

Viability of allergy (IgE) detection using an alternative aptamer receptor and electrochemical means

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Abstract

The current work describes the systematic development of a disposable electrochemical sensor based on an aptamer receptor (aptasensor). The optimal conditions for which aptamers perform have been thoroughly investigated by Enzyme Linked OligoNucleic Assay (ELONA) and manipulated to achieve sufficient sensitivity in the final sensor set-up. Parameters investigated included buffer type, reagent concentration profiling, pH, ionic effect and thermo-stability. Method transfer then allowed for the preparation of the aptasensor utilising differential pulse voltammetry as the transduction mode. The principle system chosen was for the detection of allergy (IgE), which is present in humans due to exposure to a multitude of allergens in food and/or the environment. The system incorporates a competitive format for IgE detection using a biotinylated form of the aptamer. Enzymatic role is completed by the subsequent use of an extravidin-alkaline phosphatase label for either colorimetric or electrochemical detection. Through the careful choice of conditions the aptasensor functions at levels suitable for human testing ($>300 \text{ ng ml}^{-1}$) and exhibits sensitivity similar to antibody-based sensors of this type. The thermo-stability of the aptamer is significantly better than a corresponding IgE antibody receptor. In addition the sensor using this aptamer receptor is robust ($\text{CV} < 5\%$) and varies little from day-to-day (R.S.D. 8%). Recoveries of $100 \pm 5\%$ or better were obtained for two types of ELONA. The facet that the final sensor is a disposable strip makes it a suitable candidate for decentralisation of assaying away for the laboratory environment to a point-of-care device.

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1. Introduction

Allergy is a mysterious disease. It is a chronic condition and is the commonest cause of absenteeism. It can be caused by anything one inhales, eats or touches. The term allergy describes the situation of “changed reactivity” to antigen stimulation irrespective of whether this resulted in immunity (protection) or hypersensitivity. It can be defined as inappropriate or exaggerated reactions of the immune system to substances that in the majority of people cause no symptoms. Symptoms of the allergic diseases may be caused by exposure of the skin to a chemical, the respiratory system to particles of dust or pollen (or other substances) or the stomach and intestines to a particular food. Immunoglobulin E (IgE), though a trace serum protein, is found on the surface membrane of basophiles and mast cells

in all individuals, making these cells highly sensitive to stimulation by IgE antibody–allergen interaction. This reaction is the key to IgE-mediated allergy. Total IgE serum level is widely reported as a marker of atopic diseases, such as asthma, dermatitis, and pollenosis [1]. In allergic patients, serum total IgE levels, in general, corresponds to the severity of the allergic disease and may be therefore used for monitoring of all kinds of anti-allergic therapy or allergen elimination. Non-allergic, normal individuals have IgE concentrations that vary widely and increase steadily during childhood, reaching their highest levels at age 15–20, and thereafter remaining constant until about age 60, when they slowly decline. In adults, normal concentrations of IgE may reach $240\text{--}288 \text{ ng ml}^{-1}$.

The majority of allergy testing nowadays is based on the antibody receptor. Commercially available quantitative test kits for serum IgE are based on traditional sandwich immunoassay techniques (ELISA) and use two antibodies specific to different epitopes of the IgE molecule. The minimum detectable concentration of such assays was estimated at 12 ng ml^{-1} [2–4]. Another antibody-based approach involves the use of piezoelectric crystals, where anti-IgE antibody immobilised on gold or

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silver-coated crystal serves as the receptor layer [5,6]. A similar format was employed using a surface plasmon resonance sensor that could detect IgE in a linear range from 12 to 720 ng ml⁻¹ [7]. Anti-IgE antibodies were also used for the development of an immunosensor that could determine IgE in blood samples (LOD 0.09 ng ml⁻¹) using screen-printed carbon electrode technology and amperometric detection [8].

This study describes the development of a disposable screen-printed electrode sensor for quantification of human IgE, based on an alternative receptor other than an antibody. The sensor uses an aptamer (D17.4ext) and detection is achieved by differential pulse voltammetry (DPV). The same format was also applied to an ELISA-type assay (ELONA) for preliminary optimisation work. Both systems were able to detect normal and elevated levels of human IgE with high sensitivity and reproducibility and these initial findings show promise in replacing antibodies as receptors in affinity sensors for allergy testing.

2. Experimental

2.1. Chemicals and reagents

Monoclonal human IgE antigen was purchased from DIATEC.com (Oslo, Norway) and the biotinylated aptamer D17.4ext (45-mer, 5'Biotin GCG CGG GGC ACG TTT ATC CGT CCC TCC TAG TGG CGT GCC CCG CGC 3') from RiNA GmbH (Berlin, Germany). Anti-IgE antibody species and IgE mAb were purchased from Biospecific (CA, USA). *p*-Aminophenyl phosphate (*p*-APP) was from Universal Sensors, Ireland. Extravidin-alkaline phosphatase conjugate (xAv-AP), 4-nitrophenyl phosphate di(2-amino-2-ethyl-1,3 propane-diol) salt (*p*-NPP), bovine serum albumin (BSA), anti-mouse IgG-alkaline phosphatase (IgG-AP), rabbit IgG, sodium carbonate (Na₂CO₃), sodium chloride (NaCl), magnesium chloride (MgCl₂), potassium chloride (KCl), diethanolamine (DEA), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), tris(hydroxymethyl)amino methane (Trisma base), and polyoxyethylene-sorbitan monolaurate (Tween-20), were purchased from Sigma (Dublin, Ireland). Di-sodium hydrogen phosphate anhydrous (Na₂HPO₄) and potassium di-hydrogen phosphate (KH₂PO₄) were obtained from Fluka. Deionised water was produced with an Elix Millipore system (Millipore S.A., Molsheim, France).

2.2. Apparatus

Polystyrene, flat bottom Falcon microtitre plates were purchased from Becton Dickinson Labware (NJ, USA). The ELISA spectrophotometer was a Microplate Autoreader (model EL311) from Bio-Tek Instruments Inc. (VT, USA). Incubations at elevated temperatures were carried out in a thermostat oven supplied by Heraeus Instruments. Three-electrode system strips with a 4 mm × 4 mm carbon surface were prepared using a DEK (model 247) screen-printer (Dorset, UK) together with a stainless steel mesh screen (400 counts/in., mesh orientation of 45°). The inks used in screen-printing (Electrodag® B-0851, 423-SS and Ag/AgCl, 5% AgCl, Electrodag® 477 SS) were purchased

from Acheson (Plymouth, UK). Electrochemical measurements were recorded using the BAS 100 W workstation supplied by Bioanalytical Systems (IN, USA).

2.3. ELONA procedures

The primary coating buffer was 50 mM carbonate/bicarbonate, pH 9.6, the assay buffer contained 42 mM Tris-HCl, 8 mM Trisma base, 1 mM MgCl₂, pH 7, the blocking buffer was assay buffer containing 1% BSA, the substrate buffer was 0.1 M DEA, 50 mM KCl, 1 mM MgCl₂, pH 9.8 and the washing buffer consisted of 42 mM Tris-HCl, 8 mM Trisma base, 100 mM NaCl, and 0.05% (v/v) Tween-20 at pH 7.

Various two-dimensional capture ELONAs were performed to optimise the concentration of immobilised IgE antigen and test the binding efficiency of the aptamer D17.4ext. Typically 50 µl of various concentrations of IgE antigen were immobilized to the surface of the wells, which were subsequently blocked with 200 µl of blocking buffer. Then 50 µl of various concentrations of aptamer D17.4ext followed by 50 µl of xAv-AP conjugate in assay buffer were added to the plate. Finally, 100 µl of 1 mg ml⁻¹ of *p*-NPP was used as substrate and after colour development at 37 °C, the absorbance was measured at 405 nm. After each step the plate was incubated for 1 h at 37 °C, and in between steps the wells were washed three times with 400 µl of washing buffer.

Competition assays were performed in a similar manner, with the exception of the competition step, where 25 µl of IgE antigen (3-fold dilution) and 25 µl of D17.4ext (previously determined) in assay buffer were premixed for 10 min at room temperature. Then 50 µl per well of each mixture was added to the corresponding wells and allowed to incubate for 1 h at 37 °C. The optimum xAv-AP concentration was subsequently added depending on the ELONA format (1000–3000 ng ml⁻¹).

2.4. Aptasensor procedure

Non-hydrodynamic, three-electrode set-up was used during sensor development. The current was measured by differential pulse voltammetry. The potential wave form of this method consists of small pulses of constant amplitude, superimposed upon a staircase wave form. The current is sampled twice in each pulse period, once before the pulse and at the end of the pulse, and the difference between these two current values is recorded and displayed. The initial potential was set at -100 mV, the final potential at 500 mV and the sensitivity at ≤10 µA. The remaining parameters were as follows: scan rate 20 mV s⁻¹, pulse amplitude 75 mV, sample width 17 ms, pulse width 100 ms, pulse period 200 ms and quiet time 30 s.

Initially, the concentrations of IgE antigen, D17.4ext aptamer and xAv-AP were optimised. The procedure was the same as in the ELONA optimisations (Section 2.3) with the following exceptions; the primary coating volume was 5 µl, a blocking volume of 150 µl, the aptamer and xAv-AP conjugate were added in 10 µl volumes, and the substrate was 100 µl of 0.5 mg ml⁻¹ *p*-APP per electrode. When recording the DPV signal a single drop of the substrate solution was pipetted onto a horizontally

positioned SPE ensuring all three electrodes were covered in solution. A measurement was also taken for the substrate buffer prior the addition of *p*-APP and this value subtracted from the actual response. In between steps the surface of the electrodes was sequentially washed with washing buffer and distilled H₂O and after each step the electrodes were incubated for 1 h at 37 °C. The competition assays were performed in a similar manner, with the exception of the competition step, 5 µl of IgE antigen (3-fold dilution) and 5 µl of aptamer in assay buffer were pre-mixed for 10 min at room temperature in vials and then 10 µl of each mixture were added to the corresponding electrode area.

3. Results and discussion

3.1. Aptamers as receptors

Aptamers are nucleic acid ligands that can be isolated from complex libraries of synthetic oligonucleotides through an iterative process of in vitro selection and amplification, termed systematic evolution of ligands by exponential enrichment (SELEX) [9,10]. This methodology can yield ligands against a wide array of targets ranging from small molecules, such as metal ions, organic dyes, amino acids, and antibiotics, to nucleotides, peptides, large proteins and even parasites [11–13]. The affinities of these ligands lie in the micromolar to the sub-nanomolar range and can therefore be comparable to, if not better than, those of antibodies for the same targets. A comparison of aptamers to the widely used antibody technology reveals various advantages [14,15]. These features make aptamers outstanding candidates for therapeutic [16], diagnostic [17] and analytical applications [15,18]. In particular, their use as molecular recognition elements in affinity sensing, even though limited, has been quite successful when coupled with optical [19], fluorescent [20,21] and acoustic methods [22].

Aptamers have also demonstrated high specificity and can distinguish between compounds based on differences of a single hydroxyl or methyl group, e.g. the affinity of the theophylline-binding RNA aptamer for its cognate ligand is 10,000 times that of caffeine [23]. The high affinity and specificity of aptamers to their related targets is achieved by a combination of hydrogen bonding, stacking, non-canonical base pairing and hydrophobic interactions [24–26].

A well described aptamer, specific for IgE, is D17.4 published by Wiegand et al. It was suggested that the stem-loop formation of this polyanionic oligonucleotide favours interaction with a positively charged site located in the Ce3 region of the protein [27]. However, this aptamer has a relatively fast dissociation constant of 10 nM due to its unstable three-dimensional structure, which tends to spontaneously unfold and release the analyte, even under binding buffer conditions. In order to stabilize the structure of the aptamer and increase its sensitivity, Liss et al. extended both the 5'- and 3'-ends of D17.4 by adding four base-pairs, thus allowing the formation of a longer stem. This resulted in a more stable tertiary structure and reduced the dissociation constant of the aptamer to 3.6 nM [28]. The engineered aptamer was called D17.4ext (extended) and is the one used in the development of our aptasensor.

The primary objective of the present work was to establish a quantitative technique utilizing aptamers, attempting to display the usability of these receptors towards sensing and to compare this method to existing antibody-based protocols for the detection of IgE. IgE has an importance for detection at a clinical level of 300 ng ml⁻¹ as this represents the normal human levels for adults. An allergic state would thus, increase such levels and any assaying system would therefore have to measure levels around this reference value and higher.

3.2. ELONA optimisation

The format of the ELONA follows a standard indirect method, where competition between surface-bound IgE and IgE in solution proceeds for the biotinylated aptamer D17.4ext and the signal achieved by the secondary label (xAv-AP). As with any system of this type it is essential to optimise the reagents to provide a dose-dependent response. Two-dimensional experiments, whereby optimum coating IgE and aptamer concentrations were determined for both ELONA and aptasensor formats. It was found that characteristic saturation occurring at both high coating IgE and aptamer concentrations occurred in a dose-dependant manner (data not shown). A combination of both reagents was chosen based on non-excess conditions (ca. 80% of maximum signal) and yielding a signal of ca. 1.0 A.U. At this level there is also conservation of reagents.

Aptamers are inherently susceptible to their surrounding environment and careful control of buffer conditions is needed [29]. Buffer pH, ionic strength, additional ions and protein within the assay buffer all contribute to the binding capacity of a particular aptamer for its target. All these parameters were investigated to find the best conditions yielding high affinity.

3.2.1. Presence of salts

The effect of the presence of salts in the assay buffer was initially investigated, and in particular of divalent ions required by the aptamer, to assemble into the appropriate three-dimensional conformation [27,28]. As can be seen in Fig. 1a, the binding capacity of the aptamer, in the presence of Mg²⁺, for the surface-bound IgE, was evaluated along with the non-specific binding (NSB) component of xAv-AP in the absence of aptamer. Clearly, a concentration of MgCl₂ of 1 mM yielded the best binding with low NSB across the range investigated. Lower concentrations resulted in reduced signal, due to lack of bivalent metal ions required by the ligand to attain the tertiary structure. The same effect was observed when excess concentrations of Mg²⁺ were used, showing that buffers of high ionic strength disrupt the binding between the aptamer and IgE. This is in accordance to the findings of Jiang et al., who studied the effect of ionic strength on the aptamer/protein interaction by measuring the single-molecule bond rupture force of aptamer–IgE in PBS buffer with two different concentrations of NaCl (150 mM and 1.5 M), using atomic force microscopy and the Poisson statistical method [39]. It was stated that increased concentration of NaCl in the buffer resulted in a decrease of both the mean rupture force of the aptamer–protein and the number of the bonds formed, indicating that the aptamer/IgE interaction becomes weaker in

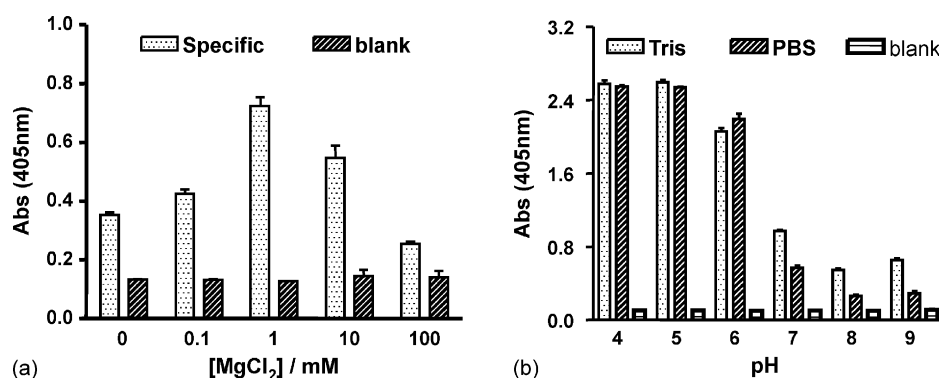


Fig. 1. Effect on aptamer binding to the immobilised IgE by (a) the addition of MgCl₂ to the buffer and (b) by changing the pH using both Tris and PBS. Blank is the non-specific binding of extravidin-AP to the surface-bound IgE (absence of aptamer).

the presence of a high salt concentration. We also performed similar experiments with Ca²⁺ ions and a similar trend was noted but not with such a dramatic change in absolute signal. The signal only increased by 6% up to 1 mM concentration of Ca²⁺ when compared to absence of this ion. Magnesium in this case was largely more effective in the binding process as this ion was used during the PCR amplification and the ligand selection process of SELEX [28]. The configuration (hexagonal) and lower ionic radius of magnesium over calcium (cubic) is a probable explanation to the more efficient binding of the ligand in the presence of Mg²⁺. Monovalent ions were also investigated. In this case NaCl and KCl (data not shown) varied up to 100 mM but little variation in aptamer binding was seen for both salts in this concentration range.

3.2.2. Buffer type

The binding of the aptamer was also compared between Tris buffer and phosphate buffered saline (PBS), the latter being the one used by Wiegand et al. for the selection of the D17.4 ligand and subsequently by Liss et al. for the development of their quartz crystal biosensor [27,28]. The variability in our case was pH, ranging between the values of 4 and 9. Between pH 4 and 6, the binding profile of the aptamer was identical for both buffers at 50 mM ionic strength. In this region the binding is optimum. From neutral to alkaline pH 7–9, the absolute signal decreases rapidly to between 40 and 20% of that at pH 5. Tris buffer sustains better binding of the aptamer over PBS in this pH region with signals accounting for double that of PBS. Using a lower ionic strength PBS buffer (10 mM), an identical trend was noted as for the 50 mM PBS case (data not shown). Low binding was observed at pH 7 or at more alkaline pH values, while signal increased to 80% of maximum at pH 6. No advantage was noted by using this lower ionic strength buffer (10 mM). The pH buffer trends are shown in Fig. 1b. Since the use of Tris buffer resulted in higher signal at neutral pH values, it was decided to utilise this buffer in all subsequent assays. It is also noted that across the pH range measured, NSB of xAv-AP (no aptamer) was minimal, accounting for <4% of the maximum signal.

An interesting corollary is that aptamer binding was more efficient at acidic pH, possibly due to increased electrostatic interactions between the ligand and its target. However, for this ELONA, where the analyte is a protein, neutral pH values are

preferential, since acidic conditions would cause damage to the proteinaceous coating and/or analyte and thus a pH value of 7 was compromised even though the overall signal was only at 50% of the maximum. Nevertheless, in situations where the analyte is an organic molecule with solubility problems at neutral pH, this type of receptor could be more useful over traditional antibodies, as denaturation of the aptamer would not occur in acidic pH.

3.3. Development of quantitative ELONAs

3.3.1. Optimised ELONA

At this point, these optimised conditions were used to develop the ELONA and Fig. 2a shows the result. Fig. 2a represents the average response from five curves ($N=5$) performed on separate days. Each point is therefore the average of 15 values and have been normalised to 100% (B/B_0) in order to compensate absorbance variations from day-to-day, mainly due to colour development time variations. Clearly the dose-dependent relationship is seen for IgE. Preliminary discussion at this point focuses on the ELONA parameter of EC_{50} which, was determined to be 961 ± 79 ng ml⁻¹ and to the linear range of the assay that was established between 303 and 8120 ng ml⁻¹. This assay will be discussed in detail in the next section in conjunction with the Improved ELONA. Needless to say, the determination of the curve and its associated accuracy, were quite good (cf. Table 1, column 1).

3.3.2. Improved ELONA

One of the typical procedures used when developing assays/sensors using antibodies as receptors, uses the inclusion of proteinaceous medium during the competition step. This has a number of effects, most notable that the antibodies now in solution have less a tendency to adsorb to the plastic or glassware they are contained. This in turn frees the antibodies with a higher equilibrium toward the solution rather than sticking to the containers. It must be noted that this however does not always improve assay performance. It may increase the absolute signal as more available antibody (receptor) is present, but this in turn can force assays to become less sensitive. Re-optimisation of assay conditions is generally recommended at this point. Based on these principles it was investigated how our previously developed

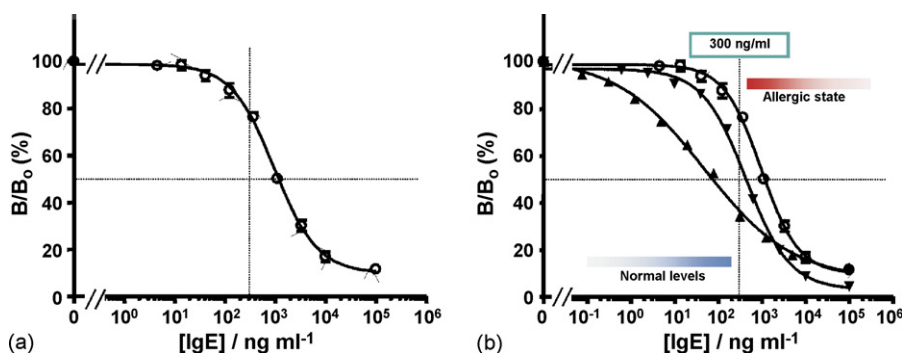


Fig. 2. Developed IgE ELONAs on microtitre plates. *Optimised ELONA* (a) curve shown is the average of five curves on different days and (b) *Improved ELONAs* for IgE with (▲, ▼) and without (○) incorporating BSA (0.1%) in competition step. Each curve is the average of at least four separate curves ($N=4$ or 5). B/B_0 is the ratio of response B , to the maximum response when no analyte, IgE is present B_0 .

Optimised ELONA would function by inclusion of a protein like bovine serum albumin. Instantly, higher absolute signals were evident through the incorporation of 0.1% BSA in the competitive buffer (under identical conditions). Thus, this allowed for a re-estimation of reagent conditions. What follows is therefore a comparison of our initial *Optimised ELONA* (Section 3.3.1) with the *Improved ELONA* system. Depicted in Fig. 2b, is a comparison of all developed ELONAs with and without the inclusion of 0.1% BSA. Having previously re-optimised the reagent conditions through aforementioned methods, assays shown in Fig. 2b were performed at least on four separate occasions ($N=4$ or 5) and averaged. Thus, each point is the average of 8–15 data points. Initially, it is noticeable that inclusion of 0.1% BSA makes the assay more sensitive, EC_{50} lowered from 961 ± 79 to 433 ± 29 ng ml $^{-1}$. Both these curves express a similar best-fit characteristic (Hillslope) when compared in Table 1 (columns 1 and 2). Curves determined with BSA present (column 2) show better sensitivity, precision, detection (33 ± 4 ng ml $^{-1}$) and correlation (0.994). In addition, the EC_{50} is now closer to the reference normal human level of 300 ng ml $^{-1}$, thus making this *Improved ELONA* more suitable for assaying samples from allergic patients close to the threshold value. By the inclusion of BSA we could also investigate the *Improved ELONA* by further decreasing the concentration of aptamer to 5 ng ml $^{-1}$ (Table 1,

column 3). Although a superfluous advantage, we can clearly measure to lower levels of IgE with the consequence of reducing the Hillslope. This is unwise, but highlights the flexibility of the aptamer, detecting IgE to the picomolar level (1.38×10^{-12} M).

Keeping in mind that the overall objective was to develop an aptasensor for IgE, it was felt that sufficient information had been gathered to proceed to the sensor development phase.

3.4. Aptasensor optimisation and development

Optimisation of the aptasensor was achieved in a similar manner as the ELONA previously, by checking all reagents including coating IgE, aptamer and label. As screen-printed electrodes (SPEs) are slightly more difficult to handle than the standard microtitre plate format, in terms of number of replicates, sequential one-dimensional assays for all parameters were performed rather than combined two-variable experiments. Characteristically, higher concentrations of reagents were needed due to the binding capacity of the carbon working area and the following conditions were chosen; coating IgE at 20 μ g ml $^{-1}$ (80% of maximum signal), aptamer at 100 ng ml $^{-1}$ and xAv-AP at a concentration of 2 μ g ml $^{-1}$ (95% of maximum signal). This combination of reagents yielded current signals in the 3 μ A range for DPV measurements. With associated backgrounds of 140 nA this gave a signal to noise ratio of 21, which effectively meant it was possible to further dilute reagents and to, at least theoretically, improve sensitivity if while using these conditions yielded an inappropriate range for the aptasensor. It was found that preliminary sensing gave curves with EC_{50} values similar to the initial developed *Optimised ELONA*, with sensitivity around the 1000 ng ml $^{-1}$ level. Subsequent profiling of the reagent concentrations diminished the absolute signal to ca. 500 nA but allowed the sensor to function with better sensitivity. Curves depicted in Fig. 3a are three such individual aptasensors prepared on separate days ($N=3$, $n=3$). The aptasensor performance is summarised in Table 2 with the LOD determined from Eq. (1) using the best-fit parameters determined from the data of the averaged curves (Fig. 3b).

$$LOD = EC_{50} \left(\frac{Max - Min}{Max - Min - 3S.D._{max}} - 1 \right)^{-1/k} \quad (1)$$

Table 1
Best-fit parameters for *Optimised* and *Improved ELONAs* for IgE^a

ELONA parameters	D17.4ext aptamer (ng ml $^{-1}$)		
	10 ^b	25 ^c	5 ^c
Top	98.9	97.0	101.4
Bottom	9.9	3.2	7.4
Hillslope	−1.018	−0.870	−0.430
EC_{50} (ng ml $^{-1}$)	961 ± 79	433 ± 29	49 ± 17
LOD (ng ml $^{-1}$)	126 ± 7	33 ± 4	0.25 ± 0.06
Linear range (ng ml $^{-1}$)	303–8120	103–2407	3–736
R.S.D. ^d (%)	2.4	2.6	3.2
R^2	0.973	0.994	0.984

^a Absorbance values measured at 405 nm ($n=8$).

^b Competition step without the presence of BSA.

^c Competition step with the presence of 0.1% BSA.

^d Average R.S.D. across all data points of the curve.

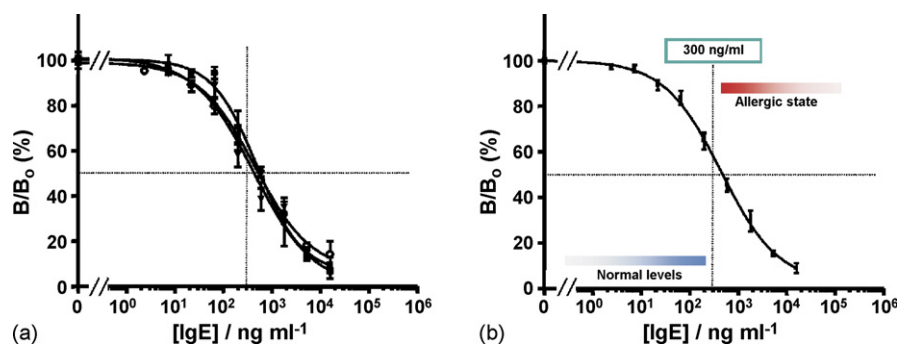


Fig. 3. Aptasensor for IgE on disposable screen-printed electrodes using DPV detection. Curves shown in (a) are three separate curves on different days ($n=3$), while (b) is the averaged data shown in (a). B/B_0 is the ratio of response B , to the maximum response when no analyte, IgE is present B_0 .

The essential characteristic of this developed aptasensor lies in the EC_{50} value (451 ng ml^{-1}) which, when compared to the *Improved ELONA* of Section 3.3.2 (433 ng ml^{-1}), shows a similar level and suitable for measuring levels found in real patient samples. The EC_{50} is the point of the curve which is most accurately determined (point of inflection) and thus a value of $451 \pm 38 \text{ ng ml}^{-1}$ suggests that elevated levels of IgE in humans can be adequately measured with this aptasensor (remembering the reference value of 300 ng ml^{-1}). In conjunction with a favourable Hillslope (-0.788 ± 0.131), this assay does not need to reach lower levels of IgE. We must remember that altering the present protein conditions of the sensor would contribute to loss of absolute signal (ca. 500 nA) and render the accuracy of measurements irreproducible (through loss of suitable signal:noise ratio). Thus, the sensor, using this aptamer, seems at the limit of its functionality using the current conditions. As shown earlier, inclusion of BSA in the aptamer binding buffer led to better sensitivity when assayed by ELONA. This modification could also be used for the sensor to further reduce the EC_{50} value closer to the reference value of 300 ng ml^{-1} but the authors feel it is not currently required. While good results were obtained in terms of best-fit parameters, investigation continued in order to measure recovery from unknown samples.

3.5. Recovery experiments using improved ELONA

Having developed, what we termed as *Improved ELONA* (using 25 ng ml^{-1} aptamer) and its 5 ng ml^{-1} counterpart, it was intended to measure unknown samples of IgE within the linear

ranges highlighted in Table 2 (columns 2 and 3). Table 3 shows the results for 16 samples (4 samples $\times$ 4 assays) and also for 12 other samples (4 samples $\times$ 3 assays) utilizing both developed *Improved ELONAs* corresponding to two different linear ranges. For the 16 samples, recoveries varied between 94 and 108%, with averaged results for each sample between 99 and 103%. Similarly for the 12 samples, recoveries again varied between 94 and 106% or $100 \pm 5\%$ for averaged values. This exhibits the ability of either *Improved ELONA* to accurately assess IgE levels in unknown samples. The judicious choice of aptamer concentration allows for either low levels (using 5 ng ml^{-1}) or elevated levels of IgE determination (using 25 ng ml^{-1}) with equal precision and accuracy. It is worth mentioning that this lower level detection is slightly redundant but does highlight the flexibility of the aptamer receptor.

3.6. Stability test (aptamer versus antibody)

Stability is the ability of an in vitro diagnostic reagent, when kept under specified conditions, to retain, throughout the shelf life its characteristics and/or performance within limits specified. It was attempted to compare the stability of a monoclonal antibody receptor to the D17.4ext aptamer used within this study. Using an indirect format, the antibody system was optimised for surface-bound IgE, anti-IgE and secondary anti-species (anti-mouse IgG-AP) to yield an adequate signal by ELISA. This was then compared to the developed indirect ELONA using the aptamer. In order to minimize possible operator error, the optimum coating, blocking and buffer storage was performed prior to the freezing of the microtitre plates. By freezing the plates, we eliminated any possible errors between stock solutions prepared on different days, i.e. for the coating IgE and block (day-to-day variation), incubation time and temperature differences and thus, the stability was largely focused to either antibody (IgE mAb) or aptamer (D17.4ext). On any particular day, coated and blocked plates were removed from the freezer, thawed and washed as usual. Samples of IgE mAb and D17.4ext aptamer (continually stored in stock at 37°C) were then added to the plate and subsequently developed by the appropriate secondary labels. The commercially available secondary labels are inherently stable and we can exclude them as possible sources of instability under the lifetime of this test. In this study, we also applied an

Table 2
Best-fit parameters for optimised aptasensor for IgE determination^a

Sensor parameter	Average $\pm$ S.D.
Top	100.8
Bottom	5.7
Hillslope	-0.788 ± 0.131
EC_{50} (ng ml^{-1})	451 ± 38
LOD (ng ml^{-1})	23 ± 4
R.S.D. ^b (%)	5.7 ± 0.8
R^2	0.972

^a Values shown are for three separate assays ($N=3$).

^b Average R.S.D. across all data points of the curve.

Table 3
Recovery experiments performed on *Improved ELONA* for IgE determination^a

Assay ^b	Real concentration (ng ml ⁻¹)	Sample 1 100.00	Sample 2 40.00	Sample 3 8.00	Sample 4 2.50
1	Calculated value	105.62	38.81	7.88	2.36
	Recovery (%)	105.62	97.03	98.48	94.60
2	Calculated value	103.83	38.82	8.52	2.67
	Recovery (%)	103.83	97.04	106.56	106.96
3	Calculated value	104.68	41.13	7.68	2.69
	Recovery (%)	104.68	102.83	95.97	107.76
4	Calculated value	102.74	39.28	8.21	2.62
	Recovery (%)	102.74	98.20	102.59	104.87
	Average recovery (%)	103.26	99.52	99.26	101.81
Assay ^c	Real concentration (ng ml ⁻¹)	875.00	350.00	140.00	56.00
1	Calculated value	820.51	329.30	137.14	58.90
	Recovery (%)	93.77	94.09	97.95	105.18
2	Calculated value	845.41	334.02	138.02	58.70
	Recovery (%)	96.62	95.43	98.59	104.82
3	Calculated value	904.22	343.71	136.07	59.32
	Recovery (%)	103.34	98.20	97.19	105.92
	Average recovery (%)	97.91	95.91	97.91	105.31

^a Absorbance values measured at 405 nm ($n = 3$).

^b Optimised ELONA using 5 ng ml⁻¹ aptamer.

^c Optimised ELONA using 25 ng ml⁻¹ aptamer.

accelerated Arrhenius approach (heat-stressed testing), through long-term storage of stock solutions of both receptors at 37 °C, before making appropriate dilutions and adding to the plates. Accelerated Arrhenius testing is designed to increase the rate of chemical degradation or physical change by using exaggerated storage conditions as part of the formal definitive storage program. This data in addition to long stability studies may be used to assess longer-term chemical effects at non-accelerated conditions and to evaluate the impact of short-term periods outside ideal storage conditions [30].

Fig. 4 shows the profile of antibody versus aptamer stability. Rapid degradation of the IgE mAb can be seen, from an initial signal of 1.987–0.370 A.U. in just 13 days at 37 °C, demonstrating clearly the loss of activity of the monoclonal antibody. The D17.4ext aptamer remained consistent without fluctuation in absolute signal (ca. 1.5 A.U.) under the conditions chosen. The aptamers stability to heat was very clearly illustrated and considering that we used an accelerated condition (elevated storage temperature) these results point to a longer overall stability of aptamer and can be extrapolated to normal storage conditions (14 days at 37 °C is equivalent to 24 months at 4 °C) [30]. In addition, the non-specific binding was assessed in Fig. 4, whereby the coating IgE was replaced by rabbit IgG protein of a similar molecular size. In all cases it can be seen that there was little non-specific contribution (<6%) by the aptamer to binding to the IgG. Zero control was also included in this test whereby the elimination of the receptor, leads to the degree of binding of the secondary labels to the coating. For the mAb system there seems a higher non-specific binding effect (ca. 20% of maximum signal) when compared to the aptamer one (10%).

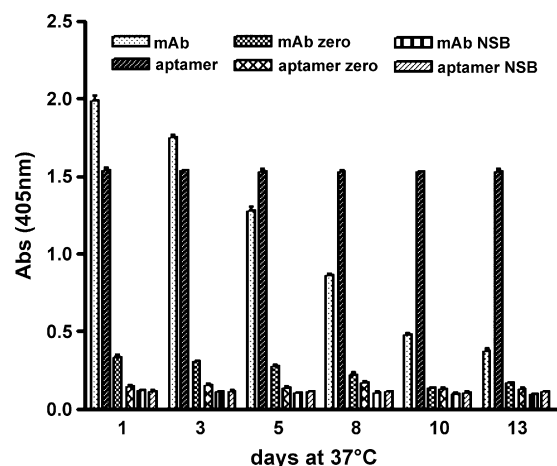


Fig. 4. Stability test of IgE mAb and D17.4ext aptamer under accelerated Arrhenius conditions. Receptor solutions were kept at 37 °C and assayed by microtitre plate under optimised conditions ($n = 3$). Zero receptor and non-specific binding controls are also included.

4. Conclusions

Aptamers as receptors for sensing purposes has begun to flourish in recent years with their application finding its way to a variety of formats. In particular their use has been largely noticed in quartz crystal sensing [28,31], microtitre-based assays [29,32,33], surface plasmon resonance [34] and fluorescence [35,36]. To this point, they have not been exploited in electrochemical methods to any great extent with the authors recognizing only the recent communication of Hianik et al. [37]. The

authors believe that the current work highlights the usability of the aptamer receptor, although with careful, but not excessive control of the assaying conditions. No more so than the majority of antibody systems. Aptamers may have advantages in the area of stability, with easy regeneration using harsh conditions, unsuitable for antibodies and also in their thermo-stability (no affect on aptamer folding) [28]. This aptamer binding, functions better in acidic environment which, could be used for analytes displaying favourable properties (solubility) in such conditions. There are grounds for continuation of this work, particularly in the sensor area, and the authors are investigating the operational capacity of the aptasensor using the *Improved ELONA* conditions in particular to recovery experiments in a matrix, such as blood (serum, plasma). This investigation is underway. We feel that this work represents the beginning of electrochemical aptasensors, exhibiting their feasibility and highlighting their comparative sensitivity to their antibody predecessors in this application [8]. Closer collaboration between aptamer developers and end-users could tailor these receptors for greater benefit in a similar fashion as the original work of Yalow and Berson on immunoassays in the late 1950s [38].

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Professor George G. Guilbault is internationally recognised as one (if not the one) of the “founding fathers” within the area of development and use of biosensors. Thus, he has a number of “scientific firsts”, such as having developed the first biosensors with immobilised enzymes (amperometric as well as potentiometric) and the first biosensor for isoenzymes. He is an extraordinarily prolific scientist, who has published more than 435 papers in chemistry journals covering an unusually wide scope, and authored 12 books and 75 book chapters. His scientific career began in Loyola University where in 1958 he received his BSc (Magna cum Laude) and completed his MSc and PhD while at Princeton University in 1961. He is a most sought after speaker, and has altogether given over 550 presentations in all corners of the world. He has received numerous awards for his research accomplishments, and via his international contacts he has had close collaboration with many highly reputed scientists in different countries. His current position is within University College Cork, Ireland where his enthusiasm for research, despite his years, has never diminished.