Multi-Functional Nanocavities Fabricated Using Molecular Imprinting and Post-Imprinting Modifications for Efficient Biomarker Detection

Hirobumi SUNAYAMA*, Toshifumi TAKEUCHI1,2
1Graduate School of Engineering, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan
2Center for Advanced Medical Engineering Research & Development (CAMED), Kobe University, 1-5-1 Minatojimaminami-machi, Chuo-ku, Kobe 650-0047, Japan

Abstract
Antibodies and enzymes are currently considered the gold-standard molecular recognition elements as they facilitate the construction of biosensing systems and exhibit high specificity and affinity toward target molecules. However, the low stability of such systems and high associated production cost limit the practical applications of antibodies and enzymes, thereby necessitating the development of alternative molecular recognition elements. Molecularly imprinted polymers (MIPs) are synthetic polymer receptors that are capable of molecular recognition. These polymers contain binding cavities of various shapes and sizes that are complementary to the target molecule and aid in the capture of target molecules. However, although the original procedure for generating MIPs, developed before 2000, is simple, the resulting binding activity and selectivity are inferior to those of antibodies. Meanwhile, post-imprinting modification (PIM) involves site-directed chemical modification of functional monomer residues within the molecularly imprinted cavities to alter MIP functionality. In this review, we provide an overview of sophisticated PIM techniques for developing highly sensitive MIPs that can be used to recognize biomarker proteins. Toward this, we draw heavily on information from our own recent work. This article has the potential to provide important insights that would aid the development of synthetic polymer materials for biosensing.

Keywords: Molecularly imprinted polymers; Post-imprinting modifications; Bio-sensing; Synthetic receptors

1. Introduction
Molecularly imprinted polymers (MIPs) are synthetic polymers capable of recognizing specific molecules [1-5]. MIPs are synthesized via a simple procedure. In the first step, the target molecule (or a derivative/analog of the molecule) and the functional monomer interact, via intermolecular interactions, to form a complex. Next, the complex is copolymerized in the presence of co-monomers and cross-linkers to generate the cross-linked polymer matrix. In the final step, the target molecule is removed using a suitable washing solution, resulting in the generation of molecularly imprinted cavities in the polymer matrix. The shape and size of the created cavities are similar to those of the template molecule. Moreover, the functional groups derived from a functional monomer are suitably arranged to facilitate the rebinding of the target molecule. MIPs have been shown to effectively bind a wide range of targets, including amino acids, hormones, herbicides, antibiotics, peptides, proteins, viruses, and bacteria. Thus, MIPs have applications in diverse fields, with potential for use as a substitute for various naturally occurring materials that are frequently used as molecular recognition elements, including antibodies and enzymes. However, MIPs often exhibit simple functionality with only moderate affinity and selectivity. Although only a few MIPs have been reported to exhibit high affinity and selectivity toward target molecules, developing MIPs with sophisticated functionalities, similar to those of proteins, remains a significant challenge.

To address this issue, we developed a post-imprinting modification (PIM) strategy that involves chemically
modifying the functional groups present within the imprinted cavity to develop multi-functional MIPs (Fig. 1) [6,7]. This PIM technique is inspired by the post-translational modifications observed during protein biosynthesis, and involves the use of functional monomers bearing modifiable parts for the preparation of MIPs. Reversible bonds, such as disulfide, imine, and oxime bonds, as well as amino, hydroxyl, and carboxyl groups are incorporated into the functional monomers to generate multi-functional MIPs. Following the synthesis of MIPs, the functionalizable moieties present within the imprinted cavities are transformed into alternate functional groups or are modified to alter specific functionalities, including switching on/off binding activity, fluorescent signaling ability, photo-responsive ability, and catalytic activity. Indeed, we have reported various multi-functional MIPs prepared using the PIM strategy and have summarized their properties in a previously published review article [7]. In the current review, we describe the recently developed multi-functional nanocavities fabricated using the molecular imprinting and PIM, focusing on MIPs suitable for the highly sensitive and selective detection of biomarker proteins.

We employed the PIM strategy to reduce non-specific binding. Specifically, we designed and synthesized 4-[2-(N-methacrylamido)ethylaminomethyl]benzoic acid (MABA), a functional monomer [10] (Fig. 2), bearing a benzoic acid unit capable of interacting with target molecules, and secondary amino groups. The secondary amino moiety forms the modifiable functional group, the properties of which are exploited using the PIM method. Prostate-specific antigen (PSA) was selected as the target biomarker as increased PSA (> 10 ng/mL) indicates the progression of prostate cancer [11]. A PSA-immobilized substrate was prepared by utilizing a cyclic di-ester formation reaction between phenylboronic acid (introduced on the substrate) and the cis-diol of the sugar chain on PSA. As a result, PSA was immobilized on the substrate with the sugar chain facing down, this would be helped to form homogeneous imprinted cavity [12,13]. A polymer matrix was fabricated on the PSA-immobilized substrate through the surface-initiated atom transfer radical polymerization (SI-ATRP) [14]. 2-Methacryloyloxyethyl phosphorylcholine (MPC) was used as a bio-compatible co-monomer [15] and N,N'-methylenebisacrylamide (MBAA) was used as a hydrophilic cross-linker during the process. A PSA-imprinted cavity was generated after the removal of the PSA template. The protein binding activity of the synthesized MIP was assessed using a surface plasmon resonance (SPR) sensing system. Meanwhile, human serum albumin (HSA) and immunoglobulin G (IgG) were used as the reference proteins for determining the selectivity of the prepared MIP. Although the MIP was found to exhibit a high binding affinity toward PSA ($K_a = 9.15 \times 10^8$ M$^{-1}$), it did not demonstrate selectivity (Fig. 3), indicating the presence of both high- and low-affinity cavities in the MIPs. Next, to suppress off-target protein binding, capping of the functional monomer residues within the low-affinity cavities was performed in the following manner. A low concentration of PSA was added to occupy the high affinity cavities and protect the functional monomer residues. Subsequently, the capping reagent, oligo(ethylene glycol)carboxylic acid-reactive ester (NHS-PEG12-Me), was added to the MIP to react with the amino groups present in the MABA residues within the low-affinity cavities. Oligo(ethylene glycol) was selected as the capping reagent because of its biocompatibility [16-18]. The bound PSA molecules were then removed by washing, effectively regenerating capped of synthesized MIPs for target molecules [9]. Moreover, the formation of the unstable complex results in the formation of heterogeneous imprinted cavities in the polymer matrix, some of which exhibit high affinities toward target molecules, while the remaining cavities have low affinities. Low-affinity cavities can potentially induce non-specific binding, resulting in MIPs with low selectivity. It is, therefore, important to reduce the number of low-affinity cavities to ensure the production of highly specific MIPs.

Fig. 1. Preparation of multi-functional MIPs using the PIM technique. a) Formation of complexes between the functional monomers bearing modifiable moieties and target proteins. b) Polymerization in the presence of co-monomers and cross-linkers to yield cross-linked polymer matrix. c) Removal of template protein and cleavage of the reversible bond. d) Introduction/exchange of functional groups.

2. Capping treatment

The simplicity of the non-covalent-imprinting technique makes it an attractive strategy for the development of MIPs[8]. In this technique non-covalent interactions, such as electrostatic interactions, hydrogen bonding, and van der Waals interactions, are exploited to form the functional monomer–template molecule complex, the instability of which may contribute to the observed heterogeneous affinity.

of synthesized MIPs for target molecules [9]. Moreover, the formation of the unstable complex results in the formation of heterogeneous imprinted cavities in the polymer matrix, some of which exhibit high affinities toward target molecules, while the remaining cavities have low affinities. Low-affinity cavities can potentially induce non-specific binding, resulting in MIPs with low selectivity. It is, therefore, important to reduce the number of low-affinity cavities to ensure the production of highly specific MIPs.

We employed the PIM strategy to reduce non-specific binding. Specifically, we designed and synthesized 4-[2-(N-methacrylamido)ethylaminomethyl]benzoic acid (MABA), a functional monomer [10] (Fig. 2), bearing a benzoic acid unit capable of interacting with target molecules, and secondary amino groups. The secondary amino moiety forms the modifiable functional group, the properties of which are exploited using the PIM method. Prostate-specific antigen (PSA) was selected as the target biomarker as increased PSA (> 10 ng/mL) indicates the progression of prostate cancer [11]. A PSA-immobilized substrate was prepared by utilizing a cyclic di-ester formation reaction between phenylboronic acid (introduced on the substrate) and the cis-diol of the sugar chain on PSA. As a result, PSA was immobilized on the substrate with the sugar chain facing down, this would be helped to form homogeneous imprinted cavity [12,13]. A polymer matrix was fabricated on the PSA-immobilized substrate through the surface-initiated atom transfer radical polymerization (SI-ATRP) [14]. 2-Methacryloyloxyethyl phosphorylcholine (MPC) was used as a bio-compatible co-monomer [15] and N,N'-methylenebisacrylamide (MBAA) was used as a hydrophilic cross-linker during the process. A PSA-imprinted cavity was generated after the removal of the PSA template. The protein binding activity of the synthesized MIP was assessed using a surface plasmon resonance (SPR) sensing system. Meanwhile, human serum albumin (HSA) and immunoglobulin G (IgG) were used as the reference proteins for determining the selectivity of the prepared MIP. Although the MIP was found to exhibit a high binding affinity toward PSA ($K_a = 9.15 \times 10^8$ M$^{-1}$), it did not demonstrate selectivity (Fig. 3), indicating the presence of both high- and low-affinity cavities in the MIPs. Next, to suppress off-target protein binding, capping of the functional monomer residues within the low-affinity cavities was performed in the following manner. A low concentration of PSA was added to occupy the high affinity cavities and protect the functional monomer residues. Subsequently, the capping reagent, oligo(ethylene glycol)carboxylic acid-reactive ester (NHS-PEG12-Me), was added to the MIP to react with the amino groups present in the MABA residues within the low-affinity cavities. Oligo(ethylene glycol) was selected as the capping reagent because of its biocompatibility [16-18]. The bound PSA molecules were then removed by washing, effectively regenerating capped.
MIPs with high affinity cavities. The capped MIPs exhibited reduced non-specific binding (in reference proteins) and significantly increased selectivity. Moreover, the binding activity of the capped MIP was retained after the capping treatment ($K_a = 1.24 \times 10^9 \text{M}^{-1}$). Hence, the relatively simple and feasible process of capping can potentially be employed for producing highly selective and specific synthetic receptors.

Studies on the capping treatment of MIPs revealed the importance of introducing tunable functional groups into an imprinted cavity to modulate the specific binding properties of MIPs. Although modification procedures are necessary for the preparation of template molecules, the covalent-imprinting approach is a promising strategy for delivering modifiable groups to an imprinted cavity [4,5]. In our example, PSA was conjugated to a polymerizable group via the formation of a disulfide bond between the two components and used as a template. Next, the PSA-imprinted polymer film was prepared on a polymerizable-group-modified PSA-immobilized substrate using the SI-ATRP in the presence of MPC, MBAA, and a functional monomer containing phenylboronic acid (FM-B) for sugar chain recognition. The disulfide bond was reduced, and the resultant polymer was washed to remove the PSA moiety. Throughout this process, thiol groups were generated within the imprinted cavity. Additionally, 4-((2-[[3-(pyridyldithio)propionylamino]ethyl]amino)methyl)benzoic acid was designed and synthesized to be used as a post-imprinting reagent (PIR, Fig. 4) that introduced interacting groups during the PIM process. The pyridyldisulfide moiety, capable of conjugating with thiol groups exposed on the surface walls of the imprinted cavity [19,20], as well as the (4-aminomethyl)benzoic acid moiety (which is similar to...
Chromatography

Fig. 4. Preparation of fluorescent signaling PSA-imprinted polymers following the multi-step PIM technique using PIR. a) Polymerization in the presence of FM-B, MPS, and MBAA. b) Removal of the protein moiety by reducing the disulfide bond. c) Introduction of interacting groups using PIR. d) The fluorescent dye conjugation process.

MABA) were used as the PIRs, which were introduced via a disulfide exchange reaction. The binding activity of the synthesized MIP was subsequently examined using the SPR sensing system. The results revealed PSA specific and selective binding ($K_a = 6.76 \times 10^9 \text{ M}^{-1}$) (Fig. 5a). Furthermore, the role of cross-linking monomers in the MIPs was investigated. Non-cross-linked polymers were found to induce non-specific binding, a phenomenon that can be attributed to the flexibility of the polymer chain. Thus, increasing the degree of monomer cross-linking in the polymer matrix served to enhance selectivity, while maintaining the PSA affinity (Fig. 5b). However, a significantly high degree of cross-linking could also induce non-specific binding as the increased number of amide components derived from MBAA, which formed hydrogen bonds, may facilitate non-specific binding [21].

The generation of fluorescent signaling MIPs using MABA and fluorescent dye as the PIM, has been previously reported [22,23]. This process required multi-step PIMs to introduce a fluorescent reporter molecule (Alexa Fluor 647) into an imprinted cavity. The fluorescent signaling capacity of the resultant MIPs was examined using a custom-made fluorescent measurement system equipped with a fluorescent microscope and a computer-controlled liquid handling robot [24,25]. Results show a decrease in the fluorescence intensity upon increasing the PSA concentration. Moreover, the degree of change in fluorescence intensity was greater when PSA was added to the MIP than when IgG was used as the additive (Fig. 5c). These results indicate the successful introduction of fluorescent reporter molecules into the PSA-imprinted cavity, while achieving sensitive transduction of PSA binding events to fluorescent changes (apparent $K_a = 4.58 \times 10^9 \text{ M}^{-1}$).

Fig. 5. Binding properties of MIPs. a) SPR responses of MIPs toward varying PSA concentrations. b) Selectivity of MIPs under varying degrees of cross-linking (0%, 10%, and 20%). c) Fluorescent responses exhibited by fluorescent dye-introduced MIP toward PSA and IgG.
Additionally, α-fetoprotein (AFP)-imprinted polymer films, prepared by covalent-imprinting and the PIM strategies, exhibiting fluorescent signaling properties, have also been reported [26]. AFP is a biomarker used to detect hepatocellular carcinoma and other liver-related diseases [27,28]. In the reported study, AFP was modified using 2-iminothiolane to introduce thiol groups on the surface. It was further modified using 2-(2-pyridyl)ditioethyl methacrylate, yielding an AFP connecting the polymerizable groups via a disulfide bond. Following immobilization of the modified AFP on the Br group and phenylboronic-acid-modified substrate—where one was for ATRP and the other for the formation of a cyclic di-ester with the sugar chain of AFP—the SI-ATRP was used to copolymerize the molecule with MPC, MBAA, and pyrrolidyl methacrylate (PyM). PyM was then used as an electrostatic interaction monomer and the AFP-imprinted cavity was generated by treating the polymer with tris(2-carboxyethyl)phosphine (TCEP) and sodium dodecyl sulfate solutions, resulting in disulfide bond cleavage and removal of the AFP moiety. A thiol-reactive fluorescent reporter dye (Alexa Fluor 647 C2 maleimide) was then reacted with the thiol group exposed on the surface of the imprinted cavities resulting in the generation of MIPs with fluorescent signaling capacity. The fluorescent intensity changes were observed with addition of AFP. The apparent binding constant ($K_a$) was calculated as $7.6 \times 10^{10} \text{ M}^{-1}$ and the limit of detection was 0.45 ng/mL. Moreover, the fluorescently labeled MIP was functioned in diluted serum. Hence, the introduction of a cavity-selective fluorescent reporter dye via the PIM strategy may represent an attractive method for the production of fluorescence-labeled MIPs exhibiting signaling properties for the highly sensitive and selective detection of biomarker proteins.

Recently, the PIM strategy has also been used for sensing extracellular vesicles (EVs) [29]. EVs are produced and released by nearly all cell types, including cancer cells, and serve as a means to communicate with other cells. EVs comprise exosomes, microvesicles, and apoptotic bodies [30,31]. Exosomes are lipid bilayer-enclosed nano-sized vesicles (diameter: 30–200 nm) that contain microRNAs, mRNAs, and proteins. These EVs have attracted increasing attention as they have been found to play a vital role in maintaining intercellular communication. Moreover, proteins and glycans derived from the original cell surface are present on the surface of EVs, allowing them to function as tumor biomarkers.

For preparation of EV-imprinted polymer, at first the Br and nitrilotriacetic acid (NTA) groups were introduced on an Au-coated glass chip, using the self-assembled monolayer (SAM) formation technique. (Fig. 6c). The NTA moiety formed a complex with Ni$^{2+}$ ions and captured the His-tagged protein G. This process was followed by the introduction an anti-CD9 antibody into the system (Fig. 6d). The target EVs secreted from the prostate cancer cells (PC-3) were
subsequently immobilized on the surface via an antibody-
antigen reaction pathway (Fig. 6e). Immobilized EVs were
modified using thiolated oleyl poly(ethylene glycol), a cell
membrane modifier, to introduce a modifiable moiety into
the scaffold during the PIM step (Fig. 6a). The resulting
sample was further modified using 2-(2-pyridyl)dithioethyl
methacrylate (Fig. 6b), thereby effectively introducing
modifiable disulfide bonds and polymerizable groups on the
surface of the EVs (Fig. 6f). SI-ATRP was then performed to
surround the EVs with poly MPC brushes (Fig. 6g). Next, the
EVs, antibody, and protein G were removed from the
polymer by de-complexing Ni²⁺ and reducing the disulfide
bonds. The process yielded polymer nanocavities containing
NTA moieties and thiol groups on the inside (Fig. 6h). The
His-tagged protein G was used to introduce the antibody, as
various antibodies can be potentially introduced into the
cavities. However, further modification of the antibodies is
not needed for the effective execution of the process. During
the PIM process, the His-tagged protein G was introduced
into the NTA moiety, followed by introduction of the
antibody. A fluorescent signaling dye was coupled with the
thiol groups present within the cavity (Fig. 6i) yielding
antibody-conjugated fluorescent signaling nanocavities with
the potential to detect EVs (Fig. 6j). The sensing abilities of
the prepared chips were then investigated and the chip was
found to exhibit EV concentration-dependent fluorescent
changes with high affinity ($K_d = 6.0 \times 10^{-16} M$). In fact, the
sensitivity of the proposed system was approximately 1000
times greater than that of conventional ELISAs. Meanwhile,
considering that antibodies and fluorescent dyes could
accumulate inside the nanocavity, higher sensitivity could be
achieved by increasing the local concentration of the
functional molecules. By introducing both the fluorescent
signaling dyes for EV binding readout, and antibodies for
capturing EVs, into the nanocavity, the EVs were effectively
detected in a single step. Meanwhile, conventional ELISAs
involve the execution of multiple steps for detecting EVs.
Hence, this facile, rapid, and highly sensitive EV-sensing
method can be potentially used for the early diagnosis of
various cancers. Indeed, the EV sensing chip reportedly
detected cancer-related EVs in tear samples collected from
breast cancer patients.

EV-imprinted nano-cavities conjugated with antibodies
and fluorescent reporter dyes achieved highly sensitive
detection of EVs. However, they have certain limitations
such as the difficulty in using EVs as a template for
fabricating MIP, because EVs are heterogeneous in size,
surface properties, and interiors. In addition, it is difficult to
obtain the purified sample. Therefore, silica nanoparticles,
which are similar in size to EVs, were used as a template for
preparing the nano-cavity [32]. They are easier to handle,
with the possibility of size control and the surface properties
are amenable to modifications such as the addition of
functional groups. Silica nanoparticles (diameter 200 nm)
modified with carboxylic acid was conjugated with
hexahistidine peptides and methacryloyl groups via disulfide
bond and used as the template. Ni-NTA group and bromo
group were conjugated to the Au-coated glass chip followed
by immobilization of the template silica nanoparticles. The
procedures for preparing the antibodies conjugated
fluorescent signaling nano-cavities were as described
previously. This sensing system exhibited higher affinity
towards PC-3 secreted EVs ($K_d = 2.4 \times 10^{-16} M$). We
successfully used this system, for the first time, to detect
cancer-related intact EVs in tears. We could clearly
differentiate between the samples from healthy donors and
breast cancer patients, and between the samples collected
before and after total mastectomy. This strategy could
accelerate research on the bio-related lipid bilayers, such as
EVs, viruses, bacteria, and cells, for important applications
in the various fields of life science.

4. Multi-colored MIPs for simultaneous detection of
multiple target proteins

The capping treatment described herein can be used to
introduce various functional groups into the desired cavities.
Meanwhile, a covalent-imprinting strategy-based PIM is a
promising approach for introducing modifiable groups to the
imprinted cavities. Dual-colored MIPs for the simultaneous
detection of two biomarker proteins were developed by
combining these two methods [33]. Specifically, AFP and
PSA were modified using a functional monomer (FM1), the
process for which yielded the polymerizable-group-modified
AFP and PSA molecules (Fig. 7). The modified molecules
were then used to develop dual-imprinted polymer films. The
SI-ATRP was conducted for developing the polymer films in
the presence of AFP and PSA-immobilized substrates.
Reduction of disulfide bonds helped remove the protein
moieties present in the polymer matrix, leaving the AFP- and
PSA-imprinted nanocavities with exposed thiol groups
inside. To achieve the cavity-selective introduction of
fluorescent dyes, a low concentration of AFP was added to
the polymer film, which then occupied the AFP-imprinted
cavity to protect the exposed thiol groups. Subsequently, the
fluorescent dye Alexa Fluor 594 C2 maleimide was added to
react with the thiol groups present within the PSA-imprinted
cavity. The protected AFP-imprinted cavity was then
regenerated by removing the bound AFP via a washing
procedure. The procedure that was used to protect the PSA-
imprinted cavity was similar to that described for the AFP-
imprinted cavity. The fluorescent dye Alexa Fluor 647 C2
maleimide was then introduced into the PSA-imprinted
cavity and the bound PSA moieties were subsequently
removed, yielding PSA-imprinted cavities labeled with
Alexa Fluor 594 and AFP-imprinted cavities labeled with
Alexa Fluor 647 in the polymer matrix. Results from
fluorescence experiments revealed that each fluorescent-labeled cavity selectively translated AFP and PSA binding events to fluorescence intensity (Fig. 8). Moreover, using a 1:1 binding model and a curve-fitting software, the estimated binding constants ($K_d$) were $1.5 \times 10^{-9}$ M for PSA and $1.6 \times 10^{-9}$ M for AFP. Further, the limits of detection were calculated to be 1.67 ng/mL (PSA) and 1.80 ng/mL (AFP), indicating that the desired fluorescent dye could be successfully introduced to the desired imprinted cavity following a multi-step post-imprinting strategy that protected the functional groups present in each imprinted cavity using proteins. This strategy can be potentially used to develop

**Fig. 7.** PSA and AFP dual imprinting and cavity-selective multi-step PIMs for the introduction of different fluorescent dyes inside imprinted cavities. (a) Cleavable functional monomer (FM1) conjugated with AFP or PSA. (b) Immobilization of FM-protein conjugates on the ATRP initiator and phenylboronic-acid-modified gold substrate. (c) Preparation of polymer matrix by SI-ATRP. (d) Removal of template proteins by reducing the disulfide bond and hydrolysis of cyclic diester. (e) Protection of the AFP-imprinted nanocavity by AFP itself. (f) Introduction of the first fluorescent dye. (g) Regeneration of AFP-imprinted nanocavity by removing bound AFP via washing. (h) Protection of PSA-imprinted nanocavity by PSA itself. (i) Introduction of the second fluorescent dye, and (j) regeneration of PSA-imprinted nanocavity to yield the multi-colored fluorescent MIP.

**Fig. 8.** Fluorescence-based selectivity tests using a filter corresponding to (a) Alexa Fluor 594 and (b) Alexa Fluor 647.
three or more fluorescent signaling protein-imprinted cavities in an MIP matrix, thus, representing multi-protein analysis systems (for simultaneous detection of samples) that differ from the conventional array-type systems.

5. Protein sensing MIP nanoparticles prepared via PIMs

As described above, fluorescent signaling MIPs prepared using the PIM strategy have been fabricated on substrates. Moreover, nanoparticle-based MIPs, functionalized using the PIM approach, were developed to expand the field of the PIM-based materials. Nanoparticle-shaped materials have applications in various biological fields as they can be used to develop sensors, imaging agents, and drug delivery systems [34-36].

MIP-nanogels that emit fluorescent signals for HSA were prepared using the emulsifier-free precipitation–polymerization technique, with MABA serving as the functional monomer (Fig. 9a) [37]. Dynamic light scattering, and transmission electron microscopy (TEM) were used to characterize the nanogels, revealing the diameter of the purified nanogel to be 18 nm (Fig. 9b). Hence, the nanometer-sized polymer gels retained their nano-sized structures after the PIM process. They were conjugated with ATTO647N, a fluorescent signaling dye, and a characteristic fluorescence peak was observed at approximately 664 nm following PIM, indicating the successful incorporation of the ATTO dye inside the MIP-nanogels. Moreover, the nanogels were found to effectively bind target proteins, and selectively translate these binding events to fluorescence changes (Fig. 9c). The fluorescent signaling MIP-nanogels were found to effectively detect HSA in dilute serum samples (real serum samples) with an efficiency that was comparable to that of the conventional bromocresol purple-based method that is frequently used for quantifying HSA in clinical samples. Hence, these fluorescent MIP-nanogel-based sensing systems have the potential for application in various fields, including food analysis. In fact, we have developed a system for detecting contamination of halal food using fluorescent signaling MIP-nanogels. This system used the porcine serum albumin as the template and was found to detect pork contamination (0.1 wt.%) in beef extracts [38].

6. Conclusions

Herein, materials capable of sensing biomarker proteins, prepared following the molecular imprinting and PIM techniques, have been reported, with particular attention paid to our recent work. The capping treatment method can be potentially used to circumvent the challenges faced during non-covalent-imprinting for developing MIPs. Meanwhile, the selectivity could be enhanced by producing a hindered atmosphere around the functional group present within low-affinity binding cavities. Moreover, the method of cavity-selective functional group modification was demonstrated and a dual-colored fluorescent signaling MIP was developed. The cavities inside the developed MIPs could selectively respond to target proteins. Modifiable parts and multiple functional groups were successfully incorporated into the imprinted cavity following covalent-imprinting, resulting in high binding affinities ($K_d$ of the order of $10^{-9}$ or $10^{-10}$ M), comparable to those exhibited by natural antibodies. The method developed by combining the molecular imprinting and sophisticated PIM strategies may prove to be a reliable, affordable, and convenient method for the development of biomarker-sensing materials, while also helping to develop artificial antibodies consisting of synthetic polymers (plastic antibodies).

Acknowledgements

The authors thank Dr. Yukiya Kitayama (Osaka Prefecture University) for the useful discussions. We thank our colleagues and graduate students for their hard work in making sure that our work sees the light of the day. The work was funded by JSPS (KAKENHI) and JST (A-STEP). We thank the private companies who have funded the work presented herein. I thank the Society for Chromatographic Sciences for the Encouragement Award in 2020 (H. Sunayama).

References

[1] Haupt, K.; Mosbach, K. Chem. Rev. 2000, 100, 2495-2504.
[2] Schirhagl, R. *Anal. Chem.* **2014**, *86*, 250-61.
[3] Takeuchi, T.; Hayashi, T.; Ichikawa, S.; Kaji, A.; Masui, M.; Matsumoto, H.; Sasao, R. *Chromatography* **2016**, *37*, 43-64.
[4] Komiyama, M.; Mori, T.; Ariga, K. *Bull. Chem. Soc. Jpn.* **2018**, *91*, 1075-1111.
[5] BellBruno, J. J. *Chem. Rev.* **2019**, *119*, 94-119.
[6] Takeuchi, T.; Sunayama, H.; Takano, E.; Kitayama, Y. Post-imprinting and in-cavity functionalization. In Mattiasson, B.; Ye, L., (Eds.) *Molecularly imprinted polymers in biotechnology*, Springer, Cham, **2015**, pp 95-106.
[7] Takeuchi, T.; Sunayama, H. *Chem. Commun.* **2018**, *54*, 6243-6251.
[8] Zhang, H.; Ye, L.; Mosbach, K. *J. Mol. Recognit.* **2006**, *19*, 248-259.
[9] Umpleby Li, R. J.; Bode, M.; Shimizu, K. D. *Analyst* **2000**, *125*, 1261-1265.
[10] Matsumoto, H.; Sunayama, H.; Kitayama, Y.; Takano, E.; Takeuchi, T. *Sci. Tech. Adv. Mater.* **2019**, *20*, 305-312.
[11] Catalona, W. J.; Smith, D. S.; Ratliff, T. L.; Dodds, K. M.; Coplen, D. E.; Yuan, J. J. J.; Petros, J. A.; Andriole, G. L.; Coplen, D. E.; Yuan, J. J. J.; Petros, J. A.; Andriole, G. L.; Komiyama, M.; Mori, T.; Ariga, K.
[12] Komiyama, M.; Mori, T.; Ariga, K. *Bull. Chem. Soc. Jpn.* **2017**, *90*, 1156-1161.
[13] Suda, N.; Sunayama, H.; Kitayama, Y.; Kamon, Y.; Takeuchi, T. *R. Soc. Open Sci.* **2017**, *4*, 170300.
[14] Saeki, T.; Sunayama, H.; Kitayama, Y.; Takeuchi, T. *Langmuir* **2019**, *35*, 1320-1326.
[15] Matyjaszewski, K. *Macromolecules* **2012**, *45*, 4015-4039.
[16] Ishihara, K. J. *Biomed. Mater. Res. A* **2019**, *107*, 933-943.
[17] Otsuka, H.; Nagasaki, Y.; Kataoka, K. *Biomacromolecules* **2000**, *1*, 39-48.
[18] Nagasaki, Y. *Polym. J.* **2011**, *43*, 949-958.
[19] Schöttler, S.; Becker, G.; Winzen, S.; Steinbach, T.; Mohr, K.; Landfester, K.; Mailänder, V.; Wurm, F. R. *Nat. Nanotechnol.* **2016**, *11*, 372-377.
[20] Sunayama, H.; Takeuchi, T. *ACS Appl. Mater. Interfaces* **2014**, *6*, 20003-20009.
[21] Sunayama, H.; Kitayama, Y.; Takeuchi, T. *J. Mol. Recognit.* **2018**, *31*, e2633.
[22] Horikawa, R.; Sunayama, H.; Kitayama, Y.; Takano, E.; Takeuchi, T. *Angew. Chem. Int. Ed.* **2016**, *55*, 13023-13027.
[23] Sunayama, H.; Ooya, T.; Takeuchi, T. *Biosens. Bioelectron.* **2010**, *26*, 458-462.
[24] Sunayama, H.; Ooya, T.; Takeuchi, T. *Chem. Commun.* **2014**, *50*, 1347-1349.
[25] Takano, E.; Shimura, N.; Akiba, T.; Kitayama, Y.; Sunayama, H.; Abe, K.; Ikekuburo, K.; Takeuchi, T. *Microchim. Acta* **2017**, *184*, 1595-1601.
[26] Morishige, T.; Takeano, E.; Sunayama, H.; Kitayama, Y.; Takeuchi, T. *ChemNanoMat* **2019**, *5*, 224-229.
[27] Yachnin, S.; Hsu, R.; Heinrikson, R. L.; Miller, J. B. *Biochim. Biophys. Acta. Protein Struct.* **1977**, *493*, 418-428.
[28] Wong, G. L. H.; Chan, H. L. Y.; Tse, Y.-K.; Chan, H.-Y.; Tse, C.-H.; Lo, A. O. S.; Wong, V. W. S. *Hepatology* **2014**, *59*, 986-995.
[29] Mori, K.; Hirase, M.; Morishige, T.; Takeano, E.; Sunayama, H.; Kitayama, Y.; Inubushi, S.; Sasaki, R.; Yashiro, M.; Takeuchi, T. *Angew. Chem. Int. Ed.* **2019**, *58*, 1612-1615.
[30] Valadi, H.; Ekström, K.; Bossios, A.; Sjöstrand, M.; Lee, J. J.; Lötvall, J. O. *Nat. Cell Biol.* **2007**, *9*, 654-659.
[31] Hoshino, A.; Costa-Silva, B.; Shen, T.-L.; Rodrigues, G.; Hashimoto, A.; Tesic Mark, M.; Molina, H.; Kohsaka, S.; Di Giannatale, A.; Ceder, S.; Singh, S.; Williams, C.; Soplop, N.; Uryu, K.; Pharmer, L.; King, T.; Bojmar, L.; Davies, A. E.; Ararso, Y.; Zhang, T.; Zhang, H.; Hernandez, J.; Weiss, J. M.; Dumont-Cole, V. D.; Kramer, K.; Wexler, L. H.; Narendran, A.; Schwartz, G. K.; Healey, J. H.; Sandstrom, P.; Jørgen Labori, K.; Kure, E. H.; Grandgenett, P. M.; Hollingsworth, M. A.; de Sousa, M.; Kaur, S.; Jain, M.; Mallya, K.; Batra, S. K.; Jarnagin, W. R.; Brady, M. S.; Fodstad, O.; Muller, V.; Pantel, K.; Minn, A. J.; Bissell, M. J.; Garcia, B. A.; Kang, Y.; Rajasekhar, V. K.; Ghajar, C. M.; Matei, I.; Peinado, H.; Bromberg, J.; Lyden, D. *Nature* **2015**, *527*, 329-335.
[32] Takeuchi, T.; Mori, K.; Sunayama, H.; Takano, E.; Kitayama, Y.; Shimizu, T.; Hirose, Y.; Inubushi, S.; Sasaki, R.; Tanino, H.; Kitayama, Y.; Takeuchi, T. *Bull. Chem. Soc. Jpn.* **2021**, *94*, 525-531.
[33] Smith, B. R.; Gambhir, S. S. *Chem. Rev.* **2017**, *117*, 901-986.
[34] Takeuchi, T.; Kitayama, Y.; Sasao, R.; Yamada, T.; Toh, K.; Matsumoto, Y.; Kataoka, K. *Angew. Chem. Int. Ed.* **2017**, *56*, 7088-7092.
[35] Haupt, K.; Medina Rangel, P. X.; Bui, B. T. S. *Chem. Rev.* **2020**, *120*, 9554-9582.
[36] Tsutsumi, K.; Sunayama, H.; Kitayama, Y.; Nakamachi, Y.; Sasaki, R.; Takeuchi, T. *Adv. NanoBiom Res.* **2021**, *1*, 2000079.
[37] Cheubong, C.; Takeo, E.; Kitayama, Y.; Sunayama, H.; Nakamachi, Y.; Minamoto, K.; Takeuchi, R.; Furutani, S.; Takeuchi, T. *Biosens. Bioelectron.* **2021**, *172*, 112775.