METHOD FOR DETECTION AND QUANTIFICATION OF SIALYLATED IGE AND ITS USE IN DIAGNOSIS OF ALLERGY

The present invention relates to methods and kits for the quantification of sialylated IgE in test samples which may be derived from human or animal subjects. Methods of the invention may be used as part of diagnosis of allergy or allergic disease.

Background

Allergy is caused by hypersensitivity of the immune system to different substances (allergens) present in the environment. Allergy can manifest clinically as an allergic disease. Such diseases include food allergy, asthma, atopic dermatitis, allergic rhinitis, conjunctivitis, and chronic rhinosinusitis.

Allergic sensitisation is the process by which a person develops significant levels of specific IgE antibodies (sIgE) against an allergen through repeated exposure as a consequence of a deviation of the immune system.

Allergy diagnosis is a complex and multistep process. It begins with a clinical interview to elaborate the patient's history, and a physical examination. Additional tests are used to identify the allergenic source/s that is/are related with symptoms through detection of sIgE. Two types of tests can be performed, including *in vivo* and *in vitro*. *In vivo* tests (i.e. skin prick tests, intradermal test, etc...) are based on the demonstration of allergen sIgE by the direct exposure of the skin to allergens and measuring of the wheal size produced within 30 minutes after allergen application. *In vitro* tests detect circulating sIgE antibodies in serum using laboratory methods (ImmunoCAP™, ALEX2, etc) (1).

Frequently, practitioners initially use *in vivo* testing and, in the case of a positive response, confirm the reaction to the positive allergens by *in vitro* methods. Confirmation of allergen sensitisation and the identification of causal allergens are essentials for optimising the management of allergic conditions. However, it is known that many subjects have positive skin tests and/or positive serum sIgE without allergic symptoms because a positive allergy test result indicates only sensitisation. It does not necessarily mean clinical allergy (1). Moreover,

the concentration of sIgE determined by *in vitro* tests and/or the value of the response obtained by skin tests does not usually correlate with the severity of symptoms (1, 2).

Another limitation of sIgE tests, especially *in vivo*, is that they can produce false negatives (negative result in patients with allergic disease). This can be critical when allergic symptoms are associated with life-threatening anaphylaxis in subjects with allergy to foods or Hymenoptera venoms (1).

Apart from sIgE, the measurement of total IgE in sera was considered in early studies as the simplest way to identify subjects with allergy but it soon became evident that total IgE levels could not be considered a reliable marker of allergy status. Total IgE levels significantly higher than the normal threshold are usually associated with allergic disorders, but also with other conditions. Furthermore, low or normal total IgE levels do not exclude the presence of IgE-mediated diseases. Therefore, levels of total IgE should be carefully interpreted and not considered as an indication for the presence of allergy (1).

Thus, there is currently a lack of clinically validated *in vitro* methods that can be used as complementary tools for the diagnosis of allergy and allergic diseases.

IgE is the most glycosylated immunoglobulin in human and possesses seven potential N-glycosylation sites within the constant region of the heavy chain located in the asparagine (N) residues: N21, N49, N99, N146, N252, N264 and N275 (Uniprot P01854) (3). Site-mutagenesis studies (3, 4) have established that:

N275-linked oligomannose is required for appropriate IgE folding and binding to receptors to initiate effector functions

N264 is unoccupied; and

The remaining five sites are occupied by complex antennary glycans.

Glycosylation studies performed in samples from allergic patients and non-allergic subjects determined that 5-acetylneuroaminic acid (sialic acid) residues were enriched in the terminal position of antennary glycans of positions N49 and N146 in patients with allergy. These sialic acid moieties are linked to galactose by α-2,6 bridges. Enzymatic removal of sialic acid from

IgE produced a statistically significant reduction of the allergenic activity of the IgE from allergic mice and in *in vitro* human model experiments (5). These results demonstrated that specific sialylation patterns can distinguish the IgE of subjects with allergy from non-allergic subjects and thus can act as an important biomarker for the pathogenicity of IgE and the diagnosis of allergy.

Current methods for detecting the sialylation of proteins, including IgE, are laborious using techniques such as enzymatic digestion followed by liquid chromatography with tandem mass spectrometry (5). This method is challenging to employ in a clinical setting because IgE is the least abundant immunoglobulin in blood and therefore large volumes of blood are required to prepare enough material to test. The IgE must be effectively pure which requires a multi-step purification process. This method requires highly skilled laboratory workers, access to expensive machinery and complex analysis. Therefore, there remains a need for a simple technique to quantify sialylation of IgE which requires minimal sample processing and produces a readily interpretable readout.

A reverse lectin-based ELISA technique developed by Wu et al. for quantifying the sialylation of the IgG family of immunoglobulins requires minimal sample processing and provides a spectroscopic readout. This method utilises the interaction between sialic acid and SNA-1 lectin to specifically immobilise sialylated IgG from sera. IgG-specific horseradish peroxidase (HRP) conjugate antibodies were then used to colorimetrically detect the bound, sialylated IgG through the activity of HRP (7).

This kind of ELISA test generates non-quantitative results, and the results can be easily influenced by external factors such as temperature, handling, etc. The integration in the assay of a reference (standard) curve would improve the results by normalizing inter-assay fluctuations and, in case that a standard or reference molecule is included, the interpolation of the sample response (optical density (OD)) from the curve will generate quantitative results.

Unfortunately, there is no available reference of IgE with a known amount of sialic acid. In fact, this is an important handicap for the development of a quantitative method for measuring sialylated IgE. However, experimental methodologies capable of determining the

sialic acid content of a purified IgE preparation are now available. The generation of an easy method for the quantification of IgE sialylation would be a new and useful diagnostic tool where this could discern the allergy disease from other non-IgE mediated diseases that share similar symptoms.

Immunochromatographic Lateral Flow Assays (LFAs) are based on the biochemical interaction of antigen-antibody or any kind of probe-target. An LFA is composed of four parts: a sample pad, which is the area on which sample is dropped; a conjugate pad, on which labelled tags are combined with biorecognition elements; a reaction membrane containing a test line and a control line for probe-target or antigen-antibody interaction; and an absorbent pad, which absorbs excess fluid. In construction of an LFA, several labelling methods such as gold nanoparticles, coloured latex beads, carbon nanoparticles, etc., are used for increasing sensitivity.

The use of LFAs have attracted interest due to their friendly user formats, short assay times, low levels of interference, low costs, and being easy to operate by non-specialized personnel. Usually, LFAs use small quantities of biological fluids such as saliva or capillary blood obtained from a finger puncture. These features allow a physician to obtain the results of the test during a medical visit which permits a faster diagnosis.

The concept of a LFA using lectins in the role of the biorecognition element was presented in 2016 (8). The authors used SNA-1 lectin immobilized on the reaction membrane to discriminate α-2,6 sialic acid and other monosaccharides present in the glycosylated form of the Prostatic Specific Antigen (PSA), a biomarker related to prostate cancer. In this assay, the detection of the SNA-1 lectin captured glycosylated-PSA was later detected by an anti-PSA antibody labelled with gold nanoparticles. This evidence supports the usefulness of LFA for detecting glycoforms of proteins related to diseases such as sialylated IgE. The problem in the application of this technique on detection of sialylated IgE is the very low amounts of IgE (nanograms) in blood sera compared to the normal amount of PSA (micrograms).

The present invention provides the successful adaptation of the technique of Wu et al. for the quantification of sialylated IgE and the correlation of this parameter with allergic disease.

In addition, the invention provides a reference preparation of sialylated IgE has been established by quantification of sialic acid bound to IgE. This reference is used to perform a calibration curve of known amount of sialic-IgE. The serum is analyzed in parallel using the same assay and the OD values are transformed into ng of sialic IgE per mL of serum by interpolation from the reference curve. Therefore, this method generates a quantitative determination of this parameter.

The success of this technique is surprising given the relative scarcity of IgE in sera when compared to, for example, IgG (3). One would expect that other, more abundant sialylated proteins would mask any differences in the sialylation status of IgE. In fact, this effect has been observed when an ELISA method was translated to a LFA with no-detection of IgE in serum samples. This limitation observed in a LFA was addressed by centrifugation of serum IgE linked to anti-IgE labelled with gold-nanoparticles. In this step, molecules that interfere with IgE detection by lectin are removed allowing IgE to be recognised by SNA-1 lectin via sialic acid. The LFA kit, being a new and complementary tool for allergy diagnosis, will allow clinicians to easily measure the sialylation of IgE at the point of care without complex laboratory equipment.

Summary of the invention

In a first aspect of the invention, a method of quantifying sialylated IgE in a test sample is provided, the method comprising:

immobilising sialylated IgE in the test sample via a capture probe which is specific for sialylated glycoproteins, wherein the capture probe is immobilised; and

using an antibody-based detection technique to quantify sialylated IgE.

In a second aspect of the invention, a diagnostic method for diagnosing allergy in a subject is provided, the diagnostic method comprising using the method of the first aspect of the invention and diagnosing the subject with allergy based on the quantity of sialylated IgE in the test sample.

In a third aspect of the invention, a method of treatment of allergy or allergic disease is provided, the method comprising the steps of:
diagnosis of allergy by the diagnostic method according to the second aspect of the invention; and
treatment by immunotherapy.

In a fourth aspect of the invention, a kit is provided, the kit comprising:
a capture probe which is specific for sialylated glycoproteins wherein the capture probe is either immobilised or is readily capable of immobilisation; and
an IgE recognising antibody.

In a fifth aspect of the invention, use of the kit of the fourth aspect of the invention is provided to quantify sialylated IgE in a test sample.

Figures

The invention is now exemplified with reference to:

Figure 1 which shows analysis of serial dilutions of purified myeloma IgE and Bovine Serum Albumin (BSA) from 6.4 pg/ml to 50 µg/ml. Results are expressed in ELISA Arbitrary Units (EAU) which are equivalent to $A_{450\ nm}$;

Figure 2 which shows analysis of serial dilutions of sera from human subjects with allergic disease (A, B and C) and one non-allergic (healthy) control (the sera were serial diluted from 1/5 to 1/640; healthy control had 0.00 kU/L (9) of total IgE; results are expressed in EAU which are equivalent to $A_{450\ nm}$;

Figure 3 which shows a box-plot representation of results from human subjects with allergic disease (n = 405) and non-allergic human subjects (n = 30) (values are expressed in EAU which are equivalent to $A_{450\ nm}$; **** shows statistical differences between groups ($p < 0.0001$). Filled circles in the allergic group correspond to results from patients sensitized to venoms;

Figure 4 which shows a ROC curve generated by the analysis of sera from the same 30 non-allergic (controls) human subjects and 405 human subjects with allergic disease of Figure 3;

Figure 5 which shows correlation analysis between total IgE (kU/L) (9) and Sialic-IgE ELISA (EAU) of sera from human subjects with allergic disease (n = 404);

Figure 6 which shows the results of the analysis of levels of sialylation of IgE by an alternative method in sera from 3 subjects suffering from allergic disease and 3 non-allergic controls (the sera were serial diluted from 1/50 to 1/12150 with results expressed as $A_{415\,nm}$);

Figure 7a which shows a schematic of the method according to the invention (see Example 2);

Figure 7b which shows a schematic of an alternative method which is not part of the invention (see Example 8);

Figure 8 which shows the quantification of sialic acid bound to a purified human IgE preparation (see Example 9);

Figure 9 which exemplifies the quantification of sialic acid bound to IgE in a serum sample (see Example 10);

Figure 10 which exemplifies the application of quantification of sialic acid bound to IgE in the detection of allergy IgE-mediated disease (see Example 12);

Figure 11 which shows the design of LFA for the quantification of sialic acid bound to IgE (see Example 13);

Figure 12 which shows T/C (test/control) ratio values registered by LFA reader for different sialic-IgE concentration samples (0, 20, 100, and 200 ng/mL) spiked in buffer solution (see Example 16);

Figure 13 which shows T/C ratio values (n = 3) registered from 2 pools of sera (low and medium sialic-IgE values) by LFA reader in serum samples (A) without adding centrifugation process and (B) with centrifugation process (see Example 17); and

Figure 14 which shows sialic acid-IgE determination (T/C ratio values) by LFA test strips in 25 individual serum samples (n = 10 allergic shaded and n = 15 non-allergic subjects non-shaded) with each column representing the average of three analyses and the error bar representing the standard deviation (see Example 18).

Detailed description of the invention

In a first aspect of the invention, a method of quantifying sialylated IgE in a test sample is provided, the method comprising:

immobilising sialylated IgE in the test sample via a capture probe which is specific for sialylated glycoproteins, wherein the capture probe is immobilised; and

using an antibody-based detection technique to quantify sialylated IgE.

Sialylated IgE means an immunoglobulin from the IgE family which comprises at least one glycan further comprising a sialic acid moiety. IgE has different glycosylation sites and sialylated IgE may only comprise a single sialic acid moiety or can comprise up to 30 sialic acid moieties. Multiple sialic acid moieties may be part of the same glycan and therefore be connected to the same residue and/or may be spread over multiple glycans.

Immobilising sialylated IgE via a capture probe means binding sialylated IgE to a surface such that the remaining test sample can be removed whilst leaving the bound sialylated IgE behind, thus separating the sialylated IgE from other components of the test sample. This can be achieved by several methods known to the skilled person. The surface is a macrostructure able to bind the capture probe. This binding may be achieved through functionalisation of the surface. The surface may be a solid surface. The surface may be selected from the well of a plate or a portion thereof, a bead, a membrane, a gel or other matrix. The surface enables bound material to be retained whilst unbound material can be readily washed away.

The capture probe may be any agent able to bind sialylated glycoproteins and also bind to a surface. This includes antibodies which recognise sialylated glycoproteins, lectins which recognise sialylated glycoproteins, other proteins which recognise sialylated glycoproteins and chemical agents which recognise sialylated glycoproteins. The capture probe may bind to a surface through a covalent interaction such as an N-succinamide linkage or isopeptide bond, or a non-covalent interaction such as a specific protein interaction (e.g. biotin/streptavidin), a chelating group, hydrogen bonding, hydrophobic interactions or a combination thereof. The corresponding surface should be functionalised accordingly to facilitate or enable the binding.

Quantifying sialylated IgE means measuring the amount or concentration of sialylated IgE in a test sample. Suitable antibody-based detection techniques utilise antibodies which are specific for an epitope of the bound sialylated IgE. Quantification can produce a relative quantity or an absolute quantity. An absolute quantity may be obtained by comparing the direct result of a detection technique with the results of a standard curve to infer the absolute quantity (in mass units, moles, etc.) of sialylated IgE in the test sample. The standard curve can be prepared by performing the method of the invention on samples of known concentration of sialylated IgE. A relative quantity is not linked to a physical unit of quantity but is interpreted directly from the results of the detection technique. A relative quantity may have units of absorbance or fluorescence counts, for example.

In an embodiment, the capture probe is *Sambucus nigra* agglutinin 1 lectin (SNA-1 lectin). This tetrameric protein recognises specifically the α-2,6 link between galactose and sialic acid in glycans.

In an embodiment, the capture probe is immobilised to a solid surface. The solid surface may be the surface of a well of a plate or a bead, for example a magnetic bead, or a membrane, for example a nitrocellulose membrane. Preferably the solid surface is the surface of the well of an ELISA plate or a nitrocellulose membrane (LFA).

In an embodiment, the antibody-based detection technique comprises a colorimetric assay. Colorimetric assays measure the generation (or depletion) of a chromophore in a solution due to the presence (or absence) of a particular entity. Changes to the concentration of the

chromophore change the absorbance (OD) of the solution according to the Beer-Lambert law. Measurement of absorbance of the solution can be readily carried out using a spectrophotometer which may be integrated into a plate reader for high-throughput assays.

In one example, the antibody-based detection technique comprises a signal that can be visually detected, preferably by the use of anti-IgE antibody conjugated with gold nanoparticles and the area and/or intensity of the signal generated can be measured by using a scanner/reader.

In an embodiment, the antibody-based detection technique comprises the use of an IgE recognising antibody. The IgE recognising antibody is any antibody which specifically recognises the IgE being tested in the method. The IgE recognising antibody may be a monoclonal antibody. The IgE recognising antibody may be derived from mouse cells. Where the sialylated IgE being quantified has come from a human, the IgE recognising antibody may be obtained by immunizing a non-human animal (for example a mouse) with human IgE, isolating specific B-lymphocytes which produce the anti-human IgE antibodies from the non-human animal, fusing the B-lymphocytes with immortal myeloma cell lines to form hybridoma cell lines which produce anti-human IgE monoclonal antibodies. Where the sialylated IgE being quantified has come from a non-human animal, the IgE recognising antibody may be similarly obtained using a different species of non-human animal (rabbit, mouse, rat, dog, goat, pig etc.) to generate hybridomas.

In an embodiment, the IgE recognising antibody is conjugated to a horseradish peroxidase enzyme (HRP). HRP, in the presence of a suitable oxidant such as hydrogen peroxide, catalyses the oxidation of some organic compounds, such as diaminobenzidine (DAB), tetramethylbenzidine (TMB), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), to form chromophores which can be detected and quantified using a spectrophotometer. Compounds which can be oxidised by HRP in this manner are termed chromophore precursors. HRP can also catalyse the oxidation of luminol resulting in a chemiluminescent compound which can be detected using a fluorimeter.

In one example, the IgE recognising antibody is passively conjugated to gold-nanoparticles as described in detail in Example 14.

In an embodiment, the method comprises:

a) immobilizing SNA-1 lectin in the well of an ELISA plate;

b) adding a test sample and allowing any sialylated glycoproteins to bind to the immobilised SNA-1 lectin;

c) adding an anti-IgE antibody conjugated to horseradish peroxidase and allowing the antibody to bind to any bound sialylated IgE; and

d) adding a developing solution comprising a chromophore precursor and hydrogen peroxide, allowing oxidation of the chromophore precursor to a chromophore to occur, and detecting the production of the chromophore using spectrophotometry.

A schematic of this method is shown in Figure 7a.

The method may further comprise a blocking step between steps a) and step b) comprising the addition of an immunochemically inert substance to saturate the ELISA plate. The immunologically inert substance should interact neither with the SNA-1 lectin, the anti-IgE antibody or the conjugated horseradish peroxidase. The immunologically inert substance may be bovine serum albumin (BSA). Saturation of the plate means that any “vacant” binding sites are filled. This means that only the intended components of the test sample will be immobilised in the well via interaction with SNA-1 lectin.

The method may further comprise one or more washing steps between any previously disclosed step to remove any unbound material.

In an embodiment the chromophore precursor is tetramethylbenzidine.

In another embodiment, the method comprises:

a) immobilizing SNA-1 lectin on a nitrocellulose membrane;

b) mixing a test sample with an anti-IgE antibody conjugated with gold-nanoparticles allowing the antibody to bind to any bound sialylated IgE;

c) centrifuging the mixture of step (b) until a clear supernatant is obtained and discarding the supernatant;

d) resuspending the pellet from step (c) with buffer solution and applying the suspension to the nitrocellulose membrane with immobilized SNA-1 lectin from step (a);

e) visually detecting and quantifying the complex of sialylated-IgE/labelled anti-IgE bound to the immobilized SNA-1 lectin.

Preferably centrifugation is at 3000-8000x g for 3 to 15 minutes, more preferably 4000-6000x g for 3-10 minutes, most preferably 5000x g for 5 minutes.

In an embodiment, the test sample is blood serum or plasma from a subject. Serum is prepared from whole blood by allowing the blood to clot and then removing the clotted material by, for example, centrifugation. Plasma is prepared by obtaining blood from the subject, adding an anticoagulant to prevent clotting and removing the blood cells by, for example, centrifugation. The main difference between serum and plasma is that serum does not comprise clotting factors such as fibrinogen. It will be appreciated that both blood serum and plasma are only available *ex vivo* and thus are not part of a human or animal body.

The test sample may be diluted in a suitable buffer before the method of the invention is carried out. In this case the dilution factor should be considered in order to quantify the sialylated IgE present in the original test sample.

In an embodiment, the subject is a human subject. In an alternative embodiment, the subject is a non-human animal subject.

In a second aspect of the invention, a diagnostic method for diagnosing allergy in a subject is provided, the diagnostic method comprising using the method of the first aspect of the invention, wherein the test sample is blood serum or plasma from the subject, and determining from the quantity of sialylated IgE whether the subject suffers from allergy.

In an embodiment, where the test sample is blood serum from a human subject, the subject is diagnosed with allergy if the test sample comprises sufficient sialylated IgE such that, when tested according to a method comprising:

a) immobilizing SNA-1 lectin in a well of a standard 96-well plate;

b) diluting a test sample 20-fold into an inert buffer (preferably phosphate buffered saline);

c) adding 100 µL of the diluted test sample to the well and allowing any sialylated glycoproteins to bind to the immobilised SNA-1 lectin;

d) adding an anti-IgE antibody conjugated to horseradish peroxidase and allowing the antibody to bind to any bound sialylated IgE; and

e) adding a developing solution comprising tetramethylbenzidine and hydrogen peroxide, allowing oxidation of the chromophore precursor to a chromophore to occur,

f) halting oxidation though addition of a peroxidase inhibitor such that the total solution volume is 200 µL; and

g) detecting the production of the chromophore using spectrophotometry;

the chromophore elicits an absorbance of at least 0.55 at 450 nm.

A blocking step and/or washing steps as defined above may also be incorporated into the method. Each step may comprise an incubation period to allow binding to occur.

The maximum absorbance elicited by the chromophore may be the detection limit of the spectrophotometer which may optionally be 2.5, 2, 3, 3.5 or 4.

In one embodiment, a serial dilution of a preparation of a purified human IgE (reference) with a known amount of sialic acid linked was included in the method of the second aspect to establish a reference curve of, for example, 0.05 to 0.001 ng of sialylated-IgE per millilitre. Blood serum samples are analyzed in parallel in a serial dilution from, for example, undiluted to 32-fold dilution. The amount of sialylated IgE in the sample is calculated by extrapolation from the reference curve and multiplying by serum dilution from those points of OD comprised in the reference curve. The result is expressed as nanograms of sialylated IgE per mL of serum.

In an embodiment, if the subject is diagnosed with allergy, the diagnostic method further comprises a suitable clinical or *in vitro* method to determine if a subject is allergic to any allergy source. Suitable clinical or *in vitro* methods include those already known to the skilled person for diagnosing allergy, for example skin tests and methods to detect circulating IgE antibodies in serum.

In a third aspect of the invention, a method of treatment of allergy or allergic disease is provided, the method comprising the steps of:
diagnosis of allergy by the second aspect of the invention; and
treatment by immunotherapy.

Immunotherapy involves the administration of increasingly larger doses of an allergen extract with the aim of inducing immunological tolerance. Allergen immunotherapy modulates the immune response to the allergen rather than ameliorating the symptoms induced by an allergic reaction, and can either reduce the need for medication, reduce the severity of symptoms or eliminate hypersensitivity altogether.

In a fourth aspect of the invention, a kit is provided, the kit comprising:
a capture probe which is specific for sialylated glycoproteins wherein the capture probe is either immobilised or is readily capable of immobilisation; and
an IgE recognising antibody.

Readily capable of immobilisation means that the capture probe does not need to be modified in order to be immobilised. For example, it may comprise a tag group which is intended to interact with a functionalised surface. The capture probe may be inherently readily capable of immobilisation due to its hydrophobic/hydrophilic properties.

In an embodiment, the capture probe is SNA-1 lectin.

In an embodiment, the IgE recognising antibody is conjugated to horseradish peroxidase or gold-nanoparticles.

In a fifth aspect of the invention, use of the kit according to the fourth aspect of the invention to quantify sialylated IgE in a test sample is provided.

Examples

Example 1 - Preparation of ELISA plates for the method of quantifying sialylated IgE

A Maxisorp™ 96 well ELISA plate (Thermo Scientific™) was coated with SNA-1 (Vector laboratories) by adding 100 µl/well SNA-1 at 20 µg/ml in 0.01 M PBS (phosphate buffer solution) and incubating overnight.

The plate was washed with Buffer A (0.1 % (w/v) Tween™ 20 in 0.01 M PBS) and then blocked for 1 hour using 100 µl/well Buffer B (3% (w/v) BSA, 0.1 % (w/v) Tween™ 20 in 0.01 M PBS). The plate was then washed with Buffer A again.

Example 2 – General method for quantifying sialylated IgE

Plates were prepared according to Example 1.

Samples were prepared in Buffer A and added to the plates at 100 µL/well. Plates were incubated for 2 hours and then washed with Buffer A.

Mouse anti-human IgE monoclonal antibody conjugated to horseradish peroxidase (clone B3102E8, Southern Biotech) was diluted 500-fold in Buffer C (1 % (w/v) BSA, 0.1 % (w/v) Tween™ 20 in 0.01 M PBS) and 100 µL/well was added. The plates were incubated for 1 hour and then washed with Buffer A.

100 µL/well peroxidase substrate (k-Blue® TMB; Neogen) was added and the plates incubated for 20 minutes. The oxidation reaction was then stopped by adding 100 µl/well of concentrated H_2SO_4.

Absorbance of each well at 450 nm was measured using a plate reader.

A schematic of this method is shown in Figure 7a.

Example 3 – Establishing working range of method for quantifying sialylated IgE

Samples of myeloma purified IgE (Merck) from 6.4 pg/mL to 50 µg/mL as well as equivalent concentrations of BSA were prepared in Buffer A. Samples were added in triplicate to plates and the method of Example 2 was carried out.

The results are shown in Figure 1 and demonstrate that the method can detect sialylated IgE and the method has a linear working range of between 80 ng/mL and 2 µg/mL myeloma purified IgE. The method is specific for purified IgE as evidenced by the results for the BSA control samples.

Example 4 – Detection of sialylated IgE in serum

Sera from three allergic subjects (A, B and C) and one healthy patient without allergic disease were obtained. Total IgE concentration as determined by ImmunoCAP™ assay is shown in Table 1.

Table 1: Total IgE for the sera for subjects A-C and a healthy subject

Serum reference	Total IgE (kU/L)
A	812
B	258
C	45
Healthy	0

Serial dilutions of the sera from 5-fold to 640-fold were prepared and tested following the method in Example 2. The results are shown in Figure 2 and demonstrate that the subjects with allergic disease have elevated levels of sialylated IgE which scales linearly with dilution over the linear working range of the method. Thus, the method is able to be used with complex biological samples and does not require prior purification of IgE.

Example 5 – Confirmation of specificity for sialylated IgE

Sera from 13 patients (I – XIII) with respiratory allergy (rinhitis and/or asthma) and sensitized to house dust mites were treated with sialidase A (Agilent) diluted in reaction buffer to 0.5

U/mL during 72 h at 37 °C. The desialylated serum samples, as well as the untreated samples (maintained in the same conditions without enzyme), were diluted 10-fold and tested following the method in Example 2. The results are shown in Table 2.

Table 2: Effect of desialylation with sialidase A enzyme on the result of the method.

Serum sample	Untreated sample ($A_{450\ nm}$)	Desialylated sample ($A_{450\ nm}$)	Decrease in Absorbance (%)
I	1.369	0.190	86
II	1.153	0.420	64
III	1.134	0.459	59
IV	0.900	0.056	94
V	0.815	0.187	77
VI	0.790	0.056	93
VII	0.670	0.003	100
VIII	0.592	0.099	83
IX	0.580	0.237	59
X	0.871	0.357	59
XI	0.939	0.000	100
XII	0.718	0.280	61
XIII	0.804	0.010	99

The data shows that desialylation leads to a decrease in the absorbance of, on average 80 %. This data shows that the method is specific for sialylated IgE.

Example 6 – Comparison of subjects with and without allergic disease (small sample size)

Sera from five human subjects were analysed. Subjects 1 and 2 were clinically diagnosed with allergic asthma caused by house dust mites. Subjects 3, 4 and 5 did not suffer from allergic disease and subject 5 also did not have detectable total IgE as determined by ImmunoCAP™ assay. Total IgE (kU/L) are given in Table 3.

Table 3: Clinical information and total IgE for each serum sample

Serum reference	Clinical info	Total IgE (kU/L)
1	Allergic asthma	150.0
2	Allergic asthma	42.5
3	Healthy	135.6
4	Healthy	40.7
5	Healthy	0.0

Sera were diluted 20-fold into Buffer A and the method according to Example 2 was carried out in triplicate for each sample as well as for a Buffer A control.

The results are shown in Table 4.

Table 4: Results of sample testing for triplicate assays for sialylated IgE (% CV = $100 \times \frac{\sigma}{\mu}$)

Serum reference	**$A_{450\ nm}$**			**Average $A_{450\ nm}$**	**% CV**
1	2.487	2.346	2.205	2.346	7
2	1.398	1.499	1.316	1.404	7
3	0.229	0.226	0.271	0.242	10
4	0.286	0.322	0.337	0.315	8
5	0.232	0.241	0.275	0.249	9
Control	0.237	0.190	0.201	0.209	12

The results for sera subjects with allergic disease and without allergic disease show clear differences with the sera from subjects without allergic disease being very similar to the Buffer A control.

Example 7 –Comparison of subjects with and without allergic disease (larger sample size)

Sera or plasma from 435 subjects was obtained. The allergic disease status of the subjects is shown in Table 5.

Table 5: Allergic disease status for 435 subjects

Allergic disease status	Sample size
Respiratory allergy (rhinitis and/or asthma)	377
Allergy to Hymenoptera venom	28
No allergic disease	30

Serum/plasma samples were diluted 20-fold and tested according to the method of Example 2. The results are shown in Figure 3. The data shows a statistically significant difference between the samples from subjects with allergic disease vs those without allergic disease ($p < 0.0001$ using Mann Whitney test).

An ROC curve for the results of the method is shown in Figure 4. The area under the ROC curve is 0.878 with a 95 % confidence interval between 0.834 and 0.922 ($p < 0.0001$) indicating a strong diagnostic test. The ROC curve gives a cut-off $A_{450\,nm}$ of 0.55 to distinguish subjects with allergic disease from those without. This cut-off is the maximum of Youden's index for the test:

$$\text{Sensitivity} = \frac{\text{True positives}}{\text{True positives+False negatives}} = 0.7$$

$$\text{Specificity} = \frac{\text{True negatives}}{\text{True negatives+False positives}} = 0.9$$

$$\text{Positive predictive value} = \frac{\text{True positives}}{\text{True positives+False positives}} = 0.99$$

$$\text{Negative predictive value} = \frac{\text{True negatives}}{\text{True negatives+False negatives}} = 0.21$$

$$\text{Youden's index} = \text{Sensitivity} + \text{Specificity} - 1 = 0.6$$

The total IgE content of these 435 samples was also determined and compared to the results from the method (Figure 5). Weak correlation can be seen between the two variables and a Spearman analysis of the samples from subjects with allergic disease suggests that the variables are independent ($r=0.26$, $p < 0.0001$).

Example 8 (comparative) – Alternative assay configuration

Some ELISA assays are known to be reversible, i.e. the surface binding and recognition agents can be switched. In fact, due to the relative scarcity of IgE in blood it would make sense to enrich for IgE first before selecting for sialylated IgE.

A method was trialled where IgE was immobilised in the wells of plates using immobilised anti-IgE antibodies. Bound sialylated IgE was detected using SNA-1 conjugated to biotin and the biotin was developed using anti-biotin polyclonal antibody conjugated to an alkaline phosphatase. A schematic of this method is shown in Figure 7b.

A Maxisorp™ 96 well ELISA plate (Thermo Scientific™) was coated with anti-IgE monoclonal antibody (clon 4F4, Santa Cruz Biotechnology) at 0.25 µg mAb/well diluted in 0.01 M PBS (phosphate buffer solution) and the plate was incubated overnight.

The plate was washed with Buffer D (0.1 % (w/v) Tween™ 20 in TBS (Tris 10 mM plus NaCl 150 mM) and then blocked for 1 hour using 100 µl/well Buffer E (5% (w/v) BSA, 0.1 % (w/v) Tween™ 20 in TBS). The plate was then washed with Buffer D again.

Samples were diluted from 50 to 12,150-fold in Buffer F (1% (w/v) BSA, 0.1 % (w/v) Tween™ 20 in TBS) and added to the plates at 50 µL/well. Plates were incubated for 2 hours and then washed with Buffer D.

SNA-1 lectin conjugated with biotin (Vector laboratories) was diluted to 0.4 µg/ml in Buffer G (1% (w/v) BSA, 0.1 % (w/v) Tween™ 20, 0.1 M calcium chloride, 0.1 M magnesium chloride in TBS) and 50 µL/well was added. The plates were incubated for 1 hour and then washed with Buffer D.

50 µL/well of anti-biotin polyclonal antibody conjugated with Alkaline phosphatase (Thermo Fischer) diluted 3000-fold in Buffer F was added and the plates were incubated for 1 hour and then washed with Buffer D.

p-Nitrophenyl Phosphate (pNPP) substrate (Merck) was added at 50 µL/well and incubated for 30 minutes. The reaction was then stopped by adding 50 µl/well of 2N NaOH.

Absorbance of each well at 415 nm was measured using a plate reader.

The results are shown in Figure 6 and demonstrate that the results for samples from subjects with and without allergic disease are largely indistinguishable. Thus, this configuration of the assay is not suitable and it is surprising that the method as exemplified in Example 2 and illustrated in Figure 7a shows such clear discrimination between healthy subjects and those with allergic disease.

Example 9 – Stabilising a reference of sialylated IgE and quantitative determination of sialic acid bound to IgE

An aliquot of 175 µg of purified IgE was dried in a vacuum centrifuge and the pellet was resuspended in 25 µl of 2M acetic acid and incubated for 2 hours at 80 °C. Next, an aliquot of 5 µl of each test sample, as well as sialic acid standard, were mixed with 20 µl of DMB labelling solution and incubated for 3 hours at 50 °C in the dark. To stop the reaction, 475 µl of water was added, and an 8-point calibration curve was created with the sialic acid standard, ranging from 1.29 to 0.00026 µM. The preparation was quantified by UPLC-FLR using ACQUITY UPLC System with a column ACQUITY UPLC Glycan BEH C18, 50 x 2.1 mm, 1.7 µm. The calibration curve for sialic acid standard exhibited an excellent linearity using a log transformation of the data (see Figure 8). The amount of sialic acid in the purified IgE was extrapolated from the standard curve. Thus, it contains 10.2 µg sialic acid per mg of IgE.

Results from each test sample can be compared to the calibration curve.

Example 10 – Alternative method for quantitative determination of sialic acid bound to IgE

ELISA plates were prepared according to Example 1. Then, assays were carried out in accordance with Example 2 with the following minor changes.

First, a serial dilution of a preparation of purified human IgE (reference) with a known amount of sialic acid was included in wells of the SNA-1 lectin coated ELISA plate. The concentration of sialic acid ranged from 0.05 to 0.001 ng of sialylated-IgE per millilitre.

Second, a serial dilution of a serum sample was analyzed from undiluted to 32-fold and dispensed in the same plate in parallel with reference curve.

After reading the ODs, the amount of sialylated IgE in the sample was calculated by extrapolation from the reference curve multiplied by serum dilution from those points of OD comprised within the linear portion of reference curve, at least two dilutions were considered. The result obtained was expressed as nanograms of sialylated IgE per mL of serum. An example analysis is shown in Figure 9.

Example 11 – Validation of quantitative method for determining sialic acid bound to IgE in serum

A pool of sera from patients with allergy was used as a sample to validate the quantitative method for determination of sialic acid bound to IgE. For that, assays were performed as described in Example 10. The method was validated according to ICH Guideline Q2 for a quantitative method with the following assays: Precision (intra-assay - repeatability of the instrumental system, inter-assay - repeatability of the method and intermediate precision), linearity/range, accuracy and selectivity/specificity. Mean, standard deviation, coefficient of variation and confidence intervals were calculated with intra-, inter- and intermediate precision. Linear regression, correlation, linearity test and confidence test were calculated for the linearity assay. Percentage recovery for the accuracy test was calculated with the formula: % Recovery = (Obtained value/expected result)*100. For the accuracy assay, t-test was also calculated. Parameters, results and acceptance criteria are set forth hereinbelow.

The method showed linearity for test samples at least in the range of 0.20 – 0.01 ng sialic-IgE/mL of serum with $r2 \geq 0.99$ and CV = 11 % (acceptance criteria $r2 \geq 0.99$; CV ≤ 15 %).

For intra-assay precision, the same pool of sera was prepared once and tested 6 times on the same plate as described in Example 10. The result of this parameter was a CV of 4.88 % (acceptance criteria CV ≤ 5.81 %).

For inter-assay precision, the pool of sera was prepared 6 times, and each time tested in a different plate-reference curve. All assays were performed by the same Technician following the method described in Example 10. The result of this parameter was a CV of 7.61 % (acceptance criteria CV ≤ 8.22 %).

Intermediate precision was calculated by testing the pool of sera with three different technicians on three different days, placing the sample twice in the same plate each day. The % CV obtained was 9.18 % (acceptance criteria CV ≤ 16.44 %).

Accuracy was calculated in the same dilution sample with a recovery of 96 % (acceptance criteria 90 – 110 %).

Finally, the specificity was determined after testing two sera with total IgE ≤ 1 kU/L. The result obtained was 0.00 ng sialic-IgE/mL of serum.

These results demonstrate that the new method is suitable for quantification of sialylated-IgE (sialic-IgE) in serum.

Example 12 – Quantitative determination of sialic acid bound to IgE in samples from patients with allergy and non-allergic subjects

The quantitative method described in Example 10 and validated in the Example 11 was used to analyse sera from patients clinically diagnosed of respiratory allergy (n=41) and sera from healthy subjects (n=26). The results are shown in Figure 10. The data shows a statistically significant difference between the samples from subjects with allergic disease versus those without allergic disease which showed lower values ($p < 0.001$ using Mann Whitney test). Thus, this method is useful for discriminating between allergic patients and non-allergic subjects. The setting of a reference value to distinguish allergic conditions needs the analysis of a higher number of subjects which requires clinical validation.

Example 13 – Lateral flow assay design

A Lateral Flow Assay device for detecting the presence of sialic-IgE in a test sample is illustrated in Figure 11. The device comprises a) a sample pad and/or a conjugate pad onto which the mixture of liquid gold conjugate and test sample is placed, b) a capture membrane comprising a test line where a SNA-1 lectin is fixed and a control line where a Goat anti-mouse IgG antibody is fixed to capture the target analyte-gold conjugate complex and the gold conjugate respectively, and (c) a reservoir region positioned at the end of the capture membrane for absorbing excess fluid (adsorbent pad).

Example 14 – Conjugation of anti-IgE monoclonal antibody with Gold nanoparticle (AuNP)

40 nm gold nanoparticles (BBI Solutions) were conjugated to a mouse anti-IgE antibody (Southern Biotech) via adsorption.

In particular, AuNP-anti-IgE conjugate was prepared adjusting the pH of a colloidal gold solution to 7 using 0.1 M borate buffer and adding antibody at final concentration of 2.5 µg mL^{-1}. The mixture was incubated at room temperature for 15 minutes to facilitate antibody binding. To block any remaining non-specific binding sites, a blocking protein (such us bovine serum albumin or casein) was added at 1 % (v/v) and antibody coated particles were incubated for an additional 30 minutes at room temperature. The conjugate was then purified by centrifugation at 14,000 x g for 10 minutes, and the pellet was resuspended in conjugation buffer. The washing step was repeated to ensure the removal of any antibody or protein excess. The final AuNP-anti-IgE conjugate was diluted in a buffer containing blocking protein, sugar and surfactant and stored at 4 °C until use.

Example 15 – Preparation of the lateral flow assay device

Stock solution of SNA-1 lectin (Vector laboratories) and goat anti-mouse IgG antibodies (Jackson Immunoresearch) were diluted in HEPES and phosphate dilution buffer to a concentration of 2 mg mL^{-1} and 1 mg mL^{-1} respectively and dispensed using an automated lateral flow reagent dispenser (XYZ3060 Dispense System, Biodot) onto a nitrocellulose membrane (200CNPH-N-SS60, Advanced Microdevices). Goat anti-mouse antibodies were loaded on the control line and SNA-1 lectin was loaded on the test line. After dispensing the capture biomolecules, the membrane was dried for 30 minutes at 37 °C and stored in a hermetically sealed bag with silica gel desiccant.

To assemble the test strips, all the membranes (sample pad, conjugate pad, nitrocellulose membrane and absorbent pad) were layered onto a laminated backing card (PS-SW31-40, KINBIO). The nitrocellulose and absorbent pad (CFSP002000, Merck) were positioned at the end of the card, overlapping each other by 3 mm. A glass fiber pad (ReliaFlowTM 6614, Ahlstrom-Munksjö) was used as a conjugate pad, overlapping the nitrocellulose membrane by

4 mm. The sample pad, BT03 blood separator (Shenzhen Sekbio), was then placed to overlap the conjugate pad by 2 mm.

Once assembled, the cards were cut into 4 mm wide strips using a ZQ2002 guillotine cutter (KINBIO) and encapsulated in a cassette.

Example 16 – Detection of purified sialylated IgE by lateral flow assay (LFA)

Figure 12 represents the capacity of the LFA device for detecting sialylated IgE in a buffer solution. Different concentrations (0-200 ng/mL) of purified sialic-IgE antibody were assayed by mixing 5 µL of sialic-IgE buffer solution with 5 µL of AuNP-anti-IgE conjugate. Once mixed, in order to remove the interferents present in the serum matrix, the mixture was centrifugated (5 minutes, 5,000 x g) and the pellet was diluted in 91 µL sample buffer (10 mM PB, 50 mM NaCl, 0.25 % Tween 20, 0.01 % ProClin-300, pH 7,4). Finally, this solution was loaded onto the sample pad of the LFA device. The conjugate and the sialic-IgE-conjugate complex migrated towards the strip by capillary flow. After 15 minutes, the test strip was inserted into a lateral flow reader (iPeak^{RTM}, IUL) to measure the control line (C; goat anti-mouse IgG) and test line (T; SNA-1 lectin) colour area and intensity. With these values the T/C ratio was obtained. The results obtained by LFA show linearity in the range of concentrations analyzed ($r2 = 0.98$).

Example 17 – Matrix effect is avoided by centrifugation

An example of samples that may be tested using the disclosed device include but are not limited to serum. In order to reduce the presence of agents in serum samples which could bind to SNA-1 lectin and block the test line binding sites, a centrifugation step was incorporated into the assay procedure.

The significance of including a sample-conjugate centrifugation process was evaluated to eliminate the potential interfering agents present in the sample. Two pools of sera with different sialylation levels previously determined by quantitative ELISA method were compared: pool low (0.04 ng sialic-IgE/mL) and pool medium (0.2 ng sialic-IgE/mL). For this purpose, 5 µL of each pool of sera was mixed with 5 µL AuNP-anti-IgE and the mixture was either centrifugated (5 minutes, 5,000 x g) or left unprocessed to assess the relevance of the

centrifugation step. Subsequently, both sets of samples were diluted in 91 µL of sample buffer (10 mM PB, 50 mM NaCl, 0.25 % Tween 20, 0.01 % ProClin-300, pH 7,4) and applied to the sample well. Figure 13 shows the responses obtained by analysing pools with the LFA including the centrifugation step (B) agreed with values previously determined by ELISA method. However, LFA without centrifugation (A) generated the same low responses for both pools.

Example 18 – Quantification of sialic-IgE in serum samples from allergic and non-allergic subjects

To determine the level of sialylated IgE in serum samples from allergic and non-allergic patients, the LFA response was analysed for 25 individual serum samples from patients with allergy (n=10) and from non-allergic subjects (n=15).

For each patient sample, 5 µL of serum was mixed with 5 µL of AuNP-anti-IgE conjugate. Once mixed, the mixture was centrifuged for 5 minutes at 5,000 x g. After removing the supernatant where interferences were present, 91 µL of sample buffer was added to the pellet (10 mM PB, 50 mM NaCl, 0.25 % Tween 20, 0.01 % ProClin-300, pH 7,4) and all the diluted sample was added onto the sample well. A colorimetric quantitative immunoassay reader (IUL) was used to measure the control line and test line colour intensity and the T/C ratio values were obtained for each serum sample. Figure 14 shows the T/C ratio values obtained for each tested sample (patients with allergy shown shaded and non-allergic subjects non-shaded).

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Claims

1. A method of quantifying sialylated IgE in a test sample, the method comprising:
immobilising sialylated IgE in the test sample via a capture probe which is specific for sialylated glycoproteins, wherein the capture probe is immobilised; and
using an antibody-based detection technique to quantify sialylated IgE.

2. The method according to claim 1, wherein the capture probe is SNA-1 lectin.

3. The method according to claim 1 or claim 2, wherein the capture probe is immobilised to the surface of a well of a plate.

4. The method according to any one of the preceding claims, wherein the antibody-based detection technique comprises a colorimetric assay.

5. The method according to any one of the preceding claims, wherein the antibody-based detection technique comprises the use of an IgE recognising antibody, optionally wherein the IgE recognising antibody is conjugated to any one of a horseradish peroxidase enzyme, gold nanoparticles, coloured latex beads, or carbon nanoparticles.

6. The method according to claim 5, wherein the method comprises the steps of:
   a) immobilizing SNA-1 lectin in the well of a plate, preferably an ELISA plate;
   b) adding a test sample and allowing any sialylated glycoproteins to bind to the immobilised SNA-1 lectin;
   c) adding an anti-IgE antibody conjugated to horseradish peroxidase to step (b) and allowing the antibody to bind to any bound sialylated IgE;
   d) adding a developing solution comprising a chromophore precursor and hydrogen peroxide, allowing oxidation of the chromophore precursor to a chromophore to occur, and detecting the production of the chromophore using spectrophotometry.

7. The method according to claim 6, wherein between step a) and step b) is a blocking step comprising addition of an inert substance which saturates the ELISA plate.

8. The method according to claim 6 or claim 7 wherein the chromophore precursor is tretramethylbenzidine.

9. The method according to any one of the preceding claims, wherein the method includes a step of obtaining a calibration curve based on a reference sample of IgE with a known amount of sialic acid in order to quantify the amount of sialylated IgE in the test sample in terms of the amount of sialic acid.

10. The method according to claim 5, wherein the method comprises the steps of:

a) immobilizing SNA-1 lectin on a nitrocellulose membrane;

b) separately mixing a test sample with an anti-IgE antibody conjugated with gold nanoparticles allowing the antibody to bind to any bound sialylated IgE;

c) centrifuging the mixture of step (b) until a pellet and clear supernatant is obtained and discarding the supernatant;

d) resuspending the pellet from step (c) with buffer solution to form a suspension and applying the suspension to the nitrocellulose membrane with immobilized SNA-1 lectin from step (a) or a pre-prepared nitrocellulose membrane with immobilized SNA-1 lectin;

e) visually detecting and quantifying the complex of sialylated-IgE and anti-IgE antibody conjugated with gold nanoparticles bound to the immobilized SNA-1 lectin.

11. The method according to any one of the preceding claims, wherein the test sample is blood serum or plasma from a subject, optionally wherein the blood serum or plasma is from a human subject or a non-human subject.

12. A diagnostic method for diagnosing allergy in a subject, the diagnostic method comprising using the method of claim 11 and diagnosing the subject with allergy based on the quantity of sialylated IgE in the test sample.

13. The diagnostic method according to any one of claims 6 to 9, wherein the test sample is blood serum from a human subject and the subject is diagnosed with allergy if the test sample comprises sufficient sialylated IgE such that, when tested according to the method the chromophore elicits an absorbance of at least 0.55 at 450 nm.

14. The diagnostic method according to claim 12 or claim 13 wherein if the subject is diagnosed with allergy, the diagnostic method further comprises a suitable clinical or *in vitro* method to determine if a subject is allergic to any allergy source.

15. A method of treatment of allergy or allergic disease, the method comprising the steps of:
diagnosis of allergy according to any one of claims 12-14; and
treatment by immunotherapy.

16. A kit comprising:
a capture probe which is specific for sialylated glycoproteins wherein the capture probe is either immobilised or is readily capable of immobilisation; and
an IgE recognising antibody;
optionally wherein the capture probe is SNA-1 lectin.

17. Use of a kit according to claim 16 to quantify sialylated IgE in a test sample.