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Semicontinuous Measurement of Mite Allergen (*Der f* 2) Using a Surface Acoustic Wave Immunosensor under Moderate pH for Long Sensor Lifetime

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In this work, we developed a surface-acoustic-wave (SAW)-based immunosensor for the monitoring of house dust mite (HDM) allergen, *Dermatophagoides farinae* group 2 (*Der f* 2). The SAW immunosensor was fabricated by modifying the sensor surface with a self-assembled monolayer of ORLA85 protein and immobilized capture antibodies (cAb) for *Der f* 2. Because of the pH-insensitive property of ORLA85, the sensor surface could be used for successive measurement and regeneration of the sensor surface. In experiments, first, the conditions for cAb immobilization on the sensor surface were optimized, and then the sensor properties were investigated. These revealed the limit of detection of the SAW immunosensor for *Der f* 2 to be 6.3 ng/mL and hence, the sensor's high selectivity to *Der f* 2. Finally, the optimum pH for regeneration, at which the regeneration efficiency and sensor output were balanced, was investigated. It showed that the use of pH 4.0 HCl for regeneration allowed reproducible sensor outputs, and less damage to immobilized cAb; thus, longer sensor lifetime could be expected. The results allowed us to anticipate that the SAW immunosensor would be usable for sensitive, selective, and repeated measurement of *Der f* 2 with a longer lifetime, and it would be a promising sensor for HDM allergen monitoring.

1. Introduction

The prevalence of allergic diseases has become a global health issue. In particular, IgE-mediated type I allergic disease that is accompanied by asthma, rhinitis, and atopic dermatitis are caused by environmental factors.[1,2] House dust mites (HDM) are one of the major factors of type I allergy development,[3–6] and unlike pollens, the risk of experiencing HDM allergens increases indoors because these mites multiply and are found in household items such as bedding, mattresses, and sofas.[7] The most relevant HDM allergens are group 1 and group 2

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of *Dermatophagoides farinae* (*Der f* 1 and *Der f* 2) and *Dermatophagoides pteronyssinus* (*Der p* 1 and *Der p* 2). Because of their strong allergenicity, most mite allergy patients produce IgE antibody to both group 1 and group 2 HDM allergens, and a standard of 20 ng/mL for sensitization and development of asthma was set by the World Health Organization (WHO).[8] In order to reduce the risk of sensitization to these HDM allergens, it is effective to prevent exposure to them. In particular, airborne HDM allergen that is easily inhaled should be carefully monitored because its concentration fluctuates over time influenced by many factors including temperature, humidity, and human activities. Previously, we proposed the concept of an airborne HDM allergen monitoring system for the prevention of allergic diseases. Such a system would be comprised of two parts—an airborne HDM sampler that draws air containing HDM allergens, and a biosensor that repeatedly measures the collected HDM allergens.[9] In such a biosensor, immunoassay is an effective method of measuring HDM allergens. Conventionally, immunoassays such as enzyme-linked immunosorbent assay (ELISA) or lateral flow immunochromatographic assay are used for one-time measurement.[10–14] Other state-of-the-art methods for measuring the allergens have also been reported. Morris *et al.* reported a quartz crystal microbalance-based immunosensor for monitoring airborne cat allergens, which allowed the measurement of cat allergen from dust without separation.[15] Kim *et al.* developed a fully automated bioaerosol monitoring system using carbon nanotube field-effect transistors.[16] It enabled *in situ* capture and continuous monitoring of airborne fungal particles. Kurita *et al.* demonstrated the measurement of mite allergen in real house dust using a thin layer flow cell and magnetic beads with comparative sensitivity to ELISA within 30 min.[17] Despite such remarkable progress in the development of allergen biosensors, the difficulties of repeatedly using the same sensor surface by regenerating it after each measurement have been drawbacks in the development of an immunosensor for monitoring. Surface regeneration is usually performed under harsh conditions for proteins, which causes protein degradation and thus, the loss of sensing capability. Recently, we introduced a surface-acoustic-wave (SAW)-based immunosensor for *Der f* 1, and it allowed rapid and repeated (semicontinuous) measurement with high sensitivity and selectivity.[18] Although ten repetitions of measurement were demonstrated with high reproducibility, the regeneration solution used was pH 1.0 HCl, and there was potential damage of the immobilized capture antibody (cAb) on the sensor surface. Considering that frequent exchange of a sensor chip in the airborne allergen monitoring system is laborious, the lifetime of the sensor must be prolonged. This could be achieved if surface regeneration could be accomplished using a relatively moderate pH solution that reduces the risk of damage to proteins. In addition, it would make the monitoring system more powerful if the system could be used to monitor not only *Der f* 1 but also other various airborne allergens concurrently.

Therefore, in this work, we first developed a SAW immunosensor for *Der f* 2, and characterized the sensor properties, including the sensitivity and selectivity to *Der f* 2. Then, the influence of the regeneration solution pH on the degree of surface regeneration (regeneration rate) was investigated because the balance between measurement reproducibility and damage reduction is important in achieving monitoring with a long sensor lifetime. Finally, repeated measurement of *Der f* 2 was demonstrated using a selected pH for the regeneration in order to assess the sensor capability for the semicontinuous measurement of *Der f* 2 and the possibility of implementation in the airborne HDM allergen monitoring system.

2. Materials and Methods

2.1 Reagents and chemicals

Ethanolamine and β-mercaptoethanol (BME) were purchased from Sigma-Aldrich. Sodium dodecyl sulfate (SDS), sodium acetate and acetic acid were from Wako. *Der f* 2 antigens (Mw 14 kDa), and the capture (cAb) and detection (dAb) antibodies for *Der f* 2, were from Shibayagi. Coarse extraction solutions of another mite allergen from *Der p* 1 and ragweed-pollen allergen (*Amb a* 1) were from ITEA. A cross-linker, PEGylated bis(sulfosuccinimidyl)suberate [BS(PEG)$_5$], was from Thermo Fisher Scientific. Orla IgGBinder-G kit, including a mixed solution of protein G-fused Orla protein (ORLA85 protein) and 11-mercaptoundec-11-ylhexaethylene glycol (HS-C11-EG6, PEG-thiol), tris(2-carboxyethyl)phosphine (TCEP), and Tris-HCl buffer solution (50 mM, pH 7.5), was from Orla Protein Technologies. Phosphate-buffered saline (PBS) with Tween 20 (PBS-T) was prepared by mixing Tween 20 [0.05% (v/v)] with PBS solution (10 mM phosphate buffer, 140 mM NaCl, 3 mM KCl, pH 7.4). Acetate-buffered saline (ABS) with Tween 20 (ABS-T) was prepared by a similar protocol to that of PBS-T: acetic acid solution (10 mM) was added to sodium acetate solution (10 mM) until the desired pH was obtained, and then this ABS solution and Tween 20 [0.05% (v/v)] were mixed.

2.2 Fabrication of SAW immunosensor

The SAW device was composed of an input or output transducer, a SAW propagation path (sensing area), and a reflector. The device was fabricated by depositing 90-nm-thick gold on a 36Y-X quartz substrate, followed by a lift-off process. SAW excited at the input transducer with a center frequency of 250 MHz propagated on the propagation path to the reflector and then reflected back to the output transducer [Fig. 1(a)]. Here, a shear horizontal (SH)-SAW was adopted because it propagated along a path with less attenuation into a liquid; thus, accurate measurement was possible. In the SAW immunosensor, *Der f* 2 was detected as a mass and/or viscoelasticity change on the sensing area, which induced velocity and attenuation changes in the SH-SAW.

The SAW immunosensor was fabricated as follows. The sensing area surface was modified by a self-assembled monolayer of ORLA85 and PEG-thiol; the binding of cAb and its immobilization by cross-linking on the sensing area surface took place [Fig. 1(b)].[18–20] The surface modification of the sensing area with the SAM was carried out as per the manufacturer's protocol: the surface was initially rendered hydrophilic in order to prevent nonspecific hydrophobic adsorption of ORLA85 by incubating the sensing area with 1% (v/v) BME in ultrapure water for 5 min.[21] Afterwards, in order to form the Au-S bond between ORLA85 and the gold on the SAW device, disulfide bonds of ORLA85 were reduced by mixing the protein solution with TCEP solution (TCEP 7.5 mg/mL in Tris-HCl) and letting stand for 30 min. Then the mixed solution was applied to the sensing area, and incubated for 10 min to form the SAM. Noncovalently adsorbed ORLA85 proteins and PEG-thiols were removed by washing the surface with 1% (w/v) SDS in ultrapure water. This SAM formation process was repeated twice to increase the surface density.

As the last process in SAW immunosensor fabrication, cAb was bound and immobilized on the sensing area surface modified with ORLA85 SAM. To bind cAb to ORLA85, 100 mg/mL cAb in a buffer solution at an optimum pH was applied and left for 10 min. After removing excess

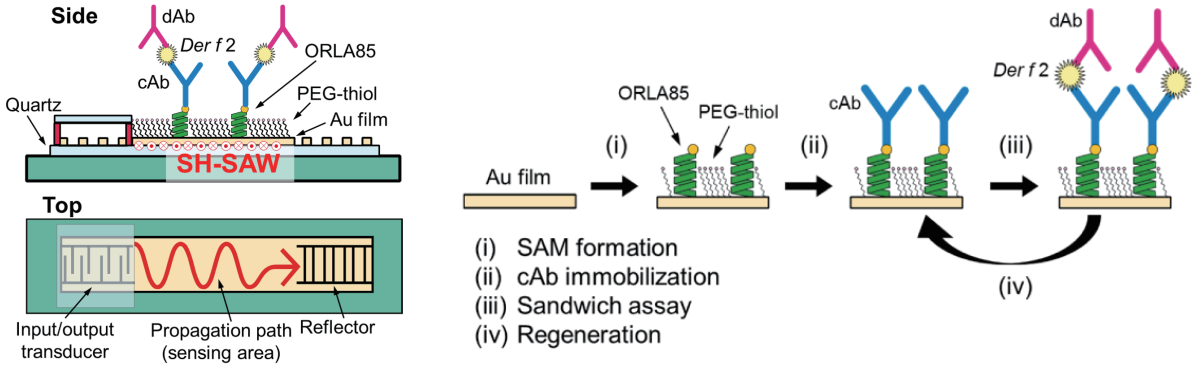


Fig. 1. (Color online) (a) Schematic diagram of the SAW immunosensor chip. SH-SAWs propagate along a propagation path and reflect back to the transducer. (b) Steps for (i), (ii) SAW chip fabrication and (iii), (iv) repeated measurement of *Der f* 2. cAb, capture antibody; dAb, detection antibody.

and unbound cAb, a cross-linker, 0.5 mM $BS(PEG)_5$ in PBS-T, was applied and left for 10 min to cross-link the cAb and protein G through a reaction of N-hydroxysuccinimide ester groups with lysine and N-terminal amino groups. Finally, 100 mM HCl and PBS-T were successively added to remove non-cross-linked cAb from the surface. This cross-linking process was repeated three times to improve the immobilization rate, that is, the ratio of cross-linked cAb to bound cAb. At the end of the cross-linking process, unreacted active esters were passivated by ethanolamine (1 M, pH 8.4).

2.3 Characterization of *Der f* 2 SAW immunosensor

For the evaluation of the sensitivity of the SAW immunosensor, two measurement methods were compared. In one method, a phase shift from the baseline after dAb binding, ΔP_{dAb}, was used as a sensor output; in the other, a differential method was used to shorten the measurement time, where the slope of the phase change during dAb binding, $\Delta P_{dAb}/\Delta t$, was used as the output.[19] The experimental procedures of these two methods were as follows. *Der f* 2 at a given concentration in PBS-T was initially applied to the sensing area and left for 10 min. After rinsing with PBS-T, 20 mg/mL dAb in PBS-T was applied and left for another 10 min, followed by rinsing with PBS-T to determine the sensor output ΔP_{dAb}; in the differential method, the incubation time of dAb was 3 min instead of 10 min. Then, the surface was rinsed. The slope $\Delta P_{dAb}/\Delta t$ was obtained by differentiating the phase change of dAb binding for 30 s by time.

To assess the selectivity of the sensor, two other environmental allergens, *Der p* 1 from mite and *Amb a* 1 from ragweed pollen, in mixed solutions with (three allergens) and without (two allergens) *Der f* 2 were measured by the differential method at the sample concentration of 100 ng/mL in PBS-T.

2.4 Semicontinuous measurement of *Der f* 2 under moderate condition

To extend the sensor lifetime as well as retain the capability of semicontinuous measurement of *Der f* 2 in the SAW immunosensor, the optimum pH for regeneration, at which the reproducibility

of the measurement could be balanced with the damage to cAb on the sensor, was investigated. HCl with pH 1.0–4.0 was used to regenerate the sensor surface after successive bindings of 100 ng/mL *Der f* 2 and 20 mg/mL dAb, and the regeneration rate $\Delta P_{Re}/\Delta P_{dAb3}$, defined as the ratio between the phase shift after 3 min of dAb binding, ΔP_{dAb3}, to the phase recovery through regeneration, ΔP_{Re}, was determined. These regeneration rates indicated degrees of surface regeneration at various pH values when using the differential method.

After optimizing the HCl pH for surface regeneration, the sensor capability for the semicontinuous measurement of *Der f* 2 was assessed by conducting five repetitions of the measurement of 100 ng/mL *Der f* 2 by the differential method, and the sensor outputs of these measurements were compared to assess the reproducibility.

3. Results and Discussion

3.1 Optimizing conditions for SAW immunosensor fabrication

In order to maximize the cAb density on the sensor surface, the relationship between the cAb solution pH and the phase shift induced by cAb binding, ΔP_{cAb}, was investigated with ABS-T (pH 4.0–5.0) and PBS-T (pH 6.0–8.0). Figure 2(a) shows the phase change when 100 mg/mL cAb in PBS-T at pH 6.0 was applied. A phase decrease as a response to cAb binding and phase recovery upon rinsing nonbound cAb were observed. The degree of phase shift from cAb binding, ΔP_{cAb}, was determined after rinsing, and these at different pH were plotted in Fig. 2(b). Throughout the entire pH range, the use of PBS-T resulted in larger phase shifts ΔP_{cAb} than those with ABS-T. For PBS-T, the shift ΔP_{cAb} was maxium at pH 5.0 and 6.0, and was about 1.9-fold larger than that in the case of ABS-T at pH 6.0. This was probably attributed to the optimum pH of IgG binding to protein G being a slightly acidic condition.[22] Considering that the buffer capacity of PBS-T ranges from pH 5.8 to 8.0, we used pH 6.0 in the subsequent experiments.

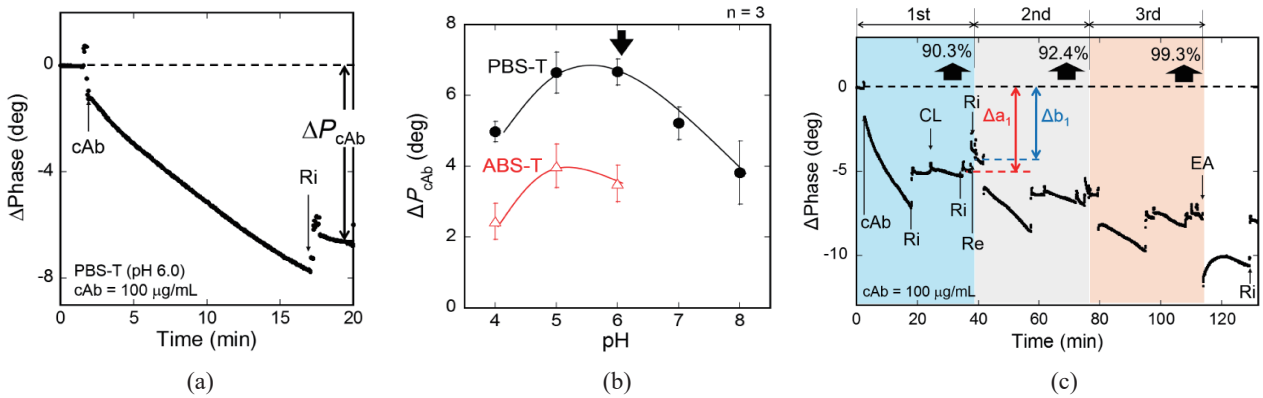
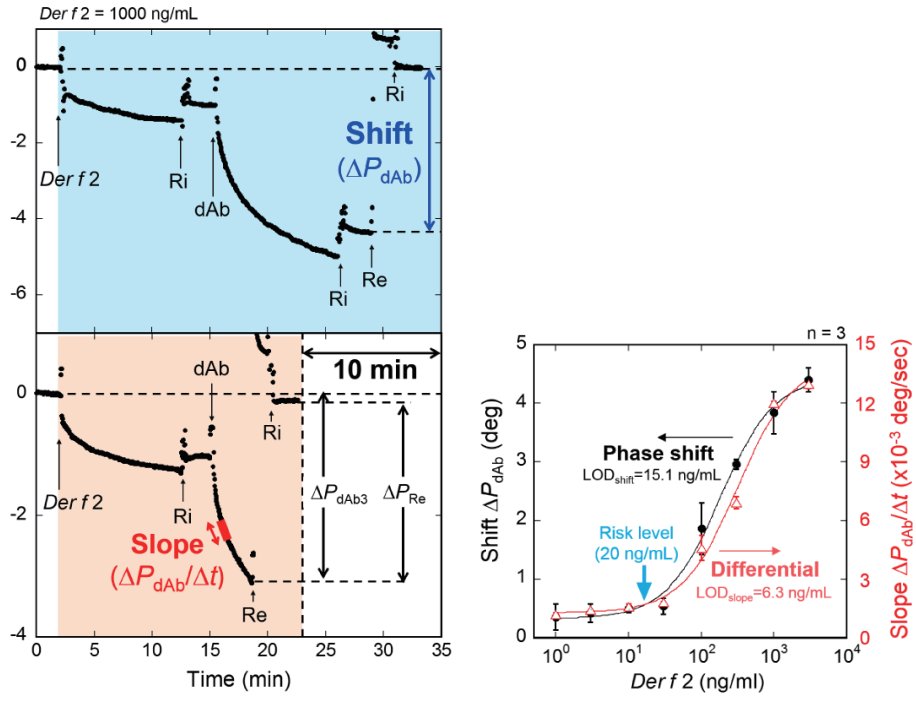


Fig. 2. (Color online) (a) Typical time course of phase during cAb binding. (b) Effects of buffer and pH on cAb binding to ORLA85 protein. Note that the error bars in this figure and all the others indicate the standard deviations. (c) Cross-linking between cAb and protein G with $BS(PEG)_5$. The cross-linking process was repeated three times to increase the immobilization rate $\Delta a/\Delta b$. CL, cross-linking; Ri, rinsing; Re, regeneration with HCl; EA, ethanolamine terminating the reaction.

Figure 2(c) shows the time course of the phase change during the binding and cross-linking of cAb. After the first cross-linking, HCl of pH 1.0 was applied to the sensor surface to remove non-cross-linked cAb, and the immobilization rate $\Delta a_1/\Delta b_1$ was 90.3%. a_1 and Δb_1 were defined as phase shifts before and after applying HCl; thus the immobilization rate indicated the degree of successfully cross-linked cAb on the surface among all bound cAb. The immobilization rate was improved to 99.3% by repeating the cross-linking three times.

3.2 Characterization of *Der f* 2 SAW immunosensor

Figure 3(a) shows the time course of the phase during *Der f* 2 measurement conducted by two different methods. The employment of the differential method resulted in a 10 min shortening of the total measurement time to 20 min because it took only 3 min for dAb binding compared with the 10 min needed in the phase shift method. In addition, although the phase shift method required rinsing in order to determine the sensor output ΔP_{dAb} before surface regeneration, it was not needed in the differential method, and dAb was removed only with HCl, which also allowed further shortening of the measurement time. Figure 3(b) shows the calibration curves of the two methods for *Der f* 2. The plots were fitted by the following equations in the range of 1.0–3000 ng/mL with coefficients of correlation of 0.997 and 0.996, respectively:



(a) (b)

Fig. 3. (Color online) (a) Time courses of phase during *Der f* 2 measurement by phase shift (upper) and differential (lower) methods. (b) Calibration curves of the phase shift method (●) and differential method (△) for *Der f* 2. ΔP_{dAb}, phase shift after dAb binding; $\Delta P_{dAb}/\Delta t$, slope of phase change during dAb binding; ΔP_{dAb3}, phase shift after 3 min of dAb binding; ΔP_{Re}, phase recovery via regeneration.

$$\Delta P_{dAb}\,(\mathrm{deg}) = A_1 + (B_1 - A_1) / \{1 + ([Der\,f\,2] / C_1)^{D1}\}, \quad (1)$$

$$\Delta P_{dAb}/\Delta t\,(\mathrm{deg/s}) = A_2 + (B_2 - A_2) / \{1 + ([Der\,f\,2] / C_2)^{D2}\}, \quad (2)$$

where $A_1 = 0.3$, $B_1 = 4.5$, $C_1 = 181$, and $D_1 = -1.2$, and $A_2 = 1.3 \times 10^{-3}$, $B_2 = 1.4 \times 10^{-2}$, $C_2 = 316$, and $D_2 = -1.1$ are the coefficients, and [*Der f* 2] is the concentration of the applied *Der f* 2 in ng/mL. The limit of detection (LOD) of each method was 15.1 ng/mL (phase shift, 1.1 nM) and 6.3 ng/mL (differential, 0.4 nM), encompassing a sensitization risk level of 20 ng/mL.[23] This LOD was determined to be the concentration at which the mean phase shift, or the slope for a blank sample, plus three times the standard deviation σ intersected the fitted curve. This result revealed that using the differential method improved the sensitivity by a factor of 2.4 along with shortening the measurement time by 10 min, and suggested that the differential method was more suited to the monitoring system.

The result of sensor selectivity evaluation is shown in Fig. 4. Almost no response was observed from samples without *Der f* 2, which validated the high selectivity of the SAW immunosensor to *Der f* 2. It suggested that parallel measurement of different HDM allergens, *Der f* 1 and *Der f* 2, would be possible by combining it with the previously developed *Der f* 1 SAW immunosensor, because the *Der f* 1 sensor also showed high selectivity to *Der f* 1.[18]

3.3 Semicontinuous measurement of *Der f* 2

Prior to demonstrating semicontinuous measurement of *Der f* 2, the influence of the pH of the regeneration solution on the regeneration rate $\Delta P_{Re}/\Delta P_{dAb3}$ was investigated. The results showed that the regeneration rate was almost 100% for pH 1.0–2.0, and it gradually declined above pH 2.0 (Fig. 5). When using pH 4.0, the regeneration rate dropped to about 48%. In the monitoring system, it

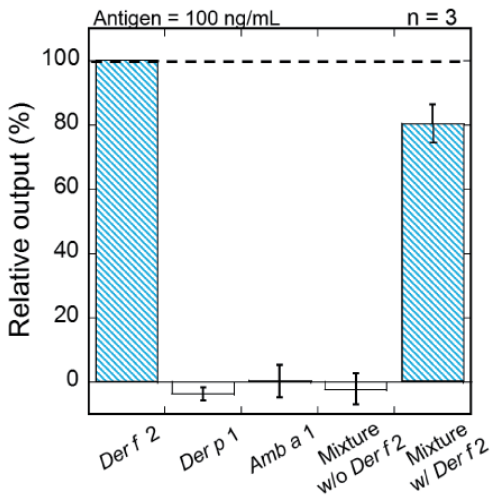


Fig. 4. (Color online) Sensor responses to various allergens and their mixture for the assessment of sensor selectivity. Hatched bars are sensor outputs from the samples containing *Der f* 2.

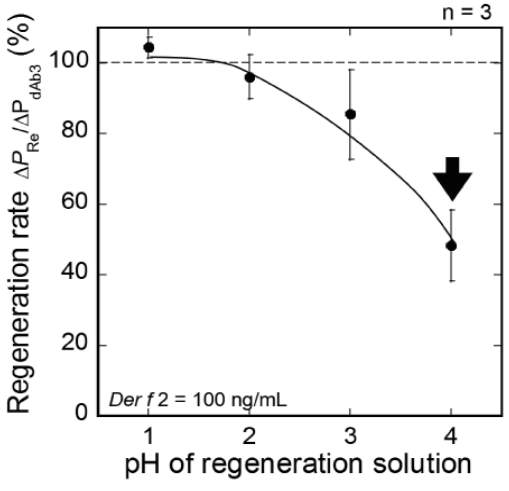


Fig. 5. Regeneration rate $\Delta P_{Re}/\Delta P_{dAb3}$ for various pH of regeneration solution.

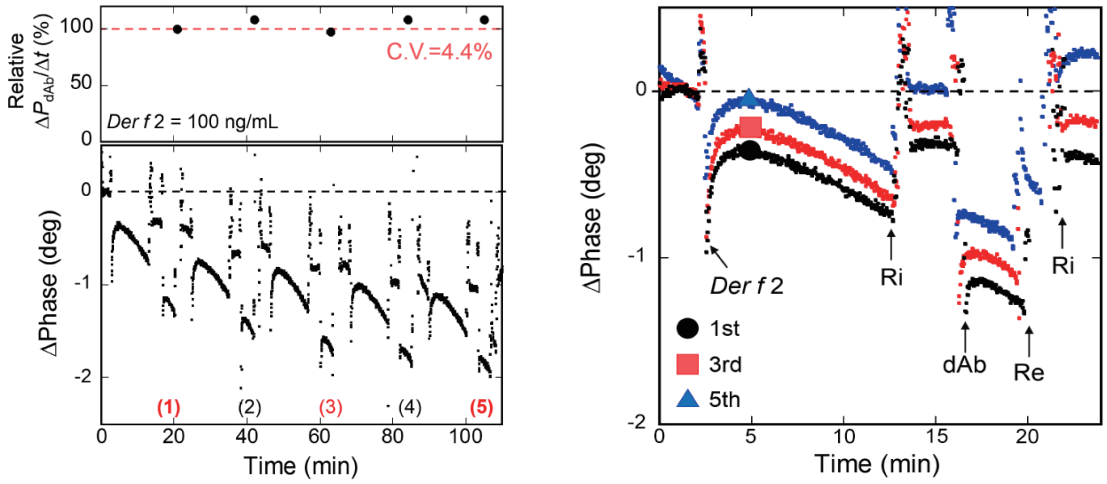


Fig. 6. (Color online) (a) Phase change over five repeated measurements of *Der f* 2 and relative sensor output at each measurement. (b) 1st (●), 3rd (■), and 5th (▲) measurements taken from (a).

is convenient for users if a sensor chip need not be exchanged for a long period of time, and it was considered that using a moderate pH solution for surface regeneration would lead to such a long sensor lifetime. Therefore, in the subsequent experiment, pH 4.0 HCl was chosen for the surface regeneration despite its low regeneration rate.

Figure 6(a) shows the time course of the phase during five repetitions of the measurement of 100 ng/mL *Der f* 2, and the relative sensor output $\Delta P_{dAb}/\Delta t$ for each measurement. It was observed that the baseline gradually declined with repeated measurement because of the imperfect regeneration and accumulation of residual molecules on the sensor surfaces, as indicated in Fig. 5, which shows a regeneration rate of 48% at pH 4.0. However, the coefficient of variation (C.V.) of the sensor outputs in the five measurements was 4.4%, which indicated a high reproducibility of measurement. Additionally, overlaid phase changes for the 1st, 3rd, and 5th measurements in Fig. 6(b) were well matched, which also supports the high reproducibility. The reason for the highly reproducible output despite the decline of the baseline was considered to be that adequate binding sites still remained for *Der f* 2; thus, the influence of the occupation of the binding site on the reproducibility for five repetitions of measurement was negligible. These results indicate the possibility of semicontinuous measurement of *Der f* 2 using the SAW immunosensor even with a regeneration solution at a moderate pH.

4. Conclusions

In this study, we developed a SAW immunosensor for semicontinuous measurement of the HDM allergen, *Der f* 2. The employment of the differential method allowed us to not only shorten the measurement time by 10 min compared with the phase shift method (30 min) but also improve the sensitivity by a factor of 2.4 (LOD of 6.3 ng/mL). The SAW immunosensor also showed high selectivity to *Der f* 2. The demonstration of five repetitions of measurement revealed that reproducible semicontinuous measurement was feasible even with a regeneration

solution at a relatively moderate pH (pH 4.0). These results allowed us to confirm that the *Der f* 2 SAW immunosensor could be used in airborne HDM allergen monitoring in conjunction with the previously reported *Der f* 1 SAW immunosensor.

Acknowledgments

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