

Ultrasensitive and rapid detection of β -conglutin combining aptamers and isothermal recombinase polymerase amplification

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Abstract Lupin is increasingly being used in a variety of food products due to its nutritional, functional and nutraceutical properties. However, several examples of severe and even fatal food-associated anaphylaxis due to lupin inhalation or ingestion have been reported, resulting in the lupin subunit β -conglutin, being defined as the Lup an 1 allergen by the International Union of Immunological Societies (IUIS) in 2008. Here, we report an innovative method termed aptamer-recombinase polymerase amplification (Apta-RPA) exploiting the affinity and specificity of a DNA aptamer selected against the anaphylactic β -conglutin allergen termed β -conglutin binding aptamer II (β -CBA II), facilitating ultrasensitive detection via isothermal amplification. Combining magnetic beads as the solid phase with Apta-RPA detection, the total assay time was reduced from 210 min to just 25 min, with a limit of detection of 3.5×10^{-11} M, demonstrating a rapid and ultrasensitive generic methodology that can be used with any aptamer. Future work will focus on further simplification of the assay to a lateral flow format.

Keywords Aptamer · Recombinase polymerase amplification · Lupin · Apta-RPA · Apta-PCR

Introduction

Annually, millions of people have allergic reactions to food, and although most food allergies cause relatively mild and minor symptoms, some of them can cause severe reactions and may even be life-threatening. In general, there is no cure for food allergies and only strict avoidance of food allergens can prevent serious health consequences. Rapid, reliable and sensitive detection of food allergens is therefore critical, and the most exploited commercial analytical methods are ELISA [1] and lateral flow assays [2]. However, the homology between proteins can present challenges for antibody-based approaches, which may be addressed by PCR [3] or, alternatively, mass spectrometry (MS) [4], which have been used extensively for the detection of and differentiation between related allergens, but both techniques are inherently laboratory based and require significant infrastructure and highly trained personnel.

Lupinus is a leguminous plant whose seeds are rich in protein with high nutritional value, and they are widely used for food production as well as animal feed. Furthermore, lupin does not contain gluten and is used in gluten-free products including bread and other bakery products [5]. However, due to various reports of anaphylactic allergic reactions induced by lupin inhalation/ingestion, lupin and lupin foodstuff products were added to the list of allergens requiring mandatory advisory labelling on foodstuffs sold in the European Union according to the Commission Directive 2006/142/EC. Lupin globulins are composed of two major subunits (α - and β -conglutin) and two minor subunits (γ - and δ -conglutin) [6]. Although there is no

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general consensus on the identification of the major allergen/s in lupin, in 2008, the β -conglutin subunit was designated as the anaphylactic lupin allergen Lup an 1 by the International Union of Immunological Societies (IUIS) [7].

There are several reports of immunoassay and molecular assay for the detection of lupin, but none of these are capable of selectively and specifically detecting the β -conglutin subunit [8, 9]. To this end, in 2012, an aptamer was selected against β -conglutin, termed the β -conglutin binding aptamer I (β -CBA I) [10]. Aptamers are artificial oligomers capable of binding to a wide range of diverse targets including proteins [11], small molecules [12], amino acids [13] or even whole cells [14] with affinities and specificities equal to and often superior to antibodies [15]. Recently, we reported the selection of a second aptamer against β -conglutin (β -CBA II) that binds another site (aptatope) with better affinity in the low nanomolar range [16, 17].

Here, we report on the use of the β -CBA II for the rapid detection of β -conglutin, using a combination of the aptamer's affinity and exploiting its nucleic acid nature to be used as a template for amplification. Different methods were compared in order to achieve a rapid and ultrasensitive assay termed aptamer-recombinase polymerase amplification (Apta-RPA) by replacing first the classic thermal cycling amplification by isothermal amplification and, subsequently, the plate for magnetic beads with the purpose of decreasing the assay time and the limit of detection (LOD).

Materials and methods

Materials

Phosphate-buffered saline (PBS; 10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4), PBS-Tween (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 0.05 % v/v Tween 20, pH 7.4) 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS) and all other reagents were purchased from Sigma (Barcelona, Spain). Magnesium chloride, sodium chloride, sodium hydroxide and hydrochloric acid were purchased from Scharlau Chemie S.A. (Barcelona, Spain). SiMAG-Carboxyl beads were obtained from Chemicell (Berlin, Germany), Nunc 96-well microplates from Thermo Scientific (Madrid, Spain) and *Tfi* DNA polymerase and 10 bp DNA ladder from Life Technologies (Barcelona, Spain). Certified™ Low Range Ultra Agarose was purchased from Bio-Rad (Barcelona, Spain). All solutions were prepared in high-purity water obtained from the Milli-Q RG system (Barcelona, Spain). All primers and probe sequences can be found in Table 1. All DNA oligonucleotides were purchased from Biomers (Ulm, Germany), and β -conglutin was extracted and purified as previously detailed [18].

Table 1 Sequences of oligonucleotides used in the reported work

Name	Sequence	MW (g mol ⁻¹)
Forward primer	5'-agctccagaagataaattacagg-3'	7090
Reverse primer	5'-gggggtcatagtagtctagttg-3'	6492
Biotin probe	5'-Biotin-ggggtcatagtagtctagttg-3'	6930
β -CBA II	5'-agctccagaagataaattacaggggccgggggt ggctcaggcaagggttgacctgtcgtagggtt gttttaacaactaggatactatgacccc-3'	29,198

Competitive enzyme-linked oligonucleotide assay on microtiter plates

To elucidate optimal parameters of enzyme-linked oligonucleotide assay (ELONA), the concentrations of coating β -conglutin and β -CBA II were optimised previously (data not shown). β -Conglutin (140 nM) in 50 mM carbonate buffer (pH 9.6) was immobilised on a microtiter plate for 30 min at 37 °C, followed by 30 min of blocking with PBS-Tween. The wells were then washed three times with 200 μ L of PBS-Tween. In individual Eppendorf tubes, serial dilutions of β -conglutin were pre-incubated with 150 nM of aptamer in binding buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM MgCl₂, pH 7.4) for 30 min at room temperature (RT) and were subsequently added to individual wells of the coated microtiter plate and left to incubate for 30 min at RT. Following incubation, the wells were washed, followed by the addition of 200 nM of biotinylated probe complementary to the 3' terminus of the aptamer for further 30 min. Subsequently, the wells were thoroughly washed and 10 nM of horseradish peroxidase-linked streptavidin (SA-HRP) was added and incubated for further 30 min. Finally, 3,3',5,5'-tetramethylbenzidine (TMB) was added and, after 10 min, the reaction was stopped with 1 M of sulfuric acid and the absorbance was read at 450 nm using a SpectraMax 340 PC microtiter plate reader. Triplicates were analysed for each sample. The data was normalised by dividing the absorbance of the sample for the absorbance of the blank (1 nM aptamer without target). The normalised data was plotted on the GraphPad Prism software, and the LOD was calculated by the formula top value - 3 $\times$ standard deviation of the top value.

Competitive Apta-PCR and Apta-RPA using the β -CBA II

The combination of microtiter-based competition and nucleic acid amplification via PCR/RPA was performed as described above until the competition assay was complete. After a thorough washing, Milli-Q water was added to each well and the aptamer was heat eluted and used as template for

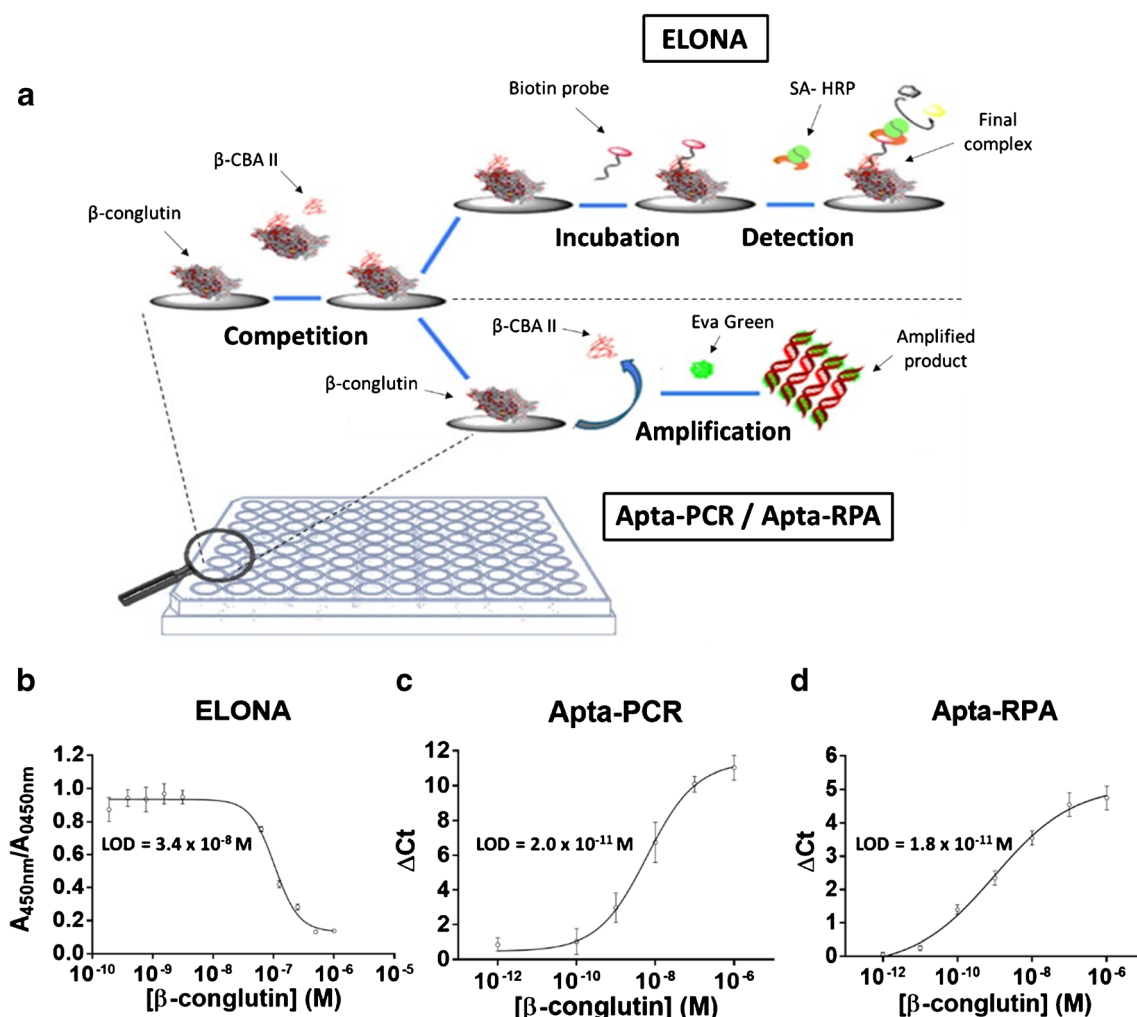


Fig. 1 (a) Scheme of ELONA versus Apta-PCR/Apta-RPA assay. (b) Calibration curve of ELONA assay. (c) Calibration curve of Apta-PCR assay. (d) Calibration curve of Apta-RPA assay

amplification. Real-time PCR was performed as previously described, but using Eva Green instead of SYBR Green [19]. Briefly, 9 μ L of master mix containing $1 \times$ *Tfi* DNA polymerase buffer, 5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μ M primers, 0.5 mg/mL bovine serum albumin (BSA) and 5 U *Tfi* DNA polymerase were mixed with 1 μ L template. After an initial step of 2 min of incubation at 95 $^{\circ}$ C, 40 cycles of PCR were carried out using the following: (i) 95 $^{\circ}$ C, 30 s for denaturation; (ii) 58 $^{\circ}$ C, 30 s for annealing; (iii) 72 $^{\circ}$ C, 30 s for elongation; and finally, 5 min at 72 $^{\circ}$ C for final elongation.

To elucidate the optimal parameters for Apta-RPA, firstly, different concentrations of aptamer (100–0.01 nM) and different temperatures of RPA amplification (35–41 $^{\circ}$ C) were tested. Afterwards, the concentration of coating β -conglutinin was also optimised. A range of concentrations of β -conglutinin (500–15.62 nM) diluted in 50 mM carbonate buffer (pH 9.6) were incubated in the wells of a microtiter plate for 30 min at 37 $^{\circ}$ C, followed by 30 min of blocking with PBS-Tween at 37 $^{\circ}$ C.

Following a thorough washing step, the β -CBA II (1 nM) was added to each well and incubated in binding buffer for 30 min at RT and the bound aptamer was eluted and used as a template in RPA. Following aptamer elution, RPA was carried out using a TwistAmp basic kit (TwistDX, Ltd., Cambridge, UK), where each 50 μ L reaction contained 29.5 μ L rehydration buffer, 2.1 μ L of forward primer (10 μ M), 2.1 μ L of reverse primer (10 μ M), 2.5 μ L Eva Green DNA binding dye (20 $\times$), 2.5 μ L magnesium acetate and one enzyme pellet. This master mix (9 μ L) was mixed with 1 μ L template to the total volume of 10 μ L. RPA reactions were executed in a cold block cooled to 4 $^{\circ}$ C. The fluorescence of the Eva Green DNA binding dye was monitored every 30 s (each cycle) for 40 min after a pre-incubation of 1 min at 37 $^{\circ}$ C in the real-time PCR machine.

In an alternative approach, magnetic beads rather than the microtiter plate were used and β -conglutinin was immobilised on the surface of carboxyl magnetic beads as previously

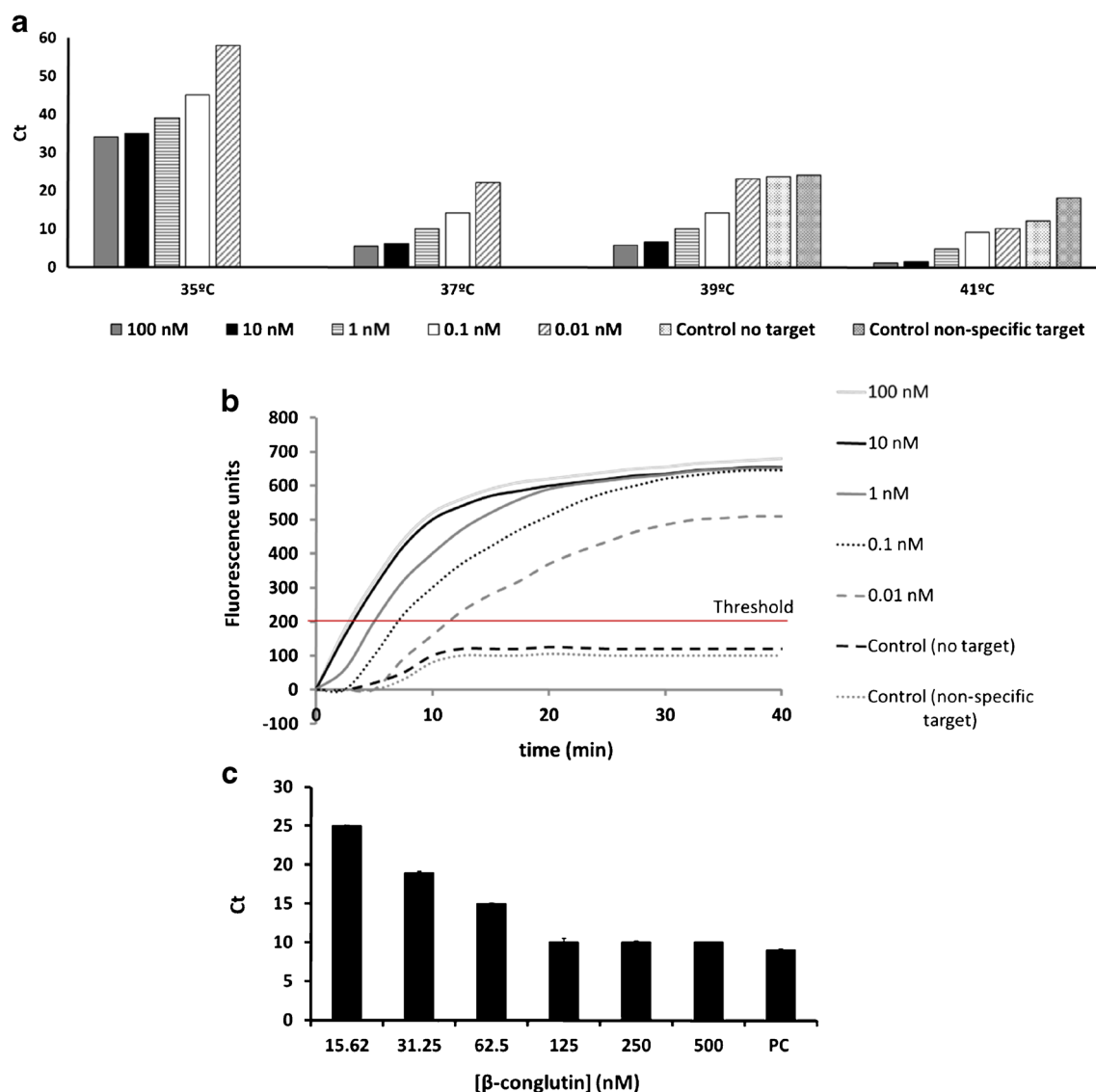


Fig. 2 Optimisation of Apta-RPA approach. (a) Optimal temperature for Apta-RPA assay. (b) Optimal aptamer concentration for Apta-RPA assay. (c) Optimal amount of immobilised β -conglutinin on the plate (PC corresponds to positive control (1 nM aptamer amplified by RPA without β -

conglutinin). Ct corresponds to the cycle where the sample crosses the threshold; Δ Ct is the difference between the sample Ct and the positive control Ct)

described [10]. The amount of functionalised beads was optimised. In individual Eppendorf tubes, a range of concentrations of β -conglutinin were pre-incubated with 1 nM of the β -CBA II for 5 min at RT. Subsequently, the functionalised beads were added to each Eppendorf tube and incubated for further 5 min. Magnetic actuation was applied, and the Eppendorf tubes were rigorously washed three times with binding buffer followed by the addition of Milli-Q water for heat elution of the aptamer for use as a template in RPA.

In all the different methods, triplicates were performed and raw fluorescence data was analysed by Applied Biosystems SDS 2.4 software and exported to GraphPad Prism software. The threshold was 200 fluorescence units, and the normalisation (Δ Ct) was calculated by the difference in the cycle

thresholds (Ct) between the sample and the blank (1 nM aptamer without target). The LOD was calculated by the formula bottom value + $3 \times$ standard deviation of the bottom value.

Results and discussion

Competitive enzyme-linked oligonucleotide assay on microtiter plates

Competitive ELONA was used as a means for comparing the detection limits and assay times achievable with the combined competition, elution and nucleic acid amplification

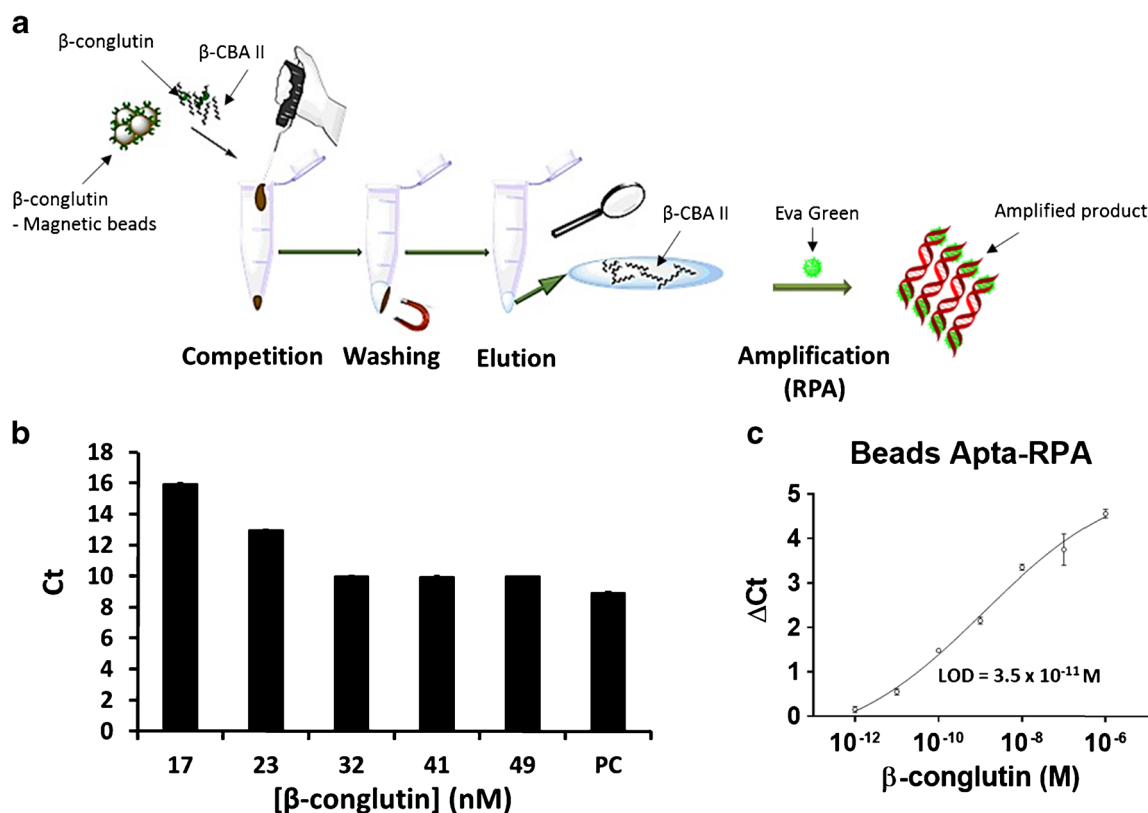


Fig. 3 Bead-based Apta-RPA. (a) Scheme of bead-based Apta-RPA assay. (b) Optimal amount of immobilised β -conglutin on the beads (PC corresponds to positive control (1 nM aptamer). Ct corresponds to the

cycle where the sample crosses the threshold). (c) Calibration curve of bead-based Apta-RPA assay (Δ Ct is the difference between the sample Ct and the positive control Ct)

approaches (Fig. 1a). The LOD obtained using a type of competitive ELONA based on immobilised β -conglutin on a microtiter plate, which competes with β -conglutin free in solution for the binding of the β -conglutin binding aptamer II (β -CBA II), effectively binding any aptamer that has not formed a complex with the β -conglutin during pre-incubation, was determined. In the presence of higher concentrations of free β -conglutin, less aptamer binds to the immobilised β -conglutin and a LOD of 3.4×10^{-8} M was achieved (Fig. 1b), with a total required assay time of 160 min.

Apta-PCR assay using the β -CBA II

In order to improve this LOD, Apta-PCR was used, where the aptamer bound to the immobilised β -conglutin was heat eluted and used as a template in real-time PCR (RT-PCR) (Fig. 1a).

The calibration curve was obtained by plotting the resulting Δ Ct (the difference between the cycles needed to cross the threshold compared with the blank (1 nM aptamer without target) versus the concentration of β -conglutin). The LOD in this case improved to 3 orders of magnitude as compared to the ELONA assay, achieving a LOD of 2.0×10^{-11} M (Fig. 1c).

The major drawback of Apta-PCR is the length of the assay and the requirement for thermal cycling, thus inherently requiring significant laboratory infrastructure. To increase the simplicity and the feasibility of the assay, an isothermal nucleic acid amplification method termed recombinase polymerase amplification (RPA) was exploited. In RPA, the need for thermal cycling used in the polymerase chain reaction is avoided and replaced by three core proteins that operate optimally between 37 and 40 °C. The first protein, a recombinase, binds to primers, forming filaments that can then recombine with homologous DNA in a duplex target, forcing displacement of the non-complementary strand and thus provoking the formation of a D-loop. The second protein is a single-stranded DNA binding protein, which attaches to the strand of DNA displaced by the primer, preventing the dissociation

Table 2 Comparison of different methodologies

Methodology	LOD (M)	Assay time (min)
ELONA	3.4×10^{-8}	160
Apta-PCR	2×10^{-11}	210
Apta-RPA	1.8×10^{-11}	100
Bead-based Apta-RPA	3.5×10^{-11}	25

of the primer and hybridisation of the duplex target. The final core protein is a strand-displacing polymerase that copies the DNA, adding bases onto the 3' end of the primer, forcing the double helix to open as it progresses. When opposing primers are used, exponential amplification occurs. Since the introduction of recombinase polymerase amplification [20], the system has been extensively used in a range of formats due to its simplicity and true isothermal nature of amplification, where no first cycle thermal denaturation step is required. Reported systems include the use of fluorescent probes for real-time amplification/detection of either DNA [21] or RNA [22], whilst other common applications include an end-point detection system using lateral flow assays [23].

Competitive Apta-RPA assay

A standard dilution curve using the β -CBA II as a template (100–0.01 nM) with different amplification temperatures (35–41 °C) was performed, and as expected, 37 °C was observed to be optimal, which was the faster assay (less cycles to cross the threshold were needed) without non-specific binding in the controls (non-specific target and absence of target) (Fig. 2a). Furthermore, amplification of 1 nM β -CBA II is the lowest amount of aptamer that is still detectable after 10 min of amplification, with signal saturation observed after 30 min (Fig. 2b), and this was thus selected as the optimum aptamer concentration for the competition assay.

Following optimisation of the RPA for the direct detection of the β -CBA II, the optimum amount of immobilised β -conglutinin required for the competitive Apta-RPA assay was elucidated using 1 nM aptamer. The aptamer was eluted from the plate by heating and was amplified following the optimised protocol described above. One hundred twenty-five nanomolars of immobilised β -conglutinin was determined to be the lowest saturating concentration and was thus selected as the optimum coating concentration for the competition assay (Fig. 2c). The assay proceeds in a type of competitive assay format, where target β -conglutinin forms a complex with the β -CBA II during pre-incubation, with any unbound aptamer being free to bind to the β -conglutinin on the surface of the microtiter plate (or magnetic beads as described below).

In comparison to Apta-PCR, RPA was observed to markedly more rapid, with amplification being completed in less than 15 min, whilst also achieving an equal LOD of 1.8×10^{-11} M, with amplification being carried out at 37 °C, thus highlighting potential exploitation in low-resource settings, on-site or at the point-of-care (Fig. 1d).

In order to further reduce the assay time, the same methodology was exploited but immobilising the β -conglutinin on

carboxyl magnetic beads via carbodiimide cross-linking rather than on microtiter plates (Fig. 3a). Magnetic beads can reduce in decreased incubation times, as stirring can be used to enhance the diffusion rate and the spherical nature of the magnetic beads can render the immobilised β -conglutinin more accessible for binding.

Different amounts of β -conglutinin-functionalised beads were used to define when all the β -CBA II was captured and 32 nM was observed to be the lowest amount of beads, providing complete capture of all the β -CBA II, and thus optimal for the competition assay with the target β -conglutinin (Fig. 3b). In this case, the LOD was observed to be 3.5×10^{-11} M (Fig. 3c) with a greatly increased pre-incubation of β -conglutinin with aptamer of just 5 min and followed by a competitive step for further 5 min. The entire assay was achieved in 25 min, as compared to 100 and 210 min when using microtiter plates and Apta-RPA and Apta-PCR, respectively, highlighting the potential of the assay format for on-site rapid analysis (Table 2). Moreover, compared to previously published assays for the detection of β -conglutinin such as using the β -CBA I in Apta-PCR [19] or its truncated form (11-mer) in FRET [24], the detection limit was improved around twofold and fourfold, respectively. The current work is focusing on transferring the detection to a lateral flow format.

Conclusions

Methodologies for the rapid and ultrasensitive detection of the anaphylactic allergen β -conglutinin have been developed and compared. Competitive ELONA with no incorporated amplification step gave a LOD of 3.4×10^{-8} M and assay time of 160 min. The use of a microtiter plate-based assay with an incorporated step where the β -CBA II and target β -conglutinin were pre-incubated, followed by competition, aptamer elution and amplification, resulted in assay times of 210 and 100 min, with detection limits of 2.0×10^{-11} and 1.8×10^{-11} M, using PCR or RPA, respectively. Finally, replacing the microtiter plates with β -conglutinin-functionalised magnetic beads coupled to RPA, the assay time was reduced to just 25 min, with a detection limit of just 3.5×10^{-11} M, being this novel assay termed competitive bead-based Apta-RPA the best strategy reported and demonstrating a rapid and ultrasensitive generic methodology that can be used with any aptamer.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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