Electronic Supplementary Information

Rational Design of Degradable Polyanion for Layer-by-Layer Assembly for Encapsulation and Release of Cationic Functional Biomolecules

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Experimental

Materials

Dehydrated *N,N*-dimethylformamide (DMF), 4,4'-oxydiphthalic anhydride (ODPA), poly(ethylene glycol) [PEG 200, weight-average molecular weight (M_w) = 200], acetic acid, LiBr, dimethyl sulfoxide-d_6 (DMSO) containing 0.05 v/v% tetramethylsilane, sulfuric acid, methanol, toluene, hexane, 1 mol L^{-1} hydrochloric acid, 1 mol L^{-1} sodium hydroxide, disodium hydrogen phosphate, sodium dihydrogen phosphate, polyethyleneimine (branched, M_w = 10 000, PEI), lysozyme from egg white, lyophilized cells of *Micrococcus lysodeikticus*, and chitosan 10 (viscosity of 0.5 wt% chitosan aqueous solution containing 0.5 wt% acetic acid is 10 cP) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dulbecco's modified Eagle medium, lysogeny broth (LB) medium, hydrogen peroxide and phosphate-buffered saline (PBS)(−), without Ca^{2+} and Mg^{2+}, were purchased from Nacalai Tesque (Kyoto, Japan). α-Cyano-4-hydroxycinnamic acid and poly(sodium

4-styrenesulfonate) (PSS, M_w = 70 000) were purchased from Sigma (St. Louis, MO, USA). Other chemicals were purchased from Tokyo Chemical Industry (Tokyo, Japan). p-Type silicon wafers were purchased from the Electronics and Materials Co., Ltd (Hyogo, Japan). The water used was high-quality deionized water (Milli-Q water, >15 MΩ cm^{-1}), produced using a Milli-Q Advantage A100 system equipped with an Elix UV 3 system (Millipore, Molsheim, France).

PPEGE Synthesis

Poly(phthalic ethylene glycol ester) (PPEGE) was synthesized by ring-opening polymerization. ODPA (3.88 g, 12 mmol) and PEG 200 (2.4 g) were dissolved in DMF (25 mL). DIEA (3.83 g, 30 mmol) was added to the DMF solution. The solution was stirred, using a magnetic stirring bar, at 30 °C. After 3 h, an aqueous solution containing 1 wt% acetic acid was added to the reaction solution to yield PPEGE as a white precipitate. The product was collected and dried under vacuum. The product was characterized using ^{1}H NMR (Varian Gemini 300) spectroscopy and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS; ultrafleXtreme-KB, Bruker Daltonics). The PPEGE molecular weight was determined using a size-exclusion chromatography system (LC2000 plus, Jasco Co., Tokyo, Japan) equipped with a 7.6 × 300 mm column (Showa Denko K. K.) and a UV detector (UV2075 plus, Jasco Co.) at 40 °C. DMF/50 mM LiBr aqueous solution (60/40, v/v) was used as the eluent, at a flow rate of 0.5 mL min^{-1}. A standard curve was produced using PSSs (Showa Denko K. K.) as molecular-weight standards. ^{1}H NMR (300 MHz, DMSO): δ (ppm) = 7.83–7.89 (m, 1.5H), 7.64–7.74 (m, 0.5H), 7.33 (s, 0.5H), 7.19–7.29 (m, 3.5H), 4.24–4.32 (m, 4H), 3.61–3.69 (m, 4H), 3.41–3.54 (m, 8H).

PPEGE Degradation

PPEGE was dissolved in phosphate buffer at a concentration of 0.5 mg mL^{-1}. The solution was

stirred, using a magnetic stirring bar, at 37 °C (or 25 °C). Aliquots (1.5 μL) were periodically withdrawn and analyzed using size-exclusion chromatography and MALDI-TOF/MS.

Preparation of Fluorescein Isothiocyanate (FITC)-Labeled Lysozyme

Lysozyme was labeled with FITC as described previously.[1] Briefly, FITC (2 mg) and lysozyme (200 mg) were dissolved in 0.1 M borate buffer (pH 9.0). After incubation for 60 min at room temperature, the pH was adjusted to 7.5 using 0.1 M boric acid. The solution was dialyzed against Milli-Q water using a cellulose membrane with a molecular-weight cutoff of 3.5 kDa for 48 h at 4 °C. The labeling degree of FITC/lysozyme was 0.023.

Preparation of Films by Layer-by-Layer (LbL) Assembly

Silicon substrates were cleaned using piranha solution. Briefly, the substrates were immersed in sulfuric acid/hydrogen peroxide (3/1, v/v) at 80 °C for 1 h, followed by extensive rinsing with Milli-Q water and methanol. Silicon substrates with positively charged surfaces were prepared by amination of the cleaned substrates using APS. The substrates were immersed in toluene solution containing 10 mM APS at 25 °C for 2 h. The substrates were washed three times with excess hexane and dried under vacuum.

Ten bilayers of PEI/PSS (terminated with a PEI layer) were deposited on the aminated silicon wafers to prepare suitably charged surfaces for the PPEGE adsorption. LbL films were fabricated using the alternate dipping method, according to the reported protocol.[2] 1) Substrates were submerged in PPEGE solution (Table S1) for 5 min. 2) Substrates were removed and immersed in a first water bath for 15 s, followed by immersion in second and third water baths for 30 and 45 s, respectively. 3) Substrates were submerged in lysozyme solution for 5 min. 4) Substrates were rinsed as described above. This cycle was repeated until the desired number of PPEGE and lysozyme layers (typically 20 each) had been deposited. Films to be used in degradation and release

experiments were stored in a desiccator.

The surface morphology of the dried (PPEGE/lysozyme)$_{20}$ film was observed using an atomic force microscope (AFM, Nano Navi E-sweep, Hitachi High-Tech Science Co., Tokyo, Japan). The AFM observation was performed in the intermittent contact mode. Silicon cantilevers with spring constant of 15 N/m were used (SI DF20, Hitachi High-Tech Science Co.).

Table S1. Preparation conditions for polymer solutions for LbL assembly.

Polyethyleneimine (PEI) solution	20 mM PEI (M_n[a] = 10 000, branched) in Milli-Q water (adjusted to pH 5 using NaOH and HCl)
Poly(sodium 4-styrenesulfonate) (PSS) solution	20 mM PSS (M_n = 70 000) in Milli-Q water (adjusted to pH 5 using NaOH and HCl)
Poly(β-amino ester) (PBAE) solution	5 mM PBAE (Mn = 28 000, PDI[b] = 1.06) in sodium acetate buffer (0.1 M, pH 5)
PPEGE solution	5 mM PPEGE (M_n = 10 600, PDI = 1.90) in phosphate buffer (0.1 M, pH 6)
Ribonuclease A, cytochrome c solutions	0.1 wt% ribonuclease A (or cytochrome c) in phosphate buffer (0.1 M, pH 6)

[a] Number average molecular weight.

[b] Polydispersity index

Synthesis of poly(β-amino ester) (PBAE)

PBAE was synthesized as previously described.[3,4] 1,3-Di-4-piperidylpropane (4.93 g, 15 mmol) was dissolved in dichloromethane (10 mL). 1,4-Bis(acryloyloxy)butane (4.76 g, 24 mmol) was pipetted into the solution. The solution was stirred, using a magnetic stirring bar, at 50 °C. After 24 h, the solution was cooled to room temperature and poured slowly into vigorously stirred *n*-hexane. The

resultant precipitate was collected and dried under vacuum to yield PBAE as a white solid. The product was characterized using ^{1}H NMR spectroscopy. The molecular weight of PBAE was determined using a gel-permeation chromatography system (Jasco LC2000 plus) equipped with an 7.6 × 300 mm column × 2 (Showa Denko K. K.) and a refractive index detector (Jasco RI2031 plus) at 40 °C. Chloroform/0.1 M piperidine was used as the eluent, at a flow rate of 1.0 mL min^{-1}, and poly(methyl methacrylate) molecular-weight standards (Showa Denko K. K.) were used to construct a standard curve. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) = 4.10 (t, J = 5.8 Hz, 4H), 2.98–2.39 (m, 12H), 1.95 (t, J = 9.0 Hz, 4H), 1.75–1.65 (m, 4H), 1.65–1.54 (m, 6H), 1.37–1.24 (m, 2H), 1.24–1.07 (m, 8H).

Degradation of LbL Films and Evaluation of Lysozyme Release

Substrates (10 × 15 mm) with LbL films were incubated in PBS buffer (100 mL, pH 6.0 or 7.4) at 37 °C (or 25 °C) and periodically removed from the solution. The substrates were dried under vacuum and examined using an ellipsometer (M-220K, Jasco Corp., Hachioji, Japan). The ellipsometric measurements were performed at more than 10 different locations on each substrate surface.

FITC-lysozyme release into the buffer solution was quantified by measuring the solution fluorescence using a fluorescence spectrophotometer (Excitation 494 nm, Emission 521 nm).

Enzymatic Activity of Released Lysozyme

Lyophilized *M. lysodeikticus* cells, which were used as substrates for lysozyme, were dispersed in phosphate buffer (50 mM, pH 7.4) at 0.25 mg mL^{-1}. The solution absorbance (at 450 nm) was adjusted to 0.6–0.7 by adding phosphate buffer. Sample solutions (0.04 mL) were added to substrate-containing solution (1.0 mL) and immediately stirred using a magnetic stirring bar. The sample absorbances (at 450 nm) were recorded for 5 min at 37 °C. The lysozyme activity was

calculated using the following equation.

$$Lysozyme\ activity\ [units/ml] = \frac{(\Delta A_{450}/\min\ of\ sample - \Delta A_{450}/\min\ of\ blank)}{0.001 \times 0.04}$$

PPEGE Cytotoxicity

The PPEGE cytotoxicity was evaluated using HeLa cells and a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Briefly, cells were plated at 5.0×10^3 cells per 100 μL in a 96-well plate using Dulbecco's modified Eagle medium with 10% fetal bovine serum, and incubated for 24 h at 37 °C. PPEGE [M_w 20 100, polydispersity index (PDI) 1.90] was added to the wells at various concentrations, followed by incubation at 37 °C for 20 h. Then Cell Counting Kit-8 solution (10 μL) was added to each well. After incubation for 1 h, the absorbance at 450 nm was measured, using a microplate reader (ARVOsx 1420 multilabel counter, PerkinElmer, Waltham, MA, USA), at 25 °C. The assay was performed five times for each sample.

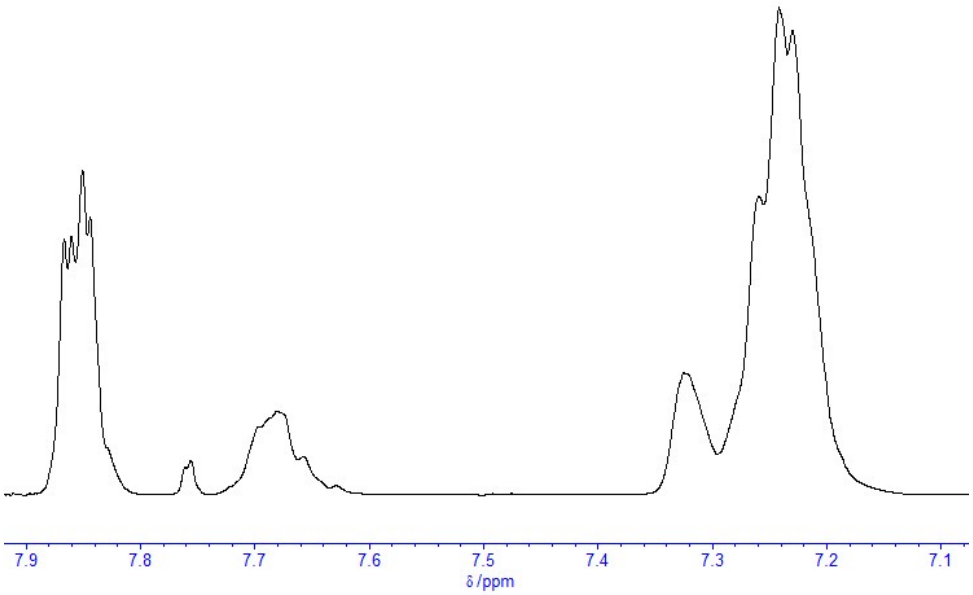
Inhibition of *B. subtilis* Growth on Lysozyme/PPEGE Films

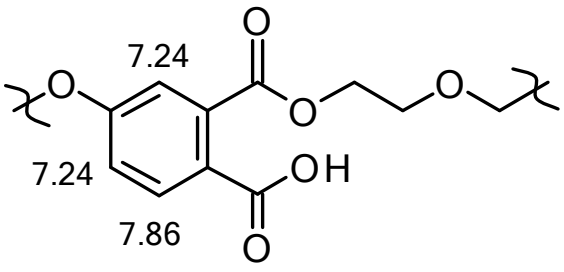
Culture medium (0.5 mL, LB medium) containing *Bacillus subtilis* (OD_{660} = 0.08) was placed on a bare silicon substrate, PPEGE/PBAE, and PPEGE/lysozyme film (15 × 30 mm). After incubation at 37 °C for 5 h, the turbidity of the culture medium was measured at 660 nm. To prevent the culture medium from evaporating, all samples were incubated in petri dishes with wet cotton.

Results

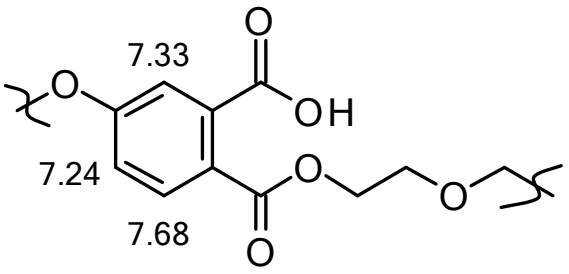
Table S2. Weight-average molecular weights (M_w) and polydispersity indices (PDIs) of PPEGEs synthesized under various conditions. (a) Effect of PEG200 concentration, (b) effect of reaction time, and (c) effect of DIEA concentration. Typical conditions were ODPA 0.5 M, PEG200 0.48 M, and DIEA 0.5 M at 30 °C for 3 h. M_ws were determined using size-exclusion chromatography relative to poly(sodium styrenesulfonate) standards.

[a]PEG 200 (mol L^{-1})	M_w	PDI
0.47	8800	2.09
0.48	15 600	2.29
0.49	13 000	2.16
0.50	9900	2.02
[b]Time (h)	M_w	PDI
1	11 700	2.13
3	15 600	2.29
5	14 900	2.26
8	13 500	2.21
[c]DIEA (mmol L^{-1})	M_w	PDI
0.5	15 600	2.29
0.7	17 600	2.29
0.9	20 100	1.90
1.1	10 500	2.02





Form 1



Form 2

Fig. S1. ^{1}H NMR spectrum of PPEGE, and two types of ester formed between ODPA and PEG 200, with NMR assignments.

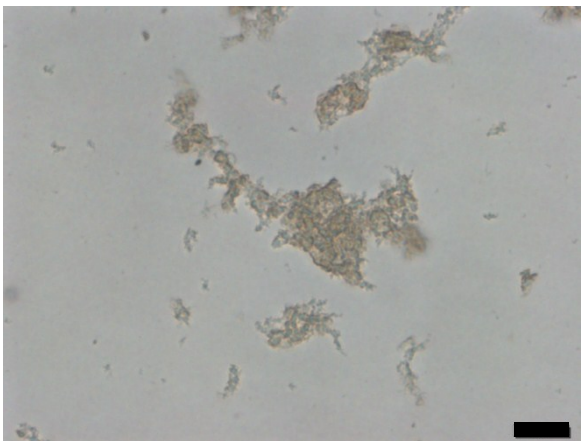


Fig. S2. Polyelectrolyte complex formation between PPEGE and chitosan on mixing equivalent volumes of 1 wt% PPEGE solution (pH 6.0) and 1 wt% chitosan solution (pH 6.0). Scale bar represents 20 μm.

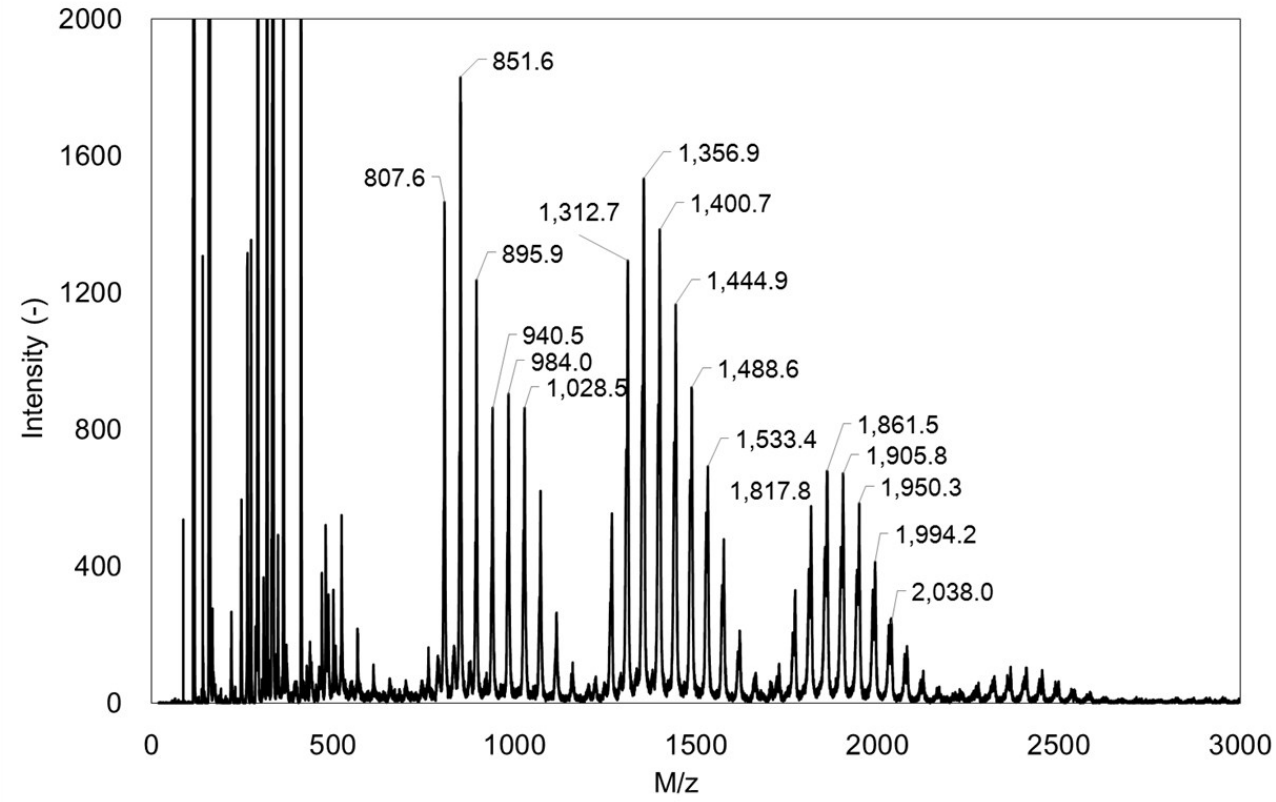


Fig. S3. MALDI-TOF/MS of PPEGE degraded in PBS (pH 7.4) at 25 °C for 7 d. Spectra were recorded in negative mode, using α-cyano-4-hydroxycinnamic acid as matrix.

For example,

・M/z 851.6 indicates ODPA-PEG200-ODPA (calc. Mw = 850.2)

・M/z 1356.9 indicates ODPA-PEG200-ODPA-PEG200-ODPA (calc. Mw = 1354.3)

・M/z 1861.5 indicates ODPA-PEG200-ODPA-PEG200-ODPA-PEG200-ODPA (calc. Mw = 1858.4)

Terminal ODPA was hydrolyzed.

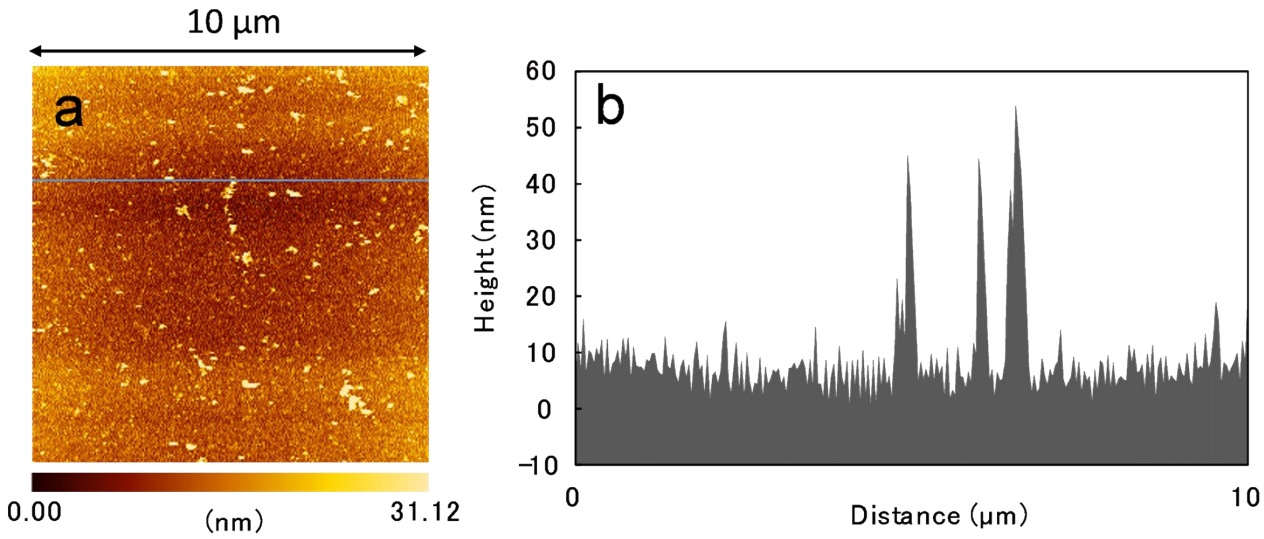


Fig. S4. a) Atomic force microscopy height image showing a 10 µm×10 µm area of $(PPEGE/lysozyme)_{20}$ film. The root mean square roughness was 5 nm for the blue line. b) Height profile of the selected blue line in the AFM height image.

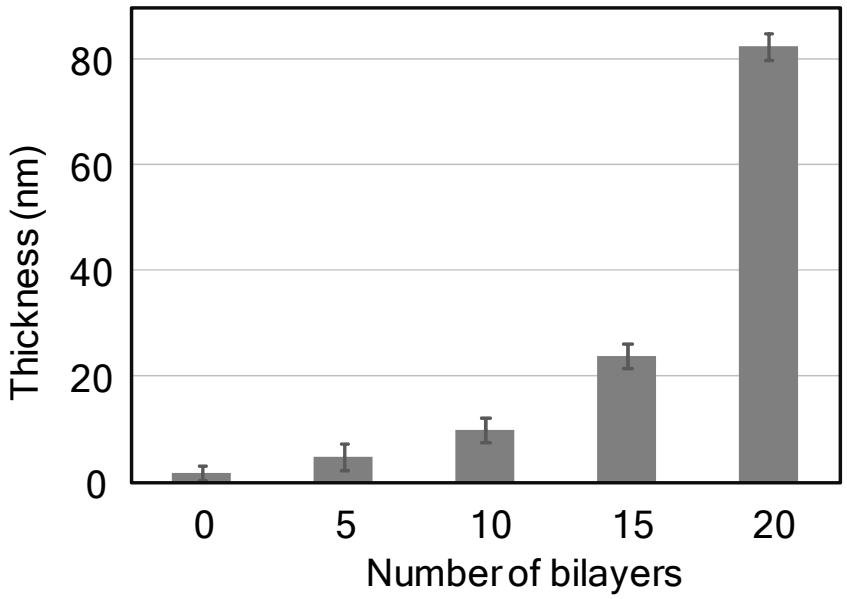


Fig. S5. Ellipsometric thickness of dry PPEGE/PBAE LbL film as function of number of bilayers.

References

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