



Ultrasensitive and rapid immuno-detection of human IgE anti-therapeutic horse sera using an electrochemical immunosensor

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ABSTRACT

Antivenom allergy disease mediated by patient IgE is an important public health care concern. To improve detection of hypersensitive individuals prior to passive antibody therapy, an amperometric immunosensor was developed to detect reactive human IgE. Whole horse IgG3 (hoIgG3) was immobilized onto the surface of carbon or gold screen-printed electrodes through a cross-linking solution of glutaraldehyde on a chitosan film. Sera from persons with a known allergic response to hoIgG3 or non-allergic individuals was applied to the sensor. Bound human IgE (humIgE) was detected by an anti-humIgE antibody through a quantitative amperometric determination by tracking via the electrochemical reduction of the quinone generated from the hydroquinone with the application of a potential of 25 mV. The optimal immunosensor configuration detected reactive humIgE at a dilution of 1:1800 of the human sera that represent a detection limit of 0.5 pg/mL. Stability testing demonstrated that through 20 cycles of a scan, the specificity and performance remained robust. The new immunosensor successfully detected humIgE antibodies reactive against hoIgG3, which could allow the diagnosis of potential allergenic patients needing therapeutic antivenom preparations from a horse.

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1. Introduction

Antivenoms are an efficient immunotherapeutic preparation that can prevent or reverse the clinical effects of circulating snake toxins [1]. With its appropriate administration, an antivenom can have a profound and rapid impact on the recovery of a victim [2,3]. They are normally composed of pepsin-refined immunoglobulin fragments, F(ab')₂, most often prepared from the plasma or serum of horses that were hyperimmunized with preparations of the particular toxin(s) in question. However, the foreign proteins comprising snake antivenoms can lead to severe life-threatening side effects, principally from immediate Type I hypersensitivity reactions. Worldwide, it has been estimated that 1–40% of the population suffer this problem [1,4–10]. However, this prevalence

could be higher due to the absence of an accurate diagnostic assay [11]. While the definitive cause of these reactions is unclear, it is most likely due to the presence of intact, whole horse immunoglobulins in the final preparations that lead to allergy-based responses mediated by patient immunoglobulin E (IgE).

The identification of circulating, reactive IgE antibodies would positively contribute to the correct diagnosis of an allergic response as a follow-up to antivenom therapy. Today, the diagnosis of antivenom allergy involves a patient history, physical examination, and confirmatory diagnostic testing to identify whether the patient's symptoms are of allergic or non-allergic origin. If an allergy is responsible for the symptoms, then the allergens responsible must be identified. The most relevant type of diagnostic testing for allergy is the provocation by the allergen. This type of testing is uncomfortable for the patient and can be dangerous. It is unadvised for patients taking certain drugs or those with skin problems. In addition, it cannot be performed for multiple allergens in a single examination [12]. With regards to allergies against therapeutic antivenoms, pre-testing is no longer recommended [13,14]. Also,

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the use of premedication before administration of antivenom is controversial and not widely practiced due to the lack of clear evidence that indicates a benefit from either steroids or antihistamines [15,16].

In human plasma, the IgG levels range from 8 to 16 mg/mL while the IgE level is much lower with a range of 1–400 ng/mL. This translates into a 10,000 to a 1-million-fold difference that complicates the development of an IgE specific diagnostic test. Advances in understanding the Type I hypersensitivity hold promise to improve in vitro techniques for allergy tests. Allergenic components of horse immunotherapeutic sera have been studied using sera from known allergic patients and was found to be restricted to the horse immunoglobulin subclass IgG3 [17]. Further refinement, through epitope mapping using overlapping synthetic peptides, revealed a prominent linear IgE-binding epitope to be represented by the amino acids of residues D177 to V189 [17]. Such an epitope could be of high diagnostic and clinical value as the basis for specific immunoassays for use in identifying patients susceptible to anaphylactic shock to antivenom therapy. However, the validation of the candidate epitopes is impeded by the lack of a rapid and reliable screening procedures, as well as the difficulty of generating epitope-specific immunochemical reagents by simple standard procedures. Therefore, with the goal of an epitope-based assay, it is mandatory to improve on existing in vitro or ex vivo allergy diagnostic systems. Among these assays, electrochemical immunosensors have received special attention due to their sensitivity, specificity, fast response, accuracy, simplicity, portability and low cost to use [18].

Electrochemical immunosensors determine the concentration of the analyte of interest by monitoring changes in the current [19–22] or changes in the electronic interfacial properties such as impedance [12,23,24] and capacitance [23] caused by the formation of immunocomplexes on the electrode surface. Unlike other techniques, electrochemical methods are not usually affected by sample impurities and the equipment required is relatively simple and of low cost. Most of the studies in immunosensors concentrate on the electrochemical detection using labeled immunogens, most commonly with enzymes, with or without nanoparticles to amplify the signal [25–27].

Here, we present the development of an immunosensing test based on electrochemical changes that represents an important improvement in the diagnosis of patients with allergies to therapies generated from horse immunoglobulin preparations. This IgG3-analytic system requires no standard curve and quantifies allergen-specific human IgE (humIgE) at sub-picogram concentrations. The horse IgG3 (holgG3) electrochemical sensor detects the presence of circulating specific IgE antibodies permitting rapid in vitro diagnosis or predisposition of IgE-mediated hypersensitivity.

2. Materials and methods

2.1. Materials

Disk-shaped (4.0 mm, Ø) screen-printed electrodes (SPEs) of carbon (SPCE/110) or gold (SPGE/220AT) with a counter electrode of the same material as the working electrode, carbon or gold, and a silver pseudo-reference electrode were obtained from DropSens. Horse serum was supplied by the National Institute of Quality Control of FIOCRUZ (Rio de Janeiro, Brazil). Goat anti-human IgE (anti-humIgE) immunoglobulin labeled with alkaline phosphatase was purchased from Kirkegaard & Perri Laboratories. SuperSignal West Pico chemiluminescent substrate from Life Technologies. Glutaraldehyde, hydroquinone, chitosan (Ch) and other chemicals were obtained from Sigma-Aldrich. All solutions were prepared

with ultrapure water obtained from a Milli-Q System (Millipore).

2.2. Serum sampling

The human serum that served as the source for IgE antibodies was collected from four selected patients, who had been victims of one or more snake bites and presented with a severe anaphylactic reaction when treated with three injections of horse antivenom (polyvalent anti-bothropic sera). The allergic response was confirmed by clinical history, a positive response to horse immunoglobulin and characterization by measuring specific IgE reactivity [17]. For non-reactive IgE controls, sera were collected from healthy individuals ($n = 5$) that never received a horse sera-based therapy. Informed consent was obtained from all patients and volunteers. Our study was approved by the Ethics Committee of the Santa Casa de Misericórdia do Rio de Janeiro (028/10) and Fernandez Figueira Hospital (FIOCRUZ, 0071/10).

2.3. Purification of horse IgG3

Ho-IgG3 was purified by affinity chromatography using jacalin-Sepharose CL4B resin as described previously [28]. Protein concentration was estimated using a percent extinction coefficient (ϵ percent) of 13.6 @ 280 nm.

2.4. Preparation of SPEs with a chitosan film containing horse IgG3

Ch films were prepared as previously described [27]. Briefly, a Ch solution was prepared with 10 mg of Ch powder dissolved in 1.0 mL of acetic acid (1.0% v/v) under stirring for 3 h at room temperature until completely dissolved, then drops of NaOH (1 mol/L) were added to a pH of 7.0. The solution was stored at 4 °C.

A Ch film was formed by placing 10 μ L of the Ch solution (0.1 mg/mL) onto the surface of an electrode followed by the addition of 10 μ L of an holgG3 solution (0.13 mg/mL, 0.26 mg/mL or 1.0 mg/mL). Electrodes stood 2 h at room temperature until the solution fully evaporated. Next, an additional 10 μ L of holgG3 at the same concentration was added followed by 10 μ L of a solution of glutaraldehyde (2.5%, v/v) to crosslink antibodies to Ch.

2.5. Application of the IgE-binding immunosensor

After immobilization of holgG3, the carbon SPE or gold SPE sensors were exhaustively washed with PBS prior to the placement of 20 μ L of patient serum at different dilutions (1:50, 1:300, 1:600, 1:1200 or 1:1800 in PBS). Next, electrodes were incubated for 1 h at 37 °C followed by two washes in PBS. Subsequently, alkaline-phosphatase (AP) labeled goat anti-hum-IgE (1:5000 in PBS) was added and the electrode incubated at 37 °C. After 1 h, the electrode was washed with PBS and hydroquinone (1.4 mM in PBS, pH 7.4) added. Measurements were obtained from cyclic voltammetry (CV) spectra at a scan rate of 0.01–0.025 V/s with a potential ranging from –0.6–0.9 V using an Ivium Compact Stat Potentiostat/Galvanostat (Ivium Technologies) interfaced with a portable notebook controlled by Ivium Soft software. Electrodes were connected through a three-electric contacts edge connector for single-working models from DropSens [29].

3. Results

3.1. Generation of a horse IgG3 immunosensor

The design of an immunosensor to detect humIgE against holgG3 was based on the use of an SPE with a working electrode made of either carbon or gold, a counter electrode and a reference

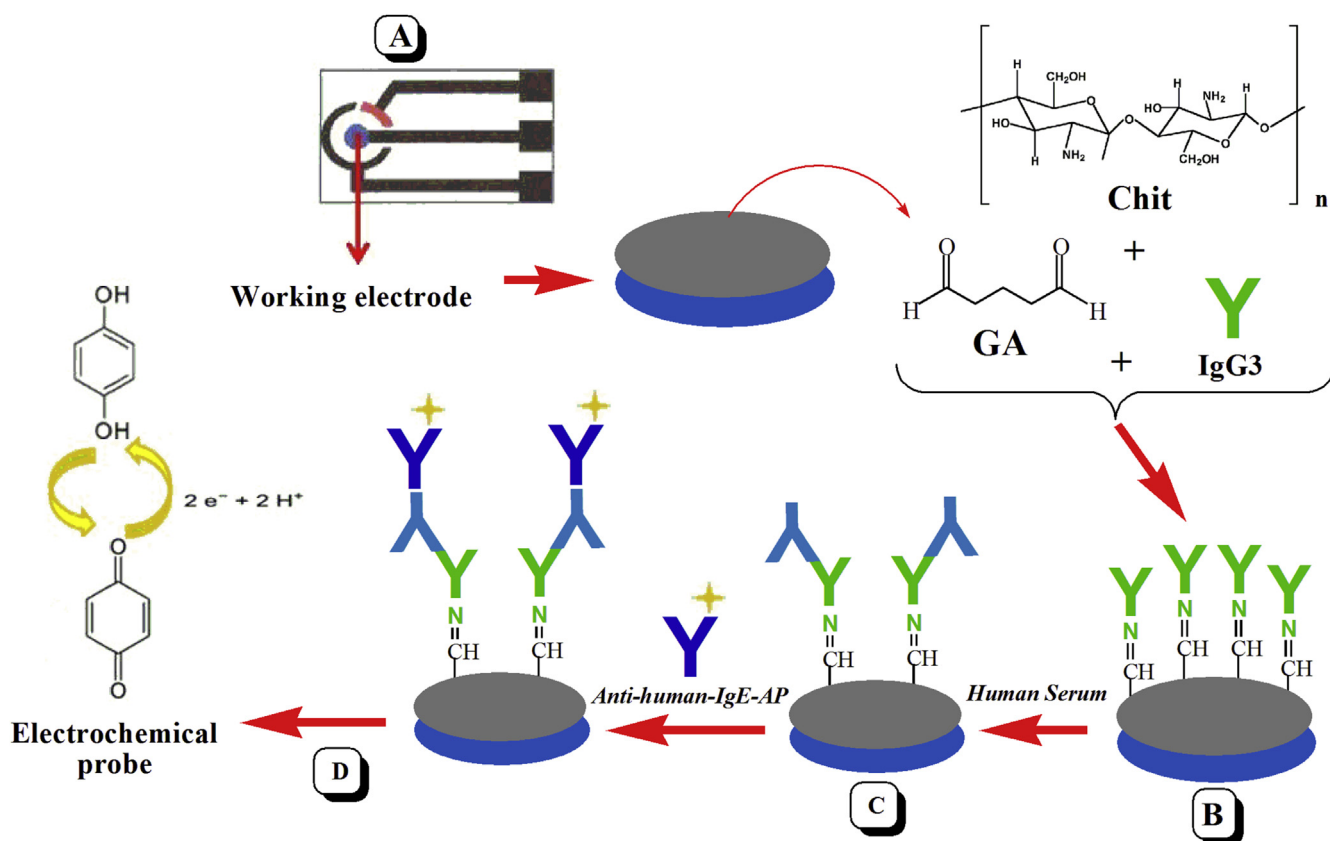


Fig. 1. Schematic representation of the electrochemical immunosensor for the detection of humIgE that bind holgG3. Panel A shows a top view of the SPEs with a silver reference electrode (top), the disk-shaped electrode of carbon or gold (middle) and the counter electrode (bottom). Panel B shows a side view of the SPE with the electrode in black and in gray the deposition of a Ch film by the combination of a Ch solution (Chit) with holgG3 (IgG3) and glutaraldehyde (GA). Panel C shows the progression of generating a signal by the binding of immobilized holgG3 with humIgE (present in the patient serum) followed by an anti-humIgE AP-labeled secondary and (D) the application of an electrical potential. Since the potential liberates two (e^-) from quinone or two (H^+) from the reduction of hydroquinone, a change in current can be measured.

electrode (Fig. 1A). A film of Ch was deposited over the working electrode through a two-step drying protocol [30]. Since holgG3 is a four-chain glycoprotein containing about 2–3 times more carbohydrate than the other horse immunoglobulin [31], it was possible to preferentially cross-link it, in addition to its prior purification by affinity chromatography, to the Ch monolayer through glutaraldehyde (Fig. 1B). With the addition of serum allergic patient (+IgE), this interacts with immobilized holgG3 on the surface of the electrode (Fig. 1C), then anti-humIgE-AP is added to investigate only the allergic immunoglobulin present in human serum. Finally, an electrochemical probe of hydroquinone is used to trace the reaction (Fig. 1D). From the redox reaction with the electrochemical reduction of the quinone generated from the hydroquinone by applying the potential of 250 mV.

In the absence of humIgE, the immunosensor with a Ch film containing holgG3 defined the baseline CV spectra in the presence of hydroquinone and unbound secondary antibodies (Fig. 2, solid line). The measured CV was approximately 10 times greater than in the absence of holgG3 in the Ch film (Fig. 2, dotted line).

3.2. Performance analysis of the IgG3 immunosensor

The behavior of the immunosensor was evaluated against increasing concentrations of the target antigen, holgG3, in the Ch film and exposure to a fixed dilution (1:50) of patient sera positive for IgE against holgG3. The CV spectra are presented in Fig. 3. In comparison to the control conditions (The measured peak current

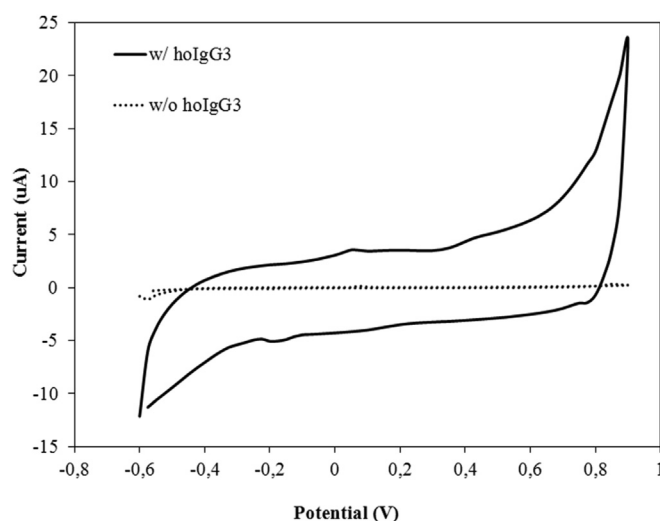


Fig. 2. Deposition of a Ch film containing horse IgG3 sensitizes carbon SPEs. A carbon SPE was coated with a Ch film containing 20 $\mu\text{g/mL}$ of ho-IgG3 entrapped by glutaraldehyde (w/ho-IgG3) or maintained uncoated (w/o ho-IgG3). The presence of the Ch film and ho-IgG3 on the surface of the carbon SPE generated a typical CV spectrum (solid line) and was tracked with a phosphate buffered solution. Addition of AP-labeled anti-humIgE and hydroquinone to uncoated carbon SPEs showed no current (dotted line). Scan rate was 0.025 V/sec over a potential range of -0.6 – 0.9 V.

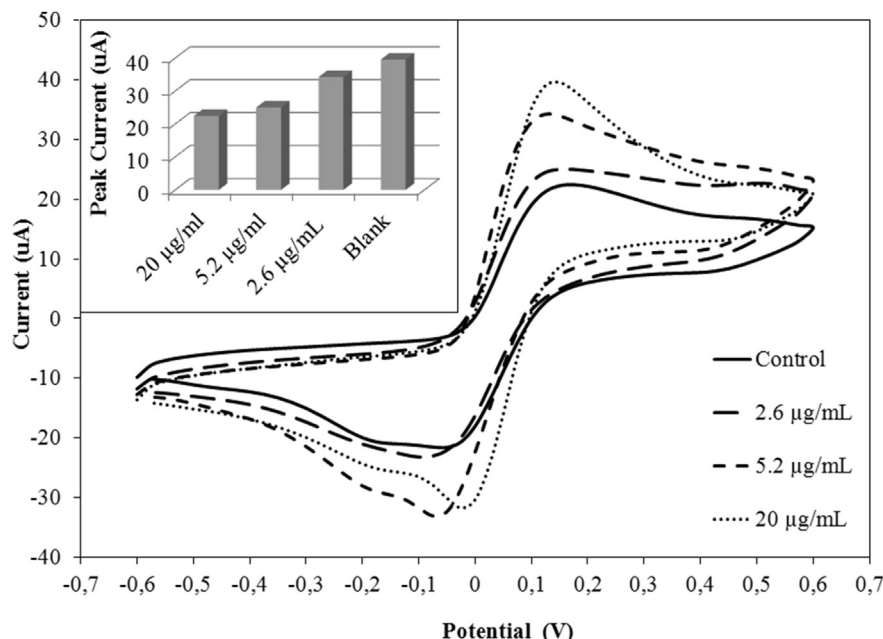


Fig. 3. Cyclic voltammetry spectra of a carbon SPE coated with Ch film and increasing concentrations of holgG3. Gold SPEs were coated with a Ch film containing different concentrations of holgG3 probed with a 1:50 dilution of sera from an allergic patient and exposed to anti-humIgE AP-labeled secondary antibody with hydroquinone. CV spectra showed decreasing current with increasing concentrations of ho-IgG3: 2.6 $\mu\text{g/mL}$ (dashed line); 5.2 $\mu\text{g/mL}$ (dash dotted line); 20 $\mu\text{g/mL}$ (dotted line); and no patient sera (solid line). Scan rate was 0.01 V/s over a potential range of -0.6 – 0.9 V.

values increased proportionally to a decreasing concentration of the holgG3 antigen deposited at the working electrode; 20 μA at 20 $\mu\text{g/mL}$ (dotted curve), 26 μA at 5.2 $\mu\text{g/mL}$ (dash and dotted curve) and 35 μA at 2.6 $\mu\text{g/mL}$ (dashed curve). The observation of decreasing peak current can be attributed to hu-IgE binding holgG3 at the electrode and the formation of enzymatically active immunocomplexes with AP-labeled secondary to humIgE that partly shielded the transduction of current due to the physical blockade at the working electrode, i.e., the larger the immunocomplex formation, the lower the current flow in this working electrode, thereby lowering the peak of oxidation current of the hydroquinone.

3.3. Detection limit and specificity of the horse IgG3 immunosensor

The limit of detection for the immunoassay was investigated by maintaining a fixed concentration of holgG3 (20 $\mu\text{g/mL}$), AP-labeled secondary and hydroquinone (1.4 mmol/L) over a dilution series of sera from a patient positive for anti-holgG3 IgE. The CV spectrum for each of the dilutions was measured along with the spectra in the absence of hu-IgE (PBS only) for each electrode. The peak current for each condition, with sera (open column) and no serum (solid column) is plotted in Fig. 4. At the lowest dilution of 1:300, the presence of humIgE showed the greatest difference in peak current as expected. This difference decreased at the higher dilutions of 1:600, 1:1200 and 1:1800. At the highest dilution, the delta value of the current (Δi) obtained by subtracting the experiment curve by the control was 2.81 μA . Since the concentration of IgE in allergenic and asymptomatic patients can be in the range of low pg/mL, the difference in the plotted peak current shows the sensitivity of the biosensor. When maintaining a fixed concentration of ho-IgG3 in the Ch film and the AP-anti-humIgE conjugate, the detection limit of the decreasing electrical current with increasing dilution of the sera was calculated to be 0.5 pg/mL of IgE at 3σ , which was in good agreement detection limits with the reference ELISA (data not shown).

The specificity of the immunosensor was examined by

comparing the current response to sera from a patient positive for IgE against holgG3 and sera from a healthy under the same conditions with a dilution of 1:600. Fig. 5 shows the CV spectra from the application of allergic patient sera (solid line), healthy sera (dash and dotted line) and no human antibodies (PBS; dotted line). In this situation, can be observed that although a small decrease occurs in the oxidation peak, healthy sera, when compared with analysis of no human antibodies (PBS; Blank), the interaction with holgG3 is much greater when analyzing allergic patient sera, i.e., the peak current value is lower when the patient positive for IgE.

3.4. Reproducibility, repeatability, and stability of the immunosensor response

The reproducibility of the immunosensor AP-anti-humIgE-serum–holgG3–CH–Ca was estimated through four independent immunosensor preparations using 20 $\mu\text{g/mL}$ holgG3 and exposing each to the same dilution of patient sera followed by AP-labeled secondary and hydroquinone. The four electrodes displayed similar current responses with a calculated relative standard deviation of 3.6% (data not shown). Likewise, under these same preparation and application conditions, the repeatability of the immunosensor was tested by 20 successive scan cycles. The CV spectrum from each scan is plotted in Fig. 6. The calculated relative standard deviation was 3.7% for the peak current. When immunosensors were immersed in PBS at 4 $^{\circ}\text{C}$ for 2 weeks, the daily measured current response of an immunosensor using fresh hydroquinone retained over 91% of its initial responses (data not shown).

4. Discussion

Passive antibody therapy (PAT) is highly effective for the immediate treatment of a variety of pathological conditions. In the case of snake bites, PAT has the additional advantage of containing a diverse mixture of antibodies that are capable of neutralizing the

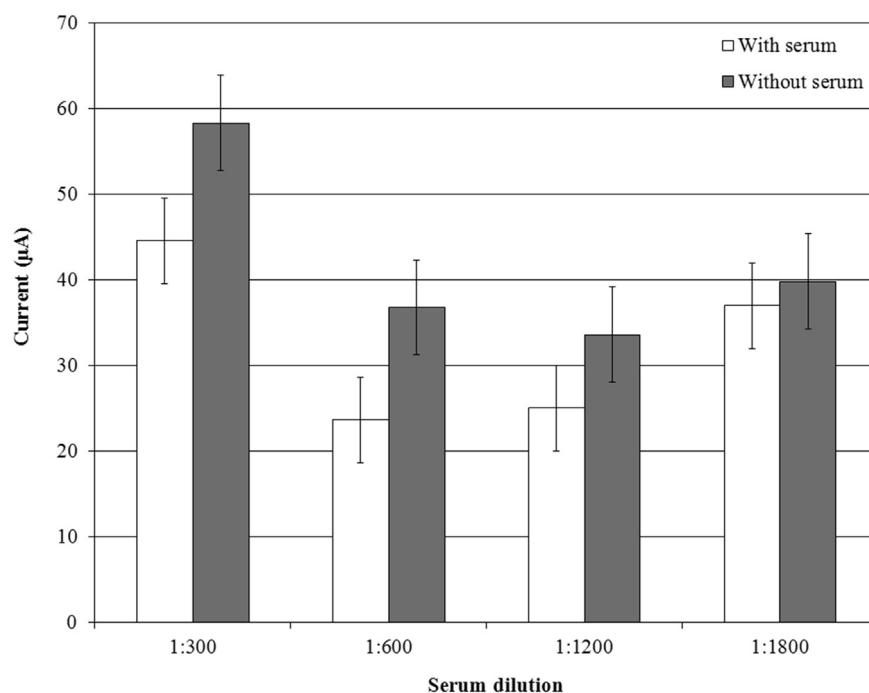


Fig. 4. Target analyte alters the cyclic voltammetry spectra of sensitized carbon SPEs. Carbon SPEs were coated with Ch film and holG3 (20 µg/mL) prior to exposure to increasing dilutions of IgE patients positive sera followed by anti-humIgE AP-labeled secondary antibody and hydroquinone. The CV spectra from each dilution (1:300; 1:600; 1:1200; and 1:1800) was used at each dilution, the peak current with patient sera (open column) was plotted next to no sera (solid column). Scan rate was 0.01 V/s over a potential range of -0.6 – 0.9 V.

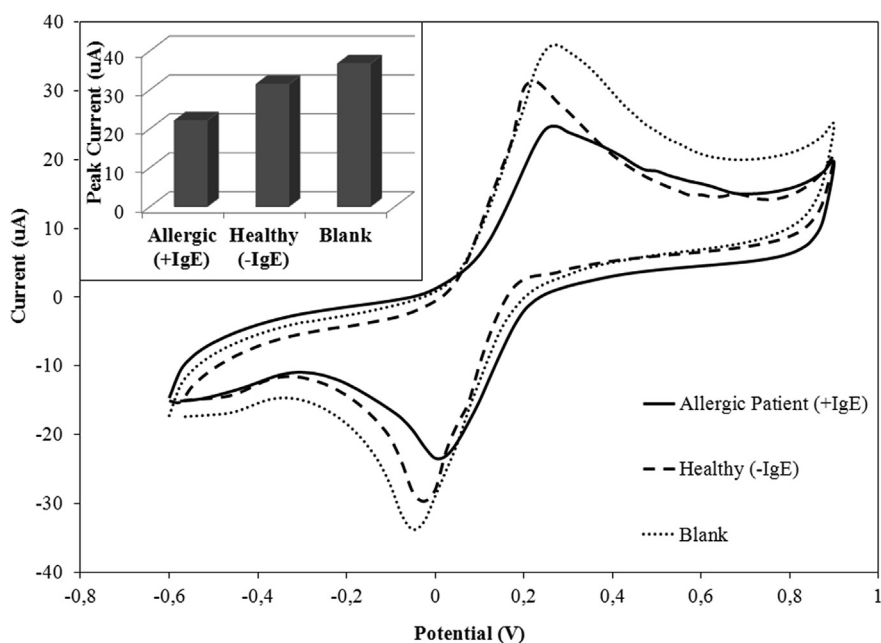


Fig. 5. Sensitized carbon SPEs recognizes reactive human IgE sera. Carbon SPEs were coated with a Ch film containing 20 µg/mL holG3 and exposed to a 1:600 dilution of sera from patients positive for anti-holGE (solid line), healthy patients (B, dashed line) or PBS (dotted line). CV spectra were collected after addition of anti-humIgE AP-labeled secondary antibody and hydroquinone using a scan rate of 0.01 V/s over a potential range of -0.6 – 0.9 V.

complex mixture of proteases that mediate the deleterious effects of the venom. Most preparations of PAT utilize horse serum as the source of the immunoglobulins that provide the therapeutic activity. However, the use of horse antibodies as a therapy in itself involves the introduction of foreign proteins that can be recognized by the immune system of the patient. The least severe reaction is

serum sickness caused by the induction of an immune response against the horse proteins 7–10 days after application of the PAT. Of greater concern is the potential for a rapid, allergic response against the PAT and anaphylactic shock. Despite its life-threatening consequences, patients normally are not screened for allergies prior to administration of PAT. To advance current technologies, we have

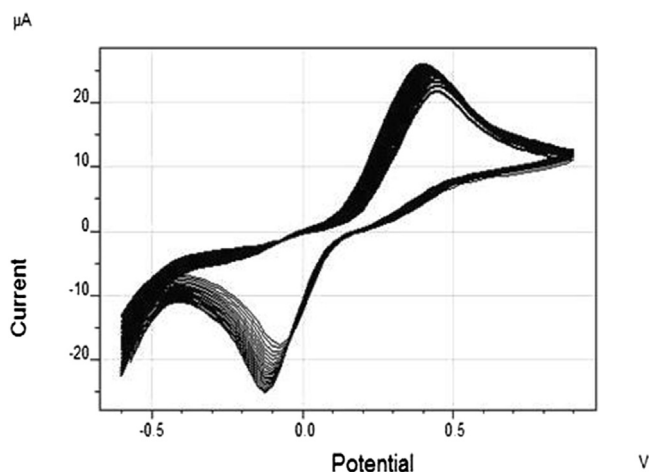


Fig. 6. Cyclic voltammetry spectra drift of activated carbon SPEs over successive scans. A carbon SPE was coated with a Ch film containing 20 µg/mL holgG3 and exposed to a 1:1200 dilution of serum from a patient positive for anti-ho-IgE followed by anti-humIgE AP-labeled secondary and hydroquinone. CV spectra were plotted from 20 successive scans at a scan of 0.01 V/s over a potential range of −0.6–0.9 V.

developed an immunosensor to detect reactive IgE in patient sera.

For an immunoassay, the antigen is the key factor to provide high sensitivity and specificity. Recently, we reported that holgG3 is the major protein allergen in equine sera used to prepare antivenom therapies [17]. While the specific linear B-cell epitope involved was limited to 177DVLFTWYVDGTEV189, the entire holgG3 molecule was chosen here as the target over the peptide. The whole immunoglobulin was advantageous due to its compatibility with cross-linking to Ch by glutaraldehyde as well as the ease and high yield from a lectin-based purification protocol [29].

To improve cost and portability, this target was combined with an SPE-based platform that utilized the redox cycling properties of hydroquinone after conversion by alkaline phosphatase activity. Hydroquinone has several attractive characteristics for use in electrochemical immunoassays. First, its electrochemical characteristics allows it to be oxidized to quinone at a low potential, which is important for its detection in the presence of biological components that can interfere at higher potentials. Another aspect is it avoids electrode passivation during cyclic voltammogram measurements in neutral or alkaline solutions [25]. Finally, the decreasing amperometric signal caused by oxidation of the hydroquinone can be significantly enhanced by the recycling of quinone [32].

Numerous tests were performed to evaluate the electrochemical detection of patient IgE reactive for holgG3. A comparison between carbon and gold SPE showed no performance differences. The working electrode could be sensitized by the deposition of a Ch film with the sequential inclusion of holgG3 and cross-linking by glutaraldehyde. The binding capacity of the Ch film for the capture molecule did not appear to be saturated by a 20 µg/mL solution of holgG3, which showed a good response (Fig. 3). In addition, the Ch film did not appear to leach holgG3 during storage for two weeks in PBS at 4 °C or during repeated scans of CV. In practice, the background current provided a direct means to evaluate that all the components necessary for a valid measurement were correctly incorporated in the immunosensor since it depended on the presence of holgG3, AP-labeled secondary and hydroquinone.

The performance of the immunosensor to detect reactive humIgE showed high selectivity when compared to patient sera without reactive IgE. It also displayed high sensitivity with a detection limit approaching 0.5 pg/mL. At this level of sensitivity, it

suggests that this immunosensor could not only identify allergic patients prior to PAT administration, it can be used as to confirm diagnoses of antivenom hypersensitivity as well as reactivity against various other PATs prepared in equine. The mechanism of the immunosensor was dependent on the interaction of the holgG3 with IgE antibodies from allergenic patient's sera, which decreased the current measured at the working electrode during CV. The reduction in the electrical current signal was a key factor for the development of this device. The excellent performance of reproducibility and sensitivity exhibited by the immunosensor indicates that this methodology could be practical and inexpensive. In addition, the electrochemical ho-IgG3 testing methods developed here appear to be superior conventional ELISA or enzymatic spectrophotometric methods due to the short detection time and the absence of pretreatment steps. Normally, 20 s were needed for measurements from the electrochemical method compared to about 10 min for spectrophotometric method and 3 h for an ELISA, both of which require sample preparations.

Ultimately, we have developed a sensor capable of specifically measuring humIgE reactive against holgG3 at high sensitivity that can be indicative of anaphylactic reactions. To our knowledge, this is the first time an SPE platform has been sensitized to directly detect humIgE. We are currently exploring methods for fabricating and processing carbon/gold platforms into array formats to combine high sensitivity, reproducibility and reliability for multiplexed biomarkers.

Conflict of interest

The authors declare that part of the results of this paper compose a patent under evaluation.

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Abbreviations

(IgE)	Immunoglobulin E
(humIgE)	human IgE
(ho-IgG3)	horse IgG3
(SPEs)	screen-printed electrodes
(Ch)	chitosan
(AP)	alkaline-phosphatase
(CV)	cyclic voltammetry

References

- [1] P.A. Christensen, Snakebite and the use of antivenom in Southern, Afr. S. Afr. Med. J. 59 (1981) 934.
- [2] J.M. Gutiérrez, R.D.G. Theakston, D.A. Warrell, Confronting the neglected problem of snake bite envenoming: the need for global partnership, PLoS Med. 3 (2006) 727.
- [3] G.K. Isbister, S. Shahmy, F. Mohamed, C. Abeyasinghe, H. Karunatilake, et al., A randomized controlled trial of two infusion rates to decrease reactions to antivenom, PLoS One 7 (2012) c38739.
- [4] P.W. Coetzer, C.R. Tilbury, The epidemiology of snakebite in northern, Natal. S. Afr. Med. J. 62 (1982) 206.
- [5] P. Consroe, N.B. Egen, F.E. Russel, K. Gerrish, D.C. Smith, et al., Comparison of a new ovine antigen binding fragment (Fab) antivenin for United States Crotalidae with the commercial antivenin for protection against venom-induced lethality in mice, Am. J. Trop. Med. Hyg. 53 (1995) 507.
- [6] C.S. Kitchens, L.H.S. Van-Mierop, Envenomation by the eastern coral snake *Microbus fulvus fulvus*, a study of 39 victims, J. Am. Med. Ass 258 (1987) 1615.

- [7] N.F. Moran, W.J. Newman, R.D.G. Theakston, D.A. Warrell, D. Wilkinaon, High incidence of early anaphylactoid reaction to SAIMR polyvalent snake antivenom, *Trans. R. Soc. Trop. Med. Hyg.* 92 (1998) 69.
- [8] S. Rawat, G. Laing, D.C. Smith, D. Theakston, J. Landon, A new antivenom to treat eastern coral snake (*Micrurus fulvius fulvius*) envenoming, *Toxicon* 32 (1994) 185.
- [9] F.E. Russell, *Snake Venom Poisoning*, Great Neck: Scholium International, 1983, p. 235.
- [10] D. Wilkinson, Retrospective analysis of snakebite at a rural hospital in Zululand, *S. Afr. Med. J.* 84 (1994) 844.
- [11] S.G. De-Simone, P. Napoleão-Pêgo, L.A.L. Teixeira-Pinto, J.D.T. Santos, T.S. De-Simone, et al., Linear B-cell epitopes in BthTX-1, BthTX-II and BthA-1, phospholipase A2's from *Bothrops jararacussu* snake venom, recognized by therapeutically neutralizing commercial horse antivenom, *Toxicon* 72 (2013) 90.
- [12] W. King, Efficacy of a screening radioallergo sorbent test, *Arch. Otolaryngol* 108 (1982) 781.
- [13] B.J. Currie, E. Canale, G.K. Isbister, Effectiveness of pressure-immobilization first aid for snakebite requires further study, *Emerg. Med. Australas.* 20 (2008) 267.
- [14] A. Thiansookon, P. Rojnuckarin, Low incidence of early reactions to horse-derived F(ab')₂ antivenom for snakebites in Thailand, *Acta. Trop.* 105 (2008) 203.
- [15] H.A. de Silva, A. Pathmeswaran, C.D. Ranasinha, S. Jauamane, S.B. Samarakoon, et al., Low-dose adrenaline, promethazine, and hydrocortisone in the prevention of acute adverse reactions to antivenom following snakebite: a randomized, double-blind, placebo-controlled trial, *PLoS Med.* 8 (2011) c1000435.
- [16] I.B. Gawarammana, S.A.M. Kularatne, W.P. Dissanayake, R.P.V. Kumarasiri, N. Senanayake, et al., Parallel infusion of hydrocortisone G chlorpheniramine bolus injection to prevent acute adverse reactions to antivenom for snakebites. A randomized, double-blind, placebo-controlled study, *Med. J. Aust.* 180 (2004) 20.
- [17] S.G. De-Simone, P. Napoleão-Pêgo, L.A.L. Teixeira-Pinto, A.R. Melgarejo, A.S. Aguiar, et al., IgE and IgG epitope mapping by microarray peptide-immunoassay reveals the importance and diversity of the immune response to the IgG3 equine immunoglobulin, *Toxicon* 78 (2014) 83.
- [18] N.G. Dolinnaya, M.R. Jan, A.N. Kawde, T.S. Oretskaya, V.N. Tashlitsky, et al., Electrochemical detection of abasic site-containing DNA, *Electroanalysis* 18 (2006) 399.
- [19] M. Campàs, J.L. Marty, Enzyme sensor for the electrochemical detection of the marine toxin okadaic acid, *Anal. Chim. Acta* 605 (2007) 87.
- [20] Z. Dai, F. Yan, J. Chen, H.X. Ju, Reagent less amperometric immunosensors based on direct electrochemistry of horseradish peroxidase for determination of carcinoma antigen-125, *Anal. Chem.* 75 (2003) 5429.
- [21] R.E. Ionescu, S. Cosnier, S. Herrmann, R.S. Marks, Amperometric immunosensor for the detection of anti-West Nile virus IgG, *Anal. Chem.* 79 (2007) 8662.
- [22] J. Wu, J.H. Tang, Z. Dai, F. Yan, H.X. Ju, et al., A disposable electrochemical immunosensor for flow injection immunoassay of carcinoembryonic antigen, *Biosens. Bioelectron.* 22 (2006) 102.
- [23] C. Fernández-Sánchez, C.J. McNeil, K. Rawson, O. Nilsson, Disposable noncompetitive immunosensor for free and total prostate-specific antigen based on capacitance measurement, *Anal. Chem.* 76 (2004) 5649.
- [24] L. Alfonta, I. Willner, D.J. Throckmorton, A.K. Singh, Electrochemical and quartz crystal microbalance detection of the cholera toxin employing horseradish peroxidase and GM1-functionalized liposomes, *Anal. Chem.* 73 (2001) 5287.
- [25] S. Michael, M.S. Wilson, R.D. Rauh, Hydroquinone diphosphate: an alkaline phosphatase substrate that does not produce electrode fouling in electrochemical immunoassays, *Biosens. Bioelectron.* 20 (2004) 276.
- [26] W.J. Parak, D. Gerion, T. Pellegrino, D. Zancher, C. Micheel, S.C. William, R. Boudreau, M.A. Le Gros, C.A. Larabell, A.P. Alivisatos, Biological applications of colloidal nanocrystals, *Nanotechnology* 14 (2003) R15.
- [27] A.G. Tkachenko, H. Xie, D. Coleman, W. Glomm, J. Ryan, et al., Multifunctional gold nanoparticle-peptide complexes for nuclear targeting, *J. Am. Chem. Soc.* 125 (2003) 4700.
- [28] S.G. De-Simone, A.L.A. Souza, A.R. Melgarejo, A.S. Aguiar, D.W. Provance-Jr, Development of ELISA assay to detect specific human IgE anti-therapeutic horse sera, *Toxicon* 138 (2017) 37.
- [29] I.C. Prado, M.E. Chino, A.L. Santos, A.L.A. Souza, L.P. Gomes, E.R. Lemos, S.G. De Simone, Development of an electrochemical immunosensor for the diagnostic testing of spotted fever using synthetic peptides, *Biosens. Bioelectron.* 100 (2018). <http://dx.doi.org/10.1016/j.bios.2017.08.029>.
- [30] V.E. Gómez, D.H. Santos, M.B.G. García, J.M.P. Carrazón, A.C. García, Simultaneous detection of free and total prostate specific antigen on a screen-printed electrochemical dual sensor, *Biosens. Bioelectron.* 24 (2009) 2678.
- [31] R.S. Nezhlin, *The Immunoglobulins: Structure and Function*, Academic Press, San Diego, USA, 1998, 269p.
- [32] F. Mizutani, S. Yabuki, M. Asai, Highly-sensitive measurement of hydroquinone with an enzyme electrode, *Biosens. Bioelectron.* 6 (1991) 305.