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Review

Oriented Immobilization of Protein Templates: A New Trend in Surface Imprinting

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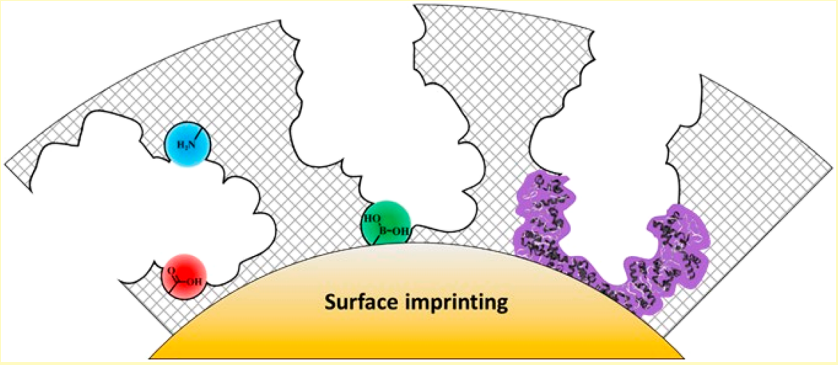

ABSTRACT: In this Review, we have summarized recent trends in protein template imprinting. We emphasized a new trend in surface imprinting, namely, oriented protein immobilization. Site-directed proteins were assembled through specially selected functionalities. These efforts resulted in a preferably oriented homogeneous protein construct with decreased protein conformation changes during imprinting. Moreover, the maximum functionality for protein recognition was utilized. Various strategies were exploited for oriented protein immobilization, including covalent immobilization through a boronic acid group, metal coordinating center, and aptamer-based immobilization. Moreover, we have discussed the involvement of semicovalent as well as covalent imprinting. Interestingly, these approaches provided additional recognition sites in the molecular cavities imprinted. Therefore, these molecular cavities were highly selective, and the binding kinetics was improved.

KEYWORDS: *surface imprinting, semicovalent imprinting, covalent imprinting, aptamer, metal chelation, oriented immobilized protein, molecularly imprinted self-assembled monolayer, chemosensor*

The importance of protein biomarkers levels determination is currently growing in light of the increasing importance of developing point-of-care (POC) diagnosis and thus POC testing devices.[1−3] Proteomics regularly provides discoveries of protein biomarkers associated with various health issues.[4−6] Therefore, a fast and reliable protocol for the determination of those biomarkers is needed. However, selective and sensitive determination of proteins is an arduous task. Various reported methods and procedures suffer from difficulties in exact protein determinations.[7] The introduction of sample preparation steps improved these determinations.[8−10]

Protein biomarkers are currently being selectively determined in biological samples mainly through immunoassays or high-performance liquid chromatography combined with mass spectrometry.[8] The excellent accuracy of these determinations comes with a high cost, including the cost of specific antibody supplies or instrumentation and experienced operation. Therefore, numerous biosensors for fast and selective quantification of protein biomarkers were devised and fabricated.[11] Most interesting protein biomarkers belong to the glycoprotein family.[12] Unfortunately, it seems challenging to generate antibodies for this family because of low immunogenicity and the lack of high-throughput methods to evaluate the glycan-binding properties.[13] Thus, an alternative research field is developing which claims to synthesize more stable artificial antibody-like receptors for the replacement of natural antibodies in these assays.[14]

One of the most promising methods for that purpose, as mentioned earlier, includes utilizing molecularly imprinted polymers (MIPs).[15] MIP-based receptors have been designed to recognize and selectively target analyte molecules, including proteins.[16−19] Usually, MIPs are synthesized by forming a

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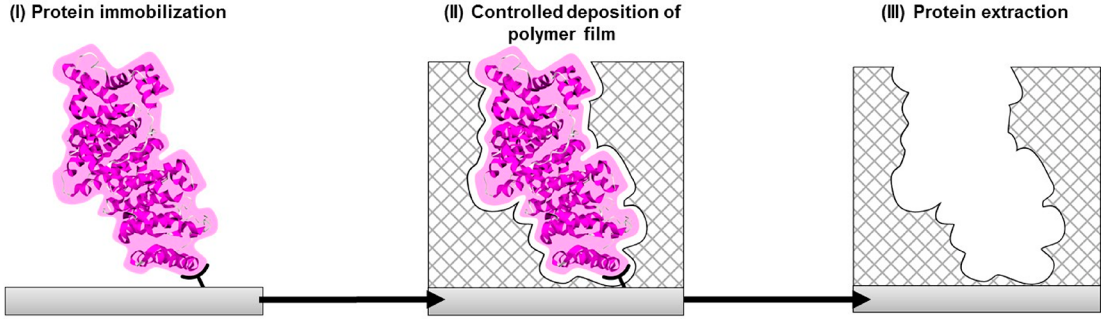
Scheme 1. Illustration of the Consecutive Steps of Surface Imprinting



polymer matrix around template molecules by copolymerization of functional and cross-linking monomers. The formation of a stable complex of the template with the functional monomer in solution before polymerization is an essential step for successful imprinting. After polymerization resulting in an MIP, subsequent template extraction from this MIP leaves empty molecular cavities with their size, shape, and orientation of chemical functionalities capable of recognizing, via binding with high affinity, complementary template molecules. This simple to follow approach results in synthetic receptors for numerous target analytes, namely, drugs, biomarkers, pesticides, and toxins.[20−23] However, typical methods used for syntheses of these receptors are not suitable for macromolecular templates, such as proteins.[24,25] For instance, maintaining the native structure of proteins in organic solvents and their permanent entrapment in highly cross-linked polymer receptors are challenging for classical imprinting.[26] Progress in this field provided a partial solution of protein mass-transfer hindrance by introducing surface imprinting[27−31] and imprinting of selected proteins parts[27] instead of complete protein molecules. This specific part is described as an epitope.[32] Moreover, low cross-linked hydrogels were used.[33]

Haupt et al. first proposed surface imprinting. For that, they used theophylline.[34] The template molecules were immobilized on the surface of a silica gel. At least 75% of the silica surface was coated with the theophylline template. After imprinting, the silica gel was dissolved to form porous polymer particles with imprinted cavities located only on the pore's surface. Later, this approach was employed for hemoglobin (Hb) imprinting on the silica surface. For this imprinting, Hb was covalently immobilized by forming imine bonds between amine groups on the Hb surface and the aldehyde groups anchored to silica. Then, those bonds were readily cleaved with oxalic acid. However, the silica surface remained here (Scheme 1).

The surface imprinting can be divided into two steps: (i) immobilization of the protein template on the solid substrate surface and (ii) deposition of a thin polymer film around the immobilized template molecules (Scheme 1).[16,17,35] The latter appears to be more important because polymer thickness directly affects the imprinting success. Careful monitoring of polymerization conditions is crucial to deposit a 5−10 nm thick MIP film, i.e., of the thickness comparable to the size of the protein template molecules (Scheme 1).

Subsequently, numerous modified approaches to protein imprinting were reported. Namely, semicovalent imprinting, application of aptamer-MIP hybrid systems, and the involvement of specially designed functional monomers were introduced. The main idea for these imprintings was the same as that for classical imprinting. However, additional steps were introduced to avoid permanent entrapment of protein molecules and to generate homogeneous molecular cavities in the resulting MIP. Of note, these steps helped in preparing MIPs with superior affinity to target proteins in many instances.[36,37] In the next section, we will outline those efforts that improved the performance of protein imprinted polymers.

■ ORIENTED PROTEIN IMMOBILIZATION

Oriented or site-directed protein immobilization is becoming popular in surface imprinting.[38−42] Proteins are site-directed assembled through specially designed functionalities. This assembly resulted in homogeneous protein attachment with a preferable orientation, which hindered protein conformation changes and exposed the protein molecule's maximum functionalities for its recognition.[34] Currently, various strategies are being exploited for oriented protein immobilization, including covalent immobilization of a protein template through interaction with the boronic acid group, metal coordinating centers coordinated by low-molecular-mass ligands, and aptamers. This section will summarize progress in the field of oriented immobilized proteins for surface imprinting.

Molecular Cavities with Aptamers for Enhancement of Interactions. Aptamers are single-stranded oligonucleotides that can fold to create three-dimensional structures.[43,44] They have emerged as promising biorecognition units in the development of sensing devices because of their recognition ability.[43] These oligonucleotides selectively recognize and bind their target analytes. Aptamers can be generated for small molecules, for molecules as large as proteins, and even for whole cells. Usually, they are selected from a combinatorial library using the systematic evolution of ligands by an exponential enrichment process.[45] Importantly, aptamers can be synthesized chemically. Unfortunately, under certain conditions, an aptamer's affinity is compromised because of numerous conformation dynamic structures. Therefore, instead of applying aptamers alone, aptamer−MIP hybrid systems were proposed.[37,46,47] That is because MIPs and aptamers can overcome together typical drawbacks of antibodies. Although both MIPs and aptamers have their advantages and disadvantages, it was demonstrated that the introduction of aptamers in molecular cavities created by molecular imprinting resulted in MIPs of superior affinity.

In this aptamer−MIP hybrid approach, aptamers are used to anchor protein molecules on the surface (including metal electrodes and nanoparticles). Controlled polymerization of functional monomers around the aptamer-anchored protein resulted in entrapment of the protein.[37,46] Careful tuning of the

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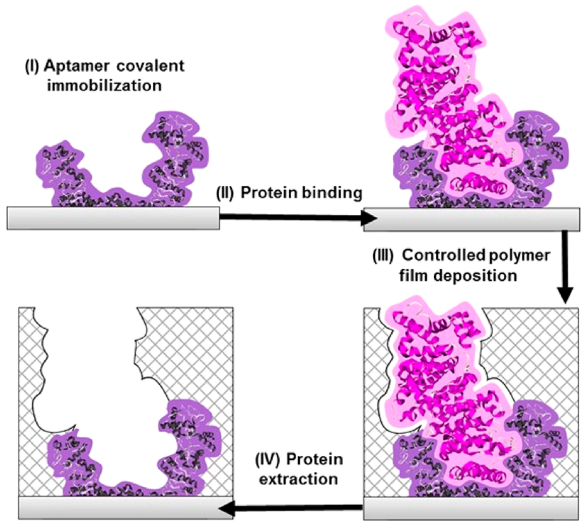
polymer thickness generated protein binding sites on the sensor surface (Scheme 2). In one such example, dopamine

Scheme 2. Illustration of Surface Imprinting of Protein Immobilized by an Aptamer



was electropolymerized around the aptamer-bound thrombin protein.[37] Polydopamine acts as a supportive scaffold for the aptamer and restricts changes in the aptamer conformation. Whether the polydopamine provided any selective interactions in molecular cavities was not explained. However, it was confirmed that the aptamer at the bottom of molecular cavities played a vital role in the selectivity of the resulting MIPs.

The most extreme example of aptamers being applied in molecular imprinting was the synthesis of an imprinted self-assembled monolayer (SAM) of two thrombin binding aptamers on gold nanoparticles (AuNPs).[48] For that purpose, two different thrombin binding aptamers with proper thymidine linkers and thiol anchoring groups were synthesized. These two aptamers formed a 1:1:1 complex with thrombin molecules. Then, this complex was deposited on the surfaces of AuNPs. Finally, thrombin molecules were removed, resulting in two-aptamer SAM coated AuNPs with the thrombin imprinted pattern. Therefore, the affinity of these particles for thrombin was very high, with a dissociation constant of 5×10^{-11} M. Moreover, the anticoagulant properties of these particles were eight times stronger than those of nonimprinted particles.[48]

Application of Protein Ligands for Directed Immobilization of Protein Template Molecules. The dissociation constant of the human apurinic-apyrimidinic endonuclease-1 (APE1) complex with avidin is as low as 3.2 nM. This value indicates that avidin is a suitable candidate for oriented surface immobilization of APE1.[49] As before, dopamine was chosen as the functional monomer for surface imprinting. The generated microcavities bound APE1 via multiple noncovalent interactions, similar to natural antibodies. The bound APE1 enzyme activity was effectively inhibited, most likely because of conformational changes or blocking of its active center. Surprisingly, the dissociation constant of the molecular cavity complex with avidin was not determined in this report. It could indicate if APE1 was mostly recognized because its interaction with avidin or the cavities imprinted in polydopamine also contributed to the selective recognition.

Similarly, a polymer material containing heparin as an additional interaction site was devised. It allowed discrimination of different vascular endothelial growth factor (VEGF) isoforms.[50] Heparins are common specific ligands for the VEGF isoforms, namely, VEGF165, and VEGF189. However, the VEGF121 isoform lacks the domain responsible for this interaction. Therefore, heparins were used as additional interaction sites in molecular cavities to decrease VEGF121 isoform binding compared with VEGF165 and VEGF189. Overexpression of two VEGF isoforms, VEGF121 and VEGF165, by human glioma U87 MG cells induced tumor-associated intracerebral hemorrhage, while expression of a third form, VEGF189, did not cause vessel rupture. Toward that, cavities devised that way allowed for sensitive and selective detection of only the VEGF165 isoform. The molecular imprinting was performed on the heparin bound surface-immobilized VEGF165 using methacrylic acid as a functional monomer to interact with the basic amino acids of VEGF165. After removing the VEGF165 template, imprinted cavities remained. The heparin moiety and acid groups were located in the molecular cavities in positions and orientations suitable for VEGF165 recognition. The dissociation constant of these cavities for VEGF165 was 34 nM, i.e., 10 times higher than that for the case of only heparin immobilized on the substrate.[50]

In another example, putida redoxin, a natural redox partner of P450cam, served as an anchor for oriented immobilization of the P450cam protein.[51] This anchor was tethered to two different materials, i.e., to gold by direct chemisorption via Au−S bonds forming through the terminal cysteine and on glassy carbon electrodes using *N*-(1-pyrenyl) maleimide. Then, an electrically insulating hydrophilic polyscopoletin MIP film was deposited around the surface-immobilized protein molecules.

Application of Low-Molecular-Mass Ligands for Oriented Protein Immobilization. The use of biotin as an immobilizing agent for surface imprinting of avidin can be considered as one of the first studies toward applying specific ligands (here biotin) for surface imprinting.[52] A two-step procedure was reported for generating surface imprinted molecular cavities. The first step involved removing the avidin template by cleaving the disulfide bond of the immobilizing agent, and the second involved direct dissolution of the bead used for protein immobilization. However, it could be more effective if, first, beads were removed and, second, disulfide bonds were cleaved.[52]

In another example, mannose sugar was used for the imprinting of concanavalin A, a carbohydrate-binding protein.[53] In comparison to only mannose immobilized surface, molecularly imprinted microcavities generated by polymer film grafting together with polymer architecture support showed ∼20-fold higher affinity. Numerous other low-molecular-mass compounds were applied for directed protein surface imprinting, including benzadimine and aprotinin for trypsin,[54,55] glutathione for glutathione-*s*-transferase-π,[56] propidium for acetylcholinesterase,[57] and mercaptoundecanoic acid for hexameric tyrosine coordinated heme protein,[58] respectively.

Molecular Cavities with the Boronate Affinity Group. For the imprinting of glycoproteins, boronic acid based functional monomers were proposed.[38,41,42,59−66] Worth mentioning, the imprinting with this functional monomer relies on the covalent bond formation between boronic acids

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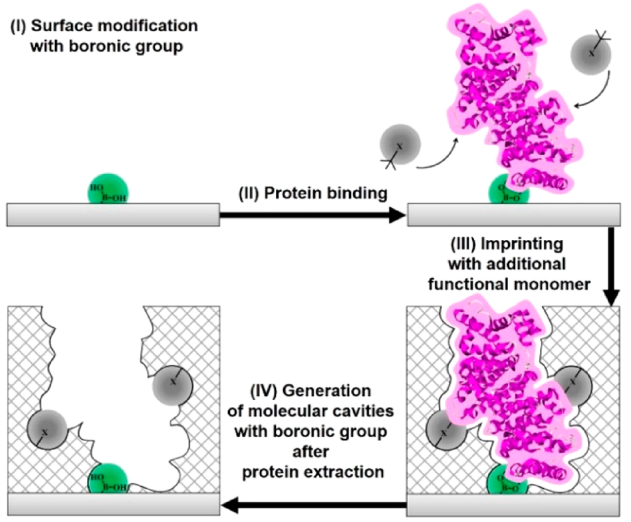
ACS Sens. 2020, 5, 3710−3720

and the template. One of the most exciting outcomes of this monomer's application is a quick release of the glycoprotein template due to the pH change. The interference of molecular cavities generated with boronic acid binding sites was low. Moreover, the dissociation constant ranged from 10^{-8} to 10^{-10} M.[59,65]

However, under certain conditions, the use of only a boronic acid functional monomer may not suffice to provide the desired selectivity because of the presence of other competing biomolecules containing *cis*-diol groups (Scheme 3).[63,67]

Scheme 3. Illustration of Surface Imprinting of Protein on the Boronic Acid Functionalized Surface



Therefore, in addition to boronic acid based functional monomer, polymerizable pyrrolidyl acrylate and 2-methacryloyloxyethyl phosphorylcholine were employed as additional functional monomers.[67] The pyrrolidyl group interacted with the protein molecule via electrostatic interactions, while the phosphorylcholine group provided biocompatibility, which decreases nonspecific adsorption of several proteins. For controlling another critical parameter of surface imprinting, the polymer film's thickness, controlled/living radical polymerization, was employed.

In another study, the C-terminus nonapeptide epitope was specially derivatized with a sugar group for controllable oriented immobilization on boronic acid functionalized substrates in a slightly modified approach.[41] Then, a thin film was grafted by polycondensation of multiple ethoxysilane derivatives containing functionalities capable of interacting with the epitope template, including aminopropyltriethoxysilane, 3-ureidopropyl-triethoxysilane, isobutyltriethoxysilane, and tetraethyl orthosilicate. Finally, the glycated epitope was removed with an acidic acetonitrile solution. The resulting MIPs could bind both full protein and only the exposed peptide epitope. Interestingly, in this approach, the boronic group was only used for template immobilization and did not recognize protein during binding. Several other examples were reported, in which boronic acid functional monomers were used for surface imprinting. However, in this approach, boronic acid recognition sites were randomly oriented in molecular cavities.[68,69]

The boronic acid centered SAMs with imprinted patterns were suggested as an alternative for typical MIP films to avoid polymerization initiators and organic solvents, which can denature the protein.[61] A newly designed SAM containing 1,2-dithiolane derivative (DHAP), in which an oligo(ethylene glycol) (OEG) moiety was covalently linked to a dithiolane moiety through an alkyl chain incorporated with two amide groups, was applied for the imprinted SAM direct deposition on the transducer surface. DHAP was self-assembled onto the gold surface for to enhance binding stability. The two amide groups incorporated in the alkyl chain of DHAP offered additional binding sites in the molecularly imprinted spots. These amide groups were interacting with proteins and, additionally, with neighboring SAM chains through H-bonding to provide improved stability of those cavities. The OEG terminal moiety decreased nonspecific protein binding on the SAM surface. Moreover, protein mass transfer to and from imprinted cavities was faster in the monolayers.

Another SAM of an orthogonally functionalized acrylamide-alkyne cysteine derivative was synthesized for surface imprinting of glycoproteins.[70] Initially, a (3-acrylamidophenyl)boronic acid–glycoprotein adduct was prepared. In the next step, acrylic moieties of the SAM were polymerized in the presence of this adduct. This way, phenylboronic acid groups were grafted onto the imprinted pattern of the SAM surface via copolymerization. Moreover, polymerization of acrylic groups provided high stability of the deposited SAM. Furthermore, the amide part of the SAM molecules was engaged in hydrogen bonding with amide groups of terget glycoprotein. Finally, alkyne units of the monomer underwent copper-catalyzed azide–alkyne cycloaddition. That was used to cap off the residual alkyne functionality, thus building an ordered pocket around the immobilized glycoprotein template molecules.

Molecular Cavities with Metal Ions. This section describes efforts at developing metal ion assisted surface imprinting for selective enrichment or sensing of histidine-tagged proteins.[36,71−73] The objective of this approach was to improve existing procedures of purification of recombinant proteins. The iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) based ligand complexes of transition metal ions, such as Zn^{2+}, Cu^{2+}, Ni^{2+}, and Co^{2+}, show a strong affinity to histidine and cysteine in aqueous solutions.[74] Therefore, a surface modified with the IDA ligand, complexed with a metal ion, firmly fixed the protein template molecules.[71] Proteins bearing exposed histidine residues and recombinant proteins fused with the polyhistidine tag are an example of proteins for which such an approach can be applied. Interestingly, when the histidine-tagged interfering 9−10 long peptide epitopes were allowed to bind to the MIP, their affinity was lower, indicating that selectivity of the recognition in final molecular cavities was not only governed by metal chelating histidine amino acids, but other parts of the protein were also playing a significant role.[71]

The IDA ligand coordinates Ni^{2+} with three valences. Hence, its other three valences are accessible for coordination by imidazole rings of histidine parts of the target proteins. However, the coordination of all three imidazole molecules is impossible because of steric hindrance.[74−77] In contrast to tridentate ligands, NTA coordinates a metal ion with four valences, leaving only two valences free for interaction with the imidazole ring.[74,75,77]

This coordination number can play an essential role in making molecular cavities useful for selective and sensitive protein fraction recognition. Besides considerable metal

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leaching, chromatographic purification of histidine-tagged proteins using an IDA matrix frequently results in purity lower than that reached in the NTA based protein purification.[74] Other than surface immobilization applications, several reports exploited functional monomers derivatized with IDA ligands to place metal coordination centers into molecular cavities. However, in surface imprinting, such comparative studies are not yet reported.

Most cells secrete exosomes and other extracellular vesicles for intercellular communication, especially cancer cells. A sandwich-type structure was proposed to generate selective molecular cavities for exosome.[78] Exosomes were immobilized on a substrate surface, which was previously modified with anti-CD9 antibodies. This antibody was immobilized on histidine-tagged protein G. The latter was immobilized on the transducer surface through Ni^{2+} complex formation with NTA groups. The polymer film deposition followed with the subsequent exosome removal. Then, postimprinting modification allowed measuring precisely the exosome binding only inside of the imprinted cavities. That way, a highly selective protein assay was possible.

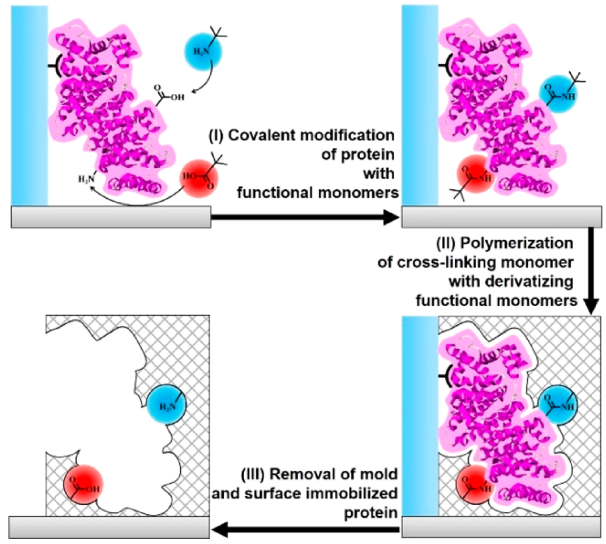
RANDOM PROTEIN IMMOBILIZATION

As mentioned in the above section, specific moieties in surface imprinting play a dual role. On the one hand, it helps to immobilize proteins before polymerization; and on the other, these moieties serve as additional recognizing sites for the target protein. Unfortunately, this approach cannot be extended to all proteins. As an alternative, recently, semicovalent imprinting was introduced.[79] This imprinting claims to generate multiple binding sites in the imprinted polymer's molecular cavities with very high precision.

Semicovalent Surface Imprinting. Molecular cavities containing homogeneous binding sites are synthesized by covalent[41,61,69,70] and semicovalent imprinting.[80−83] These syntheses are well established for the imprinting of small molecules.[84−86] Covalent protein imprinting is mostly performed with boronic acid derivatized functional monomers, described in detail in the Oriented Protein Immobilization section above, and it is limited to glycoproteins.[41,42,59,61,69,70] Recently, a semicovalent procedure was proposed, and several papers claim an improvement in this procedure (Scheme 4).[80−82] Briefly, this strategy consists of a combination of three methods, namely, (i) inverse opal structuring, (ii) surface imprinting, and (iii) semicovalent imprinting. Hard silica beads were used as a mold to prepare a highly organized opal structure. On top of this, template molecules derivatized with functional monomers were immobilized.[80] Later, these functionalized template molecules were electrochemically cross-linked with an excess of a cross-linking monomer. Removal by the dissolution of this silica mold created a macroporous structure containing molecular cavities on the surface. The recognition performance of this macroporous structure was high. This structure was advantageous for nonrestrictive diffusion of bulky protein toward respective molecular cavities. The necessity of introducing chromatographic purification for the separation of derivatized human serum albumin protein from unreacted monomers and side products before the immobilization was the disadvantage of this approach. Unfortunately, this procedure also requires a large amount of protein.

One step further, instead of derivatized protein template immobilization, unmodified protein templates were first

Scheme 4. Illustration of Surface Imprinting of Protein via Semicovalent Imprinting



immobilized over assembled in an opal structure silica beads.[81] Then, template molecules were derivatized with functional monomers. After subsequent cross-linking, molecular cavities were generated, which kept the recognizing site's orientation precisely that of the template binding sites (Scheme 4). The suitability and versatility of this synergistic imprinting were confirmed by devising MIP film based electric chemosensing systems to determine the human chorionic gonadotropin hormone. SEM imaging confirmed the MIP film macroporous structure. This film's recognition of proteins was transduced with an electric transducer, namely, an extended-gate field-effect transistor, and capacitive impedimetry. The close attachment of receptors onto the electrode surface aided the design of ultimate chemosensors. Recently, semicovalent imprinting was further improved.[82] The time and the amount of protein needed for immobilization and the number of functional monomers were optimized systematically. Justification of this optimization is simple because several biomarker proteins, which can be chosen for future imprinting, are expensive. Therefore, the amount of protein necessary for successful imprinting should be minimized. This optimization will make a new semicovalent surface imprinting cost-effective and more environmentally friendly. Moreover, the necessity of semicovalent imprinting was validated. That is, a macroporous MIP film prepared with semicovalent imprinting was much more sensitive and selective than the macroporous MIP film prepared by merely surface imprinting.[82]

The semicovalent approach was also applied using acrylic based functional monomers. Two cleavable such monomers were designed and synthesized.[63,83] Monomer possesses thiol-reactive pyridyl disulfide moiety to bond with the thiol groups on α-fetoprotein. At the same time, another contained the amine-reactive active ester to target amino groups of α-fetoprotein. A 2-iminothiolane spacer was introduced on α-fetoprotein to conjugate thiol-reactive pyridyl disulfide moiety. Notably, this derivatization was possible under mild conditions being advantageous for bulky and fragile templates.

Surface Imprinting with Additional Functional Monomers. Once again, to make cavities highly selective, additional monomers, i.e., other than carboxyl, were used. For that purpose, silane monomers with four different amino-acid-

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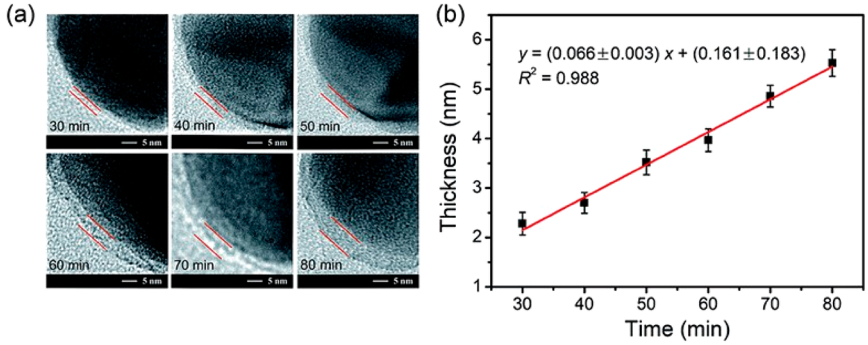

Figure 1. (a) TEM images showing growth of the polymer matrix with time (scale bar 5 nm). (b) Dependence of the polymer thickness on the polymerization time. Error bars represent the standard deviation of five parallel experiments. Reprinted with permission from ref 41. Copyright 2019 the Royal Society of Chemistry.

like functional groups were employed to synthesize antibody mimicking cavities.[87] These monomers provided the protonated amine (NH_3^+), hydroxyl (OH), benzyl (C_6H_6), and propyl ($CH_2CH_2CH_3$) groups in these microcavities. Their presence facilitated ionic and H-bonding as well as hydrophobic interactions. However, special care was needed to optimize the monomer ratio when more than one monomer was used.

Similarly, in imprinting with SAMs, derivatized thiols were synthesized to create hydrophilic imprinted spots for providing additional interacting sites.[88] Of note, traditional SAMs produce hydrophobic patterns with poor stability.[89] In the study mentioned above, the functionalities in chains additionally interacted with adjacent chains via van der Waals interactions and hydrogen bonding.[88] That improved the packing and stability of the grafted SAM. In another approach, 4-[2-(*N*-methacrylamido)ethylaminomethyl]benzoic acid was synthesized as the functional monomer to add different binding sites on the surface imprinted molecular cavities of prostate-specific antigen.[62] The carboxyl group interacted with prostate-specific antigen via electrostatic interactions.

APPROACHES FOR CONTROLLED POLYMER GROWTH

Not only protein orientation but also controlled growth of the MIP film is critical in surface imprinting.[90,91] The overgrowth of the film may cause disadvantages similar to those encountered in classical bulk imprinting. That is, the protein template might be buried deep inside of a thick deposited MIP film. Thus, template extraction and subsequent diffusion of the analyte to the imprinted cavities would be difficult. Therefore, precise control of polymer thickness is essential.

Toward that, various approaches have recently been developed, e.g., electrochemical or self-polymerization of dopamine,[37,46,65,92] chemical polymerization of aniline,[59,93] and silica polycondensation.[38,41,66,87] The polymerization time and monomers concentration in the solution for polymerization were factors that helped to tune the thickness of the grafted MIP films according to the protein size.[41] When the MIP film was prepared using tetraethyl orthosilicate (TEOS), the silica layer's thickness was linearly increasing with the polycondensation time. The silica film growth rate was 0.04 nm min^{-1}.[38] A much thicker film was prepared when TEOS concentration in the solution was higher, and the film growth (0.066 nm min^{-1}) was faster (Figure 1).[41] On average, 40−60 min sufficed to deposit a nanometer-thick MIP film.

A similar study was performed with dopamine as the functional monomer for surface imprinting of glycoproteins.[94] That is, three different glycoproteins of distinguished molecular sizes were used as model templates and varying polymerization times provided thickness-tuned surface imprinted MIP films. The binding isotherm study indicated that film overgrowth resulted in a decrease in imprinting efficiency. The optimal film thickness and the highest binding efficiency were positively correlated.

As mentioned above, dopamine self-polymerization in slightly basic solutions was frequently used to prepare thin MIP films.[37,71] Dopamine polymerization also follows a linear relation with time. Interestingly, dopamine self-polymerization was much slower in the presence of a protein template. Few reports suggested a longer polymerization time (7 h) to increase the imprinting performance.[64] In contrast, another report presented successful 80% coverage of the MIP matrix with APE1 protein in just 5 min.[49]

Moreover, electropolymerization of dopamine from solutions of relatively low concentrations was used to deposit thin MIP films. However, this approach is less frequent.[46,92,95] In this case, the number of potentiodynamic cycles controlled the thickness. Some other MIPs, prepared by electropolymerization, were also reported because of their advantage of easy control of the thickness of the deposited film.[51−53,80−82,96−99]

When acrylic-based monomers were used for surface protein imprinting, the film thickness was controlled by advanced free-radical polymerization types, namely, living polymerizations. For instance, atom transfer radical polymerization (ATRP), a catalyst-activated, controllable radical polymerization, was employed to grow ultrathin MIP films on the substrate surface.[100] This method produces no solution-phase radical species; therefore, solution-phase polymerization was avoided in the absence of chain transfer. This method allowed the precise control of the thickness of the deposited MIP films.[50,63,67,83,101,102]

OUTCOME OF CONTROLLED ORIENTATION

Various approaches aiming at oriented surface imprinting are described in detail in different sections above. Few questions arise after going through all these approaches. Is it worth considering oriented immobilization of proteins? Does it add any value to surface imprinting? However, the answer to the former question comes immediately. In surface imprinting, the MIP film's thickness must be tuned to the protein size (Scheme 5). Therefore, the protein molecule can assume any

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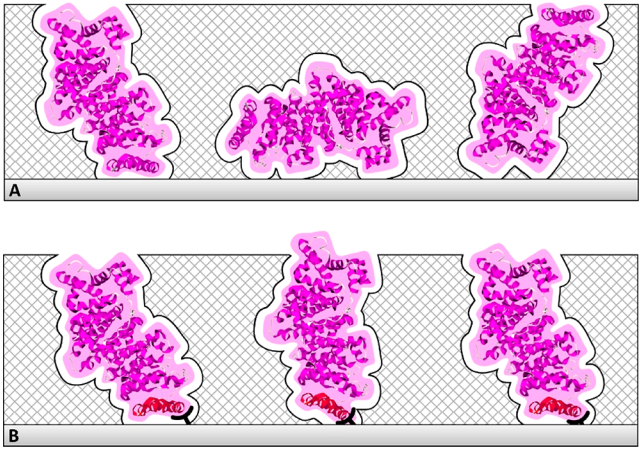
Scheme 5. Illustration of Surface Imprinting of (A) Randomly Immobilized Protein and (B) Oriented Immobilized Protein



orientation, either vertical or horizontal, against the support surface plane if this orientation is not controlled during protein immobilization (Scheme 5A). Hence, the MIP film will cover only a small part of the vertically oriented protein if the film thickness is tuned according to the horizontally oriented protein's size.

Similarly, the film will overgrow horizontally oriented proteins if the thickness is controlled according to the protein's vertical size. That can result in permanent entrapment of these protein molecules inside MIP, similar to that found in classical bulk imprinting (Scheme 5A). Either way, the efficiency of the imprinting will decrease. This point strongly justifies the need for oriented surface immobilization of protein molecules before their surface imprinting (Scheme 5B).

Few reports suggested that the final MIP's binding efficiency would be increased if oriented surface imprinting was introduced. For instance, surface imprinted nanoparticles, prepared by precipitation polymerization of ethylene glycol derivatized acrylic and other supporting monomers, exhibited a binding ability of 67.3 mg g^{-1}.[103] Integration of a metal-chelating monomer in molecular cavities resulted in a lysozyme imprinted particle with a binding ability higher than that of other reported particles.[73,104] Moreover, the binding kinetics was comparatively slower in imprinted particles containing a metal coordinating monomer. They attained maximum adsorption at ~40 min.[100] In contrast, the avidin containing molecular cavity bound 85% of the protein in solution within 5 min.[49]

Another exciting advantage of directed surface imprinting of proteins is the potential control of protein molecule functionality captured into molecular cavities. In one example, benzamidine, a well-known trypsin inhibitor, was applied for directed surface imprinting of the trypsin enzyme.[54] Core polymer microspheres were modified with benzamidine. Then, trypsin molecules were immobilized through binding with benzamidine. When an MIP film was deposited on core particles' surface through ATRP polymerization, active sites of immobilized trypsin molecules were facing down. Therefore, molecular cavities on the surface of MIP particles could recognize and bind trypsin with their active sites. Therefore, these MIP particles acted as competitive trypsin inhibitors. Because of imprinting, the MIP particle inhibiting efficiency was 1000 times higher than that of benzamine. Notably, the NIP control particle inhibiting efficiencies were noncompetitive. That was because NIP particles were binding trypsin molecules in random places, usually far from active enzyme centers.

Aprotinin, another trypsin inhibitor, was applied in oriented trypsin MIP NP synthesis using an automated solid-support procedure.[55] For that, trypsin molecules were immobilized on the aprotinin modified solid support. Then, MIP NPs were grown over trypsin molecules. This time, all active trypsin centers were facing out of the synthesized particles because of fixed orientation. For comparison, randomly oriented MIP NPs and NIP NPs were synthesized. It appeared that only randomly oriented MIP NPs acted as trypsin inhibitors with efficiencies comparable to that of benzamine. For NIP NPs and oriented MIP NPs, trypsin was not inhibited. That was because of binding trypsin molecules in oriented MIP molecular cavities, thus exposing the active enzyme centers to the solution.

Interestingly, when trypsin was stored in solution, either alone or in the presence of NIP NPs, its activity dropped by half after 72 h. In the presence of oriented MIP NPs in solution, trypsin activity was fully preserved over time. That was because oriented MIP NPs maintained the trypsin structure. Nevertheless, they prevented trypsin self-digestion.

Other than desired outcomes of oriented immobilization, there were some undesired effects. For instance, the presence of strong binding affinity based ligands and receptors in molecular cavities made template extraction challenging, even from thin MIP films.[37] As reported in the aptamer-MIP hybrid case, 15 h rinsing with an acidic solution was required for complete template extraction. Similarly, to break the specific interaction between putidaredoxin and cytochrome P450cam, MIP was washed overnight in glycine-hydrochloric acid (pH = 2.2, 1% SDS).[51]

Boronic based monomers appeared advantageous if the ease of template extraction is considered. Ester bond formation with this group is reversible and pH-dependent.[9] However, some limitations were reported for this functionality. Its binding ability is the highest for carbohydrates in the furanose form.[105] Worth mentioning, biologically relevant carbohydrates appear mostly in the pyranose form. Other than that, the literature suggests that binding is the strongest in slightly alkaline solutions (pH > 8).[13] Unfortunately, such a condition is not compatible with fragile proteins. The use of benzoboroxoles derivatives can avoid this disadvantage because these derivatives can bind sugars under physiological conditions.[13] Moreover, they can bind sugars in the pyranose form. That makes them a functionality suitable for the preparation of MIP for recognition of glycoproteins.

Opposite to this, molecular cavities prepared with metal coordination interactions were readily disrupted to facilitate template removal. For instance, simple washing with the EDTA solution resulted in both the template and metal ions removal.[71] Another approach suggested the use of imidazole to establish environment competitive for elution of histidine-containing epitope without releasing the complexed metal ion.[72]

One more important point worth discussing here is related to template extraction in oriented immobilization. Usually, extraction conditions enable breaking proteins and facilitate their extraction from molecular cavities. Therefore, it is worth considering how, under such conditions, aptamers and other specific receptors survive without losing their binding efficiency. For instance, heparin slowly loses its activity after

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immobilization.[106] Such a stability point was not discussed yet in any of the published reports.

Compared to the strategies mentioned above, the semicovalent approach does not integrate any biologically derived entity in the molecular cavity. It still follows the original idea of imprinting in polymers.[81,82] Moreover, the reusability and stability of these protein imprints should be high compared to those of aptamer and protein receptor based imprints.

CONCLUSIONS

Herein, we presented several successful attempts to immobilize proteins in a particular molecular orientation to prepare uniform molecular cavities in the MIP matrix. Immobilization of the template over the functionalized surface was facilitated by aptamer, boronic groups, and some specific ligands. In addition to controlling protein orientation, these approaches increased the efficiency of protein immobilization. That resulted in a higher performance of the ultimate MIP. Moreover, these efforts increased the number of recognition sites in molecular cavities because additional binding sites were introduced at the cavity's bottom. Several approaches claimed 60−80% MIP surface coverage by immobilized template molecules. This high coverage is essential if MIPs are applied as recognition units of chemosensors. Although several approaches are reported for ideally oriented protein immobilization, multiple binding sites generated by semicovalent surface imprinting appeared to be a promising strategy for preparing stable surface imprinted cavities. Moreover, this strategy was efficient for decreasing nonspecific binding.

For thin MIP film preparation, a procedure of dopamine self-polymerization under mild basic condition appeared advantageous compared to other procedures. Moreover, this condition was suitable for the immobilization of fragile protein templates. An easy to follow procedure that was developed made it more popular for protein surface imprinting. Then, silica's polycondensation appeared to be the second preferred choice to prepare thin and stable thin MIP films. However, the time reported to prepare these films varies a lot.

Stability can be an issue for such thin films. Therefore, film stability should be improved in the context of reusability. For comparison, surface imprinting with lithography can be more advantageous because the generated microcavities always appear on the surface after mold dissolution.

The value of the binding constant of oriented molecular cavities varies, but mostly in the nanomolar range. The presented results showed that the MIP-aptamer hybrid provided a cost-effective protein determination. Additionally, chemically synthesized stable aptamers allowed oriented immobilization of biomarker proteins to improve the hybrid recognition material's efficiency. Progress in imprinting with oriented MIP microcavities will bring antibody-like materials in the foreseeable future. These materials are promising as outstanding MIP based diagnostics tools.

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The manuscript was written through the contributions of all authors.

Notes

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ABBREVIATIONS

APE1, human apurinic-apyrimidinic endonuclease-1; ATRP, atom transfer radical polymerization; EDTA, ethylenediaminetetraacetic acid; Hb, hemoglobin; IDA, iminodiacetic acid; MIP, molecularly imprinted polymer; NIP, nonimprinted polymer; NP, nanoparticle; OEG, oligo(ethylene glycol); POC, point of care; SAM, self-assembled monolayer; SEM, scanning electron microscopy; SDS, sodium dodecyl sulfate; TEOS, tetraethyl orthosilicate; TEM, transmission electron microscopy; VEGF, vascular endothelial growth factor; NTA, nitrilotriacetic acid

VOCABULARY

Aptamer, aptamers are single-stranded oligonucleotides that can fold to create a three-dimensional structure for selective recognition of target analytes; Biomarkers, biological molecule occurring in body fluids or tissues, whose concentration reflects the conditions of processes or reactions for treatment; Chemosensor, integrated receptor-transducer device in which a chemical recognition unit provides selective quantitive analytical information; Cross-linking monomer, monomers used to provide desirable rigidity to polymer architectures in molecular imprinting; Functional monomer, monomers used to prepare complex with the template analyte through H-bonding, ionic, and van der Waals interactions in molecular imprinting; Imprinting factor, ratio of template analyte response over molecularly imprinted polymer to its response over the nonimprinted polymer; Molecularly imprinted polymer, synthetic receptor prepared by polymerization of suitable functional and cross-linking monomers in the presence of a target analyte to generate memory effect; Point of care testing device, device that provides medical testing at the location where the patient receives care

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