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Protein Nanotubes with an Enzyme Interior Surface

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Abstract: This report describes the synthesis and enzyme activities of multilayered protein nanotubes with an α-glucosidase (αGluD) interior surface. The nanotubes were prepared by using an alternating layer-by-layer (LbL) assembly of human serum albumin (HSA) and oppositely charged poly-L-arginine (PLA) into a track-etched polycarbonate (PC) membrane (pore size = 400 nm) followed by addition of αGluD as the last layer of the wall. Subsequent dissolution of the PC template yielded $(PLA/HSA)_2PLA/\alpha GluD$ nanotubes. SEM measurements revealed the formation of uniform hollow cylinders with (413 ± 17) nm outer diameter and (52 ± 3) nm wall thickness. In aqueous media, the nanotubes captured a fluorogenic glucopyranoside, 4-methyl-umbelliferyl-α-D-glucopyranoside (MUGlc), into their one-dimensional pore space and hydrolyzed the substrate efficiently to form α-D-glucose. We determined the enzyme parameters (Michaelis constant, K_M, and catalytic constant, k_{cat}, values) of the protein nanotubes. The several-micrometers-long cylinders were of sufficient length to be spun down by centrifugation at 4000 *g*, so the product could therefore be easily separated. Similar biocatalysts were prepared by complexation of biotinylated-αGluD into HSA-based nanotubes bearing a single avidin layer as an internal surface. The obtained hybrid nanotubes also exhibited the same enzyme activity for the MUGlc hydrolysis.

Keywords: enzymes · layer-by-layer assembly · nanotubes · proteins · supramolecular chemistry

Introduction

Template synthesis by using a nanoporous membrane, such as anodic aluminum oxide (AAO) or track-etched polycarbonate (PC) membrane, is an efficient procedure to construct nanocylinder architectures with various materials.[1–13] In particular, smart nanotubes comprising proteins[14–20] and DNA[21] have attracted great interest because of their potential applications as biological containers, sensors, and channels. However, this template-assisted approach involves a critical issue in the exploitation of the biomolecules as wall components: significant physical deformation of the tube structure occurs in the membrane dissolution process.[14,17,19] Recently, we demonstrated a unique procedure to prepare robust protein nanotubes through alternating layer-by-layer (LbL) assembly in a porous PC membrane by using a poly(amino acid) as an electrostatic glue.[22,23] Subsequent dissolution of the template in *N,N*-dimethylformamide (DMF) and rapid freeze-drying of the extracted core yields homogeneous hollow cylinders as lyophilized powder. The tubes typically comprise six layers in total of negatively charged human serum albumin (HSA) and positively charged poly-L-arginine (PLA) $[(PLA/HSA)_3]$. HSA is the most prominent plasma protein in the human circulatory system and serves as a transporter of insoluble endogenous and exogenous compounds.[24,25] The HSA components in the $(PLA/HSA)_3$ nanotubes retain their original ligand-binding ability in water. Therefore, the swollen walls capture cyanine dye, zinc(II)-protoporphyrin IX, and fatty acids, which are all ligands for HSA.[22] Furthermore, the bound molecules can be released by a ligand-exchange reaction. This flexibility in the molecular design of our protein nanotube is stimulating efforts to develop a cylindrical biocatalyst. Martin and coworkers prepared glucose oxidase (GOD) nanotubes in the hard AAO template by using interlayer cross-linking with a glutaraldehyde.[14] Unfortunately, many tubules were crumpled during the template-etching step in 5% phosphoric

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acid. Nevertheless, it is noteworthy that the liberated free nanotubes showed higher GOD activity than that of the template-embedded form because mass-transfer resistance is lower for the free tubes. The reaction within the enzyme-modified membrane is easy and convenient by filtration,[2,14,18] although an appropriate volume of the sample solution must be used. If the enzyme nanotubes are released from the template framework safely, then they will act as a new class of bionanoreactor that is useful in very small volumes of medium, for example, in several microliters of solution.

The α-D-glucosidase from *Saccharomyces cerevisiae* (αGluD; $M_w = 68\,500$ Da) is a typical *exo*-type carbohydrase used to cleave the α(1→4) glucosyl linkage in the nonreducing terminal of the substrate with release of α-D-glucose (Glc) [Eq. (1)].[26,27] The αGluD has an isoelectric point (*p*I) of 4.0–5.5. Therefore, the protein is sufficiently negatively charged at the neutral value of pH 7.0.

$$\text{RO-α-D-Glucose} + H_2O \xrightarrow{\alpha\text{GluD}} \text{ROH} + \text{α-D-Glucose} \quad (1)$$

In this paper, we report the syntheses, structures, and enzyme activities of two different types of nanotubes with a αGluD interior surface. The first is sequentially fabricated $(PLA/HSA)_2PLA/\alpha GluD$ nanotubes. A fluorogenic glucopyranoside is captured into the one-dimensional pore space and hydrolyzed efficiently to generate Glc (Figure 1). The

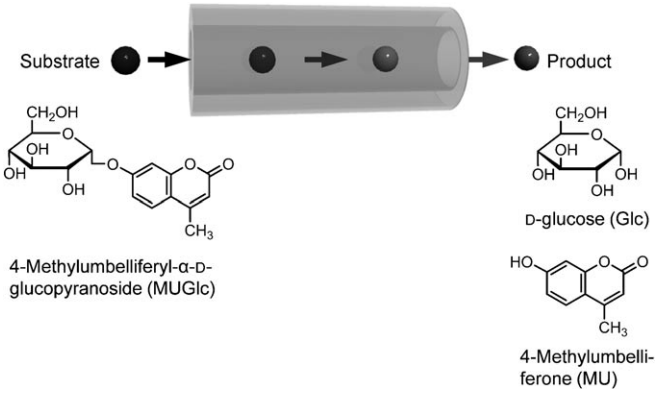

Figure 1. Schematic illustrations of protein nanotubes with an enzyme interior surface and an enzymatic reaction in the one-dimensional hollow space: exohydrolysis of the α(1→4) glucosyl bond of MUGlc.

second is prepared by using supramolecular complexation of biotin-labeled αGluD (B-αGluD) into HSA-based nanotubes bearing a single avidin (Avi) layer as an internal wall. This method will enable us to synthesize various biocylinders with the desired enzyme activity.

Results and Discussion

Synthesis and structure of $(PLA/HSA)_2PLA/\alpha GluD$ nanotubes: Protein nanotubes with an enzyme interior surface were prepared by an alternating LbL assembly of negatively charged protein (HSA or αGluD) and positively charged PLA onto the pore walls of a track-etched PC membrane (pore diameter (D_p) = 400 nm). After 2.5 cycles of LbL growth of the PLA/HSA combination at pH 7.0, a αGluD layer was deposited onto the fifth (PLA) layer through electrostatic interaction. Careful dissolution of the PC template in DMF and freeze-drying of the extracted core yielded $(PLA/HSA)_2PLA/\alpha GluD$ nanotubes as a white powder. SEM measurements revealed the formation of uniform hollow cylinders with a (413 ± 17) nm outer diameter and a wall thickness of (52 ± 3) nm (Figure 2a and b). The maximum length of the tubules (approximately 9 μm) corresponded to the channel depth of the PC membrane. If it is assumed that each HSA and αGluD layer has a single-protein thickness (approximately 8 nm),[28] the width of an individual PLA layer is estimated as 9.3 nm (Figure 2c). Normally, multilayers prepared with the LbL technique in a nanoporous template under a certain pressure are thicker than those of corresponding thin films fabricated on a flat substrate.[5,8,29–31] Jonas and co-workers demonstrated a very thick bilayer (50–120 nm) of poly(vinylbenzylammonium chloride) and poly(styrene sulfonate) (PSS).[29] In that case, the channels of the membrane are almost closed after a single cycle of deposition. The thickness was one–two orders of magnitude greater than that of the planar sheet made of the same components on a silicon wafer (1–3 nm). The PLA layer thickness (9.3 nm) estimated in our $(PLA/HSA)_2PLA/\alpha GluD$ nanotube is between the reported values for general polyelectrolyte walls fabricated in a porous membrane under pressured conditions.[8,29,30]

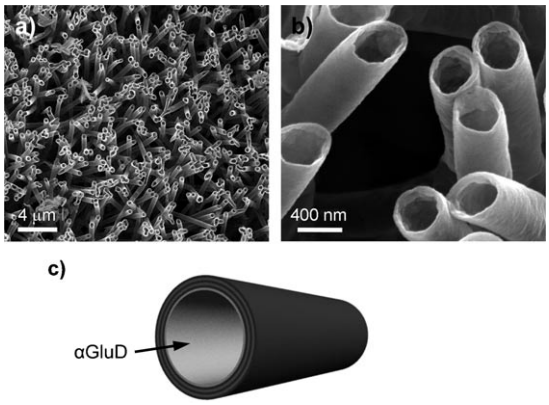

Figure 2. a, b) SEM images of $(PLA/HSA)_2PLA/\alpha GluD$ nanotubes prepared by using a porous PC template; c) schematic illustration of the six-layer nanotubes with an αGluD interior surface.

The lyophilized $(PLA/HSA)_2PLA/\alpha GluD$ nanotubes were suspended in sodium phosphate buffered (PB) solution (100 mM, pH 7.0, 1.5 mL) or deionized water, to yield a homogeneous dispersion. For observation of the nanotube morphology in water, the aqueous medium was freeze-dried in vacuo. SEM measurements revealed that all tubes were swollen considerably by water uptake and that their wall

thickness had almost doubled (to (110±8) nm) compared to that of the dried state. The only direction of swelling was toward the inside of the hollow tube. The outer diameter was unaltered. Consequently, reduction of the inner pore diameter (from 309 to 200 nm) was observed. This structure, which closely resembles that of swollen $(PLA/HSA)_3$ nanotubes,[22] is stable for 24 h without any marked difference. The average thickness of a bilayer of PLA/HSA (or PLA/αGluD) in water is estimated to be 37 nm. The dimensions of the proteins do not change very much (≤8 nm);[28] therefore, the PLA layer thickness might be 29 nm. The volume of the inner aqueous phase of one nanotube (length = 9 μm) was calculated to be 0.3×10^{-15} L (0.3 femtoliters).

The amount of αGluD in the last layer of the wall in one nanotube was estimated. The white powder of the $(PLA/HSA)_2$PLA/αGluD nanotubes (approximately 150 μg) obtained from one PC membrane (r = 2.5 cm), in which around 3.0×10^8 effective channels exist, was dispersed into acidic water (pH 3.5, 1.5 mL) to dissolve the LbL assembly. The tubules are deconstructed at this pH value below the pI values of the component proteins, to yield a clear solution.[20] From the absorbance at 280 nm, the αGluD concentration was determined to be approximately 90 nM, with the assumption that the tubes consist of the uniform multilayered structure described above. We concluded that the inner pore wall of one cylinder (length = 9 μm) is made of 2.7×10^5 molecules of αGluD enzyme.

Enzyme activity of $(PLA/HSA)_2$PLA/αGluD nanotubes: The αGluD enzyme from *Saccharomyces cerevisiae* exohydrolyzes the α(1→4) glucosyl bond of nonreducing 1,4-linked α-D-glucose residues.[26,27] We first determined the enzyme activity of free αGluD under our experimental conditions (100 mM PB solution, pH 7.0, 22 °C). A fluorogenic glucopyranoside, 4-methylumbelliferyl-α-D-glucopyranoside (MUGlc), was exploited as the substrate (Figure 1). The product of the enzymatic reaction, 4-methylumbelliferone (MU), is a coumarin derivative that gives strong emission at around 447 nm. After addition of MUGlc to the αGluD solution in PB ([MUGlc] = 20–160 μM), gradual increases in fluorescence (λ_{em} = 447 nm) and UV absorption (λ = 360 nm) were observed; these changes are both based on the hydrolyzed product, MU. By converting fluorescence intensity into UV absorbance, we determined the enzyme parameters of free αGluD for MUGlc hydrolysis: the Michaelis constant (K_M) was 0.18 mM and the catalytic constant (k_{cat}) was 960 min^{-1} (Table 1).

Next, equivalent experiments were conducted with the $(PLA/HSA)_2$PLA/αGluD nanotubes. Immediately after the mixing of MUGlc into the PB dispersion of the nanotubes (50 μg mL^{-1}), a similar fluorescence increase was found at 447 nm (Figure 3a). The appearance of the fluorescence was clearly visible (Figure 3b). By contrast, the use of $(PLA/HSA)_3$ nanotubes, without the αGluD layer, did not enhance the fluorescence at all. We reasoned that diffusion of MUGlc into the hollow cylinder occurs and the substrate is hydrolyzed on the αGluD wall. The enzyme parameters of the protein nanotubes were determined. The Lineweaver–Burk plots showed a linear slope (Figure S1 in the Supporting Information). The K_M value of the $(PLA/HSA)_2$PLA/αGluD nanotubes was nearly the same as that of free αGluD, although the k_{cat} value (25 min^{-1}) was significantly lower (Table 1). This restricted enzyme activity, which resembles noncompetitive-type inhibition, is probably attributable to the fact that some αGluD molecules were deposited onto the curved wall with unfavorable geometries. Certain geometries may lead to local distortions of the active sites.

The several-micrometer-long nanotubes are of sufficient length to spin down by centrifugation. Therefore, the product can be separated easily. We centrifuged the resultant mixture and subjected the supernatant to fluorescence spectroscopy and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) measurements. The fluorescence spectrum showed a single peak of the hydrolyzed product (MU) at 447 nm and no peaks of the proteins (HSA and αGluD) at 330 nm (Figure 4a). In the SDS-PAGE of the

Table 1. Enzyme parameters for the hydrolysis reaction of MUGlc (100 mM PB solution, pH 7.0, 22 °C).

Enzyme	K_M [mM]	k_{cat} [min^{-1}]
αGluD	0.18	960
$(PLA/HSA)_2$PLA/αGluD nanotubes	0.20	25
$(PLA/HSA)_2$PLA/αGluD thin films on a quartz plate	0.20	–[a]
B-αGluD[b]	0.08	370
$(PLA/HSA)_2$PLA/PLG/Avi–B-αGluD nanotubes[b]	0.10	15

[a] V_{max} = 7.6 μM min^{-1} under our experimental conditions. [b] The PB solution contained 10 μM HSA.

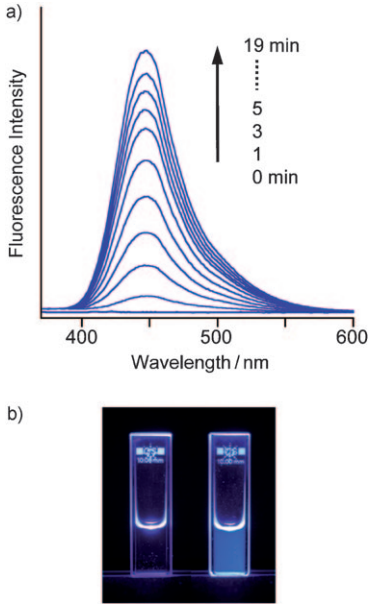

Figure 3. a) Fluorescence spectral change that occurred upon MUGlc hydrolysis by $(PLA/HSA)_2$PLA/αGluD nanotubes in PB solution (100 mM, pH 7.0; [MUGlc] = 83 μM, excitation at λ = 370 nm). b) Photographs of the MUGlc solution (20 μM) with excitation at 360 nm without nanotubes (left) and after the reaction with nanotubes (right).

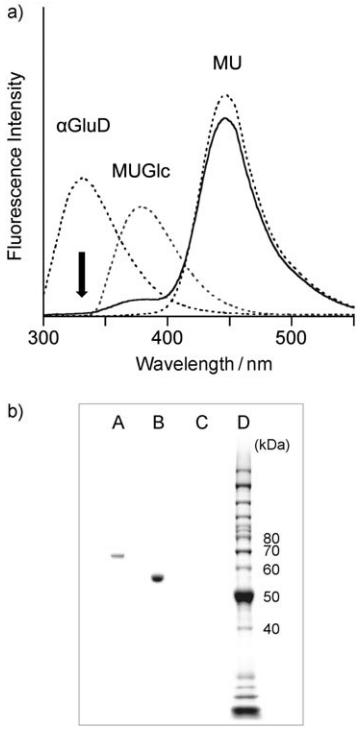

Figure 4. a) Fluorescence spectrum of the supernatant of the reaction mixture after MUGlc hydrolysis by $(PLA/HSA)_2PLA/\alpha GluD$ nanotubes in PB solution (100 mM, pH 7.0) (solid line). The dotted lines show where other possible components would appear if present (excitation at $\lambda = 280$ nm). b) SDS-PAGE results for the supernatant of the reaction mixture (300-fold concentrated; lane C) after MUGlc hydrolysis by $(PLA/HSA)_2PLA/\alpha GluD$ nanotubes in PB solution (100 mM, pH 7.0). Lane A: αGluD (0.013 %); lane B: HSA (0.013 %); lane D: protein ladder.

300-fold concentrated supernatant, no protein band appeared in the M_w region of 50000–70000 Da (Figure 4b). To confirm that the enzyme layer remains adhered onto the pore wall through electrostatic attraction after the separation steps, fluorescein isothiocyanate (FITC) labeled HSA (FITC-HSA) was employed instead of αGluD as a fluorescent component layer. The $(PLA/HSA)_2PLA$/FITC-HSA nanotubes prepared by the same procedure with the porous PC template ($D_p = 400$ nm) were colored pale yellow. SEM images showed the formation of similar hollow cylinders (Figure S2 in the Supporting Information). The tube powder was then suspended in PB solution and the mixture was centrifuged. As expected, the supernatant did not show any fluorescence at 520 nm (excitation at 488 nm) from dissociated free FITC-HSA. These findings manifest that 1) the $(PLA/HSA)_2PLA/\alpha GluD$ nanotubes retain their original multilayered structure without protein release during the hydrolysis reaction and 2) the product can be harvested in the supernatant by centrifugation at 4000 *g*.

LbL enzyme thin film on a flat substrate: To evaluate the enzyme activity of an αGluD surface on a flat substrate, a layered $(PLA/HSA)_2PLA/\alpha GluD$ thin film was prepared on a planar quartz surface by using the normal immersion procedure. The multilayer-modified plate was put into the PB solution (100 mM, pH 7.0) in a cuvette and the hydrolysis of MUGlc was measured. The αGluD adhered to the surface, which is more exposed to the solution, showed a significantly faster rate of catalysis than that of the nanotube under our experimental conditions (Figure S1 in the Supporting Information, Table 1). We reasoned that low diffusion of the substrate into the one-dimensional pore space also influences the rate of the enzyme reaction.

Enzyme activity of $(PLA/HSA)_2PLA/PLG/Avi–\alpha GluD$ nanotubes: Supramolecular complexation of biotin to avidin ($M_w = 68000$ Da) is the strongest biospecific interaction with an affinity (K) over $10^{15}\,M^{-1}$.[32] We previously demonstrated that HSA-based nanotubes with an Avi layer as the internal surface, $(PLA/HSA)_2PLA/PLG/Avi$ nanotubes (PLG: poly-L-glutanic acid), captured several biotinylated compounds into the pore, for example, FITC-biotin and biotin-labeled nanoparticles (diameter = 100 nm).[22] Numerous protein nanotubes with various enzyme activities would be produced if the Avi–biotin interaction could be used to immobilize any enzyme into the hollow cylinder.

We first conducted the introduction of biotin groups onto the αGluD molecule by using sulfosuccinimidyl-6-(6′-biotinamido-hexanoylamino)hexanoate, to generate biotinylated αGluD (B-αGluD). The average number of biotin groups per αGluD was estimated by using the general Green's method[33] to be 6.6. The hydrolysis activity of B-αGluD was slightly lower than that of the unmodified αGluD (Table 1). The B-αGluD (17.9 μM, 4.8 μL) was added to a PB dispersion (100 mM, pH 7.0)[34] of the $(PLA/HSA)_2PLA/PLG/Avi$ nanotubes (with (403 ± 10) nm outer diameter) and the mixture was incubated for 3 h at room temperature. The tubes were washed by precipitation/resuspension cycles by using centrifugation two times. SDS-PAGE of the supernatant (300-fold concentrated) showed no B-αGluD band, which indicated that most of the B-αGluD was accommodated into the nanotubes and bound onto the Avi wall (Figure 5).

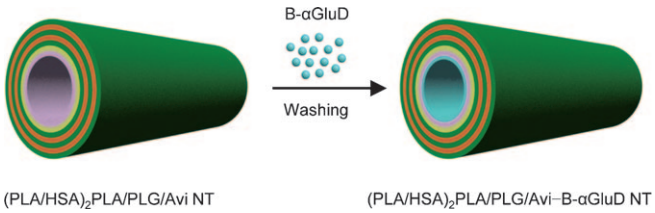

Figure 5. Preparation of protein nanotubes with an enzyme interior surface by using the avidin–biotin interaction. NT: nanotubes.

The obtained hybrid $(PLA/HSA)_2PLA/PLG/Avi–B-\alpha GluD$ nanotubes also showed hydrolysis reactivity for MUGlc. The parent $(PLA/HSA)_2PLA/PLG/Avi$ nanotubes caused no fluorescence increase of MU at 447 nm under identical conditions. The K_M value of 0.1 mM was broadly similar to that of free B-αGluD, whereas the k_{cat} value was again low, in much the same way as for the $(PLA/HSA)_2PLA/\alpha GluD$

nanotubes (Table 1). The possible explanation is that some B-αGluD molecules bind onto the Avi wall with unfavorable geometries that lead to the lower activity.

Conclusion

We have shown the synthesis of protein nanotubes with an αGluD interior surface by the LbL-assembly technique and have characterized their exohydrolysis activity for a 1,4-linked D-glucose residue. MUGlc diffused into the one-dimensional pore space of 0.3 fL volume and was hydrolyzed to Glc. The noncompetitive-type inhibition observed in the enzyme parameters could be the result of diverse geometries of the αGluD molecules on the curved surface wall and low diffusion of the substrate into the pore. Additionally, biotin-labeled αGluD was incorporated into $(PLA/HSA)_2PLA/PLG/Avi$ nanotubes, to yield similar hybrid cylinders spontaneously. The obtained tubes also exhibited activity for MUGlc hydrolysis. Simply binding specific enzymes onto the inner wall of the nanotubes by using the Avi–biotin interaction would enable us to create a new class of biocylinder catalyst. These catalysts could also be used as nanochannels for syntheses of unique biomaterials on a mesoscopic scale.

Experimental Section

Materials and apparatus: Poly-L-arginine hydrochloride (PLA; $M_w \approx 70000$ Da), poly-L-glutamic acid sodium salt (PLG; $M_w = 50000$–100000 Da), human serum albumin (HSA; recombinant product expressed by yeast species *Pichia pastoris*), HSA–fluorescein isothiocyanate conjugate (FITC-HSA), 4-methylumbelliferyl-α-D-glucopyranoside (MUGlc), and 4-methylumbelliferone (MU) were purchased from Sigma–Aldrich Corp. In addition, α-glucosidase from *Saccharomyces cerevisiae* (αGluD) and avidin (Avi) from egg white were purchased from Wako Pure Chemical Industries Ltd. The water was deionized (18.2 MΩ cm) by using water purification systems (Elix UV and Simpli Lab-UV; Millipore Corp.). The UV/Vis absorption spectra were recorded by using a UV/Vis spectrophotometer (8453; Agilent Technologies Inc.). Fluorescence emission spectra were measured by using a spectrofluorometer (FP-6500; Jasco Corp.). SDS-PAGE was performed by using 10% polyacrylamide precast gel (SuperSep Ace 10%; Wako Pure Chemical Industries Ltd.).

Template synthesis of protein nanotubes with an αGluD interior surface: The $(PLA/HSA)_2PLA/$αGluD nanotubes were prepared according to our previously reported procedure by using a track-etched polycarbonate (PC) porous membrane (Isopore membrane, $r = 25$ mm, pore diameter (D_p) = 400 nm; Millipore Corp.).[22] First, a sodium phosphate buffered (PB) solution (pH 7.0, 10 mM, 10 mL) of PLA (1 mg mL^{-1}) containing 0.1 M NaCl was filtered slowly through the membrane (0.25 mL min^{-1}) to absorb the positively charged PLA onto the pore wall. After washing away of excess PLA by water filtration, the membrane was dried in vacuo for 10 min. Second, a PB solution (pH 7.0, 10 mM, 10 mL) of HSA (2 mg mL^{-1}) was injected (0.5 mL min^{-1}) into the pores. Loosely adsorbed proteins were washed away with water. The membrane was then dried under vacuum. These pressure infiltrations were repeated for 2.5 cycles to grow the LbL thin film of $(PLA/HSA)_2PLA$. Finally, a PB solution (pH 7.0, 10 mM, 10 mL) of αGluD (2 mg mL^{-1}) was filtered through (0.25 mL min^{-1}), to produce an enzyme interior surface, followed by the washing and drying treatments. The PC membrane surface was then wiped with a cotton swab and dried in an automatic low-humidity chamber (Super Dry; Toyo Living Co. Ltd., Japan) for 12 h (humidity ≤ 1%). To release the protein-nanotube cores from the template support, the membrane was immersed into a DMF solution and the precipitates were freeze-dried in vacuo, to yield the $(PLA/HSA)_2PLA/$αGluD nanotubes as a white powder. The $(PLA/HSA)_2PLA/$FITC-HSA and $(PLA/HSA)_2PLA/PLG/$Avi nanotubes were fabricated by using the same procedure but PB solutions (pH 7.0, 10 mM, 10 mL) of FITC-HSA (2 mg mL^{-1}), Avi (1 mg mL^{-1}), or PLG (1 mg mL^{-1}) were used as appropriate.

Scanning electron microscopy: SEM measurements of the nanotubes were conducted by using the methods described previously.[20,22] The SEM observations were performed by using a scanning electron microscope (S-4300; Hitachi Ltd.) with an accelerating voltage of 10 kV. For each sample, at least 40 different nanotubes were measured to obtain average sizes for the outer diameter and wall thickness.

Enzyme activity assays: First, we evaluated the enzyme activity of free αGluD in aqueous media under our experimental conditions (pH 7.0, 22 °C). Several microliters of an *N*-methyl pyrrolidone (NMP) solution of MUGlc (5 mM) were added to the PB solution (pH 7.0, 100 mM, 1.5 mL) of αGluD (30 nM) in a 10 mm pathlength optical quartz cuvette ([MUGlc] = 20–160 μM). Immediately after injection, increases in the fluorescence at 447 nm (excited at 370 nm) and the UV absorbance at 360 nm based on the hydrolyzed product, 4-methylumbelliferone (MU), were monitored at 22 °C for 20 min. By converting the fluorescence increase into the UV absorbance, we determined the initial rate constant (V_0) for MUGlc hydrolysis: a molecular coefficient of $\varepsilon_{360} = 2.63 \times 10^3$ M^{-1} cm^{-1} for MU in PB solution was used. The K_M and k_{cat} values of αGluD were obtained from Lineweaver–Burk plots (Figure S1 in the Supporting Information). Second, the enzyme activity of the nanotubes was measured. The lyophilized powder of the $(PLA/HSA)_2PLA/$αGluD nanotubes (approximately 50 μg) was dispersed in PB solution (pH 7.0, 100 mM, 1.5 mL). The mixture was sonicated for a few seconds and transferred to a 10 mm pathlength optical quartz cuvette. Several microliters of the MUGlc solution in NMP (5 mM) were added ([MUGlc] = 20–160 μM) and the V_0 value was obtained from the fluorescence-intensity change at 447 nm (excited at 370 nm) by using the procedure described above.

LbL enzyme thin film on a planar quartz plate: A quartz plate (9 × 35 × 1 mm), which had been cleaned by using piranha solution, was first immersed into a PB solution (pH 7.0, 10 mM) of PLA (1 mg mL^{-1}) containing 0.1 M NaCl for 10 min to absorb PLA onto the smooth surface. After washing with deionized water, the quartz plate was dried with an N_2 gas flow for 10 min. The plate was then immersed into a PB solution (pH 7.0, 10 mM) of HSA (2 mg mL^{-1}) for 10 min, followed by washing and drying, to make a second layer of HSA. This was repeated for 2.5 cycles to construct the LbL assembly of $(PLA/HSA)_2PLA$. Finally, the plate was dipped into a PB solution (pH 7.0, 10 mM) of αGluD (2 mg mL^{-1}) to produce the enzyme surface. After the washing and drying process, the $(PLA/HSA)_2PLA/$αGluD thin film on the substrate was dried in an automatic low-humidity chamber for 12 h (humidity ≤ 1%). The enzyme activity of the LbL thin film was measured in PB solution (pH 7.0, 100 mM) in a 10 mm pathlength optical quartz cuvette as described above.

Preparation of biotin-labeled αGluD (B-αGluD): A freshly prepared phosphate-buffered saline (PBS, pH 7.4) solution of sulfosuccinimidyl-6-(6′-biotinamido-hexanoylamino)hexanoate (Pierce Biotechnology, Inc.; 10 mM, 108 μL) was added to a PBS solution of αGluD (3.7 mg mL^{-1}, 1.85 mL; biotin/αGluD = 20/1, mol/mol). The mixture was stirred for 30 min by using a magnetic stirrer in the dark at room temperature. The protein was then diluted with PBS solution and concentrated to the initial volume by using a centrifugal filter device (Vivaspin 20, 10000 kDa M_w cutoff; Vivascience AG/Sartorius AG) at 4000 *g* with a benchtop centrifuge (Allegra X-15R; Beckman Coulter Inc.). The dilution/concentration cycle was repeated twice and the total volume was adjusted to 2 mL. The biotin-labeled ratio of the surface Lys groups of αGluD was assayed by using Green's procedure with 2-(4′-hydroxybenzenazo)benzoic acid (HABA; Fluka Chemika GmbH).[33] The enzyme activity assay was performed by using the method described for αGluD.

Complexation of B-αGluD into the $(PLA/HSA)_2PLA/PLG/Avi$ nanotubes and enzyme activity assay: To prevent nonspecific binding of B-αGluD onto the nanotube outer surface, HSA (969 μM, 15.4 μL) was added to a PB dispersion (pH 7.0, 100 mM, 1.5 mL) of the $(PLA/HSA)_2PLA/PLG/Avi$ nanotubes (approximately 33 $\mu g\,mL^{-1}$). B-αGluD in PBS (17.9 μM, 4.8 μL) was then injected into the tube dispersion and the mixture was slightly sonicated and incubated for 3 h at room temperature. The dispersion was centrifuged for 10 min at 4000 *g* to precipitate the tubes. The supernatant was carefully discarded. The remaining nanotubes were suspended again in the PB solution of HSA (10 μM) and centrifuged. The precipitation/resuspension cycle was repeated twice and the volume was adjusted to 1.5 mL. The enzyme activity assay was conducted by using the same method as that used for the $(PLA/HSA)_2PLA/\alpha GluD$ nanotubes.

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[1] C. R. Martin, *Science* **1994**, *266*, 1961–1966.
[2] S. B. Lee, D. T. Mitchell, L. Trofin, T. K. Nevanen, H. Söderlund, C. R. Martin, *Science* **2002**, *296*, 2198–2200.
[3] S. Hou, C. C. Harrell, L. Trofin, P. Kohli, C. R. Martin, *J. Am. Chem. Soc.* **2004**, *126*, 5674–5675.
[4] M. Steinhart, R. B. Wehrspohn, U. Gösele, J. H. Wendorff, *Angew. Chem.* **2004**, *116*, 1356–1367; *Angew. Chem. Int. Ed.* **2004**, *43*, 1334–1344.
[5] M. Steinhart, *Adv. Polym. Sci.* **2008**, *220*, 123–187.
[6] Z. Liang, A. S. Sucha, A. Yu, F. Caruso, *Adv. Mater.* **2003**, *15*, 1849–1853.
[7] Y. Wang, A. S. Angelatos, F. Caruso, *Chem. Mater.* **2008**, *20*, 848–858.
[8] S. Ai, G. Lu, Q. He, J. Li, *J. Am. Chem. Soc.* **2003**, *125*, 11140–11141.
[9] Q. He, Y. Cui, S. Ai, Y. Tian, J. Li, *Curr. Opin. Colloid Interface Sci.* **2009**, *14*, 115–125.
[10] S. J. Son, J. Reichel, B. He, M. Schuchman, S. B. Lee, *J. Am. Chem. Soc.* **2005**, *127*, 7316–7317.
[11] J. Huang, T. Kunitake, *J. Mater. Chem.* **2006**, *16*, 4257–4264.
[12] C. Barrett, D. Iacopino, D. O'Carroll, G. De Marzi, D. A. Tanner, A. J. Quinn, G. Redmond, *Chem. Mater.* **2007**, *19*, 338–340.
[13] H. Zhou, S. S. Wong, *ACS Nano* **2008**, *2*, 944–958.
[14] S. Hou, J. Wang, C. R. Martin, *Nano Lett.* **2005**, *5*, 231–234.
[15] H. Hillebrenner, F. Buyukserin, J. D. Stewart, C. R. Martin, *J. Nanosci. Nanotechnol.* **2007**, *7*, 2211–2221.
[16] G. Lu, S. Ai, J. Li, *Langmuir* **2005**, *21*, 1679–1682.
[17] Y. Tian, Q. He, Y. Cui, J. Li, *Biomacromolecules* **2006**, *7*, 2539–2542.
[18] A. Yu, Z. Liang, F. Caruso, *Chem. Mater.* **2005**, *17*, 171–175.
[19] G. Lu, T. Komatsu, E. Tsuchida, *Chem. Commun.* **2007**, 2980–2982.
[20] X. Qu, G. Lu, E. Tsuchida, T. Komatsu, *Chem. Eur. J.* **2008**, *14*, 10303–10308.
[21] S. Hou, J. Wang, C. R. Martin, *J. Am. Chem. Soc.* **2005**, *127*, 8586–8587.
[22] X. Qu, T. Komatsu, *ACS Nano* **2010**, *4*, 563–573.
[23] X. Qu, N. Kobayashi, T. Komatsu, *ACS Nano* **2010**, *4*, 1732–1738.
[24] T. Peters, *All about Albumin: Biochemistry, Genetics, and Medical Applications*, Academic Press, San Diego, **1996**.
[25] U. Kragh-Hansen, *Dan. Med. Bull.* **1990**, *37*, 57–84.
[26] S. Chiba, *Biosci. Biotechnol. Biochem.* **1997**, *61*, 1233–1239.
[27] S. Howard, G. Withers, *Biochemistry* **1998**, *37*, 3858–3864.
[28] S. Curry, H. Mandelkow, P. Brick, N. Franks, *Nat. Struct. Biol.* **1998**, *5*, 827–835.
[29] H. Alem, F. Blondeau, K. Glinel, S. Demoustier-Shampagne, A. M. Jonas, *Macromolecules* **2007**, *40*, 3366–3372.
[30] D. Lee, A. J. Nolte, A. L. Kunz, M. F. Rubner, R. E. Cohen, *J. Am. Chem. Soc.* **2006**, *128*, 8521–8529.
[31] E. Donath, G. B. Sukhorukov, F. Caruso, S. A. Davis, H. Möhwald, *Angew. Chem.* **1998**, *110*, 2323–2327; *Angew. Chem. Int. Ed.* **1998**, *37*, 2201–2205.
[32] M. Wilchek, E. A. Bayer, *Anal. Biochem.* **1988**, *171*, 1–32.
[33] N. M. Green, *Methods Enzymol.* **1970**, *18*, 418–424.
[34] To prevent nonspecific adhesion of B-αGluD molecules onto the outer surface of the $(PLA/HSA)_2PLA/PLG/Avi$ nanotubes, HSA was added to the solution ([HSA] = 10 μM).

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