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Recent Advances in the Study of Protein Imprinting

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Abstract: Molecular imprinting is an important tool for generating synthetic receptors with specific recognition sites. The resulting artificial receptor has extensive applications in chromatographic stationary phases, solid phase extraction, catalysis, drug delivery and sensors. The synthesis of molecularly imprinted polymers (MIPs) specific for proteins has been proved challenging due to a number of inherent problems in protein imprinting but potentially rewarding work. Hence, this review discusses recent advances in various synthetic protocols developed to overcome the obstacles, focusing on their advantages, disadvantages and potential future direction. In addition, the case of the selection of the functional monomers has also been reviewed here.

Keywords: Molecular imprinting, protein recognition, monomer selection

INTRODUCTION

In nature, many essential biological interactions are governed by the selective recognition of biological molecules. Yet, to develop synthetic receptors with affinity and specificity mimicking these processes remains a major challenge. Nowadays, molecular imprinting technology has become an important tool in

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the preparation of synthetic receptors due to a number of distinct advantages including the durability, the specificity and the ease of mass production and the robustness of the recognition element (1). Hence molecularly imprinted polymers (MIPs) have been widely used in chromatographic stationary phase (2), solid phase extraction (3, 4), catalysis (5), drug delivery (6) and various sensors (7, 8).

The common principles of molecular imprinting are summed-up in the scheme shown in Figure 1. One or more of various types of polymer-forming components (functional and cross-linking monomers) are copolymerized in the presence of a target species that acts as a template. A complex between the functional monomers and the template is initially formed (covalently or non-covalently). After they have been polymerized, the template is removed by washing with the solvent or a combination of chemical treatment. The material containing recognition sites of a complementary shape and functionality to the template is obtained. To date, two main approaches to molecular imprinting have been developed; the covalent and the non-covalent approach. The covalent imprinting refers to molecular imprinting strategies whereby the functional monomers and template molecules are formed complexes via reversible covalent bonds prior to polymerization; the rebinding of the templates to MIPs is based on the covalent bonds. The non-covalent imprinting uses only non-covalent forces such as hydrogen bonds, ionic interactions, hydrophobic interactions and metal-chelating interactions for both the molecular imprinting process and the subsequent rebinding (9).

Until now, MIP approach has already been successfully used for the specific recognition of drugs, small analytes, peptides and sugars (10–13). But protein imprinting has met with only limited success due to several inherent limitations of the protein templates.

i. Size. The molecular size of the protein makes it difficult to slip in and out of a traditional polymer monolith. Such low cross-reactivity and permanent template entrapment results in inadequate recognition properties;
ii. Complexity. The complexity of the proteins structure, e.g., the variety of their sequence and folding motives is also the challenge of imprinting

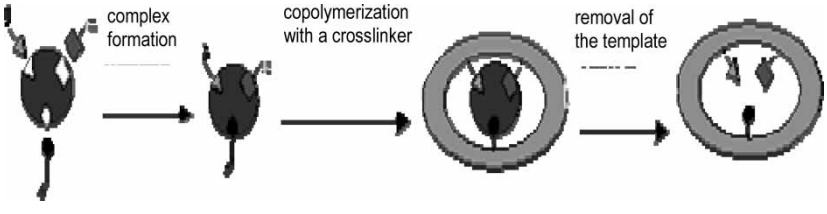

Figure 1. The imprinting process.

proteins. A high number of potential interactions between a protein and corresponding polymer will lead to an increase in nonspecific interactions, hence unsatisfactory specificity (14);

iii. Flexibility. Due to the high flexibility of conformation, the protein is sensitive to environment such as pH, salt concentration, temperature or phase, which determines that the well-defined recognition sites are hard to produce;

iv. Solubility. Most of the proteins are not stable or soluble in apolar solvents, which is not compatible with the mainstream MIP technology relied on organic solvents for the polymer preparation. Moreover, this also limits the choice of suitable monomers of imprinting proteins since most conventional monomers are insoluble or partially soluble in water.

Though protein imprinting has proved to be one of the most challenging tasks, many research groups have engaged in exploring novel synthetic strategies and new types of monomers for recognition of proteins since the MIPs of proteins have great potential for applications.

SYNTHETIC PROTOCOLS

Acrylate Chemistry

The group of Hjertén has pioneered the use of the polyacrylamide gel as imprinting matrices (14, 15). The choice was inspired by the inert nature of the polyacrylamide gel, its biocompatibility and its neutrality; thus the nonspecific interactions were expected to be minimized (16). Acrylamide (AAM) and N, N′-methylenebisacrylamide (MBA) have been used to imprint the following proteins: hemoglobin (Hb), lysozyme (LSZ), ribonuclease (RNase), cytochrome C (Cyc), human growth hormone (HGH), transferrin (Tf) and myoglobin (Mb) (14). After the gel was pressed through a sieve, the particles obtained were packed into chromatography columns. The binding affinity of each imprinted polymer was evaluated by measuring the difference in protein retention times on imprinted and non-imprinted columns. The results showed a highly selective recognition of the templates. For example, the polyacrylamide gel could resolve horse Mb from whale Mb. There is a difference in 20 residues of a total sequence of 153 residues between the two molecules. However, the recognition mechanism is still uncertain and it was hypothesized that a large number of weak bonds formed between the gel and the protein, giving an overall strong interaction and hence the success of the imprint (14).

Though the polyacrylamide gel imprinting strategy shows a higher specificity and a broader applicability compared with the metal chelating approach (see metal chelating section), it still suffers from some limitations. For example, some proteins entrapped in the deep parts of the polyacrylamide

monoliths cannot be removed successfully, which affects the imprinted efficiency to some extent. Moreover, in general bulk acrylamide materials specific for proteins relying on low cross-linking polymers tend to lose their imprinted properties rapidly and are less stable with respect to changes in environment (17). Therefore several groups have endeavored to modify the acrylamide chemistry at various aspects.

Silica was used as a rigid support and incorporated charged functional monomers in the polymeric backbone (18–20). Two functional monomers acrylic acid (AAC) and AAM, together with cross-linkers in the presence of the proteins, were polymerized on the surface of functionalized silica beads, leading to the thin layer polymer for the recognition of glucose oxidase (GOD). Binding assays showed selectivity for the imprinted protein GOD over bovine serum albumin (BSA). Such materials were then exploited to imprint LSZ by the same group (20). The resultant silica exhibited selectivity for LSZ over Hb, though non-specific binding of both proteins did occur. The recognition effect was attributed to both weak electrostatic interactions and shape conformation between the template protein and imprinted polymer particles.

The similar method for imprinting LSZ was furthered to three-dimension (3D) by Ou et al. (21). Methacrylic acid (MAA), AAM and 2-(dimethylamino) ethyl methacrylate were used for preparation of a recognition polymer targeted for LSZ binding. The resultant polymer was sieved and then washed with deionized water, NaCl solution and then water to remove the template. It is a mild elution way in contrast to the former acidic wash step. The imprinting factors, calculated as a binding ratio between MIP and control polymers, were 1.83–3.38. However, the imprinted polymer could not discriminate efficiently between different proteins in that it also bound the competitor protein, albumin ($\sim$13.3% w/w from protein solution).

Vaidya et al. optimized the cross-linker concentration to improve the cross-reactivity of the acrylamide materials (22). In the process, a complex of trypsin and its inhibitor N-acryloyl para-aminobenzamidine (Ac. PABA) was polymerized with AAM and MBA. Then the protein was extracted out of the resultant gel. The imprinting effect was demonstrated in a competitive binding assay mixture of trypsin and chymotrypsin. The Scatchad analysis of the system showed that the imprinted polymer exhibited a straight line which is a characteristic of a true receptor, while the non-imprinted polymerl exhibited a curvilinear plot, indicating that it functioned as an affinity chromatography gel. The optimization of the cross-linker concentration ensured minimum swelling, suitable pore size to allow maximum transport of the protein and imprint integrity (22).

Huang et al. developed another technique to address the low cross-reactivity of the polyacrylamide gels (23). They synthesized an amphoteric polymer by copolymerizing the two functional monomers (MAA and N-[3-(dimethylamino)propyl] methacrylamide) in the presence of the proteins BSA or LSZ with $CaCO_3$ as a pore-forming agent. The resultant gels were then treated with acid to remove the $CaCO_3$ and with a pronase to digest

the template. The chromatography experiment demonstrated that only LSZ bound to its equivalent polymer but not in the BSA imprinting system. The results showed that the use of $CaCO_3$ as a pore-forming agent facilitated the movement of the protein through the materials.

In a recent paper, BSA imprinted polyacrylamide gel beads were prepared via inverse-phase suspension polymerization by Pang et al., using AAM and MBA as polymeric matrix components and MAA as functional monomer (24). They obtained good quality spherical beads which exhibited visible macroporous structure, facilitating the movement of proteins through the material. The selectivity test showed the produced beads that had a relatively high separation factor ($\alpha = 4.71$) exhibited good recognition for BSA as compared to a challenging protein ovalbumin. The success was attributed to steric factors and multiple-point electrostatic interactions, similar to that suggested by Hjertén (14). At any rate, the strategy is a simple protocol with clear optimization for protein imprinting.

Hawkins and his coworkers investigated the effect of the washing methods on template removal (25). In the case, a sodium dodecyl sulfate (SDS, a strong cationic surfactant): acetic acid ratio of 10%:10% was found to be the most efficient, with 90% of subsequently reloaded template. This novel procedure showed superior removal efficiency compared with the in-use protocol based on trypsin digestion.

Guo and coworkers proposed a possible strategy to overcome the limited mechanical strength of acrylamide materials (26). They chose macroporous chitosan beads as a matrix. Chitosan is a natural polysaccharide; a biopolymer comprising D-glucosamine and D-acetyl-glucosamine, which is non-toxic and bio-absorbable (27). The imprinted polymers were achieved by letting AAM and the protein diffuse into the pores of the chitosan beads before polymerization. The protein was then removed after polymerization by washing with an acetic acid/SDS solution. The imprinted beads were shown to have greater capacity and higher selectivity for the template protein than the non-imprinted polymer with the same chemical composition. The same group further applied the chitosan beads in the chromatographic stationary phase, where the selectivity of Hb and BSA was demonstrated (28). The soft polyacrylamide gel was entrapped in the beads and so the obtained polymer beads would possess good mechanical strength and chemical stability, as well as hydrophilicity. However the protocol was time-consuming due to obtaining the equilibrium of mass transfer.

In conclusion, acrylate chemistry in aqueous solution is considered the general and popular technique for protein imprinting because the material is water soluble, cheap, easily produced and can be engineered to possess attractive structural parameters (25). Some success was gained with the methods described above; but we can safely deduce that it is far from an ideal strategy for working with proteins. First, the polyacrylamide gel is generally prepared with a low degree of cross-linking to ensure reasonable mass transfer, but leading to soft texture and losing the imprinted property

rapidly with respect to changes in environment. Second, some proteins entrapped in the imprinted polymers also affect imprinted efficiency. Moreover, the reliance on aqueous solvent for the imprinting step limits the choice of suitable monomers (29). As consequence, a number of other novel strategies were explored to imprint proteins.

Sol-gel Made of Organic Silanes

The first success of protein imprinting reported was achieved by the group of Glad (30). They developed a method of direct coating an imprinted polymer onto the surface of porous silica beads modified with silanes. A mixture of organic silanes prepared for imprinting glycoprotein Tf was polymerized on the surface of silica particles in aqueous solution, including a new boronate-silane functional monomer designed to interact with the carbohydrate portion of Tf. The high performance liquid chromatography (HPLC) experiment demonstrated the imprinted silica beads had superior enzymatic properties compared to control beads (40% for the imprinted beads versus 5% for the bulk beads). The beads also showed a higher affinity for its template than its competitor BSA (relative retention of 2.16). From these results, it is evident that silane imprints can discern differences in tertiary structure of proteins.

As consequence, Venton et al. explored the polysiloxane chemistry and demonstrated that the organo-functional side chains on the silanol monomers tended to associate with complementary residues on the protein surface during the polymerization process, leading to complementary binding pockets for the protein on the polymer (31, 32). The polymer prepared from 3-aminopropyltriethoxysilane (APTMS) and tetraethylorthosilicate (the molar ratio of 1:3) in the presence of the protein (urease or BSA) was able to recognize the template and showed preferentially binding ability. In order to further investigate the assembling of the monomers in the polymer, two more closely related proteins labeled with ^{125}I (^{125}I-Hb and ^{125}I-Mb) were studied. The results demonstrated that the rebound proteins did not equilibrate with the labeled solution proteins, indicating a very tight association with the polymer surface. However, a high percentage of templates remained in the polymer due to its high degree of cross-linking.

A recent paper published by Shiomi et al. exploited another approach based on the use of the organic silanes (33). They combined the silica materials and the concept of template immobilization (see section of molecular imprinting nanotechniques). In the case, the protein template Hb was covalently immobilized on the silica by forming imine bonds between amino groups on the protein surface and anchored aldehyde groups on silica. Then APTMS and propyltrimethoxysilane (PTMS) were polymerized onto the Hb-silica surface. A comparison between the imprinted silica beads obtained (MIP_i) and imprinted materials formed using conventional free Hb template (MIP_f) was made. The MIP_i showed a slightly higher binding capacity for the template, but a far better performance in

competitive binding experiments. It was able to bind the template specifically, while the competitor proteins such as Tf and chymotrypsinogen A were not absorbed. The authors indicated that the capacity of MIP_i was linked to the isoelectric point and the size of the protein as well as the characteristics of recognition cavities imprinted on the silica (33). The results proved that more homogeneous binding cavities were formed by immobilization of the template.

From this discussion we see the sol-gel made of organic silane monomers has high potential for imprinting proteins, but as yet these are the only the protocols that have been reported.

Metal Chelating

Mallik et al. demonstrated the concept of synthetic receptors composed of scaffolds carrying metal ions coordinating groups to selectively bind protein functional groups on the surface of the protein (34). They used metal ions to coordinate histidine (His) residues of a series of bis-imidazole protein analogues. Taking advantage of these results, Kempe and co-workers used silica particles coated with specific metal chelating monomer to prepare selective adsorbents for HPLC separation of proteins (35). They employed a metal chelating monomer, N-(4-vinyl)-benzyl iminodiacetic acid (VBIDA), with methacrylate-derivated silica particles, in the presence of the template RNase A and metal ions. Reference polymers were made with BSA. Imidazole groups on the surface exposed His residues combined with the monomer VBIDA to coordinate Cu^{2+} ions. During the polymerization, the metal binding ligands were positioned on the silica surface at precise distances as to enable selective rebinding of the protein in the presence of Cu^{2+} ions. The imprinted silica particles were packed into chromatography columns and tested for their selectivity. The results indicated that RNase A was recognized over the control protein LSZ using this method (35).

However, the metal chelating method was not further exploited. The main reason could be that such an approach is limited to the proteins with exposed His residues on their surface; hence it is not universally applicable for protein imprinting. Moreover, metal chelating groups provide strong anchoring points for non-specific interactions, which might impede specificity in real samples.

Molecularly Imprinted Polymers Grafted on the Surface of Microplates

Other than imprinting proteins on the derivated silica as shown above, Pilesky and co-workers proposed a novel technique for grafting imprinted polymers on the surface of microplates. They applied for small molecules, such as atrazine or epinephrine (36) and for proteins (37, 38). In this approach, affinity matrices for proteins were obtained by grafting an imprinted

3-aminophenylboronic acid (APBA) polymer on the surface of polystyrene microtiter plates in the presence of various protein templates. The authors used horseradish peroxidase (HRP), lactoperoxidase, Hb, microperoxidase as templates, which differed in mass and charge. From the rebinding experiments, it was evident that the size of the molecule and its charge affected the imprinted efficiency. The results indicated that small proteins, such as microperoxidase, had high K_d in the μM range while large proteins, such as lactoperoxidase, Hb and horseradish peroxidase, had K_d's a 1000 times smaller. The significant potential for this promising approach was demonstrated between Hb and its glycosylated derivative (HbA_{1c}), with K_d values differing by more than three-fold.

Subsequently, Rick and Chou exploited the same protocol to develop a protein sensor (39). In the case, poly-APBA was deposited onto a quartz crystal microbalance (QCM), using LSZ and Cyc as templates. The authors demonstrated a good selectivity for the template proteins, both on a single LSZ-templated polymer and on a polymer templated with a complex of LSZ and Cyc.

The same group extended the idea to create specific cavities for a structure more complex than a single protein (40). They developed a micro-contact imprinting that involves contacting the microscope cover glass on which a single layer is formed by the protein and functional monomers with a glass support carrying the cross-linkers, after polymerization removing the cover glass to allow surface washing of the polymer; hence the recognition sites for templates are formed (Figure 2). The imprinted film was formed with the functional monomer O-(4-nitrophenylphosphoryl)choline (4NPPC), the cross-linker polyethylene glycol 400 dimethacrylate (PEG400DMA) and the template C-reactive protein (CRP), a protein which is composed of five identical non-glycosylated subunits, each being formed from 206 amino acids, associating into a pentameric structure (41).

The polymer demonstrated good binding capacity and selectivity for the template protein. Under a competitive binding experiment, it was demonstrated that the polymer imprinted with CRP was able to bind 3.78 $\mu g/cm^2$ of CRP versus 0.08 $\mu g/cm^2$ of human serum albumin (HSA), a competitor protein. The authors attributed the success to little or less template entrapment on the surface of the polymer film. In a recent paper, the similar approach was carried out further by the combination with the isothermal titration calorimetry (ITC) to select the monomer and cross-linker prepared for imprinted polymers of RNase A (42). It is believed that micro-contacting imprinting has the potential for recognition of large proteins and combination with the sensor for the detection and quantification of such species.

Molecular Imprinting Nanotechniques

In recent years, nanotechniques have been introduced to the area of molecular imprinting. These nanosized imprinted materials are relatively monodisperse

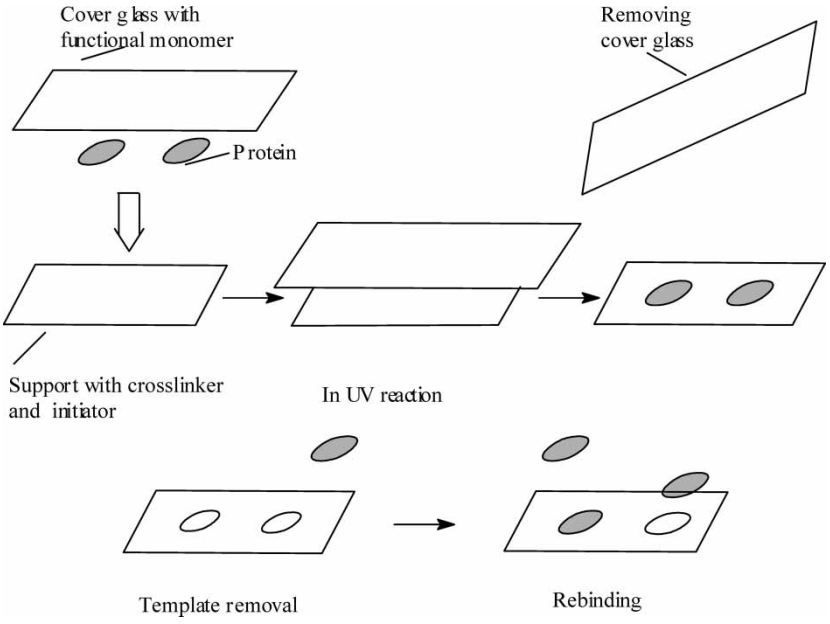

Figure 2. Schematic representation of micro-contact imprinting of a C-reactive protein (adapted from Ref. 40).

and have a moderately high imprinting surface area (43, 44) so the removal of the template molecules from the polymer matrix can be easier and the binding capacity is also improved.

In a paper, Shi et al. used radiofrequency plasma deposition to form nanostructured polymeric thin films around proteins coated with disaccharide molecules (45, 46). Firstly, the template protein was adsorbed onto a flat mica surface, which is hydrophilic and negatively charged. This was important to prevent the denaturation of the adsorbed proteins. Then disaccharides were applied in decorating the protein surface during which multiple hydrogen bonds were formed between the hydroxyl groups of the disaccharide and polar residues on the surface of the protein. This shell was coated with a fluoropolymer film applied by plasma deposition. Then the resulting film was fixed onto a glass cover by using epoxy resin, which was finally cured in an oven. Finally, the mica substrate was peeled out by harsh washing, leaving cavities complementary to the template. Several proteins including BSA, immunoglobulin G (IgG), LSZ and RNase A were imprinted by this technique (45, 46). The selectivity of an imprint for its template protein was proved by a competitive binding experiment, estimating the amount of competitor required to cause a 50% reduction in the maximum adsorption. The two proteins (LSZ and RNase A), which are similar in physicochemical properties and isoelectric points, were used to investigate the shape specificity. In the case of RNase A's

adsorption from a mixture of LSZ and RNase A, there was a 20-fold increase in selectivity for the RNase A imprint, and 26-fold for the LSZ system. The mechanism of recognition was mainly attributed to hydrogen bonding and shape selectivity. Hydrophobic interactions and van der Waals forces were also thought to be involved (45, 46).

Though remarkable affinity and selectivity have been achieved by this technique, it is complex, lacking in generic application. Li et al. reported a novel protocol for creating surface imprinting nanowires by immobilizing the imprint molecules (Hb, Cyc and albumin) onto a porous disposable alumina prior to polymerization with a mixture of AAM and MBA (47). The alumina membrane was dissolved after the polymerization, resulting in the imprinted polymer nanowires with binding sites on the surface (Figure 3). These imprinted nanowires showed a relatively large binding capacity, that, at a fixed nanowire concentration, the imprinted nanowires bound 50% of Hb versus 6.8% for control nanowires. Notably, the nanowires were able to discriminate between bovine Hb and human Hb, which have similar amino acid sequence (differing in only 15% of the 547 amino acid positions), and three-dimensional structures. It was believed that remarkable improvement of binding capacities was attributed to the relatively monodisperse nanowires resulting in a moderately high imprinting surface area (47).

To date, molecularly imprinted nanostructured materials have been seldom explored in recognition of proteins. However, various nanosized imprinted materials containing nanofibres, nanowires and nanotubes possess several remarkable advantages over normal imprinting materials: (1) easy removal of template molecules because of extremely high surface-to-volume ratio; (2) higher binding capacity because of more recognition sites in the proximity of the surface; (3) faster binding kinetics due to easy accessibility to the target molecules (48). It is evident that this promising strategy

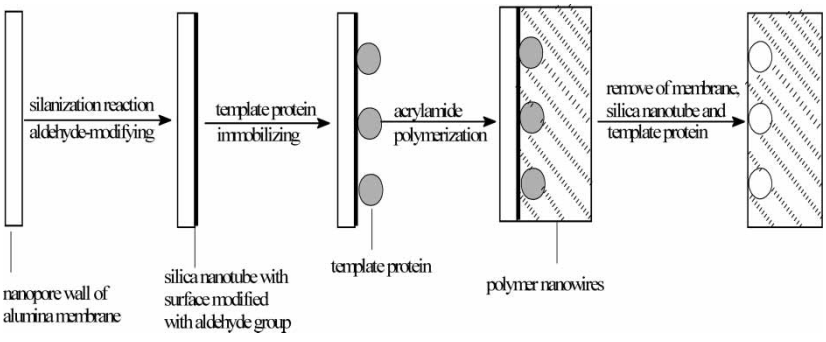

Figure 3. Molecularly imprinted polymer nanowires prepared using template immobilization.

has great potential for preparation of MIPs against proteins with high site homogeneity and large binding capacity.

Epitope Approach

In nature, antibody-antigen interaction depends on the recognition between the antibody and an antigenic site of the protein, the epitope which is short amino-acid sequence complementary to binding site of antibody. Rachkov et al. applied this concept to the protein imprinting and developed an epitope approach (49–51). In the case of imprinting, a short peptide, often exposed at the protein surface, not the whole protein was used as a template for the imprinted polymer preparation. The ultimate goal was to obtain recognition of the parent molecule.

The imprinting procedure used for epitope approach was similar to conventional methods. The polymer specific for the nonapeptide oxytocin was made of MAA and ethylene glycol di-methacrylate. A small oxytocin sequence of three amino acids proved to be enough for recognition of the whole protein (51). The obtained polymers were packed into chromatography columns to define the optimal conditions for the recognition of oxytocin. The results demonstrated selectivity and high affinity for the template molecule. The values of the separation factor, α (ratio between k' for tetrapeptide and k' for oxytocin), was dependant on the concentration of acetic acid and pH. The best α value, 3.46, was obtained at pH 6.0 and 2.5 mM acetic acid. Subsequently, this approach was repeated using an angiotensin II octapeptide in aqueous solvent. However, the resultant polymer retained for the octapeptide, not bound with the parent protein angiotensin II at all.

Later Nishino et al. processed the epitope approach to utilize "exposed" epitopes of proteins to imprint the capture sites (52). First the peptide epitopes of protein targets, Cyc, BSA, alcoholdehydrogenase (ADH) were covalently attached to a glass or silicon surface. Then monomers containing AAM, N, N-ethylene-bis-acrylamide and polyethylene glycol 200-diacrylate polymerized on the surfaces to produce a MIP film. Following separation from the functionalized surface, the polymer film bound and captured the target protein from protein mixtures. By analysis of SDS-PAGE, the films imprinted with the epitopes could selectively capture the target proteins from the protein mixture. The dominant protein that was captured corresponded to the peptide epitope that was used as the surface-attached imprint. The author attributed the binding specificity to collective hydrogen bonding (52).

As the results showed above, the epitope approach needs further exploitation. Since the imprinting is relied on the interaction between the epitope and the polymer, the small point of interaction may not give affinity strong enough between the whole protein and the polymer. Moreover, by far the templates to

be mimicked were small peptides, just 8–10 amino acids long, which also contributed to the failure of imprinting the whole protein. So attention paid to three-dimensional structure of the selected epitope will render the epitope-imprinting play a significant role in recognition of the proteins.

Advantages and Disadvantages of 3D Imprinting and 2D Imprinting

Since the first report about protein imprinting was proposed in 1985 (30), many research groups have engaged in exploring new synthetic protocols for preparation of imprints for proteins, though limited success has been achieved compared to the field of small molecules imprinting.

There are two different schools of thoughts with respect to the design of MIPs specific for proteins. One called 3D imprinting is based on MIPs ability to recognize a template by using a combination of shape complementarity and multipoint weak interactions provided by the monomers, able to form hydrophobic interactions or hydrogen bonds. The other named 2D imprinting is based on the proper placement of a few functional groups able to form focused interactions with the template. For example, the APBA (38) and metal chelating polymers (35) each have their own specific targets on a polymer surface (hydroxyl groups and His residues, respectively). Either strategy has its superiority and shortcomings.

3D Imprinting

The 3D imprinting is hypothesized that a large number of weak bonds are formed between the polymer and the protein, which give an overall strong interaction leading to a high recognition (14). Some experiments proved this theory and gained some success that the MIPs obtained had a high total capacity and relatively high site homogeneity (18–20, 24). The 3D strategy is popular as the procedure can be universally adopted. Nevertheless it has some inherent problems. Generally, it requires materials with high porosity for mass transfer of the proteins, leading to less stability and ease of losing the imprinted property rapidly with respect to changes in environment. In some cases environmental change can even result in pore collapse and permanent loss of function. Guo and coworkers proposed a possible strategy using macroporous chitosan beads as a matrix to overcome the limited mechanical strength and stability (26, 28). On the other hand, dense matrices to obtain stability and function lead to template entrapment in the imprinted polymers and some decrease of total capacity. Moreover, dense structure requires grinding to expose binding sites, which diminishes specificity greatly as larger binding sites are destroyed by mechanical process of grinding. Hawkins and his coworkers optimized the washing method for template removal (25).

2D Imprinting

An alternative approach called 2D imprinting has made great progress in the protein imprinting. It provides substantial advantages for a large size molecule, the protein. Imprinting the specific areas of proteins improves binding kinetics, avoiding the excessively protracted time needed to attain diffusion-controlled equilibrium in the interior of three-dimensional polymeric matrices (39). Furthermore, the imprinted polymers have enhanced stability due to the supports and can be integrated with the sensors. However, the 2D imprinting also reduces the total capacity of a material and is not universally adopted because of specially adapted protocols. Moreover, only a small portion of the surface of the protein as anchor points causes site heterogeneity inevitably, hence not high specificity. Shi and co-workers developed another surface imprinting procedure for protein recognition, oriented immobilization of the template on sacrificial solid supports (45, 46). Recently, taking advantage of this concept, the polymer nanowires have been applied to the recognition of proteins (47). All the experiments showed more homogeneous binding cavities were formed by immobilization of the template. Furthermore, the template which is insoluble in the polymerization matrix can be easily used and protein aggregation can be minimized.

Functional Monomers

It is assumed that a pre-polymerization complex is formed between the template protein and functional monomers. Most conventional monomers used in organic imprinting are not soluble in water, hence selection of monomers suitable for imprinting proteins is predominant and different from that used in small molecules imprinting. However, it is far from an easy task and at present, traditionally acrylate monomers are still the most popular. Some researchers encountered the materials with advantageous features such as APBA, organic silanes and the MIPs prepared with them represented notable recognition property. Recently, the analogue of the template's natural receptor has also been paid attention to in search of new types of monomers.

Selection of Conventional Monomers for Imprinting in Organic Solvent

Imprinting proteins by using traditionally acrylate monomers in organic solvent is the most popular. Hjertén et al. pioneered the use of polyacrylamide gel for specific imprint proteins (15). They attributed the difficulty in synthesizing a gel for ideal protein imprinting to the increasing number of binding sites. Therefore, it was indicated that monomers with very strong bonds should be replaced with those more weakly, and the number of bonds must

be large, giving an over-all strong binding. Inert and biocompatible AAM was believed to be appropriate monomers (16). The experiments showed that a column of low degree of cross-linking acrylamide gel could even resolve horse Mb from whale Mb. The high recognition specificity was attributed to a large number of weak interaction such as hydrogen bonds. As consequence, many groups used the similar polyacrylamide gel to entrap proteins. Some of them incorporated charged monomers such as MAA, N, N′-diethylaminoethyl methacrylate (20, 21, 24). It was postulated that the recognition of the protein can be realized through the optimum distribution of positive charges, negative charges and hydrogen bonds (21). Though some success was gained, the acrylate monomers are not ideal for working with proteins, since the polyacrylamide gel has low cross-reactivity; poor stability and mechanical strength as discussed above (see the section of acrylate chemistry).

Most researchers chose monomers from the classic work for reference and some did according to a "feel" that was due to their experience and the structure of the template. However it was lack of rational instruction to choose suitable monomers for imprinting proteins. The group of Hjertén explored a universal method for rapid screening of interactions between the potential monomers/gels and the proteins (53). It assumed that the absorption maximum of a protein changed when the protein interacted with the free monomers or the adsorbent synthesized from the monomers and did not change when the protein did not interact. The spectra of the template Hb in the absence and presence of the monomers (AAM and MBA) were recorded. The results showed the absorption maxima of spectra were different, which was indicative of an interaction between the monomers and the protein. The authors speculated that a shift in an absorption maximum of the protein was only a necessary but not sufficient condition that the gel prepared from this monomer would be selective for the protein. They repeated the experiment using albumin instead of Hb and no shift in absorption maxima was observed. It is in accordance with the previous result that the albumin was not absorbed by a polyacrylamide gel prepared in the presence of the protein. In the system of agarose and Hb, the agarose beds failed to absorb Hb selectively due to no shift in the absorption maximum of the protein. This spectroscopy method is simple and applicable generally, but only a qualitative measurement.

In a recent paper by Hsu et al. a quantitative method based on the ITC was used to select the appropriate cross-linker for the preparation of RNase A imprinted polymer (42). In the case, various cross-linkers or MIPs with different compositions were titrated in a cell containing an RNase A-coated substrate, using microcalorimeter to monitor the heat flux into or out of the titration cell. The results demonstrated the isothermal titration was well correlated with the binding experiments. The cross-linker PEG400DMA, the imprinting effectiveness (the ratio of MIP and non-imprinted polymer NIP rebinding efficiency), was relatively high (3.33) and had the largest saturated heat response difference (ΔQ_{sat}). It was as high as 100 mJ. From

the experiment it is clear that microcalorimetry has great potential for rational design of imprinted polymers for proteins.

Selection of the Analogue of the Template's Natural Receptor

Affinity interactions between active sites of enzymes and receptors are very strong and selective for a given pair of enzyme and ligand in contrast to hydrogen bonds, electrostatic interactions etc., which are conventionally used in the imprints of proteins (54). Taking advantage of the molecular imprinting, Vaidya and co-workers made use of enzyme-inhibitor interactions to synthesize imprinted polymers for trypsin (22). It is known that PABA is the natural receptor of the trypsin (55). The guanidine group present in PABA exhibits strong affinity for the aspartate group in the active site for trypsin, hence the author chose Ac.PABA as the trypsin-specific affinity monomer (Figure 4). The molar ratio required for the formation of monomer-template complex was determined by the active-site titration of the enzyme with its inhibitor. Taking into account that the excess of Ac.PABA would act as merely an affinity chromatography ligand, the trypsin Ac.PABA molar ratio was 1:127. The results showed that the imprinted polymer synthesized with affinity monomers, I(50), demonstrated superior recognition of trypsin than the non-imprinted polymer made of Ac.PABA, NI(50) and the imprinted polymer polymerized traditionally using acrylate monomers. Trypsin uptake by I(50) was 0.620 mg/g, which was almost three times higher than that of NI(50).

Chou's group used the similar concept to imprint CRP (40). It is composed of five identical non-glycosylated subunits, each formed with 206

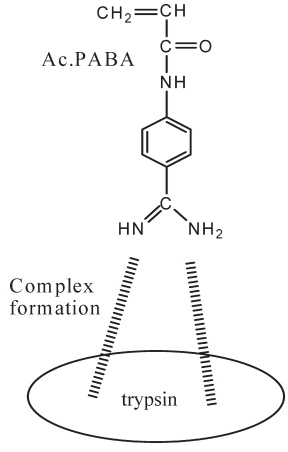

Figure 4. Scheme of the acryloid-para amino benzamidine – trypsin interaction (adapted from Ref. 22).

amino acids, associated as a flat cyclic pentameric disc, in which the overall structure has a molecular weight of approximately 115,000 (41). It is known that the phosphorylcholine (PC) is a natural ligand for CRP in a Ca^{2+}-dependent manner, in vivo. In the case, 4NPPC, the commercially available phosphorylcholine derivative of PC was chosen as a functional monomer, together with a fixed concentration of Ca^{2+} ions (56, 57). The polymer produced by the micro-contact imprinting, demonstrated a good selectivity that under competitive binding experiment, the polymer imprinted with CRP was able to bind 3.78 $\mu g/cm^2$ of CRP versus 0.08 $\mu g/cm^2$ of HSA, a competitor protein. The results proved that the use of affinity monomers was a viable means of forming imprints of significantly larger globular proteins.

Selection of Monomers with the Advantageous Features

Glad's group "encountered" organic silanes when they were searching for new types of monomers applicable to imprint proteins (30). The unique nature of the materials provides certain potential advantages for imprinting proteins. First, a plethora of organosilanes with different functional groups is available. Imprinting in aqueous solvent is based on non-covalent bonds containing ionic, hydrophobic and hydrogen interactions which are relatively weak, therefore it is necessary to use a mixture of different monomers that can interact cooperatively with different parts of the template proteins to obtain imprints with high selectivity. Second, the silanes which spontaneously hydrolyze to silanols and polymerize to organic polysiloxanes can be used in aqueous solutions. Furthermore, the rigidity of the polymers can be controlled by controlling the relative concentration of trialkoxy-, dialkoxy-, and monoalkoxysilane monomers (32). In the case of imprinting, a mixture of organic silanes was polymerized on the surface of silica particles in aqueous solution. The HPLC experiment demonstrated that the imprinted silica beads had superior enzymatic properties compared to control beads (40% for the imprinted beads versus 5% for the bulk beads). The beads also showed a high affinity for its template than its competitor (relative retention of 2.16). Undoubtedly, organic silanes will find increasing use in preparation imprints of proteins due to its novel features. However, the examples of imprinting proteins using organic silanes are few.

Another prominent material for imprinting proteins is APBA. It was first used for imprinting proteins by Piletsky et al. and the polymers obtained had a high recognition property (37, 38). Then Chou's group extended to integrate the poly-APBA with OCM (39). It is known that the APBA is able to mediate recognition through a variety of reversible covalent and electrostatic interactions. The imprinted polymer synthesized from APBA, containing no additional cross-linker, possesses an equal number of positive and negative residues, leading to well complementary matching with the template. Moreover, the APBA chemistry can be integrated into sensor systems.

Nevertheless, the interactions with respect to rebinding are highly dependent on environmental conditions (38).

CONCLUSION

The prevalent works above are listed in Table 1. From Table 1, it is evident that imprinting proteins is a reality, though there are still problems as shown next.

i. Low affinity. The majority of materials for imprinting small molecules have exceptional high affinity. For example, the dissociation constant (K_D) for biotin using computationally designed imprinted polymers ranged from 1.4 to 16.8 nM (58), in comparison with K_D's of 1.5 μM for microperoxidase, 0.54 μM for lactoperoxidase, and 0.056 μM for Hb, using a APBA matrix by the group of Bossi (38). The K_D of proteins is an order of magnitude lower than that of small molecules.
ii. Low specificity. Limited success in separation of templates from a mixture of competing proteins has been achieved (26) and on the other hand selectivity is decreased by high cross-reactivity (20, 31, 32).
iii. Limited proteins. Most of the proteins used for the templates are the monomeric proteins, generally one single chain, the sizes of which are small ($M_w < 100$ kDa) and the isoelectric point are below 7.
iv. Limited monomers. The monomers used for preparation of MIPs selective to proteins are limited to acrylate monomers, silane monomers and APBA etc. A few successful protocols with prominent efficiency are complex, labor-intensive and lack generic application (45, 59).

Since the year 1985 the first imprinted polymer specific for protein was reported, there has been a growing interest in the field of protein imprinting as the history shown in Table 2. However, the success is limited compared to the small molecules imprinting, due to the inherent limitations of the proteins. Different approaches have been developed to overcome the obstacles; the most important ones containing 3D and 2D imprinting which demonstrated fair affinity and specificity, and have been presented in this review. It is clear that further work of each method is needed, using a wider range of proteins and monomers. Furthermore it is clear that the selection of monomers for proteins imprinting is limited to a "local minimum." Experienced workers develop a "feel" for which monomers are likely to give good results, but there is a tendency sticking with prior literatures. However, some possibly unexpected monomers might work better than the tried recipes.

With this in mind, better understanding of the fundamental molecular mechanisms governing molecular imprinting procedures by studying the intermolecular interactions of proteins and functional monomers will enable the

Table 1. Examples of imprinting proteins

Type	Refs	Protein (pI, M_w/kDa)	Polymer composition	Functional interaction	Imprinting method	Separation/ detection system	Affinity	Specificity
Acrylate chemistry	(14–15)	Hemoglobin, Lysozyme, Ribonuclease, Cytochrome C, Human growth hormone (4.9, 21.7), Transferrin, myoglobin (6.8–7.2, 17.5)	Acrylamide, N,N′-methylenebis-acrylamide	A large number of weak electrostatic bonds	Polyacrylamide gel based polymers	Chromatography phase	Various	Various
	(18–20)	Glucose oxidase(4.2, 154), Lysozyme	N,N′-1,2dihydroxyethylene-bis(acrylamide), N,N′-methylene-bis-acrylamide, Acrylamide, Acrylic acid	Massed Electrostatic interaction	Modification of silica beads with acrylate based polymer	Batch binding, enzyme activity test; QCM	0.557 mg/g of polymer for glucose oxidase imprint; 0.8 mg/mL by QCM for lysozyme	Not shown for glucose oxidase system; demonstrated between hemoglobin and lysozyme on lysozyme system
	(21)	Lysozyme	Acrylamide, Methacrylic acid, 2–(dimethylamino) ethyl methacrylate	Electrostatic interaction	Polyacrylamide gel based polymers	Solution depletion	12.5–43.8% w/w	1.34–3.38

Acrylate chemistry	(22)	Trypsin(5.35–5.45, 5), Chymotrypsin(8.1–8.6, 25)	Acrylamide, N,N′-methylen bisacrylamide, N-acryloyl para-aminobenzamidine	Enzyme-ligand interactions and a large number of weak electrostatic bonds	Combination of affinity separation and molecular imprinting	Solution depletion	Capacity of 0.7 mg/g of polymer	2.92 for trypsin imprinted/non-imprinted; 1.92 for trypsin/CTrp on the trypsin specific polymer
	(23)	Bovine serum albumin, Lysozyme	Methacrylic acid, N-[3-(dimethyl amino)propyl] methacrylamide	Cooperative multiple electrostatic interactions	Amphoteric polymer for direct protein separation by HPLC	Chromatography phase	Lysozyme imprint shows enhanced rebinding of template	Slight cross-reactivity between two systems
	(26, 28)	Hemoglobin (6.8–7.0, 65)	Acrylamide	Electrostatic interaction	Macroporous chitosan beads with acrylamide matrix	HPLC; solution depletion	Adsorption capacity 12 mg/g	42.7 (hemoglobin) 1.41 (bovine serum albumin)
Sol-gel made of organic silane	(30)	Transferrin (5.9, 80–95)	Borate-silane complex	Multiple hydrogen bonds	Direct imprint of protein onto silane modified silica particles	Chromatography phase	Not shown	Relative retention of transferrin over bovine serum albumin of 2.16
	(31)	Urease (5.99, 550), Bovine serum albumin	3-aminopropyl triethoxysilane, Tetraethylortho silicate	Multiple hydrogen bonds	Traditionally imprinted sol-gel monolith	Solution depletion	60–90% rebinding	Binding factor of 1.5 for about protein specific MIPc
	(33)	Hemoglobin	3-aminopropyl triethoxysilane, Propyltrimethoxy silane	Multiple hydrogen bonds	Modified silica surface	Solution depletion	Not directly measured	Demonstrated between hemoglobin and a range of completing protein

(*continued*)

Table 1. Continued

Type	Refs	Protein (pI, M_w/kDa)	Polymer composition	Functional interaction	Imprinting method	Separation/ detection system	Affinity	Specificity
Metal chelating	(35)	Ribonuclease A (9.6, 13.7), Lysozyme (7.0, 14.3)	Metal chelating monomer, N-(4-vinyl)-benzyl iminodiacetic acid	Metal chelating	Specific monomer used to coat silica particles	Stationary chromatography phase	Capacity factor of 5.79 compared to 2.68 on ref	2.35 between ribonuclease A and lysozyme
Molecularly imprinted polymers grafted on the surface of microplates	(37–38)	Hemoglobin, Horseradish peroxidase (7.2, 44), Microperoxidase (4.7–4.9, 1.1), Lactoperoxidase (5.5, 77)	3-aminophenyl boronic acid	Reversible covalent and electrostatic interactions	Supported polymers grafted to wells of polystyrene microplate	Solution depletion	Various	Various
	(39)	Lysozyme, Cytochrome C(10.2–10.8, 12.38)	3-aminophenyl boronic acid	Reversible covalent and electrostatic interactions	Grafted layers onto gold QCM crystal	QCM microcalori-metry	Various	Various
	(40 45–46)	C-reactive protein (4.82, 115), Lysozyme, human serum albumin (4.7, 66.5) bovine serum albumin, Lysozyme, Ribonuclease A, ImmunoglobulinG (5.5–8.3, 150), Fibrinogen (5.5, 330)	O-(4-nitrophenyl-phosphoryly) choline/ PEG400dimethacrylate disaccharide-coated hexafluoropropylene on fixed support	Enzyme-ligand interaction hydrogen bonding hydrophobic interactions and Van der Wals forces	Micro-contact approach with thin films(protein stamping) thin films formed around proteins coated with disaccharide layers	Competitive binding by ELISA competitive adsorption using radiolabel target protein	3.78 ng/cm^2 for CRP 2.66 μg/cm^2 for HSA not directly measured	0.08 ng/cm^2 for human serum albumin 0.27 μg/cm^2 for C-reactive protein 5–26 depending on substrate

Molecular imprinting nano-techniques	(47)	Bovine serum albumin, Hemoglobin, Cyto-chrome c, Horseradish peroxidase	Acrylamide, N,N′-methylenebisacrylamide	Electric interactions	Polymer nano-wires via sur-face imprinting	Solution depletion	50% of hemo-globin versus 6.8% for control nanowires	Demonstrated between bovine hemoglobin and human hemoglobin
Epitope approach	(49–51)	Angiotensin αoctapep-tide attempt to bind the whole corresponding protein	Sodium acrylate/poly (ethylenglycol) diacrylate	Antigen-anti-body interaction	Peptide chain selectivity for an angiotensin αoctapeptide epitope approach	Chromato-graphy phase	0.4 μg/mL detection limited by HPLC for octapeptide	19.2–2.10 depend-ing on environment
	(52)	Cytochrome c, bovine serum albumin, alcoholdehydrogenase	Acrylamide, N,N-ethy-lenebis-acrylamide, polyethylene glycol 200-diacrylate	Collective hydrogen bonding	The film imprinted with the peptide epitope for rec-ognition the target protein	SDS-PAGE; mass spectrometry	22.4 pmol/cm^2 for Cyc, not shown for BSA and alcohol-dehydrogenase	Demonstrated between target pro-teins and the protein mixture

Table 2. The history of imprinting proteins

Time	Country	Strategy	Achievement	Refs.
1985	Sweden	2D Sol-gel	First protein imprinting reported, direct imprint of protein onto silane modified silica particles	(30)
1994	USA	2D Metal chelating	Demonstration of the concept of synthetic receptors composed of scaffolds carrying metal ions coordinating groups to selectively bind protein functional groups	(34)
1995	Sweden	2D Metal chelating	Specific metal chelating monomer used to coat silica particles	(35)
1995	USA	3D Sol-gel	Traditionally imprinted sol-gel monolith using organic silanes monomers	(32)
1996	Sweden	3D Acrylate chemistry	Polyacrylamide gel based polymers	(15)
1996	Japan	2D Acrylate chemistry	Modification of silica beads with acrylate-based polymer	(18)
1999	USA	Nanostructured thin films	Nanostructured thin films formed around proteins coated with disaccharide layers	(45)
2000	UK	2D APBA chemistry	Supported polymers grafted to wells of polystyrene microplate	(36)
2000	Japan	2D Epitope approach	Use the peptide exposed on the surface of the protein as a template	(49)
2001	India	3D Acrylate chemistry	Optimize the cross-linker concentration to overcome low cross-reactivity	(22)
2001	Sweden	3D Acrylate chemistry	Pioneer the rapid screening of interactions between the potential monomers/gels and the proteins by UV	(53)
2002	China (Taiwan)	3D Acrylate chemistry	Incorporate charged functional monomers	(21)

2004	China	3D Acrylate chemistry	Use macroporous chitosan beads made of polyacrylamide gel to overcome the less stability and soft texture	(26)
2005	UK	3D Acrylate chemistry	Optimize the washing method to overcome the difficulties of the template removal	(25)
2005	China	3D Acrylate chemistry	Use pore-forming agent to overcome the low cross-reactivity	(23)
2005	China (Taiwan)	2D APBA chemistry	Imprint two proteins simultaneously	(39)
2005	China (Taiwan)	2D APBA chemistry	Exploit a micro-contact approach with thin films	(40)
2005	Japan	2D sol-gel	Combine the 2D sol-gel and the template immobilization	(33)
2006	China	3D Acrylate chemistry	Adopt inverse-phase suspension polymerization to overcome the low cross-reactivity	(24)
2006	China	Polymer nanowires	Polymer nanowires via surface imprinting	(47)
2006	China (Taiwan)	2D Acrylate chemistry	Use ITC to select the appropriate cross-linker for polymers imprinting proteins	(42)

development of more effective imprinted polymers specific for proteins. Some sophisticated analytical techniques such as NMR, CD and molecular modeling which have accelerated the development of the natural receptors and host-guest systems are anticipated to advance the field of protein imprinting to the same extent. Finally, the most appropriate polymerization strategy containing functional monomers concentration, cross-linker concentration, buffer composition, pH, ionic strength and temperature has to be determined.

In conclusion, new synthetic protocols adapted for molecular imprinting of proteins and new monomers addressing the high density of recognition sites at proteins will be sure to produce novel MIPs of proteins.

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