Analyst

PAPER

View Article Online
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Cite this: *Analyst*, 2018, **143**, 1182

Highly sensitive colorimetric detection of allergies based on an immunoassay using peroxidase-mimicking nanozymes†

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Nanomaterials that exhibit enzyme-like characteristics, which are called nanozymes, have recently attracted significant attention due to their potential to overcome the intrinsic limitations of natural enzymes, such as low stability and relatively high cost for preparation and purification. In this study, we report a highly efficient colorimetric allergy detection system based on an immunoassay utilizing the peroxidase-mimicking activity of hierarchically structured platinum nanoparticles (H-Pt NPs). The H-Pt NPs had a diameter of 30 nm, and were synthesized by a seed-mediated growth method, which led to a significant amount of peroxidase-like activity. This activity mainly occurs because of the high catalytic power of the Pt element, and the fact that the H-Pt NPs have a large surface area available for catalytic events. The H-Pt NPs were conjugated to an antibody for the detection of immunoglobulin E (IgE) in the analytes; IgE is a representative marker for the diagnosis of allergies. They were then successfully integrated into a conventionally used allergy diagnostic test, the ImmunoCAP diagnostic test, as a replacement for natural signaling enzymes. Using this strategy, total and specific IgE levels were detected within 5 min at room temperature, with high specificity and sensitivity. The practical utility of the immunoassay was also successfully verified by correctly determining the levels of both total and specific IgE in real human serum samples with high precision and reproducibility. The present H-Pt NP-based immunoassay system would serve as a platform for rapid, robust, and convenient analysis of IgE, and can be extended to the construction of diagnostic systems for a variety of clinically important target molecules.

Received 17th November 2017,
Accepted 17th January 2018

DOI: 10.1039/c7an01866e

rsc.li/analyst

1. Introduction

An allergy indicates a disorder of hypersensitivity, in which the immune system reacts to normally harmless substances, which are called allergens. A variety of allergens can enter the human body *via* inhalation or ingestion, and cause allergic reactions of the respiratory tract, skin, and gastrointestinal organs. These reactions could induce diverse adverse symptoms, such as eye redness, itchiness, rhinorrhea, eczema, abdominal cramping, and dyspnea.[1]

To identify causal allergens, the skin prick test was traditionally used for patients with allergies; however, this method has some limitations such as inconvenience to patients, interruption by anti-histamine medications, false positivity due to dermographism, and low reliability while evaluating food allergens.[2] To overcome and compensate for these limitations, direct measurement of the serum level of allergen-specific IgE is frequently performed. For determination of IgE levels, several immunological methods have been developed, including the radioallergosorbent test (RAST), liquid-phase enzyme immunoassay, enzyme-linked immunosorbent assay (ELISA), or fluorescent enzyme immunoassay (FEIA).[3–11] The assays generally involve a single allergen or total allergen-specific immunoreaction; they yield sensitive, precise, and quantitative results for measuring total or allergen-specific IgE levels in the serum. Among them, the commercially available ImmunoCAP (Phadia, Uppsala, Sweden), which is based on ELISA performed in the solid-phase, is considered as a gold standard for the diagnosis of allergy.[5] The ImmunoCAP test

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†Electronic supplementary information (ESI) available. See DOI: 10.1039/c7an01866e

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employs a polymeric cellulose membrane, which enables a high binding capacity of allergens on it, consequently resulting in enhanced sensitivity to determine the target IgE molecules. This technology, which provides quantitative measurements of the IgE levels in blood, has also been approved by the Food and Drug Administration (FDA).[12,13] Although this method enables the selective and sensitive detection of the target IgE molecules, its performance is limited due to the inevitable drawbacks of using natural enzymes, including their intrinsic instability during long-term operation or storage, and relatively high costs involved in their preparation and purification.

To overcome these limitations, we herein developed an efficient colorimetric assay system, based on enzyme-mimicking nanomaterials (nanozymes).[14–16] Diverse kinds of nanozymes, which show a considerable amount of enzyme-like activity with high stability, have been reported and applied to the development of colorimetric immunoassay systems.[17–19] Among them, Pt-based nanozymes have attracted exceptional interest due to their super catalytic activity to oxidize a chromogenic substrate into a colored product.[20–25] To maximize the peroxidase-like activity of Pt-based nanozymes, we also recently reported hierarchically structured Pt nanoparticles (H-Pt NPs) that show superior peroxidase-like activity under operating conditions because of their extremely large surface area that could be used for catalytic events.[26] The H-Pt NPs, conjugated to antibodies against total or specific IgE, were found to exhibit excellent selectivity for the target IgE when used in the ImmunoCAP test, as a replacement for enzyme–antibody conjugates. Moreover, by employing the new method, the levels of the target IgE molecules can be conveniently determined by simply measuring the intensity of blue color generated by the H-Pt NP-mediated colorimetric reaction of the applied peroxidase substrate, 3,3′,5,5′-tetramethylbenzidine (TMB) with H_2O_2. The clinical utility for determining diverse levels of both total and specific IgE present in real human blood serum samples was also evaluated.

2. Materials and methods

2.1 Materials

Chloroplatinic acid hexahydrate ($H_2PtCl_6{\cdot}6H_2O$), sodium borohydride ($NaBH_4$), sodium citrate, citric acid, horseradish peroxidase (HRP), 3,3′,5,5′-tetramethylbenzidine (TMB), bovine serum albumin (BSA), human serum albumin (HSA), and glucose were purchased from Sigma-Aldrich (Milwaukee, WI), and 35% hydrogen peroxidase (H_2O_2) solution was purchased from Junsei Chemical Co. (Japan). All chemicals were of analytical grade, and used without further purification. All solutions were prepared with double distilled water purified by a Milli-Q purification system (Millipore).

2.2 Synthesis of H-Pt NPs

H-Pt NPs were prepared according to our previously reported seed-mediated growth method in aqueous solution.[27] Pt nanoparticle seeds were first prepared through the reduction of chloroplatinic acid using sodium borohydride. In the reaction, 9 mL of 0.2% chloroplatinic acid solution was added to 116 mL of boiling deionized (DI) water, and incubated for 1 min. Then, 2.75 mL of 1% sodium citrate–0.05% citric acid solution was added, followed by a 1 min incubation period. Then, 1.38 mL of an aqueous solution containing 0.08% sodium borohydride and 1% sodium citrate–0.05% citric acid was added into the reaction mixture. After incubation for 10 min, the mixture was cooled to room temperature (RT) to produce a seeding solution. The seeding solution (10 mL) was diluted with 90 mL of DI water, followed by the addition of 0.5 mL of 0.4 M chloroplatinic acid solution and 5 mL of 1% sodium citrate–1.25% L-ascorbic acid solution. The resulting solution was heated to its boiling point, incubated for 30 min, and then cooled to RT. Finally, the H-Pt NPs were purified by thoroughly washing them thrice with DI water. The morphology of the H-Pt NPs was examined using scanning electron microscopy (SEM) with an S-4200 field-emission SEM (Hitachi), transmission electron microscopy (TEM) with a JEOL JEM-2200FS, and dynamic light scattering (DLS) with a Malvern Nano-ZS.

2.3 Determination of peroxidase activity and stability

To investigate the peroxidase-like activity of the H-Pt NPs, the catalytic oxidation of TMB in the presence of pre-determined concentrations of H_2O_2 was performed in a transparent 96-well plate as follows: a solution containing diverse kinds of nanomaterials including H-Pt NPs (20 μL, 0.1 mg mL^{-1}), the TMB substrate (20 μL, 0.5 mM), and H_2O_2 (20 μL, 100 mM) in a sodium acetate buffer (140 μL, 0.2 M, pH 4.0) was added into each well and incubated at RT for 3 min. After the reaction, the well-plate was used to capture color images. Spectrophotometric measurements were carried out in a scanning mode, or at 652 nm using a microplate reader (Synergy H1, BioTek, VT), after separating the nanomaterials from the mixtures in each well by centrifugation at 10 000*g* for 3 min.

The stability of the H-Pt NPs and HRP was evaluated in sodium acetate buffer (0.2 M, pH 4.0) and sodium phosphate buffer (0.2 M, pH 6.0), respectively, under static conditions at RT. First, the initial activity was determined. Subsequently, the residual activity of each sample was determined by measuring the absorbance at 652 nm as described above. The relative activity (%) was calculated by obtaining the ratio of the residual activity to the initial activity of each sample.

2.4 Preparation of antibody-conjugated H-Pt NPs

Antibody conjugated H-Pt NPs (Ab-H-Pt NPs) were prepared by facilitating the adsorption of the antibodies onto the surface of the H-Pt NPs. Antibodies against IgE (anti-IgE, Abcam) were used for conjugation. Briefly, 250 μL of H-Pt NPs (1.0 mg mL^{-1}) in DI water and 250 μL of anti-IgE (200 μg mL^{-1}) were mixed, and 500 μL of storage buffer (PBS solution (pH 7.4) containing 0.1% Tween-20 and 3% BSA) was added to the mixture to block nonspecific binding sites. The Ab-H-Pt NPs were incubated for 1 day at 4 °C with gentle shaking, then washed thrice

with PBS buffer and stored at 4 °C with their final concentration being 0.1 mg mL^{-1}.

2.5 H-Pt NP-based immunoassay to detect IgE on the ImmunoCAP system

For the determination of total IgE, each ImmunoCAP, which contained the antibody toward total IgE (anti-IgE) on its membrane, was inserted into each well of the 96-well plate, and 200 μL of washing buffer prepared as per the manufacturer's instructions was added to each well and incubated for 30 min to remove the coating of ImmunoCAP. After the plates were washed with the PBS solution, 200 μL of sample solutions containing human IgE were added to the ImmunoCAP-inserted wells and incubated for 1 h at RT to allow the antigen–antibody interaction. The reacted ImmunoCAP was then washed several times and further incubated with 200 μL of Ab-H-Pt NPs (0.1 mg mL^{-1}) for 2 h at RT. After thorough washings with the PBS solution, a color development reaction was carried out using 250 μL of development solution containing 0.5 mM TMB as the chromogen, with H_2O_2 (100 mM) in PBS solution (pH 4.0). After 5 min, the color-generating reaction was immediately stopped by removing the ImmunoCAP and transferring the reacted solution into a new 96-well plate; this was used to capture color images. Spectrophotometric measurements were carried out at 652 nm using a microplate reader (Synergy H1, BioTek, VT). For the determination of specific IgE, ImmunoCAPs containing the specific allergen on their membrane were used instead of ImmunoCAPs containing anti-IgE; the other procedures were performed as described previously.

In the experiments of HRP-based immunoassay to detect IgE on the ImmunoCAP system, an HRP-conjugated monoclonal antibody against human IgE from mice (0.2 mg mL^{-1}) was employed instead of Ab-H-Pt NPs, and the development solution containing 2 mM H_2O_2 was used instead of 100 mM H_2O_2. The other procedures were the same as those used for the immunoassay using the H-Pt NPs.

Diagnoses of allergies by determining the total or specific IgE levels using the H-Pt NP-based immunoassay were carried out with real blood serum samples, which were collected from local hospitals. Clinical serum specimens were prepared by centrifuging blood samples at 2000*g* for 10 min at 4 °C, followed by the collection of the supernatant. The collected serum samples were stored at −80 °C until use. The actual concentrations of IgE in clinical blood serum samples were determined by standard ImmunoCAP procedures using Phadia 250 instrumentation (Thermo Scientific). To assess the reliability and reproducibility of this method for the determination of total and several specific IgE levels, three parallel assay experiments were performed, and their within-assay variation was determined. The recovery rate [recovery (%) = measured value/actual value × 100] and coefficient of variation [CV (%) = SD/average × 100] were determined to evaluate the precision and reproducibility of the assays.

3. Results and discussion

3.1 Construction of H-Pt NPs for colorimetric determination of IgE

As an effective nanozyme to replace the signaling enzyme, β-galactosidase, in the ImmunoCAP diagnostic system, H-Pt NPs, which were proven to exhibit a great ability for catalyzing redox reactions due to the high catalytic power of the Pt element and their large surface area, were synthesized following previous reports.[26,27] The synthesized H-Pt NPs were monodispersed with a narrow size distribution of a diameter of ~30 nm, and had rough, porous, and extremely large surfaces, which can be utilized for the catalytic events (Fig. 1). Relevant antibodies against human IgE were subsequently conjugated to the surface of H-Pt NPs *via* a simple physical adsorption method. These Ab-H-Pt NPs bind to the target human IgE in a sample *via* a mechanism similar to that of the sandwich-type immunoassay. Finally, when H_2O_2 and the selected peroxidase substrate, TMB, are applied, the H-Pt NPs catalyze the quick oxidation of TMB to generate a blue-colored product (Fig. 2).

3.2 Investigation of the peroxidase-like activity of H-Pt NPs

We first examined the peroxidase activity of H-Pt NPs by performing the representative peroxidase-catalyzed TMB oxidation reactions in the presence of H_2O_2, while monitoring the responses by using absorption spectroscopy (Fig. 3). The results of this experiment show that the H-Pt NPs exhibit much more intense TMB oxidation rates than the other nanozyme systems that we have previously developed, such as bare Pt NPs, bare Fe_3O_4 magnetic nanoparticles (MNPs), aminopropyl-triethoxysilane-modified MNPs (APTES-MNPs), mesoporous silica particles entrapping 40 wt% MNPs (MSU_40%), and graphene oxide (GO).[28–31] The extremely high peroxidase activity of H-Pt NPs was also previously demonstrated through its comparison with the activity of horseradish peroxidase

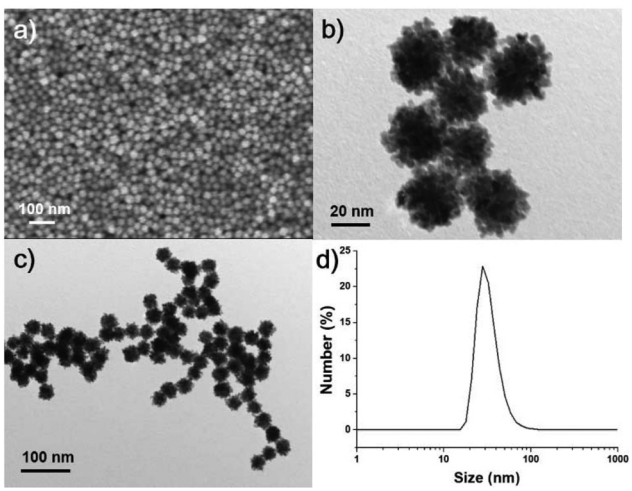

Fig. 1 Characterization of H-Pt NPs. (a) SEM and (b, c) TEM images of H-Pt NPs, and (d) hydrodynamic size distribution of H-Pt NPs by DLS.

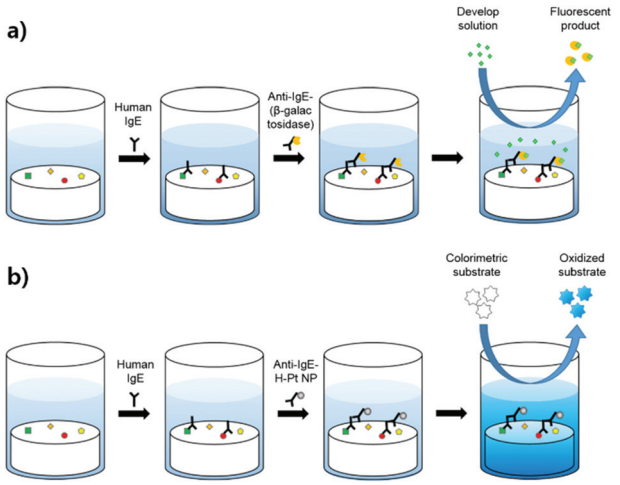

Fig. 2 Schematic illustration of the (a) conventional and (b) peroxidase-mimicking H-Pt NP-based ImmunoCAP diagnostic systems for the quantification of human IgE.

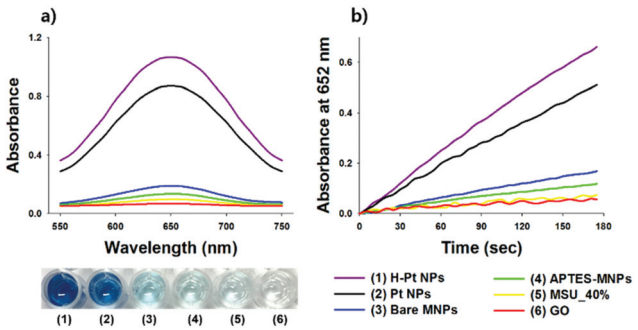

Fig. 3 (a) Absorption spectra and (b) their corresponding images from the representative color-generating reaction of TMB in the presence of H_2O_2 using diverse nanozymes, including H-Pt NPs. (b) Time-dependent absorbance changes at 652 nm in the presence of the nanozymes.

(HRP), an enzyme that is widely used in immunoassays for signal amplification.[26] Observations made in studies on the effects of the initial concentrations of TMB, H_2O_2, and reaction time on the catalytic activity of H-Pt NPs showed that 0.5 mM TMB, 100 mM H_2O_2, and 5 min reaction time were the ideal assay conditions which were employed in further analyses (Fig. S1†). H-Pt NPs were also highly stable over a long incubation period at RT, whereas the HRP lost all of its activity within 1 day of incubation. This demonstrates the robust nature of H-Pt NPs, which facilitates their practical applicability (Fig. 4).

3.3 Colorimetric determination of IgE using an H-Pt NP-based ImmunoCAP assay system

The feasibility of the peroxidase-mimicking nanozyme, H-Pt NPs, to detect IgE was demonstrated by integrating them into the ImmunoCAP assay system, as a replacement for the natural signaling enzyme. The ImmunoCAP system was generally prepared by using β-galactosidase conjugated to antibodies

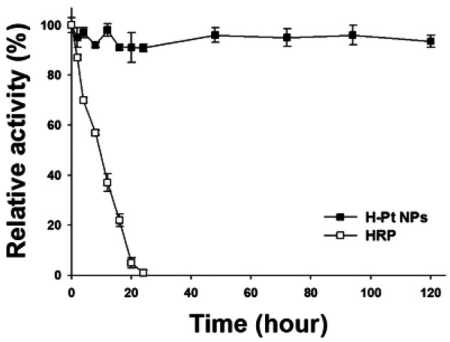

Fig. 4 Catalytic stabilities of the H-Pt NPs and HRP against incubation time at RT. The error bars represent the standard deviations derived from three independent measurements.

against human IgE, to assess the binding of IgE with specific allergens on the membrane of the ImmunoCAP. However, the intrinsic instability of β-galactosidase, which depends on the various environmental parameters including temperature, pH, and storage time, frequently produces false positive results, and limits its utilization during long-term operation or storage. The aforementioned unique superiorities of H-Pt NPs, including their high activity and stability, led us to hypothesize that they can efficiently replace β-galactosidase in the ImmunoCAP-based allergy detection system. In addition, the conventional ImmunoCAP assay based on β-galactosidase needs special instrumentation for the identification of fluorescent responses, while H-Pt NPs can oxidize versatile chromogenic substrates such as TMB, in the presence of H_2O_2, making the reaction procedures much more simple and convenient due to the ability of visual discrimination. Overall, we expect that this H-Pt NP-based ImmunoCAP diagnostic assay for allergies would enable convenient, reliable, and sensitive quantification of the target IgE at a lower cost in comparison with the conventional natural enzyme-based ImmunoCAP system.

Through the immunoassay procedures described in the Materials and methods section, H-Pt NPs conjugated to specific antibodies against total IgE or specific IgE were applied to the ImmunoCAP system using standard IgE samples, which were provided by manufacturers. Although various surface coatings on nanozymes have been reported to bring about a significantly reduced catalytic activity,[17–19] the current antibody conjugation protocol produces nanoparticles retaining greater than 80% of the original activity (Fig. S2†). We first performed an experiment to evaluate the specificity of the H-Pt NP-based assay to detect the target IgE. As shown in Fig. 5a and b, significant absorption intensities corresponding to the oxidized TMB were rapidly (within 5 min at RT) observed in the wells containing the target IgE molecules, whereas the wells containing negative control samples did not produce significant color changes. This confirms the specific binding of the Ab-H-Pt NPs to the target IgE molecules present on the membrane of the ImmunoCAP system. When the concentrations of both total IgE and specific IgE increased, the

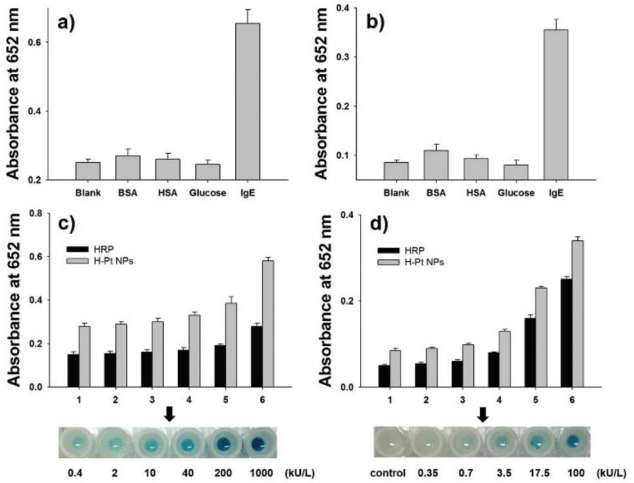

Fig. 5 Absorption intensities of well plates for the H-Pt NP-based ImmunoCAP assay to specifically detect (a) total IgE and (b) specific IgE, and absorption intensities of the blue color signal and their corresponding images for the immunoassay to detect (c) total IgE and (d) specific IgE at various concentrations by using H-Pt NPs and HRP. For (a) and (b), the employed concentrations of total and specific IgE are 2.42 μg mL^{-1} (1000 kU L^{-1}) and 0.242 μg mL^{-1} (100 kU L^{-1}), respectively, while those for negative control samples are all 10 μg mL^{-1}. The error bars represent the standard deviation derived from three independent measurements.

intensity of the blue color signal, corresponding to the oxidized TMB substrate, correspondingly increased, which was also detected by the naked eye alone (Fig. 5c and d). Most importantly, by the color signal development for 5 min, the level of the absorption intensity produced by the H-Pt NPs was higher than that produced by the HRP used in the conventional ELISA system; additionally, our H-Pt NP-based system was able to quantify the target IgE very rapidly within 5 min, whereas the conventional ELISA usually requires several tens of minutes for color signal development.[32] Based on the linear calibration plot, the limit of detection (LOD) for total IgE was determined to be as low as 0.25 kU L^{-1} in the dynamic linear range from 0.4 to 1000 kU L^{-1} (Fig. 6a). For specific-IgE, the LOD was determined to be as low as 0.17 kU L^{-1} in the linear range from 0.35 kU L^{-1} to 17.5 kU L^{-1} (Fig. 6b). These LOD and linear range values, which are associated with describing

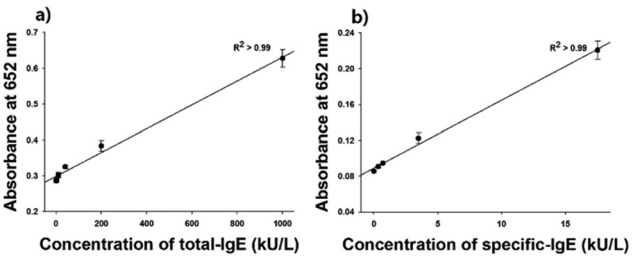

Fig. 6 Linear calibration plots of the H-Pt NP-based ImmunoCAP assay system for the quantification of (a) total IgE and (b) specific IgE.

the immunological determination of human IgE, are among the best results obtained so far (Table S1†).[33,34]

3.4 Determination of IgE using clinical blood samples

Finally, we evaluated the actual diagnostic ability of the H-Pt NP-based ImmunoCAP assay system by using real human blood samples that contain diverse levels of total IgE or four kinds of specific IgEs corresponding to d1 (*Dermatophagoides pteronyssinus*), d2 (*Dermatophagoides farinae*), e1 (cat dander), and e5 (dog dander). The actual levels of total or specific IgE in the serum samples were first determined by standard ImmunoCAP procedures using Phadia 250 instrumentation (Thermo Scientific). As a result, total and specific IgE levels were quantified with excellent precision, yielding CVs in the range of 3.6–8.3% and 2.3–8.3%, with recovery rates varying from 95.4 to 101.3% and from 95.5 to 102.9%, respectively (Tables 1 & 2), verifying the excellent reproducibility and reliability of the method. The results demonstrate that the H-Pt NP-based ImmunoCAP assay system for the determination of IgE could be a promising analytical platform capable of diagnosing allergies in clinical settings.

Table 1 Detection precision for H-Pt NP-based total IgE detection from real clinical serum samples

Sample no.	Expected (kU L^{-1})	Average	SD	CV (%)	Recovery (%)
1	496	483.2	37.70	7.8	97.4
2	471	471.5	30.63	6.4	100.1
3	920	896.4	32.99	3.6	97.4
4	125	126.6	9.43	7.4	101.3
5	351	339.9	28.28	8.3	96.8
6	430	421.5	16.49	3.9	98.0
7	415	406.5	18.45	4.6	97.0
8	117	111.6	7.07	6.3	95.4
9	68.4	66.6	4.71	7.1	97.4
10	194	186.6	9.43	5.1	96.2

Table 2 Detection precision for H-Pt NP-based specific IgE detection from real clinical serum samples

Sample no.	Allergen	Expected (kU L^{-1})	Average	SD	CV (%)	Recovery (%)
1	e5	59.5	60.5	1.66	2.7	101.7
2	d1	4.08	4.1	0.26	6.4	100.5
	d2	9.96	9.7	0.70	7.2	97.4
3	d1	29.9	29.5	1.79	6.1	98.7
	d2	26.0	26.2	1.32	5.0	100.8
	e1	>100	118.4	6.22	5.3	N.D.
	e5	7.78	8.0	0.30	3.8	102.8
4	d1	20.6	21.2	1.47	6.9	102.9
	d2	16.6	16.8	0.66	3.9	101.2
	e5	38.2	36.5	3.04	8.3	95.5
5	d1	25.1	25.4	1.14	4.5	101.2
	d2	62.1	60.8	1.37	2.3	97.9

d1: *Dermatophagoides pteronyssinus*, d2: *Dermatophagoides farinae*, e1: cat dander, e5: dog dander, and N.D. indicates "not determined".

4. Conclusion

We herein developed an efficient colorimetric immunoassay for detecting and determining the concentration of IgE based on the high peroxidase activity of H-Pt NPs. The results of this investigation demonstrate that the H-Pt NP-based ImmunoCAP assay system has high sensitivity, stability, and precision for the detection of the corresponding IgE. Since the current system enabled the rapid visual detection of the target IgE, it should find practical applications in facility-limited or point-of-care testing environments, without the addition of natural enzymes. Because the H-Pt NPs can rival natural signal enzymes due to their high activity, stability, robustness, and low cost by a facile scale-up, the analytical platform can be further extended to many potential applications as a convenient diagnostic tool for clinical analysis.

Conflicts of interest

The authors declare no financial or commercial conflict of interest.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (Ministry of Science, ICT & Future Planning (MSIP)) (NRF-2017R1C1B2009460) and by the Gachon University Gil Medical Center (grant number 2014-22).

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