Molecular imprinting of proteins emerging as a tool for protein recognition

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This article gives the recent developments in molecular imprinting for proteins. Currently bio-macromolecules such as antibodies and enzymes are mainly employed for protein recognition purposes. However, such bio-macromolecules are sometimes difficult to find and/or produce, therefore, receptor-like synthetic materials such as protein-imprinted polymers have been intensively studied as substitutes for natural receptors. Recent advances in protein imprinting shown here demonstrate the possibility of this technique as a future technology of protein recognition.

Introduction

Recent advances in materials sciences have helped develop intelligent materials bearing various functions and performances. Synthetic materials with molecular recognition ability can be used as separation media, adsorbents, chemical sensors, selective catalysts, and so on. Molecular imprinting, a templated polymerization technique, is a promising method to prepare such functional polymers selective for target molecules.[1] To obtain selective molecularly imprinted polymers (MIPs) with highly selective molecular recognition ability for target molecules, design of functional monomers for suitable adduct formation with template molecules is critical. There are two distinct methods to prepare imprinted polymers. One is non-covalent molecular imprinting, in which intermolecular interactions such as hydrogen bonding, electrostatic interactions, hydrophobic interactions, π–π stacking, van der Waals forces, *etc.* are used to form functional monomer-template adducts in solution and are co-polymerized with cross-linkers. The other is covalent molecular imprinting, in which target molecules are conjugated with functional monomers by covalent bonds. In both systems, the template molecules are removed from the obtained polymers after the polymerization, to yield binding sites toward the corresponding target molecules complementary in shape, size and functional group orientation. Non-covalent methods are easy to perform and many kinds of functional monomers are available, but the homology among generated binding sites in terms of affinity and selectivity is commonly low, depending upon the stability of functional monomer-template molecule complexes during the polymerization. Covalent methods generally provide more homogeneous binding sites but usually the cleavage of template molecules is not easy. The two methods have merits and defects, and therefore, careful choice should be required, according to the chemical characteristics of the target molecules.

Recently, recognition of bio-molecules has drawn much attention, and proteins, saccharides, DNAs, cells, and viruses have been considered to be the next targets in molecular recognition chemistry. Generally speaking, recognition of proteins seems to be difficult, because they fold into three dimensional structures and are vulnerable to harsh conditions such as high/low pH, temperatures, high salt concentrations, and so on. In order to achieve molecular imprinting of proteins, such obstacles should be overcome. In this article, some highlights of recent advances in

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molecular imprinting of proteins are described and they should help give a clue as to how to obtain highly selective protein-imprinted polymers as a reliable tool of protein recognition.

N,*N*′-Methylene bisacrylamide-based protein-imprinted hydrogels

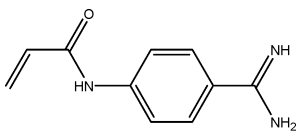
In protein-imprinting, non-covalent molecular imprinting is commonly used. Although proteins are not good template molecules because of their flexible structures and instability in organic solvents, many efforts have been recently made to prepare protein-imprinted hydrogels by using a hydrophilic crosslinker, *N*,*N*′-methylene bisacrylamide (MBAA).

For protein imprinting, acidic and/or basic functional monomers have been frequently used in order to bind proteins by electrostatic interactions. Minoura *et al.* reported glucose oxidase-imprinted hydrogels prepared on silica gel. They used acrylic acid (acidic monomer) and dimethylaminopropyl acrylamide (basic monomer) as functional monomers.[2,3] Silica gel was used as a support to obtain robust and large surface areas of the polymer matrices. Glucose oxidase bound most strongly to the polymer when the polymer had a positive charge and the same charge values as the protein.

Unlike Minoura's system, Ou *et al.* reported support-free MBAA-based lysozyme-imprinted hydrogels prepared by co-polymerization of methacrylic acid and 2-(dimethylamino)ethyl methacrylate as functional monomers.[4] The polymers were extensively studied by adsorption experiments and isothermal titration calorimetry (ITC).[5] ITC experiments thermodynamically proved the creation of lysozyme binding sites by the imprinting process, *i.e.* the binding of the template protein (lysozyme) gave differences in the binding enthalpy between the imprinted and the non-imprinted polymers, while any major changes in the adsorption enthalpy were observed for cytochrome c.

Chitosan is a natural polysaccharide composed of β-(1–4)-linked D-glucosamine and *N*-acetyl-D-glucosamine. Various functional materials based on chitosan have been reported, since it is biocompatible and biodegradable, and works as a cationic polyelectrolyte. Hemoglobin-imprinted polymers have been prepared by using acrylamide as a functional monomer.[6] Porous chitosan beads crosslinked by epichlorhydrin were treated with maleic anhydride to introduce polymerizable vinyl groups, and after the addition of hemoglobin as a template, acrylamide as a functional monomer and MBAA as a crosslinker, polymerization was carried out. The resulting beads were washed with acetic acid containing sodium dodecyl sulfate to remove hemoglobin, yielding hemoglobin-imprinted chitosan beads. Hemoglobin and albumin were separated chromatographically by a hemoglobin-imprinted polymer-packed column.

The use of functional monomers capable of specific binding for the particular amino acid residues, catalytic sites, *etc.* could be good for obtaining specific imprinted binding sites for target proteins. Enzyme inhibitors are good candidates of specific functional monomers for enzyme imprinting. Vaidya *et al.* developed MBAA-based trypsin imprinting by using a trypsin inhibitor, *N*-acryloyl *p*-aminobenzamidine, as a functional monomer.[7] The obtained imprinted polymer bound trypsin more strongly than chymotrypsin. Imprinted polymer containing 50% crosslinker exhibited a linear Scatchard plot,[8] meaning that homogeneous binding sites are obtained under the conditions and the crosslinker content is crucial for the imprinting efficacy.



N-acryloyl p-aminobenzamidine

Miyata *et al.* prepared protein-responsive hydrogel by combining a functionalized lectin and an antibody as functional monomers.[9] A glycoprotein, tumor-specific marker AFP was used as a target protein. A ternary complex was formed among AFP, lectin and the antibody corresponding to AFP, and then the complex was cross-linked with MBAA to obtain AFP-imprinted hydrogel. The imprinted gel shrunk when AFP was added, in which both lectin and the antibody bind to the target glycoprotein. In contrast, when ovalbumin, which can be bound to only lectin, was added, no change was observed (Fig. 1). The volume of the swelling was dependent on the concentration of the target,

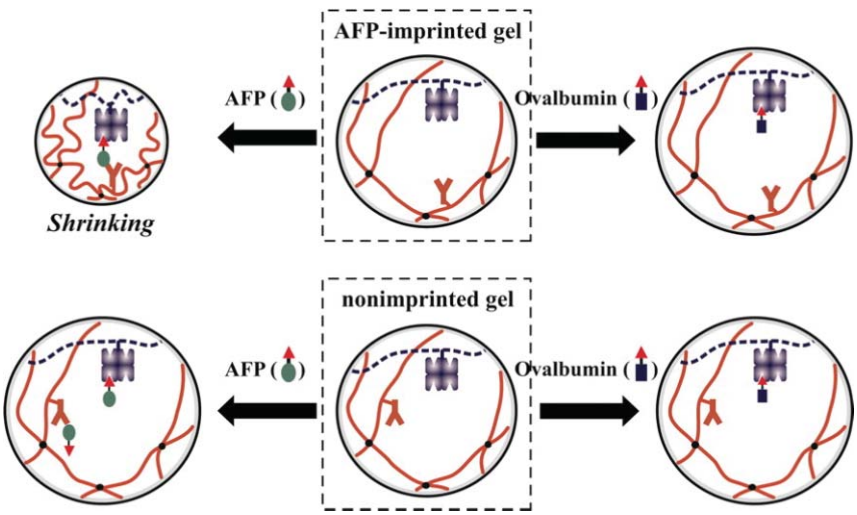


Fig. 1 Schematic representation of glycoprotein-responsive behaviors of tumor marker (AFP)-imprinted and non-imprinted gels for AFP and ovalbumin. (Reproduced from ref. 9: Copyright 2006 The National Academy of Sciences of the United States of America.)

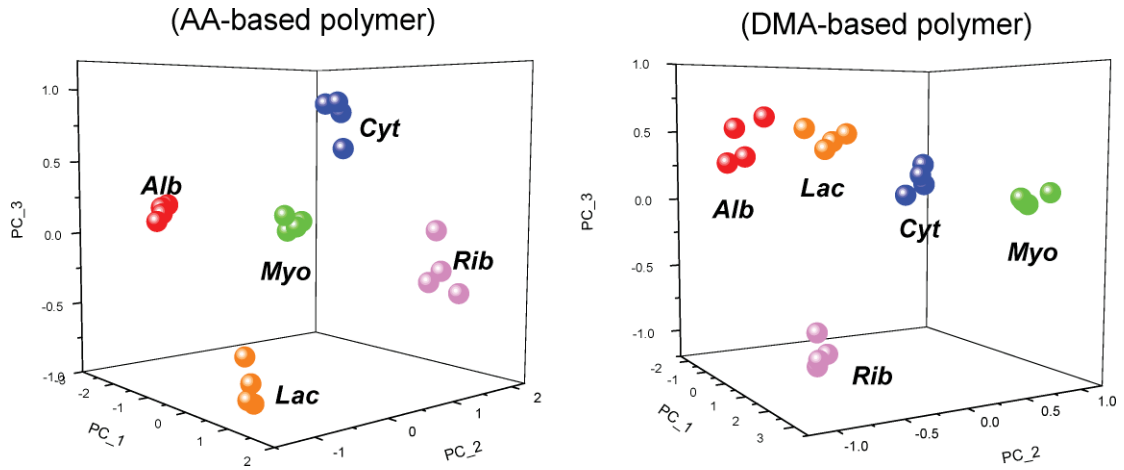


Fig. 2 PCA score plots showing the discrimination of four trials of five different proteins based upon bound amounts of acrylic acid (AA)-based and 2-dimethylaminoethyl methacrylate-based DMA-based polymers. Alb: albumin, Cyt: cytochrome c, Lac: lactalbumin, Myo: myoglobin, Rib: ribonuclease A. Alb and Myo are non-templated proteins. (Reproduced from ref. 12 with permission from Royal Society of Chemistry.)

where cooperative binding by lectin and the antibody was clearly evidenced.

Pattern-based recognition of various bioanalytes has been conducted by differential receptor array systems in nature, *e.g.* the mammalian nose for the senses of smell, where odor can be recognized by the combination of the olfactory receptor responses. Such differential receptor arrays can be constructed artificially by using synthetic receptors.[10] This strategy can be applied to the molecular recognition of proteins by using plural imprinted polymers.[11] Takeuchi and co-workers have studied the binding profiles of guest proteins toward plural protein-imprinted polymers, where acrylic acid (AA) or 2-dimethylaminoethyl methacrylate (DMA) was used as a functional monomer, and MBAA was used as a cross-linker.[12] Protein-imprinted polymers were prepared in the presence of cytochrome c, ribonuclease A, and lactalbumin as template proteins. Non-imprinted polymer was also prepared in the absence of templates. Each polymer had the highest binding affinity for the corresponding template protein. When principle component analysis (PCA) was carried out toward the binding data, and the PCA scores were plotted on a three-dimensional scale, each protein could be clearly distinguished from each other (Fig. 2). This means that the protein-imprinted polymer array can be applied to protein profiling by pattern analysis of binding activity for each polymer. In the case of the DMA-based polymers, the binding profiles obtained also showed clear fingerprinting of proteins on the imprinted polymer array. Unlike natural antibodies, binding properties of imprinted polymers can be changed easily by varying template proteins, functional monomers, and reaction conditions employed, *i.e.* imprinted polymers with a diverse range of binding characteristics can be easily obtained to construct differential receptor array systems. These results suggest that protein-imprinted polymer arrays may be an alternative tool in proteomics to antibody-based microarrays.

Recently, efforts have been made to visualize protein specific cavities for MBAA-based imprinted polymers. The imaging of the adsorption and desorption processes have been investigated by using FITC-albumin and hemoglobin, where confocal microscopy was employed for FITC-albumin and two-photon confocal microscopy was used for protein autofluorescence of hemoglobin.[13] Transmission electron microscopy imaging was also reported for hemoglobin-imprinted polymer, where a critical point drying based sample preparation technique was employed.[14]

According to our experiences, a hydrogel prepared by co-polymerizing acrylamide and MBAA sometimes possesses its intrinsic binding property for proteins, and it may cause non-specific binding. This binding can be weakened by the addition of salt, thus it may be due to weak ion-exchange ability. Matsunaga *et al.* reported that molecularly imprinted polymers selective for lysozyme were prepared on surface plasmon resonance (SPR) sensor chips by radical co-polymerization with acrylic acid and MBAA.[15] The presence of NaCl during the polymerization and the re-binding tests affected the selectivity, revealing that the optimization of NaCl concentration in the pre-polymerization mixture and the re-binding buffer could improve the selectivity in the target protein sensing. If more hydrophilic crosslinkers than MBAA that can retain more free water were employed, this kind of non-specific binding could be reduced, yielding more selective protein imprinted polymers.

Ethylene glycol dimethacrylate-based protein imprinted polymers

Ethylene glycol dimethacrylate (EDGMA) is a common crosslinker that has been preferably used to prepare imprinted polymers for small lipophilic molecules. EDGMA-based polymers are fairly hydrophobic and suitable for imprinted polymers with the use of apolar solvents, in which hydrogen bonding is a main driving force to achieve the specific binding. Therefore, when water is used as a medium, hydrophobic interaction can be dominant; *e.g.* EDGMA-based atrazine imprinted polymers worked in a reversed phase mode during solid-phase extraction with water.[16] In spite of the hydrophobic property that may cause non-specific binding in aqueous solution, EDGMA-based protein imprinted polymers have been reported and they seem to work.

Ersöz *et al.* reported EDGMA-based histidine-imprinted beads prepared by suspension polymerization with a copper(II) complex of *N*-methacryloyl-L-histidine and L-histidine[17] (Fig. 3). An association constant of L-histidine was estimated to be 58 300 M^{-1}. The resultant metal complex-based histidine imprinted polymer showed higher affinity to L-histidine than its antipode. The polymer also showed higher affinity for surface histidine exposed proteins such as cytochrome c than for ribonuclease A. This looks like "epitope imprinting" described later in this article.

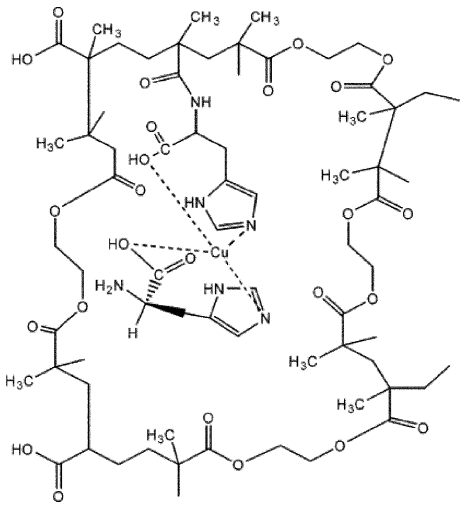


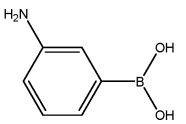
Fig. 3 Schematic representation of binding sites in the L-histidine-imprinted polymer for the recognition of surface histidine exposed proteins. (Reproduced from ref. 17 with permission from American Chemical Society.)

Tong *et al.* prepared superparamagnetic ribonuclease A-imprinted polymeric particles (700 to 800 nm) using methyl methacrylate and EDGMA as the functional and cross-linker monomers.[18] Redox-initiated miniemulsion polymerization was employed in this case. Although good selectivity toward ribonuclease A over lysozyme was observed for the imprinted particles in the competitive rebinding tests, lysozyme itself also bound considerably to the particle and even the bound amount was greater when re-binding tests were carried out individually. They mentioned that the hydrophobic effect was probably the main form of interaction responsible for the template rebinding to the imprinted sites in an aqueous media.

Ribonuclease A-imprinted polymeric nanoparticles were prepared by miniemulsion polymerization using methyl methacrylate and EDGMA as the functional and cross-linker monomers.[19] To prevent denaturation of the protein, poly(vinyl alcohol) was used as a co-surfactant and the amount was optimized by measuring circular dichroism spectra to check the conformation of ribonuclease A. The prepared imprinted nanoparticles showed better selectivity than those prepared through non-optimized miniemulsion polymerization, suggesting that protein structural integrity is important in protein imprinting. Unfortunately, the selectivity toward ribonuclease A over albumin was not superior. This may be due to the use of methyl methacrylate that has a less selective binding ability, thus the screening of monomers to optimize the system would lead to the enhancement of the present selectivity and the reduction of non-specific binding.

3-Aminophenylboronic acid-based imprinted polymers

3-Aminophenylboronic acid (APBA) can be polymerized under mild aqueous conditions and is expected to interact with various saccharides and amino acid residues. The redox polymerization can be initiated chemically or electrochemically. Bossi was the first to report that this polymer is suitable for protein imprinting and various protein imprinted polymers were prepared in a microtiter plate format, including microperoxidase, horseradish peroxidase, lactoperoxidase, and hemoglobin.[20] Rick prepared poly(APBA)-based lysozyme and cytochrome c imprinted polymers on the gold surfaces of quartz crystal microbalance (QCM) electrodes.[21] They also prepared APBA-based protein-imprinted polymers on screen-printed platinum supports by cyclic voltammetric deposition.[22] Microcalorimetry experiments proved that the specificity could be induced by the imprinting process, where the enthalpy changes were observed associated with the rebinding.[23]



3-Aminophenylboronic acid

Recently, Turner and co-workers successfully imprinted thermal- and fluoro-alcohol-induced β-lactoglobulin isoforms in poly(APBA)-thin films on QCM chips.[24] Each imprinted polymer showed better template selectivity, compared with the corresponding non-imprinted polymer. Interestingly, distinct conformations of the same protein can be recognized by the polymers, suggesting that protein conformational imprinting could provide a way to detect non-native pathogenic proteins.

Protein-imprinted organic–inorganic hybrid materials

At an early stage, Mosbach and his co-workers reported imprinted polysiloxane-coated silica for a glycoprotein, transferrin.[25] A boronic acid derivative was used as a functional monomer to form a cyclic ester with a sugar moiety of transferrin. The resulting polysiloxane copolymer showed preferential binding of transferrin over albumin. Kempe *et al.* used a polymerizable metal complex as a specific functional monomer, namely a copper complex of *N*-(4-vinyl)-benzyliminodiacetate, which can be bound to histidine residues of template proteins by coordinate bonding.[26] A ribonuclease A-imprinted polymer was prepared on the surface of methacrylated silica gel by using this metal chelating monomer. Ribonuclease A was separated from lysozyme on the imprinted polymer-coated silica gel in liquid chromatography.

Recently, by using sol-gel reactions, albumin-imprinted polymers coupled with QCM were prepared by Yao *et al.*[27] QCM chips treated with thioglycolic acid were immersed into a mixture of albumin and an initial sol composed of tetraethoxysilane, phenyltrimethoxysilane and methyltrimethoxysilane. After the repetitive adsorption process, a submicrometre-ordered thin film was formed on the QCM chips. Finally, the albumin-imprinted

sol-gel thin film-coated QCM chips were obtained by washing out albumin with hot water. The imprinted films showed selective recognition for albumin and discriminated albumin from hemoglobin, peroxidase and trypsin. Although the authors mentioned that the binding occurs because of the conformation compatibility and electrostatic force, rather hydrophobic interactions could play important roles in this case, since higher binding was observed even in the presence of salt and pH dependence was not observed from pH 4.5 to 9.2.

Protein-imprinted xerogels have been prepared by Bright and co-workers, which are capable of signal transduction of the protein binding events into fluorescence change of the reporter molecules located inside the imprinted cavity (Fig. 4).[28] The proposed protein-responsive chemical sensing materials were prepared in array format and applied for the detection of interleukin-1 alpha and beta. This technique lies in sol-gel derived xerogels prepared in the presence of target proteins. An initial sol was prepared with tetraethoxysilane, methyltrimethoxysilane, *n*-octyltrimethoxysilane, 3-aminopropyltriethoxysilane, and bis(2-hydroxyethyl)aminopropyltriethoxysilane, and mixed with the target protein. Then, the sol solution was pin printed onto a slide glass, and after xerogel was formed, the template protein was removed by washing with concentrated urea or diluted phosphoric acid, yielding the imprinted cavities.

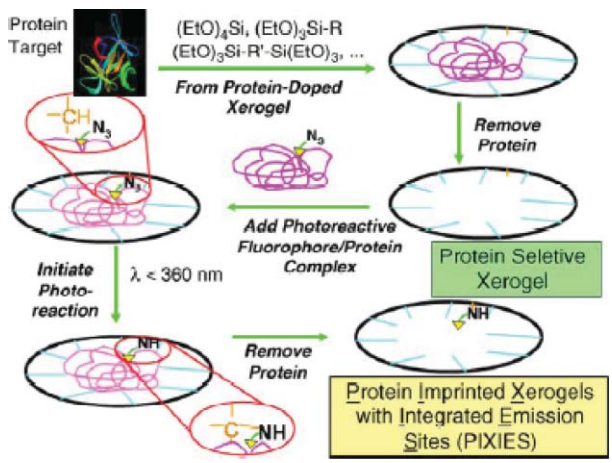
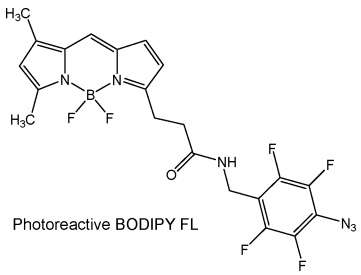


Fig. 4 Preparation of protein-imprinted xerogels capable of signal transduction of the protein binding events into fluorescence change. (Reproduced from ref. 28 with permission from Elsevier.)

BODIPY FL (4,4-difluoro-5,7-dimethyl-4-bora-3*a*,4*a*-diaza-*s*-indacene-3-propionic acid) was used as a fluorescent reporter molecule and coupled with an aryl azide (4-azido-2,3,5,6-tetrafluorobenzyl amine) to obtain a photoreactive BODIPY FL. The obtained fluorophore was non-covalently bound to the target protein (the mechanism was not described in the literature), and then mixed with the resultant imprinted xerogel. In this case, the target protein worked as a carrier to deliver the photoreactive fluorophore at the imprinted cavity. A photochemical reaction was carried out to immobilize the fluorophore around imprinted binding sites to construct the imprinted xerogel with integrated emission sites.



The resultant xerogels appeared to be highly sensitive and selective; human interleukin-1 alpha could be recognized by the imprinted xerogel with a selectivity factor of 147 (a signal of interleukin-1 alpha divided by that of interleukin-1 beta) and the detection limit was 1.4 pM. Since the system consists of a programmable liquid handler, a pin printer and an imaging system, it could be expected to be used as a high throughput screening method. It should be noted that since the aryl azide-BODIPY FL may be grafted not only to the xerogel but also to the protein itself, some binding sites have no BODIPY FL, resulting in a decrease of sensitivity. Another point is that BODIPY FL can form adducts with various proteins, therefore BODIPY FL located inside/around the binding cavity may lead to non-specific binding. Nevertheless, the system showed high selectivity and affinity, which may be due to the fact that the target protein could interact with 3-aminopropyltriethoxysilane (by electrostatic interaction) and *n*-octyltrimethoxysilane (by hydrophobic interaction) during the imprinting process, and they should be fixed at appropriate positions to create highly selective binding sites.

Liquid-phase deposition (LPD) is a rather new soft-solution process for preparing metal oxide thin films from aqueous solutions.[29] LPD proceeds in solution through the two equilibrated steps below: (a) metal fluoride is hydrolyzed to form a metal oxide and release a fluoride ion; (b) the fluoride ion produced is trapped by boronic acid. As a result, the equilibrium is shifted to the right-hand side and the hydrolysis is accelerated. Thereby a metal oxide thin film is gradually deposited on various kinds of substrates homogeneously.

$$MF_x^{(x-2n)-} + nH_2O = MO_n + xF^- + 2nH^+ \quad \text{(a)}$$

$$H_3BO_3 + 4H^+ + 4F^- = HBF_4 + 3H_2O \quad \text{(b)}$$

Takeuchi *et al.* prepared protein-imprinted organic–inorganic hybrid materials toward an acidic protein, pepsin, using titanium oxide-LPD (Fig 5).[30] Therein, pepsin–poly-L-lysine adducts were formed electrostatically in a titanium oxide-LPD treatment solution and were co-deposited with titanium oxide onto SPR sensor chips during the LPD process, yielding a pepsin-imprinted organic–inorganic hybrid thin film. As a reference, a non-imprinted film was also prepared without the addition of pepsin. The binding studies toward pepsin, lactalbumin, albumin and chymotrypsin clearly showed that the imprinted film had high selectivity for pepsin. An appropriate molar ratio of poly-L-lysine and pepsin in the complexation process could keep the native structure of pepsin, which was examined by circular dichroism spectroscopy, and such conditions should be used to construct pepsin selective binding sites. The chips prepared without poly-L-lysine were also prepared in the presence and absence of pepsin. They showed similar binding profiles toward

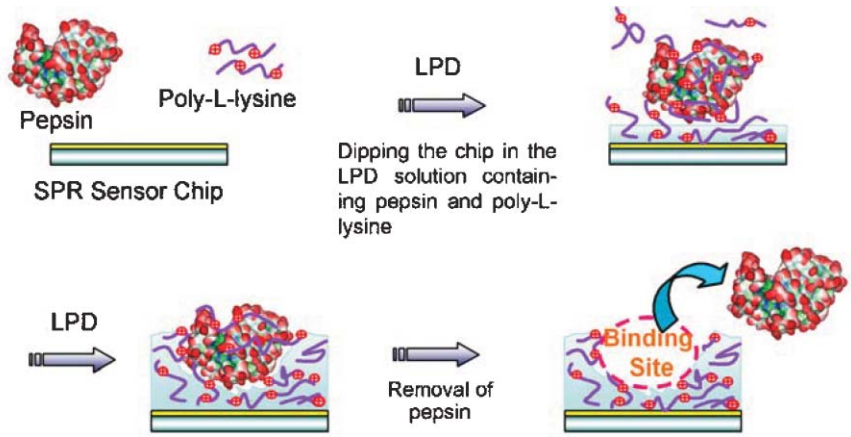


Fig. 5 Schematic illustration of the pepsin-templated LPD process. (Reproduced from ref. 30 with permission from American Chemical Society.)

guest proteins and have no specificity for pepsin, meaning that simple pepsin-templated titanium oxide films are inappropriate for protein recognition.

Various LPD-based imprinted organic–inorganic hybrid materials can be prepared by simply mixing target proteins and a diverse range of organic polyion compounds capable of interacting with target proteins in the LPD treatment solution. Therefore, the proposed LPD-based protein imprinting may be more convenient than sol-gel reaction-based processes that should have appropriate metal oxide derivatives, if protein–polyion complexes can be formed stably in the corresponding LPD treatment solutions.

Protein imprinting using immobilized templates

Difficulties in protein imprinting are not only their instability under harsh conditions but also their thermodynamic motions in solution, therefore, immobilized templates may be effective to improve the imprinting efficiency. Shiomi *et al.* have successfully used an immobilized protein as a template.[31] Hemoglobin was immobilized on glutaraldehyde-treated aminopropyl silica through imine bonds between amino groups of hemoglobin and aldehyde groups on the silica. 3-Aminopropyltrimethoxysilane and propyltrimethoxysilane were then polymerized on the hemoglobin-immobilized silica, followed by washing with oxalic acid to remove hemoglobin. The imprinted silica showed selective binding for hemoglobin, and the selectivity was superior to the imprinted silica prepared with free hemoglobin. Competitive re-binding tests in the presence of hemoglobin, myoglobin, transferrin, and chymotripsinogen confirmed the selective binding of hemoglobin. Recently, this immobilized protein system has been extended to APBA-based albumin imprinting.[32]

Chou also reported an imprinting method combining immobilized proteins and micro-contact printing techniques (Fig. 6).[33] In this case, a protein layer was formed as a stamp on a hexamethyldisilazane-treated microscope cover glass by hydrophobic interaction. Stamps of lysozyme, ribonuclease A and myoglobin were prepared and contacted on a 3-(trimethoxysilyl) propyl methacrylate-grafted glass slide carrying neat functional and crosslinking monomers. After polymerization, the cover glass was removed to obtain protein surface imprinted polymer thin films on the slide glass. Firstly, the template recognition abilities of

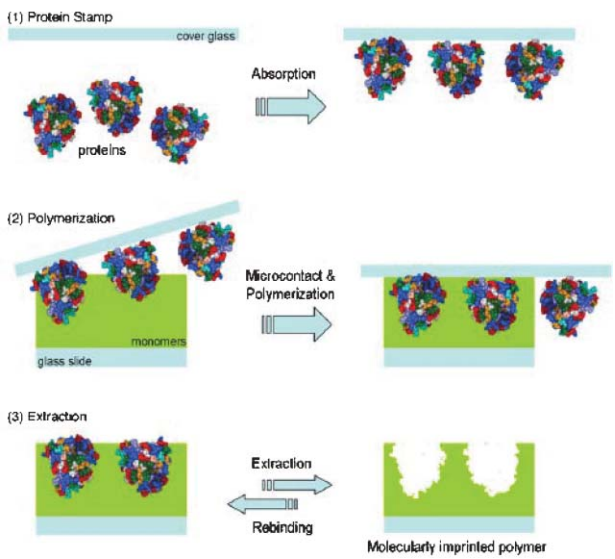


Fig. 6 Microcontact molecular imprinted polymer preparation procedures. (Reproduced from ref. 33 with permission from Elsevier.)

MIPs prepared with only crosslinkers (no functional monomers) of dimethacrylic acid ester with ethylene glycol repeat units ($n = 1$, 4, 9 and 13) were evaluated. Among the crosslinkers, tetraethylene glycol dimethacrylate ($n = 4$) gave the most selective lysozyme binding, while polyethylene glycol 400 dimethacrylate ($n = 9$) was most selective for ribonuclease A and myoglobin. From the screening of functional monomers by microcalorimetric titration toward the protein stamps with neat functional monomers as titrants, including methyl acrylic acid, 2-hydroxyethylmethacrylate, and dimethylaminoethyl methacrylate, *N*-vinyl pyrrolidone, styrene, methyl methacrylate and 4-vinyl pyridine, styrene showed a good selectivity for lysozyme and ribonuclease, while for myoglobin, the highest affinity functional monomer was methyl methacrylate.

These results reveal that the micro-contact approach is a convenient way to prepare many MIPs, and the selection of monomers for protein imprinting can be easily conducted. Nevertheless, several issues should be pointed out on this approach: the direct adsorption of proteins on glass substrates to prepare the protein stamps often lead to denaturation of proteins; the MIP preparation using highly concentrated crosslinkers would be unfavorable since

the interaction of proteins with functional and/or crosslinking monomers may occur under non-aqueous environments during the imprinting process; because neat functional and crosslinking monomers were used to prepare MIPs, a dense crosslinking network could be formed, where ethylene oxide groups of ethylene glycol repeat chains may work as hydrogen bonding-based binding sites rather than water containable matrices.

Li and co-workers prepared protein-imprinted polymer nanowires using immobilized protein templates.[34] Firstly silica nanotubes were prepared within pores of alumina membranes (100 nm in diameter) by using 3-aminopropyltrimethoxysilane, followed by a glutaraldehyde treatment to yield the amine-reactive surface bearing aldehyde groups. Immobilization of protein was then carried out; *i.e.* the membrane was treated with a template protein solution to form imine bonds between the silica nanotubes and the protein. After the protein-immobilized membrane was immersed in a mixture of acrylamide and MBAA, polymerization was carried out. Finally, the membrane and silica nanotubes were dissolved by NaOH to yield the MBAA-based protein-imprinted polymer nanowires (Fig 7). When hemoglobin was used as a template, the resultant imprinted nanowires (2 mg) could bind amounts of hemoglobin seven times higher than non-imprinted nanowires. Albumin, cytochrome c and peroxidase were also used as templates, and the resulting imprinted nanowires showed higher binding capacity toward the corresponding template proteins. The surface imprinted nanowires have a large surface area and are well-dispersed in aqueous media, therefore the imprinted nanowires could be applied to a diverse range of analytical fields.

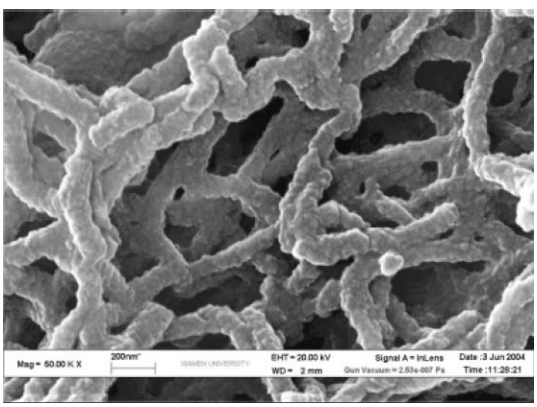


Fig. 7 SEM image of MBAA-based protein-imprinted polymer nanowires. (Reproduced from ref. 34 with permission from American Chemical Society.)

Crystallized proteins are regularly oriented with no mobility and are considered to be a kind of immobilized protein, therefore the transcription of the surface by functional monomers may be easier than conventional molecular imprinting using dissolved free proteins with thermodynamic molecular motion. Matsunaga *et al.* used a crystallized lysozyme as a template.[35] Lysozyme crystals were placed on a cellulose ester membrane, on which were added acrylic acid as a functional monomer, 2-methacryloyl oxyethyl phosphocholine as a co-monomer to reduce non-specific binding, MBAA as a cross-linker, and polyethylene glycol as a precipitant to prevent the crystal from dissolution (Fig. 8). Onto the substrate, a vinylated gold substrate (SPR chip) was placed, and then polymerization was carried out. As references, conventional

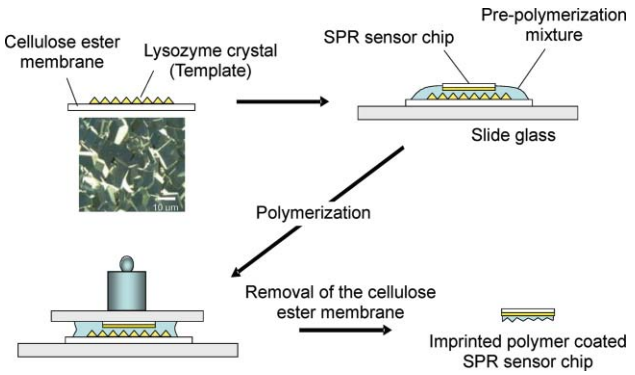


Fig. 8 Preparation procedure of a lysozyme crystal-imprinted thin film. (Reproduced from ref. 35 with permission from Chemical Society of Japan.)

lysozyme-imprinted polymer was prepared by using dissolved lysozyme, and a non-imprinted polymer was also prepared in the absence of lysozyme. The crystallized lysozyme-imprinted polymer had much higher selectivity than the conventional and non-imprinted polymers. Recently, imprinting of protein crystals has been conducted in organic solvents.[36] Organic solvents may affect the conformation of proteins on the surfaces, therefore, careful operations should be conducted in this case.

Two-dimensional protein imprinting

Britt *et al.* achieved ferritin imprinting by using self-assembling ternary lipid films prepared as Langmuir monolayers consisting of cationic dioctadecyldimethylammonium bromide (DOMA), nonionic methyl stearate (SMA), and poly(ethylene glycol) bearing phospholipids (PEG-P) (Fig. 9).[37] The cationic DOMA in the ternary lipid monolayer could be restructured according to the surface charge of adsorbed ferritin at the air/water interface, *i.e.* the lipids were reordered to accommodate ferritin by self assembling. When the ferritin adsorbed monolayer was transferred to a hydrophobic support, no more reconstruction occurred and after the removal of ferritin, the protein-sized pockets were created on the monolayers. This imprinted monolayer showed up to a 6-fold increase in ferritin adsorption compared with the corresponding control monolayers prepared without ferritin. Regarding the selectivity, albumin was tested with the ferritin imprinted monolayers and albumin was unfavorably bound to them. In contrast, albumin-imprinted monolayers showed low binding toward ferritin. Although 2-D protein imprinting often gives rise to such size-dependent non-specific binding, this would be a promising method for protein imprinting, if more biomimetic layers could be prepared by optimization of lipid ratios and careful design of components for specific binding site construction and non-specific binding reduction.

Epitope-imprinting for sequence selective recognition of peptides and proteins

Recently important studies on molecular recognition of proteins have been reported, called "epitope imprinting" by Rachkov *et al.*[38] This technique involves imprinting for a small peptide, whose structure represents a small exposed fragment of oxytocin. The

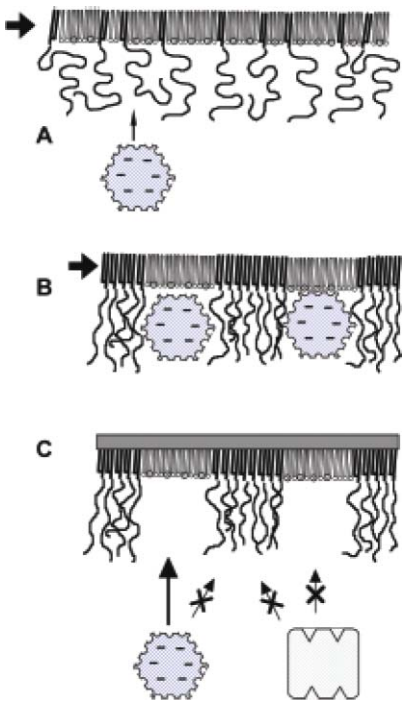


Fig. 9 Schematic illustration of 2D-ferritin imprinting. (A) Ferritin adsorption. (B) The lipids were reordered to accommodate ferritin by self assembling. (C) Immobilization of the monolayer to a solid hydrophobic support, and generation of the imprint sites by removing ferritin. (Reproduced from ref. 37 with permission from American Chemical Society.)

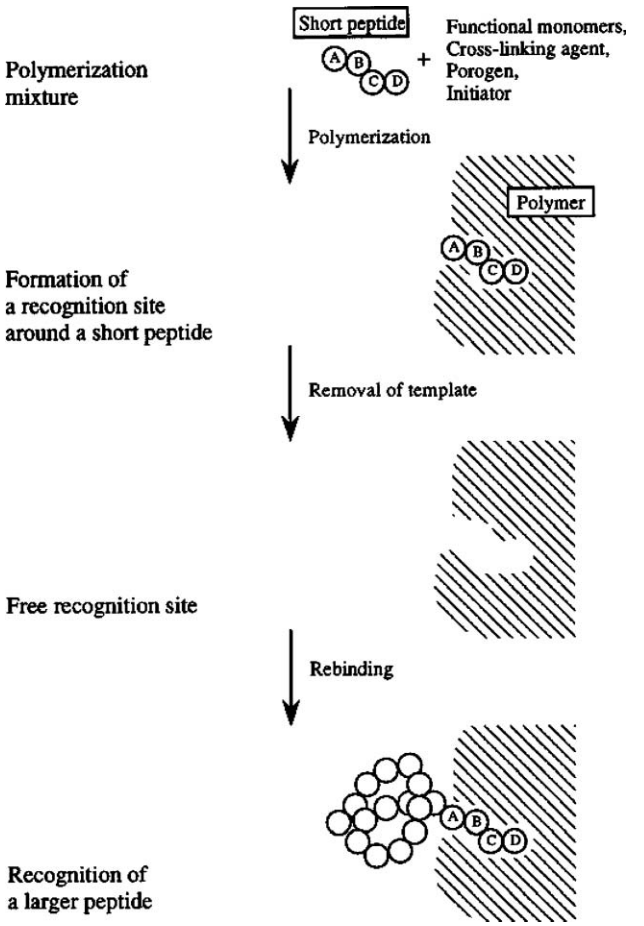


Fig. 10 Schematic representation of the epitope approach for the preparation of oxytocin-imprinted polymer. (Reproduced from ref. 38 with permission from Elsevier.)

resulting polymers efficiently recognized both the template peptide and oxytocin that possess the same C-terminal part of the structure (Fig. 10). Shea *et al.* chose C-terminal 9-mer peptides of target proteins as epitopes and prepared the peptide-imprinted polymers against the immobilized epitopes.[39] The polymer films obtained had high selectivity for the target proteins including cytochrome c, alcohol dehydrogenase and albumin.

Epitope imprinting was also applied to the preparation of MIP-based QCM sensor chips for the diagnosis of dengue virus infection.[40] Acrylic acid, acrylamide, *N*-benzylacrylamide and EDGMA were polymerized in the presence of a linear epitope (15-mer peptide) of Japanese Encephalitis virus NS1 (dengue nonstructural protein 1) to form a thin film on *N*,*N*′-diBoc-L-cystine dibenzylamide treated QCM sensor chips. For serum samples from patients, the sensors could detect dengue virus in 20 to 30 min at a $\mu g\ L^{-1}$ scale and the sensor responses were correlated with ELISA results (correlation coefficient: 0.73). The results illustrate the potential of epitope-imprinted polymers on sensitive sensor systems for diagnostic and biotechnological applications.

These data clearly show that such epitope-imprinting approaches are effective in providing sequence-specific recognition of proteins. Epitope imprinting could open a new way to prepare imprinted polymers for the recognition of various kinds of unknown or unidentified proteins, if small parts of proteins could be known from the corresponding DNA sequence analyses. This convenient technique may become essential for proteomics as a future technology in biotechnology and medicine.

Conclusions

Today, protein-profiling and its application to diagnostics for diseases draw much attention, thus proteomics is an important technology in medicine and biotechnology. However, expression of antibodies still requires elaborate and complicated processes. Therefore, for protein detection and analyses, simpler, easier and higher throughput techniques should be developed. Synthetic materials could be ideal for next-generation protein analysis due to their stability, repeatability, and inexpensiveness. To achieve this goal, more sophisticated and precise recognition systems should be integrated into the current protein imprinting technologies. Although molecular imprinting of proteins still needs to be improved, it is now surely emerging as a powerful tool for protein recognition, where biochemists, polymer chemists and organic/inorganic chemists should collaborate to design and produce highly organized systems for molecular recognition.

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