Cytocompatible Coating of Yeast Cells with Antimicrobial Chitosan through Layer-by-Layer Assembly

Beom Jin Kim,[†] Insung S. Choi,[†,*] and Sung Ho Yang[‡,*]

[†]*Center for Cell-Encapsulation Research, Department of Chemistry, KAIST, Daejeon 305-701, Korea. *E-mail: ischoi@kaist.ac.kr*

[‡]*Department of Chemistry Education, Korea National University of Education, Chungbuk 363-791, Korea. *E-mail: sunghoyang@knue.ac.kr*

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Layer-by-layer (LbL) assembly has been widely used for the fabrication of multilayered thin films composed of macromolecules, based on the electrostatic interactions or hydrogen bonding.[1,2] The method has been applied to coating living cells with not only organic macromolecules but also inorganic nanomaterials such as nanoparticles, nanotubes, and graphene oxide.[1,3–5] The LbL process is relatively compatible to the living cells because it occurs under mild conditions, including aqueous solution, low concentration of macromolecules, room temperature, and ambient pressure. Although the process and conditions are mild enough for living cells, toxicity of macromolecules or nanomaterials limited use of themselves for cell coating. In some cases, toxicity of the material is relied on the types of cells; one is cytocompatible to microbial cells while the one is highly toxic to mammalian cells.[6,7] Therefore, the cytocompatibility of LbL components is a prerequisite for successful cell coating. For instance, high molecular weight or bio-related macromolecules, and surface-stabilized nanomaterials have been preferred as biocompatible components for LbL coating.[1,3–5,8–10]

The most difficult obstacle is originated from use of cationic polymer which can easily attach to negatively charged cell surfaces and cause damage to cell membrane.[11,12] Despite the toxicity of cationic polymers, use of them is mostly inevitable because they are necessary components for LbL assembly. In this work, we developed a simple but versatile method for coating living cells with toxic macromolecules by LbL assembly. Although there are many toxic macromolecules such as poly-L-lysine, poly(arginine), or protamine, we chose chitosan (CTS) because its antimicrobial property is well known and it is degradable by specific enzyme, chitosanase, which is beneficial to examine viability of the cells according to the change of molecular weight of polymers. Living yeast cells were individually coated with LbL multilayers composed of CTS, which is cationic and antimicrobial. By protective coating of the cells with compatible multilayers, the CTS was successfully used as an LbL component for cell coating while maintaining viability of the cells.

To establish the coating process for yeast cells, formation of LbL multilayers was investigated on the planar substrate as a model system for cell surface. The gold substrate was modified to be negatively charged by self-assembled monolayers with 16-mercaptohexadecanoic acid because the yeast cell has negative zeta-potential.[13,14] Poly-L-glutamic acid (PGA) was selected as a counter pair for CTS, which has been utilized for fabrication of LbL multilayers.[15] The pH of each polymer solution was adjusted to 5.5 for CTS solution and 7.2 for PGA solution., At each pH, CTS (pK_a = ~6.5) is positively charged and PGA (pK_a = ~4.9) is negatively charged, thus LbL multilayers can be formed by electrostatic interactions between the polymers.[16,17] The thickness of multilayer was measured by ellipsometer, as a function of LbL deposition steps (Figure 1 (a)). The thickness increased exponentially by the number of deposition cycles, and the five bilayers of CTS and PGA, referred to as $(CTS/PGA)_5$, reached 33 nm. This exponential growth regime has already been observed and explained in a previous report on QCM measurements; its mechanism is relied on not only diffusion of polyelectrolytes through the film but also the weak interaction between dissolved polyelectrolyte and top layer of the film.[15,18] In addition, it is known that alternation of pH condition and increase of surface roughness contribute to the exponential growth of the films.[19,20]

Enzymatic degradation of LbL multilayers has been demonstrated for various applications such as delivery systems and medical devices.[21] Different from other stimuli such as pH, chemicals, and temperature, enzymatic hydrolysis has distinct advantages in terms of biocompatibility because the enzyme can degrade only a targeted component in multilayer films under physiological conditions.[22] For biocompatible degradation of CTS/PGA film, we selected chitosanase which is capable of endohydrolysis of β-1,4-linkages between *N*-acetyl-D-glucosamine and D-glucosamine residues in CTS. Thickness of the film gradually decreased with duration of chitosanase hydrolysis (Figure 1(b)). The thickness decreased by over 46% (33 to 18 nm) for first 3 h but only 13% for the next 21 h. It means that the films were degraded rapidly when initially exposed to chitosanase. The result can be explained by transformation of interlayer structures according LbL cycles. The structure is composed of islands partially covering the surfaces at

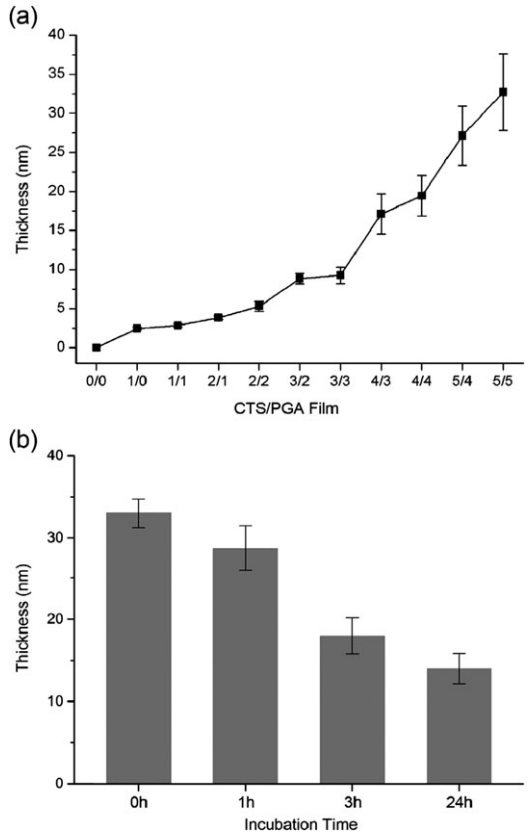

Figure 1. Ellipsometric thickness of CTS/PGA films on gold substrates. (a) Growth of the film thickness as a function of LbL steps. (b) Degradation of CTS/PGA(5/5) films with chitosanase as a function of time.

the initial point of LbL, but it changed to a blend of two components covering whole surfaces as LbL cycles repeat. The transformation mechanism has been suggested by Mano *et al.*; the blend structure would be formed by polyelectrolyte diffusion in and out of the film.[22] In their report, the blended structure also contributes to enhanced accessibility of the enzyme, chitosanase, resulting in rapid degradation.

Before coating yeast cells with CTS/PGA film, rhodamine B was linked to CTS to visualize the films with red fluorescence. The yeast (*Saccharomyces cerevisiae*) cells were coated by alternately immersing them in solution of rhodamine-linked CTS (CTS-R) and that of PGA for 5 min. The LbL cycle repeated five times, resulting in yeast@[CTS-R/PGA]$_5$. The strong red fluorescence indicated that the cells were coated with CTS-R and PGA but the cells were seriously aggregated (Figure 2(a)). The cell aggregation usually occurs when the cell membrane has been weakened and the cell is disabled or dead. Interestingly, the aggregated cells were dissociated by degrading CTS-R with chitosanase (Figure 2(b)). To verify the toxic effect of CTS-R/PGA coating, toxicity of CTS, PGA, and chitosanase was tested by fluorescein diacetate (FDA) (Figure 2(c)–(f)). After 30 min incubation of yeast cells in CTS solution (2 mg/mL), it was observed that their viability decreased remarkably and the cells were aggregated. In contrast, PGA and chitosanase were safe to yeast cells. The results imply that the direct contact of CTS triggers cell death as well as the cell aggregation because of antimicrobial property of CTS. To maintain the viability of yeast cells, the coating process should be modified to avoid a direct contact between CTS and the cells.

It is known that some cationic polyelectrolytes including CTS are antimicrobial by attaching to negatively charged surface of microbial cells, resulting in damages on cell membranes and the leakage of intracellular constituents.[23] To prevent the cells from being contacted with the toxic CTS layer, we applied a protective primer layer on the cell and then formed CTS-R/PGA multilayers. Poly(diallyldimethyl ammonium chloride) (PDADMAC) and poly(sodium 4-styrenesulfonate) (PSS) were selected as protective layers because PDADMAC is not toxic to the yeast cells in spite of its cationic character and PDADMAC/PSS films had been widely used for coating microbial cells including yeast cells while maintaining cell viability.[1,3–5,24,25] To prevent direct deposition of CTS-R on the cell surface, the yeast cells were coated [PDADMAC/PSS]$_2$ followed by [CTS-R/PGA]$_2$[CTS-R]. In other words, each yeast cell was coated with [PDADMAC/PSS]$_2$[CTS-R/PGA]$_2$[CTS-R], referred to as yeast@[PDADMAC/PSS]$_2$[CTS-R/PGA]$_2$[CTS-R]. Ring-shaped red fluorescence confirmed that the cells were individually coated with LbL multilayers and green fluorescence from FDA did that most of them are alive (Figure 2(g)). In contrast to yeast@(CTS-R/PGA)$_5$, the coated cells were not aggregated and mostly alive while maintaining 94% viability presumably because [PDADMAC/PSS]$_2$ prevented direct contact of CTS-R and deposition number of CTS-R reduced from 5 to 3. The protective effect of PDADMAC/PSS multilayer is clear and antimicrobial electrolytes could be used for cell surface modification with a help of a simple protective coating. Although both CTS and PDADMAC are positively charged, the former is toxic but the later is compatible to yeast cells. The dramatic difference could be explained by two reasons. It was well known that high molecular weight polymer is less toxic to the cell because it is less permeability to cell membrane. PDADMAC, synthetic polymer, has relatively high and narrow range of molecular weight (Mw: 100 000–200 000) while CTS could has high polydispersity index (PDI) and may contain low molecular weight chains because it is extracted from organisms. On the other hand, yeast cell walls composed of polysaccharide could interact stronger with CTS rather than PDADMAC because CTS is a type of polysaccharide.

The degradation of CTS-R/PGA multilayer was examined by exposing the coated cells to chitosanase (Figure 2(h)). The red fluorescence from CTS-R/PGA coat was noticeably decreased after 3 h, which means that the CTS-R/PGA coat was degraded by chitosanase. However, it was observed that green fluorescence from FDA decreased remarkably after the degradation of CTS-R/PGA coat. The cell viability was decreased by treatment of chitosanase although the toxicity

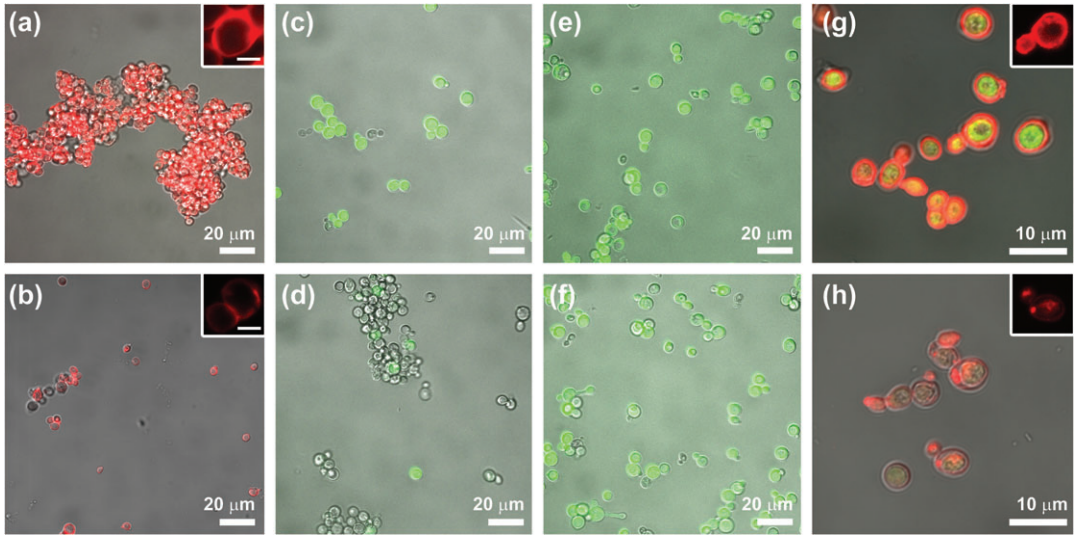

Figure 2. Confocal micrographs: (a) yeast@[CTS-R/PGA]$_5$ and (b) that after degradation with chitosanase, (c) native yeast and the cells after 30 min in the presence of (d) CTS (2 mg/mL), (e) PGA (2 mg/mL), and (f) chitosanase, (g) yeast@[PDADMAC/PSS]$_2$[CTS-R/PGA]$_2$[CTS-R], and (h) the cells after degradation with chitosanase. The cell viability was visualized with FDA staining. Scale bar (inset of a, b): 3 μm.

test had confirmed that chitosanase was not toxic to native yeast cells. The discordance could be explained by the change in molecular weight of CTS caused by degradation. The degraded CTS-R is permeable to the PDADMAC/PSS multilayer and contacts with cell membrane because its molecular weight has been reduced by degradation while the protective multilayer could block the high molecular weight CTS-R before degradation. Regardless of the protective layer, it is also known that low molecular weight cationic polymers are more toxic to the cells than high molecular weight ones[26] because of increased mobility and ionic interactions in small chains, leading to effective binding to the cell membrane.[27]

It was demonstrated that living cells were individually coated with toxic macromolecules by priorly coating them with protective layers. The protective layers enabled to introduce antimicrobial CTS to the surface of yeast cells. The method is simple but widely applicable to the other types of cells as well as various LbL components. In addition, it was found that the protective effect of primer layers depends on molecular weight of LbL components. The results would contribute to expanding application of various antimicrobial materials, not only CTS, to cell surface modification.

Experimental

Materials. Chitosan (CTS, medium molecular weight, Aldrich, Iceland), poly-L-glutamic acid sodium salt (PGA, Sigma, USA), poly(diallyldimethyl ammonium chloride) solution (PDADMAC; Mw: 100 000-200 000; 20 wt % in H_2O, Aldrich, USA), poly(sodium 4-styrenesulfonate) (PSS, Aldrich, USA), chitosanase from *Streptomyces griseus* (>50 units/mg protein, Sigma, USA), rhodamine B isothiocyanate (mixed isomers, Sigma, USA), sodium chloride (NaCl, ≥99.5%, Jin Chemical Pharmaceutical Co., Ltd., Korea), 16-mercaptohexadecanoic acid (Aldrich, USA), ethanol (absolute for analysis, Merck, Germany), hydrochloric acid (HCl, 37 wt %, Junsei, Japan), sodium hydroxide (NaOH, Junsei, Japan), fluorescein diacetate (FDA, Sigma, USA), adenine hemisulfate salt (Sigma, USA), Difco™ YPD broth (YPD broth, BD, USA) were used as received. The syringe filter (GD/X PVDF membrane, pore size: 0.45 μm, diameter: 25 mm, Whatman, USA) was used for the filtration of CTS solution. YPAD broth liquid media were prepared with 50 g of YPD broth and 100 mg of adenine hemisulfate in 950 mL of deionized (DI) water and used after autoclaved (15 min, 121°C). Ultrapure water (18.3 MΩ cm) was purified by Human Ultrapure System (Human Corp., Seoul, Korea).

Preparation of CTS and PGA Solution. The aqueous 0.15 M NaCl solutions of CTS (2 mg/mL, pH 5.5) and PGA (2 mg/mL, pH 7.2) were prepared. The pH of the CTS solution was adjusted by adding 1.0 M HCl and 2.0 M NaOH, and the solution was filtered using the syringe filter. On the other hand, the pH value of PGA solution was 7.2 without any treatment.

Formation and Degradation of CTS/PGA Multilayers on the Gold Substrate. The planar gold substrate was immersed for overnight in an ethanolic solution of 16-mercaptohexadecanoic acid (2 mM) for forming self-assembled monolayers (SAMs) on the substrate. After washing the SAM-coated gold substrate, the multilayers film was formed by first immersing the substrate in the CTS solution for 10 min, and then the substrate was immersed in the PGA solution for 10 min, with intermediate washing with DI water. The immersion and washing steps were repeated until five bilayers were formed. At each step, the substrate was dried under a stream of argon, and the thickness of the film was measured by ellipsometer. The formed (CTS/PGA)$_5$ film was degraded by chitosanase hydrolysis. To hydrolysis of CTS, the stock solution of chitosanase was prepared by reconstituting a 10 unit vial with 300 μL of DI water, and

10 μL of the stock solution was diluted with 200 μL of sodium acetate buffer (100 mM, pH 5.5). After that, the $(CTS/PGA)_5$ film was immersed in the diluted chitosanase solution and incubated at 37 °C. The decreased thickness of the film was measured by an ellipsometer.

Functionalization of CTS with Rhodamine B (CTS-R). The CTS was functionalized with rhodamine B.[28] 300 mg of CTS was dissolved in 12 mL of 0.5 M HCl solution with 1.2 mg of rhodamine B isothiocyanate, and the solution was mixed for 5 min. After that, 30 mL of DI water was added, and the final pH was adjusted by 2.0 M NaOH solution. The product solution was purified by dialysis.

Coating of Yeast Cells with $(CTS\text{-}R/PGA)_5$ or $(PDADMAC/PSS)_2$–$(CTS\text{-}R/PGA)_2$–(CTS-R). For yeast@$(CTS\text{-}R/PGA)_5$, the yeast cells were firstly immersed in the aqueous 0.15 M NaCl solutions of CTS-R (2 mg/mL, pH 5.5) for 5 min, followed by washing with the aqueous 0.15 M NaCl solution; and then the cells were immersed in the aqueous 0.15 M NaCl solutions of PGA (2 mg/mL, pH 7.2) for 5 min, with intermediate washing with the aqueous 0.15 M NaCl solution. These alternative steps were repeated five times. In the case of yeast@$(PDADMAC/PSS)_2$–$(CTS\text{-}R/PGA)_2$–(CTS-R), the yeast cells were immersed alternately in the aqueous 0.15 M NaCl solutions of PDADMAC (2 mg/mL) and PSS (2 mg/mL) for 5 min each. After that, the cells were immersed alternately in the aqueous 0.15 M NaCl solutions of CTS-R (2 mg/mL) and PGA (2 mg/mL) for 5 min. After repeating twice, the cells were lastly immersed in the CTS-R solution. The washing step was always performed after each immersion step.

Degradation of Coated Cells. $(CTS\text{-}R/PGA)_5$ or $(PDADMAC/PSS)_2$–$(CTS\text{-}R/PGA)_2$–(CTS-R) coat on the cell surface were degraded by immersing the coated cells in chitosanase solution at 37 °C for 3 h. The treated cells were washed with 0.15 M NaCl solution three times, and then the degraded coats were characterized by confocal laser-scanning microscopy.

Cell Viability Test. The viability of yeast cells was investigated by FDA assay, where FDA is hydrolyzed to the green-fluorescent fluorescein by esterases in the metabolically active cells. The stock solution of FDA (10 mg/mL) was prepared by dissolving FDA in acetone, due to the insolubility of FDA in water. 2 μL of the stock solution was mixed with 0.5 mL of the yeast suspension (phosphate buffered solution: 50 mM, pH 6.5). After 30 min incubation with shaking, the cells were washed with aqueous 0.15 M NaCl solution three times and then characterized by confocal laser-scanning microscopy.

Characterizations. The thicknesses of all films on the gold substrates were measured with a Gartner L116s ellipsometer (Gaertner Scientific Corporation, Chicago, IL, USA) equipped with a He–Ne laser (632.8 nm) at a 70° angle of incidence. A refractive index of 1.46 was always used. The images of stained yeast cells were taken by a LSM 510 META confocal laser-scanning microscope (Carl Zeiss, Germany).

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