Molecular Recognition

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A Programmable Signaling Molecular Recognition Nanocavity Prepared by Molecular Imprinting and Post-Imprinting Modifications

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Abstract: *Inspired by biosystems, a process is proposed for preparing next-generation artificial polymer receptors with molecular recognition abilities capable of programmable site-directed modification following construction of nanocavities to provide multi-functionality. The proposed strategy involves strictly regulated multi-step chemical modifications: 1) fabrication of scaffolds by molecular imprinting for use as molecular recognition fields possessing reactive sites for further modifications at pre-determined positions, and 2) conjugation of appropriate functional groups with the reactive sites by post-imprinting modifications to develop programmed functionalizations designed prior to polymerization, allowing independent introduction of multiple functional groups. The proposed strategy holds promise as a reliable, affordable, and versatile approach, facilitating the emergence of polymer-based artificial antibodies bearing desirable functions that are beyond those of natural antibodies.*

Proteins play important biological roles, such as the transportation of molecules, the transduction of stimuli-responsive signals, and the mediation of catalysis involved in metabolism and biosynthesis. These functions are made possible by post-biosynthetic processes called posttranslational modification, that is, the formation of more complex functionalized adducts by the conjugation of non-protein groups.[1] This strategy is advantageous because the biosynthetic process for fabricating apo-type scaffold proteins and the subsequent modification process for yielding holo-type functionalized proteins proceed independently under their respectively optimized conditions, enabling the preparation of complicated and sophisticated functional proteins. The finely tuned performance of conjugated proteins and other function-acquired proteins are of great interest for applications to various life science and bio-production processes.[2] However, these proteins are not sufficiently stable for use in industrial applications, and their bio-production is problematic from the standpoint of cost and quality control.

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In this context, synthetic materials capable of molecular recognition and additional bio-relevant functions have been attracting significant attention as substitutes for naturally occurring functionalized proteins.[3] In particular, molecularly imprinted polymers (MIPs) are well-known as artificial polymer receptors and have been used as affinity separation media, molecular recognition elements for sensors, and specific target-capturing materials in ligand binding assays.[4] Molecular imprinting involves a template molecule inducing the formation of nanocavities capable of molecular recognition by co-polymerizing crosslinker(s) and co-monomer(s) in the presence of functional monomer–template molecule complexes. Templating involves covalent linkages and/or noncovalent interactions, and the template molecule is either the target molecule or a derivative. After polymerization, the template molecule is removed, resulting in molecularly imprinted cavities complementary in shape, size, and alignment of functional groups suitable for rebinding the target molecule and/or its structurally related derivatives. Because of the simplicity of the process, MIPs have a good reputation as functional materials, but the functionality on most MIPs reported to date is rather simple and far from the complex functions of natural proteins.

Recently, we reported a biomimetic molecular imprinting process for the creation of new classes of synthetic multifunctional materials for small molecules capable of molecular recognition, signal transduction, and photoresponsiveness. These materials are acquired by site-directed post-polymerization modifications at predetermined positions within the imprinted cavities (post-imprinting modification, PIM), in which prosthetic groups are introduced into these cavities either covalently or non-covalently to improve binding activity, thus mimicking post-translational modification in biosystems.[5] This modification can help achieve the transformation of functional groups and/or site-directed introduction of additional functions in the imprinted cavities, such as on/off switching, signaling, catalytic functions, and other desirable features.

Herein, we propose a strategy for fabricating antibody-relevant MIPs, followed by newly designed PIMs in which multiple programmable functional groups are independently introduced only within the molecular recognition nanocavity. This PIM step provides MIPs with functions capable of differentiating from natural proteins. Herein, we demonstrate fluorescent signaling antibody-like MIPs for α-fetoprotein (AFP), a biomarker to detect hepatocellular carcinoma and other cancers of the liver. Our strategy is to use covalent-type protein-imprinting involving the formation of the template molecule comprising AFP covalently coupled with two different cleavable functional monomers. Subsequently,

multi-step PIMs independently introduce two functional groups within the AFP-imprinted cavity: one is an interactive site specific for binding AFP and the other is a fluorescent probe for transducing the AFP binding event into a fluorescence change. We cleaved each type of cleavable group independently and introduced either interactive sites or fluorescent probes after each cleavage, resulting in cavities that contained both interactive sites and fluorescent probes.

Two cleavable functional monomers were designed (Figure 1) to realize a precisely site-directed functionalization process by PIMs. Functional monomer 1 (FM1) possesses a thiol-reactive pyridyl disulfide moiety for reacting with the thiol groups on AFP, and this moiety is aligned with an oxime

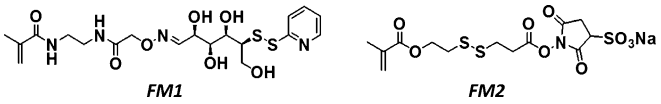

Figure 1. Chemical structures of the designed cleavable functional monomers FM1 and FM2.

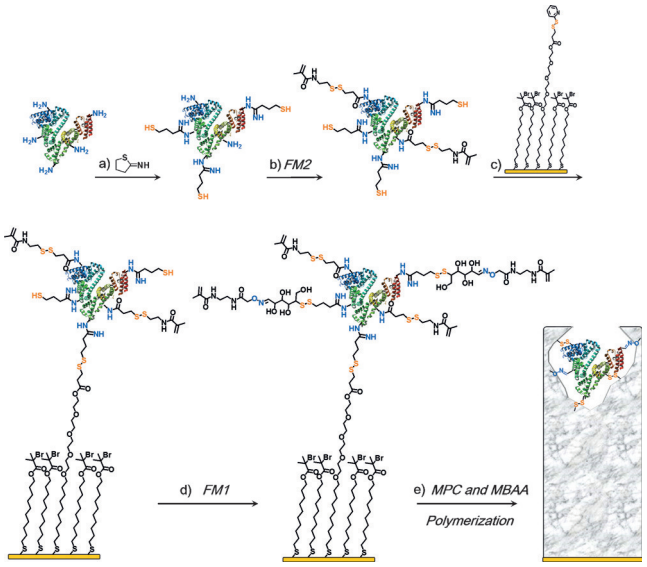

Figure 2. Preparation of the AFP-imprinted polymer layer on the sensor chips.

linkage for later introduction of a fluorescent probe that can transduce the binding event of the target protein into a fluorescence change. Functional monomer 2 (FM2) possesses an amine-reactive active ester moiety for reacting with the amino groups of AFP, and this ester moiety is aligned with a disulfide linkage for the later introduction of interactive sites for the amine groups of AFP. To conjugate FM1 with AFP, thiol groups were introduced on the surface amino groups of AFP using 2-iminothiolane.[6] The advantage of this reagent is that the reaction proceeds under mild conditions, and the number of cationic charges is unchanged on the thiolated AFP due to the generation of amidine groups after the ring opening reaction, thus possibly helping prevent denaturation of the AFP (Figure 2a). The number of introduced thiol groups was determined using Ellman's reagent[7] to be 5.4 (Supporting Information, Table S1).

First, FM2 was coupled with the thiolated AFP by forming an amide bond with the remaining amino groups on the thiolated AFP (Figure 2b). The number of conjugated FM2s was determined by MALDI-TOF mass spectrometry to be approximately 5 (Supporting Information, Figure S1). There are 42 lysine residues in AFP,[8] so the conducted modifications may not affect the structure of AFP. Indeed, circular dichroism spectra showed that the secondary structure of the conjugate remained unchanged (Supporting Information, Figure S2). The FM2-coupled thiolated AFP was then immobilized on gold-coated substrates for surface plasmon resonance (SPR) sensing and quartz crystal microbalance (QCM) sensing. Grafted onto the surface of the chip was a mixed self-assembled monolayer, comprising initiator (2-bromoisobutyl) groups for atom transfer radical polymerization using a surface-initiated activator generated by electron transfer (SI-AGET ATRP)[9] and pyridyl disulfide groups that are reactive for thiol groups under mild conditions (Figure 2c). Subsequent conjugation of FM1 with the thiol groups on the FM2-coupled thiolated AFP provided the immobilized template protein on the substrates, thus covalently imprinting AFP (Figure 2d). Since immobilization of the template protein onto the substrate suppresses the thermal motion of AFP during polymerization, the shapes of imprinted cavities could be expected to be homogeneous.

The AFP-imprinted polymer thin layer (MIP_{10}, where the subscript "10" indicates 10% crosslinking) was prepared on the template-immobilized substrates (Figure 2e) by SI-AGET ATRP of a biocompatible monomer, 2-methacryloyloxyethyl phosphorylcholine (MPC), and a hydrophilic crosslinker, *N*,*N*′-methylenebisacrylamide (MBAA), where poly(MPC) was reported to reduce non-specific binding of proteins,[10] thus we employed MPC instead of commonly used acrylamide that is capable of weakly interacting with protein. The reaction was conducted in 10 mM phosphate buffer (pH 7.4) for 1 h at 40 °C to provide a crosslinking ratio of 10%. The thickness of the obtained polymer thin layer was estimated to be approximately 14 nm by X-ray reflectivity (Supporting Information, Figure S3), consistent with the previously reported value.[11] The template molecules were then removed by hydrolysis of the oxime bond in FM1, followed by reduction of the disulfide linkage in FM2 by tris(2-carboxyethyl)phosphine (TCEP), leaving only the oxiamino groups and the thiol groups within the AFP-imprinted cavities. The removal ratio of AFP was roughly estimated using a QCM-D sensor chip to be more than 90% by determining the ratio of the decreased frequency value resulting from the removal (5.5 Hz) to the increased frequency value due to immobilization of AFP (6.0 Hz). Finally, the fluorescent signaling AFP-imprinted cavities were fabricated using multi-step PIMs (Figure 3). The first PIM step involved the introduction of a carboxy group as an interactive site at the thiol residues derived from FM2, where the positions of the introduced carboxy groups corresponded to that of the amino groups on AFP (since FM2 is coupled via amino groups on AFP). In the second PIM step, amine-reactive cyanine 5 (Cy5) was introduced as the signal transduction site at the oxiamino residues derived from FM1.

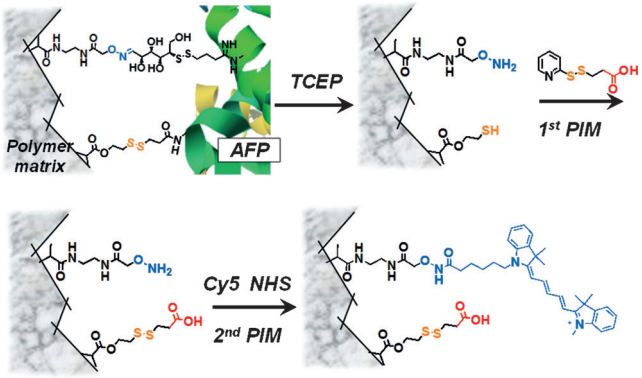

Figure 3. Multi-step PIMs within the AFP-imprinted cavity.

The effect of the carboxy groups as the electrostatically interactive sites toward AFP within the MIP_{10} cavities was investigated by introducing hydroxy groups rather than carboxy groups into the FM2 residues, then determining the binding activity of AFP for the hydroxylated binding cavities in the hydroxylated MIP_{10}-immobilized sensor chips using SPR sensing. As show in Figure 4a, strong affinity was observed with the MIP_{10} bearing carboxy groups within the cavities, with an apparent affinity constant (K_a) of $1.4\times10^{9}\,M^{-1}$, estimated using a 1:1 binding model (Supporting Information, Figure S4).

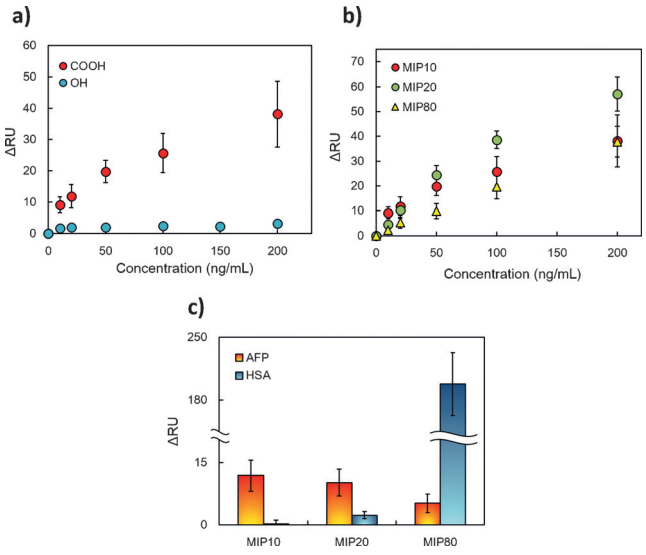

Figure 4. a) AFP binding activity of carboxylated MIP_{10} and hydroxylated MIP_{10}; b) AFP binding activity of MIPs; c) selectivity of MIPs for AFP and HSA (20 ng mL^{-1}).

In contrast, the affinity significantly decreased when these interactive sites were replaced with hydroxy groups: an apparent affinity constant could not be estimated due to the very small response, confirming that the first PIM using disulfide linkages can fabricate highly sensitive binding cavities for AFP, with the locations of the interactive sites matching the locations of the amino groups on AFP. Generally, PIM allows us to exchange functional groups reversibly,[12] and thus, the affinity for AFP can be regulated by changing the functional groups to develop the desirable binding activity as necessary.

The crosslinking ratios of MIPs may affect their binding activity, and therefore, we prepared MIPs with crosslinking ratios of 20% (MIP_{20}) and 80% (MIP_{80}), along with the original MIP_{10} (10%). These MIPs were used to investigate the binding behaviors of AFP and human serum albumin (HSA), which has similar values of isoelectric point ($pI=4.7$) and molecular weight ($Mw=66$ kDa), and 39% amino acid sequence homology[8] with AFP ($pI=4.75$ and $Mw=71$ kDa), using SPR. The SPR response reflects the total amount of AFP bound on the sensor chip. The affinities of these MIPs were similar for AFP, with estimated K_a values of $6.8\times10^{8}\,M^{-1}$ (MIP_{20}), $7.8\times10^{8}\,M^{-1}$ (MIP_{80}), and $1.4\times10^{9}\,M^{-1}$ (MIP_{10}), as mentioned above (Figure 4b; Supporting Information, Figure S4). However, non-specific binding of HSA to MIP_{20} and MIP_{80} was much higher than to MIP_{10}: 11 times and 880 times higher, respectively, than that of MIP_{10} (Figure 4c). This may be due to hydrophobic interactions resulting from a lower content of MPC and a higher content of MBAA. Therefore, the minimum crosslinking ratio required for maintaining the imprinted cavity should be used in order to achieve high selectivity toward the target protein AFP.

The detectability of AFP binding by fluorescence was investigated by preparing MIP_{10} on QCM sensor chips. These chips were mounted under a flow cell with a window for measuring fluorescence (Supporting Information, Figure S5), allowing measurement of the QCM response based on the total binding of AFP on the chips and the fluorescence change caused by the binding event of AFP on the same MIP_{10}. An increase in the concentration of AFP caused a decrease in the frequency of the QCM chip (Supporting Information, Figure S6) and quenching of the fluorescence (Figure 5a) owing

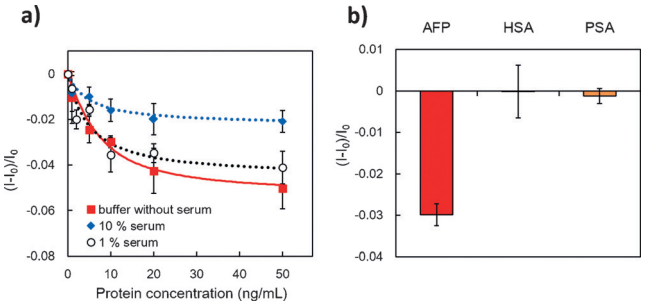

Figure 5. a) Binding isotherms of MIP_{10} in 10 mM phosphate buffer pH 7.4 containing 0%, 1%, and 10% human serum and b) selectivity of MIP_{10} prepared on a QCM chip towards AFP, HSA, and PSA (10 ng mL^{-1}), evaluated by fluorescence microscopy.

to interaction between Cy5 and AFP, as previously reported.[13] The shapes of both apparent binding isotherms were similar, but the K_a values were very different: $1.0\times10^{7}\,M^{-1}$ based on QCM detection (Supporting Information, Figure S5) and $1.5\times10^{10}\,M^{-1}$ based on fluorescence detection (Supporting Information, Figure S7-a). The fluorescence-based affinity constant estimate is 1500 times that of the QCM-based estimate and 11 times that of the SRP-based

estimate. Furthermore, the fluorescence-based K_a value was not significantly affected by the presence of human serum: $1.5\times10^{10}\,\text{M}^{-1}$ for 1% serum (Supporting Information, Figure S7-b) and $1.4\times10^{10}\,\text{M}^{-1}$ for 10% serum (Supporting Information, Figure S7-c). The highly selective fluorescence detection of AFP was confirmed (Figure 5b) by observing almost no binding of HSA or prostate-specific antigen (PSA), a biomarker for prostate cancer. The obtained sensitivity and selectivity compare favorably with previously reported methods for the detection of AFP.[14] Furthermore, no additional reagents and/or special devices are required for detection, such as secondary antibodies labelled with enzymes, reagents required for enzymatic reactions,[14a] or surface plasmon-field enhanced fluorescence devices,[14b] shortening the operation time to 5 min for injection and 5 min for washing. Therefore, MIP_{10} could be highly advantageous for clinical use.[14c]

These results suggest that programmed modification by site-directed PIMs should provide a highly sensitive response to the binding of AFP, due to highly specific in-cavity fluorescence labelling by the PIM. To demonstrate that the introduced Cy5 in the cavity leads to high sensitivity, we prepared MIP_{10} with reduced amounts of Cy5 in the cavity by using Cy5-NHS diluted with acetyl sulfo-NHS (which has no fluorescence, but kept the total concentration of reactive groups constant). The fluorescence intensity of MIP_{10} decreased linearly with decreasing molar fraction of Cy5, showing that the PIM reaction proceeds quantitatively, and the amount of Cy5 introduced is sufficiently low that concentration-dependent quenching is observed under the conditions employed (Figure 6a). As shown in Figure 6b,

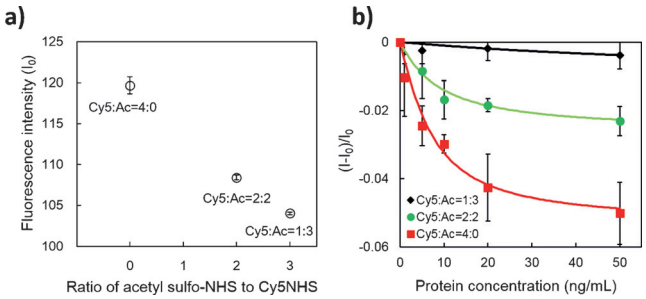

Figure 6. a) Effect of the amount of Cy5 introduced into MIP_{10} on the initial fluorescence intensity (I_0), and b) fluorescence response of the MIP_{10} conjugated with the different amounts of Cy5 towards varied concentrations of AFP.

a decrease in the amount of introduced Cy5 resulted in a lower fluorescence change and decreased sensitivity. The K_a for MIP_{10} with half the amount of introduced Cy5 was $9.2\times10^{9}\,\text{M}^{-1}$ (Supporting Information, Figure S8-a), and for one-fourth the amount of introduced Cy5, the K_a was roughly estimated to be $5.0\times10^{8}\,\text{M}^{-1}$ (the fluorescence intensity was too small to calculate K_a precisely; Supporting Information, Figure S8-b). Consequently, the reason for the higher sensitivity achieved by Cy5 labeling compared to SPR and QCM detection is the site-directed introduction of multiple Cy5 molecules at predetermined positions within the imprinted cavity generated by the PIM process.

In conclusion, the proposed MIPs not only enable the highly sensitive and selective detection of AFP, they also shorten the operation time by not requiring additional reagents or enzymatic reactions. Furthermore, introducing a reporting dye into the binding domains of antibodies to create signaling antibodies is quite difficult, whereas a well-designed PIM can introduce the signaling function specifically in the binding cavities to achieve sensitive and selective detection. MIPs have long been considered less useful than natural antibodies, but the present method involving covalent-type protein imprinting using cleavable functional monomers, followed by programmed PIMs, clearly demonstrates the possibility of providing sophisticated functional MIPs. Tailored in-cavity functionalization can be separately achieved in a pre-determined manner from the formation of imprinted cavities, and post-functionalization by PIMs can be independently conducted in a site-directed manner. This is the first reported strategy for acquiring various functions and tuning and/or transforming the acquired functionality of a MIP in a controlled tailor-made manner. We believe that these findings will lead to the emergence of a new generation of MIPs and represent a breakthrough for applying MIPs to a diverse range of scientific and industrial fields in which antibodies are primarily used, eventually inducing a paradigm shift in bioscience and bio-industry.

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