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Open-Sandwich Molecular Imprinting: Making a Recognition Matrix with Antigen-Imprinted Antibody Fragments

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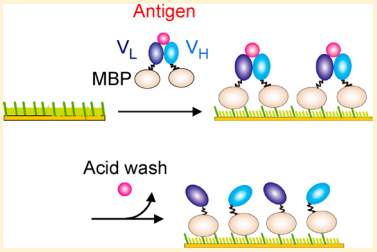
Supporting Information

ABSTRACT: A novel antibody−polymer conjugation method termed open-sandwich molecular imprinting (OS-MIP) has been proposed to produce a specific recognition matrix in the presence of a target antigen. The resultant carboxymethyldextran matrix conjugated with two separate antibody variable region fragments imprinted with the cognate antigen showed higher antigen-binding capacity than non-imprinted ones and was successfully used to sensitively monitor multiple antigen binding/desorption events by a surface plasmon resonance biosensor. Furthermore, when each fragment was labeled with different fluorophores before conjugation, the fluorescence signals of the matrix made by OS-MIP clearly showed an antigen concentration dependent increase in Förster resonance energy transfer between the two dyes. By using a combination of various methods for detecting interaction, OS-MIP will be a useful platform for detecting various targets from small molecules to proteins with high sensitivity and specificity.



INTRODUCTION

In analytical fields such as immunodiagnostics and environmental analysis, technological demands are mounting for quick and accurate methodologies for identifying various molecules, ranging from small organic compounds to larger proteins. To address these needs, development of a compact and robust analytical device that affords real-time sensing is essential. Although powerful analytical instruments such as GC/MS and LC/MS/MS already exist in the market, due to their size, they are far from being suitable for *in situ* use for point-of-care tests. Besides these, alternative methods such as ELISA utilizing antigen−antibody reactions are compatible with miniaturized portable apparatuses. However, with these methods, collected samples must be carefully analyzed by experts, which makes real-time monitoring and/or simultaneous concurrent measurements difficult.

As a means to implement rapid and specific detection of various analytes *in situ*, molecular imprinting has been proposed for decades.[1] Although conventional molecularly imprinted polymers (MIP) have been able to recognize and rapidly separate small analytes by template-assisted polymerization, strong and specific recognition of larger proteins in aqueous solution has been elusive[2,3] without the help of natural antibodies.[4] However, even with the help of antibodies, a conventional method utilizing an antibody−lectin pair in an MIP matrix to detect a glycoprotein CEA can only emit a small and slow signal on the basis of gel shrinkage,[5] and it is unclear whether the method can be applied to the detection of other (smaller or larger) antigens.

As an alternative, we propose a novel method for making a reaction matrix, which we call open sandwich molecular imprinting (OS-MIP). OS-MIP enables specific recognition, as well as real-time detection of various targets in a small reaction volume. Instead of using whole antibody molecules, we used isolated antigen recognition domains of antibodies V_H and V_L. On the basis of the nature of these domains, where the association between them is weak in the absence of antigen but markedly strengthened by a specific antigen, many targets, not only proteins but also various small molecules, were specifically detected with a high degree of sensitivity in a noncompetitive manner (open sandwich immunoassay, OS-IA).[6,7] We attempted to apply this phenomenon to make a novel matrix of protein (antibody fragments)-carboxymethyl (CM)-dextran polymer conjugate for the detection and sensing of various molecules *in situ*.

EXPERIMENTAL SECTION

Cloning of anti-HEL Gene. Two mice were immunized with 1 mg/mL hen egg lysozyme (HEL) in Freund's complete adjuvant five times in two week intervals, and sacrificed to obtain the spleen 3 days after the last immunization. Total RNA was extracted from the spleen using Isogen (Nippon Gene, Toyama, Japan) and used for RT-PCR to amplify V_H and V_L cDNAs. The primers used for amplifying variable region sequences are based on Thomas Grunwald and Greg Winter, "Primer set for generation of highly diversified mouse phage display libraries", MRC Centre for Protein Engineering, Cambridge, UK (2000). The V_H and V_L genes were inserted

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to phagemid pIT2 [8] to make a single-chain Fv (scFv) library, and an antigen-specific scFv-encoding vector pIT2-LxE16 was selected by phage display, the detail of which will be described elsewhere.

Construction of Vectors. An expression vector pET-MBPp-V_H(HEL)-His10 encoding a MBP-V_H(HEL)-His_{10} protein, in which *E. coli* maltose binding protein (MBP) and a His-tag containing ten histidine residues (His_{10}) are, respectively, fused to N- and C-termini of anti-hen egg lysozyme (HEL) V_H, was constructed by using the pET-MBPp-V_L(BPA)-His6 encoding V_L of an anti-bisphenol A antibody[9] tagged with MBP and six histidine residues at its N- and C-termini, respectively. To replace a His_6 tag of pET-MBPp-V_L(BPA)-His6 with a His_{10} tag, PCR product amplified with primers, 5′-AAAAAAAGCGGCCGCGGAG*CATCA-TCACCATCACCACCACCACCACCAC*TGAGATCCGG-3′ (underlined: *Not*I site; italicized: His_{10} sequence) and 5′-CCAATGCTTAATCAGTGA-3′ using pET-MBPp-His6 as the template was digested with *Not*I and *Sca*I, and inserted between *Not*I and *Sca*I sites of pET-MBPp-V_L(BPA)-His6, yielding pET-MBPp-V_L(BPA)-His10. PCR amplification of the V_H(HEL) gene fragment was performed by using pIT2-LxE16 encoding an scFv gene for anti-HEL antibody as a template, with primers 5′-CTTTCTATGCGGCCCAGCCGGCCATGGCCGAK-GTSVAGCTTCAGGAGTC-3′ (underlined: *Sfi*I site) and 5′-AAAAAAGCGGCCGCGCTCGAGACGGTGACCGTGG-3′ (underlined: *Not*I site). The V_H(HEL) fragment was digested with *Not*I and *Sfi*I, and ligated to pET-MBPp-V_L(BPA)-His10 digested with the same enzymes, obtaining pET-MBPp-V_H(HEL)-His10. An expression vector pET-MBPp-V_L(HEL)-His10 encoding a MBP-V_L(HEL)-His_{10} protein was constructed similarly to pET-MBPp-V_H(HEL)-His10, except that V_L(HEL) gene fragment was amplified using pIT2-LxE16 with primers AAAAAAGGCCCAGCCGGCCATGGCGTCG-ACGGATATTTGATGAC (underlined: *Sfi*I site) and TTTCTCGTGCGGCCGCACGTTTTATTTCCAACTTTG (underlined: *Not*I site) instead of V_H(HEL) fragment.

Preparation of MBP-V_H and MBP-V_L Proteins. *E. coli* OverExpress C41(DE3) cells were transformed with either pET-MBPp-VH(HEL)-His10 or pET-MBPp-VL(HEL)-His10, and single colonies were used to inoculate 4 mL of LB medium containing 100 μg/mL ampicillin and 0.1% glucose and cultured overnight at 30 °C. Four milliliters of the small-scale culture was used to inoculate 800 mL of LB medium containing 100 μg/mL ampicillin, and cultured at 30 °C. When an OD_{600} of the culture reached 0.5, isopropyl-β-thiogalactopyranoside (IPTG) was added up to 500 μM and further cultured overnight at 30 °C. The bacterial culture was then separated into supernatants and pellets of *E. coli* by centrifugation. The protein was independently collected from the supernatants by ammonium sulfate precipitation and from the pellet by ultrasonication of bacterial cells. From the supernatant, 344 g of ammonium sulfate was added to about 800 mL of the culture supernatant and the mixture was stirred overnight at 4 °C. Subsequently, the protein was collected by centrifugation and the pellet was suspended in 30 mL of Talon buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.0). From the pellet, the pellet was suspended in 30 mL of Talon buffer and then subjected to ultrasonication, followed by centrifugation, to collect a supernatant. The supernatant was dialyzed against Talon buffer. Each protein collected in Talon buffer was applied to a column (ϕ16 mm × 15 mm) filled with Talon affinity resin (Clontech Laboratories, Inc., Mountain View, CA). Subsequently, the resin was washed with the Talon buffer, and then Elution buffer (Talon buffer containing 500 mM imidazole) was added to elute the protein. The purified protein was confirmed by SDS-PAGE. The buffer of the eluate was changed to HBS-N (10 mM HEPES, 150 mM NaCl, pH 7.4), and then glycerol was added at a final concentration of 16%. The obtained solution was stored at −80 °C. MBP-V_H(BGP)-His_6 and MBP-V_L(BGP)-His_6 proteins were prepared as described previously.[10]

Preparation of V_H(HEL) and V_L(HEL) Proteins. To 1 mL of the purified MBP-V_H(HEL)-His_{10} or MBP-V_L(HEL)-His_{10} solution (in HBS-N, ∼1000 μg/mL), 20 units of Genenase (New England Biolabs, Inc., Ipswich, MA) was added to digest the linker containing HY sequence. The mixture was allowed to react at 25 °C for 5 h. After the reaction, the protein was purified with Talon affinity resin as above, and the buffer of the eluate was changed to HBS-N.

Preparation of the scFv(HEL) Protein. *E. coli* HB2151 strain was transformed with pIT2-LxE16 plasmid and the resulting single colony was used to inoculate 4 mL of LB medium containing 100 μg/mL ampicillin and 0.1% glucose. After overnight cultivation at 30 °C, the culture was added to 800 mL of LB medium containing 100 μg/mL ampicillin, and cultured at 30 °C until OD_{600} reached 0.5−0.6. Then, IPTG was added up to 400 μM and further cultured at 16 °C for 16 h. The bacteria were removed by centrifugation, and the protein was recovered from the supernatant and purified as described above.

Open-Sandwich Molecular Imprinting (OS-MIP). To perform imprinting of V_H/V_L with corresponding antigen before conjugation with CM-dextran (CMD) polymer, ∼10 μM each of V_H and V_L fused with or without MBP as prepared above were mixed with 0.1−1.0 mol equiv antigen in HBS-N at 25 °C for 3 h. Thereafter, the conjugation/immobilization reaction was performed as below.

Immobilization of Antibody Fragments and Evaluation of Antigen Binding Activity by SPR Biosensor. To immobilize and evaluate the binding activity of antibody fragments, SPR biosensor Biacore 3000 (GE Healthcare, Tokyo, Japan) was used. The proteins (diluted to ∼200 μg/mL in 10 mM NaOAc, pH 4.5) were immobilized onto a sensor chip CM5 (GE Healthcare) by amine coupling according to manufacturer's recommendations. Briefly, CM dextran in the sensor chip was activated by the 1:1 mixture of 0.4 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and 0.1 M *N*-hydroxysuccinimide in water for up to 7 min, and after brief washing with HBS-N, it was treated with the proteins for 7 min, before deactivation of excess reactive groups by 1 M ethanolamine−HCl, pH 8.5, for 7 min and washing with 10 mM glycine−HCl, pH 1.5, for 1 min. Afterward, either hen egg lysozyme or osteocalcin C-terminal peptide in HBS-N was added to the flowcells as an antigen at 25 °C at 10 μL/min, and its binding and dissociation kinetics (10 min each) were monitored. The RU value obtained with a control flowcell without immobilized protein was subtracted to determine specific binding.

To monitor repeated bindings, the flowcells after reaction with antigen in HBS-N for 10 min and dissociation in HBS-N for 10 min were serially washed for 1 min each with 10 mM glycine−HCl, pH 1.5, and 10 mM NaOH. The cycles were repeated, and the maximum increases in RU were recorded. During the measurement, the sensor chip surface was kept wet with HBS-N, while during longer storage periods, it was kept

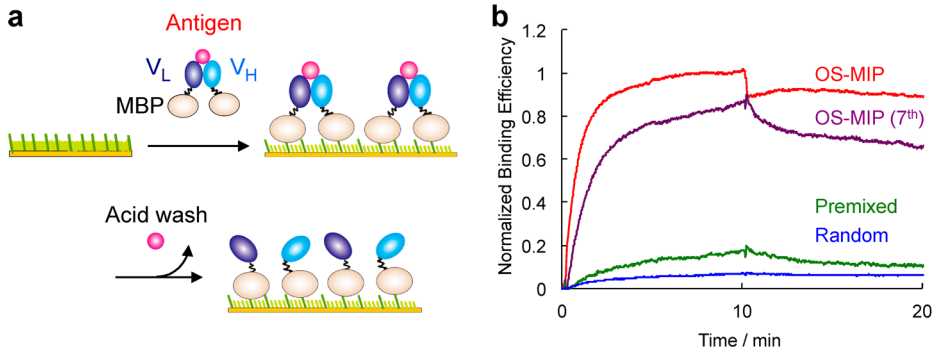

Figure 1. OS-MIP. (a) Scheme of OS-MIP. (b) SPR sensorgrams obtained for the flowcells immobilized with MBP-fused V_H and V_L fragments by the methods shown. HEL (100 nM) in HBS-N was introduced from 0 to 10 min, and dissociation in HBS-N was monitored. The sensorgrams obtained with OS-MIP (red and magenta for the first and the seventh run, respectively) are compared with those for premixed (green) and randomly immobilized (blue) surfaces.

dry at 4 °C. To calculate K_d values, *BIAevaluation 4.1* software (GE Healthcare) was used.

Thermodynamic Parameters. To derive entropic and enthalpic parameters of each antibody fragment and their mixture, SPR analysis was performed at four different temperatures (15−45 °C) using BIAcore T100 (GE Healthcare). The proteins were immobilized on a Series S sensor chip CM5 (GE Healthcare) and used for kinetic analyses as described above. The relationship between the calculated K_d value and temperature was used to derive ΔG and ΔH values using the *BIAevaluation* software.

Fluorescence Labeling. Fluorescence labeling of each antibody fragment was performed with one of the following succinimide esters: Alexa Fluor 555 (Molecular probes), Alexa Fluor 647 (Molecular probes), or HiLyte Fluor 555 (Anaspec). The antibody fragment (~0.8 mg/mL in 10 mM sodium phosphate, pH 7.0) was mixed with an appropriate amount of dye-succinimide in HBS-N and rotated at 25 °C for 30 min. The mixture was separated from free dye by gel-filtration. The *F*/*P* ratio of the protein was calculated by spectroscopy according to the manufacturer, which was controlled to fall between 1 and 2. The labeled proteins were stored at 4 °C.

Immobilization to a CMD-Modified Glass Plate. To immobilize antibody fragments onto a glass surface to enable optical observation, a Biacore 3000 surface prep unit (GE Healthcare) and CMD-modified glass slide were employed. An alkaline-treated glass slide (Matsunami Glass Co.) was washed in an ultrasonic acetone bath with ddH_2O and treated with UV/O_3 cleaner to clean its surface. The plate was then immersed once in 3-aminopropylethoxysilane and baked at 120 °C for 1 h.

A 1% CMD (M_w: 1000 k, Meito Sangyo, Aichi, Japan) solution in ddH_2O was mixed with 1 vol 5 mM 1-ethyl-3-(3-dmiethylaminopropyl)carbodiimide (EDC)-HCl, and the mixture was applied to the glass slide above, spin-coated, and incubated at 25 °C. The plate was sequentially washed with 100 mM NaOH and ddH_2O to produce a CMD-modified glass slide. The CMD-glass slide was placed on the Biacore 3000 surface prep unit, and the fluorescence-labeled antibody fragments were immobilized as above. The plate was washed serially with 10 mM glycine-HCl, pH 1.5, and 10 mM NaOH and used for FRET measurement.

FRET Measurement. Sample solution with or without antigen was applied to the CMD-glass slide immobilized with labeled antibody fragments, and incubated for 1−10 min at 25 °C. The slide was washed with HBS-N and dried briefly with an air-spray. The fluorescence intensities at 570 and 675 nm were measured with a fluorescence image analyzer (FLA-8000, Fuji Film) at an excitation wavelength of 532 nm. The FRET efficiency was calculated as (F_A/F_D), The antigen dependency of (F_A/F_D) was calculated as $\Delta(F_A/F_D) \times 100$ (%), where the (F_A/F_D) just before antigen addition was subtracted to obtain $\Delta(F_A/F_D)$. Three measurements for each matrix with two different ligand densities (375 and 627 pg/mm^2 for OS-MIP, and 396 and 648 pg/mm^2 for premixed condition) were performed. After each measurement, the slide was washed with 10 mM glycine−HCl, pH 1.5, for 1 min for regeneration. The limit of detection was determined as the antigen concentration that gives $\Delta(F_A/F_D)$ higher than its 3 SD.

For real-time fluorescence measurement, the fluorescence time course upon the addition of sample solution to the CMD-slide was monitored by a photomultiplier tube H5784−02 (Hamamatsu Photonics, Hamamatsu, Japan), equipped with a 667 nm band-pass filter, with excitation provided by an LD-excited green solid-state laser (λ = 532 nm). The time course of fluorescence intensities after injection of either HBS-N alone or 1 μM HEL in HBS-N was monitored to derive the fluorescence ratio of the two samples.

RESULTS AND DISCUSSION

Fabrication of OS-MIP. The scheme of OS-MIP is shown in Figure 1a. Many antibody variable regions (Fv = V_H + V_L) devoid of corresponding constant regions retain a property whereby they are more stable in the presence of antigen than in its absence (open sandwich principle).[6] We ventured to utilize this property for making a functional antibody−polymer conjugate that can specifically capture antigen only when the matrix is imprinted with antigen during the conjugation reaction. In other words, when a Fv−polymer conjugate is made in the presence of antigen, a high proportion of the two variable region fragments V_H and V_L would be immobilized as a functional complex. However, when the same conjugate is made in the absence of antigen, the proportion of functional V_H/V_L complex would be considerably low, leading to nonfunctional conjugate without full antigen-binding capability. To make the proposed matrix, first we obtained the genes for antibody variable region fragments V_H and V_L that specifically recognize hen egg lysozyme (HEL) from immunized mice. We successfully obtained the genes, which showed high homologies (88% and 94% amino acid identity in V_H and V_L, respectively) to corresponding genes from a known anti-HEL antibody

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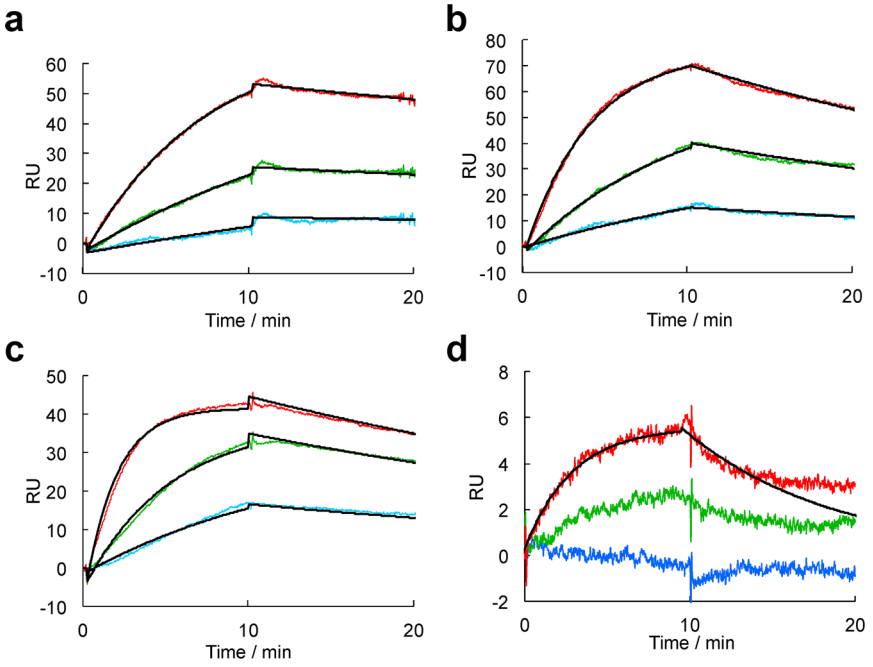

Figure 2. SPR sensorgram for the OS-MIP complex immobilized with anti-HEL (a) MBP-V_H/MBP-V_L (b) MBP-V_H/V_L, and (c) V_H/V_L. Blue, green, and red lines show signals with 3, 10, and 30 nM HEL, respectively, with fitted curves. (d) The same for anti-BGP MBP-fused Fv immobilized by OS-MIP. Blue, green, and red lines show signals with 1, 10, and 100 nM BGP-C7, respectively.

HyHEL-10.[11] From our previous results, [6] it was probable that the Fv (=V_H + V_L) also showed antigen-dependent stabilization.

Therefore, the obtained genes were used to prepare fusion proteins with maltose binding protein (MBP), and these fusion proteins were then used for chemical conjugation with CM dextran immobilized on a CM5 surface plasmon resonance (SPR) sensor chip by amine coupling. We used MBP as a fusion partner because we expected its stabilizing effect as a molecular chaperon.[12] However, it was found later that MBP is not essential in OS-MIP itself (see below). We used the following three conditions: (i) V_H and V_L were conjugated serially ("random" in Figure 1b); (ii) both proteins were premixed and conjugated (premixed); and (iii) both proteins were premixed with template (HEL) before conjugation (OS-MIP). When the sensor matrices after conjugation were acid-washed and evaluated for their HEL binding responses with the Biacore 3000 SPR biosensor, the matrix immobilized with the Fv mixed with template showed a markedly higher HEL binding response than the former two matrices prepared without template (Figure 1b). Compared with the matrices made under random and premixed conditions, the matrix made by OS-MIP showed a 14- and 5.7-fold higher maximum resonance unit, respectively. In addition, when this matrix was subjected to six antigen binding/acid-wash cycles and subjected to the same measurement, the response showed only a slight (14.3%) decrease, indicating its practical reusability.

SPR Analysis of OS-MIP. We further characterized the nature of the matrix made by a template-assisted conjugation by SPR-based kinetic and thermodynamic analyses. As controls, we prepared V_H and V_L fragments devoid of the MBP moiety, as well as the single chain Fv fragment (scFv), in which the V_H and V_L fragments are connected by a flexible $(G_4S)_3$ linker. As shown in Figure 2a–c, the matrices made with three combinations of variable region fragments (MBP-V_H/MBP-V_L, MBP-V_H/V_L, and V_H/V_L) in the presence of template HEL showed similar SPR responses to the analyte HEL. When these data as well as those for scFv were subjected to global fitting kinetic analysis, it was found that the association rate (k_{on}) of the interaction was significantly increased (up to 4.1-fold) when the ligands were each devoid of MBP. The resultant equilibrium dissociation constant K_d was as low as 1.4 nM, which was similar to that of scFv (Table 1). From this result, it was revealed that the MBP moiety is not essential for creating specific recognition matrix by OS-MIP. Considering the molecular weights for MBP-V_H (MBP-V_L) and V_H (V_L) of ~60 k and ~15 k, respectively, this result suggests sufficient mobility of the ligands made by OS-MIP upon binding with the analyte, implying ligand-dependent association of V_H/V_L as depicted in Figure 1a.

Table 1. Thermodynamic Parameters Obtained for the Interaction between Antigen HEL and the Ligands

ligand	MBP-V_H/MBP-V_L	MBP-V_H/V_L	V_H/V_L	scFv (V_H-V_L)
k_{on} (10^4/Ms)	6.7	13	28	38
k_{off} (10^{-4} s^{-1})	2.2	4.7	4.1	3.5
K_d (nM)	3.3	3.7	1.4	0.94
ΔH (kJ/mol)	−128	−132	−117	−170
ΔS (J/K·mol)	−260	−260	−240	−380

Thermodynamic Analysis of OS-MIP. To further analyze the nature of these matrices, we obtained thermodynamic parameters ΔH and ΔS by the SPR analysis with Biacore T100 at four different temperatures. As shown in Table 1, while the derived ΔH and ΔS values for matrices made by OS-MIP remain essentially the same (−125.7 ± 7.8 kJ/mol for ΔH, 253.3 ± 11.5 J/K·mol for ΔS) irrespective of tethered MBP, the absolute values were considerably smaller than those obtained for the scFv (−170 kJ/mol and −380 J/K·mol, respectively). The smaller entropy loss of OS-MIP suggests a higher degree of

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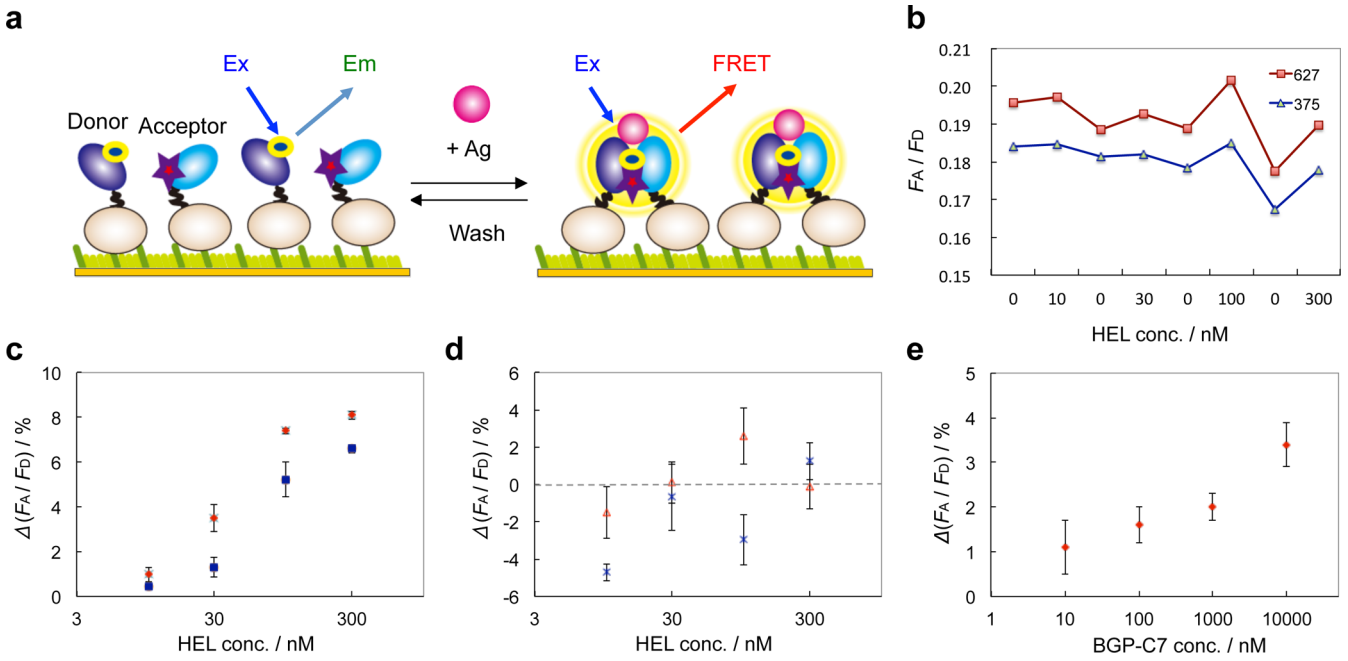

Figure 3. OS-MIP-based antigen sensor: (a) scheme; (b) example of raw data used to derive $\Delta(F_A/F_D)$ for immobilized anti-HEL Abs (Alexa555-MBP-V_H and Alexa647-MBP-V_L) made by OS-MIP at indicated ligand density (pg/mm^2); (c) dose–responses for HEL detection. Incubation time with antigen solution was 1 min (blue) or 10 min (red). The antigen-dependent change in F_A/F_D, taken as an index for FRET is shown. Average of six measurements (three each for two different amounts of immobilized ligand) with 1 SD is shown. (d) Results obtained with the matrix made under the premixed condition are shown as in (c) with open symbols. (e) Result for BGP detection with HiLyte555-MBP-V_L and Alexa647-MBP-V_H. The incubation time with antigen BGP-C7 was 10 min.

V_H/V_L prearrangement attained by OS-MIP than scFv, in which V_H and V_L fragments are connected by a flexible linker. Again, the apparent absence of the effect of MBP on ΔH and ΔS is considered to reflect the minor role of MBP in OS-MIP.

Application to Anti-Peptide Antibody. To assess the generality of the OS-MIP approach, we prepared the corresponding antibody fusion proteins derived of another antibody that recognizes the C-terminal fragment of human osteocalcin (bone gla protein, BGP).[13] BGP is known as a marker for bone metabolism and also suspected for its roles in insulin regulation[14] and male fertility.[15] Upon the binding of BGP or its C-terminal peptide, the Fv of this antibody is markedly stabilized. The matrix containing MBP-fused V_H/V_L was made in the presence of the BGP C-terminal peptide BGP-C7 (MW: 894) and measured for its antigen-binding response with an SPR biosensor as above (Figure 2d). In spite of lower signal due to lower molecular weight of the analyte, dose-dependent binding was surely observed with a calculated K_D of 4.68×10^{-8} M. On the contrary, we could not observe significant antigen binding when the antibody fusion proteins were immobilized in the absence of BGP peptide (data not shown). From these results, we concluded that the OS-MIP approach for making a specific recognition matrix has a generality in terms of applicable antigen–antibody pairs.

Application to FRET-Based Biosensor. To take advantage of the strong and specific binding of the matrix made by OS-MIP, we next examined its possibility as a fluorescence-based optical biosensor. Generally, Förster resonance energy transfer (FRET) is a useful technique to monitor intermolecular interaction in solution, and it has been successfully applied to detect many interactions in a small volume, such as an intracellular environment where bound per free (B/F) separation is impossible.[16,17] However, while FRET has also been proven useful to monitor the conformational change of proteins, it has been considered difficult to detect the interaction between immobilized molecules. To this end, we labeled the MBP-fused V_H and V_L fragments of anti-HEL antibody with fluorochromes Alexa555 and Alexa647, respectively, by amine coupling and used them for OS-MIP prepared similarly on a transparent glass slide. We expected that the CM dextran-tethered Alexa555-V_H and Alexa647-V_L would work, respectively, as a donor and an acceptor of FRET, which should be strengthened by the bridging antigen (Figure 3a).

When the two matrices with different ligand densities were made and tested for their antigen binding by SPR, a similar, albeit several-fold increase in K_d, antigen-binding affinity to that made with nonlabeled proteins was observed (Table S1, SI Figure S1a). Next, for fluorescence measurement, OS-MIP matrix was made on a glass slide and measured for the emission intensity ratio of two fluorochromes upon illumination of green laser (532 nm). To determine the antigen dose–response, the fluorescence intensity at the two wavelengths (F_D and F_A for 570 and 675 nm, respectively) was determined before and after antigen addition, and the change in their ratio (F_A/F_D) was taken as the signal. During these measurements, the same surfaces with two different ligand densities were regenerated by an acid wash and used repeatedly for each measurement (Figure 3b). As a result, the obtained HEL dose-dependency of the signal $\Delta(F_A/F_D)$ clearly showed a low limit of detection (LOD) of 30 nM HEL for 1 min incubation, and as low as 10 nM LOD for 10 min incubation (Figure 3c). It is worth noting that the intermeasurements error of $\Delta(F_A/F_D)$ was within ±5% when detected at 10 min, showing similar performance to SPR measurements. As a control, when the matrices were made similarly but without antigen (the "premixed" condition), the

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obtained signal did not show reliable antigen dependency (Figure 3d).

Furthermore, detection of the BGP peptide was attempted using the OS-MIP matrix made with fluorescence-labeled MBP-fused anti-BGP antibody fragments. The SPR results showed similar antigen-binding kinetics of the HiLyte555-labeled MBP-V_L/Alexa647-labeled MBP-V_H mixture to those obtained with nonlabeled proteins (SI Table S1, SI Figure S1b). The dose–response relationship of the OS-MIP matrix made on a glass slide for FRET measurement showed a good correlation with an LOD of 100 nM BGP-C7 within 10 min (Figure 3e).

Taken together, these data clearly show the practical utility of FRET-based antigen sensors made by OS-MIP. The obtained response is fast and reproducible. Moreover, such non-competitive and label-free antigen detection by FRET signal *in situ* would be hard to achieve by using conventional monoclonal antibody-based matrices, partly due to the lack of suitable fluorochrome pair with large (>100 Å) Förster distance R_0, which should be large enough if one wants to detect a sandwich complex made of two full-sized antibodies bridged by a typical protein antigen.

When the FRET response attained here is not enough and one wishes higher response in this OS-MIP based sensor, use of fluorochrome pairs giving more efficient FRET, such as those with Europium chelate,[18] and/or use of a more reliable fluorolabeling method such as position specific labeling during *in vitro* translation[19] will further increase the intensity and reproducibility of obtained FRET response.

Real-Time FRET Detection with OS-MIP. We also tried real-time detection of FRET-derived fluorescence using an OS-MIP matrix imprinted by HEL. With the use of anti-HEL HiLyte555-labeled MBP-V_L and Alexa647-labeled MBP-V_H fragments together with a handmade monitoring device (SI Figure S2), we were able to observe a significant time-dependent change in fluorescence intensity (SI Figure S3). Compared with the control sample without antigen, we could see a clear FRET-derived signal increase for the sample containing 1 μM HEL, which peaked within 10 min of HEL injection.

CONCLUSION

We combined the OS immunoassay concept with that of MIP, thus proposing a novel antibody-based recognition matrix that can be imprinted by a target antigen. Unlike conventional MIP, available targets include not only small molecules such as peptide but also larger proteins. Moreover, we successfully extended this concept to SPR- and FRET-based biosensors, and the affinity and sensitivity attained with the sensor were in the $\sim 10^8$/M range, which well exceeds that of most conventional MIP-based systems. Especially, the results of the FRET-based sensors suggest that they can detect different antigens *in situ* as a real-time fluorescence signal, which is hard to achieve with conventional antibody-based matrix. By utilizing the OS principle, the present strategy will be applicable to the detection of a range of antigens from proteins to small chemicals, probably on a protein chip in an arrayed format. The antibody V_H/V_L fragments have a merit that they can be immobilized at higher density than full-size antibodies to attain higher signal, and those obtained from synthetic phage-display libraries will enable detection of various targets even if they are toxic to immunized animals.[20] Furthermore, affinity maturation of antibody fragments is readily available by using phage display technology, if further enhancement of detection sensitivity is needed.[21] Hence, this method will be a strong analytical tool, possibly implemented as a portable and easy-to-use real-time analyzer, provided with a fertile library of available antibody reagents and sensitive sensing devices.

ASSOCIATED CONTENT

Supporting Information

A table for characterization of immobilized ligands, and supporting figures for FRET experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

BGP, bone gla protein (osteocalcin); ELISA, enzyme-linked immunoadsorbent assay; FRET, Förster resonance energy transfer; HEL, hen egg lysozyme; MBP, maltose-binding protein; MIP, molecularly imprinted polymers; OS-MIP, open-sandwich molecular imprinting; SPR, surface plasmon resonance; V_H, antibody heavy chain variable region; V_L, antibody light chain variable region.

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