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FEATURE ARTICLE

Beyond Natural Antibodies - A New Generation of Synthetic Antibodies Created by Post-Imprinting Modification of Molecularly Imprinted Polymers

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The molecular imprinting technology yields artificial materials capable of antibody-like molecular recognition. Molecularly imprinted materials are attractive because procedures for their preparation and use are comparatively simple. The number of research reports concerning molecularly imprinted polymers (MIPs) have been increasing yearly, attracting a great deal of interest in various fields. However, as most MIPs have been generated by relatively simple methods developed from the 1970s to the 2000s, resulting in MIPs bearing a single function, their capabilities are limited compared to those of multi-functionalised naturally occurring materials. Proteins are biosynthesised through multiple steps, including fabrication of peptide backbone and subsequent post-translational modifications that introduce additional functionalities, finally producing the mature protein. Post-imprinting modification (PIM) is an innovative strategy for generating MIPs analogous to biosynthetic proteins. New functionalities are introduced, in a site-directed manner, into a molecular imprinted cavity. Monomer residues in the cavity are chemically modified to incorporate new features, such as on/off switching of binding activity, fluorescence signalling, photoresponsivity, and finely tuned binding characteristics. In this Feature Article, we provide an overview of multifunctional MIPs prepared via PIMs developed earlier and the currently used state-of-the-art ones.

Introduction

The critical role of antibodies in the immune system requires their specificity for particular antigens. These antigen–antibody reactions are frequently used in quantitative analytical methods for diagnostics, food analysis, and environmental assessment. Recently, antibody-based medicines targeting proteins overexpressed in cancer cells have been developed as cancer therapies[1]. These antibody–drug combinations can be delivered to tumour cells effectively due to their highly specific molecular recognition capability. However, antibodies have drawbacks that are intrinsic to many other proteins. For example, they are prepared using tedious and time-consuming procedures. Furthermore, antibodies are relatively unstable, expensive, and have a limited shelf life for quality assurance. Thus, alternatives to antibodies, such as synthetic polymers with comparable molecular recognition capabilities, are highly desirable. Considerable research efforts have been directed towards generating synthetic materials as antibody alternatives. The resulting attractive materials include affibodies[2], polynucleotide aptamers[3], polypeptides[4], and synthetic (polymer) receptors[5].

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The beginnings of molecular imprinting: the limitations

In the 1940s, the challenge to create an “artificial antibody” or “plastic antibody” originated with the idea that synthetic materials capable of molecular recognition could be prepared by imprinting a target molecule on a synthetic polymer[6]. The first successes came from the work of the Wulff group (Germany)[7] and the Mosbach group (Sweden)[8] in the 1970s, resulting in “molecular imprinting,” a technique for creating a binding cavity in an organic polymer matrix[9]. Molecularly imprinted polymers are prepared in three steps (Fig. 1). In the first step, a template molecule (the target molecule or an analogue) is complexed with functional monomers via covalent or non-covalent interactions. In the second step, the template-functional monomer complex is co-polymerized with a cross-linking monomer (and co-monomer) to yield a polymer matrix. Finally, the resulting polymer matrix is washed to remove the template and create the binding cavity. Since the co-polymerization step is carried out using a template-functional monomer complex, the imprinted cavity is complementary in size and shape to the template molecule. Functional groups within the cavity, derived from functional monomers, are located in suitable positions for rebinding the template molecule. Therefore, a tailor-made molecular recognition

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cavity reflecting the size, shape, and surface functionality of the template molecule is formed in the organic polymer matrix. MIPs with high affinity and selectivity toward target molecules can be produced by optimizing the design and combinations of template molecules, functional monomers, and cross-linkers. Thus, the use of synthetic polymer material has inherent advantages.

MIPs for various target molecules such as sugars, amino acids and derivatives, agrichemicals (herbicides), peptides, proteins, and viruses have been reported. Recently, MIP nanoparticles have been developed for use as a drug-delivery system[10]. MIPs have been predicted to be alternatives to naturally occurring materials because of their demonstrated capabilities and simplicity of production. However, their capabilities remain far short of the complex and sophisticated functionality of antibodies and enzymes. Conventional MIPs, prepared by technology developed during the period between the 1970s and the 2000s, usually have a single functionality, such as molecular recognition. This methodology is consequently limited in its ability to create materials comparable to natural proteins.

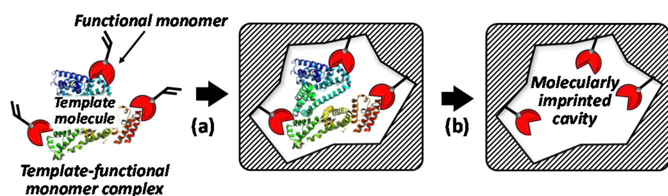

Figure 1. Schematic illustration of molecular imprinting concept. (a) Polymerisation with a crosslinker, (b) Removal of a template molecule.

Next-generation MIPs inspired by post-translational modifications of proteins

In nature, proteins are biosynthesised in ribosomes. Genetic information from DNA is transcribed into mRNA, then, in a ribosome, polypeptides are synthesised based on the sequence information carried by mRNA (translation), using amino acids transported by tRNA. The synthesised polypeptide is then folded into a 3-D structure and undergoes site-specific post-translational modifications, including glycosylation, phosphorylation, ubiquitination, and cleavage, resulting in a mature protein with versatile functions. Proteins thus acquire complex functions by a series of post-translational modifications occurring under optimal conditions.

Similar to *in vivo* protein synthesis, it was hypothesised that multifunctional MIPs could be generated by post-polymerisation modifications analogous with post-translational modifications of proteins. Site-specific chemical modifications in the binding cavities could be made to introduce additional functions and/or regulate existing functionality. We referred to this strategy as “post-imprinting modification” (PIM)[11]. PIM was designed as a site-directed chemical modification of functional monomer residues within a cavity. Functional monomers bearing a modifiable moiety, such as a reversible bond (e. g. disulphide bond or imine bond), an amino group, or a carboxyl group, were used to prepare the MIP matrix. After generating the binding cavity, the functional monomer residues were modified via disulphide exchange, hydrolysis of an imine bond, or chemical modification of amino or carboxyl groups to customise the functionalities of prepared MIPs (Fig. 2). Since this strategy is designed to modify a pre-incorporated functional group on a functional monomer residue in an imprinted cavity, introduction of undesired functionality can be avoided. Chemical modification can be carried out without loss of molecular recognition capability or inducement of non-specific binding. Furthermore, since cross-linked polymer matrices are chemically and physically stable, a wide range of chemical reactions can be used for PIMs. PIMs have the potential to introduce diverse functionalities on an MIP. In this Feature Article, we review research on multifunctional MIPs prepared by PIMs since its conception to the current advancements.

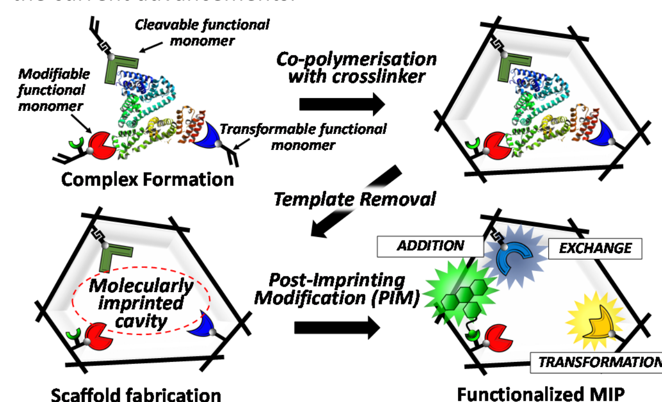

Figure 2. Schematic illustration of molecular imprinting and post-imprinting modifications (PIMs).

Functional groups change within an imprinted cavity by PIMs

The functional groups (binding sites) derived from functional monomer residues within an imprinted cavity play an important role in molecular recognition because binding activity depends strongly on the interactions between the target molecule and the functional groups. However, functional groups used in polymerisation steps are not always stable. Although a functional group that interacts strongly with a target molecule is desirable, strongly interacting groups may induce non-specific binding through random incorporation into polymer matrices[12]. We have designed a template–functional monomer complex, which is stable and inactive during the polymerisation step and is then converted to a suitable functional group after the template-removal step to enable target-molecule recognition by PIMs.

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For example, we have successfully demonstrated dopamine-MIPs generated by disulphide bonds using PIMs (Fig. 3)[13]. The template molecule was designed to consist of an allyl disulphide moiety and a catechol moiety connected by 4-vinylphenyl boronic acid by a cyclic diester bond. It was co-polymerised with styrene and divinylbenzene (DVB). The imprinted cavity was generated by reduction of the disulphide bond and hydrolysis of the cyclic diester. Finally, the free thiol group within the imprinted cavity was treated with hydrogen peroxide to transform it to a sulphonic acid group in a PIM. As a result, the binding cavity was generated into which the phenyl boronic acid moiety (to interact with the dopamine catechol moiety) and the sulphonic acid moiety (to interact with the dopamine amino group) were simultaneously incorporated. We found that the dopamine-binding affinity of the MIP after PIM was 400 times higher than before. Selectivity testing using tyramine, catechol, 3,4-dihydroxyphenylacetic acid, homovanillic acid, epinephrine, and norepinephrine, as reference compounds, showed that the MIP after PIM binds to dopamine selectively. These results indicated that high MIP affinity and selectivity were achieved by the introduction of the sulphonic acid moiety within the imprinted cavity by PIM. Other reported examples of PIM using oxidation–reduction reactions are an artificial enzyme (molecularly imprinted catalyst) for methanolysis of atrazine[14], and molecularly imprinted polymer nanoparticles for a bisphenol A (BPA)-sensing system combined with surface plasmon resonance (SPR)[15].

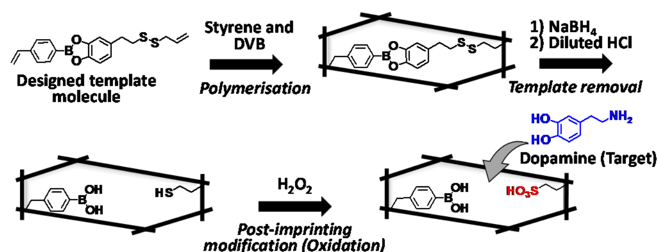

Figure 3. Preparation of a dopamine-imprinted cavity via oxidation treatment as an example of a PIM. Adapted from ref. 13 with permission from The Royal Society of Chemistry, copyright 2006.

Imprinted cavities in MIPs for proteins were also tailored by disulphide exchange reaction using a cleavable functional monomer bearing a carboxy group non-covalently interacting with proteins, ({[2-(2-methacrylamido)ethyldithio]ethylcarbamoyl}methoxy)acetic acid (MDTA)[16] (Fig. 4). Lysozyme-imprinted polymers were prepared, and after the cleavage of the disulfide bonds to expose free thiol groups, thiol-reactive amine, carboxylate, sulfonate, and oligoethylene glycol were sequentially introduced into the imprinted cavities via disulfide bonding. Each functional group demonstrated different binding behaviours; negatively charged groups showed higher binding activity, whereas neutral and positively charged groups significantly decreased the binding activity due to the positively charged lysozyme (pI: 11.2). Furthermore, the binding activity after repetitive introduction was almost the same as that of the original one, suggesting that the functional groups within the binding cavity could be exchanged reversibly within the remaining spatial structure of the binding cavity, even after repetitive exchange treatments.

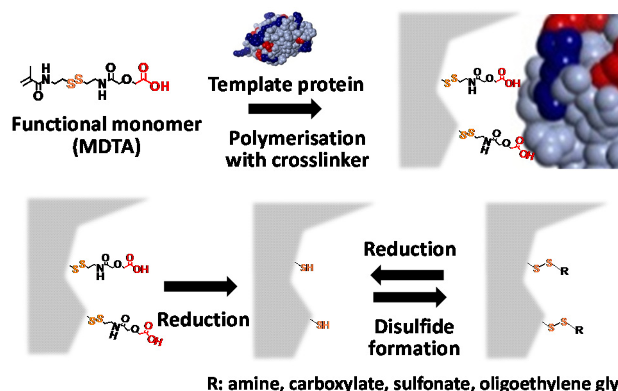

Figure 4. Schematic illustration of MIPs prepared using MDTA and functional group exchange within the binding cavity by PIM via reduction and reformation of disulphide linkage. Adapted from ref. 16 with permission from John Wiley & Sons, Inc., copyright 2018.

Tao et al. reported the preparation of horseradish peroxidase (HRP)-imprinted polymers with the MDTA-like cleavable functional monomer, followed by exchanging the carboxy group into phenylboronic acid by thiol-ene Click reaction[17] (Fig. 5). “Click Chemistry” is useful for PIM, however, in this case, interaction sites with HRP was the carboxy group of the functional monomer during polymerisation, following this the interaction sites were changed to boronic acid groups that interact with glycans on HRP. This discrepancy in the interaction sites between the imprinting process and the binding process may interfere with HRP recognition.

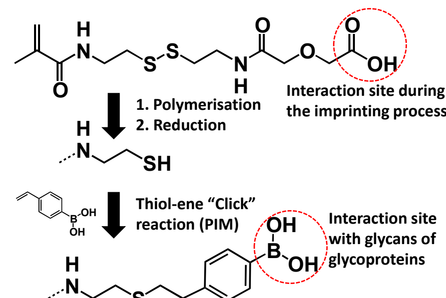

Figure 5. Functional group exchange by Click Chemistry-based PIM reported by Tao et al.[17]

Introduction of fluorophores by PIM

Functional materials for detecting molecular markers for diagnostics, food-quality analysis, and environmental analysis are of increasing importance. Antibody immunoassays are used in these applications, but these procedures are time-consuming, as they require additional reagents such as secondary antibodies or enzyme substrates for enzymatic reactions, and washing steps. Therefore, MIPs with signalling capability are expected to be more desirable analytical tools, requiring simpler handling procedures and no additional

reagents. For example, an MIP-based high-performance detection system for the tumour marker α-fetoprotein (AFP) has been developed[18].

MIPs with a fluorescent functional monomer have been reported to facilitate quantification of binding events[19]. For such applications, high fluorescence background and a low signal-to-noise ratio (sensitivity), which may be produced by functional monomer residues randomly located in polymer matrices, are concerning. Hence, we attempted to introduce a fluorescent reporter molecule into an imprinted cavity by PIM. For this purpose, two functional monomers, FM1 and FM2, were designed and synthesised (Fig. 6). FM1 includes an oxime moiety for a reversible bond and a pyridyl disulphide group to react with a thiol on the target protein. FM2 includes a reversible disulphide bond and a carboxylic acid active ester for conjugation with an amino group on the target protein.

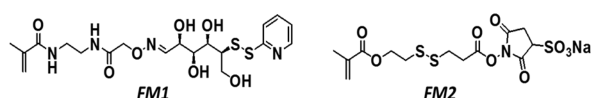

Figure 6. Chemical structures of the designed functional monomers FM1 and FM2 containing reversible bonds (oxime and disulphide, respectively) for PIMs.

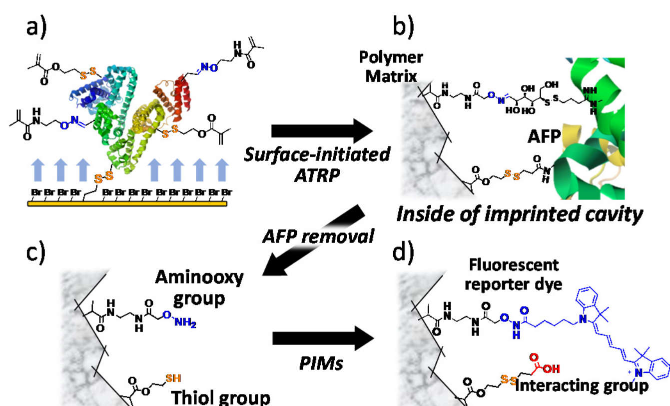

Figure 7. Schematic illustrating the preparation of an AFP-imprinted polymer film followed by post-imprinting modifications to introduce an interacting group and a fluorophore. Adapted from ref. 18 with permission from John Wiley & Sons, Inc., copyright 2016.

The functional monomers FM1 and FM2 were conjugated with AFP, and the resulting template molecule was immobilised on the sensor chip surface (Fig. 7a). The polymerisation reaction was carried out by the surface-initiated atom transfer radical polymerisation method to control polymer thickness by reaction time, yielding a polymer film of ca. 10 nm (Fig. 7b). To remove the AFP moiety, reduction of the disulphide bond and hydrolysis of the oxime bond were carried out to yield an AFP-binding cavity that was complementary in size and shape to AFP. In the AFP-imprinted cavity, the amino-oxy group derived from FM1 and the thiol group derived from FM2 were established (Fig. 7c). It should be noted that the FM1 and FM2 functional groups, which conjugated with AFP, were only within the binding cavity. Therefore, the PIMs achieved a site-selective chemical modification. In this case, a carboxyl moiety as the interacting group was introduced onto the thiol residue using disulphide exchange, and the fluorescent reporter dye (Cy5) was bound to the amino-oxy group via amine coupling (Fig. 7d). Since the interacting group and the fluorophore were located only within the cavity, highly sensitive and selective detection of AFP is expected without the high background fluorescence and noise signal resulting from non-specific binding.

The sensing capability of the prepared MIP films was examined using SPR and fluorescence microscopy. For SPR, the detection limit was estimated to be 20 ng/mL (280 pM) (Fig. 8a, COOH). To investigate the effect of the PIM, the carboxyl groups introduced as interacting moieties were replaced with hydroxyl groups. Such transformation can be easily achieved in the PIM-based MIPs bearing reversibly cleavable/bondable PIM sites[16]. The MIP binding activity dramatically decreased when the hydroxyl-containing binding site was tested (Fig. 8a, OH). This result indicated that the thiol groups were located within the cavity and interacting groups had been successfully introduced into suitable positions for binding with AFP.

The detection limit for the fluorescence measurement was estimated to be 1 ng/mL (14 pM) (Fig. 8b). Although the same MIP film was used in the binding experiment, the detection limit was 20 times lower than that by SPR, comparable to a commercially available ELISA kit. By SPR, all molecules bound on the MIP were detected; however, specific AFP binding could be detected using the fluorophore because the reporter was selectively introduced into the AFP-binding cavity by PIM. Selectivity tests using human serum albumin and prostate-specific antigen as reference proteins showed that the fluorescence signal was nearly zero for the reference proteins, confirming the high selectivity of fluorescence detection for AFP (Fig 8c).

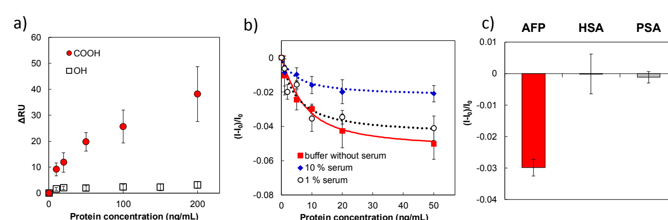

Figure 8. Binding activity of an MIP with different interacting groups (COOH or OH) in the binding cavity (a); fluorescence signalling capability of an MIP in phosphate buffer and diluted serum solutions (b); selectivity test using human serum albumin (HSA) and prostate-specific antigen (PSA) (c). Adapted from ref. 18 with permission from John Wiley & Sons, Inc., copyright 2016.

Other reported examples of PIM for the introduction of fluorophores using a cleavable monomer–protein conjugate as template include a cytochrome c-imprinted polymer film[20], prepared using a MDTA-like functional monomer covalently attached to cytochrome c. As examples using non-covalent interaction to form a cleavable monomer-protein adduct, lysozyme-imprinted polymer films were prepared using MDTA[21].

A functional monomer 4-[2-(N-methacrylamido)ethylaminomethyl]benzoic acid (MABA) was

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designed for the introduction of modifiable groups in imprinted cavities by PIM[22], which consisted of a polymerizable methacryloyl group for polymerization, a secondary amino group and a benzoic acid group as PIM sites and/or interaction sites with a template molecule (Fig. 9b). A benzene ring may provide hydrophobic interactions and π-π stacking, facilitating the approach to hydrophobic regions where electrostatic interactions could be enhanced due to the lower dielectric constant than hydrophilic regions.

Lysozyme-imprinted thin films were prepared using MABA. After the removal of lysozyme, fluorescein isothiocyanate (FITC) was introduced into the secondary amino group on MABA residues in the imprinted thin film as a fluorescent reporter dye. Compared with SPR response for the binding of lysozyme, higher selectivity was observed with the fluorescence detection, implying that the introduction of fluorescent dye by PIM provided the fluorescent MIP with specific labelling of the functional monomer residues within the imprinted binding cavity.

Other research groups have also reported post-polymerization modification techniques similar to PIM. Li et al. reported on an FRET based turn-on sensor prepared via a post-chemical modification[23], where a fluorescent dye was randomly incorporated into the MIP matrix with click chemistry after the construction of MIP. We have developed a PIM technique for introducing site-specific modifications inside imprinted cavities; therefore, such random introductions may be avoided; this would be surely attractive for MIP researchers.

Tao et al. demonstrated the photo-immobilization of the azidated BODIPY in the imprinted cavities using the azidated BODIPY-template protein adduct as a carrier[24]. Compared with our covalent conjugation-based PIM, off-target introduction may occur as the site-specific introduction depends on the specific binding of the template protein and non-specific binding possibly leads to undesirable introduction outside the imprinted cavities. This situation is the same as the case of MABA[22], therefore, the capping treatment described in the next section could help with the reduction of such fluorescence background.

Capping treatment: site-specific introduction of a reporter molecule by multi-step PIM

A major issue in the synthesis of MIPs has been the tendency of binding cavities to have heterogeneous affinity for target molecules. MIP matrices can have both high and low affinity binding cavities. These heterogeneities are caused by random localisation of functional monomer residues in polymer matrices. The problem is accentuated for MIPs prepared by non-covalent imprinting.

To solve this problem, we attempted to block the functional monomer residues in the low-affinity cavities and then site-directed modification of the residues in the high-affinity cavities was carried out by multi-step PIMs[25]. Fluorescently active MIPs specific for lysozyme were synthesised. A lysozyme-imprinted polymer film was prepared using a functional monomer with a benzoic acid moiety for electrostatic interactions with the lysozyme, and a secondary amine moiety for PIMs (Fig. 9). A diluted lysozyme solution was then added to the MIP film to protect functional monomer residues in the high-affinity binding cavities. As a first PIM (capping treatment), *p*-isothiocyanatophenyl α-D-mannopyranoside was allowed to react with the amino groups on the functional monomer residues in low-affinity cavities, leaving them unreactive to the fluorophore (Fig. 9b). High-affinity binding cavities were re-exposed by washout of the bound lysozyme, leaving the functional monomer residues available for conjugation with the reporter fluorophore in a second PIM (Fig. 9a). The protein selectivity of capped-MIP and uncapped-MIP films towards cytochrome c and ribonuclease A were examined. The selectivity factors significantly increased by 4 times (cytochrome c) and 25 times (ribonuclease A) after the capping treatment (Fig. 9c), confirming that the selectivity of MIPs generated by non-covalent imprinting can be improved by multi-step PIM-capping treatment.

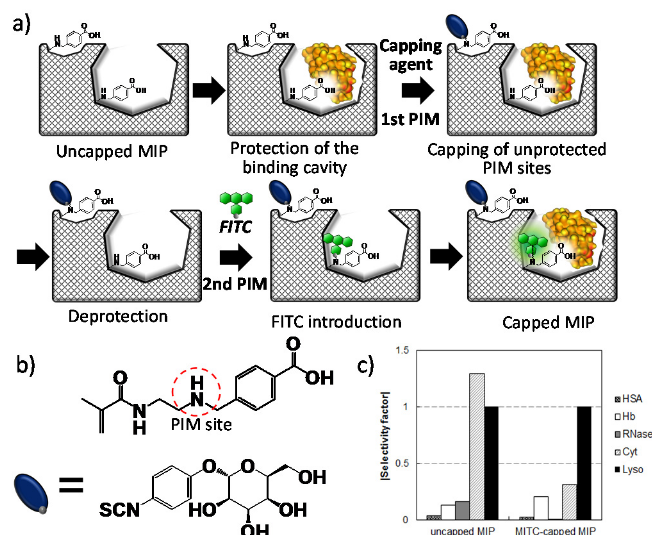

Figure 9. Schematic of site-directed introduction of fluorescent reporter dye into the high-affinity binding cavity by multi-step PIM-capping treatment (a). Chemical structures of designed functional monomer for PIM and capping agent (b). Selectivity of MIPs before and after capping treatment (c). Adapted from ref. 25 with permission from The Royal Society of Chemistry, copyright 2014.

Reconstructible molecularly imprinted cavities: conjugate protein mimics

Some natural proteins have attached prosthetic groups or cofactors. These conjugated proteins regulate their function by attachment/detachment of these non-amino acid components. For example, haemoglobin and myoglobin contain haem as a prosthetic group. Novel functional materials may be generated by mimicking the conjugated protein system. Therefore, we have attempted to generate conjugated protein mimics by molecular imprinting combined with PIMs.

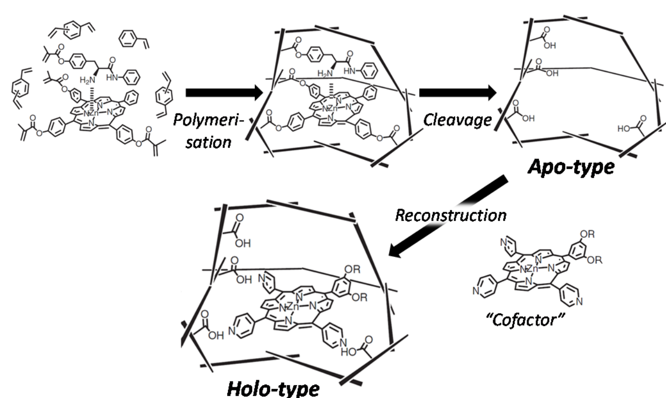

Figure 10. Conjugated protein mimics: preparation of a D-TyrAN-imprinted cavity which is reconstructible by attaching/detaching of the porphyrin derivative "cofactor". Adapted from ref. 26 with permission from The Chemical Society of Japan, Inc., copyright 2008.

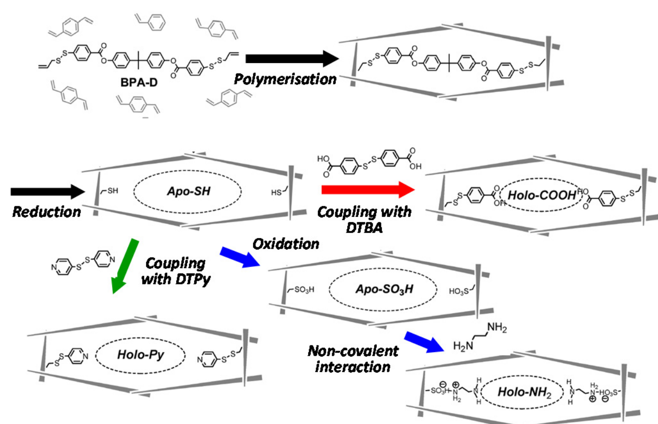

Figure 11. A bisphenol A-imprinted cavity in which the binding activity is adjusted by introducing prosthetic groups via covalent or non-covalent interactions. Adapted from ref. 27 with permission from American Chemical Society, copyright 2009.

Synthesis of conjugated protein mimics prepared by molecular imprinting and PIMs was first reported in 2008[26], in which a reconstructible imprinted cavity for D-tyrosine anilide (D-TyrAN) was produced using a porphyrin derivative, Zn^{II}-5-(3,5-dioctyloxyphenyl)-10,15,20-tri-4-pyridylporphyrin as a cofactor. In that study, methacryloyl D-tyrosine anilide was used as a template monomer and complexed with Zn^{II}-5,10,15-tris(4-methacryloyloxyphenyl)-20-phyenylporphyrin (Zn-TMPP) via axial coordination interaction. The complex was co-polymerised with styrene and divinylbenzene followed by hydrolysis of ester bonds to remove D-TyrAN and Zn-TMPP simultaneously, yielding a molecularly imprinted cavity (apo-MIP) with the size of the complex of D-TyrAN and Zn-TMPP (Fig. 10). The cavity of apo-MIP held four carboxyl groups, one derived from methacryloyl D-TyrAN and other three from Zn-TMPP. Apo-MIP lacking Zn-TMPP could not recognise D-TyrAN as the imprinted cavity was too large to bind to the target molecule. The binding ability was activated when Zn^{II}-5-(3,5-dioctyloxyphenyl)-10,15,20-tri-4-pyridylporphyrin (Zn-TPyP) as a cofactor was added to reconstruct the molecular recognition field (holo-MIP). Zn-TPyP was selected as a cofactor as its size is similar to that of Zn-TMPP, and three pyridyl groups can form a complex with carboxyl groups within the apo-type scaffold. The holo-MIP product showed specific binding activity and enantioselectivity towards D-TyrAN. It was confirmed that on/off switching of molecular recognition activity was achieved by attaching/detaching of the cofactor, Zn-TPyP. These results indicate that the functionalities of a conjugated protein could be replicated by molecular imprinting and PIMs.

The concept of conjugated protein mimics was expanded to the construction of MIPs, which could regulate their binding activity towards smaller target molecules[27]. For this purpose, bisphenol A (BPA) was used as target molecule. The template molecule (BPA-D) was designed and synthesised by a coupling reaction between BPA and two (4-allyldithio)benzoic acid molecules. After BPA-D was co-polymerised with styrene and divinylbenzene, the resulting polymer matrices were treated with $LiAlH_4$ to reduce disulphide bonds, yielding molecularly imprinted cavities (Apo-SH) containing two thiol groups in the cavity, which showed extremely low binding affinity towards BPA (Fig. 11). Using a PIM disulphide-exchange reaction, benzoic acid prosthetic groups were introduced into Apo-SH, giving Holo-COOH (Fig. 11, red arrow), which showed high binding selectivity towards BPA. However, Holo-COOH showed cross-reactivity towards 4,4-diaminophenyl methane (DAD) that can interact electrostatically with the benzoic acid prosthetic group in the cavity. The prosthetic group was then changed to a pyridine group that does not interact electrostatically with DAD, yielding Holo-Py (Fig. 11, green arrow). Although the binding affinity of Holo-Py towards BPA was slightly lower than that of Holo-COOH, the selectivity towards DAD was substantially improved. Thus, the selectivity of an MIP can be regulated by optimisation of the prosthetic groups in the binding cavity.

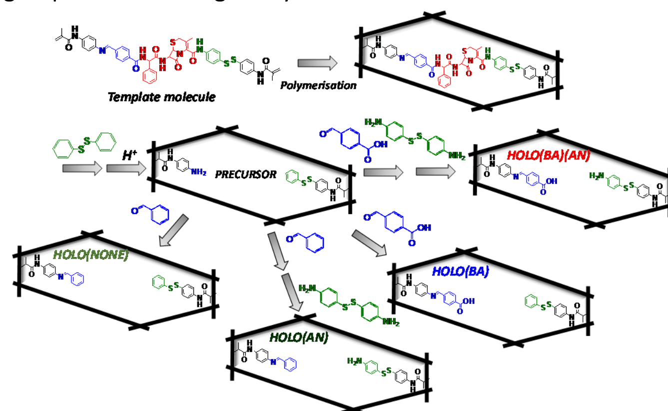

Figure 12. Schematic illustrating a β-lactam antibiotic-imprinted polymer with a cavity that is reconstructible by assembling space-filling prosthetic groups. Adapted from ref. 28 with permission from John Wiley & Sons, Inc., copyright 2014.

Prosthetic groups introduced into binding cavities via dynamically electrostatic interactions were also investigated (Fig. 11, blue arrow). Thiol groups in Apo-SH were transformed to sulphonic acid by oxidation (Apo-SO_3H), and then 1,2-diaminoethane (DAE) was introduced via electrostatic interaction to yield the Holo-NH_2. The binding activity of Holo-NH_2 depended on the DAE concentration and was decreased by addition of the monoamine compound, *n*-propylamine. These results indicated that the binding activity of Holo-NH_2

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towards BPA could be competitively tuned by the additives. Thus, the binding cavities of MIPs are sensitively structured on the molecular scale, and their binding properties can be finely controlled by introducing/removing small prosthetic groups or cofactors.

A more complicated PIM-derived system was constructed using bi-orthogonal chemical reactions to introduce two different prosthetic groups at predetermined positions in a binding cavity[28]. A β-lactam antibiotic was used as a core structure and connected with functional monomers having prosthetic groups which could conjugate with polymerisable groups via disulphide and imine bonds (Fig. 12). After co-polymerisation with triethyleneglycol dimethacrylate as a crosslinker, the core moieties connected with prosthetic groups were removed by disulphide exchange with diphenyl disulphide following hydrolysis of the imine bond under acidic conditions, yielding an apo-type molecularly imprinted scaffold (PRECURSOR). In PIMs, 4-formylbenzoic acid was allowed to react with aniline residues producing an imine bond, and the disulphide moiety was transformed into an aniline group by disulphide exchange with 4,4'-dithiodianiline. These prosthetic groups were independently introduced into the binding cavity to fill the redundant space, yielding the mature holo-MIP, HOLO(BA)(AN). These orthogonal chemical reactions, imine bond formation and disulphide exchange, progressed with high efficiency. HOLO(BA)(AN) showed high specificity for ampicillin according to a selectivity test using structurally related compounds (Fig. 13a); however, HOLO(BA), HOLO(AN), and HOLO(NONE), for which one or no prosthetic group was available, showed much less binding activity, confirming that the molecular recognition ability of HOLO(BA)(AN) was achieved by the co-operative contributions of two interacting groups (Fig. 13b). This binding activity can be switched on/off repeatedly by reversibly attaching/detaching of prosthetic groups.

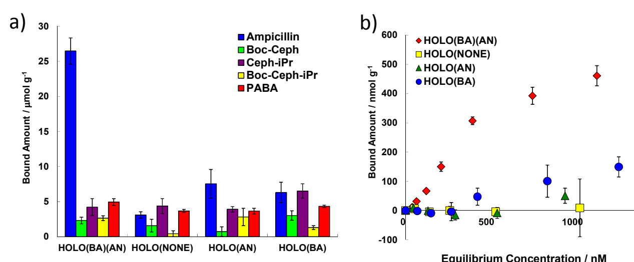

Figure 13. Selectivity (a) and binding isotherms (b) of a series of HOLO polymers. Adapted from ref. 28 with permission from John Wiley & Sons, Inc., copyright 2014.

To demonstrate generation of functional diversity in MIPs by PIMs, we investigated the feasibility of preparing MIPs with desired functions by PIMs in which the prosthetic group with a desired function is introduced into the binding cavity. First, a fluorophore for reading out a binding event was added to an MIP by PIM. 5-formylsalicylic acid (SA), a fluorescent prosthetic group, which has a similar size to that of the original prosthetic group, benzoic acid, was allowed to react with the aniline residue in the cavity. On the other disulphide side, 4-mercaptobenzoic acid was introduced as a prosthetic group to hydrogen-bond with carboxylic acid on ampicillin, yielding a holo-type MIP with fluorescence signalling capability, HOLO(SA)(MBA) (Fig. 14a). Fluorescence spectra of HOLO(SA)(MBA) were measured at an excitation wavelength of 365 nm. The fluorescence peak at around 450 nm, derived from salicylic acid, was observed, indicating that the SA prosthetic group had been successfully introduced into the polymer matrix. The fluorescence intensity increased with addition of ampicillin, indicating that HOLO(SA)(MBA) could transduce ampicillin-binding into fluorescence events (Fig. 14b). In selectivity testing using structurally related compounds, HOLO(SA)(MBA) showed the greatest fluorescence signal when ampicillin was added but weak fluorescence when other compounds were added (Fig. 14c). Thus, the molecular recognition field was maintained when the binding cavities were reconstructed by other prosthetic groups with a similar size to the original ones. We have also reported a fluorescence signalling MIP that demonstrated expansion of this concept to a fluorescence resonance energy transfer (FRET)-sensing system[29], for which two other fluorescent prosthetic groups were introduced into an imprinted cavity by multi-step PIMs.

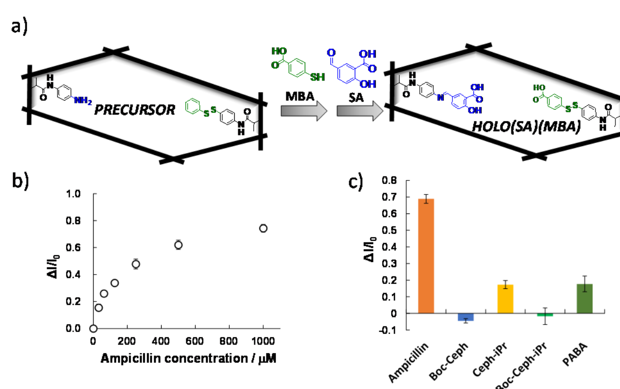

Figure 14. Schematic illustration of the preparation of a fluorescence signalling cavity by conjugation of the fluorescent prosthetic group, SA, and the interacting group, MBA (a). Relative fluorescence changes at 466 nm of HOLO(SA)(MBA) at ampicillin concentration ranging from 0 to 1000 μM. (b). Selectivity test using structurally related compounds (c). Adapted from ref. 28 with permission from John Wiley & Sons, Inc., copyright 2014.

To demonstrate the multi-functionality of an MIP generated by PIM, photoresponsivity was introduced into an apo-type scaffold. 4-Nitroso benzoic acid reacted with an aniline residue in the PRECURSOR, resulting in an azobenzene carboxylic acid (Az-BA) moiety[28]. It is well established that *cis–trans* photo-isomerisation of azobenzene can be induced by irradiation with energy levels corresponding to the π-π* transition. After the photoresponsive holo-type MIP HOLO(Az-BA)(AN) was irradiated with UV light at 365 nm, the Az-BA moiety was converted to *cis* conformation, which is expected to be an unfavourable configuration for recognising ampicillin. In fact, the ampicillin-binding activity of HOLO(Az-BA)(AN) decreased after UV light irradiation, then increased again upon irradiation with visible light, indicating that alternation of the binding activity was reversible by alternating UV and visible

light irradiation. Therefore, a reversible photoresponsive property can be introduced into a molecularly imprinted cavity by PIM.

As described above, given the circumspect design of a molecularly imprinted cavity with space available for prosthetic groups, and selection of appropriate prosthetic groups to generate a desired function, MIPs can be synthesised with diverse capabilities, such as on/off switching of binding activity, fluorescence signalling, and photoresponsivity. Recent reports have shown that tuning the functionalities of MIPs by PIM is a remarkably useful strategy to produce advanced materials with desired functions.

Conclusions and perspectives

Molecular imprinting has been gaining popularity because of the comparative simplicity of its procedures, however; the functionalities of MIPs prepared by conventional methods are limited. The discovery that a variety of functional groups can be introduced into MIPs by individual processing of the polymer-matrix fabrication and the functionalisation step of PIMs has markedly increased the possibilities for MIP functional enhancement. Site-specific transformation of functional groups showed enhancement of the specificity of MIPs towards target molecules. Introduction of spectroscopic properties has transformed MIPs into signalling MIPs, transducing the binding behaviour into spectral changes. The success of space-filing PIM has confirmed the effectiveness of molecular imprinting as a technique to prepare molecular recognition cavities complementary to template molecules and the regulation of binding properties in MIPs by using prosthetic groups and cofactors. More precisely designed PIMs could provide significantly specific synthetic antibodies comparable to the specificity of natural antibodies. The examples of PIM discussed here are a small sample of the sophisticated MIPs that have been synthesised using PIM. These reports have generated a wide range of spin-off effects in the fields of life sciences and biotechnology, in which antibodies are frequently used. The MIP–PIM approach may prove to be reliable, affordable, and convenient, facilitating the emergence of polymer-based artificial antibodies with functionalities exceeding those of natural antibodies, which can be recognized as a new generation of MIPs.

The process through which a definite number of active binding cavities per unit area or weight of MIPs can be regulated is yet to be developed. This process may enable MIPs to be transformed into more defined synthetic antibodies bearing defined density or molar concentrations of binding cavities such as natural antibodies. Such exploration is ongoing in our laboratory, and we believe that this issue may be settled in the near future by well-designed template molecules with the use of functional monomers capable of PIM treatments, allowing us to adopt MIPs for several applications.

Conflicts of interest

There are no conflicts to declare.

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Biography

Toshifumi Takeuchi

Toshifumi Takeuchi received his PhD in 1984 at the Toyama Medical and Pharmaceutical University, Toyama, Japan. Since 2003, he has been a full professor of Kobe University. Previously, he was an assistant professor at Nihon University, a post-doctoral fellow at University of Delaware and University of Hawaii at Manoa, an associate professor in Research Center for Advanced Science and Technology, University of Tokyo, and a full professor of Hiroshima City University. His research interests include design and synthesis of molecularly imprinted polymers and their analytical and biomedical applications.

Hirobumi Sunayama

Hirobumi Sunayama received his Ph.D. degree in 2013 from Kobe University, Kobe, Japan. Following this, he accepted a researcher position in the group of Toshifumi Takeuchi at Kobe University, dealing with molecular imprinting. After working for three years in Takeuchi laboratory, he joined the Department of Pharmacy, Yasuda Women's University, as an assistant professor. His current research interests include design and synthesis of advanced materials by using molecular imprinting and post-imprinting modifications.

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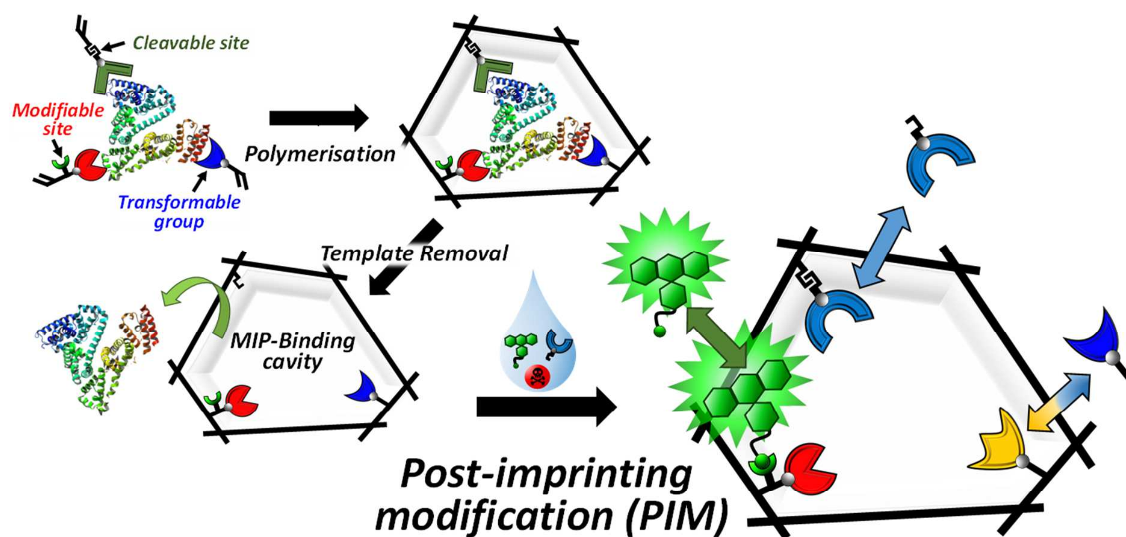

Post-imprinting modification (PIM) is an innovative strategy for generating MIPs analogous to biosynthesising proteins to introduce new functionalities in a site-directed manner.