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Molecularly imprinted protein recognition thin films constructed by controlled/living radical polymerization

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We demonstrated the synthesis of molecularly imprinted polymers (MIPs) with binding affinity toward a target protein, ribonuclease A (RNase) by atom transfer radical polymerization (ATRP) of acrylic acid, acrylamide, and *N*,*N*′-methylenebisacrylamide in the presence of RNase. The binding activity of the MIPs was evaluated by surface plasmon resonance (SPR) of the MIP thin layers prepared on the gold-coated sensor chips. The MIPs prepared by ATRP (MIP-ATRP) had a binding affinity toward RNase with larger binding amount compared to MIPs prepared by conventional free radical polymerization methods (MIP-RP). Moreover, protein selectivity was evaluated using reference proteins (cytochrome *c*, myoglobin, and α-lactalbumin) and was confirmed in MIP-ATRP of optimum film thickness determined experimentally to be 15–30 nm; however, protein selectivity was not achieved in all MIP-RP. We have shown that ATRP is powerful technique for preparing protein recognition materials by molecular imprinting.

[**Key words:** Molecular imprinting; Controlled/living radical polymerization; Proteins; Surface plasmon resonance; Molecular recognition; Quartz crystal microbalance]

Molecular recognition materials for proteins are of great importance in the field of life science, especially for use in protein separation, proteomics and diagnosis of cancers and other intractable diseases (1). Currently, biomolecules such as enzymes, antibodies, and aptamers are used as molecular recognition materials for proteins. Although highly selective binding activity can be achieved, such biomolecules are difficult to control in production as well as being unstable in harsh conditions and easily denatured. Polymer-based artificial molecular receptors serve as a more stable and versatile alternative for application to explore protein-ligand interaction in biological systems.

Molecular imprinting, a template polymerization technique, is a powerful method for preparing artificial receptors bearing predetermined binding cavities capable of recognizing target molecules. Molecularly imprinted polymers (MIPs) are obtained by the co-polymerization of functional monomers and crosslinkers in the presence of a target molecule or its derivative (template molecule) (2–7). When proteins are selected as the template molecules for MIPs, the complementary binding cavities left in the polymer matrices after removal of the templates are capable of selective protein binding according to size and chemical properties, depending upon the functional monomers used (6). In other words, the conformation of complexes formed between the template proteins and functional monomer(s) during the polymerization step of preparing MIPs is critical for the development of highly selective binding activity with strong affinity for the target protein. Previously, conventional free radical polymerization (RP) was employed for the preparation of MIPs for protein recognition; however, this method had several problems i.e., formation of heterogeneous cross-linking matrix, difficulty of the film thickness control (8,9).

Controlled/living radical polymerization (CLRP) is a powerful technique for the synthesis of well-defined polymers with narrow molecular weight distribution or complex molecular architectures such as block or star polymers (10–13). Many kinds of CLRP techniques have been discovered and developed, but atom transfer radical polymerization (ATRP) is one of most studied and highly developed of these techniques, involving radical species that are reversibly generated by the redox reaction of copper ions. It is known that CLRP can be used to synthesize more homogeneous cross-linking materials compared to those synthesized via RP (8,14). Accordingly, CLRP was applied to synthesize MIPs in order to improve the uniformity of molecular recognition sites as well as recognition ability.

To date, several methods for the preparation of MIPs by CLRP have been reported. For example, Boonpangrak et al. (15) reported that MIPs prepared by nitroxide-mediated living radical polymerization displayed more highly selective cholesterol binding compared to those prepared by RP. In our previous work (16), we also tried using reverse ATRP with $CuCl_2$ and a thermal initiator in organic solvents to synthesize the MIPs containing molecular recognition site for bisphenol A (BPA), which is believed to be an endocrine disruptor that binds to estrogen receptors. It was discovered that the MIPs obtained by ATRP had high recognition ability toward BPA compared to those obtained by conventional free radical polymerization. Moreover, very recently Salian et al. (17) investigated the role of CLRP in the formation of imprinted polymers in detail as well as elucidated that binding activity was

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clearly increased when using CLRP in place of RP methods. MIPs for protein recognition were also prepared by CLRP. Gai and coworkers (18) prepared protein-imprinted MIP by ATRP for protein separation. However, the effectiveness of the CLRP on the protein recognition ability has not been discussed compared to RP.

In this study, we synthesized MIPs toward ribonuclease A (RNase) by activators generated by electron transfer (AGET) ATRP (19,20) (henceforth referred to as MIP-ATRP) in an aqueous solution. In order to prepare MIPs bearing selective binding cavities, acrylic acid, which is often used as a functional monomer for basic compounds, was introduced into a solution containing RNase to form complexes with the protein molecules via electrostatic interactions and/or hydrogen bonding. After the addition of a cross-linker, *N*,*N*′-methylenebisacrylamide (MBAA), AGET ATRP of these monomers were carried out from 2-bromo-2-methylpropionic acid (ATRP initiator)-immobilized gold surfaces of either a quartz crystal microbalance (QCM) or surface plasmon resonance (SPR) sensor chips. QCM measurements were performed to estimate the thickness of the MIP films, and the binding activity of the MIPs was evaluated by SPR measurements. In order to compare the ATRP-based method with conventional radical polymerization, RP-based MIPs (MIP-RP) were prepared on 2,2′-azobis(2-methylpropionamidine) (V-50)-immobilized substrates, and the comparative effectiveness of AGET ATRP for the formation of selective binding cavities in MIP thin films was discussed.

MATERIALS AND METHODS

Materials RNase, ascorbic acid, $CuBr_2$, dichloromethane (CH_2Cl_2), 4,4′-dimethylaminopyridine (DMAP), tetra-sodium ethylenediamine tetraacetate (EDTA-4Na), sodium chloride (NaCl), sodium hydroxide (NaOH), ethanol, methanol, critic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Nacalai Tesque Co. Ltd. (Kyoto, Japan). Acrylic acid, V-50, MBAA, acryl amide were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *N*,*N*,*N*′,*N*″,*N*‴-Pentamethyldiethylenetriamine (PMDETA), *N*-hydroxy succinimide (NHS), 11-mercapto-1-undecanol, 11-mercapto-1-undecanoic acid, cytochrome *c*, miyoglobin, α-lactalbumin were purchased from Sigma–Aldrich (MO, USA). 1-Ehtyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Peptide Institute Inc. (Osaka, Japan) and 2-bromo-2-methylpropionicacid was purchased from Tokyo Chemical Industry (Tokyo, Japan). CH_2Cl_2 was purified by distillation prior to use in order to remove water. V-50 was used without further purification.

Preparation of MIP thin films by AGET ATRP (MIP-ATRPs) The immobilization of ATRP initiator on QCM chips (QCA-A9M-AU (P); Seiko EG&G Co. Ltd., Tokyo, Japan) were carried out as follows: After the QCM chips were cleaned by a UV–O_3 cleaner (Bioforce nanosciences) for 20 min, the QCM chips were dipped in 11-mercapto-1-undecanol (1.05 mg, 5 μmol) in ethanol (1 mL) for 1 h. The QCM chips were washed by ethanol and dried in vacuo. The QCM chips were then dipped in 2-bromo-2-methylpropionicacid (3.26 mg, 20 μmol), DMAP (3.66 mg, 35 μmol) and EDC (5.66 mg, 35 μmol) in CH_2Cl_2 (1 mL) for 2 h, and the QCM chips were washed by CH_2Cl_2 and dried in vacuo. Frequency of the QCM chips was measured by using Quartz Crystal Analyzer QCA 917 (Seiko EG&G Co. Ltd.). The concentration of 2-bromo-2-methylpropionicacid was changed from 0.2 μmol to 20 μmol.

MIP thin films were prepared by AGET ATRP as follows: RNase (13.4 mg, 1 μmol), acryl amide (0.27 mg, 3.75 μmol), acrylic acid (0.26 mg, 3.75 μmol), MBAA (7.7 mg, 50 μmol), $CuBr_2$ (0.94 mg, 4 μmol), PMDETA (1.38 mg, 8 μmol) were dissolved in a HEPES–NaOH buffer (pH 7.4) in a polystyrene (PS) container. The QCM chips were set in a Teflon cell (dip type) and dipped in the PS container. As soon as ascorbic acid (3.2 mg, 12 μmol) was added in the PS container, it was sealed by a septum. After degassing the solution by vacuum/nitrogen cycles, the PS container was set in thermostat bath at 40°C. After quenching polymerization at appropriate polymerization time by air, the QCM chips were dipped in 1 M NaCl aqueous solution for 1 h to remove RNase. In order to remove the copper ions, the QCM chips were subsequently dipped in 1 M EDTA-4Na aqueous solution for 24 h. The frequencies of QCM chips were measured both after polymerization and washing with NaCl aqueous solution. The film thickness was calculated by the following formula:

$$\text{Film thickness (nm)} = \frac{\text{deposits of PMBAA (ng)}}{\text{density of PMBAA } (1.23 \times 10^{-12}\ \text{ng/nm}^3) \times \text{surface area of quartz } (0.196 \times 10^{14}\ \text{nm}^2)} \quad (1)$$

$$\text{Deposits of PMBAA (ng)} = \text{change of frequency } \left(\text{s}^{-1}\right) \times \text{surface area of quartz } \left(0.196\ \text{cm}^2\right) \times \left(\frac{\sqrt{u \times \rho}}{2 \times F_0^2} \times \text{surface area of quartz}\right) \quad (2)$$

where F_0 is fundamental frequency (9,003,304 s^{-1}), surface area is 0.196 cm^2, u is shear stress of quartz (2.947×10^{20} ng/cm·s^2), and ρ is density of quartz (2.648×10^9 ng/cm^3).

Preparation of MIP thin films by RP (MIP-RPs) The immobilization of initiator for conventional radical polymerization on the QCM chips was carried out as follow. The QCM chips were cleaned by UV–O_3 treatment for 20 min and dipped in ethanol solution (1 mL) containing 11-mercapto-1-undecanoic acid (1.05 mg, 5 μmol) for 1 h. After washing the QCM chips by ethanol and drying in vacuo, the QCM chips were dipped in ethanol solution (1 mL) containing NHS (1.98 mg, 20 μmol) and EDC (3.26 mg, 10 μmol) for 1 h. The QCM chips were washed by ethanol and dried in vacuo, and the QCM chips were dipped in methanol solution (1 mL) of V-50 (2.7 mg, 10 μmol) (21). The QCM chips were washed by methanol and dried in vacuo. The frequency of the QCM chips was measured at each stage of modification.

The MIP-RPs were prepared by conventional radical polymerization as follows: RNase (13.4 mg, 1 μmol), acryl amide (0.27 mg, 3.75 μmol), acrylic acid (0.26 mg, 3.75 μmol), MBAA (7.7 mg, 50 μmol) were dissolved in a HEPES–NaOH buffer (pH 7.4) in the PS container. Then, the QCM chips were set in Teflon cell (dip type) and dipped in the PS container. After sealing the PS container by septum and degassing the solution by vacuum/nitrogen cycles, the PS container was set in thermostat bath at 50°C. After quenching the polymerization at appropriate polymerization times by washing with pure water, the QCM chips were dipped in 1 M NaCl aqueous solution for 1 h to remove RNase. The frequency of the QCM chips was measured after the polymerization and the NaCl aqueous solution treatment, respectively, and the film thickness was calculated. Surface morphology of the QCM chips after washing treatments was examined by scanning electron microscopy (SEM) (VE-9800, Keyence Co. Ltd.).

Preparation of MIP thin films by AGET ATRP on SPR chips Immobilization of the ATRP initiator on SPR chips [SIA Kit Au (Biacore 3000, Biacore Co. Ltd., Sweden)] were carried out under the same conditions as the QCM chips. A non-imprinted polymer film (NIP-ATRP) was also prepared by the above-mentioned procedure without RNase.

Preparation of MIP thin films by RP on SPR chips Immobilization of the initiator for conventional radical polymerization on SPR chips were carried out under the same conditions as the QCM chips. A non-imprinted polymer film (NIP-RP) was also prepared by the above-mentioned procedure without RNase.

SPR measurements Binding experiments for MIP-ATRPs, MIP-RPs, NIP-ATRPs and NIP-RPs were performed by using a surface plasmon resonance sensing system (Biacore 3000). Four kinds of different proteins (RNase, cytochrome *c*, myoglobin, and α-lactalbumin) were dissolved in a HEPES–NaOH buffer (pH 7.4) at 25°C. The conditions of SPR measurements were as follows: running buffer; 10 mM HEPES–NaOH buffer (pH 7.4, 20 μL/min), injection volume; 20 μL, regeneration solution; 10 mM HEPES–NaOH buffer containing 1 M NaCl and 5 mM NaCl aqueous solution. The amounts of bound proteins were calculated by measuring the signal intensity at 120 s after the end of each sample introduction period. Protein concentrations were varied from 0.25 to 5.0 μM. Binding isotherms were drawn by using the ΔRU at each protein concentration. The binding constants and the number of binding sites were calculated by Scatchard plots from the obtained binding isotherms.

Surface characterization using SEM Surface morphology of the obtained QCM chips was examined by SEM (VE-9800, Keyence Co. Ltd.).

RESULTS AND DISCUSSION

Fig. 1A shows MIP-ATRP film thicknesses at different AGET ATRP polymerization times on QCM chips, where a self-assembled monolayer (SAM) of 11-mercapto-1-undecanol was formed on QCM chips via strong chemical binding between the gold surface and thiol groups, and the hydroxy end groups were coupled with