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Detection of allergies using a silver nanoparticle modified nanostructured biosensor

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ABSTRACT

The IgE (Immunoglobulin E) in the serum of an asthma patient is a useful index for the detection of allergy diseases. In this study, a nanostructured biosensor with uniformly deposited gold nanoparticles (GNPs) is used as the sensing electrode for fast and low serum consuming detection of the IgE in an allergy patient's serum. To enhance the charge transfer efficiency of the biosensor, silver nanoparticles (SNPs) were deposited on the GNP layer. The group 2 allergen, Der p2, was used to indicate IgE. To ensure the specificity of detection, affinity purified goat anti-human IgE antibody was further immobilized to the IgE. After immobilizing the anti-IgE, electrochemical impedance spectroscopy (EIS) analysis was implemented to examine the concentration of the target IgE which was displayed in terms of a Nyquist plot. Blood serum samples with known allergy levels detected by the commercially available ImmunoCAP were used for verification of the sensor results. It is observed that the difference in the charge transfer resistance (ΔR_{et}) between the Der p2 immobilized electrode and the anti-IgE bonded electrode for each individual serum sample closely correlates to its ImmunoCAP class. The blood serum detection results indicate that the presented nanostructured biosensor is able to detect a patient's allergy level with low sample consumption, short sample preparation time, and quick processing.

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1. Introduction

Allergy related diseases are some of the most commonly seen immune disorder diseases in children. It has been reported that the prevalence of allergy related symptoms in the general public is around 30% [1]. The most common allergic symptoms include urticaria, allergic rhinitis, allergic conjunctivitis, asthma, and anaphylactic shock [2]. In addition, the incidence of such allergic diseases has gradually increased in many developed countries over the past decade [3]. The symptoms of the allergic response are determined by the types and concentrations of antigens in the ambient environment. Investigation has shown that dust mites, food, animal hair, insects, and pollen are the main allergens found in nature. Among these, dust mites are the major house allergen and the most significant source triggering respiratory allergies. There are reportedly 21 allergen components in dust mite allergens. The group 2 allergen, Der p2, has been reported to be the key allergen [4]. Almost 80% of child asthma patients and 87.8% of all asthma patients are allergic to Der p2 [5,6]. The recombinant DNA technology can be used to decompose a Der p2 allergen into amino-terminal sequences (1–63) and carboxyl-terminal sequences (64–129). It has been found that IgE, which is a useful index for allergy detection, is present in the serum of 80% of asthma patients, and can chemically bind with the carboxyl-terminal sequences of the Der p2 allergen. The IgE in the serum of the remaining 20% of asthma patients binds with the amino-terminal sequences [7]. Therefore, the Der p2 allergen can be used for the detection of the IgE concentration in a patient's serum. Consequently, the patient's allergy level can be examined.

The clinical detection of allergy diseases using the Der p2 allergen as the indicator to detect the presence of IgE in a patient's serum involves a complicated procedure. The detection processes can be categorized as in vitro blood tests or in vivo tests. Commercially available kits such as the ImmunoCAP, AlaSTAT, and HyTECH are commonly used for the in vitro blood test. A skin test is employed for in vivo test. The problems with these existing methods are large serum consumption (200 μL/item), relatively high cost, and long detection time. It would be of substantial interest to develop a more efficient detection device.

Recent advancements in micro/nano technologies have benefited the development of personalized detection devices that are low cost, sensitive, specific, easy to use, rapid and robust, and disposable, as well as consuming only a small sample [8–10]. The

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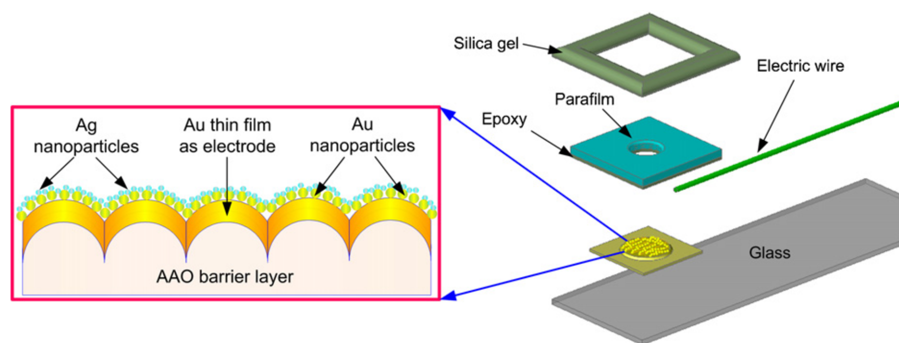

Fig. 1. Schematic illustration of the proposed nano-structured biosensor.

sensitivity of a biosensor relies on the amount of analytes that can be attached to the sensor's electrode. Nanomaterials, which can provide a substantially larger surface area than that of bulk material or thin films for better attachment of the analytes, have been used to either simplify the readout or magnify the detection signal of a diagnostic device [11–14]. Several nanomaterial-based devices have already been approved by the FDA for in vitro diagnosis [15–18].

In this study, a fast and low sample consuming detection of the IgE in the allergy patients' serum was carried out using a nanostructured biosensor [13] on which additional silver nanoparticles (SNPs) have been deposited for the enhancement of electrode conductivity. Der p2 was used as the indicator to detect the IgE. To ensure the specificity of detection, the affinity purified goat anti-human IgE antibody was further immobilized to the IgE. After immobilizing the anti-IgE on the sensor, electrochemical impedance spectroscopy (EIS) analysis was then used to detect the concentration of the target IgE which is indicated in terms of a Nyquist plot.

2. Materials and methods

2.1. Nano-structured transducer fabrication

Fig. 1 shows a schematic illustration of the structure of the nanostructured biosensor implemented for the detection of IgE in a patient's blood serum. In this device, the highly ordered nanohemisphere array of the barrier-layer surface of an anodic aluminum oxide (AAO) membrane is used as the substrate. A gold thin film is sputtered on the AAO barrier-layer substrate to serve as the electrode for (GNP) deposition and sensing. After the GNPs are electrochemically deposited on the film, the smaller SNPs are deposited on the GNPs.

The sequential fabrication procedures are as follows: preparation of an AAO barrier-layer; sputtering of an Au thin film; annealing; electrochemical deposition of GNPs; and dielectrophoretic deposition of SNPs.

2.1.1. Preparation of an AAO barrier-layer

AAO films with a nanopore diameter of around 60 nm and a thickness of 50 μm were fabricated through anodization of the washed aluminum foil in a 0.3 M phosphoric acid solution under a voltage of 90 V at 0 ± 1 °C for 2 h. The remaining aluminum under the barrier layer was then removed by an aqueous $CuCl_2$–HCl solution that was obtained by dissolving 13.45 g of $CuCl_2$ powder in 100 mL of a 35 wt% hydrochloric acid solution. A honey-comb like surface barrier-layer, with convex honey-combs of around 80 nm in diameter, was then obtained. Finally, the barrier-layer surface was immersed in a 30 wt% phosphoric acid for 40 min to further modify the shape of the honey-combs.

2.1.2. Sputtering of Au thin film

An Au thin film electrode around 10 nm was sputtered to the modified barrier-layer surface using radio frequency (RF) magnetron sputtering.

2.1.3. Annealing

The sequential annealing steps are: heating the sample to 120 °C at a rate of 5 °C/min, keeping that temperature for 60 min, finally cooling the sample to room temperature in open air.

2.1.4. Packaging

Precise packaging steps to ensure the consistency of the sensing area in each biosensor include: cut the parafilm into 2.5 × 2.5 cm^2 squares; punch a ϕ = 6 mm hole in the center of the parafilm square; bond the parafilm to the nanostructured electrode using a thin layer of epoxy; package the device using silica gel.

2.1.5. Deposition of GNPs

The electrochemical deposition of the GNPs is conducted by an SP-150 electrochemical analyzer (EC-Lab, USA). The electrolyte is produced by dissolving 1 mL of 0.02 M $HAuCl_4$ (Aldrich Inc.) solution into 39 mL of deionized water. Electrochemical deposition of GNPs was conducted under a DC −0.7 V applied voltage at room temperature lasting for 180 s.

2.1.6. Deposition of SNPs

Due to their extra high electric and thermal conductivity, SNPs have been widely applied in micro/nano systems to enhance conductivity [14,19,20]. Silver nanoparticles were dielectrophoretically deposited onto the GNPs to further enhance the device's conductivity. A 1 mM $AgNO_3$ (25 mL) solution is added to a 1 mM ice-cold $NaBH_4$ (75 mL) solution, followed by stirring the mixture at 40 °C for 2 min. Dielectrophoretic deposition of SNPs was conducted by applying a DC −0.4 V electric potential for 150 s at room temperature.

2.2. Der p2 protein analysis

A RasWin software (BioMolecular Structures Group, Glaxo Research & Development, UK) was used to verify that Der p2 contains NH_2^+ groups for the replacement of the functional group of an NHS (N-hydroxysuccinimide) and the chemical binding with the $COOH^-$ group on the 11-MUA layer.

2.3. Sample preparation

Detection of the IgE in patients' serum was carried out to examine the feasibility of the proposed nanostructured biosensor in clinical applications. The procedures for the immobilization of IgE on the sensor chip are illustrated in Fig. 2.

Table 1
ImmunoCAP detected data for the serum samples.

#	1	2	3	4	5	6	7	8	9	10	11	12	13
Value	0.25	17.6	12.1	18.3	0.46	6.03	120	18.4	0.29	2.67	0.23	0.43	6.17
Scale	0	4+	3+	4+	1+	3+	6+	4+	0	2+	0	1+	3+

Table 2
Ranges of the detected value for the Immuno CAP scale.

Scale	0	1	2	3	4	5	6
Value range	0–0.35	0.35–0.7	0.7–3.5	3.5–17.5	17.5–50	50–100	100<

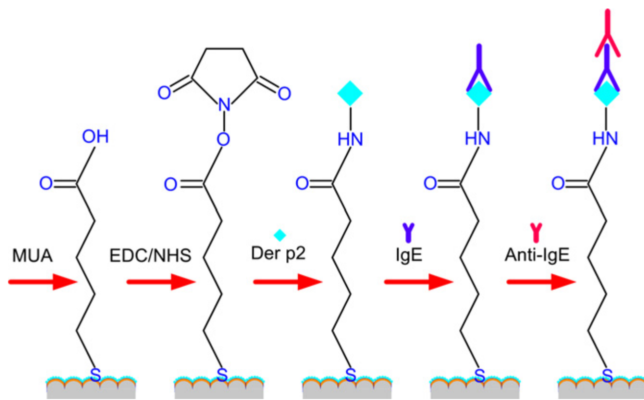

Fig. 2. Immobilization of IgE.

Thirteen patient serum samples which had already been examined by ImmunoCAP (CAP; Pharmacia Diagnostics, Uppsala, Sweden) were provided by the Taichung Veterans General Hospital, Taichung, Taiwan. The samples included four "0", one "1+", one "2+", three "3+", three "4+", one "6+" where "0" denotes that the serum contains no or undetectable IgE; "+" indicates that the allergen symptom is positive. There are total of 6 scales with "1+" being the smallest one and "6+" denoting the largest one. The detected ImmunoCAP values and the corresponding classes for the serum samples are listed in Table 1. The corresponding detected value ranges for each scale are tabulated in Table 2.

ImmunoCAP assay is a commercially available in vitro test for the detection of IgE and is the industrial quasi-standard. The CAP system is indicated in specific IgE classes (0–6) [21]. Any analytical scheme for the detection of specific IgE levels should be able to discriminate class 0 and class 1. Class 0 consists of IgE concentrations of up to 0.84 μg/L. An ImmunoCAP assay contains multiple allergens. Generally, a 100 μL of patient's serum is needed for each allergen test and a reaction time of 2.5 h is required.

The details for the immobilization of IgE on the sensor chip are as follows:

(a) The surface of the nanostructured sensor was cleansed by drenching it in ethanol, acetone and deionized (DI) water in turn, followed by an ultrasonic wave shaking for 5 min.
(b) 30 μL of a 5 mM 11-MUA (11-mercaptoundecanoic acid) solution was then dispensed on the sensor to create a self-assembled 11-MUA monolayer as an anchor membrane.
(c) Coating the sensor chip with a 30 μL mixture of NHS and EDC (1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide) (molar ratio, 1:2) for 50 min. The sensor was rinsed twice with a PBS buffer solution (pH 7.4).
(d) The sensor chip was coated with a 25 μL (50 μg/mL) dust mite allergen Der p2 solution and then incubated for 30 min. The sensor was again washed twice with a PBS buffer solution. A 20 μL 1% BSA solution was used as the blocking layer to the binding sites of those Der p2 which were not tied to the 11-MUA layer. The sample was then washed twice with a PBS buffer solution. The Der p2 was prepared according to the procedures reported by Tsai et al. [6].
(e) Coated with 25 μL of a patient's serum that had been diluted using a PBS solution at a ratio of 1:5. The sensor was incubated for 30 min before being washed twice in a PBS buffer solution.
(f) Coating a 25 μL diluted solution of the affinity purified goat anti-human IgE antibody. The diluted solution was prepared using a PBS solution at a ratio of 1:2000. The sensor was incubated for 30 min and then rinsed twice with a PBS buffer solution.

2.4. Conductivity enhancement analysis of siliver nanoparticle deposition

In this work, SNPs are deposited for the enhancement of sensor conductivity. To verify the conductivity enhancement of the SNPs, electrochemical impedance spectroscopy (EIS) analysis [22] is used to compare the conductivity of the biosensor with and without the deposition of SNP.

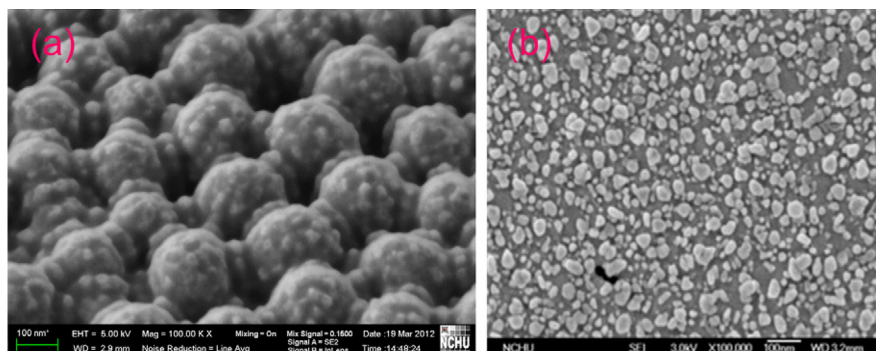

Fig. 3. SEM images of gold nanoparticles synthesized by electrochemical deposition; (a) 3D electrode; (b) flat electrode with a sodium citrate solution as the stabilizer.

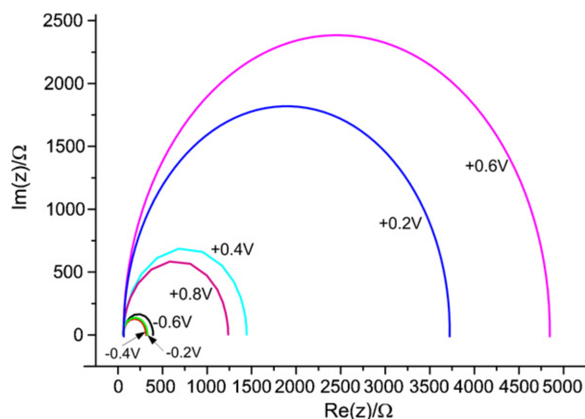

Fig. 4. Nyquist plots for the bare electrodes subjected to different applied voltages for SNP deposition; the buffer solution was a mixture of 5 mM $Fe(CN)_6{}^{4-}$ and 5 mM $Fe(CN)_6{}^{3-}$ in 100 mM 2-(N-morpholino) ethanesulfonic acid (MES) (pH 6.0).

2.5. Electrochemical impedance spectroscopy (EIS) analysis

In this study, a SP-150 electrochemical analyzer was implemented for the detection of the IgE concentration. The sensor chip, Pt film, and Ag/AgCl functioned as the working electrode, counter electrode and reference electrode, respectively, for the EIS analysis. The buffer solution was a mixture of 5 mM $Fe(CN)_6{}^{4-}$ and 5 mM $Fe(CN)_6{}^{3-}$ in 100 mM 2-(N-morpholino) ethanesulfonic acid (MES) (pH 6.0). The applied DC power and AC power were 0 V and 10 mV, respectively. The scanning AC frequency ranged between 0.01 Hz and 100 kHz.

3. Results and discussion

3.1. Device fabrication results

Fig. 3 shows the results of the electrochemical deposition of gold nanoparticles. The deposited gold nanoparticles (Fig. 3(a)) have an average diameter of about 10 nm and are uniformly and compactly deposited in a hemispheric electrode array. However, the distribution of gold nanoparticles became disorderly when a flat electrode was utilized with a sodium citrate solution as the stabilizer Fig. 3(b)). The electric flux which are uniformly perpendicular to the hemispheric Au thin film electrode prevent possible aggregations of the positive charges carrying Au nanoparticles in the electrolyte. This indicates that the GNPs can be densely deposited onto the surface of the Au thin film electrode without the necessity of any reducing agent or stabilizer.

A small amount of SNPs were deposited on nanostructured electrode to ehance the electron transfer process. The morphology of the electrode was characterized by SEM. The results indicated that there was no obvious difference between an SNP deposited electrode and an undeposited electrode. The chemical analysis electron spectroscopy measurements, carried out using a (PHI 5000, ULVAC-PHI Inc., Japan), showed a silver content of 0.3%.

3.2. Conductivity enhancement analysis results

Several voltages were applied during the electrophoretic deposition of the SNPs. Fig. 4 illustrates the Nyquist plots for the bare electrodes subjected to different applied voltages during SNP deposition.

An EIS analysis result (Nyquist plot), usually modeled by Randles's equivalent circuit [23], can be seen in the inset to Fig. 4. In the Randles's equivalent circuit, the total impedance is comprised of a series connection of an electrolyte resistance (R_s) and a parallel circuit composed of a charge transfer resistance (R_{et}) and a double layer capacitance (C_{dl}). The values of R_{et} and C_{dl} depend on the concentrations of the target molecule bonded onto the electrode surface of the sensor. In general, the value of each R_s in a Randles's equivalent circuit, as represented by a Nyquist plot, is much smaller when compared with its corresponding R_{et} value and can be neglected; the change in R_{et} when compared to that in C_{dl} is more substantial [11,13]. Hence, the fit of R_{et} is physically more meaningful than the fit of C_{dl} for the Randles's equivalent circuit of a Nyquist plot. Therefore, the Randles's equivalent circuit can be represented as:

$$\begin{aligned} Z(\omega) &= \frac{R_{et}}{1+\omega^2 R_{et}^2 C_{dl}^2} - j\frac{\omega R_{et}^2 C_{dl}}{1+\omega^2 R_{et}^2 C_{dl}^2}. \\ &= R + jX \end{aligned} \quad (1)$$

In general, the corresponding R_{et} value of each individual Nyquist plot (semicircle) can be represented by the diameter of the semicircle. It can be seen from Fig. 4 that an applied voltage of −0.4 V results in a relatively lower R_{et} value, implying that an applied voltage of −0.4 V can deposit SNP with a relatively higher electrode conductance. Therefore, a voltage of −0.4 V was selected for SNP deposition.

EIS analyses for the onductance comparisons between the SNP deposited electrodes and the GNP only electrodes with the Der p2 immobilized were carried out. The averaged R_{et} of the SNP deposited electrodes and GNP only electrodes for three experiments were calculated to be 4752 ± 2006 Ω and 9490 ± 1536 Ω, respectively. This indicates that the SNP deposited electrodes have better charge transferring characteristic than the GNP only electrodes.

3.3. Der p2 protein analysis results

The protein sequence of Der p2 is (MYKILCLSLLVAAVARDQ-VDVKD CANHEIKKVLVPGCHGSEPCIIHRGKPFQLEAVFEANQNTK-TAKIEIKASIDGLEVDVPGIDPNACHYMKCPLVKGQQYDIKYTWNVPK-IAPKSENVVVTVKVMGDDGVLACAIATHAKIRD). The protein analysis results indicate that Der p2 contains the $NH_2{}^+$ groups, comprised of amino acids such as the Asparagine, Glutamine, and Lysine. Hence, Der p2 can be firmly immobilized on the sensor chip through the SAM operation.

3.4. Blood serum detection results

Since the change in R_{et} is more substantial than that in C_{dl}, it is thus practical to represent the concentrations of the target molecule using the charge transfer resistance change (ΔR_{et}) between the Der p2 immobilized electrode and the anti IgE bonded electrode.

The blood serum detection results are shown in Fig. 5. The scale of the ImmunoCAP detection of specific IgE is related to the charge transfer resistance change ΔR_{et} between the Der p2 immobilized electrode and the anti-IgE bonded electrode. Three experimental runs were carried out for each sample. It is observed that the ImmunoCAP class can be distinguished by the EIS analysis using the proposed nanostructured biosensor. However, the clear boundary between each ImmunoCAP class have not well identified due to the limited number of patient's serum. Alternatively, a standard detection curve directly relates an ΔR_{et} value of EIS analysis to its corresponding ImmunoCAP then use Table 2 to identify the ImmunoCAP class can be a feasible solution.

Fig. 6 relates the Immuno CAP detected value for each individual serum sample (Table 1) to its EIS detected ΔR_{et}. Those patients with ImmunoCAP scales greater than or equal to "4+" are categorized as "very highly" allergic patients. The "6+" sample is an extreme case

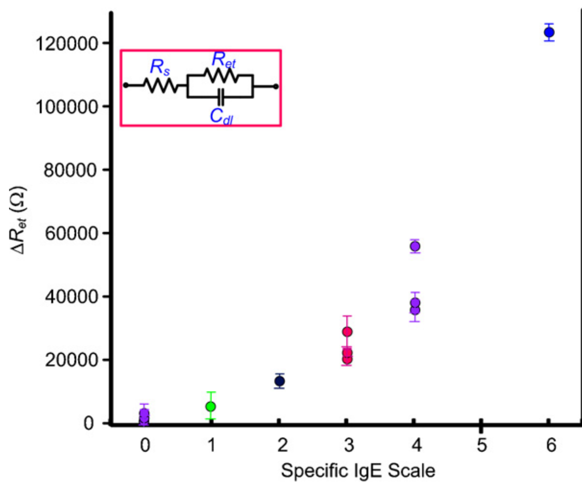

Fig. 5. Blood serum detection results ($n = 3$).

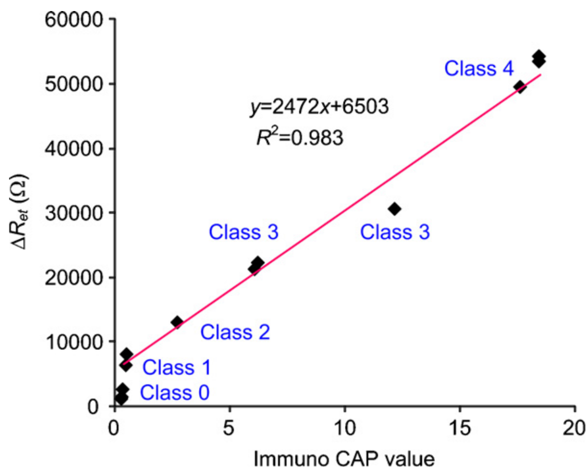

Fig. 6. Linear regression between ΔR_{et} and Immuno CAP detected value.

with an ImmunoCAP detected value of 120 and is omitted from the analysis. The data points for those samples that were detected by ImmunoCAP as having allergy symptoms (1+ ≤ scale ≤ 4+) can be linearly regressed by a standard curve, as shown in Fig. 6. In further detection, this standard curve can be used for the estimation of a sample's ImmunoCAP value, once the sample's ΔR_{et} is detected. Accordingly the corresponding ImmunoCAP class can be obtained from Table 2. Although the R^2 value for the standard curve obtained is 0.983, more experiments are required to improve the linearity of standard curve before it can be real clinically implemented.

In addition to the ImmunoCAP assay, the micro-array approaches [24–27] are used for the detection of allergen-specific IgE in human serum. The receiver-operating characteristic curve (ROC) analysis is used to discriminate between the allergy and no allergy status in well-characterized serum samples. Therefore, detection limit is not the key issue in micro-array approaches. In ref [26], a detection limit of 1.9 ng/mL for Der p1 specific IgE was reported. In this study, IgE solutions with different concentrations (1 μg/mL, 100 ng/mL, 10 ng/mL, 1 ng/mL, 100 pg/mL, and 10 pg/mL) were used for the measurement of the detection limit of the proposed device. For each concentration, three experiments were conducted. As shown in Fig. 7, IgE with concentration of 10 pg/mL could be detected. The detections of IgE solutions with concentration of 1 pg/mL also were implemented. It was found that 1 pg/mL has gone beyond the detection

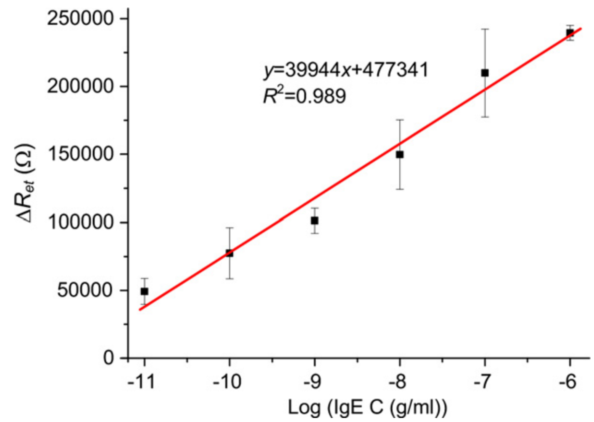

Fig. 7. Measurement of the detection limit of the proposed device; ΔR_{et} as a function of the logarithmic concentration of IgE.

limitation of the proposed sensor and is not within the linear range.

The distinguishing features of the proposed scheme over the reported work can be attributed to (1) the 3D nanostructure of the AAO film enhance the binding surface of the GNPs; (2) the symmetrical distribution of the electrical field intensity during electrophoresis deposition generates an extremely uniform distribution of the GNPs; (3) the uniform distribution of the GNPs on the hemispheric array enabled the MUA molecules to attach to individual GNPs, allowing more complete bindings of EDC/NHS molecules and Der p2 molecules. Hence the productive binding between IgE and Der p2 can be greatly enhanced.

The blood serum detection results confirm that allergy diseases can be detected using the proposed nanostructured biosensor with little sample consumption (25 μL), and short sample preparation (less than 2 h) and detection time (1 min) when compared with the ImmunoCAP assay the microarray approaches. In our future works, more samples of allergy patient's serum will be collected to establish a more complete standard curve for efficient serum detection using the proposed nanostructured biosensor scheme.

4. Conclusions

The prevalence of allergy triggered symptoms in the general public is around 30% and continues to increase year by year. Dust mites are the major house allergen and the most significant source of respiratory allergies. The group 2 allergen, Der p2, has been reported to be the key allergen. However, IgE (Immunoglobulin E) in the serum of an asthma patient can chemically bind with the carboxyl-terminal sequences of the Der p2 allergen which makes the Der p2 allergen a useful indicator for the detection of the patient's allergy level. In this study, a nanostructured biosensor with uniformly deposited GNPs as the sensing electrode was implemented for the detection of a patient's allergy level. To enhance the charge transfer efficiency of the biosensor, SNPs were deposited on the GNP layer. Blood serum samples with known allergy levels that had been examined by the commercially available ImmunoCAP were used for the verification of the sensor. It was found that the difference in the charge transfer resistance (ΔR_{et}) between the Der p2 immobilized electrode and the anti-IgE bonded electrode for each individual serum sample is closely correlated to its ImmunoCAP class. The blood serum detection results confirm that the proposed nanostructured biosensor can detect allergy diseases with small sample consumption, short sample preparation time, and little detection time.

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