

Protein profiling by protein imprinted polymer array†

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Six different protein-imprinted polymers using three template proteins with acidic or basic functional monomers resulted in unique fingerprints not only for the corresponding template but also for other proteins, allowing profiling of proteins for identification and classification.

Array-based techniques have been in great demand for protein profiling, diagnostics of diseases, and assessment of biomolecule interactions.¹ Considerable efforts have been made to develop protein sensor arrays using biomolecules including proteins, antibodies, DNA/RNA and other bio-macromolecules. Although biomolecules possess excellent properties toward molecular recognition, the screening and production of suitable biomolecules are costly, tedious and time-consuming, and their short shelf-life and the difficulty of patterned immobilization pose problems.

Recent research efforts have been directed towards preparation of synthetic polymer-based protein recognition materials because they may potentially play the role of natural receptors in practical use. It is difficult to design synthetic polymer chains with functional groups, which can be folded reproducibly to form 3-D specific binding sites for molecular recognition. Molecular imprinting (one of the available template polymerization techniques) has emerged as a method for the preparation of synthetic receptors mimicking naturally occurring molecular level recognition phenomena (Scheme 1). This technique may offer a means of creating designed binding sites possessing biomolecules-like

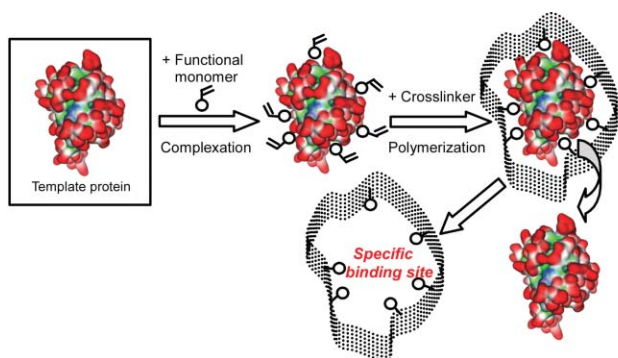
functions, provided that appropriate template molecules are designed and employed.² Recently, molecularly imprinted polymers are being used in the application area of sensor arrays for small molecular weight compounds.³ Molecular imprinting is based upon self-assembly of a template protein with polymerizable monomers bearing functional group(s) capable of interacting with the template molecule (functional monomers), followed by polymerization in order to maintain the alignment of the functional group(s). After removal of the template from the resulting polymer matrices, “tailor-made” binding sites for the template molecule can be constructed.

Molecular imprinting for biomolecules such as proteins has been intensively investigated;⁴ however, their selectivity is not as high as expected. Molecular recognition by differential receptor arrays may provide enhanced selectivity, however, there are to date no reports regarding protein imprinted polymer array-based analysis. In this communication, we propose the use of protein imprinted polymer arrays for protein classification and profiling.

We used two functional monomers in this work; typical acidic and basic monomers of acrylic acid (AA) and 2-dimethylaminoethyl methacrylate (DMA) for imprinting acidic and basic proteins mainly using electrostatic interaction. We synthesized 6 different imprinted polymers using 3 proteins to be imprinted: cytochrome C (Cyt), ribonuclease A (Rib) and α -lactalbumin (Lac), 2 functional monomers: acrylic acid (AA) and 2-dimethylaminoethyl methacrylate (DMA), and methylene bis(acrylamide) (MBAA) as a crosslinker.⁵ A pre-polymerization mixture was prepared by adding a template protein, AA or DMA, MBAA, glycosyloxyethyl methacrylate (GEMA) to a phosphate buffer of pH 7.4, and polymerization was initiated by the irradiation of UV light at 4 °C. The obtained Cyt-imprinted polymers (Cyt-MIP_{AA} and Cyt-MIP_{DMA}), Rib-imprinted polymers (Rib-MIP_{AA} and Rib-MIP_{DMA}) and Lac-imprinted polymers (Lac-MIP_{AA} and Lac-MIP_{DMA}), were ground to a fine powder and washed to remove the template proteins. Two non-imprinted polymers (NIP_{AA} and NIP_{DMA}), corresponding to the two functional monomers, were also prepared without the addition of proteins. The ratios of the recovered amounts of proteins from the resulting polymers to the initial amounts in the pre-polymerization mixtures were estimated to be in excess of 80%.⁶

Total conversions of monomers in the obtained polymers were about 90%.⁷ The specific surface area and pore size were approximately 2 to 3 m² g⁻¹ (BET method) and <5 nm (DFT method) by nitrogen gas adsorption/desorption, respectively.⁸ Copolymerization with GEMA was carried out in order to make the polymers hydrophilic and reduce nonspecific binding of proteins to polymer matrices by hydrophobic interaction.

Reproducibility of the polymer preparation was evaluated by the binding amounts of Cyt to 5 independently prepared



Scheme 1 Schematic diagram of protein imprinting.

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† Electronic supplementary information (ESI) available: Protein extraction data; total conversion data; specific surface area and pore size of the AA- and DMA-based polymers; IR data of Cyt-MIP; principal component analysis; and evaluation of the reproducibility of polymer preparation (Cyt-MIP). See DOI: 10.1039/b614532a

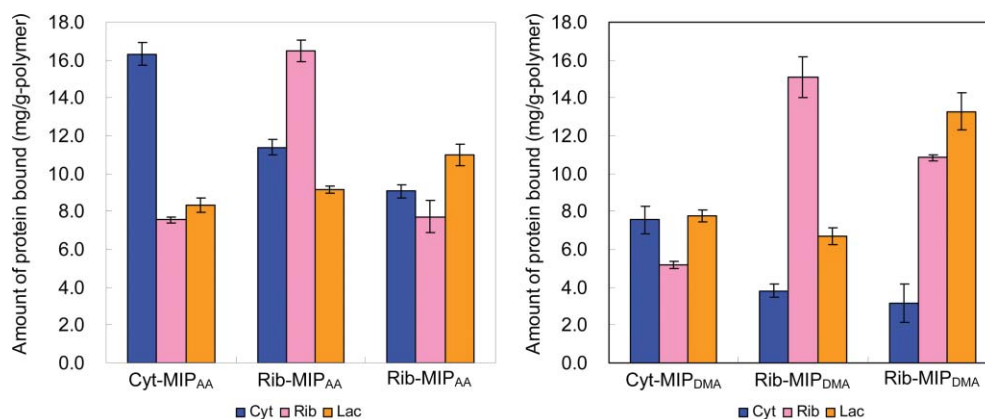


Fig. 1 Evaluation of imprinting effects on the imprinted polymers prepared. The imprint effects were evaluated by the binding behaviors of the three template proteins at pH 7.4 ($n = 4$).

Cyt-MIP_{AA}.⁹ The coefficient of variation was calculated to be 2.3%, suggesting that the MIPs can be prepared reproducibly and the MIP array may give reliable data sets for protein identification.

The AA-based acidic imprinted polymers exhibited the strongest binding for the corresponding template proteins. When DMA was used as the functional monomer, the template proteins were also bound strongly to their corresponding basic imprinted polymers, although the binding patterns were different from the AA-based polymers (Fig. 1).¹⁰ At pH 7.4, AA residues are negatively charged, thus basic proteins should be bound more strongly than acidic proteins such as Lac. On the other hand, DMA residues would be positively charged, and thus acidic proteins should be bound stronger than basic proteins such as Cyt and Rib. However, Lac-MIP_{AA} showed the strongest binding of Lac among the proteins tested (including basic proteins), and Rib-MIP_{DMA} more specifically bound Rib than other tested proteins. Thus, the present binding phenomena cannot be explained by simple ion-exchange or hydrophobic interactions with AA or DMA residues in the polymer matrices, suggesting that the imprinting process itself provides the characteristic binding.

Fingerprints of proteins were demonstrated based upon AA-based and DMA-based polymers, including Cyt, Rib, Lac, and non-templated proteins: Alb and myoglobin (Myo). Each protein exhibits an individual, unique binding pattern for the MIPs and NIPs (Fig. 2). In nature, proteins interact with each other, and it

cannot be completely excluded that when the tested proteins were bound to the polymers, the bound proteins may themselves act as binding sites in the form of ion-exchange sites and/or hydrophobic sites. However, this is considered unlikely because the apparent binding patterns could not be fully explained by simple ion-exchange or non-specific hydrophobic interactions. Therefore, the fact that template protein-selective binding was exhibited in the resulting imprinted polymers, as well as the fact that the binding patterns were significantly different from those of the NIPs, strongly suggests imprinting effects, *i.e.* the imprinting process strongly influenced the binding selectivity.

A complicated multi-dimensional data set was obtained for each protein as a result of the use of multiple imprinted polymers. It is not trivial to characterize these proteins by apparent binding differences in the raw data. In order to reduce the dimensionality of the data set, to find underlying variables, and to expose the fingerprints more clearly, principal component analysis (PCA; multivariate analysis), was applied. PCA showed clear discrimination of the five tested proteins in the AA-based imprinted polymers (Fig. 3).¹¹ Regarding the DMA-based imprinted polymers, the apparent clustering pattern was different from the AA-based system, and plots of acidic proteins such as Alb and Lac were located closely. These specific binding-based unique fingerprints that are generated by multivariate analyses may allow profiling of proteins for identification and classification.

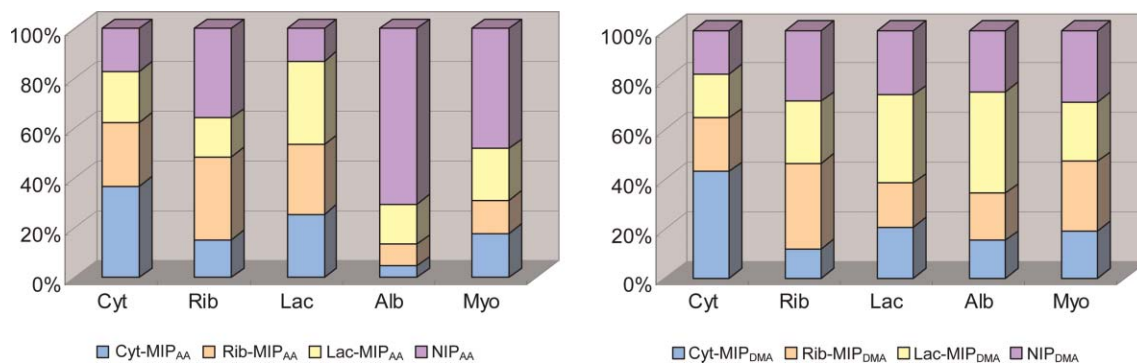


Fig. 2 Fingerprints of five proteins tested based upon AA-based and DMA-based polymers. Cyt: cytochrome C; Rib: ribonuclease A; Lac: α -lactalbumin; Alb: albumin; Myo: myoglobin. Alb and Myo are non-templated proteins. The total amount bound of each protein for the four polymers corresponds to 100%.

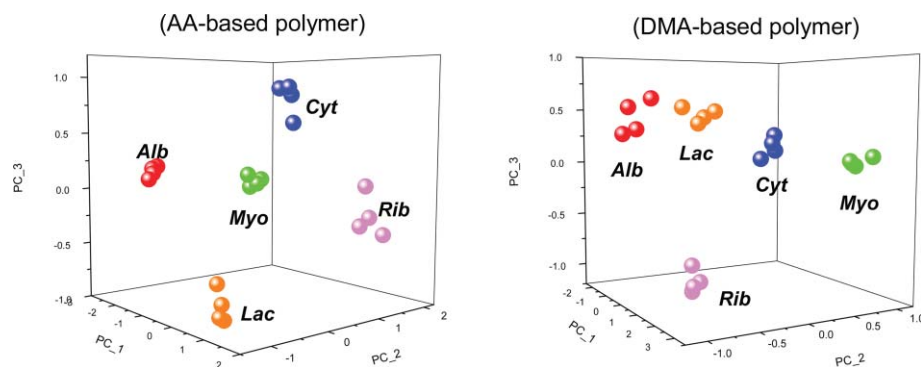


Fig. 3 PCA score plots showing the discrimination of four trials of five different proteins based upon the bound amounts of AA-based and DMA-based polymers. Cyt: cytochrome C; Rib: ribonuclease A; Lac: α -lactalbumin; Alb: albumin; Myo: myoglobin. Alb and Myo are non-templated proteins. Alb and Myo are non-templated proteins.

We have demonstrated the effectiveness of protein-imprinted polymer arrays to achieve classification of 5 tested proteins. The most attractive aspect of this technique is that even when the same functional monomers and crosslinkers are used, each polymer exhibits characteristic binding behaviors. Molecular imprinting thus has potential as a powerful method for convenient preparation of a diverse range of protein adsorbents by changing the template proteins. Patterning of *in situ* preparation of such polymers on a chip could provide new types of artificial protein chips that can characterize and profile various proteins in nature.

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- A pre-polymerization mixture (50 mM phosphate buffer pH 7.4, 7.2 mL) containing a template protein (7.5 μ mol), a functional monomer (acrylic acid or 2-dimethylaminoethyl methacrylate 0.15 mmol), glycosyloxyethyl methacrylate (1.35 mmol) and methylene bis(acrylamide) (1.5 mmol), was purged with nitrogen gas for 5 min, then, an initiator, VA-080 was added and polymerization was initiated by irradiating UV light for 18 h at 4 $^{\circ}$ C. NIP was prepared by the same way without the template protein. The resulting polymer was ground to a fine powder and washed with 0.5 M NaCl to remove the template protein. Further acrylic acid-based polymers were washed with 1 M HCl and 2-dimethylaminoethyl methacrylate-based polymers were washed with 1 M NaOH.
- The extraction of template proteins was checked by measuring absorbance at 280 nm using a spectrophotometer (280 nm Pharmacia LKB Biochrome 4060) to determine extracted proteins from the polymers (see ESI†).
- Total conversion data for all polymers are shown in ESI.† IR spectra of all polymers prepared were almost the same (typical data are shown in ESI†).
- The surface area and pore size distributions of polymers were measured by NOVA-1000 YUASA IONICS, and were determined by a Multiplot BET method and a DFT method, respectively.
- Reproducibility of the polymer preparation was evaluated by the binding amounts of Cyt to 5 independently prepared Cyt-MIP (see ESI†).
- Proteins (0.25 mg mL⁻¹) were dissolved in 50 mM phosphate buffer pH 7.4 (5 mL) containing a polymer (5 mg). After 16 h incubation with rotation at 25 $^{\circ}$ C, the mixtures were filtered to remove the polymer and the proteins in the filtrate were analyzed by HPLC consisting of an auto-sampler (Gilson 231XL, France), two pumps (Hitachi L-6320 and L-6010, Japan) and a UV/Vis detector (Hitachi L-4200). For ribonuclease A, myoglobin and albumin, TSKgel butyl-NPR (35 mm $\times$ 4.6 mm id, Tosoh, Japan) was used with a gradient elution (A: 0.1 M phosphate buffer pH 7.0, B: 0.1 M phosphate buffer pH 7.0 containing 2.5 M ammonium sulfate, 1 mL min⁻¹, A100% $\rightarrow$ B25%, 9 min). For lactalbumin and cytochrome C, TSKgel G-3000SW_{XL} (300 mm $\times$ 7.8 mm id, Tosoh, Japan) was used with an eluent of 50 mM phosphate buffer pH 7.0 containing 0.3 M sodium sulfate (1 mL min⁻¹). Detection was carried out at 280 nm. Bound protein amounts were calculated by subtracting the amount in the filtrate from the initial amount.
- PCA was performed by Multivariate Analysis Add-in for Excel v1.2 (Bristol Centre for Chemometrics) (see ESI†).