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# A gold nanoparticle-assisted sensitive SAW (surface acoustic wave) immunosensor with a regeneratable surface for monitoring of dust mite allergens

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ABSTRACT

Signal amplification in a surface acoustic wave (SAW) immunosensor is demonstrated by increasing mass and/or viscoelasticity on a delay line sensing surface with gold nanoparticles (AuNPs) for monitoring of airborne house dust mite (HDM) allergens. Streptavidin conjugated AuNPs with size of 20 nm are affinity-bound to biotinylated detection antibodies (dAb) that are already on the sensor surface together with capture antibody (cAb) and group 1 HDM allergens of *Dermatophagoides farinae* (*Der f* 1) as a result of a sandwich assay. This immunocomplex except for immobilized cAb on the sensor surface is then removed by pH change for regeneration of the surface. Surface modification of the SAW immunosensor with a self-assembled monolayer of pH resistant protein (ORLA85 protein) allows to repeat a process including immunoassay and surface regeneration for a repetitive measurement of *Der f* 1. It is revealed that capping a surface of AuNP with a monolayer of (11-mercaptoundecyl)tri(ethylene glycol) (3PEG-thiol) reduces non-specific binding to the sensor surface, and aggregation among AuNPs. The sensor output is amplified by a factor of 3 with respect to that obtained without AuNP when a molar concentration of AuNPs is about 3-fold higher than that of dAb. A limit of detection for *Der f* 1 is 2.5 ng/mL (equivalent to 100 pM), which is about 2.5-fold improvement from the sandwich assay without AuNPs. Also highly reproducible sensor outputs along with efficient regeneration through successive measurement demonstrates the capability of repetitive measurement of *Der f* 1. These results suggest that use of AuNPs under an optimum condition in the SAW immunosensor is expected to realize a sensitive and repetitive measurement of HDM allergens, which is useful for airborne HDM monitoring system.

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## 1. Introduction

Allergic diseases are one of the major issues all over the world because a number of substances in the environment can cause to induce overreaction of one's immune system. Hose dust mites (HDMs) are found especially in human habitations because they are fed by shed human skin. Some enzymes of HDM show strong allergenicity, and they are associated with allergic asthma and eczema. HDM allergens are classified by spices of the mites and groups such as *Dermatophagoides farinae* group 1 (*Der f* 1) and 2 (*Der f* 2), and *Dermatophagoides pteronyssinus* group 1 (*Der p* 1) and 2 (*Der p* 2) [1–3]. The World Health Organization (WHO) warned of the risk of exposure to HDM allergens, and set 20 ng/mL as a standard for sensitization and development of asthma [4,5]. Since HDM allergens are light, human activities such as vacuuming a room can cause the allergens to become airborne, which drastically increase the exposure risk to the allergens [6,7]. Airborne allergens are easily inhaled or come in contact with skin, and one may be sensitized. Therefore, on-site monitoring of the airborne allergen concentration and avoiding the exposure would be effective in prevention of allergic diseases. However, conventional techniques to measure HDM allergens by using immunoassays such as enzyme-linked

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immunosorbent assay or lateral flow immunochromatographic assay are designed for use in end-point assays [8–10]; thus, only single measurement is possible. Despite their advantages of sensitivity or simplicity, they are not suited to rapid and repetitive (semi-continuous) measurement that is required in the monitoring system. Although sensors employing surface plasmon resonance, quartz crystal microbalance, and electrochemical impedance spectroscopy were also reported as cutting edge research [11–14], few sensors enabled repetitive immunoassay due to difficulty of sensor surface regeneration.

Recently we proposed a concept of the monitoring system that consisted of two parts: one was for collecting airborne HDM allergens and the other was for semi-continuous measurement of the collected allergens [15–17]. Through the work, we introduced a surface acoustic wave (SAW) immunosensor that allowed repetitive measurement of *Der f* 1 by making a sensor surface regeneratable with pH resistant protein (ORLA85) monolayer [18–21]. As a shear-horizontal SAW device enables to sensitively detect changes in viscoelasticity, mass and so forth in liquid phase, it becomes a powerful tool for real-time monitoring of molecular binding events. In our previous paper of the SAW immunosensor, the limit of detection (LOD) of 6.1 ng/mL (equivalent to 240 pM) for *Der f* 1 was achieved by a standard sandwich assay [22]. In this study, we extend it to enhance the sensitivity because a final concentration of airborne HDM allergens at the sensing part in the monitoring system would be lower than that of the original due to loss through transportation of the allergens, especially at a gas-liquid interface (a border at the collecting and the sensing parts). Gold nanoparticles (AuNPs) are employed to increase total mass of immunocomplex, which is expected to boost the sensitivity of the SAW immunosensor. In the following sections, we firstly investigate an effect of capping layer on AuNP surface with a polyethylene glycol monolayer to prevent non-specific binding of AuNPs. Then dependence of signal amplification and regeneration rate on applied AuNP concentration are examined. This is because degree of surface regeneration is as important in the monitoring system as the sensitivity for repetitive immunoassay. Finally, the sensitivity of the SAW immunosensor for *Der f* 1 is assessed.

## 2. Materials and methods

### 2.1. Reagents and chemicals

Streptavidin conjugated gold nanoparticles (AuNPs) with a size of 20 nm, (11-mercaptoundecyl)tri(ethylene glycol) (3PEG-thiol), ethanolamine and β-mercaptoethanol (BME) were purchased from Sigma-Aldrich. Sodium dodecyl sulfate (SDS), sodium acetate and acetic acid were purchased from Wako. Mite allergen *Der f* 1, capture (cAb) and biotinylated detection (dAb) antibodies for *Der f* 1 were from Indoor Biotechnologies. Crosslinker, PEGylated bis(sulfosuccinimidyl)suberate ($BS(PEG)_5$), was from Thermo Fisher Scientific. Orla IgGBinder-G kit containing a mixed solution of ORLA85 protein and (11-mercaptoundecyl)hexa(ethylene glycol) (6PEG-thiol), and tris(2-carboxyethyl)phosphine (TCEP) and Tris-HCl buffer solution (50 mM, pH7.5) was from Orla Protein Technologies. Phosphate buffered saline (PBS) with Tween 20 (PBS-T) was prepared by adding Tween 20 (0.05% (v/v)) to PBS solution (10 mM phosphate buffer, 140 mM NaCl, 3 mM KCl, pH 7.4).

### 2.2. Preparation of AuNPs solution

In this study, we aimed at improving sensitivity of the SAW immunosensor by binding of AuNPs to immunocomplex and increasing a total mass on the sensor surface. The diameter of AuNP was 20 nm, and its mass was about 300-fold larger than that of dAb. Binding of AuNPs to dAb occurred via affinity-bound of streptavidin on AuNPs to biotin on dAb. In order to prevent non-specific binding of AuNPs, surface of AuNPs were modified with a capping monolayer of 3PEG-thiol by mixing a solution of AuNPs with 3PEG-thiol solution (10 μM in PBS) at a volume ratio of 3:1 for 10 min. Then, excessive 3PEG-thiol in the solution was removed by centrifugation at 8000 rpm for 20 min, followed by exchanging supernatant with PBS. This centrifugation process was repeated to increase a purity of the solution. Finally, molar concentrations of AuNPs solution were adjusted to 1, 2.5, 5, and 10-fold (0.4, 1.5, 2.8, and $5.6 \times 10^{11}$ particles/mL) higher than that of dAb solution by measuring absorbance of AuNPs solutions at a wavelength of 520 nm ($OD_{520}$) with a spectrometer (NanoDrop 2000, Thermo). Each $OD_{520}$ was 0.08, 0.2, 0.4, and 0.8, respectively.

### 2.3. Preparation of the SAW immunosensor

A SAW device was comprised of input and output transducers, a delay line and a reflector that were fabricated by deposition of 90 nm thick gold and lift-off process on a piezoelectric 36Y-X quartz substrate. Shear-horizontal (SH) SAW was excited at the input transducer and propagated back and forth along the delay line (Fig. 1a). The delay line was used as a sensing area where binding of molecules changed the velocity of SAW which induced a phase shift between input and output signals. A self-assembled monolayer (SAM) of ORLA85 protein and 6PEG-thiol was formed on a surface of the delay line. It realized well-aligned cAb and highly pH resistant surface along with an antifouling property for repetitive measurement. After sandwich assay with *Der f* 1 and dAb, AuNPs were bound to dAb via affinity-bound of streptavidin and biotin (see Fig. 1b).

A detail process of the SAM formation on the delay line was as follows: a gold surface of the delay line was rendered hydrophilic by 5 min incubation in 1% (v/v) BME in ultrapure water in order to improve specific adsorption of ORLA85 proteins; a solution of ORLA85 protein and 6PEG-thiol was applied to the delay line and left for 10 min after reduction of disulphides of ORLA85 proteins by incubating it in TCEP solution (TCEP 150 mg/mL in Tris-HCl) for 30 min; non-covalently bound ORLA85 proteins and 6PEG-thiol in a SAM were removed with 1% (w/v) SDS in ultrapure water. This process was repeated twice to increase a surface density of ORLA85 proteins on the delay line. Immobilization of cAb to ORLA85 was carried out by adding 100 μg/mL cAb in PBS-T solution at pH 6.0 and leaving it for 10 min, followed by crosslinking. In crosslinking of bound cAb, a reaction of N-hydroxysuccinimide ester groups with lysine and N-terminal amino groups occurred by applying a crosslinking agent $BS(PEG)_5$ to the sensor surface and leaving it for 10 min. Non-crosslinked cAb were removed from the surface by 100 mM HCl solution and PBS-T rinsing. This crosslinking process was repeated three times to improve immobilization rate that was determined as a ratio of crosslinked cAb to bound cAb. At the end of crosslinking, ethanolamine (1 M, pH 8.4) was applied to passivate unreacted active esters of $BS(PEG)_5$.

### 2.4. AuNP-assisted sensitive and repetitive measurement of Der f *1*

In sensitive and repetitive measurement of *Der f* 1, initially *Der f* 1 at a given concentration was added and incubated for 10 min, followed by applying 20 μg/mL dAb for 10 min to form an immunocomplex on the sensor surface. Then AuNPs were applied and incubated for 10 min. Finally HCl solution at pH 1.0 was applied to regenerate the sensor surface by removing *Der f* 1, dAb, and AuNPs. At the end of each process, the sensor surface was rinsed with PBS-T so as to remove non-specifically bound proteins and AuNPs.

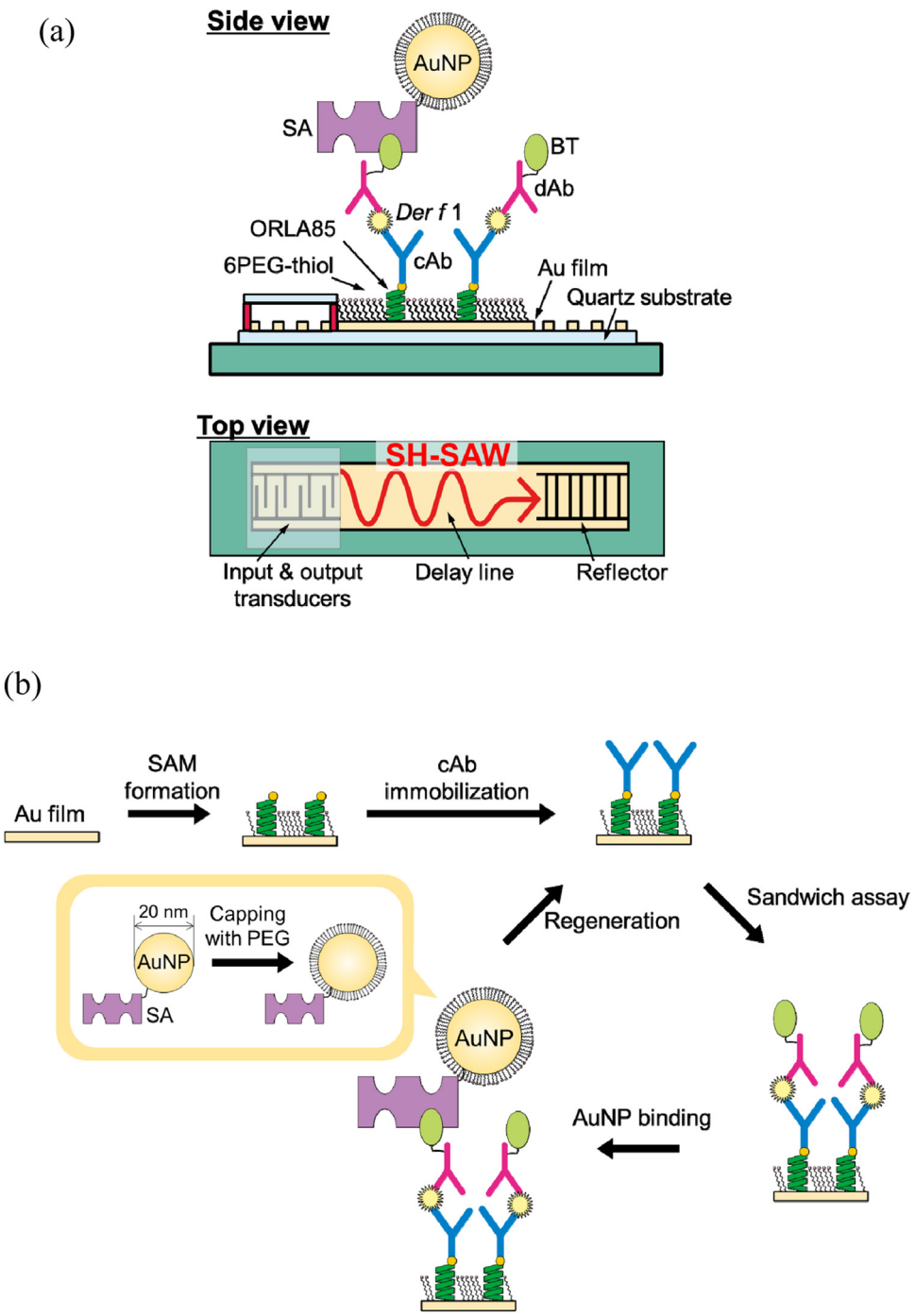



**Fig. 1.** (a) Schematic diagram of the SAW immunosensor chip. Shear horizontal (SH)-SAW propagates on a delay line between the transducers and a reflector. (b) Process of preparation of the SAW immunosensor and repetitive sandwich assay. AuNPs with and without PEG capping were used in experiments. cAb, capture antibody; dAb, detection antibody; 6PEG-thiol, (11-mercaptoundecyl)hexa(ethylene glycol); BT, biotin; SA, streptavidin; AuNP, gold nanoparticle.

## 3. Results and discussion

### 3.1. Effect of capping layer of AuNPs on repetitive measurement of Der f 1

Fig. 2 shows a sensor response during measurement of 100 ng/mL *Der f* 1 using AuNPs without capping of 3PEG-thiol. Large shift of phase *P* was observed after addition of AuNPs, while only slight shift occurred by addition of *Der f* 1 due to a small molecular weight of *Der f* 1 (25 kDa). For repetitive measurement, the sensor surface was regenerated by HCl solution at pH 1.0. However, there was little recovery of the phase *P* to a baseline because of residual AuNPs on the sensor surface. It indicated that AuNPs without capping layer bound to the sensor surface non-specifically, and they could not be removed by regeneration and rinsing. Here we defined three phase shift values, $\Delta P_{Ag}$, $\Delta P_{AuNP}$ and $\Delta P_{Re}$: $\Delta P_{Ag}$ is phase shift occurred only by *Der f* 1 binding; $\Delta P_{AuNP}$ is the phase shift induced by AuNPs binding; $\Delta P_{Re}$ is the phase recovery through the regeneration. Regeneration rate $\Delta P_{Re}/\Delta P_{AuNP}$ indicates how well the surface is regenerated, i.e., $\Delta P_{Re}/\Delta P_{AuNP} = 100\%$ is a perfect regeneration, and it was about 31% for AuNPs without capping layer.

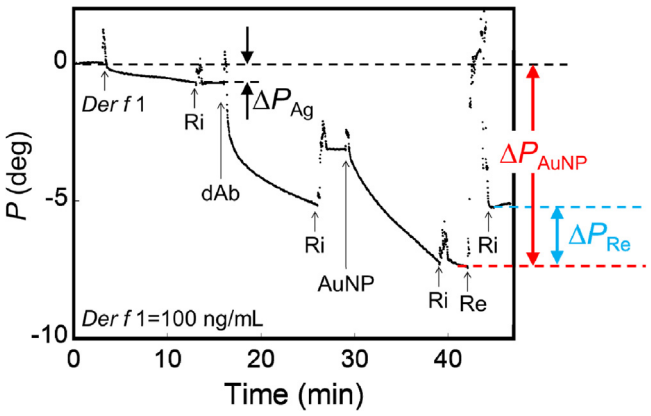



**Fig. 2.** Sensor responses during the immunoassay including AuNP biniding. AuNPs used here were without capping layer of 3PEG-thiol. Ri, rinsing by PBS-T; Re, surface regeneration by HCl; $\Delta P_{Ag}$, phase shift from *Der f*1 binding; $\Delta P_{AuNP}$, phase shift induced by AuNPs binding; $\Delta P_{Re}$, phase recovery through the regeneration.

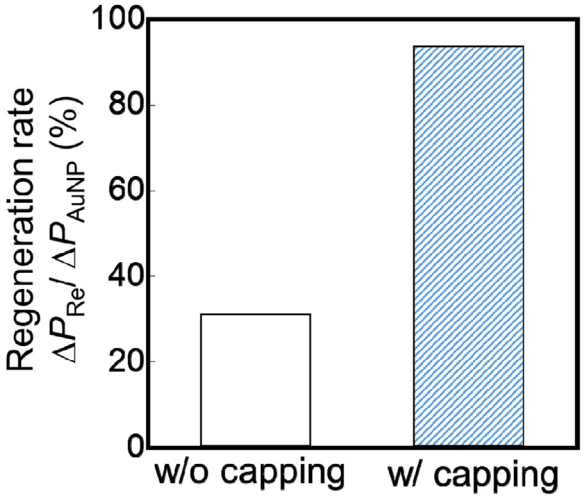



**Fig. 3.** Regeneration rates $\Delta P_{Re}/\Delta P_{AuNP}$ when using AuNP without and with capping monolayer of 3PEG-thiol.

In order to realize repetitive measurement with AuNP-assisted signal amplification, it was necessary to minimize the non-specific binding of AuNPs on the surface. It was achieved by forming a capping monolayer of 3PEG-thiol on AuNP surface, and optimizing a concentration of applied AuNPs solution. Fig. 3 shows regeneration rates after AuNPs binding in *Der f* 1 measurement $\Delta P_{Re}/\Delta P_{AuNP}$ without and with a capping 3PEG-thiol monolayer. Apparent improvement of the regeneration rate was observed when using AuNPs with the capping monolayer. It suggested that 3PEG-thiol monolayer on the AuNP surface could prevent non-specific binding to the sensor surface as well as aggregation among AuNPs. The regeneration rate $\Delta P_{Re}/\Delta P_{AuNP}$ was about 93% for AuNPs with capping layer, which was about 3-fold higher than that without the capping layer.

Then, dependence of signal amplification and regeneration rate on the concentration of AuNP solution were examined (Fig. 4). The amplification was calculated as a ratio of the sensor output signal with AuNPs to that without them. It was found that signal amplification had a linear relation to the AuNP concentration, and it reached about 400% when using $5.6 \times 10^{11}$ particles/mL which was 10-fold higher molar concentration to that of dAb (Fig. 4a). Here a signal amplification efficiency was defined:

$$\text{signalamplificationefficiency}(\%) = \text{SA}/(\text{RM} \times \text{RNM}), \quad (1)$$

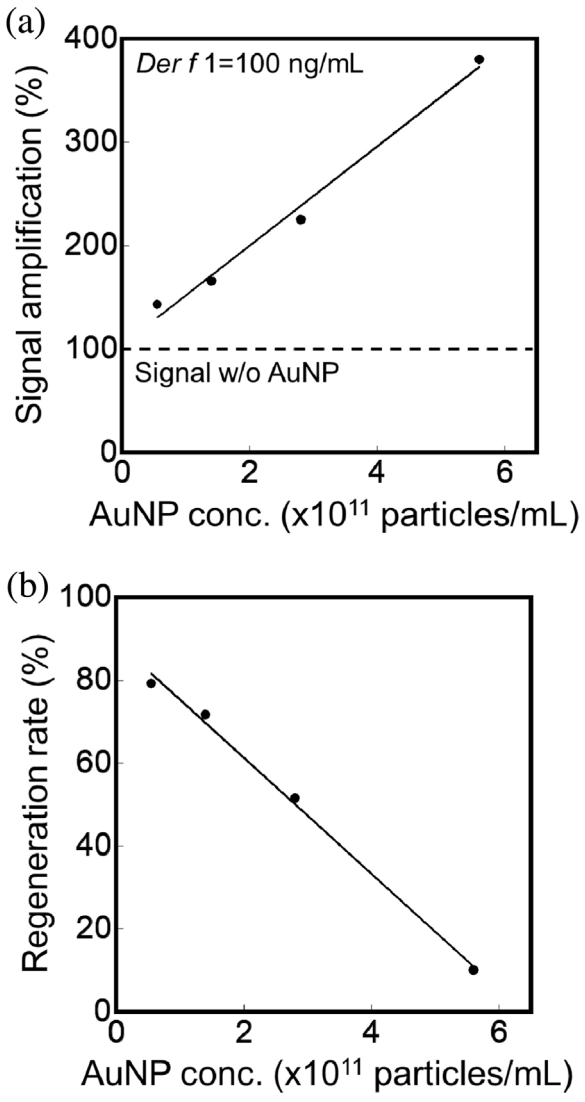



**Fig. 4.** Effects of AuNP concentration on (a) signal amplification and (b) regeneration rate.

where SA of 400% was actual signal amplification, RM of 300 was a ratio of mass of AuNP to dAb, and RNM of 10 was a ratio of the number of molecules between them (AuNP to dAb), respectively. From this equation, the amplification efficiency of AuNPs with a size of 20 nm was found 0.13%. This results indicated that mass of AuNPs less contributed to the signal amplification than expected, however further optimization, e.g., in the particle size, may improve it.

Let us now evaluate the dependency of the regeneration rate. The rate showed a linear decrease with increasing AuNP concentration, and it was about 10% when using $5.6 \times 10^{11}$ particles/mL (Fig. 4b). Here a deterioration rate of the regeneration rate was defined:

$$\text{deteriorationrate}(\%) = (100 - \text{RR})/(\text{RNM}), \quad (2)$$

where RR was the regeneration rate in%. The calculated deterioration rate of AuNPs was found 9%. This value would become higher when using AuNPs without capping of 3PEG-thiol monolayer as estimated from the result shown in Fig. 3. From the calculated amplification efficiency and deterioration rate, it could be concluded that use of AuNPs with a size of 20 nm in the SAW immunosensor was not the most efficient way to improve the sensor sensitivity especially in repetitive measurement because a high

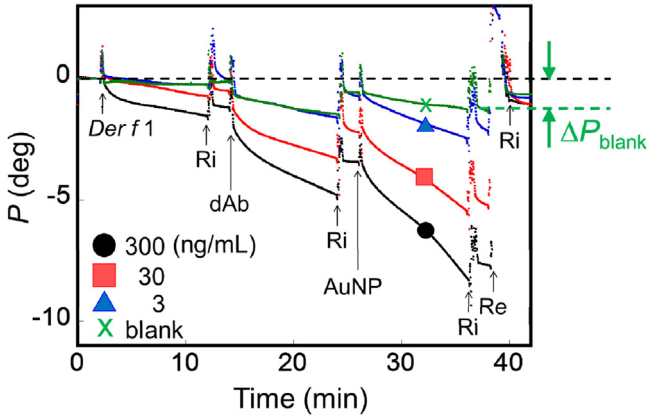



**Fig. 5.** Sensor responses to *Der f* 1with various concentrations: (●) 300 ng/mL, (■) 30 ng/mL, (▲) 3 ng/mL, and (x) blank. AuNP used here were with capping monolayer of 3PEG-thiol. $\Delta P_{blank}$, phase shift induced by AuNP binding without *Der f*1.

regeneration rate was a critical factor to maintain the baseline, thus a reproducibility of sensor output. Nevertheless, the amplification efficiency and deterioration rate will be improved if further optimization of various conditions such as particle size and length of PEG-thiol for capping layer is performed. Since the relation between the amplification efficiency and the deterioration rate was a trade-off, we chose $1.7 \times 10^{11}$ particles/mL (about 3-fold higher concentration than that of dAb) AuNPs solution in successive experiments with which the signal amplification of about 200%, and the regeneration rate of about 70% were expected.

### 3.2. *Sensitive and repetitive measurement of* Der f *1*

Fig. 5 shows sensor responses during measurement of various concentrations of *Der f* 1 using AuNPs with the capping layer. It was observed that changes of phase *P* were dependent on the concentrations of *Der f* 1. The regeneration rate was dramatically improved by capping AuNPs as discussed in Fig. 3. Amplified sensor signals were obtained after applying AuNPs with respect to that obtained with dAb. Since the sensor output did not reach saturation within 10 min of AuNPs binding, the signal would be further amplified if a reaction time for AuNPs binding was extended. On the other hand, the sensor output obtained from a blank sample (no *Der f* 1) $\Delta P_{blank}$ also became larger by using AuNPs compared to that by dAb. This was because AuNPs made affinity-bound to residual dAb that were non-specifically bound to the sensor surface. Prevention of non-specific binding of dAb is necessary to minimize a noise in the sensor output even when using AuNPs. Note that the graphs in Fig. 5 are representative data of a whole measurement of 0.3–1000 ng/mL *Der f* 1, and they were obtained by measuring a different concentration of *Der f* 1 successively. Along with this, the phase recovering to a similarly close level to the baseline after the regeneration implies that the SAW immunosensor can be used in repetitive measurement.

The sensor outputs from various concentrations of *Der f* 1 were plotted in Fig. 6. The plots were fitted with the following equations with a coefficient of correlation of 0.999:

$$\Delta P(\text{deg}) = A + (B - A)/\{1 + ([Der f\,1]/C^D)\}, \quad (3)$$

where $A = 7.0$, $B = 0.91$, $C = 18$ and $D = 0.95$ are the coefficients, and [*Der f* 1] is the concentration of applied *Der f* 1 in ng/mL. A limit of detection (LOD) with AuNP-assisted signal amplification was of 2.5 ng/mL that was equivalent to 100 pM, which was about 2.5-fold improvement from the previous study with a standard sandwich assay [22]. The LOD was determined at the concentration where a mean phase shift of a blank sample $\Delta P_{blank}$ plus three times a standard deviation σ equals the phase shift of AuNPs binding

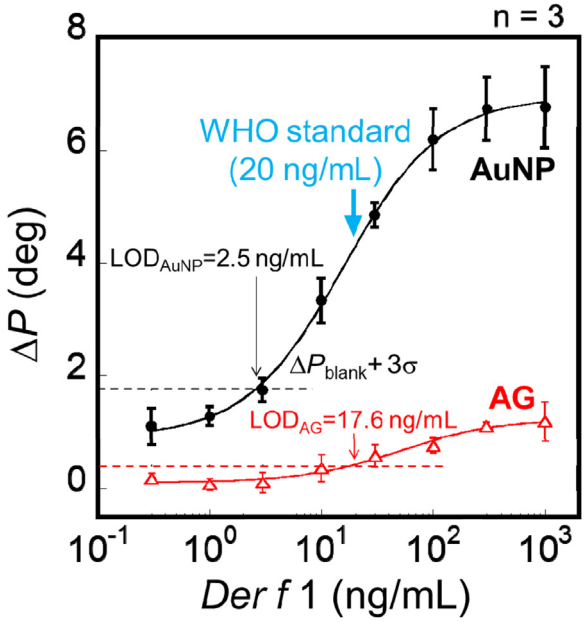



**Fig. 6.** Calibration curves of the SAW immunosensor for *Der f*1 with (●) AuNP-assisted signal amplification, and (Δ) from only *Der f* 1 antigens.

$\Delta P_{AuNP}$. For comparison, the LOD was also determined for the assay with only *Der f* 1, and it was of 17.6 ng/mL (= 704 pM). In addition, small error bars of the sensor outputs $\Delta P_{AuNP}$ from three SAW immunosensor chips (n = 3) demonstrates a high reproducibility of the output and little degradation of the senor performance through the successive measurement. These support the above mentioned capability of the repetitive measurement. In the experiments of this study, binding of dAb and AuNPs were performed successively, and it prolonged a total measurement time by 12 min (from 24 to 36 min). However, the measurement time can be shortened by pre-binding of dAb and AuNP prior to the application in the assay.

## 4. Conclusion

In this paper, signal amplification of the SAW immunosensor was demonstrated by using AuNPs with a size of 20 nm for sensitive and repetitive measurement of *Der f* 1. Capping a surface of AuNP with a 3PEG-thiol monolayer lead to prevention of non-specific binding to a sensor surface and aggregation among AuNPs. Although the sensor output was not amplified as expected from a mass of AuNP, the LOD was improved by a factor of 2.5 from that without AuNP. Choosing an optimum concentration of AuNPs and length of capping PEG-thiol is expected to further improve the LOD because signal amplification and deterioration of regeneration rate, i.e., non-specific binding of AuNPs, showed a trade-off to the concentration. Also having an adequate binding time of AuNPs would contribute to the increase the sensor output. Highly reproducible sensor outputs through a successive measurement of *Der f* 1 demonstrated the sensor capability of repetitive measurement. These results suggest that use of AuNPs is effective to amplify the sensor output, and to advance the sensitivity of the SAW immunosensor along with a capability of repetitive measurement of *Der f* 1.

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