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Authors	Yoshitaka HARADA, Yuki OHMURO-MATSUYAMA, Mika TSUNA, Hiroshi UEDA
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# An Open Sandwich Immunochromatography for Non-competitive Detection of Small Antigens

Yoshitaka HARADA,<sup>\*,\*\*</sup> Yuki OHMURO-MATSUYAMA,<sup>\*\*</sup> Mika TSUNA,<sup>\*</sup> and Hiroshi UEDA<sup>\*\*\*†</sup>

<sup>\*</sup>Food Research Center, Central Laboratory, Nippon Flour Mills Co., Ltd., 5-1-3 Midorigaoka, Atsugi, Kanagawa 243-0031, Japan

<sup>\*\*</sup>Laboratory for Chemistry and Life Science, Institute of Innovative Research, Tokyo Institute of Technology, 4259-R1-18 Nagatsuta-cho, Midori, Yokohama, Kanagawa 226-8503, Japan

Immunochromatography assay is an easy and rapid on-site detection method. However, conventional sandwich immunochromatographies using two antibodies can only detect target molecules above a threshold size. Small molecules below 1000 in molecular weight are usually detected using competitive immunoassay. However, competitive immunoassay is not suitable for visual detection of low concentration samples. Based on the principles of open sandwich immunoassay, which detects small molecules *via* interchain interaction of separated variable region fragments ( $V_H$  and  $V_L$ ) from a single antibody, we developed non-competitive open sandwich immunochromatography. Bone Gla protein (BGP)-C7, a peptide containing the seven C-terminal amino acids of human osteocalcin, was selected as the target. By using  $V_L$  fragments fixed on a nitrocellulose membrane, and colored cellulose bead-labeled  $V_H$  fragments, we specifically detected 10 ng/mL of BGP-C7. This is the first report of open sandwich immunochromatography, which is an easy and rapid method for on-site, signal-on detection of small molecules.

**Keywords** Open sandwich immunoassay, immunochromatography, bone Gla protein, lateral flow assay

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## Introduction

Recently, the importance of on-site detection methods for rapid screening of target molecules is attracting attention. Immunochromatography (IC) is a powerful tool for on-site detection of target molecules. IC involves depositing the sample solution on the test strip and provides results in a few minutes. Results can be visually confirmed without any additional equipment. Due to the simplicity of the test procedure and result confirmation, an immunochromatography assay is widely used for easy, rapid, and on-site detection. For example, an immunochromatographic pregnancy test can be purchased from a pharmacy and provides reliable results. In Japan, hospitals use IC for flu diagnosis including the typing of A and B, and provide the test results to the doctor and patient in 15 - 20 min. A rapid and easy immunochromatographic test for COVID-19, which currently poses a global health threat, would enable early and affordable diagnosis of infected patients.<sup>1</sup>

Conventional sandwich IC requires two antibodies for detection. One antibody is fixed on the membrane and captures target molecules in the sample flow. The other antibody is labeled with an enzyme or a colored particle, such as gold colloid, and flowed on the test strip with the sample where it binds with the target molecule. For successful detection by sandwich IC, target molecules must be large enough to simultaneously bind with the two types of antibodies. Therefore,

it is theoretically difficult to detect small molecules using sandwich IC.

To detect small molecules that are less than 1000 in molecular weight, competitive immunoassay is usually used. In competitive immunoassay, the target molecule (or its analogue conjugated to a carrier protein) is fixed on the test line, and competes with the target molecule in the sample for binding with the labeled antibody. To date, several competitive ICs have been reported and used for detecting small compounds, such as mycotoxins,<sup>2,3</sup> pesticides<sup>4</sup> and antibiotics.<sup>5</sup> Competitive immunoassay involves only one epitope in the target and can detect small target molecules. However, in competitive IC, the appearance of a colored test line indicates a negative result, as opposed to sandwich IC where the colored line indicates a positive result. Moreover, in competitive IC, it is difficult to differentiate between a positive and negative result when the test signal is slightly weaker than the negative control. This necessitates the use of measuring equipment, such as a colorimeter, for detecting the subtle differences between the sample and the negative control signals. The use of measuring equipment limits the on-site use of competitive IC.

Open sandwich (OS) immunoassay is a promising, non-competitive method for detecting small molecules. In OS-ELISA (enzyme linked immunosorbent assay), antigen is detected *via* interchain interactions between the two variable region fragments ( $V_H$  and  $V_L$ ) of an antibody. Both fragments are derived from a single antibody, and bind with only one epitope in the target molecule. Therefore, small molecules can be detected non-competitively. Open sandwich IC (OS-IC), which combines OS immunoassay and IC, can overcome the

<sup>†</sup> To whom correspondence should be addressed.  
E-mail: ueda@res.titech.ac.jp

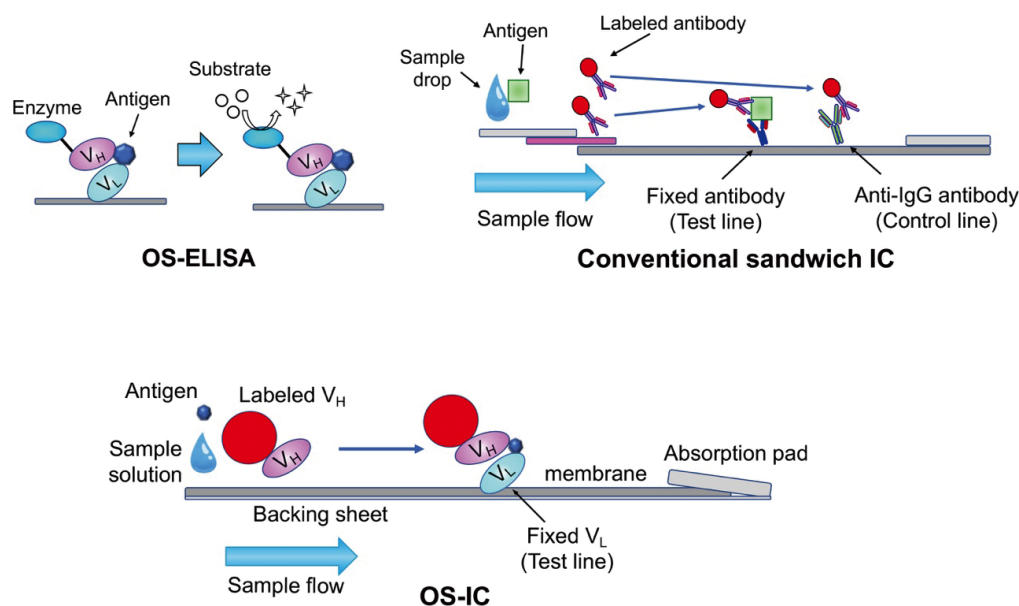


Fig. 1 Schematics of OS-ELISA, conventional sandwich IC, and OS-IC.

limitations of IC and is a promising tool for the noncompetitive detection of small molecules (Fig. 1).

We chose Bone Gla Protein (BGP)-C7 ( $M_r = 894.03$ ) as the first target for OS-IC. BGP-C7 is the septapeptide derived from the C-terminus of human osteocalcin.<sup>6</sup> Osteocalcin is a bone metabolic marker and a hormone secreted from osteoblasts that affects several organs. Detection of BGP-C7 in blood circulation is important for early diagnosis of osteoblast-associated diseases. Here, we report the development of OS-IC to detect BGP-C7.

## Experimental

### Reagents

The reagents used in this study were as follows: Nitrocellulose membrane FF120 HP, glass filter, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC), and *N*-hydroxysuccinimide (NHS) purchased from GE Healthcare (IL, USA); casein and NaN<sub>3</sub> purchased from Kanto Chemicals (Tokyo, Japan); Tris (hydroxymethyl) aminomethane (Tris) and *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) purchased from Nacalai Tesque (Kyoto, Japan); and absorption pad and bovine serum albumin (BSA) bought from Merck (NJ, USA). The backing sheet was obtained from Nippon Engineering (Tokyo, Japan). Colloidal gold was purchased from BBI Solutions, (Newport, UK). A microtiter plate (96 well) was obtained from Greiner (Frickenhausen, Germany). Alkaline Phosphatase Labeling Kit-NH<sub>2</sub>, 3-[(3-Cholamidopropyl) dimethylammonio]-propanesulfonate (CHAPS), and 2-morpholinoethanesulfonic acid (MES) were purchased from Dojindo (Kumamoto, Japan). 5-Bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (BCIP) was purchased from Roche Diagnostics (Basel, Switzerland). Colored cellulose particles (NanoAct™) were obtained from Asahi Kasei (Tokyo, Japan). Bovine serum and rabbit serum were purchased from Thermo Fisher Scientific (Tokyo, Japan). Other reagents were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

### Preparation of antibody fragments

The antibody fragments, V<sub>H</sub> and V<sub>L</sub> of anti-BGP antibody

R4A10<sup>7</sup> tagged with thioredoxin (Trx) and maltose-binding protein (MBP) to make Trx-V<sub>H</sub> and MBP-V<sub>L</sub>, respectively, were expressed in *E. coli* SHuffle T7 Express lysY, and purified as described previously.<sup>8,9</sup>

### Preparation of test strip for OS-IC

MBP-V<sub>L</sub> (2 mg/mL) in 25  $\mu$ L of 10% EtOH was applied on the tip of a nitrocellulose membrane, FF 120 HP (30  $\times$  2.5 cm), as a spot or a 1 mm line. The membrane was incubated at 40°C for 30 min to fix MBP-V<sub>L</sub>, and subsequently dipped into 50 mM sodium borate buffer (pH 8.5) containing 0.5% casein for 30 min at 25°C. The membrane was gently washed in 350 mM Tris-HCl buffer (pH 7.4) containing 0.5% sucrose and 0.05% sodium cholate, and dried overnight at 25°C. The membrane was assembled with an absorption pad on a backing sheet and cut into 5 mm wide test strips.

### Preparation of gold colloid particles-labeled Trx-V<sub>H</sub> (gold colloid-Trx-V<sub>H</sub>)

Trx-V<sub>H</sub> was electrostatically coupled to the surface negative charge of the gold colloid as follows: 13.4  $\mu$ L of Trx-V<sub>H</sub> solution (2.1 mg/mL), 3.6 mL of gold colloid solution (particle size of 20 nm), and 787  $\mu$ L of 50 mM potassium dihydrogen phosphate (pH 7.5) were mixed, and stored for 10 min at room temperature. Next, 220  $\mu$ L of PEG 20000 solution and 440  $\mu$ L of 10% BSA were gently added and mixed for blocking the gold colloid particle. This mixture was centrifuged at 8000  $\times$  *g* for 15 min at 25°C. The supernatant was removed, and the gold colloidal pellet was resuspended in storage buffer (0.05% PEG 20000, 150 mM NaCl, 1% BSA, 0.1% NaN<sub>3</sub> in 20 mM Tris-HCl, pH 9.0). Centrifugation and resuspension steps were repeated twice, and the volume of storage buffer was reduced stepwise to concentrate the gold colloid. Finally, about 300  $\mu$ L of gold colloid-Trx-V<sub>H</sub> with an absorbance of 10.0 at 530 nm was obtained.

In one well of a 96 well microtiter plate, 15  $\mu$ L of gold colloid-Trx-V<sub>H</sub>, 10  $\mu$ L of BGP-C7 (1  $\mu$ g/mL), and 75  $\mu$ L of 10 mM phosphate buffered saline (PBS), pH 7.4, were mixed. As the negative control, 10  $\mu$ L of PBS (pH 7.4) was added instead of BGP-C7. The test strip was dipped in each well, and

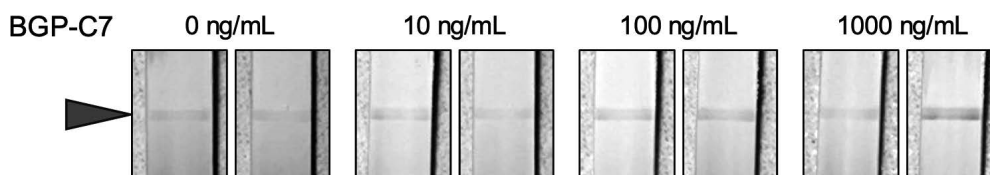


Fig. 2 Result of alkaline phosphatase-labeled OS-IC. The position of the test line is indicated by the arrowhead.

the test spot was observed after 15 min.

#### Preparation of alkaline phosphatase-labeled Trx-V<sub>H</sub> (AP-Trx-V<sub>H</sub>)

Trx-V<sub>H</sub> was labeled using the Alkaline Phosphatase Labeling Kit-NH<sub>2</sub> (Dojindo, Kumamoto, Japan) according to the manufacturer's instruction to produce AP-Trx-V<sub>H</sub>.

Sample solution containing a mixture of 1  $\mu$ L of BGP-C7 peptide in dimethyl sulfoxide (DMSO), 2.5  $\mu$ L of AP-Trx-V<sub>H</sub>, 46.5  $\mu$ L of 2% CHAPS in 10 mM PBS, pH 7.4, and development solution (0.1% CHAPS, 100 mM CAPS, 1 mM MgCl<sub>2</sub>) were prepared. Sample solution (50  $\mu$ L) and development solution (200  $\mu$ L) were dispensed into each well of the microtiter plate.

For detection using OS-IC, the test strip was dipped into the sample solution for 15 min allowing it to flow through the test strip. Subsequently, the test strip was placed on a glass filter containing 100  $\mu$ g of BCIP (alkaline phosphatase substrate) and dipped in the development solution for 40 min to allow the alkaline phosphatase reaction.

#### Preparation of colored cellulose particle (CCP-Trx-V<sub>H</sub>)-labeled Trx-V<sub>H</sub>

Colored cellulose particles, NanoAct Covalent COOH type, were used to label Trx-V<sub>H</sub>. Labeling procedure is as follows: 5  $\mu$ L of colored cellulose particle solution was mixed with 45  $\mu$ L of 100 mM MES buffer, pH 5.0, 50  $\mu$ L of 7.5% EDC, and 50  $\mu$ L of 1.15% NHS. After a 15 min reaction, the mixture was centrifuged at 12000  $\times$  g (20 min, room temperature) and supernatant was removed. The pellet was mixed with 50  $\mu$ L of MES buffer containing 10  $\mu$ g of Trx-V<sub>H</sub>. After incubation for 2 h, the solution was centrifuged at 12000  $\times$  g (20 min, room temperature) and the supernatant was removed. The precipitate was resuspended in 600  $\mu$ L of 100 mM sodium borate buffer (pH 8.5) containing 1% casein for 60 min to block the excess activated carboxyl groups on the surface of the colored cellulose particles. The mixture was centrifuged at 12000  $\times$  g (20 min at room temperature), washed with 600  $\mu$ L of 50 mM sodium borate (pH 8.5), and centrifuged again to remove the supernatant. Precipitate was diffused in 150  $\mu$ L of 50 mM sodium borate (pH 8.5) containing 0.02% casein, and 0.15% sucrose to obtain CCP-Trx-V<sub>H</sub> solution.

For the dose response measurement using OS-IC, the test strip was dipped in sample solution containing a mixture of 1  $\mu$ L of BGP-C7 in DMSO, 9  $\mu$ L of CCP-Trx-V<sub>H</sub> solution, and 90  $\mu$ L of 100 mM potassium phosphate aqueous solution (pH 7.2). The color depth on the test line was evaluated by the immunochromatography reader (IC-reader) C10066-10 (Hamamatsu Photonics; Shizuoka, Japan).

## Results and Discussion

#### OS-IC with gold colloid-conjugated Trx-V<sub>H</sub>

To find the best detection method for OS-IC, we tried three different detection methods. First, we tried gold colloid-based

detection, which is currently the most popular labeling method in IC. We immobilized maltose-binding protein (MBP)-fused V<sub>L</sub> fragment at the test spot, and conjugated gold colloid with thioredoxin (Trx)-fused V<sub>H</sub> to detect the antigen-dependent V<sub>H</sub>-V<sub>L</sub> interaction as a red spot on the test strip. Although the OS-IC with gold colloid-conjugated Trx-V<sub>H</sub> showed a clear test spot, nonspecific reaction appeared in the negative control sample without the BGP-C7 peptide. Furthermore, the test strip with fixed Trx-V<sub>H</sub> and gold colloid-labeled MBP-V<sub>L</sub> showed no reaction in the negative control sample as well as the sample containing BGP-C7 peptide (100 ng/mL) (Fig. S1, Supporting Information). To investigate the reason behind the nonspecific reaction, two additional experiments were performed. First, BSA-coated gold colloid without Trx-V<sub>H</sub> was applied to the test strip. As the nonspecific reaction was still observed, we concluded that it is not dependent on the MBP-V<sub>L</sub> and Trx-V<sub>H</sub> interaction. A second IC was performed with MBP only (without V<sub>L</sub>) fixed on the test strip, together with gold colloid-conjugated Trx-V<sub>H</sub>. In this case, the nonspecific reaction was not observed. Therefore, Trx-V<sub>H</sub> and MBP do not interact nonspecifically, implying that the nonspecific reaction is caused by the interaction between V<sub>L</sub> and the BSA-coated gold colloid. We also replaced the BSA-containing gold colloid blocking reagent with 10 mM Tris-HCl (pH 9.2) containing sodium casein (2.5%) and bovine or rabbit serum (100 mg/mL). However, there was no reduction in the nonspecific reaction. Based on the conditions tested here, we concluded that the V<sub>L</sub> and gold colloid interaction results in nonspecific reaction.

#### OS-IC with alkaline phosphatase conjugated Trx-V<sub>H</sub>

As an alternate strategy, we tried enzyme-based detection with alkaline phosphatase conjugated-Trx-V<sub>H</sub> (AP-Trx-V<sub>H</sub>). BGP-C7 peptide (0 - 1000 ng/mL in sample buffer) was applied to the test strip (Fig. 2). The blue line was clearly observed with 1000 and 100 ng/mL of BGP-C7. However, 10 and 0 ng/mL of BGP-C7 showed weak blue lines of comparable intensities. We speculate that the high activity of calf intestinal alkaline phosphatase and the weak but non-negligible nonspecific binding between AP-Trx-V<sub>H</sub> and MBP-V<sub>L</sub> resulted in a nonspecific reaction in the sample without BGP-C7.

The weak nonspecific reaction was similar to a previous report with open sandwich ELISA.<sup>9</sup> The high sensitivity of enzymatic detection can amplify even weak nonspecific reactions, resulting in a nonspecific test line. In addition, a mottled pattern of blue color was observed on the test strip. As this may result from the excess alkaline phosphatase remaining on the test strip, an extra washing step was inserted between the sample flow and test line development procedure. However, the mottled pattern showed no improvement. This may result from the nonspecific binding of AP-Trx-V<sub>H</sub> and free excess alkaline phosphatase. In this study, the nitrocellulose membrane was blocked using casein buffer. We expect that an appropriate membrane and blocking reagent might reduce this mottled pattern on the test strip.

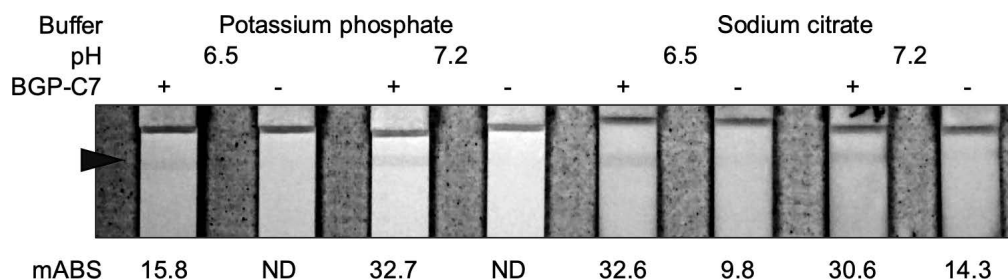


Fig. 3 IC using CCP-labeled Trx-V<sub>H</sub>. The position of the test line is indicated by the arrowhead. mABS indicate the milli-absorbance values of the test line measured with an IC-reader. The test lines of membranes without an mABS value were not detected by IC-reader. ND, not detected.

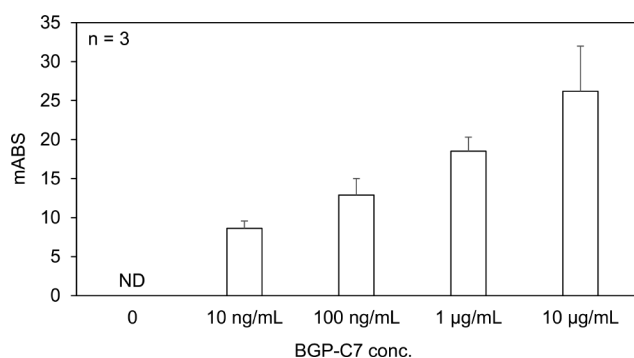


Fig. 4 Detection of BGP-C7 with CCP-Trx-V<sub>H</sub> OS-IC using an IC-reader. mABS indicates milli-absorbance. Average +1 SD of three samples is shown. ND, not detected.

#### Detection of BGP-C7 by CCP-Trx-V<sub>H</sub>

As the third strategy, Trx-V<sub>H</sub> was labeled with colored cellulose particle (CCP). To optimize OS-IC with CCP-Trx-V<sub>H</sub>, two buffers: sodium citrate (100 mM) and potassium phosphate (100 mM), at two different pH levels of 6.5 and 7.2 for both buffers, were tested (Fig. 3). We detected 10 µg/mL of BGP-C7 without any nonspecific reaction in the negative control using the potassium phosphate buffer (at both pH 6.5 and 7.2). Therefore, 100 mM potassium phosphate buffer (pH 7.2) was used in subsequent experiments. Sample solutions containing 0, 10, 100, and 1000 ng/mL of BGP-C7 peptide were applied to OS-IC with CCP-Trx-V<sub>H</sub>, and the test line was detected with an IC-reader (Fig. 4).

The OS-IC using CCP-Trx-V<sub>H</sub> successfully detected 10 ng/mL of BGP-C7 peptide without nonspecific reaction. The test lines started to appear within 10 min, and all the reactions were completed in 30 min. In addition, there was no mottled pattern on the test strip, and a uniform depth of test line color could be easily measured with an IC-reader. Alterations in membrane type or buffer composition and pH resulted in a nonspecific test line or no reaction on the test line (data not shown). Generally, IC with a low sample flow rate causes nonspecific reaction, and a high flow rate causes inadequate antigen-antibody interaction. Additionally, antigen-antibody complex formation in OS immunoassay requires simultaneous binding of V<sub>H</sub> and V<sub>L</sub> fragments and the antigen. Unlike ELISA, IC does not allow long reaction times and washing steps. Therefore, appropriate flow rate on the test strip and the buffer composition are crucial for OS-IC to detect target molecules without nonspecific reaction. Affinity and specificity of antigen-antibody interactions

depend on buffer conditions, including pH, type and concentration of salt, surfactant, and denaturant. Low concentration of urea in buffer can eliminate nonspecific reactions in IC.<sup>10</sup> The buffer conditions to eliminate nonspecific reactions in the OS-IC assay reported here may be optimized further.

#### Further improvement and potential uses of OS-IC

Although CCP-Trx-V<sub>H</sub> OS-IC could detect 10 ng/mL of BGP-C7, this limit of detection is not low enough to evaluate osteocalcin in normal human serum.<sup>11,12</sup> Further improvement in OS-IC sensitivity is required to match the OS-ELISA detection limit (1 ng/mL) for BGP-C7.<sup>9</sup> This is possibly due to the slow association kinetics of the V<sub>H</sub>/V<sub>L</sub> fragments used in this study.<sup>7</sup> Use of sensitive fluorescent labels, such as quantum dots (QDs), improves the sensitivity of IC. By using QDs, as compared to gold colloid label, the working range of competitive chloramphenicol IC was improved from 0.5 – 6 ng/mL (gold colloid) to 0.3 – 20 ng/mL (quantum dot).<sup>13</sup> Additionally, the limit of visual detection improved from 10 to 1 ng/mL. Although a UV excitation device is required to visualize the fluorescent test line, fluorescent labeling is a promising tool for improving the visual detection limit of IC.

Competitive immunoassay is usually employed to detect small antigens such as peptides. However, in competitive immunoassay, confirmation of a weak signal without specialized measuring equipment is difficult. In contrast, OS-IC allows non-competitive and qualitative detection of small antigens using only a test strip. This is the outstanding advantage of OS-IC for on-site detection of small target molecules.

Another advantage of OS-IC is its ability to detect degraded protein antigens in crude or damaged samples, as long as the epitope remains undigested. Food allergy is a common dietary concern. Occasionally, food allergy causes anaphylactic shock and life-threatening symptoms in patients. Therefore, on-site monitoring of allergen contamination in daily diet or in food manufacturing factories will empower susceptible patients to avoid intake of allergy-causing foods. Protein allergens in food are sometimes degraded during manufacturing processes, such as cooking or fermentation. Conventional sandwich immunoassay is incapable of detecting antigens when the two epitopes recognized by antibodies become separated due to degradation. OS-IC, which detects only one epitope, has a higher chance of successfully detecting antigens in degraded samples.

## Conclusions

This is the first report of the development of OS-IC for the

detection of small antigens. In the future, we expect that OS-IC will enable rapid, non-competitive detection of not only peptide antigens but also small compounds, such as toxins, pesticides, and antibiotics.

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### Supporting Information

A figure for the result of gold colloid-labeled OS-IC. This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>.

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