

Detection of Peanut-Specific IgE using Functionalized Nanoparticles

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ABSTRACT

The diagnosis of patients with clinically reactive food allergies is extremely crucial and remains a challenge for all allergists. The current technology available to diagnose the presence of peanut-specific immunoglobulin E (IgE) are highly invasive, expensive, time consuming and need trained personnel and specialized equipment for the test to be conducted. In this study we present a new technique for detecting the presence of peanut specific IgE by coating allergens on magnetic nanoparticles. Upon the isolation of the peanut specific IgEs from the sample, a colorimetric detection is utilized to assess the severity of the allergy. To evaluate the feasibility of the new technique, the functionalized particles were utilized to detect the presence of peanut specific IgE in 50 µl plasma of an allergic individual using only 100 µg of functionalized nanoparticles. Other evaluations were conducted in spiked plasma samples. The selectivity and sensitivity of the developed assay was highly specific for the peanut-specific IgE in plasma and more sensitive than conventional ELISA.

Keywords: food allergy, magnetic assay, nanoparticles, peanut allergy

Abbreviations: ELISA, Enzyme-linked immunosorbent assay; IgE, immunoglobulin E; SPT, skin prick test

INTRODUCTION

The National Institute of Allergy and Infectious Disease (NIH 2007) reported allergy as a major cause of illness in the United States, effecting millions of Americans and compromising their daily life. The National Center for Health Statistics reported in 2007 that three million US children under the age of 18 years (3.9%) have food or digestive allergy and the food allergy rate among all children younger than 18 years was 18% higher than in 1997. During the 10-year period 1997-2006, food allergy rates increased significantly among both preschool-aged children and older children (Branum and Lukacs 2007).

Severe food related allergic reactions result in an estimated 30,000 emergency room visits, 2,000 hospitalizations and 150 deaths annually (Sicherer *et al.* 2004). Furthermore, clinical data and surveys have observed an increase in the prevalence of allergy, including food allergy, over the years (Grundy *et al.* 2002; Sicherer *et al.* 2003; Branum and Lukacs 2007).

The most severe and life threatening adverse reactions to foods are associated with immunoglobulin E (IgE)-mediated hypersensitivity (Zarkadas *et al.* 1999; Jackson 2003). An allergic reaction occurs due to an abnormal immune system response to specific antigens or proteins present in food (Sampson 1999). 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 of the same allergen. During this phase the allergen binds to IgE molecules on basophiles and mast cells, and activates these cells causing the release of inflammatory molecules, such as histamine, into circulation.

The clinical manifestations of food allergies range from mild irritations to life threatening respiratory distress and shock. Symptoms occur within few minutes to hours after

consumption of the allergen present in food and generally progresses on a continuum from mild to severe reaction (Sampson *et al.* 2005).

Peanut allergy poses an enormous problem and has been shown to be the third most common food allergy in young children (Bock 1987), and the most common food allergy in older children, adolescents and adults (Sicherer *et al.* 1999; Branum and Lukacs 2007). Furthermore, it has been implicated as an allergen most capable of causing severe, life-threatening and fatal allergic reactions (Sampson *et al.* 1992; Bock *et al.* 2001). The severity and persistence of peanut allergy, and the growing prevalence of peanut allergies (Sampson 2002), emphasizes the need for the development of assays that can rapidly detect and diagnose the onset of an allergic reaction in an individual.

Currently there are three major approaches being used in the diagnosis of allergies; they are 1) Skin test or skin prick test (SPT), 2) Assays of serum IgE levels and 3) Histamine release test. The major drawbacks of these tests were that they may result in false negative or positive results, low sensitivity, and are highly invasive. Additionally, they require the detection to be done by trained personnel in controlled environments, which results in a considerable increase in cost. Hence there is an urgent need to develop reliable assays to detect the onset of food allergies in a rapid, sensitive and less invasive way that does not involve highly trained personnel and expensive equipment, making the assay cost effective.

Thus, the objective of this study was to develop a novel assay using nanotechnology as a platform to detect the presence of peanut specific IgE in plasma. In this study we choose to obtain plasma from sensitized individuals as they are at maximum risk of experiencing life threatening symptoms when exposed to peanut allergens. Based on the data from this study, we report that the magnetic assay developed using nanoparticles (NPs) that were functionalized with peanut protein extract was successfully able to detect peanut specific IgE in plasma. Additionally, when the sensi-

tivity of magnetic assay was compared to that of a traditional ELISA, magnetic assay was found to be nearly 2-fold more sensitive. Moreover, this degree of sensitivity was achieved by using only 100 μg of NPs and 50 μl of plasma.

EXPERIMENTAL METHODS

This study reports the feasibility of utilizing allergen-coated magnetic NPs to evaluate the severity of allergic attack.

Synthesis of iron oxide nanoparticles

Iron oxide NPs were synthesized in house in three main steps as described by Chatterjee *et al.* (2003). Briefly, ferrous chloride and ferric chloride was co-precipitated by sodium hydroxide. The mixture of ferrous chloride and ferric chloride were 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 in a high speed for 1 hr at 20°C and subsequently heated to 90°C for 1 hr 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 a transmission electron microscopy and was found to be 9 ± 2.5 nm. The charge of the particles was measured using a Zetasizer (Malvern Instruments, UK) and was found to be anionic.

Functionalization of nanoparticles

The produced NPs were functionalized by coating them with a positively charged polymer, polyethylenimine (PEI). The average thickness of the coat as measured by scanning electron microscope (Hitachi S-4800) imaging was 17 nm. The charge on the coated particles was verified by measuring zeta potential with the Zetasizer, nano series (Malvern, UK). The zeta potential of coated NPs was found to be 6.04 mV. The positively coated particles were then coated with whole peanut extract, by incubating the particles and extract in a 1: 1 ratio for 3 hrs at 37°C. The peanut extract was obtained by procedure outlined in (Koppelman *et al.* 2001). The coated particles were then magnetically separated from the peanut extract using a permanent magnet. The supernatant was collected and the particles, left behind, were washed five times with phosphate buffered saline (PBS), pH 7.4. After the last wash the particles were suspended in PBS to have the needed concentration of NPs/ μl of PBS. The coating on the particles was verified by three independent methods. Firstly, the zeta potential of the particles was measured before and after coating with the peanut extract. The zeta potential changed from (6.04 mV) on the peanut uncoated particles to a negative potential (-10.12 mV) after coating with the peanut extract. Secondly, the protein concentration of the peanut extract was determined, by Bicinchoninic acid (BSA) kit (Pierce, Thermo Scientific, IL), before and after the particles were coated with peanut extract. A decrease in protein concentration of the peanut extract after the NPs were coated was observed. The initial protein concentration of the extract was 4.88 $\mu\text{g}/\mu\text{l}$, however after coating the NPs the protein concentration of the extract decreased to 2.79 $\mu\text{g}/\mu\text{l}$. These data suggest that the difference in protein concentration was the amount coated onto the NPs. Thirdly, protein concentration of the coated and uncoated particles was determined by BSA assay. The protein concentration of the coated particles was found to be 254 ng/ μg of particle as compared to 13 ng for the uncoated particles. Taken together, these results strongly indicated that the NPs were successful coated with the peanut extract.

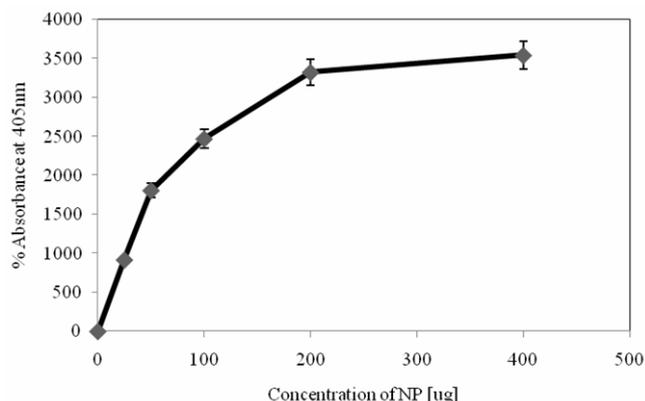


Fig. 1 Determining the amount of functionalized particles needed to detect peanut-specific IgE. Different concentrations of functionalized NPs were incubated with a fixed volume of an allergic plasma. The assay was carried out as described in the results section. The absorbance obtained was plotted relative to the concentration of the particles. The absorbance first increased with increasing concentration of the particles from 25 to 200 μg and then stabilized at 400 μg .

RESULTS

Optimizing the amount of functionalized nanoparticles needed to detect peanut-specific IgE in plasma

These experiments were carried out in microfuge tubes that had been blocked with protein free blocking buffer T-20 solution (Pierce). Briefly, different concentration (ranging from 0 to 400 μg) of the functionalized particles were incubated, with the appropriate volume of plasma from an allergic individual (Plasma Lab International, WA), for 2 hrs to facilitate the capture of peanut specific IgE. Excess substrate was washed with phosphate buffered saline and tween-20 (PBST) and the particles along with the captured IgEs were isolated with a permanent magnet. The particles were then incubated with an anti human-IgE that was conjugated to alkaline phosphatase (Sigma-Aldrich, MO). This incubation was carried out for 1 hr. The excess anti human-IgE was washed away with three PBST washes. Alkaline phosphate substrate solution, 1-step pNPP (paranitrophenol-phosphate) (Thermo Fisher Scientific, IL), was added and the tubes were incubated in the dark. After 15 min of incubation the NPs were pulled aside with a permanent magnet and 100 μl of the solution was pipetted into a 96-well plate (Thermo Fisher Scientific, IL). For each sample, in every experiment carried out, appropriate controls were run. The absorbance of the solution was read at 405 nm in a BIO-tek microtiter plate reader (BIO-tek powerwave XS, VT). **Fig. 1** shows that 25 μg of functionalized NPs was sufficient to detect the presence of peanut-specific IgE in plasma. Additionally, a dose-dependent increase in absorbance as the amount of functionalized NPS increased from 0 to 200 μg and at a higher amount (400 μg) the absorbance started to saturate. Since 100 μg of functionalized NPs fell in the linear part of the absorbance curve, this amount of functionalized particles was used for further experiments.

Optimizing the amount of peanut protein needed to effectively capture peanut-specific IgE in plasma

Particles corresponding with different amounts of peanut protein were incubated with a fixed amount of plasma from an allergic individual as described above and absorbance obtained was plotted relative to the amount of peanut protein coated on the particles. Results as depicted in **Fig. 2** demonstrated that 6 μg of peanut protein coated onto the NPs was sufficient to detect peanut-specific IgE in the given volume of plasma. Additionally, with increasing

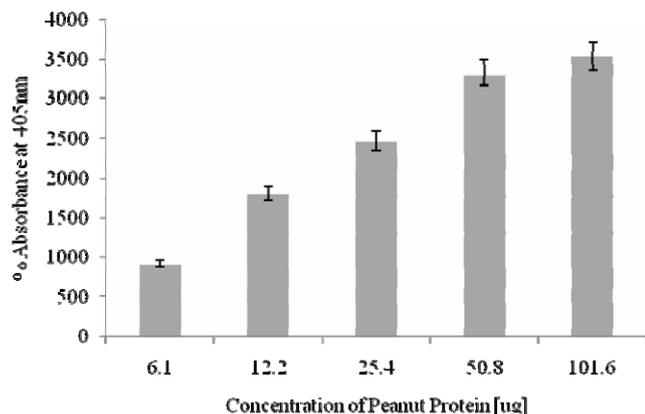


Fig 2 Determining the amount of peanut extract needed to effectively capture peanut-specific IgE. Different amounts of the peanut allergen coated on the functionalized NPs were incubated with allergic plasma. The obtained absorbance was plotted on the graph relative to the peanut protein concentration on the coated particles. The data demonstrated that 6 µg of peanut extract was sufficient to detect the presence of peanut-specific IgE in plasma.

amount of peanut protein there was an increase in the ability to the assay to detect peanut-specific IgE. However, this increases leveled off at 50.8 µg and higher concentrations, suggesting saturation in the ability of the functionalized NPs to detect peanut-specific IgE present in the given volume of plasma.

Optimizing of the volume of plasma needed for the assay to detect peanut-specific IgE

A fixed amount (100 µg) of functionalized NPs was incubated with different volumes of plasma from an allergic individual (Plasma Lab International, WA). These experiments were done according to the protocol described above and the absorbance obtained was plotted for each volume of plasma. The data indicated a dose-dependent increase in absorbance as the volume of plasma increased from 0 to 200 µl (Fig. 3A). Moreover, 50 µl of plasma was sufficient to detect significant differences between the particles incubated in the absence or presence of plasma by 450%. For experiments henceforth, 50 µl of plasma was used to further optimize the assay. The amount of peanut-specific IgE present in the allergic individuals plasma was determined by an immuno CAP assay and found to be approximately 61.5 kU/L. Using this as a reference value, the approximate amount of peanut-specific IgE present in plasma was calculated for the different volumes of plasma incubated with 100 µg of functionalized particles. These data, depicted in Fig. 3B, clearly indicated that the assay had the ability to detect peanut-specific IgE as low as 0.385×10^{-3} kU/L by 100% as compared to the negative control sample that did not have any IgE.

Testing the specificity of the functionalized nanoparticles to detect peanut-specific IgE in plasma of an allergic individual

100 µg of functionalized particles were incubated either in the absence or presence of 50 µl of plasma from the allergic individual. This experiment was repeated in triplicates and the average absorbance obtained are shown in Fig. 4 along with its standard deviation. As can be seen, the absorbance in presence of allergic plasma was 600% times more than the absorbance in the control composed of buffer solution without allergic plasma. These data demonstrated that the absorbance obtained from the samples incubated with allergic plasma was not due to the non-specific binding on anti-human IgE to the functionalized particles and the signal is extremely specific for peanut IgE present in plasma.

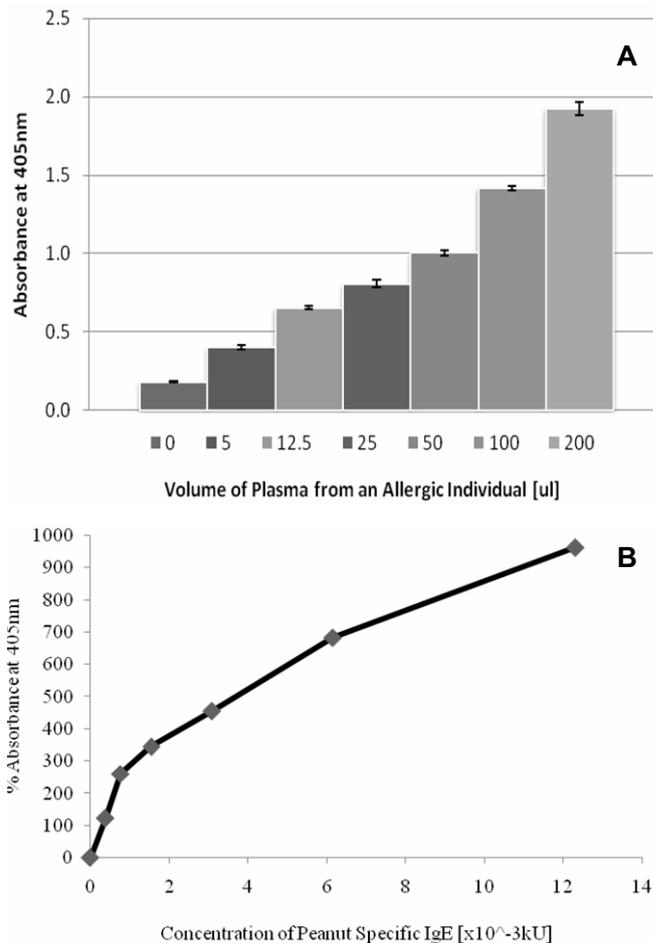


Fig. 3 (A) Determining the volume of plasma needed to detect peanut-specific IgE. In this experiment, 100 µg of functionalized NPs were incubated with increasing volume of plasma, ranging from 0 to 200 µl, in duplicates. The absorbance detected was plotted against the volume of plasma used for each condition. 50 µl of plasma was sufficient to detect significant differences between the samples and the control (0 µl of plasma) by 450%. **(B)** Determining the amount of peanut-specific IgE that can be detected with 100 µg of functionalized NPs. 100 µg of functionalized NPs were incubated with increasing concentration of peanut-specific IgEs, ranging from 0.385 to 12.32×10^{-3} kU. The absorbance obtained was plotted relative to the concentration of IgEs incubated. As can be seen in the graph our assay had the ability to detect IgE as low as 0.385×10^{-3} kU by 100% as compared to the negative control.

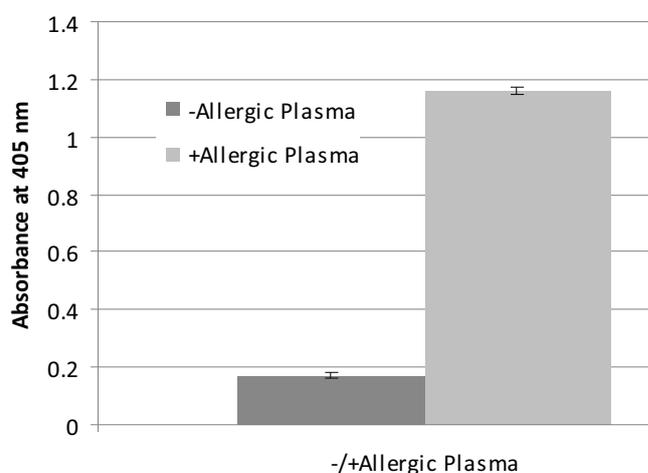


Fig. 4 Determining the specificity of the functionalized NPs. The specificity of the NPs was determined by incubating functionalized particles with peanut extract in the absence or presence of allergic plasma. As can be seen in the figure, the absorbance of the samples incubated in the presence of peanut specific IgE was found to be approximately 600% more specific as compared to the sample that was incubated in the absence of plasma. The data above is the average of three distinct experiments.

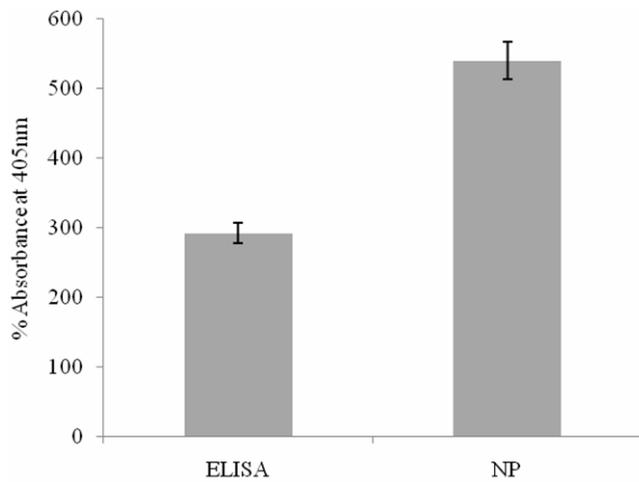


Fig. 5 Comparing the sensitivity of an ELISA and the nano assay. The sensitivity of detecting peanut specific IgE with an ELISA was compared to the magnetic assay. The absorbance using both methods relative to the negative control is depicted in the graph. Data demonstrated that the % absorbance with ELISA and NPs were 296 and 540 respectively. Thus the NPs have a much higher sensitivity to detect peanut-specific IgE in 50 μ l of plasma compared to traditional ELISA.

Comparing the sensitivity of the nano assay to a traditional ELISA

The same amount of peanut extract was coated in each well of the ELISA plate and 100 μ g of functionalized NPs. The appropriate wells of the ELISA and tubes for the nano assay were incubated with or without the allergic plasma. The samples without allergic plasma were considered to be negative controls for both, the ELISA and the assay with the NPs. These experiments were carried out in triplicate, following the determined protocol for both methods. The data obtained from these experiments clearly determined the nano assay to have more than two-fold sensitivity as compared to the ELISA (Fig. 5). Thus, magnetic assay was found to have high sensitivity and specificity for the detection of peanut-specific IgE in plasma.

DISCUSSION

The gold standard for the diagnosis of food allergy is the double-blind placebo-controlled food challenge (DBPCFC) (Bock *et al.* 1988; Bruijnzeel-Koomen *et al.* 1995; Pineda 2007; Fernandez-Rivas *et al.* 2008) accompanied with a detailed history and physical examination. However, DBPCFC is an extremely laborious procedure which can be traumatic to the patient and poses many safety issues (Perry *et al.* 2004). These concerns make it less suitable to use as a routine diagnostic tool especially in a clinical setting. Although the technology to detect and diagnose allergies has advanced tremendously through the years, there are major drawbacks to the *in vivo* and *in vitro* methods and techniques currently used.

In a clinical setting the wheal-and-flare skin test is the traditional *in vivo* test normally conducted. The skin tests are considered the gold standard for IgE antibody (Ab) detection because of its unexcelled sensitivity. This test measures the biological activity of specific IgE Abs via the activation of mast cells in the skin. Moreover, the skin test is much simple to use and is less expensive (Ten *et al.* 1995; Sun *et al.* 2009) as compared some of the other available tests. However, the major drawbacks of skin tests are ethical concerns as these test are highly invasive and the allergic individual is exposed to the very allergen causing allergic symptoms. Although these tests are performed in a control environment there is always a risk of a systemic reaction in the individual tested. Moreover, skin testing is contraindicated in pregnant women, patients suffering from severe bronchospasms, fever and dermatographism. Additionally, the

controlled environment requires the logistics and resources of highly trained personnel to perform these tasks.

Presently, the various *in vitro* methods used to determine the presence of food or allergen specific IgE in serum include, the Radio allergens sorbent test (RAST), modified RAST test, the enzyme linked assay (ELISA) and the Pharmacia CAP system. Although, these test are less sensitive in comparison to the skin prick test, they overcome major drawbacks of the skin tests. These *in vitro* tests pose no risk of systemic reactions and does not depend skin reactivity by confounding variables such as disease, age, pregnancy, or drugs taken by the patients. Therefore, *in vitro* testing has become ideally suited as a screening tool to test for allergies (King 1982; Nalebuff 1985, Tahon *et al.* 2009) and has been shown to successfully predict 90 to 95% of allergic patients (Chambers *et al.* 1997). The major drawback of the RAST and modified RAST tests are time constraints. It takes days or even weeks to obtain results from these tests and amounts to precious time lost for crucial intervention for the patient. These test are sensitive and correlate well with the skin prick test, but involves the use of radio-labelled Abs which needs the highly skilled professional to perform the test. Additionally, the facility where such test can be conducted must be equipped appropriate facilities to handle radio activity and need specialized equipment, like the gamma counter (RIA), to measure radioactivity. The need for trained personnel and specialized training, equipment and facility, increases the cost of this technique. In contrast to the RAST assay, the magnetic assay presented in this study is much more simple and does not call for the tagging of IgE with radiolabeled isotopes. This has two major advantages: firstly at a commercial level it is more cost effective since there is no need for specialized equipment and facilities. Secondly, it keeps the Abs and allergens in its most natural forms, reducing the possibility denaturing the protein and altering its biological activity. The synthesized NPs are functionalized to have an appropriate charge, opposite to the charge of the allergen that was coated onto the particles. Opposite charge on the particle and allergen, caused a strong binding of the allergen to the protein. This was also confirmed by three independent methods including the change in the zeta potential of the particles before and after the allergen was coated on to the NPs, the change in protein concentration of the extract before and after the particles were coated and the change in protein concentration of the particles itself.

ELISA overcame the need for radio-labeled antibodies, but must have the allergen or antigen immobilized to a solid surface. Most commonly the ELISAs are performed in a 96 well plate, where the antigen is coated on the bottom of the well and then incubated with the patients serum to detect the presence of specific IgEs (Engvall and Perlmann 1972; Messingham *et al.* 2009). The detection part of the assay contains an enzyme that is either directly linked to the primary Ab or introduced through a secondary Ab that recognized the primary antibody. One major drawback of the ELISA is that it needs multiple primary Abs that cannot be reused, this made the assay expensive and time consuming.

The most commonly used ELISA is the sandwich assay. In such an ELISA, the analyte to be measured is captured between two Abs, the capture Ab and the detection Ab. Additionally, there is a need for a secondary Ab conjugated to a substrate that can bind to the detection Ab. The process of generating not one, not two but three different Abs for each food allergen to be tested tremendously increases the cost of the assay. Furthermore, there is a large complex generated and with this the possibility of steric hindrance and non-specific reactions or cross-reactions of the Abs are also greatly increased. It is important to note that while generating an immunodetection assay that involves the use of Abs, the affinity and specificity of an Ab is the backbone of the assay and the Ab has to be thoroughly examined for such reactivities. Moreover, using more than one Ab may make the assay more prone to false positive results and decrease the sensitivity of the assay. The magnetic assay cir-

cumvented the need for using two or more Abs and successfully detected the presence of peanut specific IgE in plasma using only one secondary Ab that had been conjugated to alkaline phosphatase. Furthermore, the results (Fig. 4) have demonstrated that the functionalized NPs are highly specific in detecting the presence of peanut specific IgE in allergic plasma as compared to the absence of allergic plasma.

Additionally, as compared to the ELISA, where the antigen is coated to the bottom of the well making the assay two dimensional, in the magnetic assay the coated NPs are three dimensional spheres. This naturally increases the sensitivity of the assay as the functionalized particles can closely interact with the incubated substrate/sample and capture the specific IgE. As can be seen in Fig. 5, the magnetic assay was much more sensitive as compared to the traditional ELISA.

During the 1990s, in response to the drawbacks of the RAST tests, came the second-generation of IgE Ab assays which included the Pharmacia CAP system (Ewan and Coote 1990; Yunginger *et al.* 2000). The most significant advancement with this system was the development of the encapsulated hydrophilic carrier polymer to which the allergen was covalently coupled (Yunginger *et al.* 2000). This polymer was configured in the shape of a small cup and was called the CAP assay. Its allergenic protein binding capacity was superior and more user friendly as compared to other solid phase or liquid phase allergen reagents previously used such as agarose or cellulose particles. This improved Ab-binding capacity led to a more rapid assay kinetics and enhanced sensitivity of the assay. However, there still was a need for highly trained personnel to carry out the assay. In contrast, the magnetic assay was developed with functionalized NPs as the solid-phase (allergosorbent) and the advantage of using NPs is the size (nano) and shape (sphere) of the particles made extremely close interactions between the constituents of the plasma, serum or whole blood with the particle possible. As shown in Figs. 1 and 2, 100 µg of the functionalized particles detected peanut specific IgE in plasma of an allergic individual and the assay was able to detect significant differences between the negative control and sample even when the particles were functionalized with as little as 6µg of whole peanut extract. Another crucial issue while developing assays to detect the presence of specific IgEs is the sample volume. In the magnetic assay, a sample volume 50 µl of plasma (which can be obtained from a few drops of blood) was sufficient to detect the presence of peanut specific IgE in plasma (Fig. 3A).

Most of the assays currently available take a long time to give results. Initially the RAST tests took a couple of weeks for patients to receive their diagnosis. The second generation assays like the CAP system took about 5 hrs for the assay to be completed. With the magnetic assay, the current results were obtained in less than 1 hr (with a pre-labeled magnetic NPs). Further studies are still underway to optimize conditions for the total assay to be completed in a few minutes.

Thus, as compared to currently available assays to determine the presence of food specific IgE in plasma, the magnetic assay offers a more simple and easy assay to detect the presence of these IgEs. Moreover, as magnetic assay is developed using a nanotechnology platform it is much more sensitive as compared to a traditional ELISA. More importantly, the assay can successfully detected specific IgE in 50 µl of plasma which technically can be obtained from a few drops of blood. Additionally, to make this assay less invasive, currently a process to optimize the assay to detect the presence of these specific IgEs in saliva. Our preliminary results (data not shown) have successfully detected total IgE in saliva.

CONCLUDING REMARKS

A magnetic assay with ability to detect for specific IgE in plasma have been developed. The assay makes use of a single antibody to detected the presence of specific IgE in a 50

µg of plasma using 100 µg of functionalized NPs. The new assay offers a rapid, sensitive, specific and relatively inexpensive root to detect for specific allergy markers.

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