Zhang, Shelley Dougherty and Jianyu Liang

Abstract

Protein nanotubes have been successfully fabricated via sequential filtration of bovine serum albumin (BSA) solution at pH 3.8 and pH 7.0 through the nanochannels of anodic aluminum oxide (AAO) templates. The morphology of the nanostructures has been examined using SEM and TEM. Also, a simple analysis from UV/Vis spectroscopy has shown that the pH values of the solutions in our experiment did not significantly damage the bioactivity of BSA. Our future work will focus on strengthening the mechanical stability of the protein nanotubes and more precisely controlling their morphology.

Introduction

Since Iijima first discovered carbon nanotubes [1], researchers worldwide have been enthusiastically exploring the characteristics and applications of one-dimensional nanostructures composed of carbon [2], ceramics [3], metals [4] and polymeric materials [5]. Out of all the synthetic and natural materials, protein based nanotubes are particularly desirable for biomedical applications due to their ease of functionalization and intrinsic biocompatibility. Two preparation approaches, namely self-assembly and

template-assisted synthesis have been investigated for producing protein nanotubes directly. Self assembly methods have been successfully employed to fabricate protein nanotubes based on flagella [6, 7], viral capsids [8, 9], tubulin [10] and Hcp1 [11]. However, self assembly methods are typically limited to the unique chemistry and functionality of the biomolecules. In addition, the morphologies and dimensions of the self-assembled nano-tubular structures are restricted by the properties of the original bulk materials. The template-assisted method provides a highly versatile alternative. The template-assisted processes for the fabrication of protein nanotubes rely upon either the chemical crosslinking of proteins, the so-called alternate immersion method [12, 13], or electrostatic charges between protein layers, the so-called layer by layer (LbL) method [14, 15]. These mechanisms can be easily adapted to many different proteins with little to no modifications. This advantage renders template-assisted methods viable for a wide range of applications.

The LbL assembly method has long been used in the electrostatic deposition of thin films on flat substrates and surfaces of particles [16-18]. Multilayers composed of different charged species can be built up following a conventional procedure: A negatively charged solid substrate is alternately immersed in solutions of cationic polyelectrolyte and anionic polyelectrolyte [16] to build up a film consisting of alternating layers of polyelectrolytes by the electrostatic interaction between them. It is also well established that by tuning the ionic strength and pH environment of the solution, the layer thickness, surface roughness and permeability of the multilayer can be precisely controlled [19-21].

Recently, the LbL assembly approach has been applied in anodic aluminum oxide (AAO) templates to demonstrate the successful fabrication of protein based nanotubes. For example, nanotubes of cytochrome C have been fabricated inside polyethylenimine (PEI)-pretreated AAO templates by the alternate adsorption of positively charged cytochrome C with negatively charged poly-(sodium styrenesulfonate) (PSS) [14]. Similarly, human serum albumin (HSA)-based nanotubes have been synthesized by alternating HSA with poly-L-arginine (PLA) or PEI [15]. To date, the published works have been primarily focused on composite nanotubes of protein molecules and charged polymers. Only a few of

these studies have attempted the fabrication of single component nanotubes with HSA [22, 23]. In this paper we have demonstrated the fabrication of bovine serum albumin (BSA) nanotubes using LbL deposition of protein molecules inside a nanoporous AAO template.

Albumin, the most abundant plasma protein in the bloodstream, is an acidic protein with high solubility in water. It is very robust in extreme pH environments and organic solutions. It can be heated to 60 °C for up to 10 hours without catastrophic damage to its chemistry [24]. BSA consists of 583 amino acids and has a molecular weight of 66 kDa [25]. BSA has been successfully used in biological applications like HSA, but is much lower in cost since it can be readily purified from bovine blood. The isoelectric point (pI) of BSA is 4.7. Under pH 4.7, BSA molecules carry a positive charge. And above pH 4.7, BSA molecules carry a negative charge. In the present work, oppositely charged BSA molecules have been assembled into nanotubes inside the nanochannels of AAO templates in an LbL fashion. The obtained protein nanostructures have been characterized using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The effect of solution pH on BSA bioactivity has been investigated by UV/vis spectroscopy.

Experimental Section

Materials. AAO templates (13mm) were purchased from Whatman Co., with an inner pore diameter of ca. 200 nm and thickness of 60 μ m. The BSA was obtained from Sigma and was stored at 2 °C before use. All the chemicals were used as received without further purification.

Fabrication of BSA nanotubes. The AAO template was sandwiched between two cellulose membranes (MF-Millipore) and was fixed into a stainless steel microsyringe filter (Whatman Co.). 9 mL of BSA in a citric-phosphate buffer solution (2 mg/mL, 10 mM) at pH 3.8 and pH 7 were sequentially injected into the

filter at a speed of 0.5 mL/min. Between each filtration step, 9 mL of DI water was filtered through the template at a speed of 1 mL/min, followed by drying the template in air for 10 min. After three sequential filtration steps, the AAO template was washed thoroughly with DI water. The BSA aggregated on the surface of the template was carefully removed using Kimwipes before the template was dried overnight in the air.

Characterizations. Nanotube morphology was characterized using SEM (JSM-7000, JEOL) and TEM (Philips CM 12). UV/vis spectrometer (Genesys 10UV scanning spectrophotometer, Thermo Electron) was used to evaluate the absorbance of BSA molecules in a wide range of pH environments (pH 1.15~12). Zeta potential was used to measure BSA charge in the acidic and basic buffer solutions. Zeta potential was measured using a ZetasizerNano (Malvern Zen3 600).

Results and discussions:

BSA is a heart-shaped molecule comprised of three domains at its pI [26]. However, it undergoes several well-recognized conformational changes under different pH conditions. Associated with the variation of the BSA conformation, the ionic properties of albumins are also dependent on pH environment. Figure 1 shows the pH dependent zeta-potentials of BSA solutions at a concentration of 2 mg/mL, which represents the net charge that BSA molecules carry. The curve agrees well with the model from the early work by Fogh-Anderson *et al* [27]. The albumin's zeta potential decreases from 25.1 mV to zero at around pH 4.7 and becomes negative with increasing pH. BSA solutions at pH 3.8 and 7 were chosen for the alternate filtrations based on literature reviews [22]. Additionally, we demonstrated that the zeta potential is positive (12.3 mV) at pH 3.8 and negative (-17.9 mV) at pH 7 which allows the electrostatic assembly of the adsorbed BSA layers.

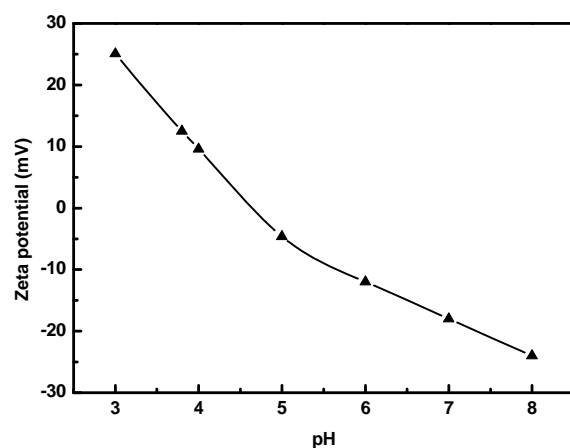


Fig. 1 pH-dependent zeta potential of BSA molecules

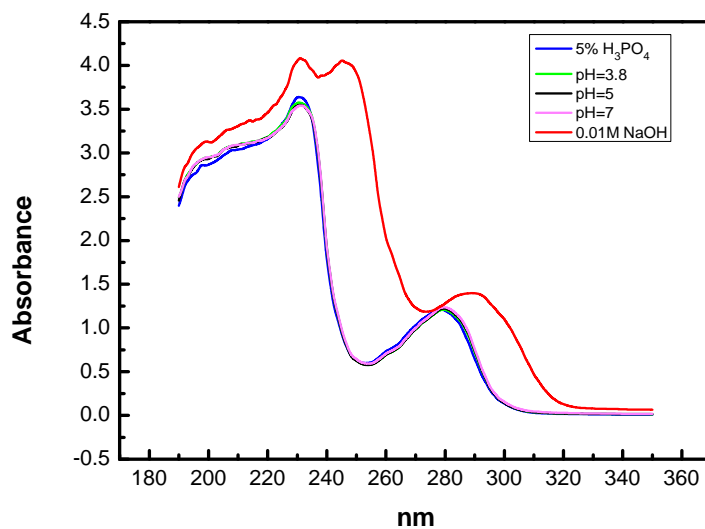


Fig.2 UV/Vis spectroscopy of BSA solution at different pH

To examine the bioactivity of BSA molecules at pH 3.8 and 7, UV/Vis spectroscopy was performed on the same solutions that we used for LbL filtrations. As shown in Figure 2, the absorption curves of

positively charged BSA at pH 3.8 and negatively charged BSA at pH 7 are overlapped with the curve obtained at its nature state (pH 4.7). It is known that without being irreversibly denatured, the BSA molecule is significantly adaptive to a wide range of pH values by frequent expansion and contraction. The pH-dependent conformations of BSA are classified as N, the native form (pH4.3-8); F, the fast-migrating form (pH2.7-4.3) and E, the expanded form (pH<2.7). In a basic environment, BSA will adapt to B, the basic form (pH8-10) and A, the aged form (pH>10) [26, 28]. Albumin can recover from structural changes caused by almost any condition except long periods of treatment with heat and alkalinity [24]. Our observation complies with the fact that at pH 3.8 (F form) and 7.0 (N form), BSA still maintains a relatively natural state, in which the molecules are not yet expanded enough to lose their original 3D structures [28, 29].

In addition, based on our previous experience of making SEM and TEM samples, AAO templates can only be dissolved by strong acids or strong bases. To explore the effect of the pH values of different solutions used to remove the AAO on the bioactivity of BSA molecules, UV/Vis spectroscopy was also used on the bulk solutions (2 mg/mL) of BSA in 5% H₃PO₄ and 0.01 M NaOH. For BSA in 5% H₃PO₄ solution, there is a slight difference in the region of 200 nm-230 nm, but no significant deviation of the UV/Vis curve has been found. However, for BSA in 0.01 M NaOH solution, the typical absorbance peak at 280 nm from tryptophan and tyrosine residues exhibits a red shift, indicating the change in the secondary structure. Also, the far-ultraviolet region below 240 nm splits into two peaks which is further evidence of the degradation of the albumin molecule caused by alkaline [30]. Consequently, 5% H₃PO₄ was preferred for the etching of AAO templates in our experiment.

In this work, positively charged BSA molecules were first adsorbed onto the anionic sites on the pore wall of the AAO templates at pH 3.8, followed by a quick washing of the loosely bound molecules and drying of the protein surface. Negatively charged BSA solution at pH 7.0 was then filtered through the membrane. Like weak polyelectrolyte multilayers obtained on flat substrates, long incubation times of at least 10 min are required to ensure a sufficient charge interaction [31-34]. This is especially important for

the first layer of protein adsorbed on the alumina surface, which plays an important role in the successful build-up of the consecutive layers [35]. In comparison, we have also tested filtrations of the same amount of BSA solution at a speed of 1 mL/min and 3 mL/min, i. e. for 9min and 3min respectively. It is not evident from SEM and TEM characterizations that stable protein multilayers have been deposited at those fast speeds.

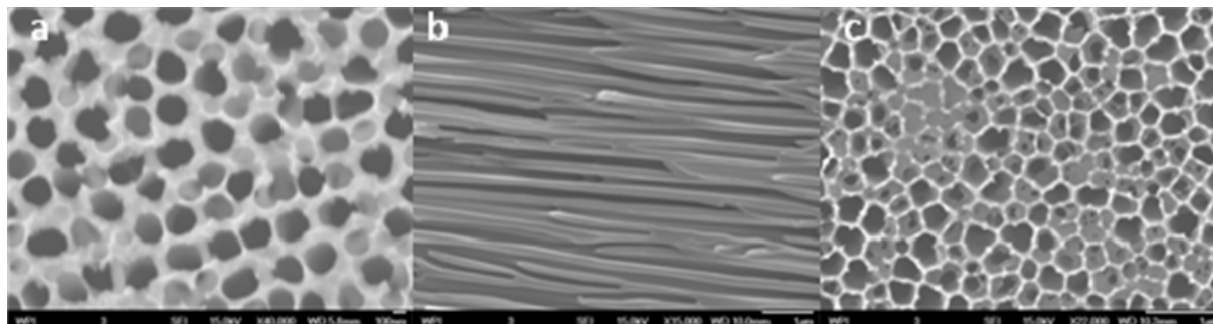


Fig.3 a) Top view of an empty AAO template; b) Cross-sectional view of an empty AAO template; c) Top view of BSA multilayers deposited inside AAO template. (The scale bars are a) 1 μ m; b) 100 nm; c) 1 μ m)

Figure 3-a, b show the top view and the cross section of the AAO template we used to fabricate BSA nanotubes. Figure 3-c is the top view of an AAO template after deposition of BSA multilayers inside the nanopores. It is seen that after six filtrations, the pores in the AAO template (Figure 3-c) are filled with protein. However, the thicknesses of the BSA multilayers are not uniform. This may be attributed to the complex morphology of the substrates provided by AAO for LbL deposition. It is known from studies on LbL assemblies on planar surfaces, that the amount of deposited polyelectrolytes significantly depends on the surface roughness [36-38] and the curvature [39, 40] of the substrate. The nanochannels in the AAO template, however, are not perfectly cylindrical pores with uniform diameter and are full of uneven

surfaces, branches and discontinuities, as seen in Figure 3-a, b. These irregularities may have directly contributed to the inhomogeneous adsorption of BSA multilayers.

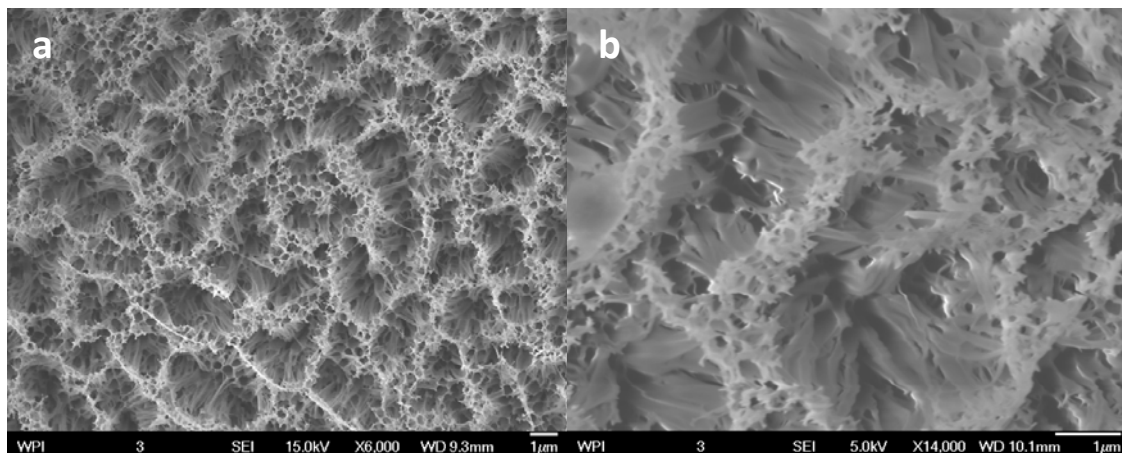


Fig. 4 SEM images of AAO template after etching with 5% H_3PO_4 for 16 hours

(The scale bars in a, b are both $1\mu\text{m}$)

Figure 4 shows an AAO template after protein deposition that was partially etched away in a 5% phosphoric acid for 16 hours at 0°C , in order to liberate the top portion of the protein nanotubes from the template. However, collapsed nanostructures were observed and individual protein nanotubes could not be differentiated. We hypothesize that this is because of the intrinsic weakness in the mechanical stability of protein nanotubes. In the early work in which LbL assemblies were performed on flat substrates, it has been demonstrated that the chain length of polyelectrolytes plays a critical part in the stability of protein multilayer. Unlike flexible polyions, the globular molecules of BSA are less capable of penetrating into each other to form a network that provides a sustainable support to the whole structure [41-44]. Consequently, the BSA nanotubes fabricated within the AAO template, which are multilayers composed of weakly attracted globular protein molecules, may have an inherent disadvantage in maintaining its structural integrity. Additionally, at both pH 3.8 and pH 7.0, electrolytic groups on BSA molecules are

only partially ionized. Therefore the charge provided by the coiled molecules of protein is limited for the electrostatic interaction between protein layers. As the aluminum oxide is being etched away, mechanical support gradually disappears and the soft and thin walls of the protein nanotubes cannot stand on their own, resulting in a structure of collapsed bundles. On the other hand, the acidic post-treatment of the protein multilayer in H_3PO_4 during the preparation of SEM samples may protonate many of the carboxylate groups within the protein layers. It may interfere with the electrostatic crosslink and mobilizes the protein chains to a certain extent, therefore further weakens the structural stability of the protein nanotubes [45].

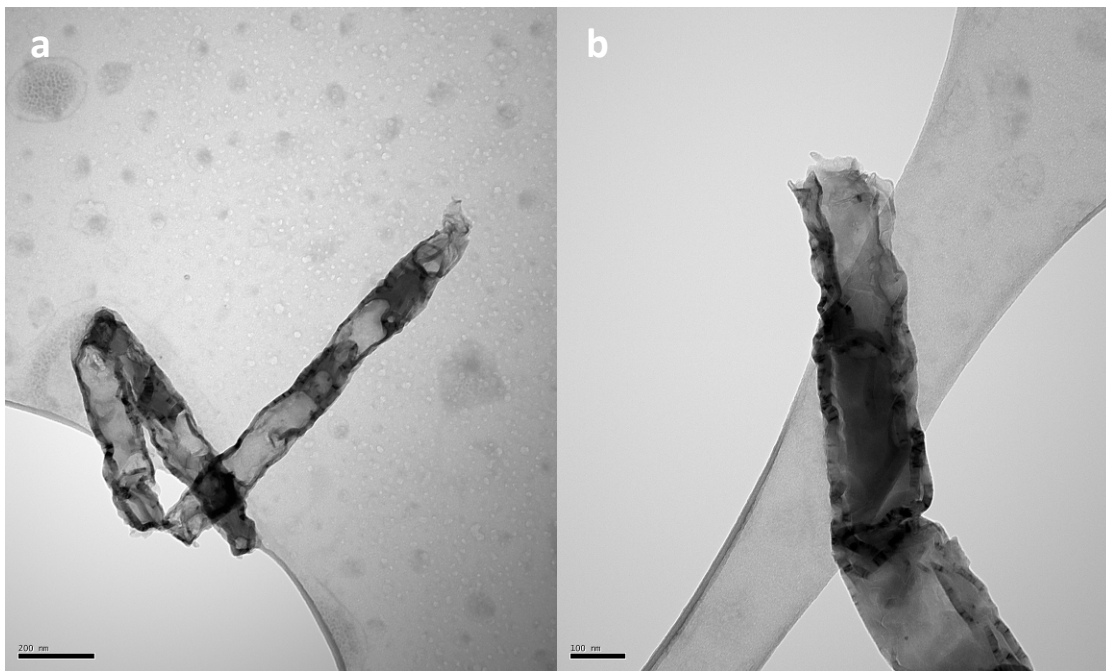


Fig. 5 TEM images of BSA nanotube (The scale bars in the pictures are a) 200 nm; b) 100 nm)

To study the morphology of individual BSA nanotubes, TEM samples were prepared by completely dissolving the AAO template containing protein multilayers in a 5% phosphoric acid solution at 0 °C for 24 hours. It is seen that although the harsh environment that is necessary to etch away the alumina can

compromise the stability of the protein multilayers, it is still possible to observe the tubular protein structure even after acidic treatment for as long as 24 hours. The walls of the obtained nanotubes are wavy due to the intrinsic softness of protein multilayers. Careful examination of the thickness of the protein nanotube walls using the image processing software imageJ reveals that the average wall thickness (measured in 15 different places) is $19 \text{ nm} \pm 1.5 \text{ nm}$, i.e. an average of 3 nm for the deposition of each single BSA layer. This is in agreement with the size of BSA molecules ($140 \text{ \AA} \times 40 \text{ \AA}$) in its unfolded form [46]. Some Y-shaped protein nanostructures are also seen from TEM characterization (See Figure 6), which replicates the branched channels in AAO templates well. This demonstrates the versatility of template-assisted method using AAO templates as scaffold.



Fig.5 TEM image of a Y-shaped BSA nanotube (The scale bar is 200nm.)

Conclusion

In conclusion, we have deposited single component BSA multilayers onto the nano-channels within AAO templates via the electrostatic interaction between oppositely charged protein molecules. A simple analysis from UV/Vis spectroscopy in both near and far ultraviolet region has demonstrated that the pH of

the solutions we used will not cause irreversible denaturation to the BSA molecules. The obtained protein nanotubes were shown by SEM to have collapsed into bundles due to the intrinsic mechanical weakness of protein multilayers and the acidic post-treatment. After the AAO template has been completely etched away, the morphology of the liberated protein nanotubes was characterized using TEM.

Our future work will focus on reinforcing the mechanical stability of our BSA nanotubes. We will also investigate various filtration conditions and AAO template dimensions, to enable us to precisely control the nanotube morphology.

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CHAPTER 4: ADDITIONAL WORK

4.1 Improvement to the mechanical stability of BSA nanotubes

Introduction:

To improve the structural integrity of the protein nanotubes, we experimented with depositing an initial layer that can covalently bind with alumina and protein molecules. We expect that this layer can serve as a flexible substrate, holding the subsequent layers of protein molecules together after the removal of AAO template. The initial layer was deposited by a well known method that has been successfully used in our lab for fabricating Glucose Oxidase nanotubes [1].

Experiment:

AAO templates were pretreated by immersing them in a 5 mM solution of 3-aminopropylphosphonic acid (APA) for 24 hours to deposit a monolayer adherent to the surface of the inner-wall of porous alumina. Both sides of the AAO templates were sputtered with Au before the immersion in order to avoid adhesion of APA on those surfaces. After rinsing with phosphate buffer solution, the APA coated template were immersed in a 2.5% glutaraldehyde (GA) solution for 12 hours to allow the deposition of a GA layer on the previous APA layer. GA has been widely used to immobilize protein through forming covalent bonding. In this case, it reacts with the amino groups of APA on one side, and binds to the free amino sites on protein with unreacted aldehyde groups on the other side. The rest of the experimental procedure was the same as described in Chapter 3 to form pure BSA nanotubes. 9 mL of BSA solutions at pH 3.8 and 7 were alternately filtered through the pretreated AAO template to deposit 4 bilayers of BSA. Between each filtration, the samples were rinsed by DI water and dried in air. After protein deposition, the samples were fully dried in air for 24 hours. Finally, the samples were treated in 5% phosphoric acid

at 0 °C for 24 hours to fully remove the AAO template.

Preliminary results:

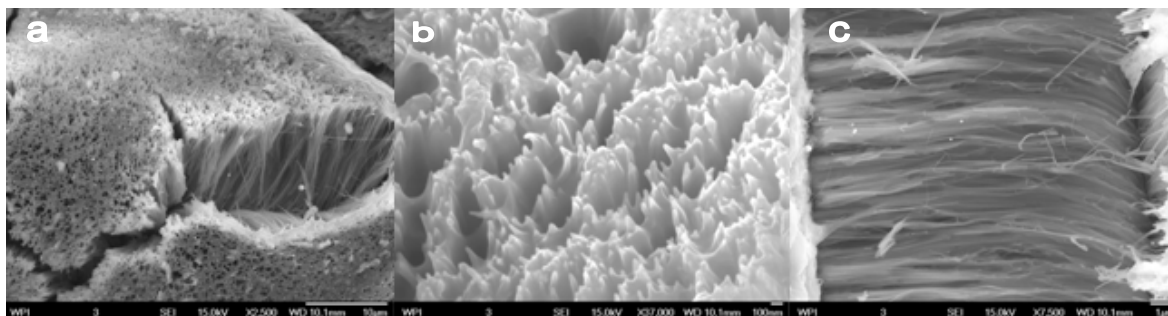


Fig.1 SEM images of BSA nanotube arrays with APA-GA pretreatment:

a, b) Top view; c) Cross-sectional view. (Scale bars are a) 10 μm; b) 100 nm; c) 1 μm.)

Figure 1 is the SEM image of protein nanotube. From both top view and cross-sectional view, we see bundles of cylindrical nanostructures. It is hard to differentiate individual nanotubes. Comparing to the SEM images of the pure BSA nanostructures obtained, it seems that by depositing APA and GA layers as the backbones that support protein nanotubes, the morphology of the tubular structures were better retained which indicates that the stability of the protein nanostructure against longtime acidic treatment might have been improved.

4.2 LbL using home-made AAO template

Experiment:

Additionally, in order to find out how well the dimensions of the nanostructure can be controlled using our method, we have tried to deposit BSA multilayers inside the homemade AAO template, which has a highly ordered pattern and a more uniform pore size compared to commercially available AAO template.

The template we used was fabricated by a conventional two-step anodization of a 99.999% pure aluminum sheet. After detachment from Al substrate and post-treatment to remove the barrier layer in 0.5 M phosphoric acid, the obtained membrane was 50 μm thick and with a pore size of 67 $\text{nm}\pm 6$ nm.

Preliminary results:

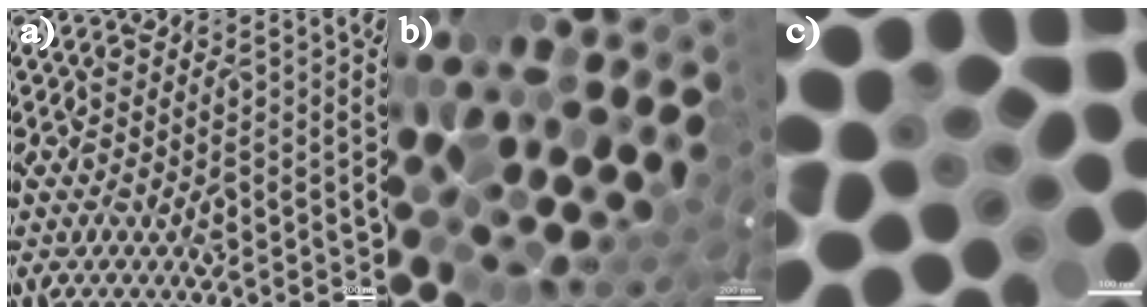


Fig. 2 a) Empty AAO template; b, c) AAO template after BSA deposition.

(Scale bars are all 200 nm).

Figure 2 shows the SEM images of empty home-made AAO template (a), and AAO template after deposition of three bilayers of BSA molecules (b, c). The amount of solution used for each time is only 6 mL (2 mg/mL, 10 mM). It is seen that for the home-made template, protein multilayers are not uniformly adsorbed in the nanopores either, which possibly because that the rectangular AAO template was not tightly sealed into microsyringe system that was used for the filtration of BSA solutions in this experiment.

For home-made AAO template, it is easy to control its pore size and thickness, by changing the anodization conditions. Therefore, the dimensions of the protein nanotubes fabricated using this template can be better controlled. More importantly, if the template is thinner and the pores are denser, the time required to dissolve the template would be significantly reduced thus less damage to the protein

nanotubes would be caused by the acidic treatment.

References

- [1] Dougherty S, Liang J and Kowalik T F, 2009, "Template-assisted fabrication of protein nanocapsules," *J. Nanopart. Res.*, 11 385-94.

CHAPTER 5: CONCLUSIONS AND FUTURE WORK

In this work, single component BSA nanotubes have been successfully fabricated by applying LbL method inside a nanoporous template. By filtering positively charged BSA solution at pH 3.8 and negatively charged BSA solution at pH 7 alternately, the walls of the protein nanotubes are deposited as multilayers on the pore wall of AAO template via the electrostatic interaction of the oppositely charged BSA molecules. From SEM and TEM images, the obtained nanostructures of BSA are soft and weak, which can be attributed to the intrinsic weakness of protein multilayers and the acidic post-treatment that is used to liberate the nanotubes from AAO template.

To strengthen the mechanical stability of BSA nanotubes, AAO templates were pretreated with APA and GA to form supporting backbones that are covalently bound to BSA nanotubes. SEM observations seem to suggest an improvement in retaining the structure of BSA nanotubes.

Finally, we have tried to deposited BSA multilayers on the home-made AAO template. With the control over the pore size and thickness of the template, this approach potentially provides us the ability to control the dimensions of BSA nanotubes more precisely.

Future works will focus on: 1. improving the mechanical stability of BSA nanotubes by incorporating different types of polymeric supporting layers; 2. providing a better control of the dimensions of protein nanotubes by varying the pore size and thickness of AAO template; 3. applying template-assisted LbL fabrication method on different kinds of proteins.