# Enzymatic Hydrolysis of a Layer-by-Layer Assembly Prepared from Chitosan and Dextran Sulfate

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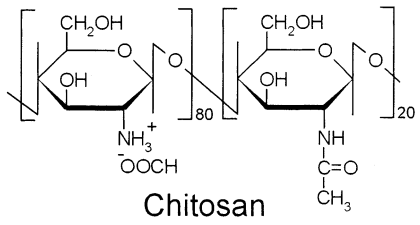
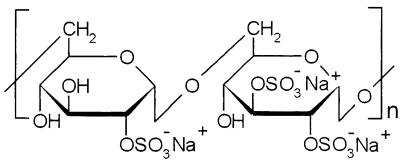
Layer-by-layer (LbL) assembly[1] permits the formation of ultrathin polymer films with a molecularly layered nanostructure and a nanometer-order thickness on surfaces. This process is based on the sequential deposition of interactive polymers from their solutions by electrostatic,[1] van der Waals,[2] hydrogen bonding,[3] and charge transfer[4] interactions. LbL assembly is attractive for applications in biomedical fields since it facilitates the creation of uniquely functional material surfaces. Hubbell et al., for example, utilized LbL assembly to produce bioinert surfaces.[5] Alternating bioactivity, derived from the outermost surface of the LbL assembly, has not, however, been previously demonstrated, although alternate anti- vs pro-coagulant activity of human whole blood on a LbL assembly between chitosan and dextran sulfate (Dex) has been achieved.[6] This alternating activity was produced when the polymers were assembled from an aqueous solution containing 1 M NaCl. Since the addition of salt to the LbL assembly process facilitated formation of a thicker film, the intact characteristics of each polymer were apparently maintained. Accordingly, further alternation of bioactivity of LbL assemblies prepared under suitable conditions is expected. Deconstruction of LbL assemblies is attractive not only for analyzing stability of the assemblies but also for technological and biomedical applications of the assemblies. Disappearance of hydrogen bonds by changing pHs[7] and of electrostatic interactions in high salt environments[7c,8] in LbL assemblies has deconstructed LbL assemblies. The biodegradability of ultrathin polymer films coated on material surfaces is one of the most important requirements for biomedical applications of these polymers. In the present study, we demonstrate the alternating enzymatic hydrolysis of an LbL assembly formed from chitosan and Dex. Chitosanase, an enzyme which hydrolyzes chitosan, was applied in this process. The chemical structures of the polymers are shown in Scheme 1.

**Scheme 1**




Chitosan




Dextran sulfate (Dex)

To realize alternating enzymatic hydrolysis, the LbL assembly between chitosan ($M_w$ 1 200 000) (Wako, Japan) with 20% chitin units and Dex ($M_w$ 500 000) (Wako, Japan) was carried out in aqueous solutions of 25% HCOOH (pH of solution = 1) and water (pH of solution = 6), respectively, containing 1 M NaCl at ambient temperature, following procedures outlined in our previous study.[6] To prepare the chitosan and Dex surfaces, the five- or six-step assembly, which was initiated with chitosan, was utilized. Although the assembly started with chitosan, the starting polymer did not affect the following alternating bioactivity. A quartz crystal microbalance (QCM) with a silver electrode was used as the main substrate for quantitative analysis of the resulting LbL assembly and its subsequent enzymatic hydrolysis. It is well-known that a QCM can be used to monitor the assembly amount ($\Delta m$) from its frequency shift ($\Delta F$). This relationship for a 9 MHz QCM with an electrode diameter of 4.5 mm can be expressed as follows according to Sauerbrey's equation[9] when $\Delta F$ is measured in the air: $-\Delta F/\text{ Hz} = 1.15\Delta m/\text{ng}$. For quantification of the reductive end for the hydrolyzed chitosan, a polystyrene dish with a diameter of 8.5 cm, and with a surface area much larger than that of the QCM, was used as substrate. After the enzymatic reaction, the Schales reagent[10] was reacted with the chitosan (as well as with Dex that was not hydrolyzed by chitosanase) in supernatant of reaction systems for 15 min at 100 °C. Hydrolysis was followed by a decrease in absorption at 420 nm.

Figure 1 shows the hydrolysis of an LbL assembly with chitosan and Dex surfaces by chitosanase ($M_w$ 30 000, 825 units $g^{-1}$). The mean thickness for the five-step (chitosan surface) and six-step (Dex surface) assembly, the density of which was assumed to be 1.2 g $cm^{-3}$,[6] was 22 ± 3 and 53 ± 8 nm, respectively. The assembly with the Dex surface was degraded with increased time, possibly due to the formation of the water-soluble polyion complex between chitosan hydrolyzed and Dex with smaller and larger molecular weights, respectively.[11] Furthermore, since the p$K_a$ of chitosan units was estimated to be 6.0,[12] the chitosan degraded may readily desorb from the film surface in the reaction solution (pH 5.6) due to relaxation of electrostatic interactions. The hydrolysis of the assembly was clearly dependent on the surface component. The hydrolysis of the assembly with the Dex surface was saturated within 10 min and was much faster than hydrolysis of the assembly with the chitosan surface, although chitosanase can hydrolyze chitosan. This unique hydrolysis may come from the electrostatic condensation of chitosanase on the outermost surface of Dex. Since the isoelectric point (pI) of the chitosanase used is 9.3, the total charge on the enzyme surface should be cationic in the present acetic acid buffer (pH 5.6). Accordingly, chitosanase seemed to be condensed on the Dex surface due to electrostatic interactions between Dex and the cationic chitosanase, leading to subsequent hydrolysis of chitosan in the underlayer of the assembly. Another possibility is that the condensed chitosanase might more readily hydrolyze chitosan due

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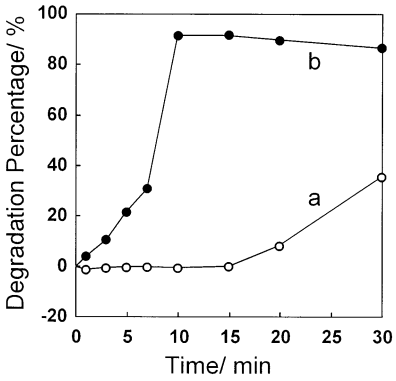


**Figure 1.** Enzymatic hydrolysis of the LbL assembly in 100 mM acetic acid buffer (pH 5.6) containing chitosanase (0.5 mg $mL^{-1}$) at 40 °C analyzed by QCM: (a) the 5-step [(chitosan-Dex)$_2$-chitosan] (chitosan surface, total thickness: 22 nm) and (b) the 6-step [(chitosan-Dex)$_3$] (Dex surface, 55 nm) assemblies. The LbL assembly was demonstrated from aqueous chitosan solution (1 mg $mL^{-1}$) containing 25% formic acid and aqueous Dex solution (1 mg $mL^{-1}$) for each immersion time of 10 min at ambient temperature in the present study. The frequency was analyzed at each step by washing a QCM with ultrapure water and drying with nitrogen gas.

to relaxation of the electrostatic repulsion between chitosanase and chitosan, the cationic charge of which has already been used for the polyion complex with Dex. On the other hand, after the hydrolysis of the assembly with the Dex surface, 260 ng of the assembly, corresponding to a mean thickness of around 7 nm, apparently remained on the substrate. This small amount of the assembly seemed to be difficult to hydrolyze or desorb due to the direct influence of the silver QCM substrate. Note that the degradation of the five-step assembly with the chitosan surface saturated to 40% after 40 min, and then approximately 435 ng (500 Hz) of the polymers remained (Figure 1a).

To confirm the above results to represent specific hydrolysis of the LbL assembly by chitosanase, similar experiments were performed using a nonspecific protein trypsinogen ($M_w$ 24 000), which has an isoelectric point (pI 9.3) similar to that of chitosanase (data not shown). Desorption of the assembly from the substrate was not observed in the presence of trypsinogen, and only adsorption onto the LbL assembly was apparent. In fact, the amount adsorbed onto the assembly with the Dex surface was much larger than that adsorbed onto the assembly with the chitosan surface, possibly due to electrostatic interactions between trypsinogen and Dex. These observations suggest that the desorption of LbL assemblies incubated in chitosanase solution is derived from the specific hydrolysis of chitosan in the assembly.

The hydrolysis process could be separated into two steps by controlling the temperature of the chitosanase solution: (1) the adsorption of chitosanase at low temperature; (2) the hydrolysis by chitosanase preserved on the assembly surface in buffer solution (without any enzyme in bulk phase) at a temperature suitable for enzyme activity, as shown in Figure 2. When the assembly was immersed in the chitosanase solution at 4 °C, at which the enzyme was inactive, the enzyme was adsorbed onto both surfaces. Subsequently, the chitosanase-adsorbed assembly was immersed into acetic acid buffer at 40 °C, at which the chitosanase was active. The chitosanase adsorbed onto the chitosan surface was slowly desorbed possibly by electrostatic repulsion without any hydrolysis (Figure 2a). On the other hand, the assembly with the Dex surface was rapidly hydrolyzed (Figure 2b). This hydrolysis was faster than the simultaneous adsorption and hydrolysis process shown in Figure 1b. In fact, the percentage degraded after 5 min was around 80%, which was much greater than desorption in the latter (around 20%). Sufficient chitosanase had remained on the LbL assembly in the former, resulting in faster hydrolysis. Furthermore, the hydrolysis of the assembly with the Dex surface significantly proceeded by the condensed chitosanase, even though the film thickness was changed from 62 to 209 nm (Figure 2c). The present two-step process suggests that the LbL assembly may be potentially useful as a protein-preserving device, which release functional molecules from assemblies or substrates, responsive to specific external stimuli.

The QCM analysis only showed desorption of the polymers. Hydrolysis was also analyzed by the relative analysis of the amount of the reductive end in the polymers in a supernatant following hydrolysis, as shown in Table 1. Since chitosanase also reacted with Schales reagent, the hydrolysis was performed according to the method outlined in Figure 2 to minimize the influence of the enzyme. Under experimental conditions, Schales reagent did not detect the enzyme. The reductive end from the assembly with the Dex surface was clearly greater than that with the chitosan surface, indicating that a greater amount of chitosan was

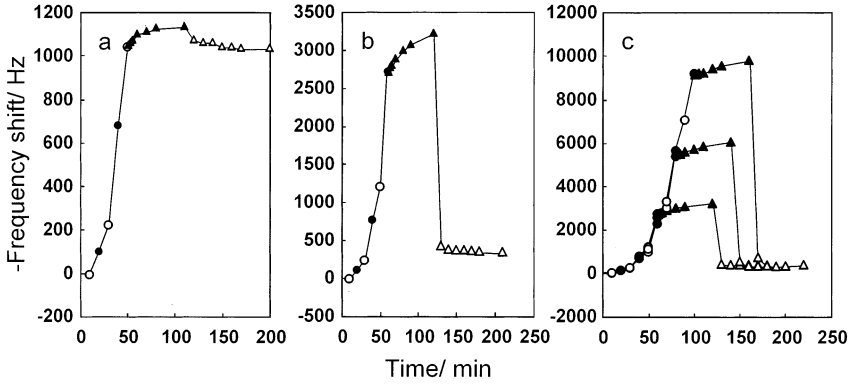


**Figure 2.** Enzymatic hydrolysis of (a) the 5-step (total thickness: 24 nm), (b) the 6-step (62 nm), and (c) the 6, 8, and 10-step (62, 122, and 209 nm, respectively) assemblies, when they were immersed in 100 mM acetic acid buffer (pH 5.6) containing chitosanase (0.5 mg $mL^{-1}$) at 4 °C (closed triangles) and subsequently in acetic acid buffer at 40 °C (open triangles). Open and closed circles indicate the immersion steps in chitosan and Dex solutions, respectively. The frequency was analyzed at each step by washing a QCM with ultrapure water and drying with nitrogen gas.

**Table 1. Quantification of the Reductive Ends in Polysaccharides by the Schales Reagent**

| surface | relative activity[a] |
|---|---|
| chitosan (5 steps) | 1 |
| dex (6 steps) | 7.8 ± 0.7 |
| (peeling off)[b] | 2.5 ± 0.1 |

[a] Reaction time was 10 min. [b] The 6-step assembly (Dex surface) was peeled off from the substrate in aqueous 0.01 M NaOH solution. The complete peeling of the polymers has been already confirmed by QCM.

hydrolyzed in the former assembly. In addition, the amount of the reductive end was greater than that of the assembly peeled off from the substrate, also indicating the effective hydrolysis of chitosan. The amount assembled on the polystyrene substrate seemed to be smaller than that assembled on the QCM, based on the QCM analysis and the results of the peeling off. Unfortunately, we did not estimate the molecular weight of the chitosan hydrolyzed. Direct analysis of the molecular weight of the hydrolyzed chitosan using suitable apparatus is now in progress.

In conclusion, the specific enzymatic hydrolysis of an LbL assembly prepared from chitosan and dextran sulfate was quantitatively analyzed using a QCM. It was found that the LbL assembly with the outermost surface with a charge opposite to the net charge of the enzyme was preferentially hydrolyzed compared to a surface with the same charge as the enzyme, possibly due to the electrostatic condensation of the enzyme, even when the polymer substrate lies under the surface layer of the LbL assembly. In other words, when an enzyme shows electrostatic repulsion against its polymer substrate, construction of an LbL assembly will facilitate rapid hydrolysis of the polymer. Since various combinations of polyelectrolytes and enzyme are possible in the LbL assembly, the present approach should have significant applications in the biomedical field. The biological activity, including enzymatic hydrolysis of the LbL assembly, can potentially be controlled by the addition of salt into the constructing polymer solutions. We are now preparing LbL assemblies on hydrogel surfaces to perform controlled release of functional molecules from the hydrogels, triggered by the hydrolysis of the LbL assembly.

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