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Nanoparticles rapidly assess specific IgE in plasma

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Abstract

Allergy is the sixth leading cause of chronic disease in the world. This study demonstrates the feasibility of detecting allergy indicators in human plasma, noninvasively, at the point of care and with a comparable efficiency and reduced turnaround time compared with the gold standard. Peanut allergy was utilized as a model due to its widespread occurrence among the US population and fatality if not treated. The detection procedure utilized magnetic nanoparticles that were coated with an allergen layer (peanut protein extract). Peanut immunoglobulin E (IgE) was detected in concentrations close to the minimum detection range of CAP assay. The results were obtained in minutes compared with the CAP assay which requires more than 3 h.

(Some figures may appear in colour only in the online journal)

1. Introduction

Allergy is the sixth leading cause of chronic disease in the world [1]. It is estimated that 1% children between age 3–4 years and 1.3% of adults is affected by peanut allergen [2]. Severe and life threatening adverse reactions to food allergens are associated with immunoglobulin E (IgE) hypersensitivity leading to anaphylactic reaction [3].

Rapid, sensitive, specific, easy to use technology for the assessment of allergy markers is of increased demand for clinicians and health care providers. Nanotechnology based assessment for proteins and cell diagnostics have provided clinicians and health care providers with the advantage of detecting targeted markers using a smaller sample volume in shorter times at the point of care [4]. The advancements of nano-based bio-diagnostics employed the ability of functionalized classes of nanomaterials that are able to target, label, and isolate biomarkers from heterogeneous biological samples [5]. In this study we present a nano-based diagnosis for peanut allergen. Principally the methodology disclosed in this work is applicable to IgE mediated allergy.

The most severe and life threatening adverse reactions to foods are associated with IgE-mediated hypersensitivity [6]. An allergic reaction occurs due to an abnormal immune

system response to specific antigens or proteins present in food. There are two phases involved in an allergic reaction, an initial phase called ‘sensitization’ occurs when a susceptible individual produces IgE antibodies against a specific allergen present in food. The second phase is ‘elicitation’, which is a robust allergic reaction in the sensitized individual on subsequent re-exposure to the same allergen. During this phase the allergen binds to IgE molecules on basophils and mast cells, and activates these cells, causing the release of inflammatory molecules, such as histamine, into the circulation [7].

The clinical manifestations of food allergies range from mild irritations to life threatening respiratory distress, asthma, anaphylactic episodes, and shock. Symptoms occur within a few minutes to hours after consumption of the allergen present in food and generally progress on a continuum from mild to severe reaction [8, 9].

Peanut allergy poses an enormous problem and has been shown to be the third most common food allergy in young children, and the most common food allergy in older children, adolescents, and adults. Furthermore, it has been implicated as an allergen most capable of causing severe, life threatening, and fatal allergic reactions [10–13]. The severity and persistence of peanut allergy, and the growing prevalence

of peanut allergies, emphasizes the need for the development of assays that can rapidly detect and diagnose the onset of an allergic reaction in an individual [14, 15].

In clinical practices, skin prick test (SPT), *in vitro* IgE measurements such as the radio allegro sorbent test (rast), ELISAs, or fluorescence enzyme assays (CAPTM-FEIA) are generally used for the detection of specific IgE. The CAP test is considered the gold standard for specific IgE tests. The drawback of the CAP test is that it requires a controlled laboratory environment with sophisticated equipment and trained personnel. In addition to that all the assays mentioned are time consuming and require a large quantity of plasma sample [15, 16]. The average time reported for each of these tests varies from a few hours to days to render results. Hence there is an urgent need to develop reliable assays to detect the onset of allergies in a rapid, sensitive and less invasive way that does not involve highly trained personnel and expensive equipment, making the assay cost effective.

Magnetic sandwich assay was earlier performed using magnetic polyacrylamide–agarose beads labeled with anti-IgE antibodies for human IgE separation. The agarose beads were micron to millimeters in size leading to a detection limit above 2 KU l^{-1} [17]. More recently IgE detection using a magnetic nanoparticle core shell in a microchip device pushed the detection limit to $1 \text{ ng ml}^{-1} \approx 1 \text{ KU l}^{-1}$ [18], however a sandwich assay with multiple antibodies is still needed. In this work we functionalized magnetic nanoparticles with a coat of peanut allergen [19]. These functionalized magnetic nanoparticles were then used to evaluate the presence of peanut IgE in plasma samples. The advantage of using functionalized magnetic nanoparticles lies in its ability to be separated from the solution by an external magnetic field. The magnetic assay developed in this work showed an ability to reliably detect up to 0.1 KU l^{-1} of allergen in a plasma solution in 5 min.

2. Methods

2.1. Synthesis of iron oxide particles

Iron oxide particles were synthesized in-house in three main steps as described by Chatterjee *et al* [20]. Briefly, ferrous chloride and ferric chloride were co-precipitated by sodium hydroxide. The mixture of ferrous chloride and ferric chloride was then mixed in a molar ratio of 1:2 in deionized water at a concentration of 0.1 M iron ions. Next, a highly concentrated solution of sodium hydroxide (10 M) was added and the solution was stirred continuously for the ions to be co-precipitated. The solution with the precipitates was stirred at high speed for 1 h at 20°C and subsequently heated to 90°C for 1 h with stirring. The iron oxide dispersion was then sonicated for 10 min at 50% amplitude using an ultrasonic homogenizer. Next, the ultrafine magnetic particles obtained were peptized by nitric acid (2 M). The precipitate was washed repeatedly with deionized water, filtered, and dried in vacuum to obtain fine iron oxide particles. The particle size distribution was tested using transmission electron microscopy and was found to be

26.6 nm. The charge of the particles was measured using a Zetasizer (Malvern Instruments, UK) and was found to be anionic.

2.2. Peanut protein extraction

10 g of peanuts were ground in 100 ml of tris buffer (pH 8.2) stirred for 2 h at room temperature. The aqueous fraction was then collected by centrifugation at $10000g$ at room temperature for 15 min. Extract was then stored at -20°C until use. The protein content of the sample was determined by bicinchoninic acid protein assay (BCA) [21].

2.3. BCA assay

A BCA assay kit was purchased from Thermo Scientific Cat. No. 23227. It was prepared and used as directed by the manufacturer; reagents A and B were mixed in a ratio of 50:1. Bovine serum albumin (BSA) (2 mg ml^{-1}) was serially diluted in a micro-titer plate followed by the addition of $180 \mu\text{l}$ of BCA reagents mixture. The plate was covered and incubated in the dark for 30 min at room temperature. Absorbance was measured at 562 nm using a Biotek Plate Reader. Protein quantification was done by plotting a standard curve from bovine serum albumin which was provided with the BCA assay kit with 2 mg ml^{-1} concentration. Total protein concentration of raw peanut extract was found to be 9.35 mg ml^{-1} and the total protein concentration of raw peanut extract coated on magnetic nanoparticles was found to be $189.6 \text{ ng } \mu\text{g}^{-1}$ of magnetic nanoparticles (MNPs). The coated peanut extract was computed based on the protein concentration measurement of the BCA assay for the supernatants after magnetically removing the nanoparticles from the solution.

2.4. Coating of magnetic particles with peanut extract

10 mg of magnetic particles were added in 1 ml of 100 mM 1X PBS and was sonicated for 2 min. From this solution $430 \mu\text{l}$ were added to $350 \mu\text{l}$ of raw peanut extract having a total protein concentration of 9.35 mg ml^{-1} (obtained by BCA assay). To this solution $220 \mu\text{l}$ of 1X PBS was added. The mixture was rotated at 360° rotation for 4 h at room temperature. The antigen bound (extract coated) magnetic particles were separated magnetically and washed with 100 mM 1X PBS buffer twice. The coated particles were resuspended in PBS buffer to obtain a final concentration of 2 mg ml^{-1} and were stored at 4°C . To ensure the coating, the total protein coated on nanoparticles was measured by doing a BCA assay, SEM imaging and zeta potential were also measured by using Nanosizer equipment. The coated particle size was measured from the SEM image and was found to be 31.18 nm.

2.5. Plasma IgE samples

Plasma samples of four patients were purchased from Plasma Lab International (Norton Avenue, USA). The samples have CAP values of: 15, 50.7, 67.4, 69.8, and 112.2 KU l^{-1} .

2.6. Conjugate IgE

Monoclonal anti-human IgE–alkaline phosphatase antibody produced in mouse was purchased from Sigma Aldrich Cat. No. A3076 and was stored at 4 °C.

2.7. Zeta potential

The zeta potential of magnetic nanoparticles was measured by a Nanosizer (Malvern Instruments, USA).

2.8. Magnetic ELISA

50 μl of 2 mg ml^{-1} MNP coated with peanut protein was taken in a 0.5 ml Eppendorf tube that was filled with 4% nonfat milk as blocking buffer and incubated at room temperature for 15 min. MNPs were separated by a 0.25 T magnetic separation rack and were washed with 1X PBS thrice. Then 200 μl of plasma sample was added to each tube and incubated for 2 h, 15 or 5 min at 37 °C; after incubation MNPs were washed three times with 1X PBS in a magnetic separation rack. After washing, 50 μl of conjugate IgE was added to nanoparticles and incubated for 1 h or 15 min at 37 °C. After incubation, nanoparticles were washed three times with PBST buffer. Following this, 100 μl of pNPP (p-nitrophenyl phosphate), which is a substrate for Alkaline phosphatase purchased from Invitrogen Cat. No. 00-2212 was added to MNPs and incubated for 15 min in the dark for color development. After incubation, 100 μl of stop solution was added and the absorbance was read at 405 nm by a Biotek plate spectrophotometer. A negative control contained nanoparticles coated with antigen except for the plasma IgE. However, secondary antibodies were also added, incubation time and volume was similar as used for sample experiments.

3. Results

3.1. Physical properties of the MNPs

Figure 1 shows the physical properties of the MNPs. The particles do not possess remanent magnetization at room temperature while they have remanent magnetization at 5 K, a typical behavior of a superparamagnetic material. The hysteresis curve was obtained by placing 10 mg of particles in a SQUID magnetometer (Quantum Design, USA). The particles' size and morphology were obtained by a Hitachi 4800 SEM machine for both naked and coated particles. Coating of nanoparticles with peanut extract results in net negative charge on nanoparticles compared to naked nanoparticles. The charge was measured by a Nanosizer (Malvern Instruments, USA). These findings from the zeta potential indicated the protein coating on the surface of nanoparticles. This interaction between protein and nanoparticles may be due to the opposite charge because nanoparticles exhibit positive charge and protein has a net negative charge (as shown in figure 2). A further coated layer was confirmed by SEM and BCA assay for total protein concentration. The coated particles' diameter increased by 17% compared to naked particles, as confirmed by SEM.

3.2. Magnetic ELISA of plasma samples with different CAP values

IgE plasma samples having CAP values 15, 50.7, 67.4, and 112.2 KU l^{-1} were used as supplied by Plasma International Lab for magnetic ELISA assay. The allergens coated on the magnetic particles associate with the IgE in the plasma by a 2 h incubation. With an external magnet the conjugated particles (allergen and IgE) are separated from the plasma solution. The complexing between the allergen and the captured IgE from plasma constitutes the primary conjugation of the magnetic immunoassay. The secondary conjugation in the assay uses monoclonal conjugate IgE labeled with alkaline phosphatase (AKP) to measure the captured IgE from plasma. The monoclonal conjugated IgE was diluted in PBS in a ratio of 1:1000 and 50 μl were added to the primary conjugate and incubated for 1 h. The magnetic immunoassay showed significant difference for all plasma samples as compared with negative control (without plasma), as shown in figure 3.

Two different dilutions of conjugate IgE, 1:1000 and 1:6000, were used to evaluate the sensitivity of the assay. Both of the dilutions were significantly different from their respective controls. The 1:1000 dilution absorbance value was 33% more than that of 1:6000. Based on the economical consideration and sensitivity we selected a 1:1000 ratio in all of the magnetic ELISA assay.

3.3. IgE standard curve

The concentration of IgE in an unknown plasma sample provided by Plasma International Lab was obtained by a standard curve which was plotted by taking the plasma sample with a CAP value 69.8 KU l^{-1} (that was also obtained from Plasma International Lab). It was diluted in PBS buffer in the following concentrations as 1.745, 3.49, 5.235, 6.98, and 8.725 KU l^{-1} . Magnetic ELISA was performed for these dilutions and for the unknown sample by the method as described in this study. The value of the unknown sample was obtained from the calibration graph shown in figure 4. The concentration of the unknown sample came to 17.17 KU l^{-1} .

3.4. Sensitivity of magnetic ELISA assay

In this experiment the sensitivity of the magnetic assay was checked by diluting the plasma sample with a CAP value of 67.4 KU l^{-1} to lower concentrations. Magnetic assay was performed with different concentration of IgE plasma samples as follows: 0.1, 1, 5, 10, 20, 40 KU l^{-1} . Absorbance was measured at 405 nm as shown in figure 5, there was a significant increase in absorbance values by increasing the concentration of IgE plasma samples.

Primary conjugation (Plasma + MNP-Allergen) incubation time was varied for different time steps ranging from 5 min, 15 min, or 2 h and we similarly varied the incubation time for all secondary conjugation (IgE + AKP) for 5 min, 15 min, or 2 h respectively. Data showed that a higher sensitivity is obtained as the incubation time is increased and also showed that at 2 h incubation all the IgE dilutions were

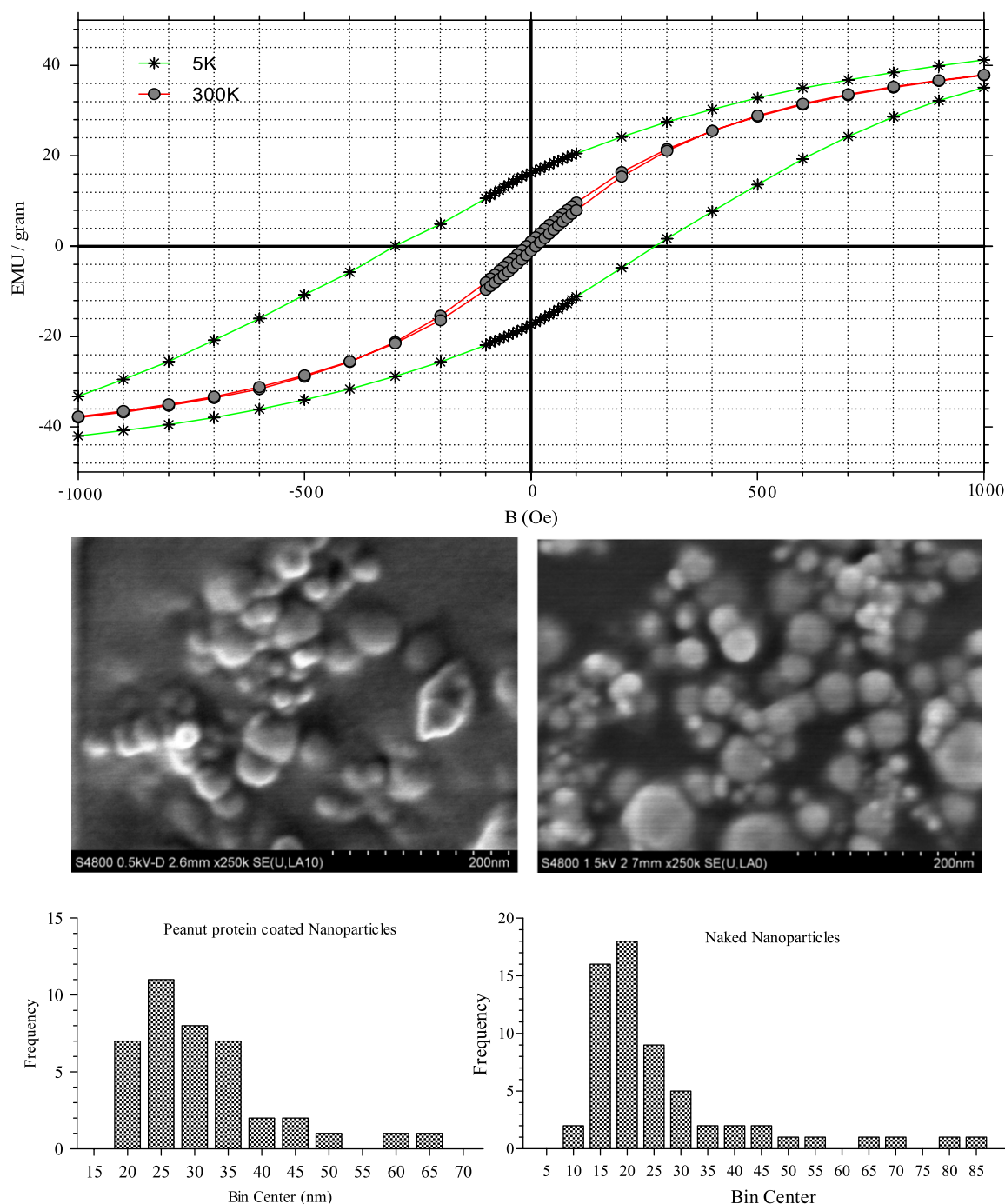


Figure 1. Nanoparticles SEM image, size distribution, and magnetic hysteresis. The particles are spherical with average diameter of 26.6 nm. The hysteresis loop at 5 K disappears at 300 K, a typical behavior of a superparamagnetic material.

detected significantly while at 15 min and 5 min incubation time, the minimum concentrations detected were 5 KU l^{-1} and 40 KU l^{-1} respectively (as shown in figure 5).

Furthermore in order to increase the sensitivity at 5 min or 15 min another experiment was carried out, we increased the amount of allergen on the MNPs from 189 to $500 \text{ ng } \mu\text{g}^{-1}$, as shown in figure 6. For 5 min incubation, an increase of 66% in signal intensity was observed when 500 ng of allergen per microgram of MNP was coated as compared to allergen $189 \text{ ng } \mu\text{g}^{-1}$ of MNP. There was also a 42% increase in signal intensity when the incubation time was set for 15 min.

Similarly $500 \text{ ng } \mu\text{g}^{-1}$ of allergen concentration on MNP was used for the 5 min assay as shown in figure 7, in which the lowest concentration of 0.1 KU l^{-1} was detected (figure 7). Hence, the presented technology is able to provide rapid detection of IgE in minutes compared to the gold standard.

4. Discussion

The study presented here used antigen coated magnetic nanoparticles to develop a novel ELISA technique for the detection of specific IgE from peanut allergic patients.

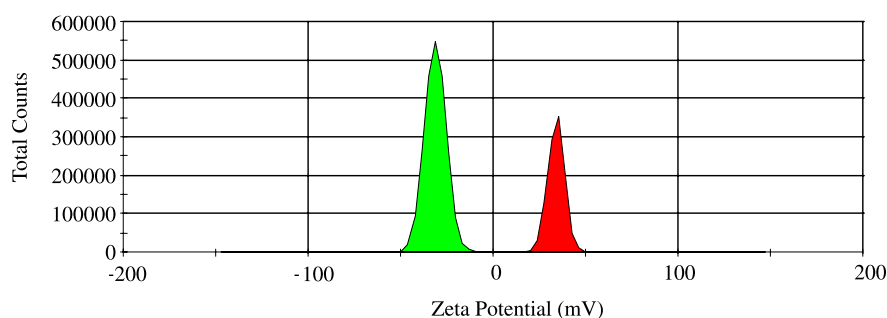


Figure 2. Zeta potential measured by a nanosizer, in this graph the right peak corresponds to naked magnetic nanoparticles and the left peak corresponds to magnetic nanoparticles coated with peanut protein. The inset shows the particle distribution and SEM image of both naked and coated particles.

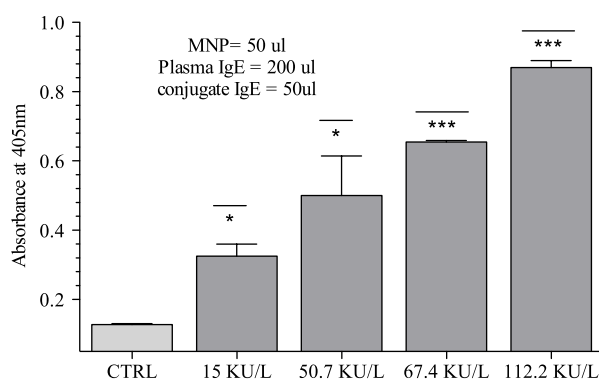


Figure 3. 50 μ l of nanoparticles was used in 200 μ l of plasma that was conjugated with 50 μ l of IgE. Each bar = mean $\pm$ STDEV; P value ≤ 0.001 is ***, P value ≤ 0.05 is *.

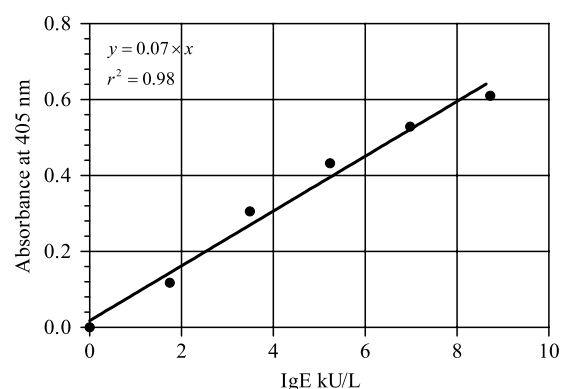
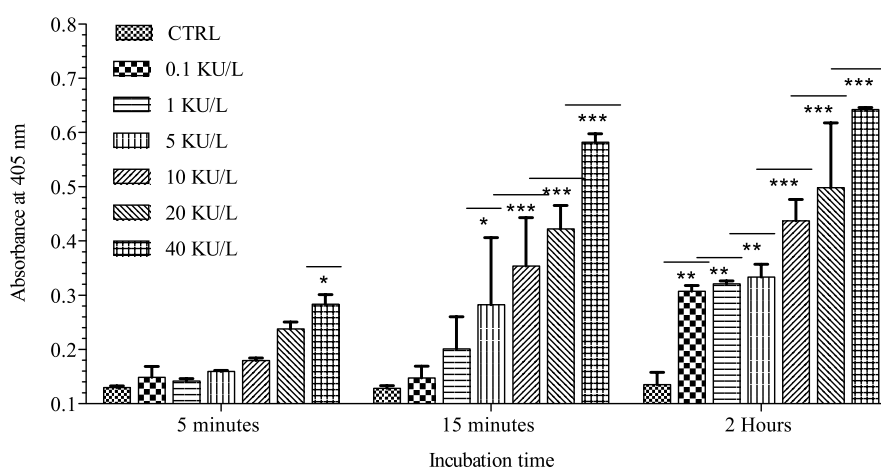


Figure 4. IgE calibration curve.

Measurements of allergen specific IgE levels are important in the diagnosis of the severity of the allergy. Techniques like the skin prick test, radioimmunoassay, and pharmacia CAP Immunoassay are well known techniques for the detection of plasma/serum IgE. The immunoCAP specific IgE blood test has been cleared by the FDA for quantitative measurements of specific IgE. Our study includes the plasma samples

of five patients allergic to peanut, obtained from Plasma International Lab with known IgE values of the standard ImmunoCAP Assay. In this technique we used magnetic iron oxide nanoparticles because of the three major characteristics they possess which include: (1) magnetism, which allows easy separation as compared to any other technique; (2) charge on magnetic nanoparticles, provides binding forces for positively charged ligand; (3) surface area, magnetic nanoparticles being



Each bar represents Mean $\pm$ STDEV, $P < 0.05$ = *, $P < 0.01$ = **, $P < 0.001$ = ***,

Figure 5. The absorbance at different incubation times for allergic plasma samples. The control is naked nanoparticles without plasma.

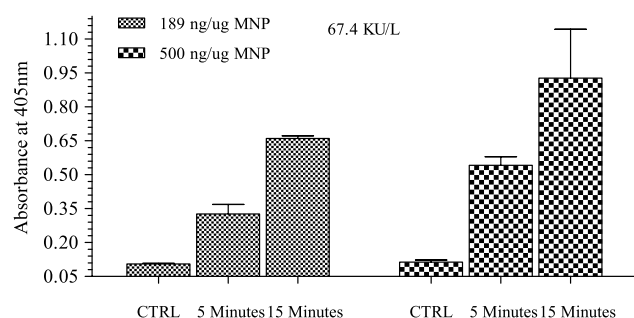


Figure 6. An increase in detection sensitivity by increasing the allergen concentration. The control is for coated nanoparticles without plasma.

spherical and smaller in diameter, e.g. 10–15 nm in size, possess maximum surface area. A combination of all three features of magnetic nanoparticles leads us to develop simple, reliable, time saving, and cost effective magnetic ELISA.

Our study does not include the clinical population. The aim of this research was to nurture a magnetic ELISA technique for IgE detection. In this regard we obtained plasma samples from unknown patients with known IgE values and those IgE values were further diluted in lower ranges until 0.1 KU l⁻¹. Our technique was able to detect all concentrations from 100 to 0.1 KU l⁻¹, to ensure the antigen coating on nanoparticles the zeta potential was measured for naked and antigen coated nanoparticles as shown in figure 1 and total protein assay confirmed the antigen coating on nanoparticles. Our ELISA method showed positive results for known IgE values as compared to negative control, as shown in figure 3. Further modifications were made to optimize the problem areas such as non-specific binding, and two different concentrations of IgE dilutions were tested. After optimization a standard curve was obtained from one of the known samples with IgE 69.8 KU l⁻¹ to identify the concentration of the unknown sample and our method showed an IgE concentration of 12.603 KU l⁻¹. This result was confirmed by Plasma International with CAP results of IgE concentration 15.7 KU l⁻¹.

By successfully detecting IgE from plasma samples with our technique, we focused on the sensitivity of this system. One of the samples with known IgE of 67 KU l⁻¹ was diluted to lower values as shown in figure 7 where 0.1 KU l⁻¹ was significantly positive compared to the negative control. According to WHO IgE reference preparation 75/502, the minimum detection limit is 0.3 KU l⁻¹, so to further increase the sensitivity of our magnetic ELISA technique we optimized time and volume in order to detect the required minimum concentration. In the ImmunoCAP assay and other techniques the minimum time duration is more than 3 h, however our improved magnetic ELISA is able to perform detection for as small as 0.1 KU l⁻¹ in minutes.

5. Conclusions

A magnetic immunoassay composed of magnetic particles coated with allergen as primary conjugate was evaluated on

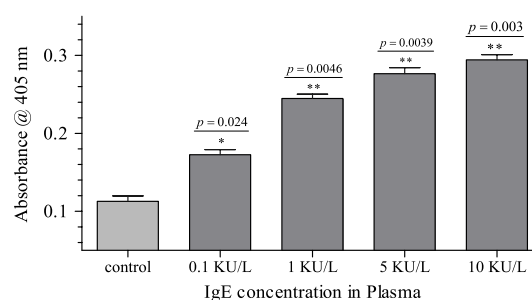


Figure 7. Detection of the lowest concentration by a 5 min assay. The control is for coated nanoparticles without plasma.

allergic human plasma samples. The assay was functionalized for testing peanut specific IgE. The assay was able to detect dilutions up to 0.1 KU l⁻¹. The assay was able to produce results in minutes. The assay sensitivity can be further improved by increasing the concentration of the allergen on the magnetic particles. The assay is scalable to other IgE mediated allergens and the test kit is basically a set of test containers, a magnet, and a spectrophotometer.

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