# Synthesis of Polymer Particles with Specific Lysozyme Recognition Sites by a Molecular Imprinting Technique

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**ABSTRACT:** To prepare silica beads covered with a lysozyme-imprinted polymer layer, we polymerized acrylamide and acrylic acid or acrylamide and *N*,*N*-dimethylaminopropylacrylamide with $(NH_4)_2S_2O_8$ in a phosphate buffer containing the lysozyme, surface-modified silica beads, and crosslinkers; the result was the formation of a polymer layer with a lysozyme recognition site on the silica-bead surface. By quantitative analysis of the supernatant of the solution containing the silica beads, we confirmed that modified silica beads, in contrast to unmodified silica beads, can selectively adsorb lysozymes. The process of binding and releasing the lysozyme to and from the modified silica beads can be repeated several times without degradation of the rebinding ability. A quartz-crystal microbalance sensor fabricated with a molecularly imprinted polymer layer with a lysozyme recognition site was prepared. When a lysozyme aqueous solution was added to the solution in which the sensor was immersed, a high level of sensitivity and response was observed. High selectivity was also demonstrated by tests with other protein solutions. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 81: 3378–3387, 2001

**Key words:** molecular imprinting; molecular recognition; lysozyme; hydrogel; quartz-crystal microbalance sensor

## INTRODUCTION

The molecular imprinting method is a useful technique for preparing host compounds for molecular recognition. The principle of this technique is schematically illustrated in Figure 1. Various kinds of host compounds have been reported to have recognition properties specific to imprinted template molecules.[1–5] However, almost all the template molecules used in these past studies have been low-molecular-weight compounds and have been insoluble in water. We have also reported that an acrylic acid (AAc)/acrylamide (AAm) polymer layer prepared by graft polymerization on the surface of silica beads exhibits antibody-like binding characteristics for glucose oxidase (Enzyme Code (EC) 1.1.3.4).[6]

In this article, we report the synthesis of a polymer layer with specific lysozyme recognition sites [EC 3.2.1.17, weight-average molecular weight ($M_w$) = 14,314]. Lysozyme is known as an enzyme that prevents food degeneration and has anti-inflammatory activity inside the body. Polymer particles with specific lysozyme recognition sites can be used for the process control of lysozyme extraction from egg whites. These particles are also of use for the diagnosis of tuberculosis, leukemia, and hepatitis, indicated by an increase in lysozyme concentration in the urine or blood, or for diagnosis of diabetes or lead poison-

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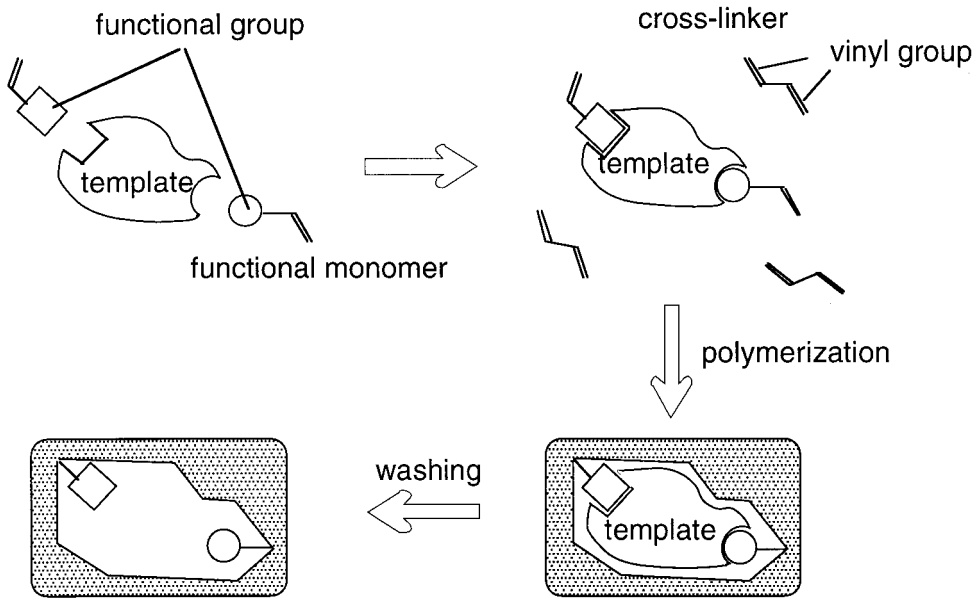



**Figure 1** Principles of the molecular imprinting technique.

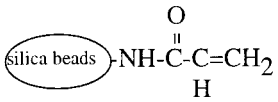
ing, indicated by a decrease in lysozyme concentration.

## EXPERIMENTAL

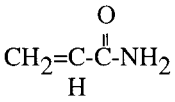
### Synthesis of a Polymer Layer on Silica Beads

The preparation method for a polymer layer on the surface of silica beads has been described elsewhere.[6] Silica beads with amino groups on their surface (Lichroprep $NH_2$, Merck, Darmstadt, Germany; particle size = 0.025–0.04 mm) were allowed to react with acryloyl chloride, and vinyl groups were then introduced onto the silica-bead surface. Two types of monomer combinations were used. One was AAm (Wako Pure Chemical Industries, Ltd., Doshomachi, Osaka, Japan) and AAc (Wako Pure Chemical Industries,

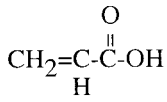
surface-activated silica beads



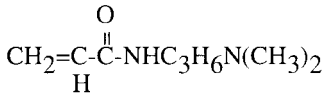

acrylamide




acrylic acid

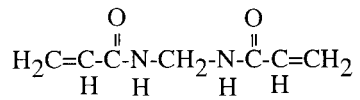



N, N-dimethylaminopropylacrylamide




cross-linker

N, N'-Methylene-bis(acrylamide)




N, N'-(1,2-Dihydroxyethylene)bisacrylamide

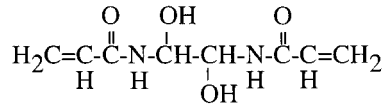



**Figure 2** Structural formulas.

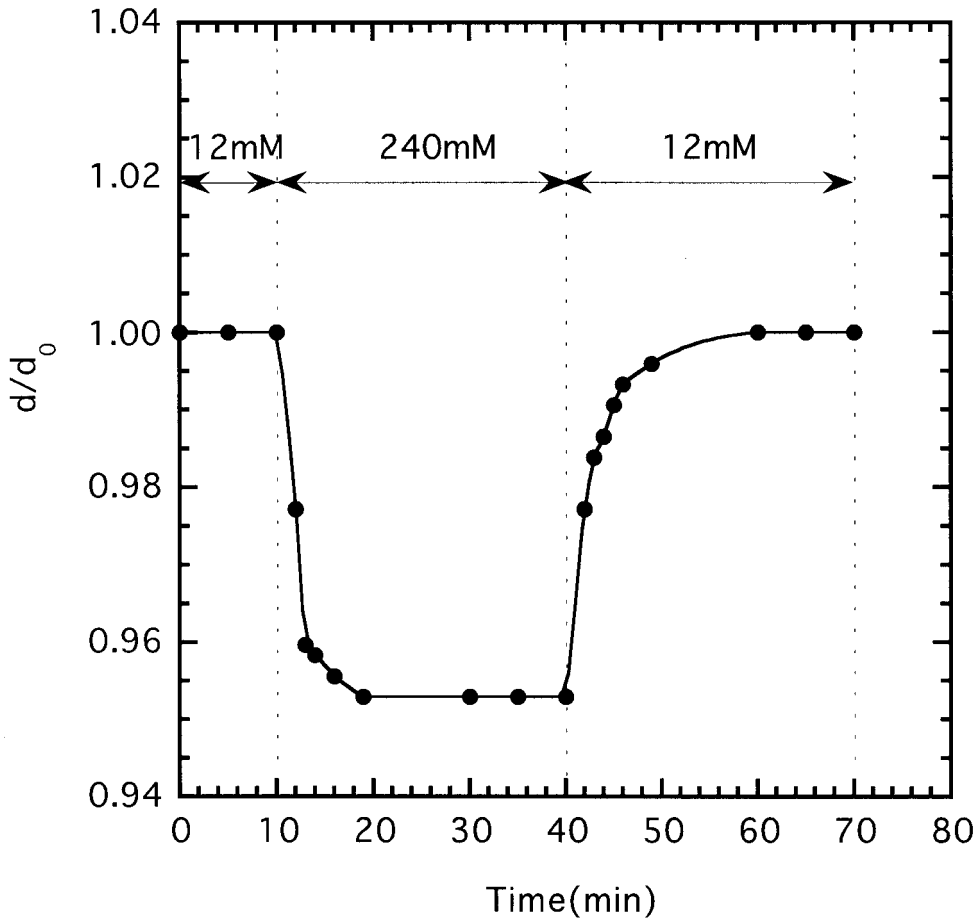



**Figure 3** Relative change in diameter of the hydrogel in response to changes in the ion strength.

Ltd.), and the other was AAm and *N*,*N*-dimethylaminopropylacrylamide (DMAPAAm; Kojin Co., Nihonbashi, Tokyo, Japan) Their chemical structures are shown in Figure 2. Two kinds of crosslinkers, *N*, *N*′-methylene-bisacrylamide (130 mg) and *N*,*N*′-(1,2-dihydroxyethylene)-bisacrylamide (120 mg), were mixed with the monomer mixture in 10 mL of a phosphate buffer solution (12 m*M*, pH 5.0). One gram of surface-activated silica beads and 15.0 mg of lysozyme were added to 2.5 mL of the phosphate buffer solution containing the monomer mixture and the crosslinkers. The polymerization was initiated by the addition of 0.18 mL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine and 0.225 mL of ammonium persulfate (40%). A polymer pellet containing the silica beads was ground and filtered through a sieve (mesh size = 0.150 mm). The nonimprinted reference polymer particles without the addition of template molecules were synthesized by the same method and with the same monomer compositions as in the case of the imprinted particles.

To obtain particles for the rebinding experiments, we had to remove the template molecules (lysozyme). We achieved this by rinsing the imprinted particles five times with a 240 m*M* phosphate buffer. The removal of lysozyme from the imprinted particle was confirmed by the quantitative measurement of the ultraviolet absorption of the phosphate buffer solution used for washing the lysozyme from the particles.

### Rebinding Test

The rebinding experiments that used lysozyme ($M_w$ = 14,314) or hemoglobin ($M_w$ = 15,126), both with almost the same molecular weight and isoelectric point (pH 7.0), were carried out under the same pH conditions (pH 5.0) as those under which the polymer-covered particles were prepared. After incubation, the particles were centrifuged, and the supernatant was used to measure the molecular weight distribution by gel filtration chromatography. The chromatography was car-

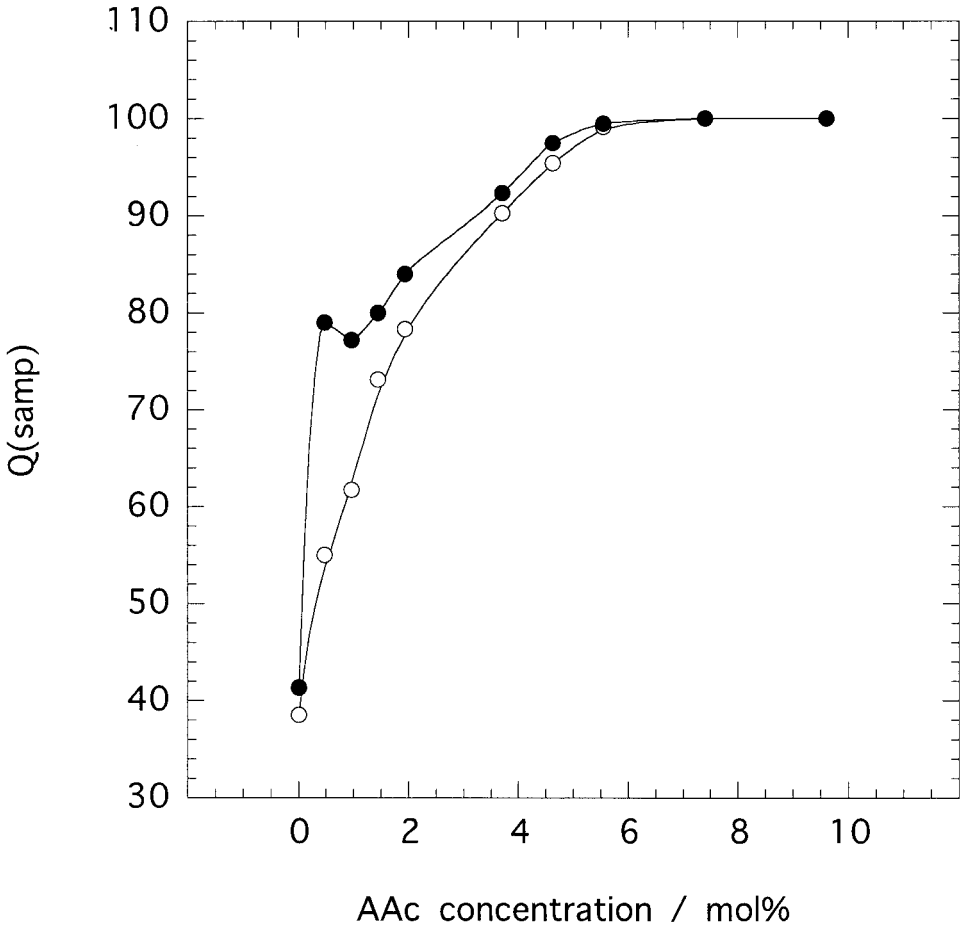



**Figure 4** Amount of protein adsorbed onto the lysozyme-imprinted polymer particles prepared with AAm and AAc: (●) lysozyme and (○) hemoglobin.

ried out with TSK gel 3000 $SW_{XL}$, Toso Co., Minatoku, Tokyo, Japan, at a flow rate of 1 mL/min with a 10 m*M* phosphate buffer solvent containing 0.2*M* NaCl at room temperature. The detection wavelength was 280 nm.

### ζ Potentials of Lysozyme and Particles Covered with a Polymer Layer

The ζ potentials (electrokinetic potential) of the lysozyme and the polymer-covered particles were measured with an electrophoretic light scattering spectrophotometer (Otuka Electronics, Hirakata, Osaka, Japan). The lysozyme and polymer-covered particles were dispersed in a 12 m*M* phosphate buffer solution by ultrasonic irradiation for 2 min. The electric mobility was measured for the supernatant in an electric field of −39 V/cm with a measurement angle of 20°. The ζ potential was calculated from the obtained electric mobility with this equation from Smoluchowski:[7]

$$U = \varepsilon\zeta/4\pi\eta \quad (1)$$

where $U$ is the electric mobility ($\mu$m cm $s^{-1}$ $V^{-1}$) and $\epsilon$ and $\eta$ are the permittivity and viscosity (in poise) of the solution, respectively.

### Preparation of the Sensor Device and the Measuring Method

A polymer layer with specific recognition sites for the lysozyme was also prepared on a quartz-crystal oscillator (HOKUTO DENKO Co., Meguro, Tokyo, Japan; fundamental frequency = 6 MHz; AT-cut) with the same procedure as that used for forming the polymer layer around the silica beads. We evaluated the sensitivity of the sensor to the lysozyme by measuring the frequency change with a universal counter (Advantest TR-5822, Shinjuku, Tokyo, Japan). In addition, the polymer layer, which did not have specific recognition sites for the lysozyme, was produced on a

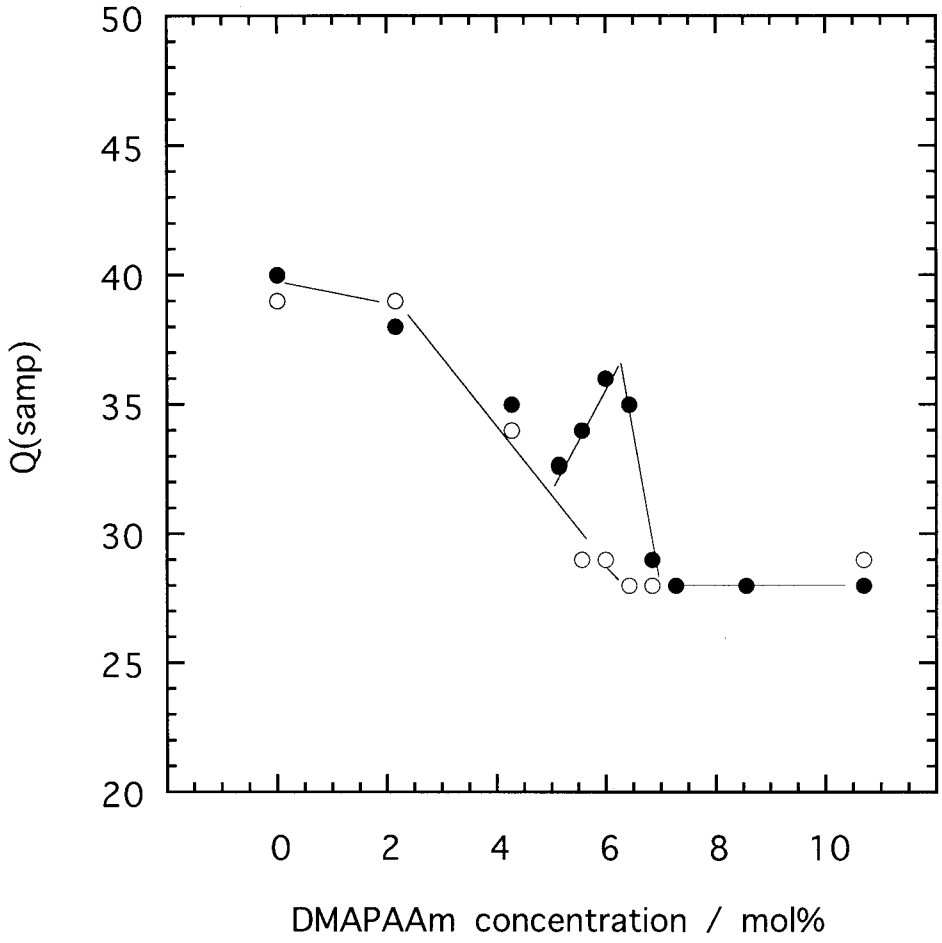



**Figure 5** Amount of protein adsorbed onto the lysozyme-imprinted polymer particles prepared with AAm and DMAPAAm: (●) lysozyme and (○) hemoglobin.

quartz-crystal oscillator as a reference and was used in the same procedure.

### Volume Change of the Polymer Layer Caused by the Difference in Ion Strength

The polymerization of the same solution as that used in the formation of the polymer layer with specific recognition sites for the lysozyme was carried out inside a capillary. The obtained hydrogel was used for the volume-change measurement. The volume change of the hydrogel caused by the difference in ion strength was measured as the change in diameter of the hydrogel in solutions of different ion strengths, and the degree of swelling was evaluated by the following equation:

$$\text{Swelling ratio} = d/d_0 \qquad (2)$$

where $d$ is the diameter of the hydrogel in a given solution and $d_0$ is the diameter of the hydrogel in the 12 m$M$ phosphate buffer solution.

## RESULTS AND DISCUSSION

### Volume Change of the Polymer Layer Caused by the Change in Ion Strength

It was suggested by Flory[8] that a hydrogel generally absorbs water according to the swelling equation. According to this equation, the amount of water absorption is determined by the balance of the osmotic pressure of ions, the affinity between the polymer and water, and the rubbery elasticity. Therefore, the volume change in the hydrogel is very dependent on the ion strength of the solution.

To remove the template molecules from the polymer layer, we rinsed the polymer-covered particles by changing the ion strength of the phosphate buffer. The polymer layer with specific recognition sites was in the form of a hydrogel that was harder than those usually used in electrophoresis. The volume change of the hydrogel in response to changes in the ion

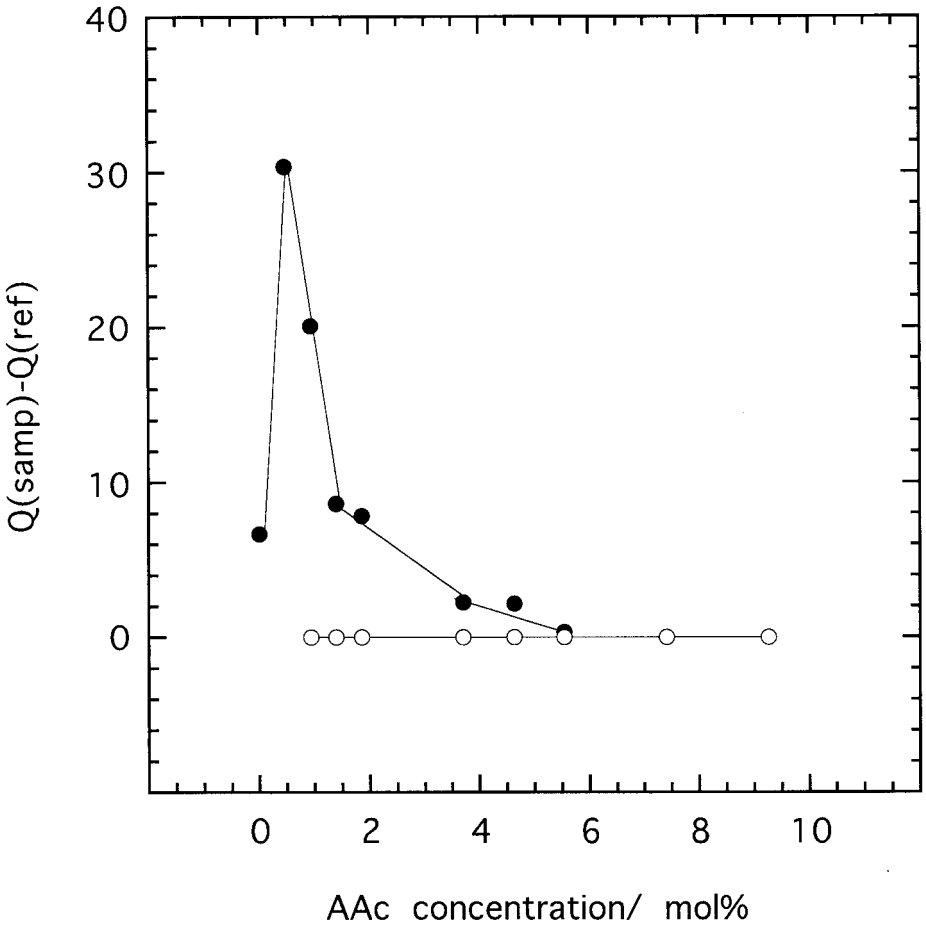



**Figure 6** Amount of specifically bound lysozyme or hemoglobin on lysozyme-imprinted polymer particles versus AAc concentrations: (●) lysozyme and (○) hemoglobin.

strength was then measured as the change in diameter of the hydrogel. The transient curve of the relative change in diameter with ion strength is shown in Figure 3. The hydrogel contracted when the solution surrounding the gel was changed from 240 m*M* phosphate buffer to 12 m*M* phosphate buffer, and it became stable in approximately 20 min. Furthermore, the diameter of the hydrogel regained its original value within 20 min when the concentration of the solution was reversed from 240 to 12 m*M*.

It was, therefore, confirmed that no influence remains when the ion strength is changed to remove the template molecule from the polymer layer as long as the ion strength is reversed to its original value.

### Recognition of Lysozymes

The lysozyme-imprinted particles were added to a solution in which a certain amount of lysozyme was dissolved. We calculated the amount of lysozyme adsorbed onto the lysozyme-imprinted polymer particles [*Q*(samp)] by subtracting the quantity of lysozyme in the supernatant from that in the original solution. To confirm the selectivity of the imprinted polymer layer, we dissolved other proteins such as hemoglobin, human albumin, or glucose oxidase in the solutions, and the same experiments as those for the lysozyme were carried out. The results with hemoglobin are plotted in Figures 4 and 5. The amount of lysozyme adsorbed onto the lysozyme-imprinted polymer layer on the particles depended on the monomer (AAc or DMAPAAm) compositions during preparation of the polymer layer on the particles. However, although hemoglobin was adsorbed on the lysozyme-imprinted polymer particles to some extent, the adsorbed amounts of hemoglobin did not appear to depend on the monomer compositions, suggesting that some nonspecific binding of the lysozyme or hemoglobin to the lysozyme-imprinted polymer particles also occurred.

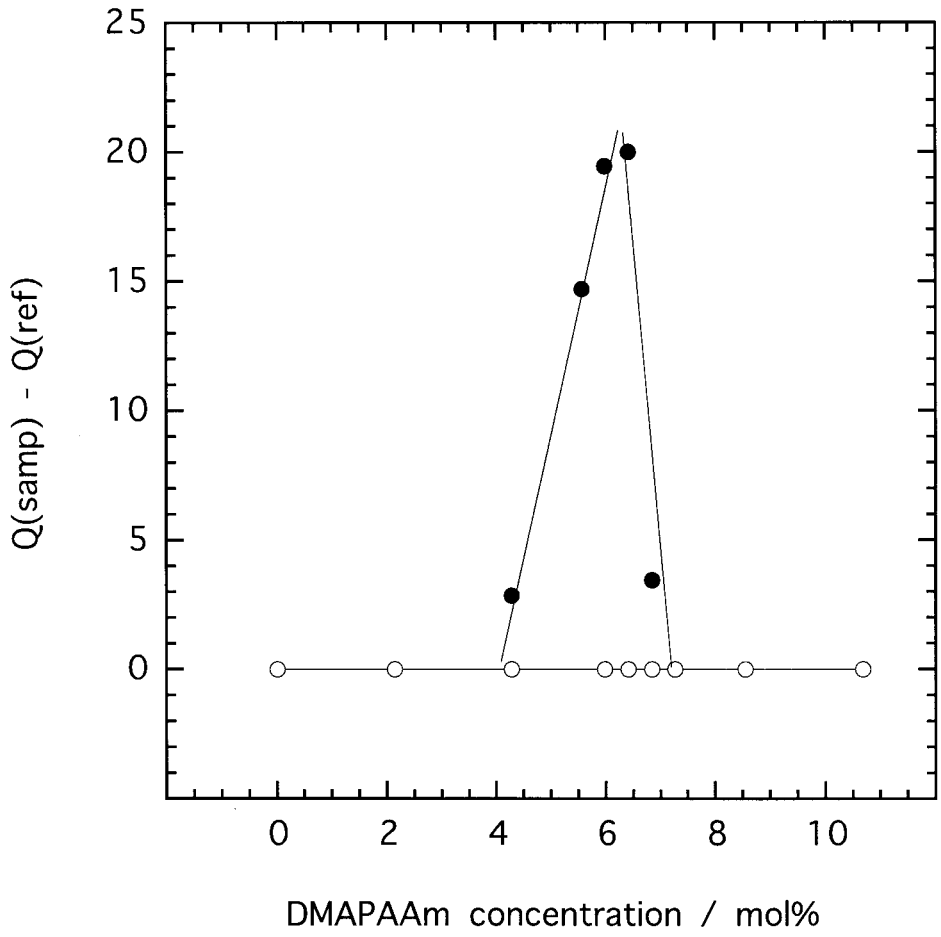



**Figure 7** Amount of specifically bound lysozyme or hemoglobin on lysozyme-imprinted polymer particles versus DMAPAAm concentrations: (●) lysozyme and (○) hemoglobin.

### Specific Binding Rate

To elucidate the effects of specific binding of the lysozyme to the lysozyme-imprinted polymer particles, we also measured the adsorption of the lysozyme or hemoglobin onto the nonimprinted polymer particles. By subtracting the amount of lysozyme adsorbed onto the nonimprinted reference polymer particles [$Q$(ref)] from that adsorbed onto the lysozyme-imprinted polymer particles [$Q$(samp)], we calculated the amount of specifically bound lysozymes. The results are shown in Figures 6 and 7. The amount of specifically bound lysozyme was the highest on the lysozyme-imprinted polymer particles prepared with 0.46 mol % AAc monomer and 5.98 mol % DMAPAAm. No hemoglobin was found to be specifically bound to the polymer particles. The results for the polymer particles prepared with approximately 0.46 mol % AAc and 5.98 mol % DMAPAAm monomer clearly showed the effects of imprinting. In the simplified model, the specified binding of lysozyme to the imprinted polymer particle is based on an electrostatic interaction; that is, the basic groups in the lysozyme molecule interact with the $COO^-$ group of AAm, and the acidic groups in the lysozyme molecule interact with the amino group of DMAPAAm. For rebinding the lysozyme at the imprinted sites in the polymer layer, the distance between the $COO^-$ group of AAm or that between the amino group of DMAAm is an important factor. Figures 6 and 7 indicate that the copolymers containing 0.46 mol % AAc and 5.98 mol % DMAPAAm had the most appropriate distance between $COO^-$ groups or amino groups. However, the amount of lysozyme specifically bound on the polymer particles composed of AAm and DMAPAAm was less than that on those composed of AAm and AAc. The difference in the amount of

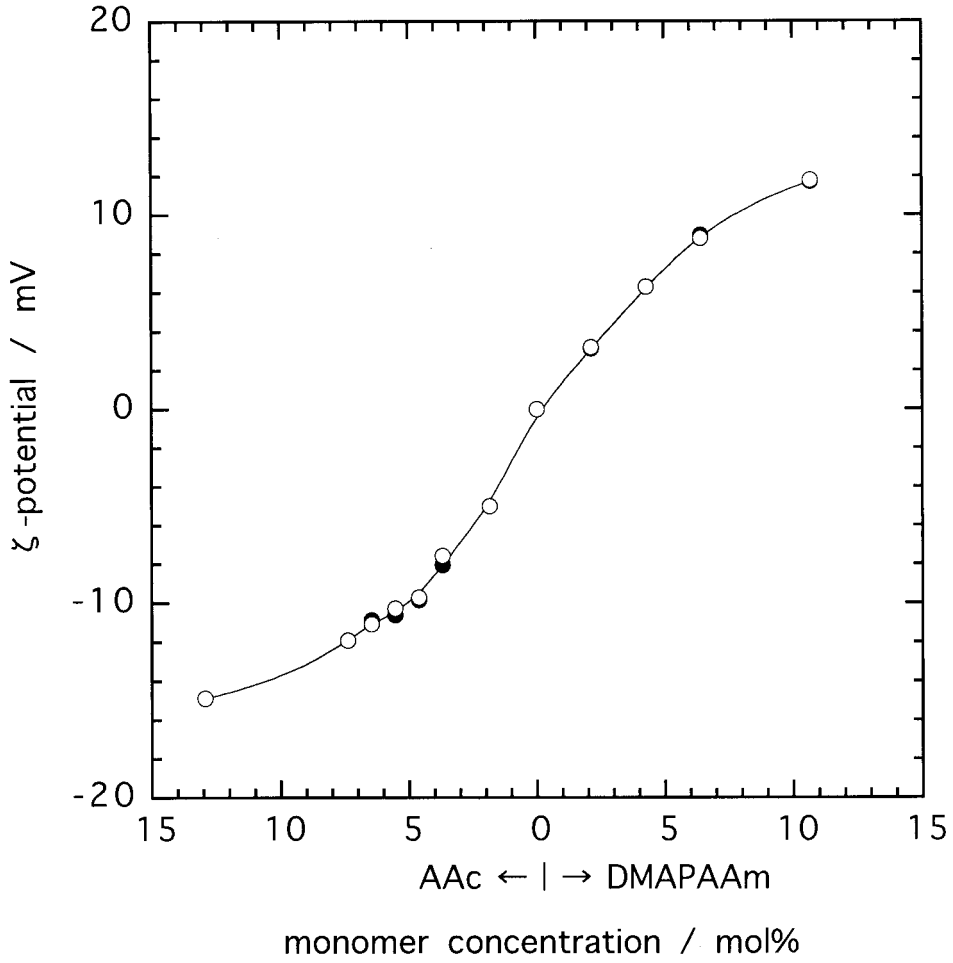



**Figure 8** $\zeta$ potential of polymer particles: (●) lysozyme-imprinted and (○) nonimprinted.

bound lysozyme between the copolymer composed of AAm and DMAPAAm and the copolymer composed of AAm and AAc was caused by the differences in charges between these two copolymers. The lysozyme had a weakly positive charge, and the AAm and DMAPAAm copolymers were also positively charged, whereas the AAm and AAc copolymers had negative charges. This difference in the sign of the charges on the surface polymer layer was likely the cause of the difference in the amounts of bound lysozyme.

### $\zeta$ Potential of the Particles and Recognition Molecules

Figure 8 shows the $\zeta$ potential of the particles with various monomer compositions in a phosphate buffer at pH 5.0. Figure 9 shows the pH dependence of the $\zeta$ potential of the lysozyme measured with an electrophoretic light-scattering spectrophotometer (Otuka Electronics). The lysozyme surface should be positively charged at pH 5.0 because its isoelectric point is pH 8.0. The observed $\zeta$ potential of the lysozyme was 8 mV. The amount of specifically bound lysozyme reached its maximum value at approximately $-4$ mV of the $\zeta$ potential for the particle composed of AAm plus AAc and at 9 mV for the particle composed of AAm plus DMAPAAm. The $\zeta$-potential value is very close to that of the lysozyme. A weak ionic interaction between the lysozyme and the particles is necessary for the lysozyme to specifically bind with the lysozyme-imprinted sites.

In the molecular design of the imprinted polymers, these results suggest that the coincidence of the $\zeta$ potential between the template molecule and that of its imprinted polymer particles is important.

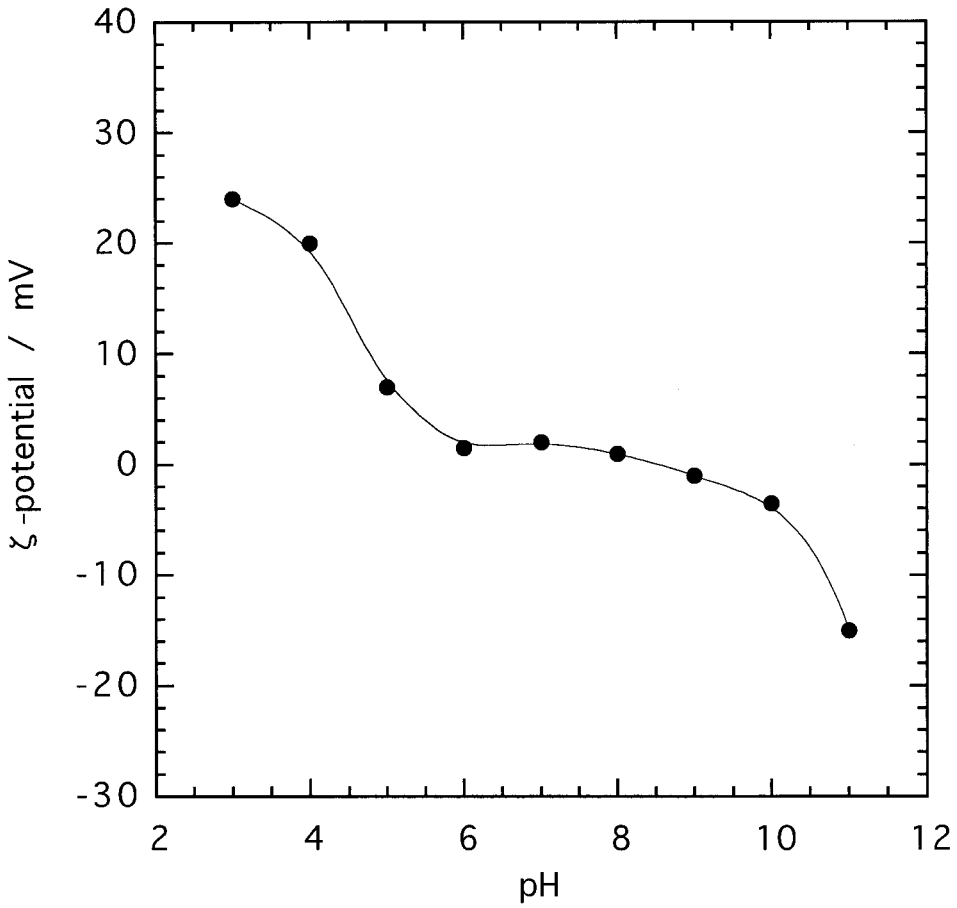



**Figure 9** Relation between $\zeta$ potential and pH for lysozyme.

### Detection of Lysozymes by a Quartz-Crystal Microbalance (QCM) Sensor

The molecularly imprinted polymer layer can be expected to be used as a sensing layer. Recently, Dickert et al.[9] reported a molecularly imprinted sensor in which imprinted polyurethanes were used for the detection of polycyclic aromatic hydrocarbons. Their detection limit was in the parts-per-thousand range. In this work, we also prepared a QCM sensor with a polymer layer that had a recognition site for the lysozyme.

This QCM method has been used by several workers to investigate the quantity of substances adsorbed on the coating material.[10–12] The frequency of the oscillating quartz crystal varies with the change in the mass according to the following equation:[13]

$$-\Delta F = k\Delta W \tag{3}$$

where $\Delta F$ is the observed frequency change (Hz), $k$ is the proportionality constant, and $\Delta W$ is the weight change on the surface of the crystal (g/cm$^2$).

Two groups of quartz crystals were coated with a polymer layer with a recognition site for the lysozyme ($\Delta F_{\text{samp}}$) and with a nonimprinted polymer layer ($\Delta F_{\text{ref}}$), respectively. The crystals were set in a 12 m*M* phosphate buffer into which a small amount of lysozyme or hemoglobin was added in a stepwise manner. In Figure 10, the difference between $\Delta F_{\text{samp}}$ and $\Delta F_{\text{ref}}$ is plotted against the amount of lysozyme added to the buffer. The result ($\Delta F_{\text{samp}} - \Delta F_{\text{ref}}$) was $-50$ Hz with the addition of 0.08 mg of lysozyme. When a hemoglobin aqueous solution was dropped into the cell, the result ($\Delta F_{\text{samp}} - \Delta F_{\text{ref}}$) of the crystals was zero. These results suggest that the influence of coexisting proteins can be excluded by the simultaneous use of a crystal oscillator with a nonimprinted polymer layer.

## CONCLUSIONS

The lysozyme-imprinted polymer layer on the surface of silica beads composed of AAm and AAc or AAm and

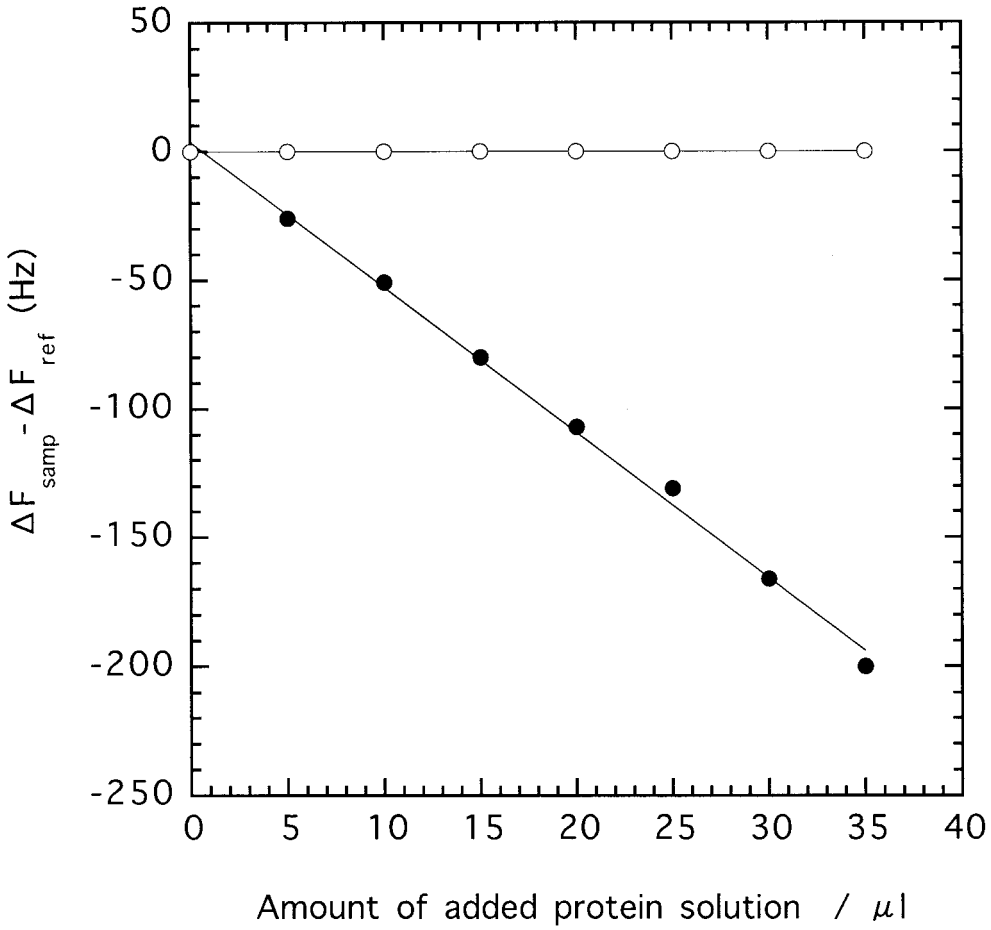



**Figure 10** Frequency change in response to the addition of a protein solution (concentration = 8 mg/mL): (●) lysozyme solution and (○) hemoglobin solution.

DMAPAAm and crosslinkers selectively responded to lysozymes. The polymer layer with the highest lysozyme-specific binding rate had nearly the same $\zeta$ potential as the lysozyme. It was confirmed that a QCM sensor fabricated with polymeric films prepared by the molecular imprinting technique could detect large molecules such as enzymes.

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