

Indoor allergen assessment quantified by a thin-layer electrochemical cell and magnetic beads



Ryoji Kurita*, Hiroyuki Yanagisawa, Osamu Niwa

National Institute of Advanced Industrial Science and Technology (AIST) Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, 305-8566 Japan

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ABSTRACT

We report the electrochemical determination of mite allergen in real house dust by using a thin layer electrochemical flow cell and magnetic beads. Der p1, which is an allergen from *Dermatophagoides pteronyssinus*, was immunochemically sandwiched between two dispersed monoclonal antibodies; one was modified on the surface of magnetic beads and the other was modified with alkaline phosphatase. After washing the beads, a small volume of *p*-aminophenol phosphate (*p*-APP) was added to produce *p*-aminophenol (*p*-AP). And then the *p*-AP concentration was measured electrochemically with a home-made electrochemical cell. The Der p1 assay was completed within 30 min and a low detection limit of 0.3 ng/mL was achieved. This is because the diffusion distance of Der p1 and the detection antibody was reduced to 22.3 μ m by using dispersed magnetic beads. Only 10 min was required to complete the entire immunoreaction, and 54% of the Der p1 was confirmed to have immunoreacted in only 1 min of mixing. Furthermore, the *p*-APP volume could be reduced using the thin-layer electrochemical flow cell. This is advantageous in terms of concentrating *p*-AP, and provides a high signal-to-noise ratio measurement in a short time. We achieved a high correlation ($r=0.967$, $p<0.001$) between our assay and a conventional enzyme-linked immunosorbent assay (ELISA) for real house dust measurements.

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1. Introduction

The prevalence of asthma and atopic disorders has increased in recent decades (Aberg, 1989; Akinbami and Schoendorf, 2002; Ninan and Russell, 1992; Peat et al., 1994; Yella et al., 2011). Exposure to indoor allergens, especially house dust mite, induces an increase in the immunoglobulin E (IgE) level in blood, and this triggers IgE-mediated type I allergies (Olkhov et al., 2012; Su et al., 2000; Ward et al., 1998; Yanase et al., 2012). Many studies have revealed the relationship between the development of allergic disease and the level of environmental exposure to house dust mite allergen. An international guideline recommends allergen avoidance for asthma management. Mite allergen exposure had traditionally been assessed by microscopic identification and by counting isolated mites, however this required considerable technical expertise, and was time consuming (Chapman et al., 1987).

Currently, the most widely used method for house dust mite detection is enzyme-linked immunosorbent assay (ELISA) on a microtiter plate (Cho et al., 2006; Lau et al., 2000; Mihrshahi et al., 2002). ELISA has a high quantitative reliability and high sensitivity with a nanogram per milliliter level detection limit. However, ELISA is a laboratory based testing method and the technology is

unsuitable for on-site allergen testing because conventional ELISA requires a large and expensive plate reader, which is used to measure slight changes in optical absorbance or fluorescent intensity. The need to further educate patients and demonstrate the importance of indoor allergens in relation to asthma has spurred the development of simple tests based on immunochromatography technology (Koets et al., 2011; Polzius et al., 2002; Tsay et al., 2002). Originally used for the medical analysis of blood or urine, immunochromatography has been employed to monitor indoor allergens. The immunochromatography technique can detect indoor allergens simply and quickly by using a palm-sized device. However, the sensitivity of immunochromatography is poor compared with that of ELISA. Furthermore, most immunochromatography techniques provide non-quantitative or semi-quantitative detection.

New and simple methods have been reported for house dust mite allergen detection that are based on electrochemistry and surface plasmon resonance (Huang et al., 2006, 2007, 2008; Sarkar et al., 2005; Tsai et al., 2011) in order to overcome problems related to sensitivity and measurement time. They showed excellent sensitivity in a short measurement time by combining gold nanoparticles (Huang et al., 2006, 2007; Tsai et al., 2011), nanostructured polycarbonate (Chen et al., 2012) and polyclonal secondary antibody (Huang et al., 2008). However, there has been a report (Sarkar et al., 2005) of real house dust sample measurements obtained using surface plasmon resonance or

* Corresponding author. Tel.: +81 29 861 6158; fax: +81 29 861 6177.
E-mail address: r.kurita@aist.go.jp (R. Kurita).

electrochemical detection. These detection approaches are known to be seriously affected by interferences caused by nonspecific adsorption and redox species in real samples. At present, the only miniaturized device for real sample measurement employs non-quantitative or semi-quantitative immunochromatography. A new method must be developed to take an account of interferences in real house dust because house dust varies widely in various environments and is not homeostatic.

In this paper, we report the electrochemical determination of mite allergen in real house dust with a homemade electrochemical cell and magnetic beads. Fig. 1(a) shows the concept behind our assay. Der p1 allergen in fecal pellets was immunochemically captured with dispersed antibodies so that the immunoreaction occurred quickly despite the use of a large sample volume. Then, the product of a labeling enzyme in a small volume substrate solution was measured with a small electrochemical cell. As a proof of concept, electrochemical results for real samples were compared with results for conventional ELISA on a microtiter plate.

2. Material and methods

2.1. Reagents

Der p1 antigen and two mouse monoclonal anti Der p1 antibodies (clone code 5H8 and 4C1) were purchased from Indoor Biotechnologies (Charlottesville, VA). Bovine serum albumin was purchased from Sigma (St. Louis, MO). A solution of streptavidin-coated magnetic beads was purchased from Invitrogen (Cat. No. 650-01). *p*-aminophenol (*p*-AP) was purchased from Kanto-kagaku (Tokyo, Japan). *p*-aminophenyl phosphate (*p*-APP) was purchased from LKT Laboratories (St. Paul, MN).

Tris buffer (pH 8.0) was prepared containing 10 mM tris (hydroxymethyl) aminomethane, 0.15 M NaCl and 5 mM MgCl₂. Tris buffer containing 0.05 (v/v)% Tween 20 was used as a washing

buffer, and tris buffer containing 0.05 (v/v)% Tween 20 and 0.1 (w/v)% bovine serum albumin was used as an incubation buffer.

Anti Der p1 antibody (clone 4C1) was immobilized with magnetic beads as follows. First, 60 µg (1.62 mg/mL, 37 µL) of antibody was biotinylated using a biotinylation reagent (Cat. No. LK-10, Dojindo, Japan). Then the biotinylated antibody was mixed gently with 3 mg of streptavidin-coated magnetic beads for 5 min in a tube at room temperature. The supernatant was removed by aspiration with the tube placed on a magnet. The magnetic beads were resuspended and washed three times with 1 mL of incubation buffer. Finally, the magnetic beads were resuspended in 1 mL of incubation buffer. Another anti Der p1 antibody (clone 5H8) was labeled with alkaline phosphatase (Choi et al., 2002) using a commercial labeling kit (Cat. No. LK-13, Dojindo). The antibody modified with magnetic beads and the alkaline phosphatase-labeled antibody were used for immunoassays at 60 and 10 µg/mL, respectively.

2.2. Fabrication and electrochemical properties of a thin-layer electrochemical flow cell

Fig. 1(b) and (c) shows schematic representations of our thin-layer electrochemical flow cell designed to assess allergen levels. The flow cell consists mainly of an acrylic block and a glass plate. The acrylic block has a funnel-shaped hole for sample injection and a screw hole for connecting an SUS pipe. First, the glass plate was sealed using an adhesive sheet with three electrode patterns. A thin titanium layer was deposited on the glass plate with a sputtering machine, and then a gold film was formed without breaking the vacuum. The total thickness of the gold and titanium film was 180 ± 10 nm. The adhesive sheet was then peeled off, and one of the electrodes was coated with silver paste as a reference electrode. To fabricate an adhesive spacer, a 50 µm thick adhesive tape was cut using a cutting plotter (Graphtec, Japan) to obtain a 20 mm × 76 mm piece. The thickness of the flow channel was as same as that of the spacer. Then a rectangular flow channel (1 mm × 48 mm) was fabricated also using the cutting plotter.

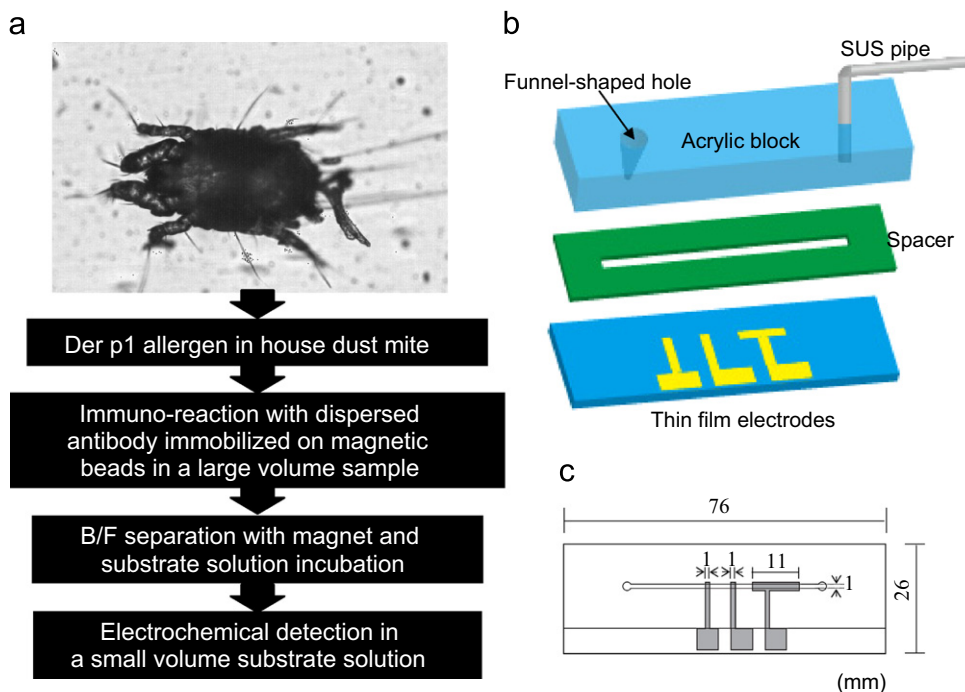


Fig. 1. (a) Outline of our assay. Der p1 allergen in fecal pellets was captured with dispersed antibodies on magnetic beads. Then immuno-complex was electrochemically detected in a small volume substrate solution. The pictured mite is *Dermatophagoides pteronyssinus*, which was found in dust in a microscope observation. (b) Schematic of an electrochemical flow cell. (c) Size of a glass plate with three thin electrodes.

Finally, the acrylic block and the glass plate were fixed together with the adhesive spacer.

We confirmed the electrochemical properties of our thin-layer electrochemical flow cell with a potentiostat (CHI Instrument, model 710) before performing an immunoassay using *p*-AP, which is the product of the labeling enzyme in this study (Ino et al., 2012; Niwa et al., 1993). Fig. S1 in the supporting information shows the scan rate dependence of the anodic peak current for 100 μ M *p*-AP. The anodic current increases linearly as the square root of the scan rate increases, suggesting that the *p*-AP oxidation in our electrochemical cell is a diffusion controlled process in the 10 to 200 mV/s range. Fig. S2 is a calibration curve for *p*-AP at a scan rate of 50 mV/s. *p*-AP can be measured with small error bars over a wide dynamic range of 1 to 500 μ M. Also, we were able to use our electrochemical cell repeatedly (more than 50 times) with a relative standard deviation of less than 5%. These results indicate that *p*-AP, namely the assay result with alkaline phosphatase, can be precisely measured with our electrochemical cell.

2.3. Assay protocol for Der p1 measurement

Fig. 2 is a schematic diagram showing the protocol of our electrochemical allergen level measurement. A 1.5 mL tube was prepared containing 30 μ L of magnetic beads modified with 60 μ g/mL of anti Der p1 antibody (a). First, 1 mL of the sample and 100 μ L of 10 μ g/mL alkaline phosphatase-labeled antibody were placed in the tube and mixed by pipetting for 1 min (b). A magnet was positioned close to the bottom of the tube, and the supernatant was removed with a pipette (c). 1 mL of washing buffer was added to resuspend the magnetic beads. This washing process was repeated twice. Next, 100 μ L of 5 mM *p*-APP was added to the tube (d). The magnet was again positioned close to the bottom of the tube (e), and the supernatant was inserted into a funnel hole in an electrochemical flow cell (f). The cell was filled with the supernatant by manually operating a syringe. Finally, a linear sweep voltammogram was obtained by scanning the potential of a working electrode from -0.3 to 0.4 V. To measure a real dust sample, various types of house dust were collected from, for example, bedroom and living room carpets with a portable vacuum cleaner. The collected dust was dissolved in 10 mL of

incubation buffer, and then the dissolved solution was analyzed using both our assay and a conventional ELISA. The ELISA protocol is detailed in the supporting information.

3. Results and discussion

3.1. Study of reaction times for immunoreaction and labeling enzyme

First, 20 ng/mL of Der p1 allergen was measured for various immunoreaction times after mixing analyte Der p1 and two antibodies. The immunoreaction time indicates the time required to form the immuno-complex shown in Fig. 2(b). Fig. 3(a) shows the measurement results for different immunoreaction times. The signal peak current increased with increasing immunoreaction time until 10 min. This is reasonable because the amount of immuno-complex increases as the immunoreaction time increases, and a high concentration of *p*-AP is produced by alkaline phosphatase (labeling enzyme) from *p*-APP. However, the peak current was saturated when the immunoreaction time exceeded 10 min. This means that the entire immunoreaction was completed in 10 min. On a conventional microtiter plate, several hours are required for a complete immunoreaction. This is because the immunoreaction on the microtiter plate occurs only at the plate bottom, moreover the analyte must diffuse a few millimeters toward the plate bottom. Since the diffusion time is proportional to the square of the diffusion length, the complete immunoreaction requires several hours. In contrast, the diffusion distance in our assay is very short. Since 9×10^7 beads were homogeneously dispersed in a 1 mL (10^{-6} m³) solution, the volume per bead is expressed as

$$10^{-6}(\text{m}^3) \div (9 \times 10^7) = 1.1 \times 10^{-14}(\text{m}^3) \quad (1)$$

and therefore, the distance between each bead is calculated as follows:

$$\sqrt[3]{1.1 \times 10^{-14}(\text{m}^3)} = 22.3 \times 10^{-6}(\text{m}) \quad (2)$$

Reducing the length scale from millimeters on a microtiter plate to 22.3 μ m in our assay shortens the incubation time from hours to 10 min. 54% of the limiting current was obtained without

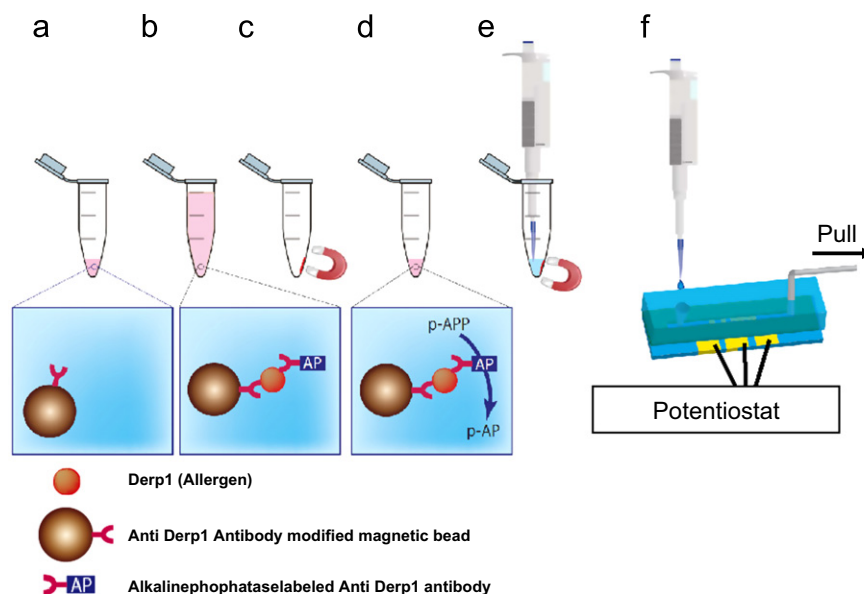


Fig. 2. Procedure of our immunoassay. (a) Prepare 30 μ L of capture antibody (anti Der p1 antibody modified magnetic beads). (b) Inject 1 mL sample and 100 μ L detection antibody (alkaline phosphatase-labeled anti Der p1 antibody), and mix for 1 min. (c) Wash the beads twice with 1 mL washing buffer while the tube is on a magnet. (d) Inject 100 μ L *p*-APP, and incubate. (e) Collect supernatant while the tube is on the magnet. (f) Inject the supernatant and perform electrochemical measurement.

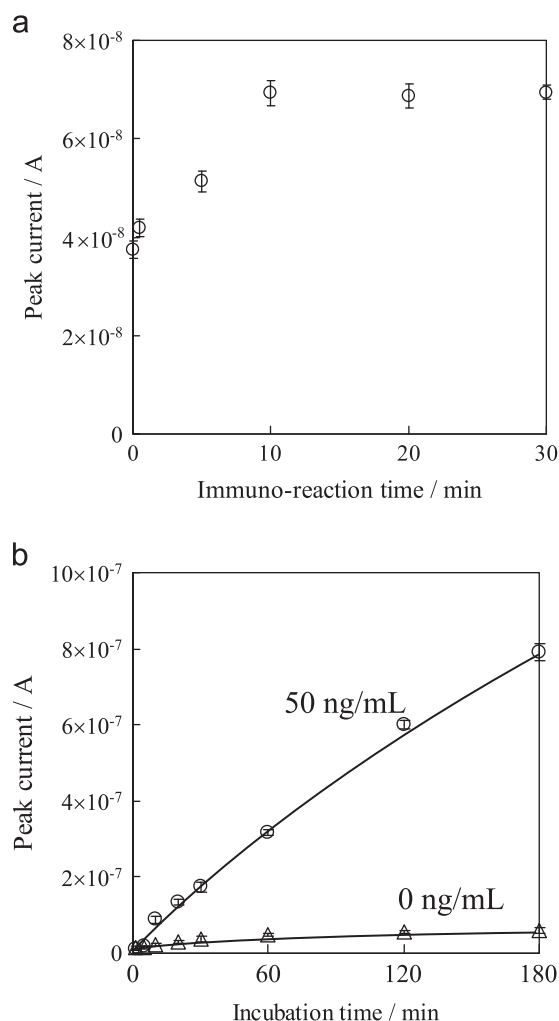


Fig. 3. (a) Variation in the peak current when the incubation time was changed after mixing the sample and the detection antibody. The Der p1 concentration was 20 ng/mL, and the scan rate was 50 mV/s. (b) Variations in the peak current for 0 (Δ) and 50 ng/mL Der p1 ($\circ$) when the alkaline phosphatase incubation time was changed. The scan rate was 50 mV/s.

any incubation time as shown in Fig. 3(a). This means that more than half of the immunoreaction occurred simply as a result of mixing and B/F separation (usually less than 1 min).

A satisfactory signal current was obtained without extra incubation time, and so we could perform the subsequent immunoreaction (process shown in Fig. 2(b) to (c)) in only 1 min without further incubation time.

Fig. 3(b) shows the variations in the peak current for 0 (Δ) and 50 ng/mL Der p1 ($\circ$) when the alkaline phosphatase incubation time was changed. The peak current increases linearly as the incubation time increases. This is because the concentration of the enzyme product (*p*-AP) increases linearly as the incubation time increases under a large excess substrate concentration (5 mM *p*-APP). In contrast, no significant increase was observed for 0 ng/mL Der p1. This indicates that the nonspecific adsorption is negligible, and a high signal-to-noise ratio can be obtained with a long alkaline phosphatase incubation time.

3.2. Calibration curves

Fig. 4(a) shows typical voltammograms of alkaline phosphatase with a 20 min incubation time when measuring 0, 1, 5, 10, 50, 100 and 250 ng/mL of Der p1. The anodic peak current at 0.22 V, which corresponds to the oxidation of *p*-AP to *p*-quinonimine, increases

when the Der p1 concentration increases. This signal increase is reasonable as the result of a sandwich-type immunoassay. Fig. 4 (b) shows a calibration curve for Der p1 obtained with our assay. The y-axis shows the peak current at 0.22 V estimated from Fig. 4 (a). We could measure the Der p1 concentration precisely with very small error bars in a total assay time of less than 30 min. The relative standard deviations were 5.3% (5 ng/mL) and 1.5% (50 ng/mL). The small error bars indicate that the quantitative performance for house dust mite is superior to that of conventional semi- or non-quantitative immunochromatographic tests. Moreover, the detection limit, which we defined as three times the standard deviation of the negative control (indicated by the dashed line in the inset), was 0.3 ng/mL. This value is two or three orders of magnitude better than that of previous house dust mite detection techniques using immunochromatography (Polzius et al., 2002). In addition, the value is better than that achieved with previous electrochemical immunoassay approaches combined with magnetic beads for other targets (IgG (Choi et al., 2002), ovalbumin (Kuramitz et al., 2012), β -galactosidase (Godino et al., 2010), dopamine (Baldrich and Munoz, 2011)). A calibration curve obtained by ELISA on a microtiter plate is shown in Fig. 4(c). Although the same detection limit (0.7 ng/mL) was realized, it required at least a few hours to complete ELISA. We were able to reduce the assay time to less than one-fifth that needed with conventional ELISA (Prester et al., 2007; Zahradnik et al., 2011) while achieving a low detection limit. A wider dynamic range was obtained with our sensor than with ELISA. This is because the current peak is linear in relation to the analyte concentration in linear sweep voltammetry. In contrast the absorbance in a high concentration range is saturated and so it deviates from the value obtained with the Beer–Lambert law. Moreover, electrochemical measurement has inherent advantages for on-site monitoring because of its high cost-performance ratio, low power and ease of miniaturization.

3.3. Real sample measurements

Fig. 5 shows the correlation between our assay results and those provided by conventional ELISA for real dust samples. The X- and Y-axes express Der p1 weight per sampling dust weight ($\mu\text{g/g}$) as in previous reports on real samples (Cho et al., 2006; Mihrshahi et al., 2002; Polzius et al., 2002; Prester et al., 2007; Sercombe et al., 2005). The concentrations of all 17 samples are provided in Table S1 in the supporting information. There was a high correlation between them with a correlation coefficient of 0.967 ($p < 0.001$). This indicates that the reliability of our assay is comparable to that of conventional ELISA despite the short assay time. Although we collected the real dust samples from various places including bedding, carpet and a corner of our laboratory, there was no significant error in our electrochemical immunoassay results. This suggests that interferences were effectively eliminated by the rinsing process on the magnet. The compatibility of the magnetic bead-based immunoassay with our thin-layered electrochemical flow cell is high, and this combined system could be readily employed for various analytes by changing the antibodies.

4. Conclusions

We reported the electrochemical measurement of Der p1 allergen in real indoor dust. We could quickly determine trace levels of allergen because the immunoreaction was performed with dispersed antibodies, which enabled the diffusion distance to be reduced by two orders of magnitude compared with conventional ELISA. Furthermore, our electrochemical flow cell enabled us

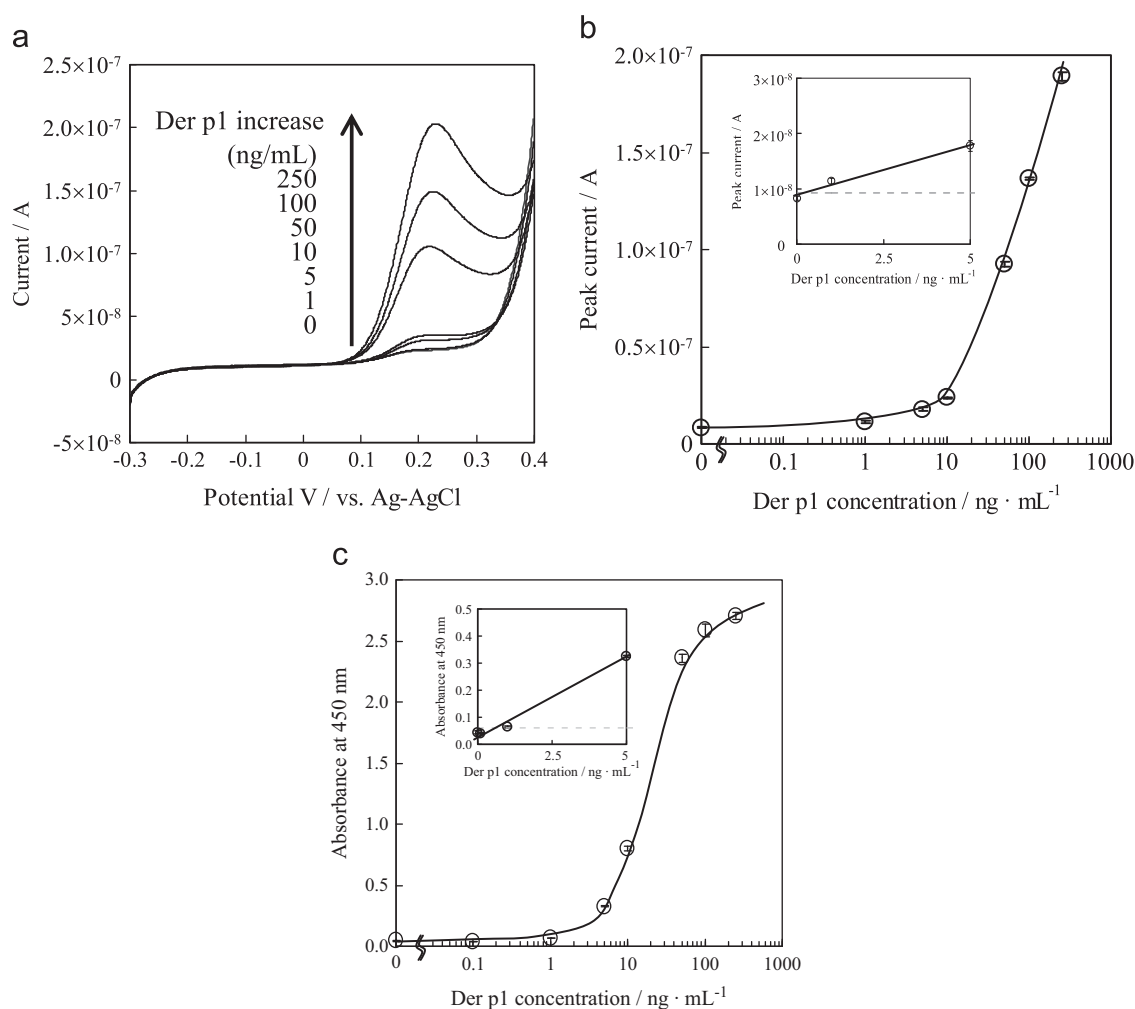


Fig. 4. (a) Variations in current when measuring 0, 1, 5, 10, 50, 100 and 250 ng/mL Der p1. All voltammograms were obtained at a scan rate of 50 mV/s. (b) Calibration curve of Der p1 obtained with the proposed assay. The inset shows an expanded calibration curve in a low concentration range. (c) Calibration curve of Der p1 obtained with conventional ELISA. The inset shows an expanded calibration curve in a low concentration range.

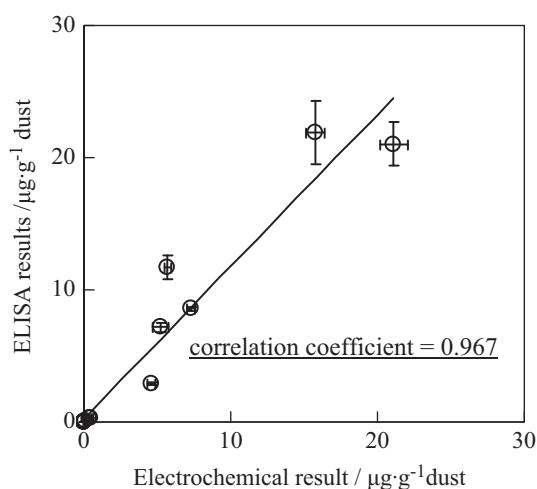


Fig. 5. Correlation between results obtained with our assay and conventional ELISA for real dust samples. The dust samples were collected in Japan during summer 2012. All the measurement results are tabulated in the supporting information.

to obtain an immunoassay result in a small volume substrate solution, which is advantageous as regards increasing the signal-to-noise ratio for the *p*-AP detection of a labeling enzyme in a short incubation time. Our quantitative allergen assessment

technique using an electrochemical cell has a high reliability comparable to that of ELISA despite its short assay time.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2013.04.010>.

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