SELECTION OF HIGH AFFINITY AND SPECIFIC APTAMER AND ITS' USE IN DIFFERENT APPLICATIONS FOR THE DETECTION OF THE ANAPHYLACTIC B-CONGLUTIN ALLERGEN

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I STATE that the present study, entitled "Selection of high affinity and specific aptamer and its use in different applications for the detection of the anaphylactic β-conglutin allergen" presented by Miriam Jauset Rubio for the award of the degree of Doctor, has been carried out under my supervision at the Department of Chemical Engineering of this university, and that it fulfils all the requirements to be eligible for the International Doctorate Award.

Tarragona, 25th February 2016,

Dr. Ciara K. O´Sullivan Dr. Markéta Svobodová Dr. Teresa Mairal Lerga

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SUMMARY

Lupin is a leguminous plant used widely in the Mediterranean region in everyday meals, traditional fermented foods, baked foods and sauces due to its' high nutritional qualities, gluten-free properties and possibility to be a substitute for soy. Lupin has recently been added to the list of allergens requiring mandatory advisory labelling on foodstuffs sold in the European Union, and since December 2008 all products containing even trace amounts of lupin must be labelled correctly. Lupin globulins consist of two major globulins termed α-conglutin and β-conglutin, and two minor globulins, γ-conglutin and δ-conglutin. There is no consensus about the allergenic properties of individual lupin globulins, but β-conglutin is the only conglutin subunit currently included in the list of the International Union of Immunological Societies (IUIS), designated as the anaphylactic Lup an 1 allergen.

Robust methods for the detection and quantification of allergens in food are necessary to ensure compliance with food-labelling regulations and to provide consumer protection. For the detection of allergenic lupin proteins in foods, sandwich enzyme linked immunosorbent assay (ELISA) is the most common method to date. However, there is no specific antibody able to specifically detect β-conglutin. Moreover, the production of antibodies is expensive and time consuming. An alternative way to detect β-conglutin is the use of highly specific aptamers. Chemically synthesized aptamers with specificity and affinity equal to those of antibodies, with higher flexibility allows them to address the ever-increasing requirements of analytical applications for food allergens. Several facets of aptamers such as their stability, ease of immobilization and labelling present them as ideal candidates for different type of applications.

The main goal of this doctoral thesis is to detect β-conglutin based on aptamer-target interactions for use in food safety applications. In order to achieve this objective, an aptamer against β-conglutin was selected and a variety of different strategies explored for the final development of rapid, sensitive, low-cost and user-friendly aptasensoric tools.

The thesis has the following structure:

The **Chapter 1** contains a general introduction which includes a brief information to food allergens and their pathogenesis, clinical disorders, diagnostics, management and prevention, highlighting lupin and the anaphylactic allergen, β-conglutin. Moreover, an overview of the state of the art of aptamers and main objective/sub-objectives are included in this chapter.

The selection of the β-CBA II aptamer against β-conglutin using SELEX with target immobilized on the surface of carboxyl magnetic beads is reported in **Chapter 2.** The affinity of the selected aptamer was confirmed by three different techniques in three different laboratories, to be in the low nanomolar range. Moreover, this aptamer in combination with the previously selected β-CBA I and its' truncated form, were then applied in a dual aptamer approach. The specific and simultaneous binding of the dual aptamer to different sites of β-conglutin was confirmed by microScale thermophoresis (MST) and surface plasmon resonance (SPR).

In **Chapter 3** SPR imaging was used to evaluate the affinity and specificity of the different aptamers selected against the β-conglutin allergen and explores the dual aptamer approach in various different sandwich formats in order to know what strategy is better for its detection. The best strategy for the dual aptamer approach was the β-CBA II as capture aptamer and 11-mer as reporter aptamer. The ability to provide parallel qualitative and quantitative detection establishes this method as a powerful tool for the study of immobilized aptamer-target interactions.

The first application using the selected β-CBA II aptamer is reported in **Chapter 4**. A comparison between enzyme linked oligonucleotide assay (ELONA), ultrasensitive Apta-PCR assay, and a novel methodology termed Apta-RPA based on isothermal amplification was carried out. This novel technique was improved by the use of magnetic beads instead of microtiter plates by decreasing time-consuming steps with picomolar detection limits.

Chapter 5 describes the proof-of-concept of a highly specific and sensitive lateral flow assay for point-of-care diagnostics. In this chapter a novel strategy for nucleic acid detection using tailed primers is described. Isothermal recombinase polymerase amplification was carried out off strip, and the duplex amplicons, flanked by two single

stranded tails were wicked onto the assay, hybridising to gold nanoparticle labelled complementary to one of the short DNA tails, and to a surface immobilised probe complementary to the other short DNA tail. The generic platform developed highlighted the possibility for extremely rapid and ultrasensitive detection of DNA/RNA for point of care diagnostics. This is the first example of lateral flow detection of DNA, combining isothermal amplification with tailed primers for binding of the amplicon and gold nanoparticle labelled DNA, thus avoiding the use of antibodies and presenting a truly inexpensive, stable assay for use in low resource settings at the point-of-care.

The assays described in Chapter 4 and Chapter 5 were then combined in **Chapter 6** to demonstrate a lateral flow strip assay for the rapid, ultrasensitive and inexpensive detection of β-conglutin. Two competitive lateral flow assays were developed and compared. Both assays were demonstrated to be highly specific and each assay had its' own specific advantages. Direct competitive β-conglutin assay on the strip is rapid, only requiring 10 minutes and is very attractive for applications where a rapid point of care diagnostic is required. On the other hand, a rapid competitive assay using functionalised magnetic beads was used, followed by elution of bound aptamer and its's use as a template for isothermal RPA and purification prior to wicking on to a lateral flow assay. The entire assay is just 50 minutes with a low femtomolar detection limit, and does not require any equipment – simply an eppendorf magnetically actuated tube holder and a Smartphone if quantitative detection is required.

Finally, **Chapter 7** presents the general conclusions of the thesis, the principal contributions and the future perspectives of this research.

RESUMEN

Lupino es una planta leguminosa con un alto valor nutricional utilizado ampliamente en la región mediterránea en comidas diarias, alimentos tradicionales fermentados, alimentos horneados, salsas y sin contener gluten, pudiendo ser el sustituto de la soja. Lupino se ha añadido recientemente a la lista de alérgenos que requieren el etiquetado obligatorio de asesoramiento sobre los productos alimenticios comercializados en la Unión Europea, y desde diciembre de 2008, todos los productos que contengan incluso pequeñas cantidades de altramuz deben estar correctamente etiquetados. Las globulinas que se encuentran en el altramuz consisten en dos grandes subunidades denominadas α-conglutina y β-conglutina, y dos menores, γ-conglutina y δ-conglutina. A pesar de no existir consenso acerca de las propiedades alergénicas de cada una de las conglutinas de lupino, β-conglutina es la única actualmente incluida en la lista de la Unión Internacional de Sociedades de Inmunología (UISI), designada como el alérgeno anafiláctico Lup an 1.

Métodos robustos para la detección y cuantificación de alérgenos en los alimentos son necesarios para asegurar el cumplimiento de las regulaciones de etiquetado y proporcionar protección al consumidor. El método más común hasta la fecha para la detección de proteínas alergénicas de altramuz en los alimentos es el ensayo por inmunoadsorción ligado a enzimas (ELISA; acrónimo del inglés *enzyme-linked immunosorbent assay)*. Sin embargo, no existe un anticuerpo específico capaz de detectar β-conglutina. Por otra parte, la producción de anticuerpos es cara y se necesita mucho tiempo para llevarlos a cabo. Una alternativa para la detección de β-conglutina es el uso de aptámeros altamente específicos. Los aptámeros son químicamente sintetizados, con especificidad y afinidad igual a los anticuerpos y con una mayor flexibilidad que les permite que se adapten a los requisitos cada vez mayores de las aplicaciones analíticas para la detección de los alérgenos alimentarios. Varios aspectos de los aptámeros como su estabilidad, facilidad de inmovilización y de conjugación con otros componentes les convierte en candidatos ideales para diferentes tipos de aplicaciones.

El objetivo principal de esta tesis doctoral es la detección de β-conglutina basada en las interacciones aptámero-diana para su posterior uso en aplicaciones de seguridad alimentaria. Con el fin de lograr este objetivo, se ha seleccionado un aptámero contra β-conglutina, así como también se han explorado diferentes estrategias para el desarrollo final de un aptasensor rápido, sensible, de bajo coste y fácil de usar.

La tesis tiene la siguiente estructura:

Capítulo 1 incluye una introducción general que contiene una breve información de alérgenos alimentarios y su patogénesis, trastornos clínicos, diagnóstico, manejo y prevención, destacando el altramuz y su alérgeno anafiláctico β-conglutina. Por otra parte, se incluye en este capítulo una visión general sobre los aptámeros y el objetivo principal y sub-objetivos de esta tesis doctoral.

La selección del aptámero β-CBA II en contra de la β-conglutina por el proceso de SELEX usando la diana inmovilizada en la superficie de perlas magnéticas con grupos carboxilos se informa en el **Capítulo 2.** La afinidad de este aptámero se confirmó mediante tres técnicas diferentes en tres laboratorios diferentes, encontrándose dentro del rango nanomolar. Además, este aptámero en combinación con β-CBA I, el seleccionado previamente, y su "forma truncada", son usados en el enfoque dúo aptámero. La unión específica y simultánea de dúo aptámero a diferentes sitios de β-conglutina fue confirmada por termoforesis de microscala (MST) y resonancia de plasmones superficiales (SPR).

En el **Capítulo 3** "SPR imaging" se utilizó para evaluar la afinidad y especificidad de los diferentes aptámeros seleccionados frente al alérgeno β-conglutina y se explora el dúo aptámero en diferentes formatos de sándwich con el fin de saber qué estrategia es mejor para su detección. La mejor estrategia para el enfoque dúo aptámero fue β-CBA II como aptámero captura y 11-mer como aptámero reportero. La capacidad de proporcionar una detección cualitativa y cuantitativa paralelamente establece este método como una herramienta poderosa para el estudio de interacciones aptámero-diana inmovilizada.

La primera aplicación en que se utiliza el aptámero β-CBA II seleccionado se expone en **Capítulo 4.** Se llevó a cabo la comparación entre el ensayo de oligonucleótido

ligado a enzimas (ELONA), el ensayo de ultrasensible Apta-PCR, desarrollado previamente en nuestro grupo, y una metodología novedosa denominada Apta-RPA, basada en amplificación isotérmica, sin la necesidad de termocicladores. Esta nueva técnica se ha mejorado por el uso de perlas magnéticas en lugar de las placas de microtitulación, con el fin de disminuir el tiempo final del ensayo, logrando un límite de detección en el rango picomolar.

Capítulo 5 describe el prototipo de un ensayo altamente específico y sensible de flujo lateral con la posibilidad de adaptarse al punto de atención de diagnóstico. En este capítulo se describe una nueva estrategia para la detección de ácidos nucleicos usando cebadores con cola. La amplificación isotérmica mediante recombinasa polimerasa se llevó a cabo fuera de la tira. Los amplicones de doble cadena flanqueados por dos colas de cadena simple resultantes de la amplificación fueron añadidos en la tira, hibridándose, por una parte, con las partículas de oro conjugadas con una secuencia complementaria a una de las colas de cadena simple y, por otra parte, con la secuencia complementaria a la otra cola, la cual se encontraba inmovilizada en la superficie de la membrana. El ensayo llevado a cabo demostró la posibilidad de la detección de ADN / ARN en el punto de atención de diagnóstico de una manera extremadamente rápida y ultrasensible. Éste es el primer ejemplo de detección de ADN mediante flujo lateral, combinando amplificación isotérmica con cebadores con cola para la unión del amplicón con las nanopartículas conjugadas con ADN, evitando así el uso de anticuerpos y presentando un ensayo verdaderamente barato y estable para ser usado en entornos de bajos recursos en el punto de atención.

Los ensayos descritos en el capítulo 4 y el capítulo 5 se combinan en el **Capítulo 6** para demostrar la detección rápida, ultrasensible y barata de β-conglutina basada en ensayo de flujo lateral. Dos ensayos competitivos de flujo lateral fueron desarrollados y comparados. Ambos ensayos demostraron ser altamente específicos y con sus específicas ventajas. El ensayo de β-conglutina competitivo directo, sólo requiere 10 minutos y es muy atractivo para las aplicaciones donde se requiere un punto de atención rápido. Por otra parte, el otro ensayo competitivo se basa en el uso de perlas magnéticas, seguido por la elución del aptámero unido y posteriormente su uso como plantilla durante la amplificación con polimerasa recombinante, siendo finalmente

añadido en la tira de flujo lateral. El ensayo completo se alcanza en 50 minutos con un límite de detección de bajo femtomolar sin requerir ningún equipamiento especial – simplemente un soporte para tubos de accionamiento magnético y un teléfono inteligente si se requiere una detección cuantitativa.

Por último, el **Capítulo 7** se presentan las conclusiones generales de la tesis, las principales contribuciones y las perspectivas de futuro de esta investigación.

LIST OF PUBLICATIONS

ARTICLES:

1) **Jauset Rubio M.,** Svobodová M., Mairal T., Schubert T., Künne S., Mayer G., O'Sullivan CK. β-conglutin dual aptamers binding distinct aptatopes. Anal Bioanal Chem. 2016 Jan;408(3):875:84. doi: 10.1007/s00216-015-9179-z. Epub 2015 Nov 19.
2) **Jauset Rubio M.,** Svobodová M., Mairal T., O'Sullivan CK. Surface plasmon resonance imaging (SPRi) for analysis of DNA aptamer:β-conglutin interactions. Methods. 2015 Oct 31. pii S1046-2023(15)30131-6. doi: 10.1016/j.ymeth.2015.10.013
3) **Jauset Rubio M.,** Sabaté del Rio, J., Mairal T., Svobodová M., O'Sullivan CK. Rapid detection of anaphylactic β-conglutin allergen based on isothermal recombinase polymerase amplification. Submitted to Molecular BioSystems.
4) **Jauset Rubio M.,** Saeed A., Svobodová M., Mairal T., Spoors J., Emanuel P., McNeil C., Keegan N., O'Sullivan CK. Ultrasensitive, rapid and inexpensive detection of DNA using paper based lateral flow assay. Submitted to Nucleic Acids Research.
5) **Jauset Rubio M.,** Svobodová M., Mairal T., McNeil C., Keegan N., O'Sullivan CK. Ultrasensitive β-conglutin detection via recombinase polymerase amplification-lateral flow competitive assay. Submitted to Nucleic Acids Research.

BOOK CHAPTER:

1) Pinto A., Polo PN., **Rubio MJ.,** Svobodova M., Lerga TM., O'Sullivan CK. Apta-PCR. Methods Mol Biol. 2016;1380:171-7. Doi: 10.1007/978-1-4939-3197-2_14.

LIST OF ABBREVIATIONS

µFFE	Microfluidic free flow electrophoresis
3SR	Self-sustained sequence replication
ALISA	Aptamer linked immobilised sorbent assay
AMD	Age-related macular degeneration
AML	Acute myeloid leukemia
AuNPs	Gold nanoparticles
bFGF	Basic fibrobast growth factor
BSA	Bovine serum albumin
CE-SELEX	Capillary electrophoresis SELEX
COLAP	Colonoscopic allergen provocation test
CTP	Uridine triphosphate
DNA	Deoxyribonucleic acid
ds-DNA	Double stranded DNA
DT1	16-(3,5-bis((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15 pentaoxahexadecane
DTT	1,4-dithiothreitol
EDC	1-ethyl-3- (dimethylaminopropyl) carboiimide
ELAA	Enzyme-linked oligonucleotide assay
ELASA	Enzyme-linked apta-sorbent assay
ELISA	Enzyme-linked immunosorbent assay
ELONA	Enzyme-linked oligonucleotide assay
ESI-MS	Electrospray ionization tandem mass spectroscopy
FDA	Food and drug administration
FDEIA	Food-dependent exercise-induced anaphylaxis
FRET	Fluorescence resonance energy transfer
HDA	Helicase dependent amplification
HNE	Human neutrophil elastase

HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IgE	Human immunoglobulin E
IL-1	Interleukin-1
IPEC	Intragastral provocation under endoscopic control
IUIS	International Union of Immunological Societies
K_D	Dissociation constant
LAMP	Loop mediated isothermal amplification
LFA	Lateral flow assay
LFTS	Lateral flow test strips
LOD	Limit of detection
MHC	6-mercapto 1-hexanol
MLPA	Multiplex ligation dependent probe amplification
MS	Mass spectrometry
M-SELEX	Microfluidic SELEX
MST	Microscale thermophoresis
NALF	Nucleic acid lateral flow
NALFIA	Nucleic acid lateral flow immunoassay
NASBA	Nucleic acid sequence based amplification
NECEEM	Non-equilibrium capillary electrophoresis of equilibrium mixtures
NGS	Next generation sequencing
NHS	N-hydroxysuccinimide
NMM	N-methyl mesoporphyrin
OAS	Oral allergy syndrome
PAD	Paper analytical devices
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PFAS	Pollen-food allergy syndrome
PKC	Protein Kinase C
POCT	Point of care test

RAPID	RNA aptamer isolated via dual cycles
RCA	Rolling circle amplification
RNA	Ribonucleic acid
RPA	Recombinase polymerase amplification
RT	Room temperature
RT-PCR	Real time polymerase chain reaction
RU	Resonance units
SA	Streptavidin
SA-HRP	Streptavidin conjugated with horseradish peroxidase
SAM	Self-assembled monolayers
SDA	Strand displacement amplification
SELEX	Systematic evolution of ligands by exponential enrichment
SIT	Allergen-specific immunotherapy
SPR	Surface plasmon resonance
SPRi	Surface plasmon resonance imaging
SPT	Skin prick test
ss-DNA	Single strand DNA
STACS	Specific target Cell-SELEX
TBA	Thrombin-binding aptamer
TCEP	Tris (2-carboxyethyl) phosphine
TMA	Transcription mediated amplification
TMB	3,3',5,5'-tetramethylbenzidine
UPT	Up-converting phosphor technology
UTP	Cytidine triphosphate
VEGF	Vascular endothelial growth factor
β-CBA I	β-conglutin binding aptamer I
β-CBA II	β-conglutin binding aptamer II

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Chapter 1

General introduction

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Chapter 1: General introduction

1.1 FOOD ALLERGENS

Food allergy is an important public health problem that affects children and adults worldwide. The term *food allergy* is used to describe an adverse, abnormal immune-mediated reaction to certain foods or food ingredient(s) that occurs in susceptible individuals that often requires a strict avoidance of the offending component [1]. In some cases, a food allergy can cause severe symptoms or even a life-threatening reaction known as anaphylaxis or anaphylactic shock [2]. In addition, the increasing intake of packed food has compelled governments to subject the food industry to more and more stringent scrutiny in order to control allergen content. Since 2003, EU regulations have obliged the compulsory labelling of foodstuffs that contain allergens from 14 majority groups of food, including the worldwide recognized big eight: milk, eggs, peanuts, tree nuts, soy, wheat, fish and shellfish, as well as celery, mustard, sesame, sulphur dioxide and sulphites, lupin and molluscs. Differences in both the allergens listed as well as the content threshold across the globe, hinders the development of analytical methods that are universally applicable [3].

1.1.1 Pathogenesis

The gastrointestinal tract encompasses the largest surface area in the human body and is composed of a single cell layer of columnar intestinal epithelial cells separating the internal sterile environment from the external world [4]. Its' main function is to process ingested food in a form that can be absorbed and used for energy and growth as well as preventing the penetration of harmful pathogens into the body. In order to achieve this, there is a gastrointestinal mucosal barrier which includes a single layer of epithelial cells joined by tight junctions and covered with a thick mucus layer that traps particles, bacteria and viruses. In addition, luminal and brush border enzymes, bile salts, and extreme pH serve to destroy pathogens and antigens that are less immunogenic. The immunogenic component consists of innate and adaptive immune

cells and factors, which also provide an active barrier to foreign antigens. However, the efficiency of this mucosal barrier in infants and young children is not optimal because of the immaturity of various components of the gut barrier and immune system, playing a role in the increased prevalence of both gastrointestinal tract infections and food allergies in the first years of life [5].

The first induction of an allergen immune response upon allergen encounter is termed *allergic sensitization* [6, 7] and can occur in the gastrointestinal tract (considered traditional or Class 1 food allergy) or as a consequence of an allergic sensitization to inhalant allergens (Class 2 food allergy) [8]. The major food allergens identified as Class 1 allergens are water soluble glycoproteins that are 10 to 70 kDa in size and fairly stable to heat, acid and proteases [9]. The majority of Class 2 allergens are comprised of conformational epitopes and therefore highly heat labile, susceptible to enzymatic degradation and difficult to isolate, often making standardized extracts for diagnostic purposes unsatisfactory. Nevertheless, cDNAs for many of these proteins have been isolated and recombinant proteins have been generated, suggesting that improved diagnostic materials will be available in the near future [10].

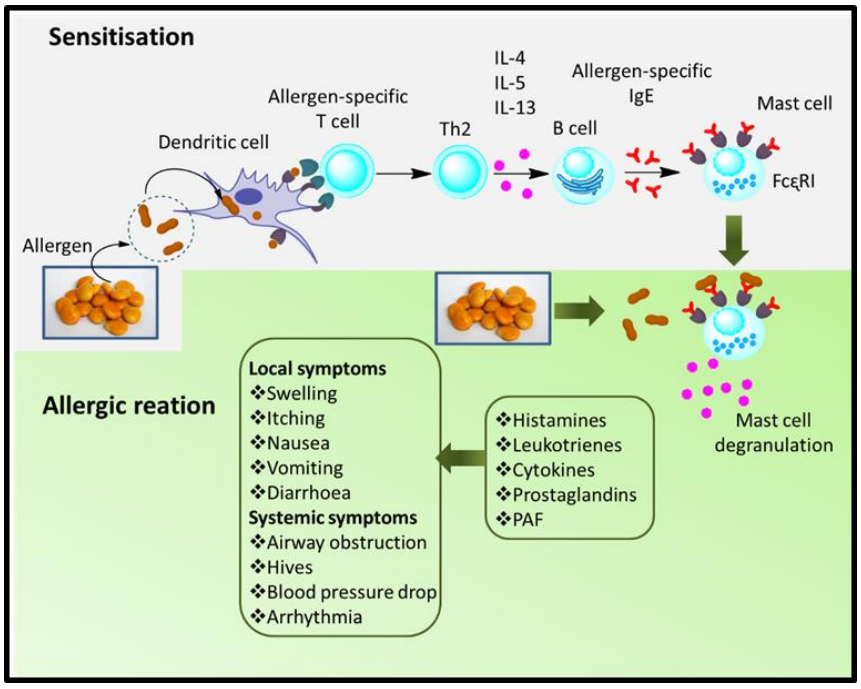

Figure 1.1: Sensitization and allergic reaction [11].

1.1.2 Clinical disorders

The clinical types of food allergy can be classified as outlined in Table 1.1. Some examples are:

- **Anaphylaxis (immediate type allergy):** this reaction can cause life-threatening signs and symptoms, including restriction and tightening of airways, the sensation of a lump in the throat that makes it difficult to breathe, rapid pulse, shock with a severe drop in blood pressure, dizziness, and loss of consciousness. Untreated anaphylaxis can result in death [12].

- **Food-dependent exercise-induced anaphylaxis (FDEIA):** in this case anaphylaxis develops only if physical activity occurs within a few hours after eating a specific food. The foods most commonly implicated in FDEIA are wheat, shellfish, tomatoes, peanuts and corn [13].

- **Oral allergy syndrome (OAS):** is a type of food allergy that affects the mouth in response to eating certain (usually fresh) fruits, nuts and vegetables. The most common reaction is an itching or burning sensation in the lips, mouth, ear canal and/or pharynx. Sometimes other reactions can affect eyes, nose and skin. OAS seems to be the most common food-related allergy in adults. The body's immune system produces IgE antibodies against pollen; in OAS these antibodies also bind to other structurally similar proteins found in plants. Another term used for this syndrome is pollen-food allergy or pollen-food allergy syndrome (PFAS) [14].

Table 1.1: The clinical disorders of food allergy [2].

Clinical types	Stage of life	Causative foods	Possibility of tolerance	Risk of anaphylaxis
Gastrointestinal Allergy in infant	Newborn infant	Cow milk	High	Low
Food allergy associated atopic dermatitis	Infant	Egg, cow milk, wheat, soybean and rice	High	High
Immediate type allergy (anaphylaxis, urticaria)	Infant-adult	Egg, milk, wheat, buckwheat, fish and peanuts	High in eggs, milk, wheat,	Very high
	Schoolchild-adult	Fish, wheat, shrimp, crab, fruits, buckwheat and peanuts	Low in soybean or others	
Food dependent exercise-induced anaphylaxis (FEIAn / FDEIA)	Schoolchild-adult	Wheat, shrimp and crab	Low	Extremely high
Oral allergy syndrome (OAS)	Infant-adult	Fruits and vegetables	Low	Low

1.1.3 Diagnosis

Diagnoses are based on careful analyses of case histories and diaries to document symptoms and offending foods [15] (Figure 1.2). Results from serologic and *in vitro* tests alone are not sufficient for the diagnosis of IgE-associated food allergies, because the presence of IgE is not always associated with symptoms [16], since some antigens in food easily react with IgE but do not have allergenic activity, and also some allergens are degraded or do not pass through the epithelial barrier in sufficient quantities. One solution is the use of provocation tests as they are useful, and confirm IgE-associated food allergy. A double-blind placebo-controlled food challenge is the standard for antigen identification [17], but it can induce severe reactions and requires careful planning and well-equipped clinical facilities.

1.1.4 Management and prevention

The primary therapy for food allergy is to avoid the causative food(s) or food ingredients. Education about avoidance includes careful attention to label reading, care in obtaining food from restaurants or food establishments, and avoidance of cross-contact of foods with an allergen during meal preparation, such as avoiding shared cutting boards, slicers and mixers. Food-labelling laws require simple English terms, i.e. milk instead of casein, to indicate the presence of specific regulated food allergens. Patients and caregivers should be trained to recognize symptoms and instructed on using self-injectable epinephrine and activating emergency services [5].

Various medications can provide relief for certain aspects of food-induced disorders:

- Anti-histamines might partially decrease symptoms of oral allergy syndromes and IgE-mediated skin symptoms [18].
- Anti-inflammatory therapies might be beneficial for allergic eosinophilic esophagitis-allergic eosinophilic gastroenteritis [19].
- Epinephrine administration for the treatment of anaphylaxis [20].

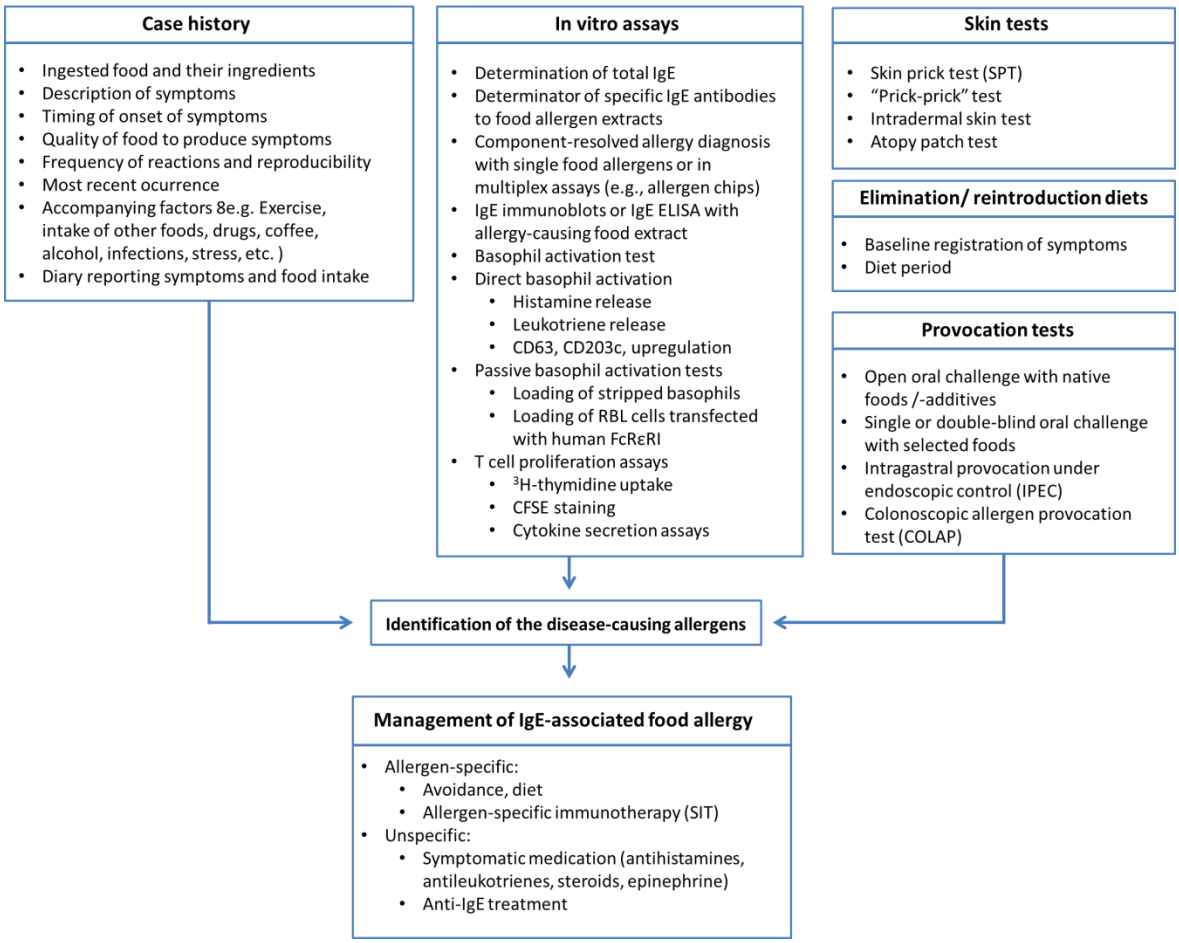

Figure 1.2: Diagnosis and management of IgE-associated food allergies [21].

1.1.5 Lupin

Lupin is a leguminous plant belonging to the genus Lupinus including more than 450 species. Seeds of various species have been used as food for over 3000 years around the Mediterranean [22] and for up to 6000 years in the Andean highlands. Lupin was eaten by the early Egyptian and pre-Incan people due to its' high protein content and were known by the Roman farmers to contribute to the fertility of the soil due to the fact that similar to other legumes they can fix nitrogen from the atmosphere into ammonia via a rhizobium-root nodule symbiosis, fertilizing the soil for other plants. This adaption allows lupins to be tolerant of infertile soils and capable of pioneering change in barren and poor-quality soils [23].

In the early twentieth century German pioneering scientists achieved the cultivation of a "sweet" variety of lupin without the bitter taste (due to the mixture of alkaloids in the seed) making it more suitable for both human consumption and animal feed [24]. Further work was carried out by the Western Australian Department of Agriculture and Food during the 1950s and 60s, with Western Australia being the most dedicated country for lupin cultivation. Today, lupin is also found in Mediterranean countries and their former colonies, especially in Spain, Portugal, Italy, Greece and Brazil, as well as Egypt, Syria, Lebanon, Jordan, Israel, Turkey, Chicago's Little Italy, and in New York City's Spanish Harlem. In Portuguese, lupin beans are known as *tremoços*, and in Spain and Argentina they are called *altramuz* (both names are derived from Arabic *al-turmus*).

However, recently the first documentation of lupin allergy was reported in a girl of a 5-years-old with peanut sensitization [25]. Due to this and other reports of lupin allergy, in December 2008, lupin and lupin derived food products were included in the EU allergen list according to the Commission Directive 2006/142/EC amending Annex IIIA of Directive 2000/13/EC (2006). In 2009, the eliciting dose (ED) for the lupin allergen was reported to be around 0,5 mg for allergic adults [26] and in a range between 0,2 and 6,4 g in peanut allergic children [27].

As mentioned previously, lupin belongs to Class 2 food allergens and lupin can thus cause reaction via ingestion among individuals allergic to peanuts (which are the major risk group) or with an unknown allergy to peanuts, and / or via inhalation as pollen-food allergy syndrome [25, 28-36]. The reported reactions of lupin allergy continue to increase and there is not yet any general consensus on the identification of the major allergen/s [37], but it seems to correspond to the globulin fraction, which is composed by two major subunits, referred to as α-conglutin and β-conglutin and two minor subunits γ-conglutin and δ-conglutin (Table 1.2). To date only the β-conglutin subunit has been designated as the lupin allergen Lup an 1, by the International Union of Immunological Societies[38].

Table 1.2: Summary of *Lupinus albus* conglutins' main features [39].

Conglutin (protein family)	Svedlerg velocity	(%) of globulins	Native protein				Upon reduction	
			M_W (kDa)	pI	Quaternary Structure	Subunits Size, kDa	Heavy Chain	Light chain
β-conglutin (Vicilin-like)	7S	44-45	143-260	5.9-6.2	Trimer	19-60	No disulphide bounds	
α-conglutin (legumine-like)	11S	35-37	330-440	5.6-5.9	Hexamer	53	31	19
						60	36	19
						66	42	19
						70	46	19
γ-conglutin	7S	4-5	200	7.9	Tetramer	47	29	17
δ-conglutin (sulfur rich)	2S	10-12	13	4.3	Monomer		9	4

1.1.5.1 High nutritional value

Lupinus angustifolius (Australian sweet lupins) are high in protein, dietary fibre and antioxidants, very low in starch, and as all such legumes, are gluten-free. Lupins can be used to make a variety of foods, both sweet and savoury, including everyday meals, traditional fermented foods, baked foods and sauces. *Lupinus albus* (the European white lupin) seeds are commonly sold in a salty solution in jars and can be eaten with or without skin. Furthermore, they are used for different foods from lupin flake, vegan sausages, lupin-tofu or baking-enhancing lupin flour. As the lupin seeds have the full range of essential amino acids and can be grown in a cool climate, lupin is increasingly becoming recognized as an alternative to soy [24, 40-43].

Table 1.3: *Lupinus albus* and *Lupinus luteus* seed composition [44].

Nutritional components	*L. albus*	*L. luteus*
Protein (g/ 100 g d.m.)	39.02 + 0.26	37.93 + 2.44
Fat (g/ 100g d.m.)	14.64 + 1.15	8.79 + 0.42
Soluble carbohydrates (g/ 100 g d.m.)		
• Sucrose	4.95 + 0.12	1.38 + 0.13
Dietary fibre (g/ 100 g d.m.)		
• Soluble	5.21 + 0.18	4.90 + 0.03
• Insoluble	34.22 + 0.08	28.78 + 0.01
• Total fibre	39.42 + 0.26	33.68 + 0.04
Starch (g/ 100 g d.m.)		
• Total	3.27 + 0.23	4.53 + 0.41
• Available	1.78 + 0.11	1.84 + 0.13
Vitamins (mg/ 100 g d.m.)		
• α-Tocopherol	0.19 + 0.01	0.48 + 0.01
• c-Tocopherol	20.10 + 0.86	11.19 + 0.63
• d-Tocopherol	0.25 + 0.02	0.38 + 0.01
• Vitamin E activity	2.21 + 0.11	1.61 + 0.06
• Thiamin	0.36 + 0.01	1.49 + 1.12
• Riboflavin	0.61 + 0.04	0.85 + 0.04
• Vitamin C	6.48 + 0.09	2.56 + 0.13

In addition, lupin proteins have several biological activities, for example the blood glucose lowering effect of γ-conglutin has been demonstrated to interact with the mammalian hormone insulin, producing a significant decrease in glycaemic levels [45]. Lowering of plasma cholesterol and triglyceride, anti-hypertensivity and inhibition of the angiotensin are other biological properties attributed to lupin proteins [46-49].

1.1.5.2 Isolation of lupin proteins

Different methods had been reported for the isolation and extraction of conglutin subunits [50-54]. The most recent report is based on steps of centrifugation with different buffers to achieve the globulin fraction. With an objective of isolating the four subunits, anion-exchange chromatography was first carried out to separate γ-conglutin, which is not retained in the column due its' isoelectric point (pI) [55], whilst β-conglutin and its' precursor are eluted between 0.3-0.45 M NaCl and α-conglutin and δ-conglutin are co-eluted at 1M NaCl. The isolation of α-conglutin and δ-conglutin was achieved by size exclusion chromatography as the main difference between both subunits is the size (α-conglutin is around 330 – 430 kDa and δ-conglutin 13 kDa). All the proteins were then analysed using peptide mass fingerprinting to confirm their isolation comparing with Swissprot and Genbank databases [56].

1.1.5.3 Detection

There are several reported publications of immuno and molecular methods for the detection of lupin. In the case of immuno-based detection of lupin the use of antibodies in a sandwich enzyme-linked immunosorbent assay (ELISA) has been reported and there are commercial kits available. The microtiter plates of these kits are provided with pre-coated capture antibody and examples include the lupin-Check ELISA kit by Immutest, AgraQuant ELISA lupin by Romer Labs, Lupin ELISA kit by Immunolab GmbH and by ELISA systems. However, these methods lack in specificity and cannot distinguish between the different globulin subunits.

The molecular analyses reported to date have been based on genomic DNA sequences coding for conglutin genes, where primers and probes were designed for the amplification of 153 bp and 150 bp fragments of α-conglutin and δ-conglutin, respectively[57]. In addition, the real time polymerase chain reaction (RT-PCR) technique has also been reported for the detection of lupin [58-60]. In 2011, Mustorp *et al.* reported Multiplex ligation dependent probe amplification (MLPA) able to specifically and simultaneously detect the eight allergens: sesame, soy, hazelnut, peanut, lupin, gluten

mustard and celery. Ligated probes were amplified by PCR and amplicons were detected using capillary electrophoresis [61].

Finally, some preliminary approaches for the development of high-performance liquid chromatography/electrospray ionization tandem mass spectroscopy (HPLC/ESI-MS/MS) for the detection and label-free semi-quantitative detection of the main lupin conglutins was recently published, but requires powerful bioinformatic tools [62].

In conclusion, most of these assays are not rapid and the majority are qualitative, relatively expensive and require specialised instrumentation and trained personnel. Moreover, none of reported techniques can detect each conglutin subunit specifically. For this reason, in 2012 in our group, the first aptamer against the Lup an 1 allergen, β-conglutin, was developed with high specificity and affinity (K_D= 3.6 x 10^{-7} M) [39]. The selected 93-mer aptamer was used to develop a competitive Apta-PCR assay which combines competitive microtiter plate assay with real-time PCR, achieving limits of detection in the picomolar range [63]. This highly specific aptamer was subsequently truncated from an original 93-mer with K_D 3.6 x 10^{-7} M to an 11-mer with K_D 1.7 x 10^{-9} M[64] and this truncated 11-mer aptamer was then flanked on either the 5' or 3' terminus with a donor dye, as well as being flanked on the opposite terminus with an acceptor dye to construct dimeric FRET-based probes and used for detection of β-conglutin achieving even lower limits of detection and requiring just one-minute assay time [65].

1.2 APTAMERS

Aptamers were discovered twenty-six years ago by three independent research groups [66-68]. The name aptamers comes from the Latin "aptus", meaning to fit. Aptamers are single stranded oligonucleotides (DNA or RNA) with the ability to fold into stable three-dimensional structures, characterised by stems, loops, hairpins, bulges, triplexes and quadruplexes, that allow them to interact with a wide variety of target molecules from single molecules to complex target mixtures or even a whole organism [69]. They bind selectively to their target through electrostatic interactions, hydrogen bonding, Van der Waals forces, base stacking or a combination of these [70]. The process used for the

selection and isolation of these aptamers is called systematic evolution of ligands by exponential enrichment (SELEX) characterized by the repetition of successive steps consisting of selection (binding, partition and elution), amplification and conditioning.

Aptamers have become increasingly important molecular tools for diagnostics and therapeutics. In particular, aptamers offer several advantages over their antibody counterparts for their flexibility to adapt to different assay formats. The main advantages of aptamers are that once they are selected they can be synthesized with high reproducibility and purity from commercial sources and are chemically stable, which offers great flexibility in the design of novel assays with high sensitivity and selectivity [71-74]. Moreover, it is also possible to produce aptamers to specific regions of targets, which is sometimes difficult for antibodies, since the animal-immune system is inherently generated towards specific epitopes on target molecules. Furthermore, aptamers can also recover their native conformation following denaturation. Aptamers in general possess excellent selectivity and affinity toward their targets, binding with dissociation constants (K_D) ranging from picomolar to nanomolar. Additionally, even small variations in the target molecule may disrupt aptamer binding (e.g., the aptamers for theophylline and L-arginine can discriminate closely related chemical structures by factors as high as four orders of magnitude) [75, 76].

1.2.1 SELEX process

A generic SELEX process involves incubation of a random nucleic acid library, generally 10^{13}-10^{15} different sequences with a target [77]. Sequences which bind to the target are then partitioned and any non-binding or low affinity sequences are discarded. Binding sequences are eluted and amplified, generally using the polymerase chain reaction (PCR). From the resulting double stranded DNA (ds-DNA), single stranded DNA (ss-DNA) or transcribed RNA is generated, and this enriched pool of selected oligonucleotides is used in the next round of SELEX. Rounds are repeated until sequence enrichment occurs at which point aptamer candidates can be sequenced and characterized (Figure 1.3).

Classical SELEX is a well-established approach; however, large rounds of SELEX (8-15 rounds) are needed for the selection, which is time-consuming, laborious and sometimes ineffective particularly as there is no standardized protocol [78, 79] and optimisation of conditions is dynamic throughout the SELEX process. However, significant progress has been achieved in optimizing the different steps of SELEX.

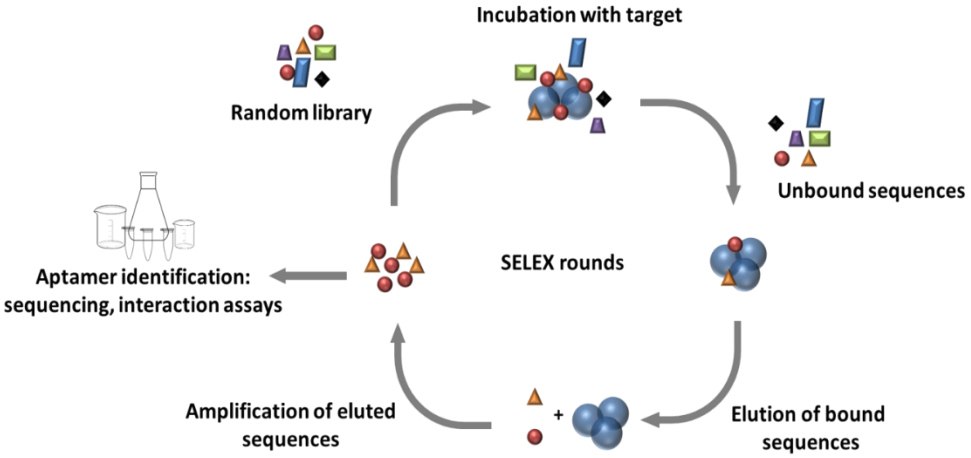

Figure 1.3: Schematic overview of DNA SELEX.

1.2.1.1 Starting libraries

The design of the library should provide an optimal starting point for the SELEX process. The most frequently used library consists of a central random region of typically 30 – 60 nucleotides flanked by primer sequences of around 20 nucleotides. This random region should contain all four bases in an equal distribution. Many researchers reported that an equal distribution increases the sequence space and consequently an enhanced possibility to select aptamers with the desired binding properties [80]. An alternative strategy involves the use of biased libraries which are degenerated sequences of a known binding motif. This technique is useful to isolate mutated sequences to facilitate a deeper understanding of the interaction with the target or to improve binding characteristics [81]. Frequently, the main drawback is that the synthetic libraries are not

of the required quality and recently, next generation sequencing (NGS) and bioinformatic analysis has been used to ensure library quality as well as a tool for monitoring SELEX.

Another approach to improve library diversity and increase stability of oligonucleotides, was the use of modifications, including 2'-aminopyrimidines in 2' position of pyrimidines [82, 83], 2´fluoropyrimidines[84, 85], 2´O-methyl nucleotides [86, 87], position 5 of pyrimidines [88, 89], and 4 of pyrimidines using thiol UTP and CTP [90], amongst others.

Another factor to consider is the length of the primers as the primer sequence can contribute up to more than half of the sequence used in the starting library depending on the length of the random region. The primer regions can interact non-specifically with the target or limit the complexity of the nucleic acid library by interacting with the random region. Several protocols have been developed to address this, including tailored [48] and dual SELEX [91] . These strategies are characterized by the use of conserved sequences (7 – 10 nucleotides) which are sequestered by self-complementary sequences, thus minimizing the risk that they become part of the target-binding motif. An alternative strategy is primer-free DNA aptamer selection [92], which employs endonuclease cleavage of the doubled stranded DNA template. The library is reconstituted after selection by ligation with primer annealing sites. This protocol was used to isolate aptamers against HIV reverse transcriptase [93].

1.2.1.2 Alternative rapid SELEXs

Different methodologies have been reported with the aim of reducing the time required to perform the selection of aptamers, by omitting steps from the traditional protocol. One example is RAPID (RNA aptamer isolated via dual cycles), a technique based on two binding and separation steps, but without amplification of the selected RNA libraries by reverse transcription, PCR and transcription [94]. Examples of aptamers selected by this methodology are against UBLCP1 and CHK2 proteins where the time to complete the selection decreased from 355 h (in the conventional SELEX) to 84 h achieving identical sequences in the top five candidates in both SELEX approaches.

Capillary electrophoresis SELEX (CE-SELEX) is another methodology used for the isolation of aptamers against protein targets [95-98]. This technique takes advantage of the efficient separation of free nucleic acids from aptamer/target complexes, thus reducing the cycles needed for the isolation of high affinity aptamers to four or sometimes even less. CE-SELEX has also been applied for aptamers against small molecules like N-methyl mesoporphyrin (NMM), which is challenging due to the minimal differences in the mobility shift between aptamer and complex [99].

An alternative capillary electrophoresis SELEX is Non-SELEX which applies non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) to achieve partitioning, with just three rounds of separation required. The time to complete the selection is just one hour [100-103]. MonoLex is based on a similar principle, which includes just one chromatographic separation step and no consecutive *in vitro* selection cycles [104, 105]. However, these methodologies allow the application of very low reaction volumes restricting the overall amount of the starting library. A microfluidic free flow electrophoresis (µFFE) device solves this problem, as it allows loading around 300-fold more library through a continuous flow compared to capillary electrophoresis. IgE aptamer was identified using this technique after one round, indicating that µFFE is a very efficient separation method [106].

1.2.1.3 SELEX on a chip

The first automated selection protocol based on a BiomeK 2000 pipetting robot (Beckman Coulter) was published in 1998 [107], followed by modifications and improvements [108-110]. In 2009, microfluidic SELEX (M-SELEX) was first reported. M-SELEX is based on a miniaturized system for the partitioning step where magnetic capture is achieved in microchannels and micro-magnetic separation is applied to select aptamers which bind the target proteins - streptavidin, botulinum neurotoxin type A and PDGF-BB, achieving K_D in the nanomolar range [111-113]. Recently, the first platform integrating magnetic separation, micropumps, micromixers and temperature control systems for enzymatic amplifications reactions for aptamers against the influenza A/H1N1 virus was reported. This is the first demonstration of screening influenza virus-specific aptamers

using microfluidic SELEX technology, which may be expanded for the rapid screening of aptamers against other pathogens for future biomedical applications [114].

1.2.1.4 Capture-SELEX

Stoltenburg *et al.* proposed an alternative separation method for small molecules since there is little difference in the molecular weight or charge of the complex compared to the aptamer alone and the strategies reported above cannot be employed. This technique is termed Capture-SELEX and in this case the nucleic acid library is immobilized on magnetic beads and aptamers are eluted by specific binding to the target [115]. The library was designed with a short fixed hybridization sequence referred to as a docking sequence. The library is captured on magnetic beads via a complementary sequence to the docking sequence and aptamers were specifically eluted by small molecule interaction. An example is an aptamer against Kanamycin A, with low micromolar affinity which was selected as proof of concept [116].

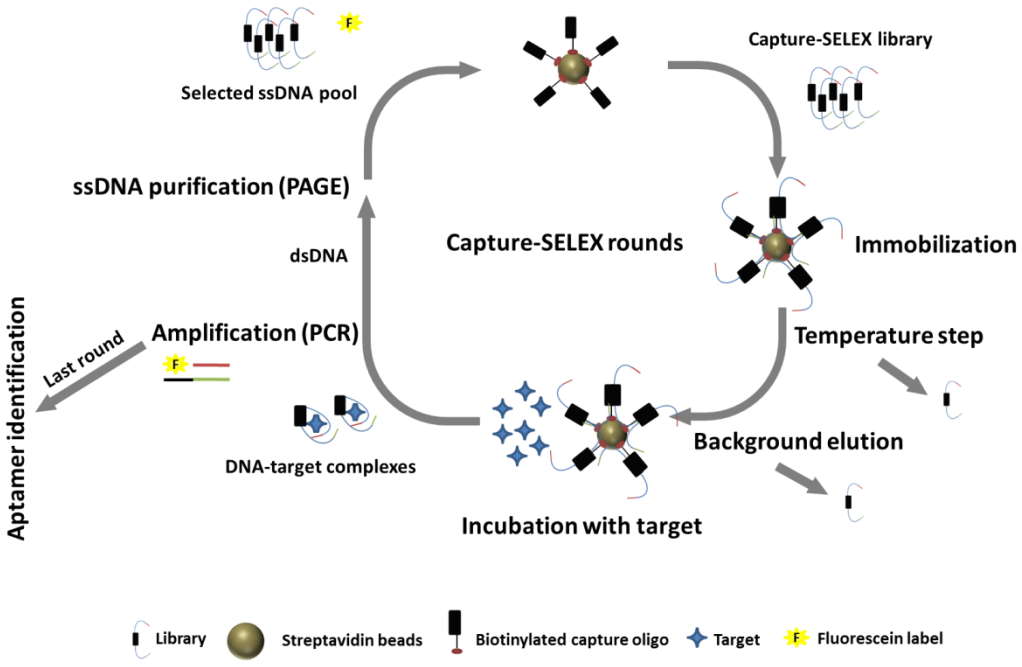

Figure 1.4: Schematic overview of Capture-SELEX.

1.2.1.5 Cell-SELEX

Cell-SELEX exploits an entire cell as the target. Extracellular cell surface proteins or some unknown structures of the cells can be used as targets. Thus, whole cell targeting aptamers can be selected through SELEX without having indepth knowledge about the surface proteins of the cells, which permits the discovery of novel biomarkers for primary diagnostic and imaging proposes [117]. Recently, Meyer *et al*. performed a variety of Cell-SELEX methods by selecting aptamers against specific cell surface proteins in their native forms termed Specific Target Cell-SELEX (STACS) [118].

1.2.1.6 High-throughput identification of aptamers

Conventional SELEX is based on cloning and Sanger sequencing to isolate candidate aptamers giving access to the most frequent clones. Identical sequences are counted and subsequently aligned in order to group similar motifs. Using NGS, the procedure followed is similar: the huge amount of raw data is analysed in terms of counting sequences and ranking them in order of frequency [111, 119, 120]. These sequences can be analysed with high resolution and be ranked and filtered by cut-off of read numbers to select candidate aptamers for further testing. The major advantage of using NGS is minimizing the number of SELEX cycles for the identification of aptamers, as in the course of multicycle SELEX evolution pressure shifts from the side of high affinity sequences to sequences which are more efficiently amplified, favouring shorter as well as structurally less stable sequences [111, 121, 122].

Another parameter to consider during the analysis of the raw data is that the most potent sequences are not necessarily those with high frequencies and for this reason it is important to apply clustering of related sequences. The clustering is performed independently from their copy number. Sequences which are members of the same clusters or families can be analysed using alignment tools [123, 124] or tools based on secondary structure predictions [125-128] with the aim of identifying essential motifs such as protruding stems and loops regions that are determinants of aptamer affinity [129-132].

1.2.2 Aptamers as diagnostic and therapeutic agents

1.2.2.1 Diagnostics

The most popular platform for clinical diagnostics, environmental monitoring and food quality control is the enzyme-linked immunosorbent assay (ELISA). Sandwich ELISA consists of an antigen (target), an antigen capturing antibody and a detection antibody that produces a signal when the antigen is present. However, ELISA assays have various drawbacks including batch-to batch variation in the production of polyclonal antibodies and it is tedious and challenging to generate specific monoclonal antibodies, especially against non-immunogenic molecules. All these problems can be addressed using aptamers, resulting an improved ELISA-enzyme-linked apta-sorbent assay (ELASA), also referred to as enzyme-linked aptamer assay (ELAA), enzyme linked oligonucleotide assay (ELONA), and aptamer linked immobilised sorbent assay (ALISA). One advantage of aptamers in ELAA is their stability. Unlike antibodies, which can suffer from permanent degradation when exposed to extremes of pH or heat, aptamers can be easily regenerated [133]. Another advantage is the immobilization of the aptamer on the surface of the solid support, which is via controlled orientation, which enables high target binding efficiency. Normally, the aptamer is functionalised with a terminal group such as a biotin or amine moiety [134]. Sometimes an oligonucleotide spacer is added to the terminal functional group to create flexibility and these spacers may increase target protein binding and improve levels of detection attainable [134-137]. Finally, aptamers can be used in different types of configurations in ELAA, classified as direct ELAA (Figure 1.5a), indirect ELAA (Figure 1.5b) and sandwich ELAA. In sandwich ELAA, the capturing antibody or aptamer is immobilised onto the microtiter plate and then the sample containing the target is added, followed by addition of an enzyme-labelled detection antibody/aptamer and subsequently substrate that will result in signal emission. Different configurations can be used, including aptamer-target-antibody, antibody-target-aptamer, aptamer-target-aptamer, aptamer-target-capture antibody-detection antibody and aptamer-target-capture antibody-aptamer (Figure 1.5c).

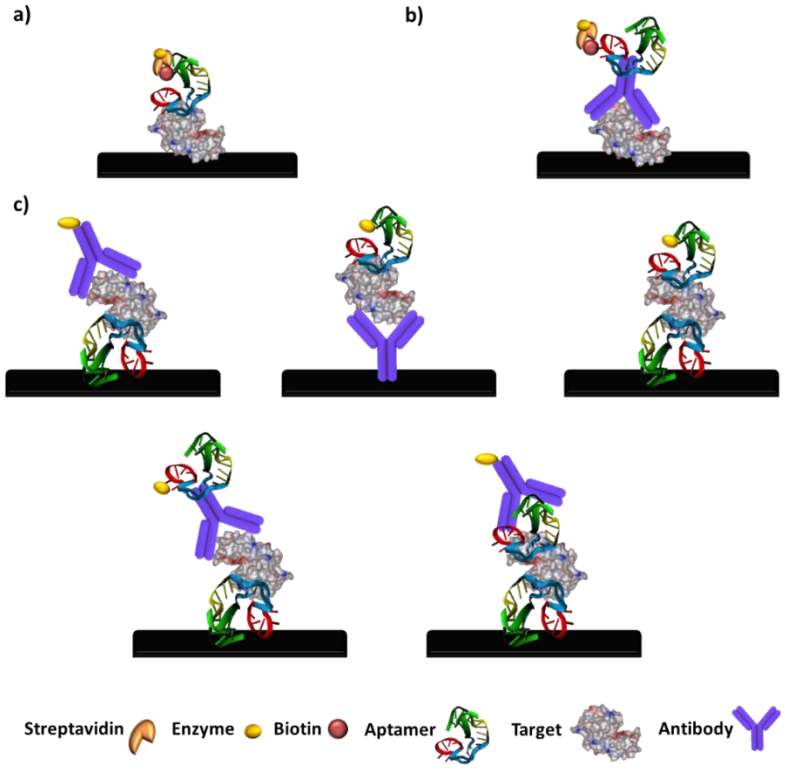

Figure 1.5: a) Direct ELAA; b) Indirect ELAA; c) Different configurations based on Sandwich assay ELAA

In the aptamer–target–aptamer configuration, two different aptamers against the target are used for capturing and detection purposes. This may facilitate a higher affinity as well as a higher specific for the target than antibodies, and the reporter aptamers can be easily labelled. However, there are very few reports of aptamer pairs.

The first mention of dual aptamers that bound distinct epitopes of human thrombin was reported in 1997 [138] and these thrombin dual aptamers has been used in several analytical techniques and diagnostic applications [139-148]. In addition, four individual aptamers have been demonstrated to bind to different binding sites on the adenocarcinoma A549 cell, but these have not yet been used in a combined detection assay [149]. Aptamer couples have also been detailed for cancer cells including aptamers for the differentiation of prostate cancer cells and prostate non-cancerous cells, which have been used both for detection [150, 151] and therapy [152], as well as a combination of aptamers against each of the MUC1 and HER2 breast cancer cells [153], with potential for

use as screening tools. The same aptamer has been used as both capture (primary) and reporter (secondary) against the cancer related platelet derived growth factor in diverse detection strategies [154, 155]. There are a few recent examples of the exploitation of dual aptamers for the detection of *Staphylococcus aureus* [156] as well as prion proteins, where one aptamer binds to 23 – 90 of the N-terminal, whilst the other binds to 90 – 231, part of the β-sheet structure of the PrP [157]. Other examples include dual aptamers against different binding sites of vaspin [158] and the VEa5 and Vap7 aptamers selected against diverse aptatopes on the vascular endothelial growth factor ($VEGF_{165}$) target. In the examples cited, the simultaneous recognition of an analyte by two aptamers generally provides a higher binding affinity and specificity to their target as compared to the use of any of the individual aptamers alone [152, 158, 159].

Furthermore, various aptasensor formats exploiting different modes of transduction have been developed.

a) **Colorimetric platforms** can be attractive approaches due to their simplicity and low cost for sensing, and are particularly suitable for point of care tests (POCT). The most common methods used are based on gold nanoparticle (AuNP) – based colorimetric aptasensors. Different formats have been exploited, including observation of localized AuNPs in solution or on a solid support. The targets are captured by aptamers immobilized on a solid support and the detection of the target can be observed by the secondary aptamer-AuNPs conjugates binding to the target in a sandwich assay. An example of this is the lateral flow assay where the support used is a cellulose membrane [160, 161]. A second approach is based on the networking of AuNPs by sandwich binding. The formation of AuNPs network results in a red-purple colour change due to a decrease of AuNPs inter-particle distance. Platelet-derived growth factor receptor (PDGFR) was detected using this method, achieving a LOD of 3.2 nM by the competitive binding of PDGF aptamer and PDFGR to PDGF [162]. The main problem of both formats is the lack of available dual aptamers against the same target, with only a few reports as described above. To overcome this limitation, complementary DNA has been proposed to construct a AuNPs network. Two probes partially complementary to parts of the aptamer are individually immobilized on

AuNPs. In the absence of target, the aptamer leads to networking and aggregation of AuNPs via hybridization with the probes resulting in a change of colour from red to purple. In the presence of target, the network is destroyed by the target binding to the aptamers, resulting in a change of colour from purple to red. Some reports include the detection of adenosine and cocaine, which could be detected using the naked eye [163].

b) In contrast with colorimetric assays, **fluorometric assays** are not easily applicable to POCTs, but they do offer advantages in terms of sensitivity as well as the wide range of fluorescent and quenching materials available, and their ease of conjugation to aptamers [164, 165]. Different assays have been reported, including aptabeacons using hairpin structured aptamers [166, 167], aptamer based fluorescence resonance energy transfer (FRET) assays [65, 168-171], target-induced displacement fluorescence assays [172, 173], intercalating dye-based label free fluorescence assay [161, 174, 175] as well as an excimer signalling method[176]. AuNPs have also been widely used in fluorometric aptasensors due to their superquenching properties [177]. Based on these properties Chang and co-workers developed a FRET based-aptasensor using AuNPs for the detection of PDGF with a limit of detection of 8 pM [178].

c) **Electrochemical aptasensors** have received considerable attention for the facile and cost-effective transduction of aptamer interactions. Different strategies were developed such as simple binding of aptamers to targets [179-182], target induced conformational change [183-185], target induced displacement of labelled complementary DNA [133, 186-188], and applications of intercalating redox molecules such as methylene blue [189, 190], [191]. The incorporation of nanoparticles such as AuNPs or magnetic nanoparticles, has the potential to amplify electrochemical signal. In the case of AuNPs, the approaches can be classified in two strategies. The first approach is the enlargement of electrode surface by attachment of AuNPs on the electrode, which can increase the amount of capture probes on the electrode, thus enhancing the electrochemical signal. An example is the detection of cocaine using AuNPS self-assembled on a gold electrode with a LOD 10-fold lower than that of the aptasensor without AuNPs modification [192]. An alternative approach is the use of AuNPs as a label for the amplification of electrochemical signal, which has been exploited for the detection of MUC1, an epithelial tumor marker, based

on the conjugation of the hairpin structured MUC1 aptamer labelled with biotin and horseradish peroxidase (HRP). In the present of target, the hairpin structure of the aptamer was stretched by binding to MUC1 and the conjugate (AuNPs-aptamer-HRP) was immobilized on a streptavidin modified electrode by the binding of biotin at the end of the aptamer. In the absence of target, no immobilization of the complex is achieved as the hairpin structure of the aptamer prevents the binding between biotin and streptavidin [193]. Magnetic nanoparticles also enable signal amplification by simple magnetic pre-concentration of targets. Tan and co-workers demonstrated the detection of acute leukemia cells [194, 195]. Specific aptamers were conjugated to magnetic nanoparticles and $[Ru(bipy)_3]^{2+}$ doped nanoparticles. The combination of the two aptamer conjugated nanoparticles enabled the performance of selective cell separation from complex biological samples, achieving high sensitivity and a short assay time.

d) **Surface plasmon resonance (SPR)-based aptasensor**: this technology offers label-free detection and real time quantitative analysis, and provides binding constant determination. SPR is a mass-sensitive biosensor which detects mass changes associated with the change in refractive index at the surface due to the molecular binding events. SPR detection of aptamer – protein interactions is well established and widely used, with examples including tubulin [196], thrombin and thyroid transcription factor 1 [197], HIV-1 Tat protein [198], C-reactive protein [199], human immunoglobulin E (IgE) [200] and adipokines [201, 202] by a single site binding configuration. Additionally, AuNPs have been combined with SPR aptasensors to enhance the signal, as in the case of IgE which produced the lowest LOD for detection of human IgE, achieving 2.07 ng/ml [200]. Recently, there is increasing interest in using SPR for small molecules. Wang and co-workers initiated adenosine detection using AuNPs based on surface inhibition detection [203].

e) **PCR techniques:** aptamers, even complexed with their target can be amplified using the polymerase chain reaction (PCR) and based on this property different techniques have been applied in aptasensors including real-time PCR (RT-PCR) analysis [204, 205], Apta-PCR [63, 142, 206-208] and rolling-circle amplification [209-212], achieving ultrasensitive detection limits.

Despite all these applications of aptamers in diagnostics, to date there are no successful products on the market and more intensive efforts for commercial applications of the aptamers are required. Lateral flow aptamer assays are particularly attractive for POCTs as the manufacturing process is well established with low cost and without the necessity of equipment and trained personnel.

1.2.2.2 Therapeutics

Another promising area of applications of aptamers is in therapeutics. The advantages are: (1) the aptamers can interact tightly and specifically with their targets and their size gives them multiple opportunities to discriminate between epitopes on related proteins, even closely related proteins, such as a protein kinase C (PKC) isozymes that are 96% identical [213]; (2) aptamers can disrupt the function of their targets such as the case of the Anti-HIV reverse transcriptase aptamers [214] or RNA and RNA modified aptamers selected to bind basic fibrobast growth factor (bFGF), which inhibit bFGF binding to cell surface receptors at 1 nM [215, 216]. Another example is the case of anti-vascular endothelial growth factor (VEGF) aptamers selected from a modified RNA pool which inhibits receptor binding [217]; (3) aptamers can also be used to inhibit or modify the metabolism associated with the target. Some examples include anti-thrombin aptamers, which have been shown to block blood clotting [218, 219]; anti-human neutrophil elastase (HNE) aptamers inhibit interleukin-1 (IL-1) induced [220]; as well as aptamers against the Rous sarcoma virus that can inhibit infection when they are pre-incubated with the virus [221] and finally, anti-IgE aptamers that can inhibit IgE-mediated serotonin release from cells in tissue-culture [222].

Although aptamers for therapeutic applications are still limited, to date Pegaptanib (marketed as Macugen), an anti-VEGF aptamer that recognizes the majority of human VEGF-A isoforms, has been approved by the FDA for the treatment of age-related macular degeneration (AMD) [223], and AS1411, a targeting aptamer for the treatment of acute myeloid leukemia (AML) is under Phase II clinical investigation [224]. Some of the most obvious disadvantages of aptamers as drugs have already been solved. For instance, while natural RNA and DNA molecules are relatively unstable in serum or

within cells, chemically modified nucleic acids including pyrimidines containing 2'-amino moieties, are recognized by T7 RNA polymerase and AMV RT, and can be incorporated into the selection process. A modified anti-bFGF aptamer increased its' stability at least 1000-fold compared to unmodified RNA [215]. Another restriction can be the size of the aptamers, and their consequent cost of production. Fortunately, many aptamers can form specific interactions with their targets via domains of 30 to 40 nucleotides and as small as just 11 nucleotides.

Table 1.4: Examples of selected aptamers for applications in diagnostics.

Target	Method	Limit of detection (LOD)	Reference
Adenosine	Colorimetric assay	Qualitative	163
	Label free fluorescent functional DNA sensor	6 µM	175
	Electrochemical sensing platform based on switching signalling aptamers	2×10^{-8} M	133
	Label-free electrochemical aptasensor based on target-induced displacement	1 nM	189
	SPR based on surface inhibition detection	$1 \times 10^{-9} - 1 \times 10^{-6}$ M	203
Adenosine monophosphate (AMP)	Aptamer induced disassembly of fluorescent and magnetic nano-silica sandwich	0.1 µM	170
Adenosine triphosphate (ATP)	Structure-switching / fluorescence-dequencing mechanism	K_D: 600 µM	173
	Target-responsible electrochemical aptamer switch (TREAS)	10 nM – 1 mM	185
	Rolling-circle amplification	1 µM	209
Adenosine triphosphate (ATP) and α-thrombin	Multifunctional label free electrochemical biosensor	ATP: 1×10^{-8} M α-thrombin: 1×10^{-11} M	186
Cancer cells	Lateral flow assay	Visual: 4000 Ramos cells Strip reader: 800 Ramos cells	160
	Apta-PCR	77 cells	206
Cocaine	Aptamer-based folding fluorescent sensor	10 – 4000 µM	171
	Excimer signalling approach	1 µM	176
	Electrochemical integration of switching aptamer.	10 µM	183
	Electrochemical aptasensor with gold nanoparticles modification	0.5 µM	192
C-reactive protein	SPR	0.0005 ppm	199
Endothelial growth factor ($VEGF_{165}$)	Sandwich aptamer microarray (SAM)	Qualitative	144
HIV- 1 Tat protein	SPR	0.12 – 2.5 ppm	198
Human immunoglobulin E (IgE)	SPR	Direct: 3.44 ng/ml Sandwich: 2.07 ng/ml	200
L-argininamide	Flourescence-labeled aptamer	K_D: 95 µM	169
Lysozyme	Label-free impedance spectroscopy	140 fmol / 10 µl	181
MUC 1 (epithelial tumor marker)	Hairpin structured aptamer with biotin and HRP electrochemical sensor	2.2 nM	193
Muc 1 and HER2 breast cancer cells	Dual aptamer-functionalized silica nanoparticles	1 cell / 100 µl	153
Platelet derived growth factor (PDGF)	Electrochemical dual aptamer-based sandwich biosensor	0.3 pM	154
	Aptamer-based fluorescence detection	4 pM	155
	FRET based aptasensor using AuNPs	8 pM	178
	Colorimetric aptasensor	3.2 nM	162
	Real time rolling-circle amplification	0.4 nM	211
Platelet-derived growth factor B-chain (PDGF-BB)	Rolling-circle amplification in electrochemical platform	10 fM	212

Table 1.4: (continued)

Target	Method	Limit of detection (LOD)	Reference
Prions proteins	Surface-enhanced Raman scattering (SERS)	3 – 12 x 10^{-9} M	157
Prostate cells	Electrochemical detection using dual-aptamer probe	1 x 10^{2} cells	151
Retinol binding protein	SPR	K_D: 0.2 µM	201
Theophylline	Fluorescing molecular switches	0.01 – 2 mM	166
	Label-free electrochemical sensor	0.2 µM	190
Thrombin	Surface plasmon resonance (SPR)	0.1 nM	139
	Enzyme linked aptamer assay (ELAA)	25 fM	141
	Apta-PCR	450 fM	142
	Fluorescent-based aptamer assay	20 pM	143
	Sandwich aptamer microarray (SAM)	Qualitative	144
	Sandwich aptamer microarray (SAM)	Qualitative	145
	Colorimetric assay	1 nM	146
	Fluorescent quantum dots nanoparticles	0.8 pM	147
	Lateral flow assay	2.5 nM	161
	Structure-switching / fluorescence-dequencing mechanism	K_D: 400 nM	173
	Impedimetric aptasensor	2 nM	180
	Impedance assay	1 x 10^{-14} – 1 x10^{-13} M	182
	Electrochemical assay using methylene blue tagged	Low nanomolar	184
	RT-PCR	10 pM	204
	Apta-PCR	450 fM	142
	Rolling-circle amplification	10 pM	210
Thyroid transcription factor 1	SPR	K_D A: 3.36 x 10^{-9} M K_D C: 3.25 x 10^{-8} M	197
Tubulin	SPR	K_D: 10 µM	196
UO (+2)	Label free fluorescent functional DNA sensor	3 nM	175
Vaspin	SPR platform	3.5 ng/ml in buffer 4.7 ng/ml in serum	158

1.2.3 Aptamers for Food Quality Control

Food safety has garnered increasing public attention [225], and as as plethora of harmful substances can be found in foods, effective food analysis is of critical importance. The amount of harmful substances is usually in the level of micrograms, nanograms and even picograms, thus requiring highly sensitive detection methods. Many analytical instruments have the disadvantages of expensive cost, complex operation, and long detection time required, as well as being inherently laboratory based. Thus, aptamers, given their advantages of high specificity and sensitivity, wide range of potential target molecules, low cost, *in vitro* synthesis, safety, and reliability,

have promising applications in food safety testing. Currently, various aptamers have been selected in this field.

1.2.3.1 Microorganisms in Food

The pollution of food microorganisms in meat, poultry, eggs, and other foods is increasing annually. Traditional methods for the detection of food pathogens are based on plate count, which has the disadvantages of a labor intensive detection process and a long detection time [226]. Several aptamers have been reported for the detection of different serotypes of Salmonella. Pan *et al.* have reported the direct selection of aptamers for *Salmonella enteric* serovar Typhi with the selection of a RNA aptamer specific for IVB [227]. Joshi e*t al*. have selected and assessed the DNA aptamers for the capture and detection of *Salmonella enteric* serovar Typhimurium [228]. Moreover, an electrochemical biosensor with a limit of detection as low as 3 CFU/ml was reported by Ma *et al.* [229]. For the detection of *Campylobacter jejuni*, a quantum dot-based sandwich assay for use with plastic-adherent DNA aptamer-magnetic bead, was reported, with detection limits of as low as 10 – 250 CFU in various food matrices [230]. Listeria spp. has been detected in foods such as chicken and beef using an aptamer and antibody-functional optical fiber biosensor with an LOD of 10^3 CFU/ml [231]. Suh and Jaykus selected Lbi – 17, which could be used to detect the pathogen at concentrations of < 60 CFU/ 500 µl buffer, in the presence of a heterogeneous cocktail of non-Listeria bacterial cells [232]. Lee *et al.* established a sensitive method to test *Escherichia coli* using aptamers and RT-PCR amplification technology [205]. In addition, a lateral flow biosensor to detect *E. coli* at concentration of 10 CFU was developed [233]. An aptamer-quantum dot conjugate to *Bacillus thuringiensis* spores was used to detect this pathogen at concentrations of about 10^3 CFU /ml [234], whilst Hen *et al.* employed aptamer-based surface-enhanced Raman spectroscopy and designed an innovative label-free platform for the detection and discrimination of *Bacillus anthracis* spores in orange juice at concentrations of 10^4 CFU spores[235]. Finally, aptamers against *Staphylococcus aureus,* which allowed the detection of the pathogen at 7.6 x 10^2 cells / ml in spiked milk has been reported by He *et al.* [236].

1.2.3.2 Food allergens

Allergy is an abnormal immune-mediated reaction occurring in susceptible individuals after consumption of a certain food or food ingredient as it is described previously. The selection of aptamers for this group of ingredients is emerging. There are just a few aptamers selected to bind allergenic proteins, and only a couple of them have been tested for analytical applications. For instance, the aptamer developed to bind the protein Ara H1 from peanut has been used as a capture reagent in a sandwich assay using fiber optic coated surface plasmon resonance (SPR). An antibody acted as the secondary recognition element but the wavelength shift was too small and an amplification scheme was implemented using protein A-modified with gold nanoparticle. However, the mixed sandwich approach was less sensitive than a previous one using two antibodies [237]. Another example are the aptamers developed against an immunotoxic peptide from gliadin from wheat [207, 238]. A competitive electrochemical magnetoassay was developed without cross-reactivity with non-triggering celiac disease proteins from soy, rice or maize [239]. Highly specific aptamers also were selected against to the food allergen Lup an 1, β-conglutin, showing no cross-reactivity with other flour ingredients or with other conglutins fractions of lupin [39]. Two methodologies were reported for the detection of this allergen, an ultrasensitive technique based on the real time amplification of the eluted aptamer referred as Apta-PCR and a fluorescence resonance energy transfer (FRET) assay with a limit of detection in picomolar range in both cases [63, 65].

1.2.3.3 Heavy metal ions

Industrial emissions of cadmium, mercury, lead, and other heavy metal ions into soil and rivers can cause pollution of the environment, accumulating in crops, seafoods and other raw materials that can enter into the human food chain, causing acute or chronic food poisoning of humans and damaging human health. The sources of heavy metals ions in food mainly comes from the use of pesticides, industrial pollution, the pollution of raw materials, additives and packaging materials in food processing [240]. Examples of aptamers against heavy metals using aptamer-functionalized colloidal

photonic crystal hydrogen (CPCH) films were developed for the detection of Hg (+2) and Pb (+2) [241]. These aptasensors could be used in the screening of a broad range of metal ions in food, drugs, and the environment with high selectivity and reversibility.

1.2.3.4 Biotoxins

Biological toxins are microbial metabolic products, which mostly have carcinogenic, teratogenic and mutagenic effects. Therefore, it is extrmely important to have reliable and sensitive biotoxin tests in order to prevent the entrance of toxins into the food chain [242]. Aptamers specific for these particular targets, including ricin, cholera toxin, staphylococcal enterotoxins, ochratoxin (OTA) and abrin toxin have been selected in the past years, and by using these aptamers, different detections systems have been developed[243-249].

1.2.3.5 Antibiotics

Antibiotics are chemical substances that have the capacity to inhibit the growth of bacteria or even destroy them. They are often added in the aquaculture industry in order to cure animal diseases or promote animal growth, and thus there are antibiotic residues in many agricultural, animal and plant products [250]. Accumulation of antibiotics in human body will cause very serious consequences [251]. Aptamers against oxytetracycline, tetracycline, danofloxacin, neomycin, kanamycin are some of the examples reported [252-257].

1.2.3.6 Organic dyes

Organic dyes are added to food in order to conceal bad food appearance and rotting. The most common additive in the tightly rolled dried skin of bean milk, bean vermicelli, flour, and bamboo shoot is sodium formaldehyde sulfoxylate, which plays a roles in the whitening, preservation, and increasing the taste of foodstuffs, disguising food degradation, which can cause harm to the kidney, liver, central nervous system, as well as disturbing immune function and digestive system. Another example is malachite green, which is widely used in aquaculture as an insect repellent, and has carcinogenic

effects, being very harmful to human health [258]. However, at present, SELEX has not been extensively applied to this field, and only Stead *et al.* presented a robust assay for the detection of malachite green in fish tissue [259].

1.2.3.7 Pesticide Residue

Pesticides, which are the most widely used agrochemicals especially in developing countries have posed a great threat for food safety and human health if their residues and relevant metabolites in food matrixes exceed their maximum tolerance levels. Examples are aptamers against acetamiprid and organophosphorus pesticides [260, 261].

1.2.3.8 Other compounds

Natural or synthetic hormones are frequently used in meat and dairy industries to increase the productivity. 17β-estradiol is an endocrine disruptor that may affect the reproduction of humans and animals and thus its' routine monitoring in water samples is required. Another endocrine disruptor is bisphenol A, used in polycarbonate plastic products, such as baby bottles. There are major concerns regarding bisphenol A exposure to fetuses, infants and young people. Aptamers have been developed for the detection of both targets in real samples. 17β-estradiol was detected in water, meanwhile bisphenol A in milk with a limit of detection of 33 fM and 5 nM respectively [262, 263]. Finally, melamine is used as a food adulterant and melamine-induced nephrotoxicity is now a global concern [264]. This substance can be used in plastics that may be in contact with food and / or added to dairy products for infants in China, to replace proteins due to its' high nitrogen content. Jiang *et al.* and Liang *et al.* reported different techniques for the detection of melamine using high affinity and specific aptamers [265, 266].

Table 1.5: Examples of selected aptamers used in Food Quality Control.

Target	Method	Limit of detection (LOD)	Reference
Microorganisms			
Salmonella	RT-PCR	Pull down assay: $10^1 – 10^2$ CFU S. typhimurium / 9 ml Recirculation format: $10^2 – 10^3$ CFU/ 25 ml	228
	Electrochemical biosensor	3 CFU/ml	229
Campylobacter jejuni	Plastic cuvette-adherent technology	10 – 250 CFU/ml	230
Listeria spp.	Antibody-aptamer functionalized fiber-optic biosensor	10^3 CFU/ml	231
	Aptamer magnetic capture (AMC) – qPCR	260 CFU/ 500 µl	232
Escherichia coli	Immunomagnetic separation and RT-PCR	10 E. coli / ml	205
	Lateral flow biosensor	10 CFU	233
Bacillus	Aptamer-quantum dot based fluorescence assay	10^3 CFU /ml Bacillus thuringensis	234
	Aptamer-based surface enhanced Raman spectroscopy	10^4 CFU/ml Bacillus antrancis	235
Staphylococcus aureus	Aptamer-fluorescent silica nanoparticles	7.6 x 10^2 cells/ml	236
	Electrochemical dual-aptamer based sandwich	1 CFU /ml	156
Food allergens			
Ara H1	Fiber-optic surface plasmon resonance (FO-SPR) biosensor	75 nM	237
Gliadin	Apta-PCR	2 nM (100 ng/ml)	207
	Electrochemical competitive enzyme-linked assay	0.5 ppb	239
β-conglutin	Apta-PCR	85 pM	63
	FRET	150 pM	65
Heavy metal ions			
Hg (+2)	Aptamer-functionalized colloidal photonic crystal hydrogel (CPCH)	10 nM – 1mM	241
	Aggregation of gold nanoparticles	250 nM	187
	Label free fluorescent functional DNA sensor	30 nM	175
	FRET	5 nM	168
Pb (+2)	Aptamer-functionalized colloidal photonic crystal hydrogel (CPCH)	1 nM – 1 mM	241
	A-basic site containing DNAzyme and aptamer for label free fluorescent detection	4 nM	174
	Label free fluorescent functional DNA sensor	8 nM	175
	Electrochemical detection via an electrode bound DNAzyme assembly	0.3 µM (62 ppb)	188
	FRET	300 pM	168
Biotoxins			
Ricin	Electrochemical biosensor	2 nM	245
Cholera toxin	Electrochemiluminescence	40 ng/ml	244
Staphylococcal enterotoxins	Electrochemiluminescence	10 pg/ml	244
Ochratoxin (OTA)	Fluorescent aptasensor	0.098 nM in buffer 0.113 nM in grape juice 0.152 nM serum	248
	Hybridization chain reaction and fluorescent perylene probe	0.10 pM	249
	Fluorescent polarization based displacement assay	5 nM	172
Abrin toxin	Molecular light switching reagent $[Ru(phen)_2 dppz]^{2+}$	1 nM	243

Table 1.5: (continued)

Target	Method	Limit of detection (LOD)	Reference
Antibiotics			
Oxytetracycline (OTC)	Fluorescent aptasensor	0.1 nM	254
	Interdigitated array electrode chip	1 – 100 nM	179
Tetracycline	Photoelectrochemical aptasensor	5.3 nM	255
Donafloxacin	Selection (SELEX)		253
Neomycin	SPR biosensor	10 nM	257
Kanamycin	Aptamer-based signal on bio-assay	0.3 nM	252
	Colorimetric detection	2.6 ng/ml	256
Organic dyes			
Malachite green	Fluorescence	5 nmol/L	259
Pesticide Residues			
Acetamiprid	Selection (SELEX)	K_D: 4.98 µM	260
Organophosphorus • Isocarbophos • Omethoate • Phorate • Profenofos	Aptamer-based surface enhanced Raman scattering method	 3.4 µM (1 ppm) 24 µM (5 ppm) 0.4 µM (0,1 ppm) 14 µM (5 ppm)	261
Other compounds			
17 β-estradiol	Photoelectrochemical aptasensor	33 fM	262
Bisphenol A	Electrochemical aptasensor	5 nM	263
Melamine	Resonance scattering using aptamer- modified nanosilver	0.02 – 1.06 µg/L	265
	Aptamer-nanogold catalytic resonance scattering	0.98 µg/L	266

1.3 THESIS OBJECTIVES

In December 2008 lupin was added to the list of substances requiring mandatory advisory labelling on foodstuffs sold in the European Union. To date, there is no existing antibody specific to the Lup an 1 allergen, the β-conglutin subunit of lupin. Our group recently reported the selection of the first aptamer against this allergen. This work overviews the selection and characterization of a second aptamer (β-CBA II) against β-conglutin and its' use in different applications with the aim to develop a specific and highly sensitive aptasensor. The main contribution of this thesis to the state of the art is the use of the selected aptamers in many different applications and the development of different aptasensors, which can serve to guide future research that would improve the knowledge gained in the field of aptasensors.

Overall objective:

The development of analytical tools for the detection of the anaphylactic β-conglutin allergen based on the use of aptamers.

Specific objectives:

The overall objective of the thesis was carried on through the following specific objectives:

1. Selection and characterization of a second β-conglutin binding aptamer II (β-CBA II) which binds to a different aptatope on the β-conglutin target protein compared to the previously selected β-CBA I.
2. Comparative study of interactions between β-conglutin and its' aptamers performed using surface plasmon resonance imaging (SPRi).
3. Ultrasensitive detection of β-conglutin using novel strategies including Apta-PCR and Apta-RPA.
4. Exploitation of lateral flow assays for the rapid, cost-effective and ultrasensitive detection of β-conglutin.

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Chapter 2

β-conglutin dual aptamers binding distinct aptatopes

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2.1 ABSTRACT

An aptamer was previously selected against the anaphylatic allergen ß-conglutin (β-CBA I), which was subsequently truncated to an 11-mer and the affinity improved by two-orders of magnitude. The work reported here details the selection and characterisation of a second aptamer (β-CBA II) selected against a second aptatope on the β-conglutin target. The affinity of this second aptamer was similar to the 11-mer, and its affinity was confirmed by three different techniques at three independent laboratories. This β-CBA II aptamer in combination with the previously selected β-CBA I was then exploited to in a dual aptamer approach. The specific and simultaneous binding of the dual aptamer (β-CBA I and β-CBA II) to different sites of β-conglutin was confirmed using both microscale thermophoresis and surface plasmon resonance where β-CBA II serves as primary capturing aptamer and β-CBA I or the truncated β-CBA I (11-mer) as a secondary signalling aptamer, which can be further exploited in enzyme-linked aptamer assays and aptasensors.

2.2 INTRODUCTION

Antibodies have been exploited in affinity-based detection systems for a wide variety of applications ranging from food quality control and environmental monitoring to clinical diagnostics and are routinely used in different types of enzyme linked immunosorbent assays (ELISA). Sandwich ELISA is a highly robust assay that uses antibodies recognising two different epitopes on the target molecule and has been highly exploited since its inception for the detection of a wide range of medium-large molecules. Furthermore, recombinant antibodies have been designed to bind to adjacent, non-overlapping epitopes simultaneously, demonstrating higher affinity and specificity [1-3].

Aptamers are nucleic acids that can bind to a wide range of diverse targets, from small molecules to proteins and even cells, with high affinity and specificity due to their specific 3D structure [4]. The process used for the selection and isolation of these aptamers is termed systematic evolution of ligands by exponential enrichment (SELEX) characterized by the repetition of successive steps consisting of selection (binding, partition and elution) and amplification [5, 6].

In comparison to antibodies, aptamers have several advantages as recognition elements. They are chemically synthesised and easily modified for wide range of applications. The thermostability and high flexibility of aptamers offer the possibility to be adapted to different assay formats. The first mention of dual aptamers that bound distinct epitopes of human thrombin was reported in 1997 [7], and thrombin dual aptamers has been used in several analytical techniques and diagnostic applications [8-17]. Whilst the vast majority of reported aptamers are single aptamers against a single aptatope on its cognate target, there are reports of targets with multiple aptamers against various aptatopes. Four individual aptamers have been demonstrated to bind to different binding sites on the adenocarcinoma A549 cell, but these have not yet been used in a combined detection assay [18]. Aptamer couples have also been detailed for cancer cells including aptamers for differentiation of prostate cancer cells and prostate non-cancerous cells, which have been used both for detection [19, 20], and therapy [21], as well as a combination of aptamers against each of the MUC1 and HER2 breast cancer cells [22], with potential for use as screening tools. The same aptamer has been used as both capture (primary) and reporter (secondary) against the cancer-related-platelet-derived growth factor in diverse detection strategies [23, 24]. There are a few recent examples of the exploitation of dual aptamers for the detection of *Staphylococcus aureus* [25] as well as prion proteins, where one aptamer binds to 23-90 of the N-terminal, whilst the other binds to 90-231, part of the β-sheet structure of the PrP [26]. Other examples include dual aptamers against different binding sites of vaspin [27] as well as the VEa5 and Vap7 aptamers selected against diverse aptatopes on the vascular endothelial growth factor ($VEGF_{165}$) target. In the examples cited, the simultaneous recognition of an analyte by two aptamers generally provides a higher binding affinity and specificity

to their target as compared to the use of any of the individual aptamers alone [21, 27, 28]. Moreover, the use of dual aptamers can be easily implemented to a sandwich platform with improved performance due to the specific characteristics of aptamers over the commonly used dual antibody ELISA format [29].

Leguminous plants including peanut, soybean and lupin are typical examples of foods containing a considerable number of potent allergenic proteins [30-33]. The globulins consisting of two major subunits (β- and α-conglutin) and two minor subunits (γ- and δ-conglutin) are represented by the majority of storage proteins found in lupin seeds [34]. Although there is no consensus regarding the allergenic properties of individual lupin conglutins [35, 36], β-conglutin has been reported to be one of the most frequent causes of severe food-associated anaphylaxis [37-39] and in 2008 was identified by International Union of Immunological Studies as the Lup an 1 allergen [40].

We have previously selected an aptamer against β-conglutin, termed the β-conglutin binding aptamer (β-CBA I) which showed high affinity and specificity to the target [41]. This highly specific aptamer was used to develop a competitive Apta-PCR assay achieving a detection limit of 8 x 10^{-11} M [42]. The β-CBA I was subsequently truncated from the original size of 93 nucleotides with a K_D 3.6 × 10^{-7} M to just 11 nucleotides, with this truncation resulting in a two-order of magnitude improvement of the K_D to 1.7 x 10^{-9} M [43]. This 11 nucleotide aptamer (11-mer), the shortest aptamer reported to date, was subsequently exploited in FRET based assay, achieving picomolar detection limits, with the only required end–user intervention being sample addition and quantitative detection achieved in just one minute [44].

Here we detail the selection and characterisation of a second aptamer selected against β-conglutin, which we demonstrate binds to a different aptatope. The aptamer was selected using SELEX based on magnetic bead separation and aptamer candidates were subsequently analysed using ion torrent sequencing. The affinity of the selected β-CBA II was determined in three independent laboratories using the different methodologies of surface plasmon resonance, radiolabelling filter-binding assay and microscale thermophoresis. Microscale thermophoresis was also used to explore if the

β-CBA I andβ-CBA II aptamers had different binding sites on the β-conglutin cognate target.

2.3 MATERIALS AND METHODS

2.3.1 Materials

Phosphate buffered saline (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4), 1-ethyl-3-(dimethylaminopropyl) carboiimide (EDC), N-Hydroxysuccinimide (NHS), and all other reagents were purchased from Sigma (Barcelona, Spain). Sodium Chloride, sodium hydroxide 2 M, hydrochloric acid 6 M were purchased from Scharlau Chemie S.A. (Barcelona, Spain). SiGMAG-Carboxyl beads were obtained from Chemicell (Zaragoza, Spain) and the Tfi DNA Polymerase and 10 bp DNA ladder from Invitrogen (Spain). Certified TM Low Range Ultra Agarose and Precision Plus Protein TM Standards were purchased from Bio-Rad (Barcelona, Spain). Oligonucleotides (HPLC purified and provided lyophilized) were purchased from BIOMERS (Ulm, Germany). All solutions were prepared in high-purity water obtained from the Milli-Q RG system (Barcelona, Spain).

2.3.2 Aptamer selection

Proteins from *Lupinus albus* seeds were extracted, purified and characterised, obtaining a pure isolate of β-conglutin. β-conglutin was linked to SiGMAG-Carboxyl magnetic beads via carbodiimide coupling, and the bioconjugation was confirmed using peptide mass fingerprinting (PMF) as previously described [41].

The DNA library used consisted of 94-mer DNA sequence containing a random region of 50 nucleotides flanked by primer binding regions; 5'-agc tcc aga aga taa att aca gg- n(50)-caa cta gga tac tat gac ccc-3'.

In the initial round of selection, the DNA library pool (300 pmol) was denatured at 95ºC for 5 min and immediately cooled to 4ºC, after which 5 µl of magnetic beads functionalised with β-conglutin was added and the volume was brought to a total volume of 100 µl with binding buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM $MgCl_2$, pH 7.4), and the mixture incubated for 30 minutes at 18ºC using tilt rotation.

Following incubation, the beads were washed several times with 500 µl of binding buffer. After the first round, a negative selection step was introduced prior to the positive selection step, in order to remove non-specific binders. Selected DNA was incubated with 5 µl of control beads (magnetic beads with no protein attached and ethanolamine blocked), and as before, the volume was brought to 100 µl with the binding buffer for incubation at 18ºC for 30 min, and the unbound DNA was subsequently used for the positive selection step. The obtained library pool was amplified by PCR and single stranded DNA (ssDNA) was generated by a combination of asymmetric PCR and exonuclease digestion using PTO forward primer and addition of T7 Gene 6 exonuclease as previously described [45]. Highly purified ssDNA was obtained using the QUIAX II kit and the ssDNA obtained from each round was used as the initial ssDNA pool for the following round of SELEX.

Evolution was followed after each round of SELEX using pilot PCR of nucleic acids that bound to the matrix (negative selection) and nucleic acids that bound specifically to β-conglutin (positive selection). A small aliquot of the SELEX pool was amplified for 4-14 PCR cycles, in order to elucidate the optimal number of amplification cycles. Following PCR, the amplified products from the negative and positive selection were analysed using agarose gel electrophoresis, with an indication of successful evolution being inferred by an increasing width of the band for positive selection and a decreasing band for negative selection. Having observed an indication of success the SELEX pool from the seventh round was sequenced using ion torrent sequencing, and analysed using Galaxy software to identify any consensus motifs.

2.3.3 Binding affinity studies

Surface plasmon resonance (SPR) was performed using the BIAcore 300 (Biacore Inc.). Proteins of interest (β-conglutin, α-conglutin, γ-conglutin) were immobilised on separate channels of a CM5 sensor chip activated with EDC/NHS (30 µl of a 1:1 mixture of EDC (400 mM) and NHS (100 mM)), followed by injection of 200 µg/ml protein at a flow rate of 5 µl/min. Following immobilisation of the proteins, any unreacted NHS esters were deactivated via injection of an excess of ethanolamine hydrochloride (1 M).

Unbound proteins were then washed from the surface using 2 M NaCl, 10 mM NaOH. The aptamer candidates were diluted in binding buffer to a final concentration of 1 µM and injected during 6 min at a flow rate of 5 µl/min followed by a 3-min stabilisation and 10-min dissociation. The binding was analysed through the corresponding changes in the refractive index of the optical signal, and expressed as resonance units (RU).

Microscale thermophoresis (MST) binding experiments were carried out with 5 nM of β-CBA II aptamer labelled with a Cy5 fluorophore in the 5' end in binding buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM $MgCl_2$, 0,1% Tween, pH 7.4) with a range of concentrations of β-conglutin (0.056 – 1845 nM) at 20% MST power, 20% LED power in standard capillaries on a Monolith NT.115 pico device at 25°C (NanoTemper Technologies, Munich, Germany). A ligand-dependent quenching effect was detected, and the raw fluorescence was thus used for analysis. The recorded fluorescence was normalised to fraction bound (0 = unbound, 1 = bound), and processed using the KaleidaGraph 4.5 software and fitted using the K_D fit formula derived from the law of mass action.

For the radiolabelling filter-binding assay 1 µl (0.5 nM) of aptamer labelled with a radioactive phosphate in the 5' end, in binding buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM $MgCl_2$, 1 mg/ml BSA, pH 7.4) was incubated with a range concentrations of β-conglutin (0,001 – 1000 nM) at room temperature (RT) for 30 min. The nitrocellulose filter was incubated with 0.3M KOH for 15 minutes at RT followed by incubation with washing buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM $MgCl_2$, pH 7.4) for a further 15 min. Finally, the radiolabelled aptamer-β-conglutin sample was applied to the filter, washed and transferred to the exposure cassette, and the phosphor screen was placed on the top of the filter for 2 h. Imaging was achieved using a Phosphorimager (Fuji FLA3000 phosphorimager) and the data was processed using AIDA and GraphPad Prism programs.

Duplicates were performed for each binding assay.

2.3.4 Truncation studies

Two truncation approaches were pursued; in the first, the primer region at either the 3' terminus or 5' terminus was removed, whilst in the second approach, various different truncated sequences postulated to be loops via the simulated 2D structure generated using m-fold [43].

2.3.5 Competitive assay to demonstrate duality of ß-CBAI and ß-CBAII aptamers

In order to probe whether the β-CBA I and β-CBA II aptamers had the same or different binding sites, a competitive assay was carried out using MST, and following confirmation of duality, SPR was carried out to monitor sandwich aptacomplex formation.

In MST analysis, 500 nM β-conglutin was pre-incubated in binding buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM $MgCl_2$, 0,1% Tween, pH 7.4) with 5 nM of Cy5-β-CBA II. This complex was then incubated with increasing amounts of 11-mer aptamer (0,061 – 500 nM). As controls Cy5-β-CBA II alone and β-conglutin/Cy5- β-CBA II were included in the experiments. The analysis was performed at 20% MST power, 20% LED power in standard capillaries on a Monolith NT.115 pico device at 25°C (NanoTemper Technologies, Munich, Germany). Two types of data analyses were performed. Due to the previously detected β-conglutin dependent quenching effect on the Cy5-β-CBA II aptamer, the raw fluorescence was checked for changes in the fluorescence upon addition of increasing amounts of 11-mer aptamer. In a second analysis the thermophoresis and temperature jump was used to detect changes in the thermophoretic movement of the Cy5-β-CBA II-β-conglutin complex upon addition of increasing amounts of 11-mer aptamer and the recorded fluorescence was normalized to fraction bound (0 = unbound, 1 = bound), processed using the software KaleidaGraph 4.5 and fitted using the K_D fit formula derived from the law of mass action. Duplicate measurements were performed for each experimental setup.

To confirm these studies, SPR was performed with BIAcore 3000. Streptavidin was immobilized on separate channels of a CM 5 sensor chip activated with EDC / NHS (30 µl of a 1:1 mixture of EDC (400 mM) and NHS (100 mM) followed by injection of 200

µg/ml streptavidin at a flow rate of 5 µl/min. Following immobilisation of the streptavidin, unreacted NHS esters were deactivated via injection of an excess of ethanolamine hydrochloride (1 M). Biotinylated aptamer (10 µM) was injected into one channel. Unbound aptamer or streptavidin (control) was then removed from the surface via washing with 2 M NaCl, 10 mM NaOH. β-conglutin was diluted in binding buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM $MgCl_2$, pH 7.4) to a final concentration of 100 nM and injected for 6 min at a flow rate of 10 µl/min followed by a 3-min stabilisation time and 2-min dissociation. Subsequently, 1 µM of the reporter aptamer diluted in binding buffer was injected for 6 min at a flow rate of 5 µl/min, again followed by 3-min stabilisation and 10-min dissociation. The binding was analysed through the corresponding changes in the refractive index of the optical signal, and expressed as resonance units (RU). The chip was regenerated by 2 M NaCl, 10 mM NaOH. A channel without immobilised capture aptamer was used as a control.

2.4 RESULTS AND DISCUSSION

2.4.1 Selection of β-conglutin binding aptamer II

Selection of the β-conglutin binding aptamer II (β-CBA II) was performed using magnetic partitioning. Following the second round of SELEX, prior to incubation with the protein-functionalised magnetic beads, a negative selection step was included, where the amplified DNA, followed by ssDNA generation was initially incubated with naked magnetic beads (i.e. beads blocked with ethanolamine but without target). This negative selection was subsequently included in every round of SELEX to remove the non-specific sequences that bind to the beads rather than the target, thus increasing the selectivity of ssDNA to β-conglutin. Evolution was monitored after each round of SELEX by carrying out Pilot PCR with ssDNA eluted from both negative and positive selection. After six rounds of SELEX, the vast majority of the ssDNA was binding to the target, where not only was there an increasing difference in the amount of DNA obtained from each round of negative and positive selection, but additionally a decreasing number of PCR cycles were required to amplify the DNA (Supporting information, Figure SI-2.1).

Gel electrophoresis analysis of each of the negative and positive rounds of selection gave a clear indication that there was evolution of the pool towards the target, and ion torrent sequencing from the enriched DNA pool of the seventh round was carried out and consensus motifs identified. As can be seen in Table 2.1, this analysis identified just five major aptamer candidates, implying a successful evolution.

Table 2.1: Aptamer candidates obtained by Ion Torrent sequencing.

Candidate	Sequence	MW (g mol^{-1})
Seq. 1	5'-agc tcc aga aga taa att aca ggt gag ggc ggg cgg gtg gtt gta ata tga tcg aat ggt ata tgt gtg ttt gca act agg ata cta tga cccc-3'	29282
Seq. 2	5'-agc tcc aga aga taa att aca ggt ggg tgg gtg ggc ggt gga att tag cgg cgg agc tct gtg tgt gtt agg gca act agg ata cta tga cccc-3'	29341
Seq. 3	5'-agc tcc aga aga taa att aca ggg gcc ggg gtg gct cag gca agg ggt tga cct gtc gta ggg att gtt tta aca act agg ata cta tga cccc-3'	29198
Seq. 4	5'- agc tcc aga aga taa att aca ggg gat cag taa ggt tga gac ggc ctg aat cta tcg tgg aga cca cgc gac gca act agg ata cta tga cccc-3'	29098
Seq. 5	5'- agc tcc aga aga taa att aca ggt cac gct ccg cgt acg gtg ggc cgg cga ggg aat agc ggc gcg cga aaa tca act agg ata cta tga cccc-3'	29101

2.4.2 Binding affinity studies

These five aptamer candidates for β-conglutin were evaluated in a screening manner using SPR with a BIACORE instrument. β-conglutin (positive) and α- and γ-conglutins (negative) were immobilised on a CM5 chip, with another control being a blocked, non-functionalised surface. The five aptamer candidates were then flowed over these surfaces and Sequence 3 was observed to have significant affinity for β-conglutin whilst having no interaction with the other globulin subunits, demonstrating the specificity of this aptamer candidate (Figure 2.1). The dissociation constant (K_D) of Sequence 3, now termed β-CBA II was estimated using the Langmuir model, via the

analysis of the binding with a range of concentrations (62 – 2000 nM) of β-conglutin. The resulting K_D was 24 nM, and a good fit for the model was obtained as demonstrated by the Chi^2 value of 1.84 (Figure 2.2a).

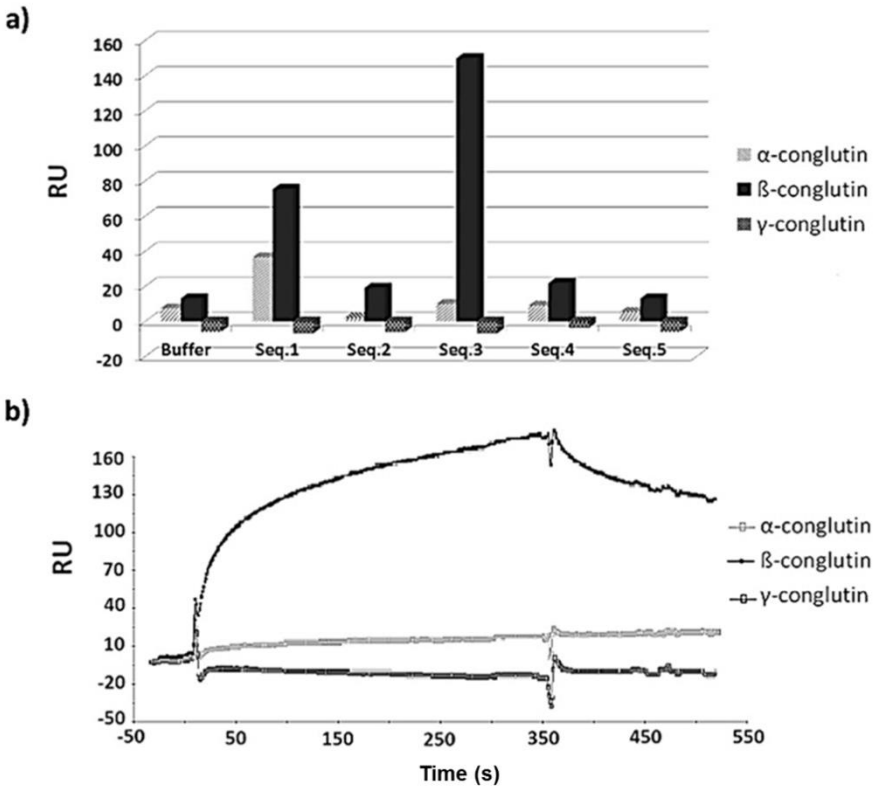

Figure 2.1: SPR experiments: (a) showing the specificity and cross-reactivity of selected aptamer candidates (2 µM), RU *resonance units*, (b) β-CBA II high affinity and high specificity against β-conglutin.

In order to corroborate the results obtained, evaluation of the affinity of the selected aptamer was performed in two independent laboratories (2Bind GmBH (www.2bind.de) and Mayer's Lab (www.mayerlab.de), using the different methodologies of microscale thermophoresis and filter radiolabelling, with MST allowing determination of the K_D in free solution, whilst radiolabelling exploits immobilisation of the β-conglutin, with both using labelled aptamer.

MST was carried out with Cy5 labelled β-CBA II in constant range (5 nM) and wide range of β-conglutin (0,056 – 1845 nM). A ligand dependent quenching effect was detected, showing decreasing fluorescence with a concomitant increase in aptamer concentration. This quenching was corroborated using denatured and native β-conglutin, where no difference in fluorescence was observed in denatured samples (high and low concentrations of β-conglutin), whilst a significant difference in fluorescence was observed with native β-conglutin (Supporting Information Figure SI-2.2). Consequently, raw fluorescence was used for the analysis. MST confirmed that the β-CBA II aptamer binds with high affinity and specificity to β-conglutin with a K_D of 12 nM) (Figure 2.2b).

Radiolabelling filter-binding assay uses a filter of nitrocellulose paper, which is negatively charged and immobilises proteins via electrostatic interaction. DNA is inherently negatively charged due to the phosphate backbone and will thus be repelled by the filter, and only high affinity aptamer that binds to β-conglutin will be present on the filter following interaction and washing. Subsequently, the presence of bound aptamer will be detected via the radioactive phosphate found in the 5' terminus of the aptamer. A constant amount of aptamer is mixed with a range of concentrations of β-conglutin (0,001 - 1000 nM). A non-specific library was used as one negative control, with the other negative control being bare filter exposed to either aptamer or non-specific library. The high affinity binding of the β-CBA II aptamer to β-conglutin was again confirmed, and a K_D of 1.81 nM obtained (Figure 2.2c).

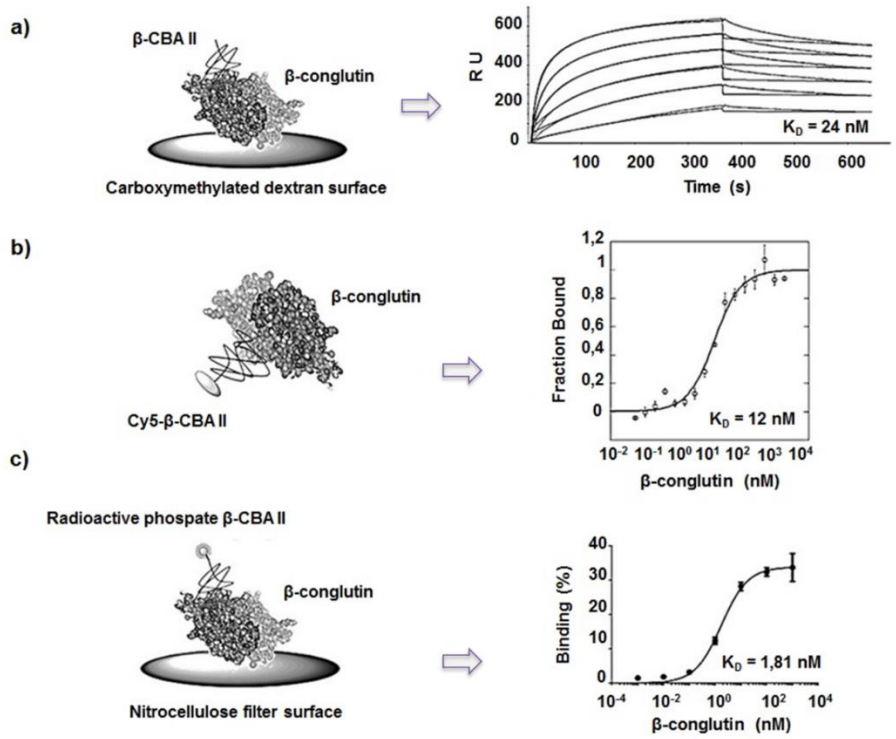

Figure 2.2: β-CBA II dissociation constants (K_D). (a) Schematic and calculation of β-CBA II K_D by SPR, *RU* resonance units; (b) Schematic and calculation of β-CBA II K_D by MST; (c) Schematic and calculation of β-CBA II K_D by radiolabeling filter binding assay.

2.4.3 Truncation studies

SPR was used to evaluate the affinity of the various truncated sequences, which were flowed over to immobilized β-conglutin on the surface of a CM5 chip. The first approach for the truncation of the β-CBA II evaluated was to remove the primer sequences at either 3' terminus (1), or the 5' terminus (2) or at both termini (3) (Table 2.2a). In another approach, the β-CBA II aptamer was postulated using the m-fold program (Supporting Information Figure SI-2.5) and all the loops predicted in the 2D predicted structure of the aptamer were selected as truncated sequences (Table 2.2b).

Table 2.2: Truncation studies: (a) Full-length aptamer sequence (β-CBA II) and truncated species obtained after removal of the 3' primer (1), or the 5' primer (2), or both primers (3). Truncated aptamer species modelled according to the predicted secondary structure of the aptamer sequence β-CBA II.

a)

Label	Sequence	Nucleotides	MW (g mol^{-1})
β-CBA II	5'-agc tcc aga aga taa att aca ggg gcc ggg gtg gct cag gca agg ggt tga cct gtc gta ggg att gtt tta aca act agg ata cta tga ccc c-3'	94	29198
1	5'-ggc cgg ggt ggc tca ggc aag ggg ttg acc tgt cgt agg gat tgt ttt aac aac tag gat act atg acc cc-3'	71	22046
2	5'-agc tcc aga aga taa att aca ggg gcc ggg gtg gct cag gca agg ggt tga cct gtc gta ggg att gtt tta a -3'	73	22777
3	5'-ggc cgg ggt ggc tca ggc aag ggg ttg acc tgt cgt agg gat tgt ttt aa -3'	50	15625

b)

Label	Sequence	Nucleotides	MW (g mol^{-1})
β-CBA II	5'-agc tcc aga aga taa att aca ggg gcc ggg gtg gct cag gca agg ggt tga cct gtc gta ggg att gtt tta aca act agg ata cta tga ccc c-3'	94	29198
A	5'-ggg ccg ggg tgg ctc agg-3'	18	5638
B	5'-ggg gtg gct-3'	9	2811
C	5'-cct gtc gtc gg-3'	11	3324
D	5'- ttg ttt taa caa-3'	12	3634
E	5'-ggg gtt gac ctg tcg tag gga ttg ttt taa caa cta gga tac tat gac ccc-3'	51	15760
F	5'-ggg gta ccc c-3'	10	3029

As can be seen in Figure 2.3, the sequences present at the 5' terminus (2) play a role in the binding as a lower K_D was obtained (K_D = 2,6 x 10^{-7} M), whilst the sequences present in 3' terminus are observed to essential for the binding (1), and the studied truncated sequences were not observed to improve binding.

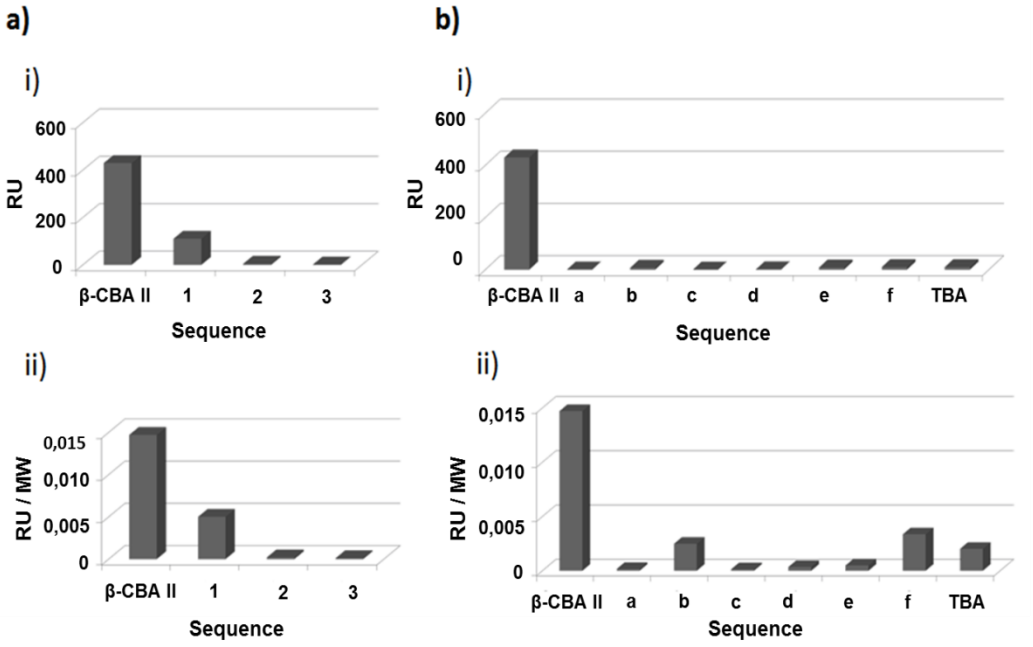

Figure 2.3: SPR experiments showing the interaction between truncated aptamer sequences obtained after removal of primer regions (a) or based on the analysis of simulated structures (b) to β-conglutin protein immobilised on the surface of the CM5 Biacore chip and DNA control: thrombin-binding aptamer (TBA). The binding of sequences is represented in resonance units (RU) (i) and normalized by the molecular weight of each sequence (ii).

2.4.4 Competition assay to determine duality of β-CBAI and β-CBAII aptamers

The aim of the competitive assay was to determine if the two β-conglutin binding aptamers (11-mer truncated β-CBA I and β-CBA II) bind to the same or different aptatopes on the protein. The strategy of the competition assay was to keep a saturated

complex of β-conglutin (500 nM) and Cy5-β-CBA II (5 nM) constant, whilst titrating the 11-mer aptamer. Two types of analyses were performed: firstly, fluorescent measurement as the Cy5-β-CBA II showed a ligand-dependent quenching effect as previously described and, secondly, the thermophoresis incorporating a temperature jump.

The analysis in the fluorescence showed that the quenching effect is still present, indicating that β-CBA II remains bound at all concentrations of the 11-mer β-CBA I (Supporting Information Figure SI-2.3). The thermophoresis analysis incorporating a temperature jump showed a binding event with a K_D of 4.3 x 10^{-9} M (Figure 2.4a), in agreement with the previously reported K_D of 1.3nM for the 11-mer. The competition assay clearly indicates that both aptamers bind to different binding sites on the surface of β-conglutin.

In order to confirm that both aptamers could simultaneously bind to the β-conglutin, a sandwich assay using β-CBA II as capture aptamer, followed by the addition of 100 nM β-conglutin and 1 μM reporter aptamer (β-CBA I, β-CBA I-11-mer, β-CBA II or scrambled 11-mer) was detected by SPR. The SPR analysis showed that both aptamers could bind simultaneously to β-conglutin as there is an increase in the sensorgram when full length β-CBA I or 11-mer is added comparing with scrambled 11-mer where no significant change is achieved or the same aptamer β-CBA II where a decrease is observed as both aptamers, capture and reporter, are competing for the β-conglutin (Figure 2.4b). Additional analysis was carried out to ensure that there was no binding between the two aptamers in the absence of β-conglutin (Supporting Information Figure SI-2.4).

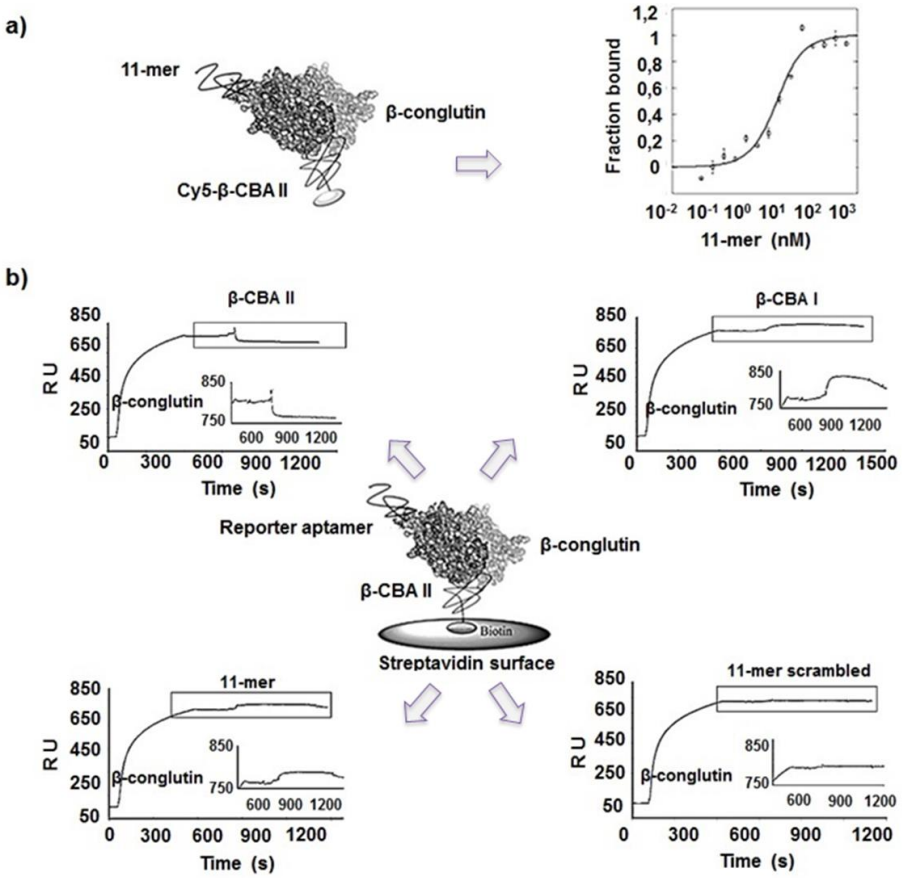

Figure 2.4: Dual-aptamer based sandwich assay: (a) Competitive binding assay by MST, using constant complex (β-congutin-β-CBA II aptamer-Cy5) and adding different concentrations of 11-mer aptamer. (b) Sandwich assay by SPR: the binding assays were performed using 10 µM biotin-β-CBA II aptamer as capture aptamer immobilised on streptavidin CM5 chip, 100 nM β-conglutin and 1 µM reporter aptamer (β-CBA II, β-CBA I, 11-mer or scrambled 11-mer).

2.5 CONCLUSIONS

In conclusion, we report on the selection of a second aptamer, β-CBAII, against the anaphylactic allergen β-conglutin with high affinity and specificity. Microscale thermophoresis technology and radiolabelling filter binding assay confirmed the affinity and specificity of the β-CBAII. Competition assays monitored using microscale thermophoresis (MST) demonstrated that the previously selected and truncated β-CBA

I, and the newly selected β-CBA II bind to different aptatopes on the protein target. Finally, SPR was used to demonstrate the formation of sandwich aptacomplexes of immobilised β-CBAII with β-conglutin and either full-length β-CBA I or truncated 11-mer. Future work will focus on exploiting this sandwich aptacomplex in biosensor and apta-amplification approaches.

2.6 ACKNOWLEDGMENTS

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2.8 SUPPORTING INFORMATION

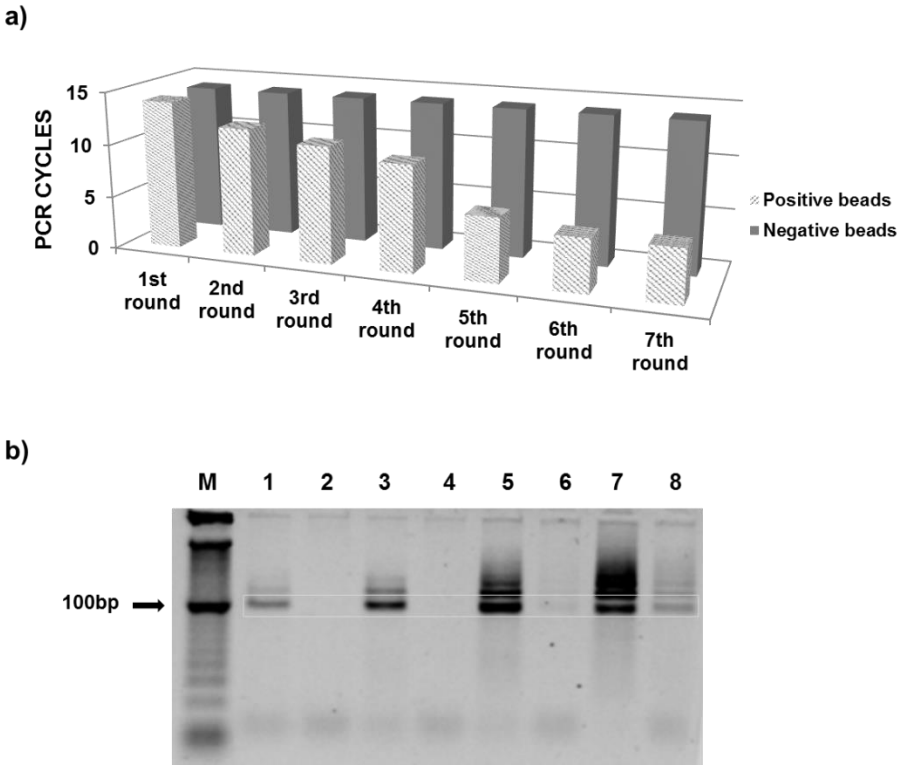


Figure SI-2.1: Evolution studies: (a) Number of PCR cycles needed in each round of selection. (b) Pilot PCR from 6th round of SELEX: M, marker (bp); lane 1, positive beads 4 PCR cycles; lane 2, negative beads 4 PCR cycles; lane 3, positive beads 6 PCR cycles; lane 4, negative beads 6 PCR cycles; lane 5, positive beads 8 PCR cycles; lane 6, negative beads 8 PCR cycles; lane 7, positive beads 10 PCR cycles; lane 8, negative beads 10 PCR cycles.

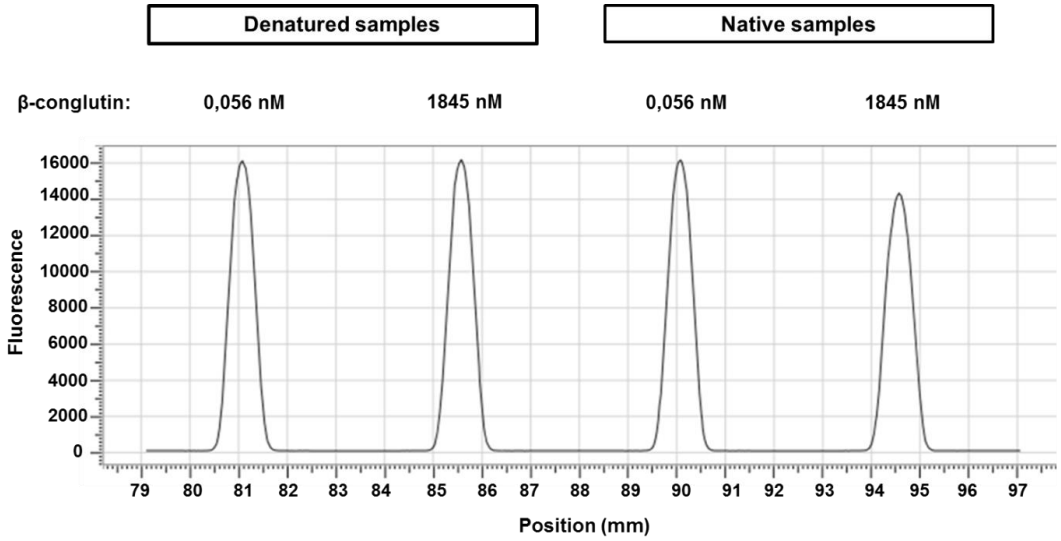


Figure SI-2.2: Capscan comparing the fluorescence between denatured and native samples.

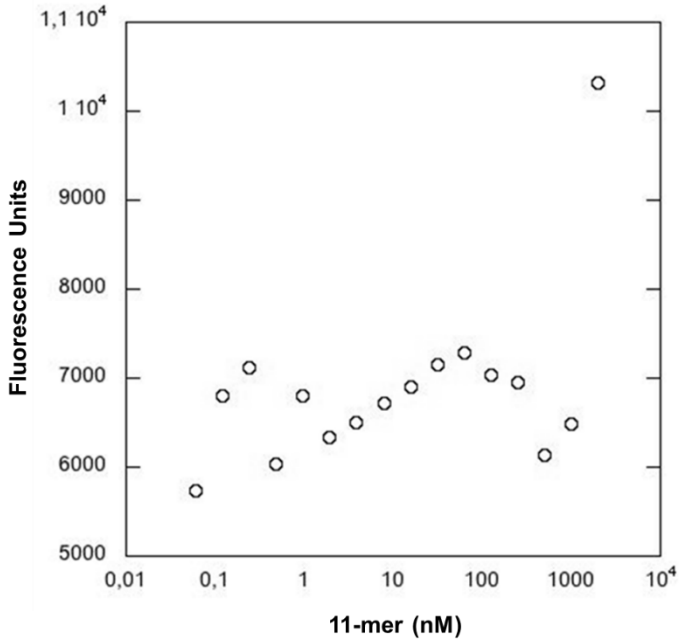


Figure SI-2.3: Quenching effect between β-CBA II and β-conglutin upon addition of 11-mer.

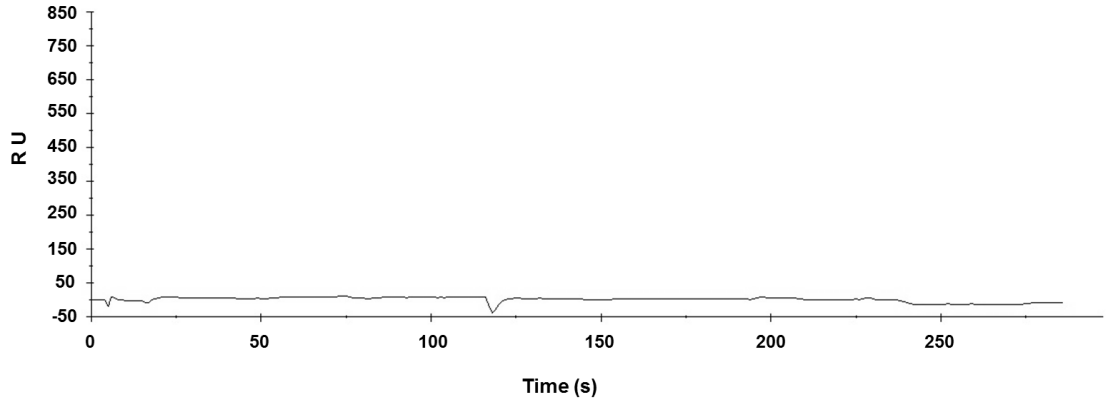


Figure SI-2.4: SPR binding assay between β-CBA II and 11-mer.

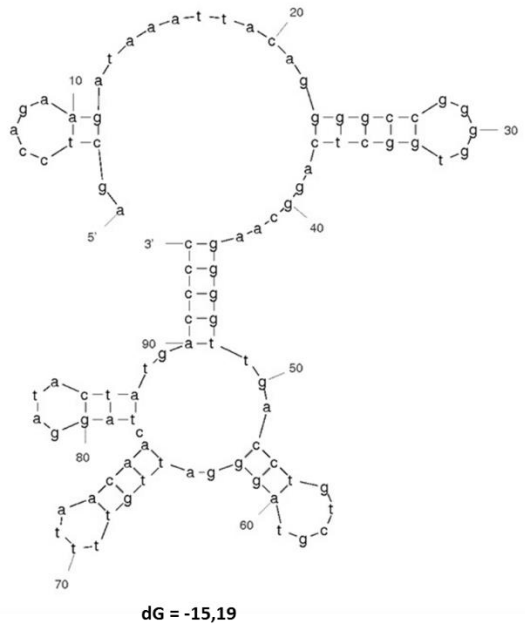


Figure SI-2.5: Predicted secondary structure of β-CBA II aptamer by m-fold software.

Chapter 3

Surface plasmon resonance imaging (SPRi) for analysis of DNA aptamer:β-conglutin interactions

(Methods. 2015 Oct 31. pii: S1046-2023(15)30131-6)

UNIVERSITAT ROVIRA I VIRGILI
SELECTION OF HIGH AFFINITY AND SPECIFIC APTAMER AND ITS' USE IN DIFFERENT APPLICATIONS FOR THE DETECTION OF THE ANAPHYLACTIC B-CONGLUTIN ALLERGEN
Miriam Jauset Rubio

Chapter 3: Surface plasmon resonance imaging (SPRi) for analysis of DNA aptamer: β-conglutin interactions

3.1 ABSTRACT

Surface plasmon resonance imaging (SPRi) is a label-free detection method that offers a suitable and reliable platform for the real time monitoring of biomolecular interactions. In the work reported here, SPRi was used to evaluate the affinity and specificity of three different aptamers selected against the Lup an 1 anaphylatic allergen β-conglutin (β-conglutin binding aptamers I and II (β-CBA I and β-CBA II)), as well as an 11-mer truncated version of β-CBA I. Thiol modified aptamers were immobilised on a gold substrate through a self-assembling process and the use of different blocking strategies to prevent non-specific binding were evaluated. Dissociation constants of 20, 13 and 1 nM were determined for β-CBA I, β-CBA II and the 11-mer truncated aptamer, respectively. The three aptamers were then studied in various different sandwich formats and the β-CBA I/11-mer and β-CBA II were observed to bind to different aptatopes on the target protein. Each of the aptamers were then used either as surface immobilised aptamer, or as reporter aptamer, and added with the protein target ß-conglutin in either a sequential of simultaneous manner, and the changes in SPR signal monitored. The preferred approach for formation of a sandwich aptacomplex was with immobilised β-CBA II, followed by addition of pre-incubated β-conglutin and 11-mer, whilst addition of the 11-mer following addition of the β-conglutin, resulted in displacement of the bound target. The ability to provide parallel qualitative and quantitative detection establishes SPRi as a powerful tool for the study of immobilised aptamer – target interactions.

3.2 INTRODUCTION

Aptamers are synthetic nucleic acids selected by process termed SELEX (systematic evolution of ligands by exponential enrichment), which lead to the selection of ligands with high specificity and affinity to their cognate targets [1,2]. Due to their

specific ability to undergo conformational changes upon target binding, their high stability, specificity and low cost, they have rapidly garnered interest as an ideal recognition element for analytical tools, including biosensors [3-8]. Surface plasmon resonance (SPR) is a label-free detection method, which has emerged as a suitable and reliable platform for monitoring aptamer:target molecular interactions [9-15]. The simultaneous detection of multiple biomolecules is of increasing interest [16-18] and the combination of protein arrays with SPR provides a label-free alternative for multiplexed kinetic analysis.

In surface plasmon resonance imaging (SPRi), a large sensing surface area is exposed to a polarised light beam, allowing high-throughput screening applications not achievable with classic SPR systems [19]. This technique allows real-time analysis, and is time and reagent-efficient in comparison to the other end-point alternative techniques [20,21], as well as being suitable for the detection of proteins, DNA and even whole living cells [18-20,22-28].

The dual detection of a target analyte has long been exploited in antibody based sandwich assays and can contribute to increased specificity and sensitivity. However, there are only a few reports of dual aptamers binding different aptatopes on a target molecule, which may be attributable to the tendency of the SELEX methodology to result in evolution of the starting library towards one particular aptatope [9].

We have recently selected dual aptamers against two different binding sites on β-conglutin, and here we demonstrate the flexibility of real-time SPRi to evaluate the duality of these selected aptamers. The aptamers studied were the β-CBA I aptamer, 93 bases in length, the β-CBA II aptamer, 94 bases in length, and an 11-mer, which is a truncated version of the β-CBA I aptamer. A range of thiol modified aptamer concentrations and the use of different approaches to eliminate non-specific binding were evaluated in order to find the optimal conditions to monitor aptamer:target interactions. Subsequently, a microarray functionalised with specific aptamers, or, control oligonucleotides were developed and used to monitor the formation of various sandwich aptacomplexes using SPRi, whilst also evaluating the affinity of the different aptamers.

3.3 MATERIALS AND METHODS

3.3.1 Reagents and chemicals

Phosphate buffered saline (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4), DT1, a bipodal alkanethiol, (16-(3,5-bis ((6-mercaptohexyl)oxy)phenyl)-3,6,9,12,15-pentaoxahexadecane) was purchased from SensoPath Technologies (Bozeman), 6-mercapto 1-hexanol, Denhardt's solution, and all other reagents were purchased from Sigma (Barcelona, Spain). Sodium Chloride, sodium hydroxide, hydrochloric acid and ethanol were purchased from Scharlau Chemie S.A. (Barcelona, Spain). Oligonucleotides (HPLC purified and provided lyophilised) were purchased from BIOMERS (Ulm, Germany). All solutions were prepared in high purity water obtained from Milli-Q RG system (Barcelona, Spain). The running buffer used was 10 mM phosphate, 138 mM NaCl, 2.7mM KCl, 1.5 mM $MgCl_2$, pH 7.4.

3.3.2 SPRi set-up

The detection of ß-conglutin was monitored as outlined schematically in Figure 1, using an SPR imager apparatus (SPRi-Plex II, HORIBA Scientific-GenOptics), equipped with an incoherent light source (λ = 635nm). The system is equipped with a 200 µL sample loop, continuous flow pump and an in-line degasser. Initially, instrument calibration is carried out where a variation of the refractive index and then a variation of the reflectivity is actuated, and this reflectivity variation for each spot is measured and used to calculate calibration co-efficients to normalise spot responses in the kinetics measurements, using 20 mM phosphate buffered saline (PBS). The experiments were carried out at 25ºC. Acquisition of the reflectivity signal, registered with a 12-bit camera, was initiated following baseline stabilisation. Reflectivity measurements were averaged for several spot replicates, with the initial reactivity being subtracted from the raw data to provide values for the reflectivity shift obtained upon binding of biomolecules.

In order to re-use the SPRi chip by complete removal of the immobilised aptamer from the surface, it was immersed in acetone and sonicated for 15 minutes, followed by a further immersion and sonication in isopropanol, and then thoroughly washed with Piranha's solution (3:1 concentrated sulphuric acid and 30% v/v hydrogen peroxide) for

10 minutes. Finally, the chip was washed with distilled water, ethanol and dried with a nitrogen stream.

3.3.3 Aptamer Immobilisation

Thiol modified aptamers (β-CBA I, β-CBA II or 11-mer) and control DNA (details of sequences can be found in Table 3.1a), were immobilised on the surface of sensor spots on a gold slide via chemisorption. The aptamers were prepared at different concentrations (1 µM, 10 µM and 40 µM) in 20 mM PBS pH 7.4. Replicate spots were printed for each sequence, using a 500 µm needle via manual droplet deposition and incubation for 30 min in a 70% humidity chamber. For the study of the best strategy to eliminate non-specific binding, the aptamer was co-immobilised with either 6-mercapto 1-hexanol or the bipodal alkanethiol DT1 using a 1:100 molar ratio (Aptamer:alkanethiol). Alternatively, the sensor surface was blocked by immersion in Denhardt's solution for 20 min at 37°C, protected from light. Finally, the slide was immersed in 20 mM PBS buffer for a further 5 minutes, rapidly rinsed with water and dried with a nitrogen stream.

Table 3.1: Sequences and Molecular Weights of Capture and Reporter Aptamers.

a)

	Capture sequences	MW (g mol^{-1})
β-CBA I	5'-thiol C6-agc tga cac agc agg ttg gtg ggg gtg gct tcc agt tgg gtt gac aat acg tag gga cac gaa gtc caa cca cga gtc gag caa tct cga aat-3'	29100
β-CBA II	5'-thiol C6- agc tcc aga aga taa att aca ggg gcc ggg gtg gct cag gca agg ggt tga cct gtc gta ggg att gtt tta aca act agg ata cta tga cccc-3'	29394
11-mer	5'-thiol C6-ttttttttttttttttttttggtgggggtgg-3'	9789
Control Oligomer	5'-thiol C6- agc tcc aga aga taa att aca ggt cac gct ccg cgt acg gtg ggc cgg cga ggg aat agc ggc gcg cga aaa tca act agg ata cta tga cccc-3'	29297

b)

	Reporter sequences	MW (g mol^{-1})
β-CBA I	5'-biotin-agc tga cac agc agg ttg gtg ggg gtg gct tcc agt tgg gtt gac aat acg tag gga cac gaa gtc caa cca cga gtc gag caa tct cga aat-3'	29341
β-CBA II	5'-biotin-agc tcc aga aga taa att aca ggg gcc ggg gtg gct cag gca agg ggt tga cct gtc gta ggg att gtt tta aca act agg ata cta tga cccc-3'	29635
11-mer	5'-biotin-ggtgggggtgg-3	3947

3.3.4 Real time detection of β-conglutin

Serial dilutions of β-conglutin (5 - 500 nM) prepared in running buffer, were tested to determine the dissociation constant of each of the aptamers. The experiments were performed at 25ºC and all the injections were dispensed via addition of 400 µl of volume to the injection loop, with 200 µl being the final volume injected at a flow rate of 50 µl/min. The aptamer-functionalised surface was regenerated using 12 mM NaOH with 1.2 % v/v EtOH after each β-conglutin injection. The data was acquired using SPRi Analysis 2.0 software and Scrubber Gen2, furnished by Horiba Scientific-GenOptics. The reflectivity values were averaged over the replicates of each spot and plotted against time.

3.3.5 Dual aptamer sandwich

Serial injections were tested to study the kinetic formation of the sandwich aptacomplex. All reagents were prepared in running buffer and dispensed at 50 µl/min for 4 min using the same conditions described above. β-conglutin (100 nM), biotinylated secondary aptamer and streptavidin-horseradish peroxidase (SA-HRP), were added in different combinations as detailed below:

1) Sequential injection:

β-conglutin was primarily injected followed by injection of secondary aptamer, and finally injection of SA-HRP.

2) Co-injections:

(i) injection of β-conglutin followed by the simultaneous injection of secondary aptamer that had been pre-incubated with SA-HRP;

(ii) simultaneous injection of β-conglutin that had been pre-incubated secondary aptamer, followed by injection of SA-HRP and finally

(iii) simultaneous injection of a mixture of β-conglutin, secondary aptamer and SA-HRP.

3.4 RESULTS AND DISCUSSION

3.4.1 Optimisation of SPR imaging conditions

Aptamers were directly immobilised on the gold SPRi sensor surface via chemisorption through their thiol moieties, and a range of concentrations were evaluated in order to identify the optimal aptamer coating considering the size of the spots around 500 µm., which was defined to be 40 µM where a clear spot can be observed, whereas no spot was detected using a concentration of 1 µM (Supporting Information Figure SI-3.1). Non-specific adsorption of the analyte as well as matrix proteins on the gold sensor surface can lead to a perturbation of results and it is thus critical to reduce background interference, concomitantly increasing the signal to noise ratio and promoting specific binding between the immobilised aptamer and its' cognate target. To this end, two different blocking strategies were compared. In the first approach the use of mixed self-assembled monolayers (mixed SAM) of co-immobilised aptamer and hydroxyl-terminated alkanethiols were used, whereas in the second approach the use of a blocking agent was explored. 6-mercapto 1-hexanol is one of the most commonly used blocking agents, being a short alkanethiol molecule containing a sulphur head and a terminal hydroxyl group [29]. An alternate alkanethiol evaluated was DT1, a bipodal PEGylated alkanethiol, which prevents non-specific binding via its' PEG moiety [30,31]. In both cases, the monolayers formed are dense and compact, properties of high value for optical biosensors. Each of these alkanethiols were co-immobilised with the aptamer, but presumably due to the sticky nature of the target β-conglutin, no visible difference between specific (aptamer functionalised surface) and control (blocked gold surface) spots indicating high non-specific binding of the protein directly to the gold surface (Supporting Information Figure SI-3.2). Furthermore, a backfilling rather than co-immobilisation approach for creating the aptamer SAM was evaluated but no improvement in elimination of non-specific binding was observed. Thus, the use of an alternate blocking strategy was explored, using Denhardt's solution, which is a mixture of blocking agents commonly used in membrane-based hybridisation protocols. The solution contains 1% w/v Ficoll's reagent, 1% w/v polyvinylpyrrolidone, and 1% w/v bovine serum albumin. This blocking agent was observed to be very effective, with the

surface surrounding the spot remain black whilst the sensor spots were grey, indicative of elimination of non-specific binding of the protein to the surface (Supporting Information, Figure SI-3.2c).

Regeneration agents, including various concentrations of NaCl and NaOH were evaluated in order to regenerate the aptamer-functionalised sensor spots, and the optimal conditions determined to be 12 mM NaOH containing 1.2 % v/v EtOH. Aptamer functionalised chips (stored at 4ºC) could be re-used for several months simply by rinsing with regeneration buffer. Chips could be re-used 4 times by removal of the coating aptamer by sonication in acetone and isopropanol and washing with Piranha's solution. However, after 4 cycles, the gold surface of the chip was too damaged to be used further.

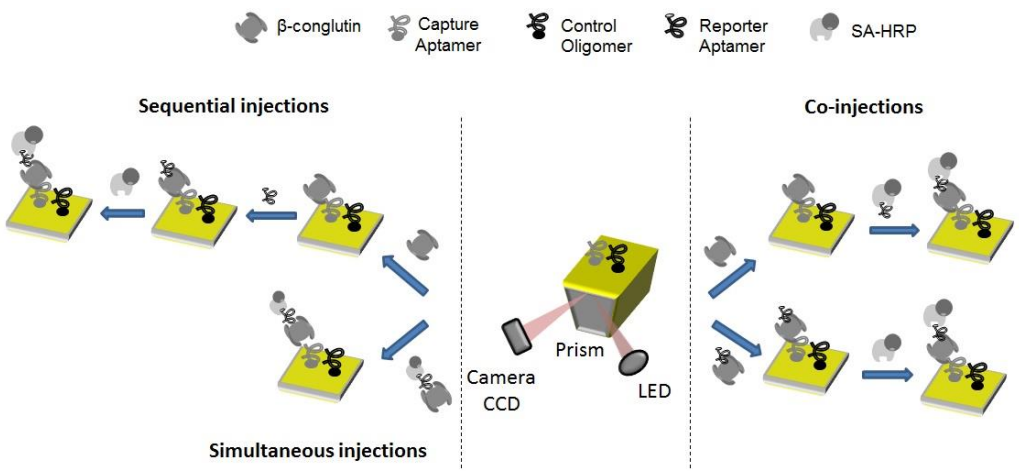


Figure 3.1: Schematic description of approaches for formation of sandwich aptacomplex studied. The microarray was functionalised with each of the two long aptamers, β-CBA I and β-CBA II, as well as the truncated version of the β-CBA I, the 11-mer, along with control oligomers, and exposed to the cognate target, β-conglutin, and each of the aptamers studied, either in a sequential or simultaneous fashion. The molecular interactions were followed in real time using Surface Plasmon Resonance imagin (SPRi).

3.4.2 Binding assays

The interactions between the various aptamers (β-CBA I, β-CBA II and 11-mer) and their cognate target, β-conglutin, were monitored in real-time. Negative controls were also analysed, where in one control, sensor spots functionalised with a sequence of the same number of bases as β-CBA I and β-CBA II aptamers were used, and in another control, non-functionalised sensor spots were blocked with Denhardt's solution. Following injection of 250 nM β-conglutin, a specific signal was observed on each spot containing either β-CBA I, β-CBA II or 11-mer aptamers, while non-specific interactions on control sensor spots were negligible (Figure 3.2).

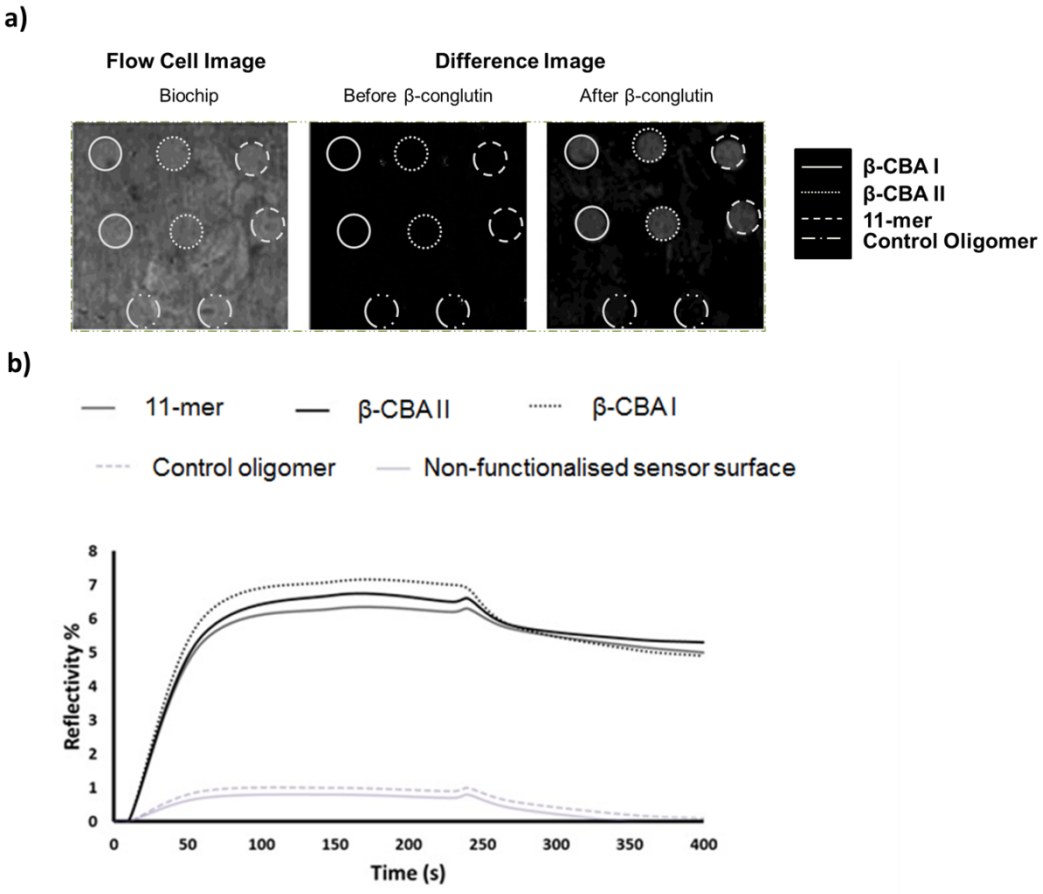
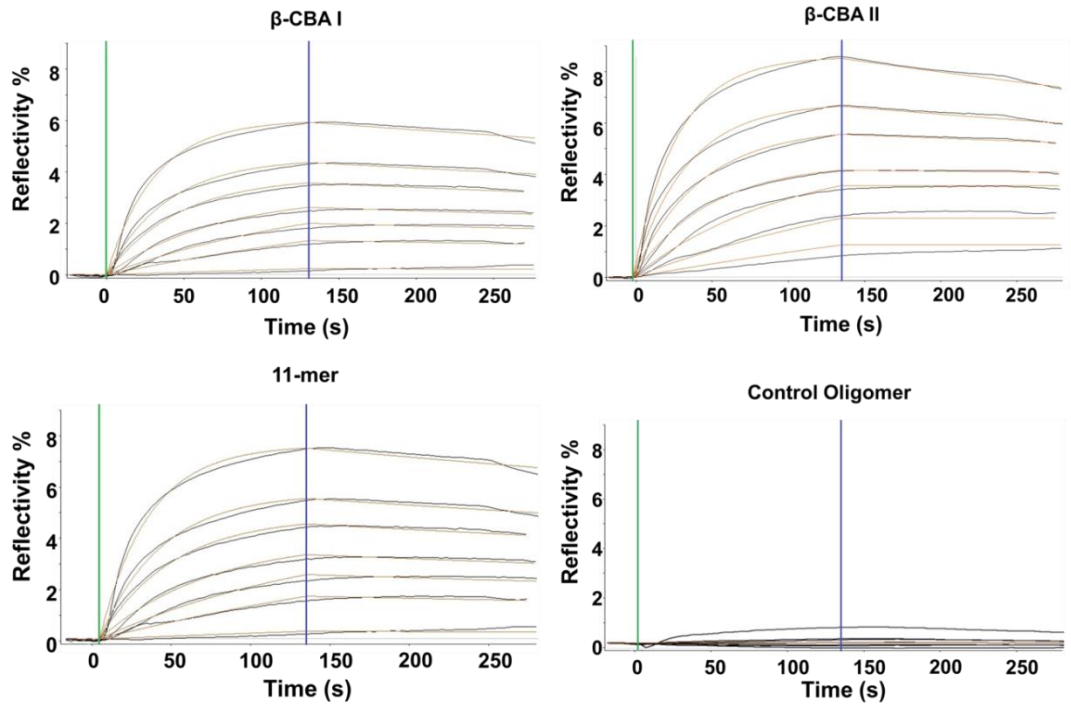


Figure 3.2: Real time label free detection of β-conglutin a) Spots before and following injection of β-conglutin; b) Sensorgram following aptamer:β-conglutin interaction.

The dissociation constants (K_D) were determined by analysing the binding of a range of β-conglutin concentrations with 5 replicates of β-CBA I, β-CBA II and 11-mer aptamers immobilised on the sensor surface, subtracted from non-functionalised sensor surface spot and calculated by fitting each part of the interaction with a mono-exponential curve using the ScrubberGen2 software. The resulting K_D were 20 nM, 13 nM and 1 nM, for each of β-CBA I, β-CBA II and 11-mer, respectively and a good fit to the model was obtained (Figure 3.3).



	K_D	Rs SD
β-CBA I	20 nM	0,150
β-CBA II	13 nM	0,090
11-mer	1 nM	0,125

Figure 3.3: SPRi sensograms obtained for the β-conglutin (500 - 5 nM) passed over the gold sensor surface functionalised with a) β-CBA I, b) β-CBA II, c) 11-mer and d) control oligomer. When the Rs SD becomes green the fit is accepted. Green line: injection time; Blue line: dissociation time.

3.4.3 Dual aptamer sandwich

The aptamers were primarily evaluated to probe if they bound to different aptatopes on the β-conglutin. It is expected that the β-CBA I and truncated 11-mer have the same binding site but believed that the β-CBA II bound to a different binding site. Indeed this was observed, and in order to elucidate the optimum configuration for the sandwich formation each aptamer was tested as either capture or reporter probe (β-CBA II, β-CBA I or 11-mer). When acting as capture probe, a 20T chain was added to the 11-mer to help extend it from the sensor surface, and for all three aptamers under study, biotinylated analogues were used as reporter aptamers. In order to achieve a signal enhancement in the sandwich formation, streptavidin (SA), which can bind to the biotin of the reporter aptamer, can be used as reported previously [22]. However, due to the higher molecular weight of β-conglutin in this study streptavidin conjugated to horseradish peroxidase (SA-HRP) was used to attempt to achieve a larger signal enhancement.

Various configurations of reagent addition were evaluated in order to understand the preferred methodology for optimal sandwich aptacomplex formation (Figure 3.4). In the first approach studied, where each of the components of the aptacomplex is added sequentially, the β-CBA II shows the highest level of β-conglutin binding (Supporting Information, Table 1). However, upon addition of either 11-mer of β-CBA I following interaction of β-conglutin with immobilsed 11-mer or β-CBA I, rather than the anticipated no interaction being observed, there is effective displacement of the bound β-conglutin, which is most pronounced in the case of immobilised 11-mer and reporter 11-mer. This phenomena can also be observed with immobilised and reporter β-CBA II. In this format, sandwich formation is only observed for immobilised β-CBA I and report β-CBA II, with the SA-HRP not observed to enhance signal.

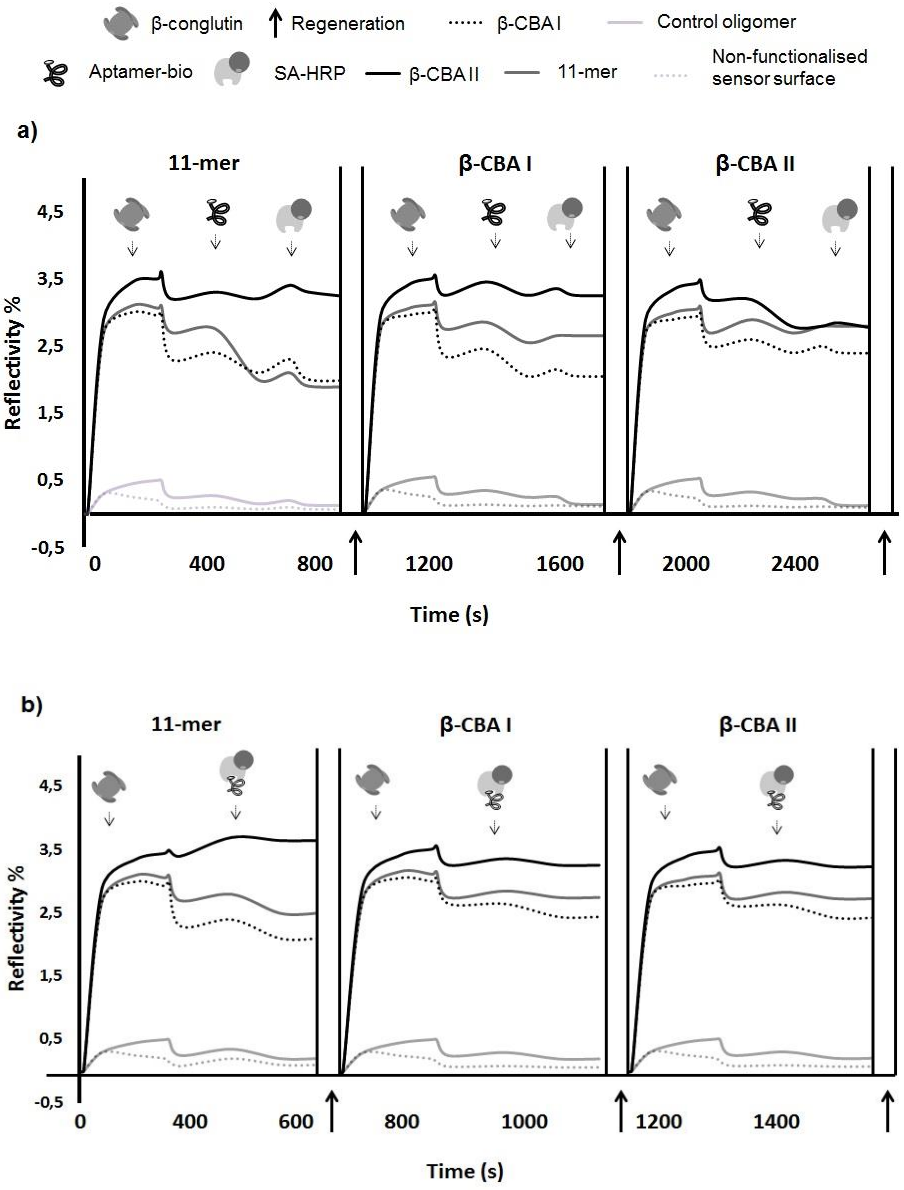
In the second format studied, where β-conglutin is added, followed by a mixture of biotinylated reporter aptamer and SA-HRP, similar binding levels of the target to the immobilised aptamer as detailed above, are observed. The displacement observed in the first format is again observed for the β-CBA I/11-mer pairs, but not for the β-CBA II. Some further differences are observed. The immobilised β-CBA I does not form a

sandwich with the β-CBA II, and indeed some displacement of bound β-conglutin is observed upon addition of the β-CBA II-SAHRP complex, perhaps attributable to the reporter aptamer having a higher affinity and the formation of the complex with the SA-HRP forcing the aptamer to unfold and more effectively bind to the β-conglutin, possibly explaining why this displacement is not observed when added alone. Additionally, the immobilised β-CBA II effectively forms a sandwich with the biotinylated 11-mer, indicating that the large SA-HRP molecule, whilst it may assist in unfolding the β-CBA II when used as a reporter, it hinders the 11-mer from displacing the β-conglutin.

When the β-conglutin is pre-incubated with reporter aptamer, considerable improvement in the formation of the sandwich aptacomplex is observed with immobilised β-CBA II and particularly with the reporter 11-mer, and to a much less extent with β-CBA I. In the reverse format, with immobilised 11-mer or β-CBA I and reporter β-CBA II, a sandwich is formed, but to a much lower degree. Interestingly, no displacement phenomena is observed for the β-CBA I/11-mer pair, and in fact interaction is observed. However, knowing that these aptamers bind to the same binding site, it could be postulated that β-conglutin that does not bind to these reporter aptamers during pre-incubation, binds to the immobilised aptamers, thus giving rise to the increase in reflectivity signal. The addition of SA-HRP does not contribute to signal enhancement.

In the final format studied, where the target, reporter aptamer and SA-HRP are pre-incubated and added to the sensor surface, the highest response is observed with immobilised β-CBA II and reporter 11-mer, in agreement with the other formats studied. In order to directly compare the results obtained for immobilised β-CBA II with reporter 11-mer, we normalised the data to take the molecular weight of each reagent added into consideration, thus facilitating a direct comparison between the formation of the different sandwich aptacomplexes, whilst assuming the same refractive index for β-conglutin, SA-HRP and the aptamers (Figure 3.5). It is clear that sequential injections of β-conglutin, reporter aptamer and SA-HRP resulted in the lowest signals, which can be attributed to the heterogeneous nature of the interaction as well as to problems of steric hindrance where aptatopes may become less accessible when the aptacomplex forms

tethered to the surface. When the β-conglutin and reporter aptamers are allowed to combine in a homogenous nature i.e. pre-incubation in solution, considerably higher signals are observed, and this can be explained by the easier access the aptamer has to its' aptatope on the β-conglutin in the solution phase and when the protein is not already bound to the surface-immobilised coating aptamer.



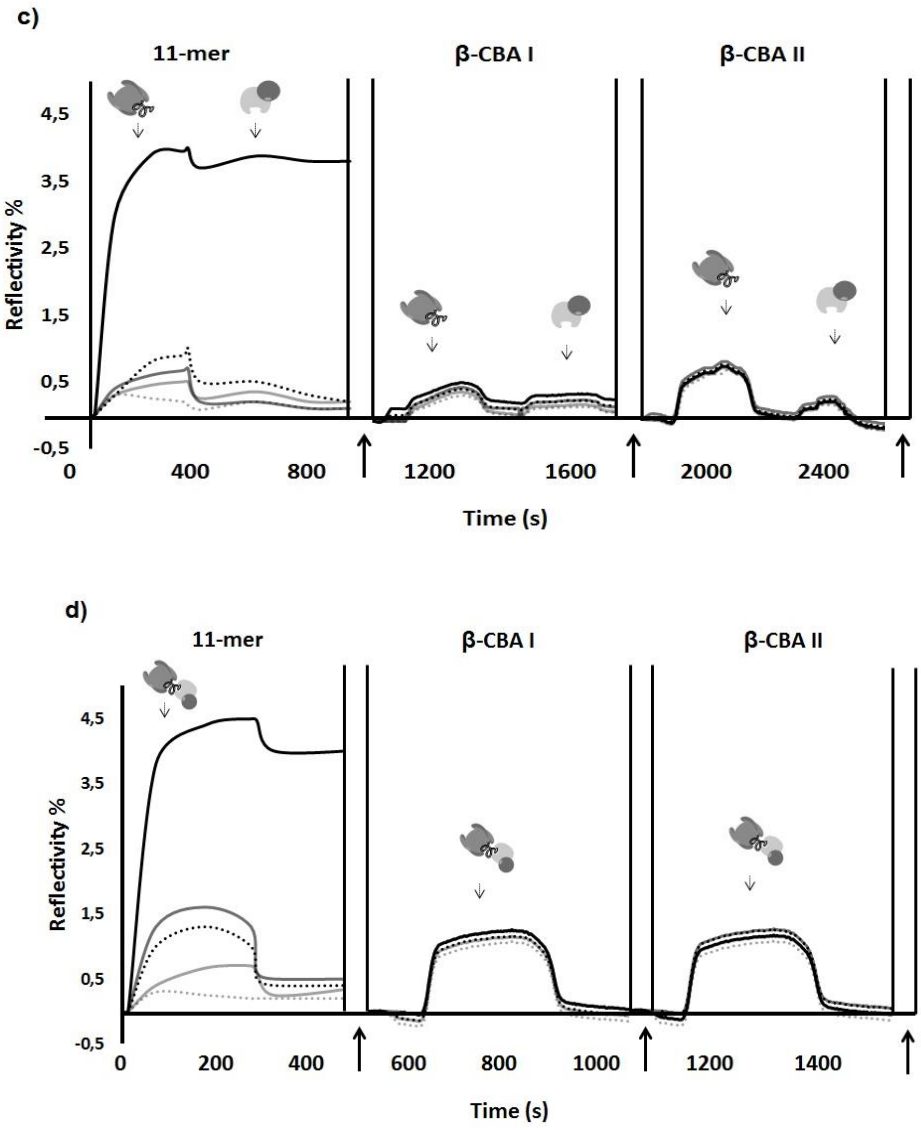


Figure 3.4: SPRi reflectivity shift after: a) Sequential injections of β-conglutin, biotinylated aptamer and streptavidin horseradish peroxidase; b) Addition of β-conglutin followed by complex of biotinylated aptamer pre-incubated with SA-HRP; c) Addition of β-conglutin pre-incubated with biotinylated aptamer followed by addition of SA-HRP; d) Addition of pre-incubated β-conglutin, biotinylated aptamer and SA-HRP. Immobilised aptamer indicated by solid grey line (20T-11-mer); solid black line (β-CBA II) and dashed line (β-CBA I), and secondary aptamer labelled on top of each set of sensorgrams. Regeneration following formation of each sandwich aptacomplex was achieved using 12 mM NaOH with 1.2 % v/v EtOH.

Previous reports monitoring the interaction of the thrombin binding aptamers with its' target in sandwich format using SPRi indicated a decrease in the reflectivity and acceleration of the dissociation rate after the addition of the same, but also the alternative reporter aptamer in the sequential injections methodology, achieving the highest signal amplification by the pre-incubation of thrombin with reporter aptamer and SA which seems to favour the sandwich format [22]. In our case, the preferred sandwich aptacomplex is the pre-incubation of β-conglutin with reporter aptamer without the presence of SA-HRP. Furthermore, no interaction between aptamers existed without the presence of β-conglutin (data not shown).

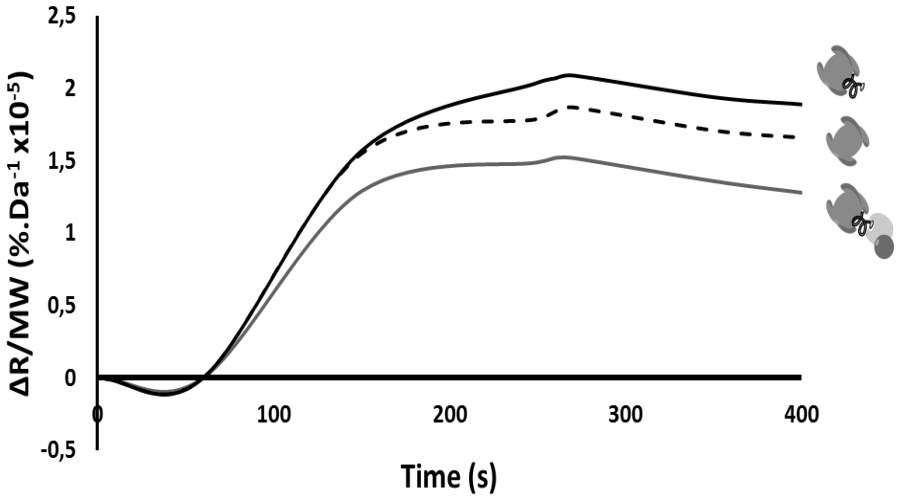


Figure 3.5: SPRi reflectivity shift normalised by the molecular weight of the injected complex using β-CBA II as capture aptamer and 11-mer as reporter aptamer.

3.5 CONCLUSIONS

Real-time label-free SPR imaging facilitates the study of aptamer:target interactions, allowing optimisation of assay formats, determination of dissociation constants as well as cross-reactivity studies. In the work reported here, the interaction of β-conglutin binding aptamers and their cognate target was monitored. The K_D values obtained using SPRi were in agreement with values previously obtained using alternate

methodologies, highlighting the flexibility of the technique. Additionally, SPRi was used to identify the preferred format of the sandwich aptacomplex to be the use of the long β-CBA II aptamer as the immobilised coating aptamer and the short 11-mer aptamer as the reporter aptamer, with a pre-incubation of the β-conglutin with the 11-mer prior to addition to the surface-tethered aptamer. SPRi is a powerful technique that can be used to monitor molecular interactions where multiple interactions can be monitored simultaneously and in real-time and could find application in monitoring SELEX evolution, evaluation of cross-reaction and optimisation of optimum operational parameters, as well as allowing determination of dissociation constants.

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3.7 SUPPORTING INFORMATION

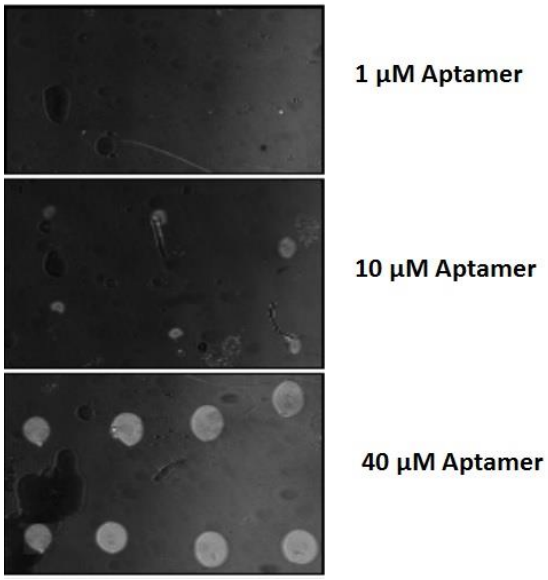

Figure SI-3.1: Optimisation of coating aptamer concentration

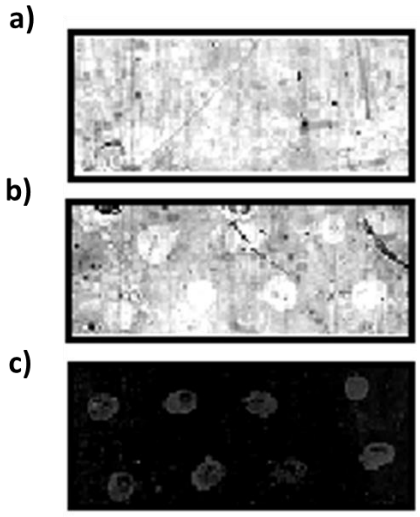

Figure SI-3.2: Optimisation of blocking conditions: a) Mixed SAM of aptamer and DT1; b) Mixed SAM of aptamer and 6-mercapto 1-hexanol; c) Following immersion in Denhardt's solution.

Table SI-3.1: Percentage reflectivity changes observed upon aptamer-target-aptamer interactions.

a)

	Reflectivity %								
	β-conglutin	**Reporter aptamer (11-mer)**	**SA-HRP**	**β-conglutin**	**Reporter aptamer (β-CBA I)**	**SA-HRP**	**β-conglutin**	**Reporter aptamer (β-CBA II)**	**SA-HRP**
β-CBA I	2,30	2,10	2,00	2,30	2,01	2,00	2,31	2,42	2,40
β-CBA II	3,20	3,21	3,32	3,21	3,21	3,20	3,20	2,80	2,85
11-mer	2,69	2,00	1,90	2,70	2,50	2,60	2,70	2,71	2,79
Control Oligomer	0,25	0,15	0,13	0,26	0,21	0,1	0,25	0,20	0,12
Non-functionalised sensor surface	0,08	0,07	0,07	0,08	0,07	0,69	0,08	0,07	0,07

b)

	Reflectivity %					
	β-conglutin	**Reporter aptamer (11-mer) + SA-HRP**	**β-conglutin**	**Reporter aptamer (β-CBA I) + SA-HRP**	**β-conglutin**	**Reporter aptamer (β-CBA II) + SA-HRP**
β-CBA I	2,30	2,10	2,60	2,40	2,62	2,40
β-CBA II	3,40	3,65	3,20	3,19	3,21	3,20
11-mer	2,70	2,50	2,70	2,70	2,72	2,70
Control Oligomer	0,25	0,21	0,25	0,19	0,25	0,20
Non-functionalised sensor surface	0,08	0,09	0,08	0,07	0,07	0,06

c)

	Reflectivity %					
	β-conglutin + Reporter aptamer (11-mer)	**SA-HRP**	**β-conglutin + Reporter aptamer (β-CBA I)**	**SA-HRP**	**β-conglutin + Reporter aptamer (β-CBA II)**	**SA-HRP**
β-CBA I	0,52	0,20	0,18	0,24	0,11	0,11
β-CBA II	3,71	3,80	0,28	0,34	0,08	0,08
11-mer	0,20	0,11	0,20	0,23	0,16	0,05
Control Oligomer	0,25	0,23	0,11	0,17	0,06	0,06
Non-functionalised sensor surface	0,09	0,10	0,08	0,14	0,11	-0,06

d)

	Reflectivity %		
	β-conglutin + Reporter aptamer (11-mer) + SA-HRP	**β-conglutin + Reporter aptamer (β-CBA I) + SA-HRP**	**β-conglutin + Reporter aptamer (β-CBA II) + SA-HRP**
β-CBA I	0,40	0,01	0,11
β-CBA II	4,02	0,09	0,02
11-mer	0,50	0,10	0,11
Control Oligomer	0,25	0,01	0,01
Non-functionalised sensor surface	0,21	-0,08	-0,07

UNIVERSITAT ROVIRA I VIRGILI
SELECTION OF HIGH AFFINITY AND SPECIFIC APTAMER AND ITS' USE IN DIFFERENT APPLICATIONS FOR THE DETECTION OF THE ANAPHYLACTIC B-CONGLUTIN ALLERGEN
Miriam Jauset Rubio

Chapter 4

Rapid detection of anaphylactic β-conglutin allergen based on isothermal recombinase polymerase amplification

(Submitted)

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Chapter 4: Rapid detection of anaphylactic β-conglutin allergen based on isothermal recombinase polymerase amplification

4.1 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 on three methods for the rapid detection of the Lup an 1 allergen, exploiting the affinity and specificity of a DNA aptamer selected against β-conglutin, termed the β-CBA II aptamer, with the inherent nucleic acid nature of aptamers, facilitating ultrasensitive detection via thermal cycling or isothermal amplification. A competition type assay was carried out between immobilized β-conglutin and a range of sample β-conglutin concentrations free in solution which compete for binding to the solution-phase β-CBA II aptamer. Following competition, any aptamer bound to the surface-immobilized β-conglutin was eluted and subsequently used as a template in either the polymerase chain reaction (PCR) or the isothermal recombinase polymerase amplification (RPA), in assays termed Apta-PCR and Apta-RPA. Using a microtiter plate to immobilise the β-conglutin, detection limits of 2 x 10^{-10} M and 8.1 x 10^{-11} M, and total assay times of 210 and 100 minutes were achieved for Apta-PCR and Apta-RPA, respectively. Using magnetic beads as the solid-phase and combining with Apta-RPA detection, the total assay time was reduced to just 50 minutes, with an LOD of 9.9. x 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.

4.2 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 and lateral flow assays [1-4]. However, homology between proteins can present challenges for antibody based approaches, which may be addressed by PCR or, alternatively, mass spectrometry (MS), which have been used extensively for the detection of, and differentiation between related allergens [5, 6], but both techniques are inherently laboratory based, require significant infrastructure and highly trained personnel [7-9].

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 [10]. Furthermore, lupin does not contain gluten and is used in gluten-free products including bread and other bakery products [11]. However, due to various reports of anaphylactic allergic reactions induced by lupin inhalation/ingestion, lupin and lupin foodstuffs 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) [12]. Although there is not a 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 [13].

There are several reports of immuno and molecular assays for the detection of lupin, but none are capable of selectively and specifically detecting the β-conglutin subunit [14-18]. To this end, in 2012 an aptamer was selected against β-conglutin, termed the β-conglutin binding aptamer, (β-CBA I) [19]. Aptamers are artificial oligomers capable of binding to a wide range of diverse targets including proteins [20, 21], small molecules [22-

[24], amino acids [25] or even whole cells [26] with affinities and specificities equal to and often superior to antibodies [27, 28]. Recently, we reported the selection of a second aptamer against β-conglutin (β-CBA II) that binds another site (aptatope) with nanomolar affinity [29]. The specificity and the affinity of β-CBA II aptamer has previously been evaluated using different methodologies including surface plasmon resonance (SPR), microscale thermophoresis (MST) and radiolabelling binding filter assay, and dissociation constants in the low nanomolar range obtained [30].

Here, we report on the use of the β-CBA II aptamer 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, facilitating ultrasensitive detection [31-37]. In this approach, β-conglutin was immobilised either in the wells of a microtiter plate, or on the surface of magnetic beads, and this solid-phase β-conglutin competed with a range of concentrations of solution phase β-conglutin for binding to the β-CBA II aptamer. Following competition, aptamer bound to the surface-immobilised β-conglutin was eluted and subsequently used as a template in nucleic acid amplification. Classic thermal cycling amplification was compared with isothermal amplification. Recombinase polymerase amplification (RPA) is a highly effective isothermal amplification method that requires no initial denaturation step, inherently overcomes the requirement for thermal cycling with optimum operation being at 37ºC, and is extremely rapid, and amplification can be completed in just 15 minutes. The detection limits achievable, as well as the assay times for the thermal cycling and isothermal approaches were explored.

4.3 MATERIALS AND METHODS

4.3.1 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) carboiimide (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). SiGMAG-Carboxyl beads were obtained from Chemicell (Berlin, Germany), NUNC 96 well microplates from ThermoScientific (Madrid, Spain) and *Tfi* DNA Polymerase and 10 bp DNA ladder from Life Technologies (Barcelona, Spain). Certified™ Low Range Ultra Agarose and Precision Plus Protein™ Standards were purchased from Bio-Rad (Barcelona, Spain). All solutions were prepared in high purity water obtained from the Mili-Q RG system (Barcelona, Spain). All primers and probe sequences can be found in Table 4.1. All DNA oligonucleotides were purchased from BIOMERS (Ulm, Germany) and β-conglutin was extracted and purified as previously detailed [38].

Table 4.1: Sequences

Name	Sequence	MW (g mol^{-1})
Forward primer	5'-agctccagaagataaattacagg-3'	7090
Reverse primer	5'-ggggtcatagtatcctagttg-3'	6492
Biotin probe	5'- Biotin-ggggtcatagtatcctagttg-3'	6930
β-CBA II aptamer	5'-agctccagaagataaattacaggggccggggtggctca Ggcaaggggttgacctgtcgtagggattgttttaacaactagg atactatgacccc-3'	29198

4.3.2 Competitive enzyme linked oligonucleotide assay on microtiter plate

To elucidate optimal parameters of ELONA, the concentrations of coating β-conglutin and β-CBA II aptamer were optimized previously (data not shown). β-conglutin (140 nM) in 50 mM Carbonate buffer pH 9.6 was immobilized on a microtiter plate for 30 minutes at 37°C, followed by 30 minutes of blocking with PBS-Tween. The wells were then washed three times with 200 µl 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 minutes at room temperature (RT) in the presence of 3 µg/ml salmon sperm DNA, and subsequently added to individual wells of the coated microtiter plate and left to incubate for 30 minutes 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 a further 30 minutes. Subsequently, the wells were thoroughly washed and 10 nM of horseradish peroxidase linked streptavidin (Streptavidin-HRP) was added and incubated for a further 30 minutes. Finally, 3,3',5,5'-Tetramethylbenzidine (TMB) was added and after 10 minutes the reaction was stopped with 1 M of sulfuric acid and the absorbance read at 450 nm using a SpectraMax 340PC microtiter plate reader.

4.3.3 Competitive Apta-PCR and Apta-RPA using β-conglutin binding aptamer (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, Mili-Q water was added to each well and the aptamer was heat-eluted and used a template for amplification. Real-time PCR was performed as previously described using SYBR Green as an intercalator for detection of fluorescence [37].

To elucidate the optimal parameters for Apta-RPA, firstly the concentrations of coating β-conglutin and β-CBA II aptamer were optimized. A range of concentrations of β-conglutin (500 – 15.62 nM) diluted in 50 mM carbonate buffer (pH 9.6) were incubated in the wells of a microtiter plate for 30 minutes at 37ºC, followed by 30 minutes blocking with PBS-Tween at 37ºC. Following thorough washing step, β-CBA II aptamer was added to each well and incubated in binding buffer for 30 minutes at RT and the bound aptamer eluted and used as a template in RPA. Different concentrations of aptamer (100 – 0.01 nM) and different temperatures of RPA amplification (35, 37, 39 and 41ºC) were also tested. Following aptamer elution, RPA was carried out using a TwistAmp basic kit (TwistDX, LTd. Cambridge, United Kingdom), where each 50 µl reaction contained 29.5 µl rehydration buffer, 2.1 µl of forward primer (10 µM), 2.1 µl of reverse primer (10 µM),

2.5 µl Eva Green DNA binding dye (20x), 2.5 µl magnesium acetate and one enzyme pellet. This master mix (9 µl) was mixed with 1 µl aptamer to the total volume of 10 µl. RPA reactions were executed in a cold block cooled to 4 ºC. The fluorescence of the Eva Green DNA binding dye was monitored every 20 seconds (each cycle) for 40 minutes after a pre-incubation of 1 minute at 37ºC.

In an alternative approach, magnetic beads rather than the microtiter plate were used and β-conglutin was immobilized on the surface of carboxyl magnetic beads as previously described [19]. The amount of functionalized beads was optimized. In individual eppendorf tubes a range of concentrations of β-conglutin were pre-incubated with 1 nM of the β-CBA II aptamer for 10 minutes at RT. Subsequently, the functionalized beads were added to each eppendorf and incubated for a further 30 minutes. Magnetic actuation was applied and the eppendorfs rigorously washed three times with binding buffer followed by the addition of Mili Q water for heat-elution of the aptamer for use as a template in RPA.

4.4 RESULTS AND DISCUSSION

4.4.1 Competitive enzyme linked oligonucleotide assay on microtiter plates

Competitive ELONA was used a means for comparing the detection limits and assay times achievable with the combined competition, elution and nucleic acid amplification approaches. The limit of detection (LOD) obtained using a type of competitive enzyme linked oligonucleotide (ELONA) based on immobilized β-conglutin on a microtiter plate, which competes with β-conglutin free in solution for the binding of 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 immobilized β-conglutin and a LOD 3.4 x 10-8 M was achieved (Figure 4.1b), with a total required assay time of 160 minutes.

4.4.2 Apta-PCR assay using β-conglutin binding aptamer II (β-CBA II)

In order to improve this LOD, Apta-PCR was used [37], where the aptamer bound to the immobilized β-conglutin was heat eluted and used as a template in RT-PCR (Figure 4.1a).

The calibration curve was obtained by plotting the resulting ΔCt (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 2-orders of magnitude as compared to the ELONA assay, achieving a LOD of 2 x 10^{-10} M (Figure 4.1c).

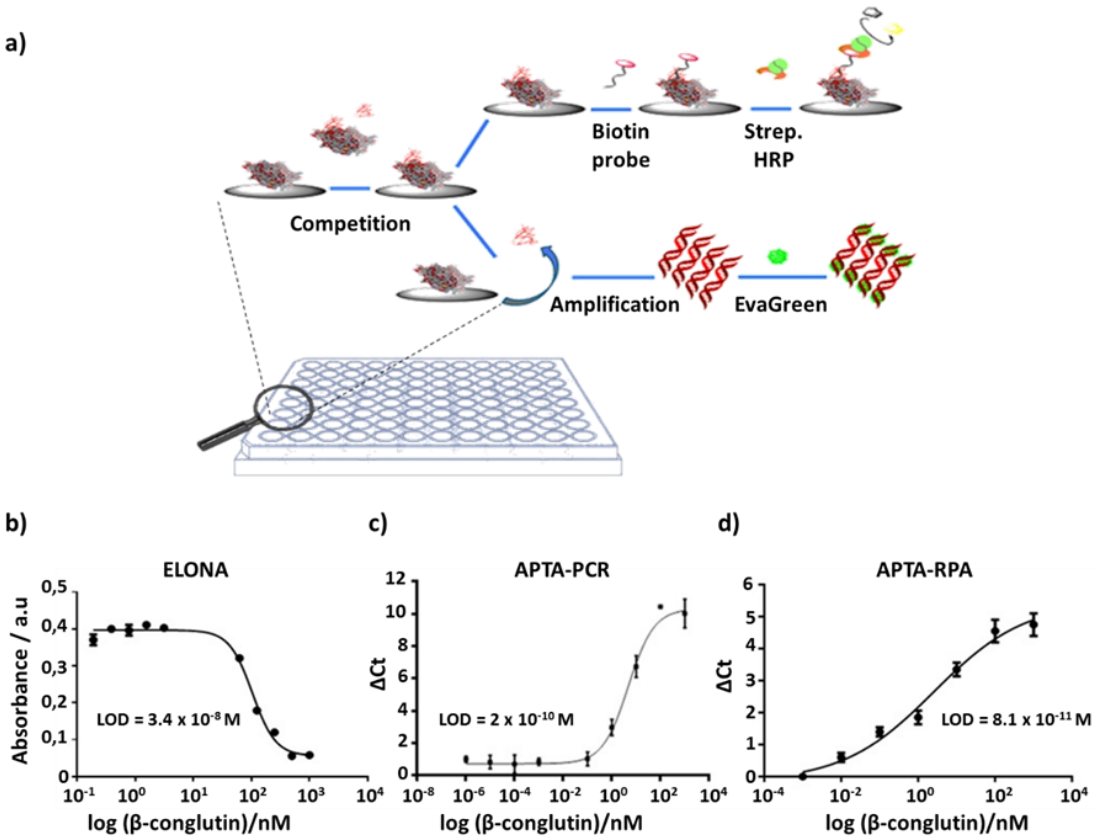

Figure 4.1: a) Scheme ELONA vs. APTA-PCR/APTA-RPA assay; b) LOD ELONA assay; c) LOD APTA-PCR assay; d) LOD APTA-RPA assay.

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 37ºC and 40ºC. 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 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 open the double helix as it progresses. When opposing primers are used, exponential amplification occurs. Since the introduction of recombinase polymerase amplification [39], 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 [40-42] or RNA [43-45], whilst other common applications include end-point detection system using lateral flow assays [46-48]. Elegant and simple solutions using DVDs as platforms have been also reported, with the detection been carried out using a slightly modified DVD optical player [49-51].

4.4.3 Competitive Apta-RPA assay

A standard dilution curve using the β-CBA II aptamer as a template with different amplification temperatures (35ºC – 41ºC) was performed, and as expected 37ºC was observed to be optimal. Furthermore, amplification of the β-CBA II was carried out at a range of concentrations (100 – 0.01 nM), and as can be seen that 1 nM aptamer is the lowest amount of aptamer that is still detectable after 10 minutes' amplification, with

signal saturation observed after 30 minutes, and this was thus selected as the optimum aptamer concentration for the competition assay (Figure 4.2a).

Following optimization of the RPA for the direct detection of the β-CBA II aptamer, the optimum amount of immobilized β-conglutin required for the competitive Apta-RPA assay was elucidated using 1 nM aptamer. The aptamer was eluted from the plate by heating and amplified following the optimized protocol described above. 125 nM of immobilized β-conglutin was determined to be the lowest saturating concentration and was thus selected as the optimum coating concentration for the competition assay (Figure 4.2b). The assay proceeds in a type of competitive assay format, where target β-conglutin forms a complex with the β-CBA II aptamer during pre-incubation, with any unbound aptamer being free to bind to the β-conglutin on the surface of the microtiter plate (or magnetic beads as described below).

A selectivity study was carried out to investigate the ability of the RPA to differentiate between specific aptamer (β-CBA II aptamer), a non-specific sequence (control DNA aptamer) and the absence of a target.

In comparison to Apta-PCR, RPA was observed to markedly more rapid, with amplification being completed in just 10 minutes, whilst also achieving an improved LOD of 8.1×10^{-11} M, with amplification being carried out at 37ºC, thus highlighting potential exploitation in low-resource settings, on-site or at the point-of-care (Figure 4.1d).

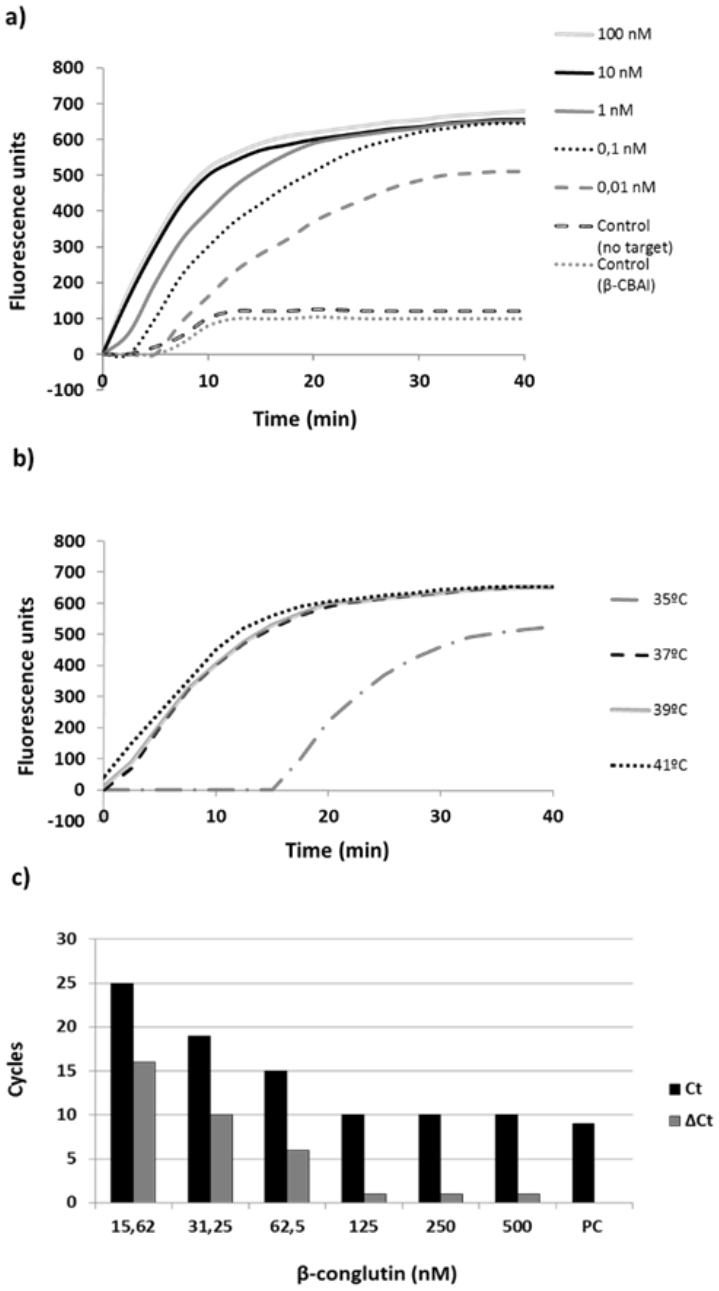

Figure 4.2: Optimization of Apta-RPA: a) optimal aptamer concentration for Apta-RPA assay; b) optimal temperature for Apta-RPA assay; c) optimal β-conglutin immobilized on the plate (PC corresponds to positive control (1nM aptamer amplified by RPA without β-conglutin). Ct corresponds to the cycle where the sample crosses the threshold; ΔCt is the difference between the sample Ct and the Positive control Ct).

In order to further reduce the assay time, same methodology was exploited but immobilizing the β-conglutin on carboxyl magnetic beads via carbodiimide cross-linking rather than on microtiter plates (Figure 4.3a). Magnetic beads can reduce in decreased incubations times, as stirring can be used to enhance the diffusion rate and the spherical nature of the magnetic beads can render the immobilized β-conglutin more accessible for binding.

Different amounts of β-conglutin functionalised beads were used to define when all β-CBA II was captured and 10 µl 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-conglutin. (Figure 4.3b). In this case the LOD was observed to be 9.9×10^{-11} M (Figure 4.3c) with a greatly increased pre-incubation of β-conglutin with aptamer of just 10 minutes. The entire assay was achieved in 50 minutes, as compared to 100 minutes and 210 minutes when using microtiter plates and Apta-RPA and Apta-PCR, respectively, highlighting the potential of the assay format for on-site rapid analysis. Current work is focusing on transferring the detection to a lateral flow format.

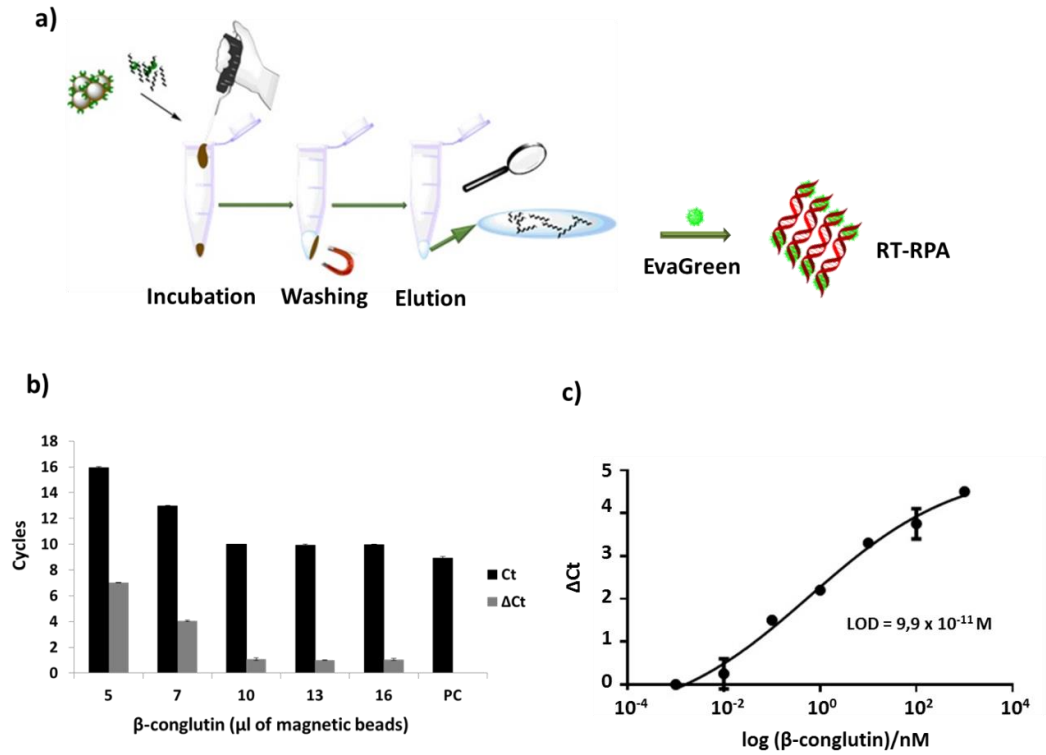

Figure 4.3: Beads APTA-RPA: a) scheme beads APTA-RPA assay; b) optimal amount of beads (PC corresponds to positive control (1nM aptamer). Ct corresponds to the cycle where the sample crosses the threshold; ΔCt is the difference between the sample Ct and the Positive control Ct); c) LOD Beads APTA-RPA assay.

4.5 CONCLUSIONS

Methodologies for the rapid and ultrasensitive detection of the anaphylactic allergen β-conglutin have been developed. Competitive ELONA with no incorporated amplification step gave a LOD of 3.4 x 10^{-8} M. The use of a microtiter plate based assay with an incorporated step where the β-CBA II aptamer and target β-conglutin were pre-incubated, followed by competition, aptamer elution and amplification, resulted in assay times of 210 and 100 minutes, with detection limits of 2 x 10^{-10} M and 8.1 x 10^{-11} M, using PCR or RPA, respectively. Replacing the microtiter plates with β-conglutin functionalized magnetic beads, the assay time was reduced to just 50 minutes, with a detection limit of just 9.9 x 10^{-11} M.

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Chapter 5

Ultrasensitive, rapid and inexpensive detection of DNA using paper based lateral flow assay

(Submitted)

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5.1 ABSTRACT

Sensitive, specific, rapid, inexpensive and easy-to-use nucleic acids tests for use at the point-of-need are critical for the emerging field of personalised medicine where companion diagnostics are essential, as well as for application in low resource settings. Here we report on the development of a point-of-care nucleic acid lateral flow test for the direct detection of isothermally amplified DNA. The recombinase polymerase amplification method is modified slightly to use tailed primers, resulting in an amplicon with a duplex flanked by two single stranded DNA tails. This tailed amplicon facilitates detection via hybridisation to a surface immobilised oligonucleotide capture probe and a gold nanoparticle labelled reporter probe. Assay parameters are optimised and a range of target DNA concentrations tested. A Smartphone camera was used to take an image of the strip followed by Image J application termed IJ_mobile to calculated the intensity of the bands, and a detection limit of 1 x 10^{-11} M (190 amol), equivalent to 8.67 x 10^5 copies of DNA was achieved, with the entire assay, both amplification and detection being completed in less than 15 minutes, carried out at a constant temperature of 37ºC. The use of the tailed primers obviates the need for hapten labelling and consequent use of capture and reporter antibodies, whilst also avoiding the need for any post-amplification processing for the generation of single stranded DNA, thus presenting an assay that can facilely find application at the point of need. Future work will focus on direct functionalisation of the membrane with the capture probe, and integration of the recombinase polymerase amplification with detection on a single membrane.

5.2 INTRODUCTION

Lateral flow assays (LFA) are very simple and highly successful rapid analytical platforms derived from the latex agglutination test developed by Singer and Plotz in 1956 for the serological diagnosis of rheumatoid arthritis [1]. Lateral flow tests, or immunochromatographic tests, were first reported in the early 1980s [2] and were commercially launched by Unipath in 1984 with the first product being the urine-based pregnancy test [3]. Since then, hundreds of lateral flow assays have been reported and commercialised with applications for detection of infectious diseases, cancer, cardiac diseases, toxins, pathogens, pesticides, metal ions as well as for pharmaceuticals and drugs, as has been extensively reviewed [4-5].

LFAs are typically composed of a nitrocellulose membrane, sample pad, conjugate pad, wicking of absorbent pad and backing pad [6]. The absorbent pad provides a capillary based driving force, with the backing pad providing mechanical support. Nitrocellulose membranes are most commonly used as they facilitate a support capable of use for both reaction and detection, with capture biomolecules e.g. antibodies, are deposited on the nitrocellulose to form the test and control lines via a combination of electrostatic interactions, hydrogen bonds and/or hydrophobic interactions [7]. The point-of-care (POC) market is rapidly expanding, believed to be worth US$15 billion in 2011 and predicted to have an annual compound growth of 4% to reach US$18 billion by 2016 [8-9]. The World Health Organisation has provided guidelines for these POCTs, which are referred to as ASSURED, and are shown in Table 5.1.

Table 5.1: Definition of ASSURED diagnostics

Affordable – for those at risk of infection
Sensitive – minimal false negatives
Specific – minimal false positives
User-friendly – minimal steps to carry out test
Rapid & Robust - short turnaround time and no need for refrigerated storage
Equipment-free – no complex equipment
Delivered – to end users

Nucleic acid testing has important applications in food safety analysis, environmental monitoring and increasingly in medical diagnostics. Meeting the emerging paradigm of medicine, where pharmacogenomics and individualised theranostics are garnering increasing importance for patient stratification and avoidance of adverse drug effects, there is a mature need for rapid, inexpensive, highly sensitive and facile-to-use companion diagnostic tests for the qualitative/quantitative detection of nucleic acids. Meeting this requirement, there are a large number of paper analytical devices (PAD) that have been developed for detection of PCR products using lateral flow assays. There are two mains types of lateral flow nucleic acid tests, referred to as nucleic acid lateral flow (NALF) and nucleic acid lateral flow immunoassay (NALFIA), where NALF directly detects DNA exploiting capture and labeled reporter oligonucleotide probes, whereas NALFIA detects hapten labeled DNA using capture and labeled reporter antibodies or streptavidin. The first example was a NALFIA, reported in 2000 for the detection of *Cryptosporidium parvum*, where they used biotin and FITC labeled forward and reverse primers for PCR and detecting the duplex using immobilized anti-FITC antibodies and streptavidin coupled to colored latex microparticles [10], which was quickly followed by a similar approach for the detection of *Staphylococcus aureus*, in this case using immobilized streptavidin and anti-fluorescein antibodies labeled to

gold nanoparticles [11], and since then a plethora of NALFIAs have been reported using different hapten labels, including digoxigenin (dig), FAM, FITC and biotin [4]. There are far fewer examples of NALF, which can be attributed to the kinetics of hybridisation in lateral flow being far more complex as compared to the formation of hapten-antibody complexes. Corstjens *et al.* first reported the use of up-converting phosphor technology (UPT) reporters using a signal enhancement tool in a NALFIA using dig and biotin hapten labels [12], and subsequently modified the approach for the detection of an asymmetric PCR product using a biotinylated capture probe immobilised via streptavidin coated on the nitrocellulose strip and a UPT labelled reporter probe, representing the first example of a NALF [13]. In the same year, Glynou *et al.* reported the first example of a NALF, where a biotinylated oligonucleotide probe was used as a capture probe, and a gold nanoparticle labelled oligonucleotide probe was used as a reporter, but required enzymatic tailing of probes [14]. Soon after, Baeumner *et al.* detailed the use of a liposome labelled oligonucleotide reporter probe and biotinylated capture probes, using polyethersulphone membranes, with the liposome-oligo complex being formed off strip and subsequently wicked to the detection zone [15]. Liu's team has published a series of papers detailing various formats of NALF, achieving a LOD of 0.5 nM using gold nanoparticles [16] and improving this LOD to 50 pM via the use of horseradish peroxidase adsorbed on gold nanoparticles linked to the reporter probe as a means of signal amplification, in both cases for a synthetic DNA target [17]. He subsequently reported the simultaneous lateral flow detection of proteins and nucleic acids, again used oligo capture and reporter probes [18] and recently improved on these previous reports, using carbon nanotube labelled reporter probes, and using pre-mixed streptavidin-biotinylated probe as the capture probe, achieving an LOD of 40 pM for a synthetic DNA target [19].

Whilst some approaches have been reported to improve the sensitivity of NALFs, via the use of locked nucleic acid gold nanoparticle reporter probes [20] or exploiting oligonucleotide-linked gold nanoparticle aggregates [21], to date all NALFs require prior or integrated nucleic acid amplification. Whilst PCR is the most commonly reported method of amplification combined with lateral flow, there are an increasing number of

reports combining isothermal amplification with lateral flow detection, moving nearer to achieving ASSURED devices that can truly be used at the point-of-need.

Isothermal amplification offers the possibility of being able to carry out on-site analysis of DNA, and several techniques have been reported and exploited in the last decade either carrying out amplification followed by detection, or combining amplification and detection [22] such as the nucleic acid sequence based amplification (NASBA), transcription mediated amplification (TMA), self-sustained sequence replication (3SR) [23-25], helicase dependent amplification (HDA) [26], rolling circle amplification (RCA) [27,28] and loop mediated isothermal amplification (LAMP) amplification [29-30]. Devices using LAMP integrating purification, amplification and detection have been reported [31], but these devices use fluorescence or turbidity measurements, which limits the application of LAMP for multiplexing, and furthermore, LAMP is highly dependent on the extremely careful design of multiple complex primers [32]. The recombinase polymerase amplification is a very attractive alternative, that overcome all of the drawbacks of the other isothermal approaches [12]. An increasing number of reports detailing different formats of RPA are appearing and the technique gives true promise for application at the point-of-need, via sensors or lateral flow formats.

As can be seen in the Table in Supporting Information (Table SI-5.1) there are more than a hundred reports of the combination of isothermal amplification with lateral flow detection, the vast majority exploiting LAMP, and almost exclusively using NALFIA formats, with a couple of exceptions, such as the detection of the single stranded DNA products from a two-stage exponential amplification reaction (EXPAR) using a biotinylated capture probe immobilized on Neutravidin and a microsphere bead labeled reporter probe [33]. The use of LAMP combined with NALF is also reported, but requires a post-LAMP denaturation step at 95ºC prior to detection [34].

The goal of this study is to develop a point-of-care nucleic acid lateral flow test for the direct detection of RPA products. Tailed primers are exploited, resulting in an amplicon with a duplex flanked by two single stranded DNA tails, allowing detection via

hybridisation to a surface immobilised oligonucleotide capture probe and a gold nanoparticle labelled reporter probe. Assay parameters are optimised and a range of target DNA concentrations tested. The use of the tailed primers obviates the need for hapten labelling and consequent use of capture and reporter antibodies, whilst also avoiding the need for any post-amplification processing for the generation of single stranded DNA, thus presenting an assay that can facilely find application at the point of need.

5.3 MATERIALS AND METHODS

5.3.1 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) carboiimide (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). Pierce™ maleimide activated plates, 8-well strip, from Pierce (Madrid, Spain) and DNA oligonucleotides were purchased from BIOMERS (Ulm, Germany). All primers and probe sequences can be found in Table 5.2.

Table 5.2: Sequences used in this study

Name	Sequence
Capture probe maleimide plates	5'-gtcgtgactgggaaaactttttttttttttt-C6 thiol-3'
Reporter probe maleimide plates	5'-HRP-actggccgtcgttttaca-3'
Capture probe test line	5'- gtcgtgactgggaaaactttttttttttttt-Biotin-TEG-3'
Capture probe control line	5'-tgtaaaacgacggccagtttttttttttttttt-Biotin-TEG-3'
Reporter probe lateral flow (DNA 2)	5'-actggccgtcgttttacatttttttttttttt-C6 thiol-3'
Reporter probe lateral flow (DNA 1)	5'-actggccgtcgttttaca-C6 thiol-3'
DNA	5'-agctccagaagataaattacaggggccggggtggctcaggcaaggggttgacctgt cgtagggattgttt taacaactaggatactatgacccc-3'
Forward primer	5'-gttttcccagtcacgac-C3-agctccagaagataaattacagg-3'
Reverse primer	5'-tgtaaaacgacggccagt-C3-ggggtcatagtatcctagttg-3'

5.3.2 Preparation of microtiter plates

Maleimide plates were prepared by pipetting 100 µl of 200 nM thiolated capture probe prepared in 10 mM phosphate, 138 mM NaCl, 2.7 mM KCl (PBS, pH 7.4) and left to incubate overnight at 4ºC. The plates were subsequently washed with PBS containing 0,05% (v/v) Tween 20 (PBS-Tween) and any remaining maleimide groups were blocked with 100 µM 6-mercapto-1-hexanol in deionized water adding 200 µl per well for 1 hour before washing the plate thoroughly with PBS-tween.

5.3.3 Recombinase polymerase amplification (RPA) reaction

RPA was performed in a tube following the indications provided in the TwistAmp Basic kit (TwistDX, Cambridge). Briefly, master mix was prepared in a tube with 480 nM of each primer, template DNA (94 bases) of the desired concentration, 14 mM magnesium acetate and 1x rehydration buffer. The reaction proceeded at room temperature for 20 minutes / 37ºC for 15 minutes (Figure 5.1).

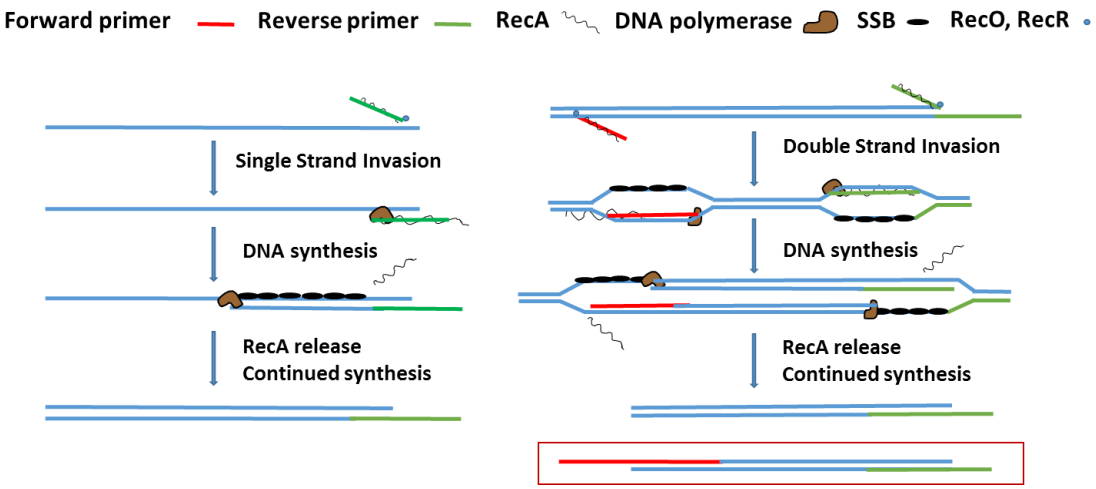

Figure 5.1: Schematic representation of liquid-phase RPA with tailed primers.

5.3.4 Enzyme linked oligonucleotide assay

The resulting RPA product was added to the functionalised maleimide plates (50 µl per well) for 30 minutes at room temperature under shaking conditions, followed by a washing step with PBS-Tween, and subsequent addition of 50 µl of 10 nM reporter probe labelled with HRP to each well and incubation for a further 30 minutes. After a final washing step, the presence of HRP was measured following addition of 50 µl of TMB substrate, and 50 µl 1 M H_2SO_4 5 minutes later. The absorbance was read at 450nm (SpectraMax 340PC384, bioNova Scientifics S.L.). To check the sensitivity of the assay, amplification was carried out with different starting concentrations of DNA (100 nM, 10 nM, 1 nM, 0.1 nM, 0.01 nM, 0 nM). The Limit of Detection (LOD) was calculated using GraphPad Prism Software. Triplicate measurements were performed for each concentration.

5.3.5 Preparation of reporter probe-AuNPs conjugation

Gold nanoparticles (AuNPs) with an approximate average diameter of 13 nm were prepared by citrate reduction of $HAuCl_4$, as previously described [35]. Conjugation of reporter DNA to AuNPs was achieved via mixture of 100 µl of reporter DNA probe with 1 ml of AuNPs. The solution was left to incubate for 24 hours at 1000 rpm in a thermomixer and salt was introduced each 20 minutes until a concentration of 0.7 M was reached. Subsequently, the mixture was again left to incubate for 24 hours under the same conditions. Finally, the conjugate was centrifuged at 15000 rpm for 30 minutes and the pellet was re-suspended three times in deionized water in order to clean the conjugate and remove any free DNA. The conjugate was then re-suspended in deionized water to the desired volume. Additional functionalities were tested in an effort to increase the yield of conjugation such as co-immobilization with 6-mercapto-1-hexanol (MCH) (ratio DNA:MCH 1:10, 1:100) and pre-treatment of thiolated DNA with reducing agents (TCEP and DTT) [36-37].

The conjugates were evaluated and characterised by agarose gel and spectrophotometer. The gel was performed using Ultra low pure agarose (3%) in 1x TBE buffer and run for 30 minutes at 100 V. To stain the gel, GelRed (Biotium, Barcelona,

Spain) was used. Spectrophotomer (Cary 100 Bio Uv-visible spectrophotometer, Agilent) was used to scan from 800 nm to 200 nm.

5.3.6 Preparation of lateral flow test strip

The test strip was made by manually cutting in strips of 4 mm. The membrane used was FF170HP nitrocelloluse (Whatman, Germany) and the absorbent pad was glass cellulose (Whatman, England). The test and the control lines were prepared by drawing a line with an eppendorf tip containing a solution of 20 pmol streptavidin and 60 pmol of the respective biotinylated probe in PBS buffer, which was then incubated for 1 hour at room temperature. Subsequently, the membrane was allowed to dry at room temperature for 1 hour, followed by a blocking step with 1% w/v skimmed milk powder and 0.1% w/v empigen detergent for 15 minutes, under shaking conditions. The membrane was left to dry, again at room temperature for approximately 2 hours and then stored in the fridge until use. The test strips were assembled according to Figure 5.4a.

5.3.7 Lateral flow assay

Ten microliters of reporter probe conjugated with AuNPs was mixed with 1 µl RPA product, and 8 µl of 10x SSC, 3.5% v/v Triton X-100, 0.25% v/v SDS, 12.5% formamide to obtain a final concentration of 4x SSC buffer, 1.4% v/v Triton X-100, 0.1% v/v SDS, 5% formamide and incubated for 3 minutes at RT, before application to the test strip.

In order to test the sensitivity of the assay, RPA was carried out with different concentrations of DNA (300 nM, 30 nM, 3 nM, 0.3 nM, 0.03 nM, 0.003 nM, 0 nM). A Smartphone camera was used to take an image of the strip, and analysed using a smartphone application based on Image J software termed IJ_mobile to calculated the intensity of the bands. These values were plotted in GraphPad Prism software in order to obtain the limit of detection of the assay (LOD). The data was normalized by subtracting the value obtained for the blank measurement. Triplicate measurements were performed for each concentration.

5.4 RESULTS AND DISCUSSION

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 37ºC and 40ºC. 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 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 open the double helix as it progresses. When opposing primers are used, exponential amplification occurs.

The combination of RPA and lateral flow detection has been reported since 2013, and the use of body heat to perform amplification has been demonstrated [38] and RPA-LF has been used for the detection of HIV-1 [39-40], Canine Visceral Leishmaniasis [41], *Orientia tsutsugamushi, Rickettsia typhi* [42], plasmodium [43], intestinal protozoa [44], *cryptosporidiosis* [45], Yellow Fever Virus [45], *Penaeus stylirostris* [46], *Plasmodium falciparum* [47], *Entamoeba histolytica* [48],*Schistoma haematobium* [49], little cherry virus [50] and plum pox virus [51]. All these reports exploit RPA amplification followed by lateral flow strip detection based on the use of two different kits. The first is the TwistAmp nfo kit (TwistDX) combined with commercial Hybridetect strips (Milena) [39-49, 52-53] and the other is the Amplify RP kit (Agdia) [50-51]. In both cases antibodies are used as capture probes in test and control lines and a probe modified with a hapten, sometimes a fluorophore such as FAM, is used as a reporter probe. The lateral flow strips are sometimes read with a commercial scanner in combination with specific software to achieve better detection limits.

As a proof of concept for the detection of DNA amplified via RPA using tailed primers maleimide coated microtiter plate were used to immobilise thiolated capture probe. RPA amplicons, duplex flanked by two single stranded tails, were added to the wells of the microtiter plate and hybridised to the immobilised capture probe, and then

to the reporter probe conjugated with horseradish peroxidase (HRP) (Figure 5.2a). The tailed amplicon was successfully detected and the limit of detection (LOD), calculated by GraphPad Prism software, defined as the blank plus three times the standard deviation of the blank was calculated to be 6 x 10^{-12} M (Figure 5.2b).

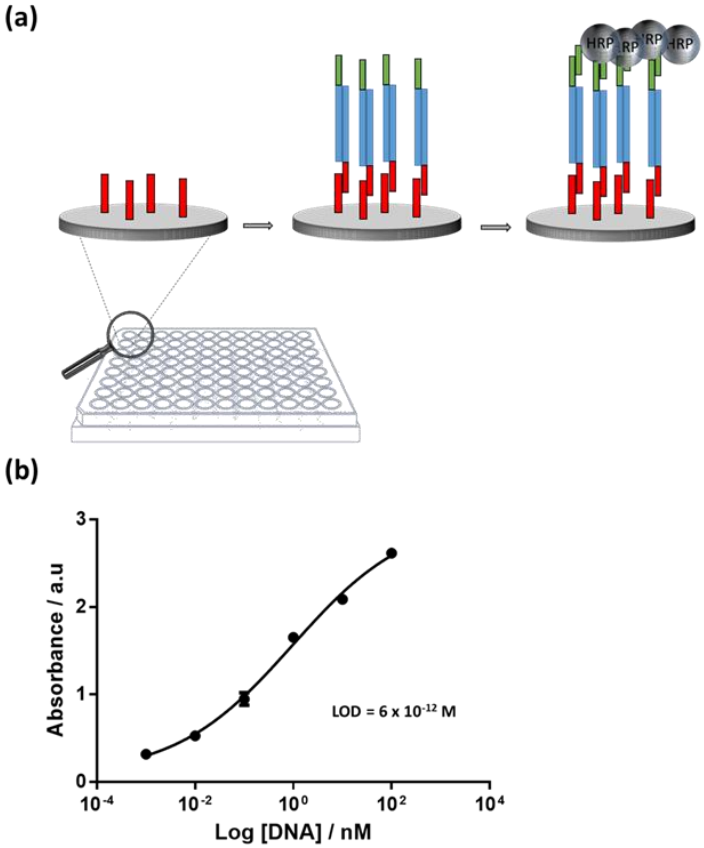

Figure 5.2: Maleimide coated microtiter plate assay. (a) schematic representation of hybridisation of immobilised capture probe and HRP reporter probe with single stranded tailed RPA amplicon; (b) calibration curve using different amounts of RPA DNA amplified using tailed primers.

Different methods were evaluated for the preparation of the reporter probe conjugated with gold nanoparticles [54-56]. Co-immobilization was explored as a means of controlling the probe surface density, as it is well known that spacing between the immobilised probes minimises steric hindrance, favouring accessibility to the complementary DNA strand. The ratio usually used for DNA to the short alkanethiol, mercaptohexanol (MCH) ranges between 1:10 and 1:100 [37]. Additionally, to ensure that the thiol moiety on DNA is free, treatment with reducing agents such as DTT or TCEP is often incorporated in the conjugation protocol, this breaking any disulphide bridges that may have formed between probes. Some reports claim that the best results are achieved with DTT, meanwhile others prefer TCEP due to the fact that it does not compete or react with thiolated compounds, thereby eliminating the need to remove it prior to conjugation with AuNPs [36]. Finally, the direct immobilization of the DNA probe on to AuNPs, with no co-immobilized alkanethiol or use of reducing agents, was tested. Thiolated DNA 1 and DNA 2 (DNA 1 incorporating a 15-mer poly-T spacer) were compared.

To characterize the different methodologies two strategies were studied. 1x TBE agarose gel 3% was prepared to carry out electrophoresis analysis of the prepared conjugates, allowing analysis and by naked eye as well as by UV following staining with GelRed. As can be seen in Figure 5.3a. in Lanes 2-5, the use of mercaptohexanol as a co-immobiliser, without pre-treatment with any reducing agent, did not result in a successful DNA-AuNP conjugation. Likewise, the use of a DTT reducing agent was not observed to result in a successful conjugation, perhaps due to the DTT competing with the thiolated DNA for immobilization. However, a visual difference was observed in the case of TCEP pre-treatment to reduce any disulphide bonds, as well as direct conjugation with both DNA probes, with better results in both cases being observed for DNA 2, the DNA reporter with an incorporated 15T spacer. Free DNA unconjugated to the AuNPs would be expected to migrate to lower than the 100 bases band of the ladder, but as can be seen for both the DNA 1 and DNA 2 reporter probes, there is a clear retardation in their migration along the gel. This can be attributed to both the increased size of the DNA-AuNP complex, as well as to each AuNP bearing multiple copies of the DNA probe.

Furthermore, it can be observed that the AuNPs linked to the DNA 2 probe bear far more DNA than the AuNPs linked to DNA 1, presumably due to the 15T spacer allowing an optimal spatial orientation of the DNA 2 probe on the AuNP surface, thus facilitating increased surface accessibility for enhanced chemisorption for a higher number of DNA probes.

The DNA-AuNPs conjugates were also evaluated using UV-spectroscopy, by scanning the wavelength from 200 to 800 nm. Two peaks, one at 260 nm for DNA and another at 520 nm for AuNPs, are expected to be observed. TCEP pre-treatment and direct immobilization in the absence of any co-immobilizer were again observed to provide the optimal conjugates (Figure 5.3b). Again, pre-treatment with DTT resulted in no linkage of DNA to the AuNP, but this could be attributable to the DTT competing with the thiolated DNA for chemisorbing to the gold.

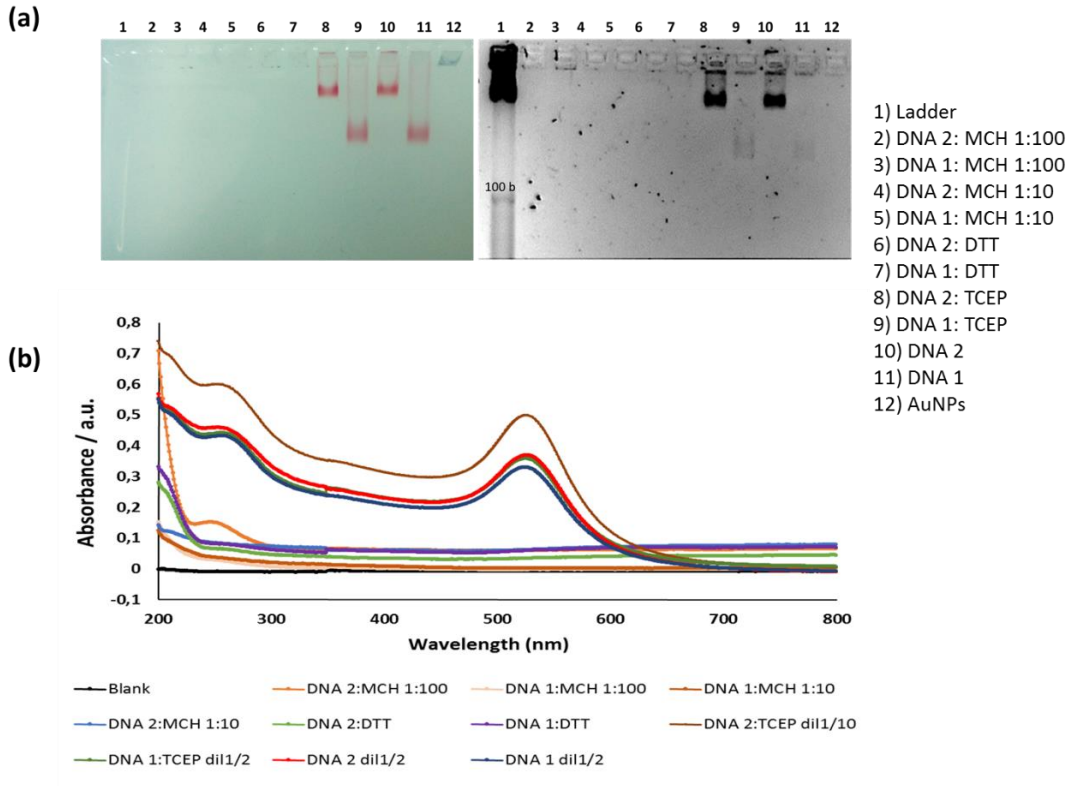

Figure 5.3: Analysis of reporter probe-AuNP conjugation using (a) agarose gel highlighting unsuccessful DNA-AuNP conjugation using MCH co-immobiliser (Lanes 2-5)

or DTT as reducing agent (Lanes 6-7), whilst successful conjugation is demonstrated via the slower gel migration of DNA-AuNP conjugates without immobiliser/reducing agent (Lanes 10, 11), with the highest level of AuNP loading with DNA observed using TCEP as reducing agent (Lanes 8, 9); (b) spectrophotometer analysis of gold nanoparticle-DNA conjugates highlighting peaks obtained at 260 nm (DNA) and 520 nm (Au NP).

5.4.1 Lateral flow assay

Once detection of the duplex flanked by single stranded tails via hybridisation to a surface tethered capture probe and a labelled reporter probe had been demonstrated using a microtiter plate format, the system was successfully transferred to a lateral flow assay format. The lateral flow was based on the immobilization of two biotinylated capture probes for each of the test and control lines. On the test line, the immobilised probe is complementary to the tail in the 5' region of the amplified DNA, meanwhile in the control line, the probe is complementary to the reporter probe conjugated with AuNPs. This reporter probe is also complementary to the other tail on the 3' end of the amplified, tailed DNA. Thus, the reporter probe conjugated with gold nanoparticles will bind to the amplified DNA forming a sandwich on the test line and with the capture probe on the control line, generating in both cases a red line visible to the naked eye (Figure 5.4a).

In order to improve assay performance, three different membranes were evaluated to find the optimal material for creation of the lateral flow strips. Unistart CN95 is cellulose nitrate, and has large pores, with a fast flow rate from 90-135 s/ 4 cm. This membrane is recommended for blood or serum tests being applicable to the detection of cells and bacteria. Alternatively, FF170HP is a nitrocellulose membrane ideal for use with low viscosity samples with a flow rate of 156 s/ 4 cm. Finally, Biodyne B membrane, a nylon membrane which contains exclusively positive charges, providing the highest possible binding capacity for negatively charged molecules such as nucleic acids. This membrane was used to immobilize the capture probes directly onto the membrane without DNA modification, but no successful results were observed, which may be attributed to the DNA being planarly linked to the membrane surface, rendering

it inaccessible for hybridisation with the tailed target. The best option was FF170HP membrane, which resulted in control and test lines of the highest intensities.

Different approaches for immobilisation of the streptavidin and the biotinylated capture probes on the membrane were evaluated. Three methodologies were compared: (a) dispensing streptavidin, allowing to dry, followed by addition of the biotinylated capture probe; (b) pre-incubation of streptavidin with the biotinylated capture probe, and dispensing of the pre-formed complex onto the membrane; (c) pre-incubation of streptavidin with the biotinylated capture probe followed by centrifugation using 30k microcon to eliminate the excess of biotinylated capture probe before dispensing of the pre-formed complex onto the membrane. The most intense bands where observed with pre-incubation without centrifugation.

In order to establish the sensitivity of the NALF, different concentrations of DNA were amplified using RPA and detected, with the test line being visible to the naked eye to concentrations as low as 30 pM (Figure 5.4b). Built-in cameras in mobile phones have been used as imaging platforms [57-58] as well as for the detection of disease biomarkers and infectious pathogens [59-64], and here a Smartphone camera was used to take an image of the strip followed by an application based on Image J software termed IJ_mobile to calculate the intensity of the bands, and these values then were plotted using GraphPad Prism software in order to obtain the limit of detection of the assay (LOD). The data was normalized by subtracting the value obtained in the blank, and the LOD achieved was 1×10^{-11} M, with a complete assay time combining amplification and detection of 15 minutes, with the entire assay being carried out at 37 ºC, but similar results and assay time was also observed if the entire amplification and detection assay is carried out at 22 ºC (room temperature) (Figure 5.4c).

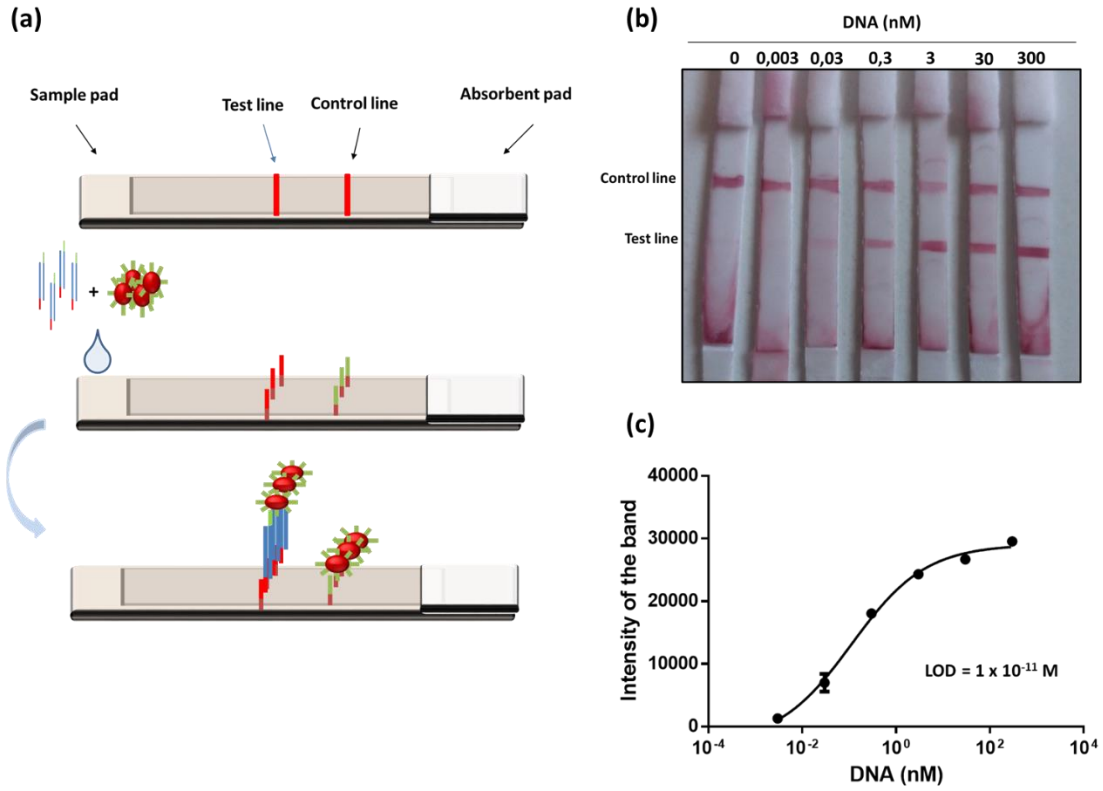

Figure 5.4: Lateral flow assay. (a) schematic representation of RPA-NALF; (b) images of NALFs with varying concentrations of RPA amplified DNA; (c) extrapolated calibration curve and LOD of the assay.

The combination of the tailed primers with isothermal recombinase amplification is a very elegant solution for rapid, cost-effective and highly sensitive nucleic acid lateral flow assays. The entire assay, including amplification and detection, was completed in just 15 minutes, which is a considerable improvement on the majority of the reported combinations of amplification and detection, which typically require at least 45 minutes (Supporting Information Table SI-5.1). This is attributable both to the efficiency of the RPA, as well as the rapid hybridisation kinetics of the tailed amplicon with the surface immobilised probe and the reporter probe. DNA hybridisation kinetics is based on a first step of collisional kinetics, where the DNA target randomly collides with the DNA probe and a discrete state where complementarity between some bases are found – this complementarity may rapidly dissociate and the collisional kinetics

proceed until better complementarity is found between target and probe, followed by a second step of a rapid DNA zippering process. The single stranded tails render enhanced kinetics as there is less requirement for collisional kinetics as there is a lower number of bases available for hybridisation. In practise, it is visually observed that hybridisation is instantaneous with the red colour forming at the test line in just 30 seconds following sample addition – and the further time is simply to allow all non-incorporated reporter probes to wick to the absorbent pad. Furthermore, the use of the tailed primers facilitates the use of oligonucleotide capture and reporter probes, avoiding the use of hapten labelling and antibodies, having a significant impact on cost, and can also be expected to have a marked impact on storage stability time as well as storage requirements. The costs/strip, on a laboratory research scale for the RPA-NALF reported here is 1,15€, compared to 5,22€ for hapten-antibody NALFIA, 4,14€ for hapten-protein (i.e. streptavidin), or 4,37€ per strip for the Milenia Hybridetect kit (Table 5.3). Imaging of the developed colour was based on an application based on Image J software termed IJ_mobile to calculate the intensity of the bands. Future work will focus on directly functionalising the nitrocellulose membrane with the DNA capture probe, as well as integrating amplification and detection on a single lateral flow assay. Real-time and accelerated storage stability studies will also be carried out.

Table 5.3: Comparison of lateral flow assay costs

Strategy	Test line	Reporter line	Conjugate	Price
Tailed primers	Biotin-DNA	Biotin-DNA	AuNPs-Thiol-DNA	1,15 €/strip (assay)
Labelled primers & antibodies	Anti-biotin antibody	Anti-rabbit IgG antibody	AuNPs-anti-FITC antibody	5,22 €/strip (assay)
Labelled primers & antibodies	Streptavidin	Anti-rabbit IgG antibody	AuNPs-anti-FITC antibody	4,14 €/strip (assay)
Milenia Hybridetect kit	Biotin-ligand	Polyclonal anti-rabbit antibody	AuNPs-anti-FITC antibody	3,12 €/ strip (assay) + 1,25€ (FAM-probe + Biotin-primer)

5.5 CONCLUSIONS

We have reported on the first example of a recombinase polymerase amplification-nucleic acid lateral flow, exploiting tailed primers which result in duplex amplicons flanked by single stranded DNA tails. These DNA tails facilitate extremely rapid hybridisation with AuNP labelled reporter probe, as well as immobilised capture probe, and contribute not only to a decreased assay time, but also to a markedly reduced assay cost. The combination of RPA, tailed primers and nucleic acid lateral flow truly address the requirement for ASSURED diagnostics at the point-of-need.

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5.8 SUPPORTING INFORMATION

Table SI-6.1: detailing combined isothermal amplification and lateral flow detection

Target	Method	Capture probe	Reporter probe	LOD	Time	Reference
E. coli O157:H7	Aptamer mediated strand displacement amplification coupled to lateral flow	Test line:DNA Control line:DNA	AuNPs-DNA	10 CFU		*W. Wu et al. Analytica Chimica Acta (2015), 861: 62-68*
cDNA permease phosphate *Emiliania huxleyi* (668 nt)	Asymmetric PCR coupled to lateral flow	Test line: Avidin-Biotin-DNA Control line: Avidin-Biotin-Control DNA	DNA-phosphor particles (UTP)	160 pg		*P.L. Corstjens et al. Analytical Biochemistry 312 (2003): 191-200*
Genes encoding virulence factors of Shiga toxin-producing *E. coli*	Carbon nanoparticles with PCR tagged primers coupled to lateral flow	Test line: Specific antibody anti-tag Control line: Biotinylated antibody	Carbon nanoparticles-neutravidin	0.1 – 0.9 ng/µl DNA		*P. Noguera et al. Analytical and Bioanalytical Chemistry (2011) 399: 831-838*
Synthetic DNA	Carbon nanotube coupled lateral flow	Test line: SA-biotinDNA Control line: SA-biotin-control DNA	Carbon nanotube-amine DNA	0.1 nM (visual) 40 pM (software)		*W. Qiu et al. Biosensors and Bioelectronics 64 (2015): 367-372*
Trypanosoma mRNA	Chemiluminiscence lateral flow	Test line: SA-biotin-DNA (dA) Control line: SA-biotin-DNA (dT)	HRP-SA-biotin-DNA	Sub-fM		*Y. Wang et al. Biosensors (2012), 2: 32-42*
P16 gene (methylated DNA)	Competitive quantitative PCR coupled to lateral flow	Test line: Antibodies anti-rhodamine, digoxin, FITC, Hex Control line: Goat anti-mouse antibody	AuNPs-anti-biotin antibody	Qualitative		*W. Xu et al. Biosensors and Bioelectronics 80 (2016) 654-660*

c-DNA	Conformational change of hairpin oligonucleotide coupled to lateral flow	Test line:SA Control line: SA-Biotin-control DNA	AuNPs-Hairpin DNA with biotin	Qualitative		*Y. Huang et al. Biosensor and Bioelectronics 61 (2014): 598-604*
Cauliflowe mosaic virus promotor	Cross-priming amplification technology coupled to lateral flow	Test line: Anti-FITC antibody Control line:Biotin	Latex particles-SA	30 copies		*X. Huang et al. Analytical and Bioanalytical Chemistry (2014), 406: 4243-4249*
Human Immunodeficiency virus (HIV)	Dialysis based concentration method in lateral flow	DNA dissolved in Streptomycin	AuNPs-DNA	0.1 nM	15 min	*R. Tang et al. Talanta 152 (2016): 269-276*
Mycobacterium tuberculosis	EXPAR	Test line: Neutravidin-DNA Control line: Neutravidin-DNA	Microspheres-DNA-neutravidin	Qualitative	70 min	*K. Roskos et al. Plos One (2012), 8 (7):e69355*
Tryposoma mRNA	Fluorescent nanoparticles coupled lateral flow	Test line: SA-biotin-DNA Control line: SA-biotin-DNA	$Ru(bpy)_3^{2+}$ doped silica nanoparticle-DNA	0.066 fmols		*Y. Wang et al. Biomedical Microdevices (2013), 15:751-758*
Human papillomavirus	Fluorescent probe based LATE-PCR coupled to lateral flow	Test line:DNA	Fluorophores-DNA	$10\text{-}10^2$ copies /µl		*Y. Xu et al. Analytical Chemistry (2014), 86:5611-5614*
E. coli	FTA card extraction coupled to LAMP and lateral flow	Test line:SA Control line: SA-biotin-DNA	AuNPs-DNA	10 CFU/ml	1 h	*J.R. Choi et al. Lab on chip (2016), 16:611-621*
Streptococcus equi	IsoAmp SE (thermophilic helicase-dependent reaction) coupled lateral flow	Test line: Anti-FITC antibody Control line:Biotin	AuNPs-SA	50 copies		*S. Artiushin et al. Journal of Veterinary Diagnostic Investigation (2011), 23(5): 909-914*
R166H-mutant gene in Epidermolyte hyperkeratosis (EHK)	Isothermal displacement polymerase chain reaction coupled to lateral flow	Test line: Anti-biotin antibody Control line: Second antibody	AuNPs-anti-dig antibody	0.08 pM		*Y. He et al. Biosensors and Bioelectronics (2012), 31 (1): 310-315*
Salmonella	Isothermal strand displacement amplification	Test line:DNA Control line: Control DNA	AuNPs-DNA	10^1 CFU		*Z. Fang et al. Biosensors and Bioelectronics 56 (2014): 192-197*
Nucleic Acids	Isothermal strand	Test line:	AuNPs-hairpin DNA	0.01 fM		*P. Lie et al. Chemical*

	displacement polymerase chain reaction coupled lateral flow	Anti-tag antibody Control line:SA				*Communications (2013), 48: 236-238*
Staphylococcus aureus	Isothermal strand displacement polymerase chain reaction coupled lateral flow	Test line: pDNA + T20 Control line: T20 Biotin	Polysterene blue bead-SA	10 copies		*B.J. Toley et al. Analyst (2015), 140: 7540-7549*
α-thalassemia	Isothermal strand displacement polymerase reaction coupled to lateral flow	Test line:SA Control line: Goat anti-mouse antibody	AuNPs-anti-digoxin antibody	0.01 fmol/L		*L. Yu et al. The Journal of Molecular Diagnostics (2013) 15 (6): :776-782*
Infectious hypodermal and hematopoietic necrosis virus	LAMP couple to lateral flow	Test line: Antibody anti-biotin Control line: Antibody anti-FITC antibody	AuNPs-Anti-FITC antibody	100 copies	50 min	*N. Arunrut et al. Journal of Virological Methods 171 (2011) 21-25*
T. evansi Type B	LAMP coupled lateral flow	Test line: Antibody anti-biotin Control line: Antibody anti-FITC antibody	AuNPs-Anti-FITC antibody	Qualitative	75 min	*Z.K. Njiru et al. Experimental Parasitology 125 (2010): 196-201*
Shrimp Taura syndrome virus	LAMP coupled lateral flow	Test line: Antibody anti-biotin Control line: Antibody anti-FITC antibody	AuNPs-Anti-FITC antibody	Qualitative	70 min	*W. Kiatpathomchai et al. Journal of Virological Methods 153 (2008): 214-217*
Shrimp hepatoprancreatic parvovirus	LAMP coupled lateral flow	Test line: Antibody anti-biotin Control line: Antibody anti-FITC antibody	AuNPs-Anti-FITC antibody	1 ng	75 min	*T. Nimitphak et al. Jornal of Virological Methods 154 (2008): 56-60*
Mycobacterium tuberculosis	LAMP coupled lateral flow	Test line: Antibody anti-biotin Control line: Antibody anti-FITC antibody	AuNPs-Anti-FITC antibody	5 pg		*T. Kaewphinit et al. BioMedical Research International (2013): 926230*
Synthetic DNA	LAMP coupled lateral flow	Test line:SA Control line: SA-biotin-DNA	AuNPs-DNA	3 x 10^3 copies		*J.R. Choi et al. Analyst. doi:10.1039/c5an02532j*

Canine Parvovirus	LAMP coupled lateral flow	Test line: Biotin-ligand Control line: Anti-rabbit antibody	AuNPs-anti-FITC antibody	10^{-1} $TCID_{50}$/ml		*Y.U-Ling SUN et al. Journal of Veterinary Medical Science (2014) 76 (4):509-516*
Human infective trypanosome	LAMP coupled lateral flow dipstick	Test line: Biotin-ligand Control line: Anti-rabbit antibody	AuNPs-anti-FITC antibody	0.01 tryp./ml		*Z.K Nijiru et al. Diagnostic Microbiology and Infectious Disease 69 (2011): 205-209*
Hendra virus	LAMP coupled to lateral flow	Antibodies	Latex beads-antibody	10^{-5} dilution		*A.J Foord et al. Journal of Virological Methods 181 (2012) 93-96*
African swine fever virus	LAMP coupled to lateral flow	Antibodies	AuNPs-antibodies	10^{-4} dilution		*M.E. James et al. Journal of Virological Methods 164 (2010) 68-74*
White spot syndrome virus	LAMP coupled to lateral flow	Test line: Biotin-ligand Control line: Anti-rabbit antibody	AuNPs-anti-FITC antibody	1 pg total DNA template	50 min	*W. Jaroenram et al. Molecular and Cellular Probes 23 (2009): 65-70*
Cyprinid herpes virus-3	LAMP coupled to lateral flow	Test line: Biotin-ligand Control line: Anti-rabbit antibody	AuNPs-anti-FITC antibody	Qualitative		*H. Soliman et al. Molecular and Cellular Probes 24 (2010) 38-43*
Peneaeus monodon nucleopolyhedo virus	LAMP coupled to lateral flow	Test line: Biotin-ligand Control line: Anti-rabbit antibody	AuNPs-anti-FITC antibody	Qualitative	75 min	*T. Nimitphak et al. Molecular and Cellular Probes 24 (2010): 1-5*
Vibrio vulnificus	LAMP coupled to lateral flow	Test line: Biotin-ligand Control line: Anti-rabbit antibody	AuNPs-anti-FITC antibody	1.5 x 10^3 CFU/ml		*T. surasilp et al. Molecular and Cellular Probes 25 (2011): 158-163*
Plasmodium falciparum / Plasmodium vivax	LAMP coupled to lateral flow	Test line: Biotin-ligand Control line: Anti-rabbit antibody	AuNPs-anti-FITC antibody	1 pg genomic DNA	1.5 h	*S. Yongkiettrakul et al. Parasitology International 63 (2014):777-784*

Candidatus Liberibacter asiaticus	LAMP coupled to lateral flow	Test line: SA Control line: Anti-rabbit antibody	AuNPs- anti-FITC antibody	10 pg	45 min	*L.A. Rigano et al. BMC Microbiology 2014, 14:86*
Capripoxvirus DNA	LAMP coupled to lateral flow	Test line: Biotin-ligand Control line: Anti-rabbit antibody	AuNPs- anti-FITC antibody	Qualitative		*L. Murray et al. BMC Veterinary Research (2013), 9:90*
Infectious spleen and kidney necrosis virus	LAMP coupled to lateral flow	Test line: Biotin-ligand Control line: Anti-rabbit antibody	AuNPs- anti-FITC antibody	Qualitative		*W. C. Ding et al. Archives of Virology (2010) 155: 385-389*
E. ictaluri	LAMP coupled to lateral flow	Test line: Anti-biotin antibody Control line: Second antibody	AuNPs- anti-FITC antibody	8 cells		*Y. Chander et al. Frontiers in Microbiology (2014), 5: 395*
Staphyloccocus pseudintermediu s	LAMP coupled to lateral flow	Test line: Biotin-ligand Control line: Anti-rabbit antibody	AuNPs-anti-FITC antibody	10^1 gene copies		*O. Diribe et al. Journal of Veterinary Diagnostic Investigation (2014), 26 (1): 42-48*
Mushroom species *Amanita phalloides*	LAMP coupled to lateral flow	Test line: Biotin-ligand Control line: Anti-rabbit antibody	AuNPs-anti-FITC antibody	Qualitative	2 h	*F. Vaagt et al. Journal of Agricultural and Food Chemistry (2013), 61(8):1833-40*
Phytophthora ramorum and P. kernoviae	LAMP coupled to lateral flow	Test line: Antibodies anti-dig/ anti-FITC Control line:Biotin	Latex particles-SA	Qualitative		*J. A. Tomlinson et al. Phytopathology (2010), 100 (2):143-149*
Mycobacterium tuberculosis	LAMP coupled to lateral flow	Test line: Anti-dig antibody Control: Biotin-DNA (dA)	Red-dyed carboxylate modified polystyrene microspheres-DNA (dT)-neutravidin	Qualitative	70 min	*K. Roskos et al. Plos One (2012), 8 (7):e69355*
Vibrio parahaemolyticus	LAMP coupled to lateral flow	Test line: Biotin-ligand	AuNPs- anti-FITC antibody	120 CFU/ml		*P. Prompamorn et al. Letters in Applied Microbiology (2011), 52, 344-351.*

		Control line: Anti-rabbit antibody				
Vibrio cholera	LATE-PCR coupled to lateral flow	Test line: Neutravidin-Biotin-DNA Control line: Goat anti-mouse antibody	AuNPs-anti-FITC antibody	1 pg genomic DNA		*G. Yang et al. Journal of Microbiology Methods 118 (2015) 99-105*
Cholera toxin gene	LATE-PCR coupled to lateral flow	Test line: Neutravidin-biotin-DNA Control line: Goat anti-mouse antibody	AuNPs-anti-FITC antibody	0.3 ng synthetic DNA 1 pg pure genomic DNA		*G. Y. Ang et al. Biosensors and Bioelectronics 38 (2012): 151-158*
DNA in plasma	Lateral flow	Test line:SA Control line: SA-Biotin-DNA	Blue dye doped làtex bead-DNA	100 pM	15 min	*X. Mao et al. Talanta 114 (2013) 248-253*
Salmonella	Lateral flow	Test line: Anti-SA antibody + SA	AuNPs- DNA	5 fmol	30 min	*C-C. Liu et al. Food Chemistry 141 (2013): 2526-2532*
Synthetic ssDNA	Lateral flow	Test line: SA-Biotin-DNA Control line: SA-Biotin-Control DNA	HRP-AuNPs-DNA	0.01 pM		*Y. He et al. Biosensors and Bioelectronics 26 (2011): 2018-2024*
Human genomic DNA	Lateral flow	Test line: Biotin-DNA Control line: Biotin-control DNA	HRP-AuNPs-DNA	1.25 fM	15 min	*X. Mao et al. Analytical Chemistry (2009), 81: 1600-1668*
Human immunodeficiency virus type 1 (HIV-1)	Lateral flow	Test line: SA-biotin-DNA Control line: SA-biotin-DNA	AuNPs-oligonucleotides aggregates	0.1 nM		*J. Hue et al. Lab on Chip (2013), 13: 4352-4357*
MicroRNA-215	Lateral flow	Test line: SA-biotin-DNA Control line: SA-biotin-DNA	AuNPs-DNA	60 pM	20 min	*X. Gao et al. Biosensors and Bioelectronics (2014), 54:578-584*

E. coli	Lateral flow	Test line: Anti-0111 antibody Control line: Goat anti-rabbit IgG antibody	AuNPs-antibody	1.8 x 10^3 to 5.6 x 10^5 CFU/ml	22 h	*Y. Terao et al. Journal of Food Protection (2013), 76 (5): 755-761*
E. coli O157:H7	Lateral flow	SA-Biotin-DNA	AuNPs-LNA	0.4 nM		*S. K. Rastogi et al. Chemical Communications (2012), 48: 7714-7716*
Micro-RNA	Lateral flow based on biotin-bridge with silver-enhancement reagent	Test line: Anti-avidin antibody Control line: DNA probe	AuNPs-DNA	Semi-quantitative 5 amol	70 min	*S.Y. Han et al. Talanta 99 (2012): 375-379*
HIV	Lateral flow with enhancement solution	Test line:DNA Control line:DNA	AuNPs-DNA	10.5 $\log_{10}$ copies		*B. A. Rohrman et al. Plos One (2012), 7 (9):e45611*
RNA	Liposomes as signal enhancement in lateral flow	Test line: SA-biotin-DNA	Liposomes-DNA	Qualitative	6 h	*K. A Edwards et al. Analytical and Bioanalytical Chemistry (2006), 386:1335-1343*
B. cereus	Microdevice PCR-UTP coupled lateral flow	Test line: Anti-dig antibody Control line:Biotin	UTP-Avidin	Qualitative		*J. Wang et al. Lab on Chip (2006), 6:46-53*
P. aeruginosa	Multiplex LAMP coupled lateral flow	Test line: Antibodies anti-FITC, hex, dig Control line: Secondary antibody	AuNPs-anti-biotin antibody	20 CFU/ml	50 min	*Y. Chen at al. Biosensors and Bioelectronics 81 (2016): 317-323*
MRSA	Multiplex PCR coupled lateral flow	Test line: DNA Control line: Biotin-label control probe	AuNPs-SA	Qualitative	3 h	*S. Nihonyanagi et al. Inflamation (2012), 35 (3): 927-34*
Tuberculosis	Multiplex PCR coupled lateral flow	Test line: Anti-FITC antibody Anti-Dig antibody Control line: Anti-mouse IgG antibody	AUNPs-anti-biotin antibody	10^4 copies		*H. Kamphee et al. Plos One (2015), 10(9):e0137791.*

Intestinal protozoa	Multiplex RPA coupled to lateral flow	Test line: Anti-tag antibody Control line: Biotin	AuNPs-SA	Giardia: 39 genes E.hystolica: 11 genes Cryptosporidium: 36 genes		*Z. Cranell et al. Analytical Chemistry (2016), 88: 1610-1616*
Classical swine fever virus	Multiplex RT-LAMP couple to lateral flow	Test line: Anti-FITC, anti-dig antibodies Control line: Secondary antibody	AuNPs-anti-biotin antibodies	100 copies		*V. K. Chowdry et al. Journal of Virological Methods 197 (2014) 14-18*
Cassava Brown streak virus (CBSV)	Multiplex RT-LAMP coupled to lateral flow	Antibodies	AuNPs-Antibodies	Qualitative	50 min	*J.A. Tomlinson et al. Journal of Virological Methods 191 (2013) 148-154*
Human papillomavirus Type 16 infection	PCR coupled lateral flow	Test line: Avidin Control line: Goat anti-mouse IgG antibody Index line: Plain phosphor particles	UPT (up-converting phosphor technology)-monoclonal antibody against dig hapten	Low attomole range		*P. Corstjens et al. Clinical Chemistry (2001), 47(10):1885-93.*
C1196T polymorphism toll-like receptor 4 (TLR4)	PCR coupled to lateral flow	Test line: SA-Biotin-(dA) Control line: Biotin-albumin	QD-SA	1.5 fmol	15 min +PCR	*E.A Sapountzi et al. Analytica Chimica Acta 864 (2015): 48-54*
Plasmodium spp.	PCR coupled to lateral flow	Test line: Anti-dig antibody Control line: Biotinylated goat anti-mouse IgG	Carbon nanoparticles-neutravidin	0.3 parasites /µl		*P.F. Mens et al. Diagnostic Microbiology and Infectious Disease 61 (2008): 421-427*
Multiple bacterial contamination	PCR coupled to lateral flow	Test line: Anti-FITC antibody Control line: Anti-avidin antibody	AuNPs-avidin	10^1 copies		*J. Wang et al. Analytical Sciences (2012), 28 (3): 237-41*
Plasmodium falciparum / Plasmodium vivax	PCR coupled to lateral flow	Test line: Anti-tagged antibody	Carbon suspension-SA	0.978 (95%) P. vivax 0.980 (95%) P. falciparum		*P. F. Mens et al. Malaria Journal (2012), 11:279*
Bonana bunchy top virus (BBTV)	PCR coupled to lateral flow	Test line: SA-biotin-DNA	AuNPs-DNA	0.13 aM		*J. Wei et al. ACS Applied Materials and Interfaces*

		Control line: SA-biotin-control DNA				*(2014), 6: 22577-22584*
DNA	PCR coupled to lateral flow	Type 1: Test line: SA Control line: DNA (dT) Type 2: Test line: DNA (dA) Control line: BSA-biotin	Type 1: Carbon-nano strings -DNA (dT) Type 2: Carbon nano strings-anti-biotin antibody	25 fmol		*D.P. Kalogianni et al. Analytical and Bioanalytical Chemistry (2011), 400: 1145-1152*
Shiga toxin	PCR coupled to lateral flow	BSA-DNA	Dyed carboxyl polymer microspheres-DNA	0.1-1 pg		*Y. Terao et al. Journal of Food Protection (2015), 78 (8): 1560-1568*
Avian Influenza virus (AIV)	PCR coupled to lateral flow (signal enhancement with electrical field)	Test line: SA-biotin-DNA Control line: Goat anti-mouse antibody	AuNPs- anti-dig antibody	0.1 ng		*J.C Wu et al. Sensors (2014), 14: 4399-4415*
Plasmodium falciparum	PCR primer extension (PEXT) coupled to lateral flow	Test line: Antibodies anti-PEXT labelled products Control line	Carbon nanoparticles-neutravidin	Qualitative		*A.P. Moers et al. Antimicrobial Agents and Chemotherapy (2015), 59 (1):365-71*
cDNA permease phosphate Emiliania huxleyi (668 nt)	RCA coupled lateral flow	Test line: Avidin-Biotin-DNA Control line: Avidin-Biotin-Control DNA	DNA-phosphor particles (UTP)	0.1 fmol		*P.L. Corstjens et al. Analytical Biochemistry 312 (2003): 191-200*
Plasmodium DNA 18S	RPA coupled lateral flow	Test line: SA Control line: Secondary antibody	AUNPs-anti-FITC antibody	5 copies /µl		*M.S. Cordray et al. Malaria Journal (2015) 14(1):472*
Orf virus	RPA coupled lateral flow	Test line: Biotin ligand Control line: Anti-rabbit antibody	AuNPs- anti-FITC antibody	80 copies	25 min	*Y. Yang et al. Virology Journal (2016), 13(1):46*
Cryptosporidiosis	RPA coupled lateral flow	Test line: SA	AuNPs-anti-FITC antibody	Qualitative	30 min	*Z.A. Crannell et al. Analytical Chemistry*

		Control line: Anti-rabbit antibody				*(2014) 86:2565-2571*
Penoeus stylirostris densovirus and virus-related sequences in shrimp genome	RPA coupled to lateral flow	Test line: Biotin-ligand Control line: Secondary antibody anti-rabbit	AuNPs-anti-FAM antibody	100 copies	35 min	*W. Jaroenram et al. Journal of Virological Methods (2014), 208:144-151*
Schistosoma haematobium	RPA coupled to lateral flow	Test line: Biotin-ligand Control line: Secondary antibody anti-rabbit	AuNPs-anti-FAM antibody	100 fg		*A. Rosser et al. Parasites and Vectors (2015), 8:446*
Rickettsia typhi / Orientia tsutsugamusgi	RPA coupled to lateral flow	Test line: Biotin-ligand Control line: Secondary antibody anti-rabbit	AuNPs-anti-FAM antibody	Qualitative	35 min	*C-C. Chao et al. Plos Neglected Tropical Diseases (2015), 9(7):e0003884*
HIV	RPA coupled to lateral flow	Test line: Anti-biotin antibody Control line: Secondary antibody anti-rabbit	AuNPs-anti-FAM antibody	10 copies		*B. A. Rohrman et al. Lab on Chip (2012), 12 (17):3082-3088*
Plasmodium falciparum	RPA coupled to lateral flow	Test line: Anti-dig antibody Control line: Second antibody Anti-rabbit antibody	AuNPs-anti-carboxifluorescein antibody	100 fg	15 min	*Kersting et al. Malaria Journal (2014), 13:99*
Canine visceral Leishmaniasis	RPA coupled to lateral flow	Test line: Anti-biotin antibody Control line: Second antibody	AuNPs-anti-FAM antibody	40 parasites/ml		*Castellanos-Gonzalez et al. The American Journal of Tropical Medicine and Hygiene (2015), 93(5):970-975*
Entamoeba hystolica	RPA coupled to lateral flow	Test line: Anti-biotin antibody Control line: Second antibody	AuNPs-anti-FAM antibody	2.5 fg		*G. Nair et al. The American Journal of Tropical Medicine and Hygiene (2015), 93(3):591-595*

Shrimp infectious myonecrosis virus	RT-LAMP coupled lateral flow	Test line: Anti-biotin antibody Control line: Second antibody anti-FITC antibody	AuNPs- anti-FITC antibody	Qualitative	75 min	*T. Puthavibool et al. Jounal of Virological Methods 156 (2009): 27-31*
Japanese encephalitis virus	RT-LAMP coupled lateral flow	Test line: Anti-biotin antibody Control line: Second antibody anti-FITC antibody	AuNPs-anti-FITC antibody	50 pg	70 min	*J. Deng et al. Journal of Virological Methods 213 (2015): 98-105*
Myonecrosis virus	RT-LAMP coupled lateral flow	Test line: Anti-biotin antibody Control line: Secondary antibody	AuNPs-anti-FITC antibody	Qualitative		*T.P. Andrade et al. Journal of Fish Diseases (2009), 32:911-924*
Laem-Singh virus	RT-LAMP coupled to lateral flow	Test line: Antibody anti-biotin Control line: Antibody anti-FITC antibody	AuNPs-Anti-FITC antibody	100 copies RNA	1 h	*N. Arunrut et al. Journal of Virological Methods (2011) 71-74*
Infectious bursal disease virus	RT-LAMP coupled to lateral flow	Test line: Antibody anti-biotin Control line: Antibody anti-FITC antibody	AuNPs-Anti-FITC antibody	10^{-1} PFU	70 min	*S.M. Tsai et al. Journal of Virological Methods 181 (2012) 117-124*
Shrimp yellow head virus	RT-LAMP coupled to lateral flow	Test line: Antibody anti-biotin Control line: Antibody anti-FITC antibody	AuNPs-Anti-FITC antibody	0.1 pg RNA	55 min + extraction	*S. Khunthoy et al. Journal of Virological Methods 188 (2013) 51-56*
Macrobrachium rosenbergii nodavirus	RT-LAMP coupled to lateral flow	Test line: Antibody anti-biotin Control line: Antibody anti-FITC antibody	AuNPs-Anti-FITC antibody	2 pg	60 min	*T. Puthavibool et al. Molecular and Cellular Probes 24 (2010):244-249*
Influenza A	RT-LAMP coupled to lateral flow	Test line: Anti-FITC antibody Control line: Biotin	AuNPs-SA	10^{6} copies	45 min	*N.M. Rodriguez et al. Analytical Chemistry (2015), 87: 7872-7879*

Foot and mouth disease virus (FMD)	RT-LAMP coupled to lateral flow	Test line: Anti-FITC antibody Control line: Anti-mouse IgG antibody	AUNPs-anti-biotin antibody	10^1 copies /µl		*R.A. Waters et al. Plos One (2014), 9 (8):e105630*
Nervous necrosis virus	RT-PCR coupled to lateral flow	Test line:SA Control line: DNA (dA)	AuNPs- DNA (dT)	270 pg RNA		*D.K Toubanaki et al. Molecular and Cellular Probes 29 (2015): 158-166*
Cryptosporidium pavum	RT-PCR coupled to lateral flow	Test line: Anti-fluorescein antibody	AuNPs-SA	10 oocysts /0.5 ml		*D. Kozwich et al. Applied and Environmental Microbiology (2000), p. 2711-2717*
Little cherry virus 2	RT-RPA coupled to lateral flow	Test line: Biotin-ligand Control line: Secondary antibody anti-rabbit	AuNPs-anti-FAM antibody	Qualitative		*T.A. Mekuria et al. Journal of Virological Methods (2014), 205:24-30*
Plum pox virus	RT-RPA coupled to lateral flow (AmplifyRP test)	Test line: Anti-FAM antibodies Control line: Secondary antibody	AuNPs-anti-biotin antibodies	1.0 fg RNA	20 min	*S. Zhang et al. Jounal of Virological Methods 207 (2014) 114-120*
Plasmodium falciparum	Semi-nested PCR coupled to lateral flow	Test line: Avidin Control line: Anti-mouse antibody	UCP-mouse anti-Dig antibody	1.35 ng DNA		*S.Y. Ongagna-Yhombi et al. Malaria Journal (2013), 12:74*
Stem cells	Strand displacement amplification coupled to lateral flow	Test line: DNA Control line: Control DNA	AuNPs – DNA	100 hPSCs	80 min	W. Wu et al. Analytica Chimica Acta 881 (2015): 124-130

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Chapter 6

Ultrasensitive β-conglutin detection via recombinase polymerase amplification-lateral flow competitive assay

(Submitted)

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Chapter 6: Ultrasensitive β-conglutin detection via recombinase polymerase amplification-lateral flow competitive assay

6.1 ABSTRACT

In the work reported here, we evaluated different methodologies for the lateral flow based detection of β-conglutin, in search of robust, simple, rapid, ultrasensitive and user-friendly lateral flow assays. In one approach we developed a competitive based lateral flow aptamer assay, where β-conglutin immobilised on the test line of a nitrocellulose membrane and β-conglutin in the test sample compete for binding to AuNP labelled aptamer. The control line exploits immobilised DNA probe complementary to the labelled aptamer, forcing displacement of the aptamer from the β-conglutin-aptamer complex. In a second approach, the competition for aptamer binding takes place off-strip, between the target in the test sample and β-conglutin immobilised on magnetic beads. Following competition, aptamer bound to the immobilised β-conglutin is eluted and used as a template for isothermal recombinase polymerase amplification. The RPA exploits the use of tailed primers, resulting in an amplicon of a duplex flanked by single stranded DNA tails, and the amplicon is rapidly and quantitatively detected using a nucleic acid lateral flow with immobilised capture probe and gold nanoparticle labelled reporter probe. The two approaches are compared in terms of detection limits and assay time required, with the competitive lateral flow being completed in just 5 minutes achieving a detection limit of 55 pM (1.1 fmol), and the combined competitive-amplification lateral flow being completed in 48 minutes, with a detection limit of 9 fM (0.17 amol).

6.2 INTRODUCTION

Food allergies is one of the most important health problems in developed countries, affecting more than 2-3% of people [1, 2], and to date no effective therapies have been developed, with the only means of preventing an adverse allergic reaction

being strict avoidance of the food allergen [3, 4]. However, non-ingredient cross-contaminations with allergenic components, hidden allergens, have resulted in severe allergic reactions [5, 6]. It is thus critical that food is correctly labelled and in order to have confidence in food labels, highly sensitive and specific assays for the detection of food allergens are essential. A variety of methods have been performed for the detection of food allergens including the polymerase chain reaction (PCR) [7], enzyme-linked immunosorbent assay (ELISA) [8] as well as mass spectrometry (MS) [9-11], but these methods are time-consuming and require expensive equipment, significant infrastructure and trained personnel. Lateral flow assays are an attractive alternative for the detection of food allergens, as they are inexpensive, rapid, portable and extremely simple to operate. Lateral flow immunoassays have been developed for a plethora of food allergens, including casein, β-lactoglobulin [12], glycinin [13], crustacean proteins [5, 14], fish proteins [15, 16], nuts [6] and fungal alpha-amylase, used as a flour supplement in the baking industry [17], as has been extensively reviewed [18, 19].

There are two mains types of lateral flow nucleic acid tests, referred to as nucleic acid lateral flow (NALF) and nucleic acid lateral flow immunoassay (NALFIA), where NALF directly detects DNA exploiting capture and labeled reporter oligonucleotide probes, whereas NALFIA detects hapten labeled DNA using capture and labeled reporter antibodies or streptavidin. There are far fewer examples of NALF, which can be attributed to the kinetics of hybridisation in lateral flow being far more complex as compared to the formation of hapten-antibody complexes. Since the first reports of NALF by Corstjens [20], Glynou [21] and Bauemner [22], Liu's team has published a series of papers detailing various formats of NALF, achieving a LOD of 0.5 nM using gold nanoparticles [23] and improving this LOD to 50 pM via the use of horseradish peroxidase adsorbed on gold nanoparticles linked to the reporter probe as a means of signal amplification, in both cases for a synthetic DNA target [24] and recently reported on the use of carbon nanotube labelled reporter probes, and pre-mixed streptavidin-biotinylated probe as the capture probe, achieving an LOD of 40 pM for a synthetic DNA target [25].

Lupin is a leguminous plant consumed extensively in the Mediterranean region due to its' nutritional benefits, being an attractive alternative for gluten-free foodstuffs. It is widely used in a variety of foods, including traditional fermented foods, baked foods and sauces. In addition, lupin can be grown from temperate to cool climates, and is becoming increasingly recognized as an attractive alternative to soy [26-30]. β-conglutin is the predominant conglutin subunit, termed as the anaphylactic Lup an 1, has been demonstrated to be a potent allergen since 2008, when it was added to the list of allergens requiring mandatory labelling list [31]. The allergic reaction to lupin ranges from mild to severe anaphylactic reactions [32-36]. Whilst there are commercially available kits for the detection of lupin, including the lupin-Check ELISA kit by Immutest, AgraQuant ELISA lupin by Romer Labs, Lupin ELISA kit by immunolab GmbH and by ELISA systems, none of them are specific for the detection of β-conglutin and instead detection all globulin subunits.

To meet this caveat in the specific detection of the Lup an 1 anaphylactic allergen, we have developed aptamers specifically against the β-conglutin subunit [37-39]. Aptamers, short single-stranded DNA or RNA ligands that often adopt a three-dimensional shape for interaction with a wide range of target analytes, with the same, or better, affinity and specificity as antibodies, and are being exploited in areas traditionally occupied by antibodies [40-43]. Aptamers offer multiple advantages over antibodies, as summarised in Table 6.1.

Table 6.1: Advantages of aptamers over antibodies

Aptamer	Antibody
Binding any target from ions to whole cells, including non-immunogenic or toxic targets	Common proteins or haptens. Difficult for non-immunogenic or toxic targets
In vitro selection under variety of conditions	Selection limited to physiologic conditions by animal immunization
Efficient production with chemical synthesis with low cost	The production is time-consuming and costly
No variation batch to batch	Variation batch to batch
Long shelf life	Short shelf life
Affinity in low nanomolar to picomolar range	Affinity in low nanomolar to picomolar range
Easily modified with other active groups in scale with a low cost	Difficult and expensive to be modified
Tolerant or able to recover at pH and temperature	Sensitive to or unable to recover at pH and temperature
Good reusability through a reversible conformation switch	Poor reusability due to irreversible conformation changes
Easy orientation immobilization through various modifications	Difficult orientation immobilization

Due to their attractive properties, aptamers are finding increasing application in lateral flow assays. Lateral flow assay platforms typically consist of a sample pad, conjugate pad, test pad and an absorbent pad, with the absorbent pad providing the driving force to draw the sample through the lateral flow via capillary action [44]. Lateral flow aptamer assays take advantage of sandwich, competitive or strand displacement formats. There have been several reports of lateral flow aptamer assays, with targets including thrombin [45, 46], *E. coli* [47], *E. coli O157:H7* [48], Ramos cells [49], IgE, *Staphylococcus aureus* [50], *Salmonella enteritidis* [51], arboviruses [52], adenosine, cocaine [53], and ochratoxin A [54, 55] (Table 6.2).

Table 6.2: Examples of lateral flow aptamer assays

Target	Format	Test line	Control line	Conjugate	LOD	References
ATP	Displacement by fluorescence changes	Aptamer gated silica nanoparticles loaded rhodamine B dye	Aptamer mutated gated silica nanoparticles loaded rhodamine B dye	----------	69 µM	*Özalp V.C., Analyst 2016, doi: 10.1039/c6an00273k*
E. coli O157:H7	Mediated strand displacement amplification	DNA	DNA	AuNPs-DNA	10 CFU /ml	*Wu W., Analytica Chimica Acta 2015, 62-68*
Salmonella	Mediated strand displacement amplification	DNA	DNA	AuNPs-DNA	10^1 CFU /ml	*Fang Z., Bionsensors and Bioelectronics 2014, 56, 192-197*
Chikungunya virus / Tick-bone encephalitis virus (TBEV)	Sandwich	SA-biotin-aptamer	----------	AuNPs-SA-biotin-DNA complementary		*Bruno J.G., BMC Research 2012, 5, 633*
Thrombin	Sandwich	SA-biotin-aptamer	Sa-biotin-DNA complementary primary aptamer	AuNPs-primary aptamer	2.5 nM	*Xu H., Analytica Chemistry 2009, 81, 669-675*
PBP2a protein / IgE	Direct detection	Anti-IgE antibody	Anti-M13 antibody	Aptamer-avidin phage	0.68 pM	*Adhikari M., Analytical Chemistry 2015, 87, 11660-11665*
Thrombin	Aptamer-cleavage reaction	SA	SA	Aptamer-AuNPs-DNA 1 / DNA 2 - AuNPs / DNA 2-AuNPs-HRP	4.9 pM	*Qin C., Analyst 2015, 140, 7710*
Ramos cells	Sandwich	SA-biotin-TE02 aptamer	SA-biotin-Control DNA	AuNPs-aptamer	800 Ramos cells	*Mao X., Analytical Chemistry 2009, 81, 24, 10013*
Ochratoxin A	Competitive	SA-biotin-cDNA	SA-biotin-polyT	AuNPs-aptamer	1 ng /ml; 0.18 ng/ml (with a reader)	*Wang L., Biosensors and Bioelectronics 2011, 26, 6, 3059-3062*
Ochratoxin A	Competitive	SA-biotin-cDNA	Sa-biotin-polyT	QD-aptamer	1.9 ng/ml	*Wang L., Chemical Communications 2011, 47, 5, 1574-1576*
E. coli	Sandwich	Amino end-labeled aptamer	Anti-digoxigenin antibody	AuNPs-aptamer / QD-aptamer	3000-6000 E.coli cells, 300-600 E.coli cells (with a reader)	*Bruno J.G., Pathogens 2014, 3, 2, 341-355*
Aflatoxin B_1	Competitive	SA	Anti-Cy5 antibody	Cy5-DNA and biotin-aptamer	0.1 ng/ml	*Shim W.B., Biosensors and Bioelectronics 2014, 62, 288-294*
HCV core antigen	Competitive	SA-biotin-cDNA	SA-biotin-polyT	AuNPs-aptamer	100 pg/ml; 10pg/ ml (with a reader)	*Wang C.F., Nucleosides, Nucleotides, Nucleic Acids 2013, 32, 2, 59-68*
Ramos cells	Sandwich	SA-biotin-aptamer	SA-biotin-cDNA complementary to aptamer	AuNPs-aptamer	4000 Ramos cells; 800 Ramos cells (with a reader)	*Liu G.D., Analytical Chemistry, 81, 24, 10013-10018*
Thrombin	Sandwich	Antibody	SA-biotin-cDNA complementary to aptamer	AuNPs-aptamer	0.25 nM	*Shen G.Y., Clinical Biochemistry 2013, 46, 16-17, 1734-1738*
Adenosine	----------	SA	----------	Aptamer-AuNPs aggregates	20 µM	*Liu J., Angewandte Chemie 2006, 45, 47, 7955-7959*
Cocaine	----------	SA	----------	Aptamer-AuNPs aggregates	20 µM	*Liu J., Angewandte Chemie 2006, 45, 47, 7955-7959*

The sandwich formats take advantage of platforms developed for NALFs, where biotinylated capture aptamer is immobilised on a streptavidin coated test line, and the target sandwich between this aptamer and a gold nanoparticle (AuNP) labelled reporter aptamer, with the streptavidin coated control line containing a biotinylated DNA probe complementary to the AuNP labelled aptamer, an approach that has been used for the LF detection of thrombin [46] and Ramos cells [49]. An alternative approach has been developed for the detection of *E. coli*, where the capture aptamer is aminated and linked via UV cross-linking to the nitrocellulose membrane, with the reporter aptamer having dual labels of biotin and digoxigenin (dig) – the biotin for linking to streptavidin coated AuNPs or quantum dots, and the dig label for capture on the control line by an anti-dig antibody [47], and for the detection of arboviruses, biotinylated capture aptamers were immobilised via streptavidin, and biotinylated reporter aptamers were linked to streptavidin coated AuNPs [52].

Competitive type lateral flow assays have also been reported for ochratoxin [54, 55], and the HCV core antigen [56]. In these formats, competition occurs between AuNP labelled aptamer with an addended polyA tail, and a DNA probe complementary to the aptamer sequence immobilised on streptavidin coated test lines on the membrane. With increasing concentrations of target in the sample, less aptamer is available for binding to the complementary DNA sequence. The control line consisted of a biotinylated polyT DNA probe which binds to the polyA tail of the aptamer, even if it is bound to target. In the case of the aflatoxin, a slightly modified approach was pursued, with biotinylated aptamer and a DNA probe partially complementary to the aptamer forming a complex in the absence of aflatoxin, which was detected on the streptavidin coated test line. In the presence of target, the complex does not form and the Cy5 labelled DNA probe is captured by anti-Cy5 antibody on the control line [57].

A combination of aptamer detection and subsequent aptamer amplification has been reported for the ultrasensitive detection of targets [58-64]. An alternative detection approach exploited for the detection of *Salmonella enteritis* and *E. Coli O157:H7* on lateral flow aptamer assays taking advantage of a combination of the aptamer's affinity and exploiting its' nucleic acid nature to be used as a template for amplification.

Ultrasensitive detection is achieved via strand displacement amplification (SDA) followed by lateral flow detection. In both cases, two aptamers were used – one for capture of the bacterial target and the other as a reporter aptamer, which was eluted and used as a template for SDA. Following amplification, the single stranded DNA product was detected in a typical NALF format, being sandwiched between a biotinylated capture probe and a AuNP linked reporter probe on the test line, with the control line containing a DNA probe complementary to the AuNP labelled reporter probe.

In the work reported here, we evaluated different methodologies for the lateral flow based detection of β-conglutin, in search of robust, simple, rapid, ultrasensitive and user-friendly lateral flow assays. In one approach we developed a competitive based lateral flow aptamer assay, where β-conglutin immobilised on a nitrocellulose membrane and β-conglutin in the test sample compete for binding to AuNP labelled aptamer. In a second approach, the competition for aptamer binding takes place off-strip, between the target in the test sample and β-conglutin immobilised on magnetic beads. Following competition, aptamer bound to the immobilised β-conglutin is eluted and used as a template for isothermal recombinase polymerase amplification. The RPA exploits the use of tailed primers, resulting in an amplicon of a duplex flanked by single stranded DNA tails, and the amplicon is rapidly and quantitatively detected using a nucleic acid lateral flow with immobilised capture probe and gold nanoparticle labelled reporter probe. The two approaches are compared in terms of detection limits and assay time required.

6.3 MATERIALS AND METHODS

Phosphate buffered saline (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4), 1-ethyl-3-(dimethylaminopropyl) carboiimide (EDC), N-Hydroxysuccinimide (NHS), and all other reagents were purchased from Sigma (Barcelona, Spain). Magnesium chloride, sodium chloride, sodium hydroxide, hydrochloric acid were purchased from Scharlau Chemie S.A. (Barcelona, Spain). SiGMAG-Carboxyl beads were obtained from Chemicell

(Zaragoza, Spain), All solutions were prepared in high purity water obtained from the Mili-Q RG system (Barcelona, Spain). All DNA oligonucleotides were purchased from BIOMERS (Germany) and β-conglutin was extracted and purified as previously detailed [65]. All primer and probe sequences can be found in Table 6.3.

Table 6.3: Sequences used in this work

Name	Sequence
Capture probe test line	5'- gtcgtgactgggaaaactttttttttttttt-Biotin-TEG-3'
Capture probe control line	5'-tgtaaaacgacggccagtttttttttttttt-Biotin-TEG-3'
Reporter probe lateral flow	5'-actggccgtcgttttacatttttttttttttt-C6-thiol-3'
Aptamer (β-CBA II)	5'-agctccagaagataaattacaggggccggggtggctcaggcaaggggttgacctgt cgtagggattgttttaacaactaggatactatgacccc-3'
Forward primer	5'-gttttcccagtcacgac-C3-agctccagaagataaattacagg-3'
Reverse primer	5'-tgtaaaacgacggccagt-C3-ggggtcatagtatcctagttg-3'
Complementary aptamer	5'-Biotin-ggggtcatagtatcctagttgttaaaacaatccctacgacaggtcaaccccttgcctg agccaccccggc ccctgtaatttatcttctggagct-3'
Reporter aptamer (β-CBA II)	5'- thiol-C6-agctccagaagataaattacaggggccggggtggctcaggcaaggggttgac ctgtcgtagggattgttt taacaactaggatactatgacccc-3'

6.3.1 Competitive lateral flow assay

Gold nanoparticles (AuNPs) with an approximate average diameter of 13 nm were prepared by citrate reduction of $HAuCl_4$ as previously reported [66]. 100 µl of thiolated β-CBA II aptamer modified in the 5' end was treated with 1 µl 10mM TCEP and 2 µl 500mM Acetate buffer pH 5.2 for 1 hour under tilt rotation at room temperature (RT), followed by the addition of 1ml AuNPs and incubated for 16 hours in dark conditions under tilt rotation. 10 µl of 500mM Tris acetate buffer pH 8.2 and 100 µl 1M NaCl were then slowly added to the solution (10 µl every 20 minutes) and again incubated for 24 hours in dark conditions and under tilt rotation. The solution was then centrifuged at 16000 rpm for 25 minutes, washed three times with deionized water and re-suspended in 30 µl deionized water.

The test strip was prepared by manually cutting in strips of 4 mm. The membrane used was FF170HP nitrocellulose (Whatman, Germany) and the absorbent pad was glass cellulose (Whatman, England). The test and the control line were created by drawing a line with an eppendorf tip containing the biomolecule to be deposited. In the case of control line, a pre-incubated (1 hour at RT) mixture of biotinylated complementary sequence to the aptamer (60 pmol) 20 pmol streptavidin in PBS buffer was immobilized onto the membrane by containing. For the test line 2 mg/ml of β-conglutin in PBS buffer was dispensed onto the membrane. Following drying of the membrane, the blocking step was carried out with 1% w/v Skimmed Milk powder and 0.1% v/v Empigen detergent for 15 minutes, under shaking conditions. The membrane was left to dry at room temperature for approximately 2 hours and then stored in the fridge until use.

Nine microliters of the aptamer-AuNP conjugate were mixed with 2 µl sample and 9 µl Binding buffer 2x to achieve a final concentration of Binding buffer 1x (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM $MgCl_2$, pH 7.4) for 2 minutes at RT. The sample was then applied to the sample pad and allowed to flow through the test strip.

In order to test the sensitivity and specificity of the assay, a range of concentrations of β-conglutin (0-20 µM) and as well as bovine serum albumin (BSA) and streptavidin (SA) (final concentration in both cases of 2 µM) were analysed.

The control line was based on the use of a DNA complementary to the AuNP labelled aptamer sequence. In the case of low concentrations of target β-conglutin, the majority of the reporter aptamer would bind to the immobilised β-conglutin, but with a small excess that would migrate to the control line. In the case of medium to high concentrations of the β-conglutin, small amounts of the reporter aptamer binds to the immobilised β-conglutin, with the rest being complexed with the target β-conglutin, which migrates beyond the test line. We postulated that the AuNP labelled aptamer would have more affinity for immobilised complementary to the aptamer found on the control line than for it's cognate target, β-conglutin, effectuating a displacement of the labelled aptamer. To test this theory, we carried out a series of control experiments.

Control Displacement Experiment 1: Maleimide microtiter plates were prepared by pipetting 100 µl of 200 nM thiolated sequence complementary to the β-CBA II aptamer prepared in 10 mM phosphate, 138 mM NaCl, 2.7 mM KCl (PBS, pH 7.4) and left to incubate at 4ºC overnight. The plates were subsequently washed with PBS containing 0.05% (v/v) Tween 20 (PBS-Tween) and blocked with 100 µM 6-mercapto-1-hexanol (MCH) via addition of 200 µl per well for 1h at room temperature (RT), prior to washing the plate with PBS-tween. An additional blocking step was carried out using 1% w/v skimmed milk with 0.1% v/v empigen detergent for 30 minutes at RT. During the last blocking step, a range of concentrations of β-conglutin (10 µM, 100 nM, 1 nM, 10 pM and 0 pM), were pre-incubated with a constant amount of biotinylated aptamer (150 nM) and streptavidin-horseradish peroxidase (10 nM SA-HRP) in in binding buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM $MgCl_2$, pH 7.4). This pre-incubated complex was then added to the plate and incubated for 15 min – effectively adding the β-conglutin-aptamer complex to a surface functionalised with the complementary to the aptamer. TMB substrate was subsequently added and following 5 minutes colour development, the reaction was stopped by the addition of 1M H_2SO_4, followed by reading the absorbance at 450 nm. Two replicates were carried out to confirm the results.

Control Displacement Experiment 2: Maleimide microtiter plates were functionalised with thiolated sequence complementary to the β-CBA II aptamer as described above. During the final blocking step, a range of concentrations of β-conglutin immobilized on carboxylated magnetic beads (0 µl, 5 µl, 10µl, 20µl and 40 µl) was pre-incubated with a mixture containing different amounts of biotinylated aptamer (10 nM, 50 nM and 100 nM) and a constant amount of streptavidin-horseradish peroxidase (10 nM SA-HRP) in binding buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM $MgCl_2$, pH 7.4). Three different strategies were evaluated: (1) All the solution containing beads and supernatant was added to wells of the microtiter plate; (2) the beads were placed magnetically isolated and only the supernatant was added to the wells of the microtiter plate; (3) the beads were magnetically isolated, and only the beads re-suspended in binding buffer were added to wells of the microtiter plate. In all the cases

the incubation time on the plate was 20 min, prior to addition of TMB substrate and subsequent addition of 1M H_2SO_4, followed by reading the absorbance at 450 nm. Two replicates were made in order to confirm the results.

6.3.2 Combined competitive assay, recombinase polymerase amplification and lateral flow

In the first step of the assay, a competitive type-assay was carried out. β-conglutin was immobilized on the surface of carboxyl magnetic beads, as previously described [38]. In individual eppendorf tubes a range of concentrations of β-conglutin were pre-incubated with 1 nM of the β-CBAII aptamer for 10 minutes at room temperature. Subsequently, 10 µl of functionalised beads were added to each eppendorf and left for a further 20 minutes incubation. Magnetic actuation was applied and the eppendorfs rigorously washed three times with 500 µl binding buffer followed by the addition of deionized water and a 5-minute sonication to elute the aptamer for its' use as a template in recombinase polymerase amplification (RPA).

RPA was performed in a tube following the indications provided in the TwistAmp Basic kit (TwistDX, Cambridge, England). Briefly, master mix was prepared in a tube containing 480 nM of each tailed primer, template DNA of the desired concentration, 14 mM magnesium acetate and 1x rehydration buffer. The reaction took place at room temperature for 20 minutes.

The protocol for the conjugate of reporter DNA probe with AuNPs was prepared as previously described [67], with minor modifications. Briefly, 100 µl of reporter DNA probe was mixed with 1 ml of AuNPs. The solution was allowed to incubate for 24 hours at 1000 rpm in a thermomixer. 1M NaCl was gradually introduced every 20 minutes until a concentration of 0.7M was reached. The mixture was again incubated for 24 hours under the same conditions. Finally, the conjugate was centrifuged at 15000 rpm for 30 minutes and the pellet was washed three times in deionized water in order to clean the conjugate and remove any free DNA. The conjugate was then re-suspended in deionized water in the desired volume.

The lateral flow strip was prepared as described above. The test and the control line were created by dispensing solutions of 20 pmol Streptavidin pre-incubated (1 h at RT) with 60 pmol of each respective biotinylated probe in PBS buffer. Ten microliters of the reporter probe-AuNP conjugate was mixed with 1 µl sample, RPA product, and 8 µl of 10x SSC, 3,5% Triton X-100, 0,25% SDS, 12,5% Formamide to obtain a final concentration of 4x SSC buffer, 1,4% Triton X-100, 0,1% SDS, 5% Formamide, and incubated for 2 minutes at RT. The sample was then applied to the sample pad and allowed to migrate through the test strip.

In order to test the sensitivity and the specificity, the assay, it was carried out with a range of concentrations of β-conglutin (0 – 20µM) and also with a non-specific DNA sequence (1 µM).

6.4 RESULTS AND DISCUSSION

6.4.1 Competitive lateral flow assay

The competitive lateral flow assay was based on the detection of β-conglutin via competition between the target immobilized on the membrane and the target in the solution. To elucidate optimal parameters of aptamer-AuNPs conjugate to perform the assay, different dilutions were tested in the absence of target and in the presence of a high target concentration (10 µM), finding a dilution of 1 in 2 of the stock AuNP conjugate to be the optimum concentration for the assay (Figure 6.1).

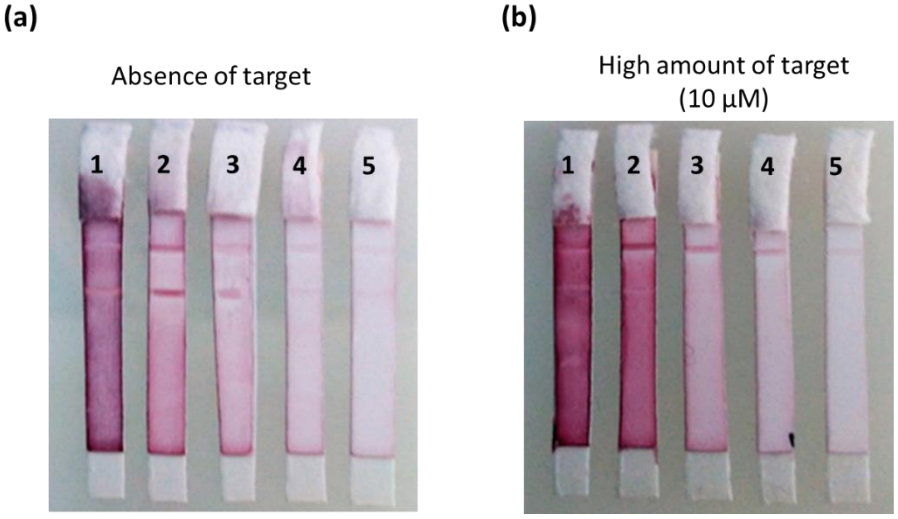

Figure 6.1: Optimisation of aptamer-AuNPs conjugate concentration: (a) absence of target; (b) high amount of target (10 µM); 1, 2, 3, 4 and 5 corresponds to stock concentration, dilution 1 in 2, 1 in 4, 1 in 8 and 1 in 16, respectively.

With increasing concentrations of β-conglutin in solution, less aptamer is free to bind to the immobilized β-conglutin on the membrane (Figure 6.2). As a result, there is a decrease in the intensity of the test line, and at concentrations of greater than 10 µM no test line was visible at all. At all concentrations a red stripe is observed at the control line. This can be explained by the fact that in the absence, or at very low concentrations of β-conglutin, the AuNP-aptamer mainly binds to immobilised β-conglutin and there is a small excess that migrates to the control line. In the presence of β-conglutin, the AuNP labelled aptamer binds both to the target β-conglutin and the immobilised β-conglutin, and at concentrations of 10 µM and greater, all the aptamer-AuNP conjugate binds to target (solution) β-conglutin, but in all cases a red stripe is observed at the control line. This control line was designed using the hypothesis that the aptamer-AuNP has more affinity for its' complementary DNA sequence than for its' cognate aptameric target, and this could be exploited to effectuate displacement of the aptamer-AuNP from the β-conglutin complex to bind to the immobilised complementary sequence.

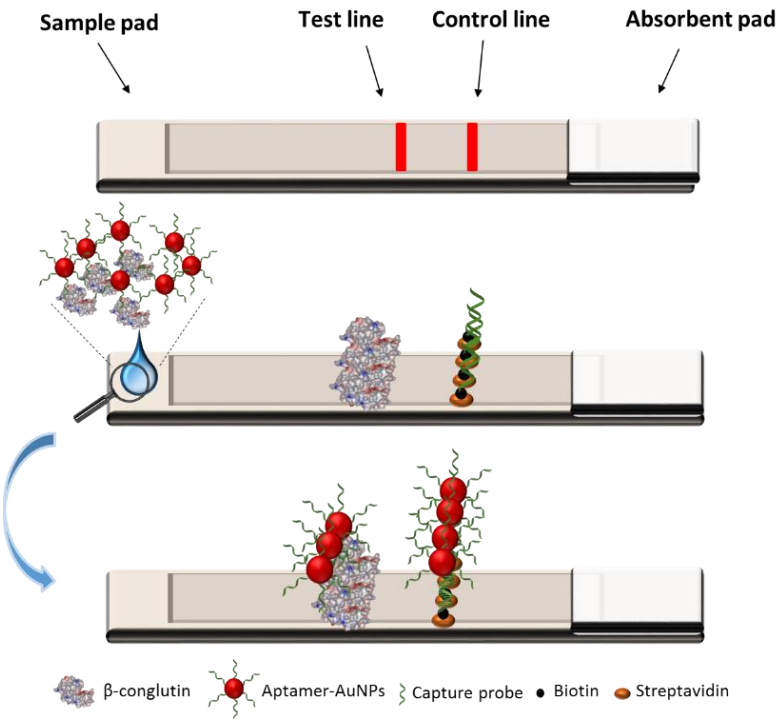

Figure 6.2: Schematic representation of competitive β-conglutin on the strip.

In order to corroborate this hypothesis, two different control experiments were performed. In the first control, in an attempt to mimic lateral flow conditions, but replacing aptamer-AuNP with aptamer-HRP, a thiolated DNA sequence complementary to the β-CBA II aptamer was immobilized in the wells of maleimide plates.

A range of concentrations of β-conglutin was mixed with a pre-incubated mixture of a constant concentration of biotinylated aptamer and SA-HRP. As can be seen in Figure 6.3a, independent of the concentration of β-conglutin, all biotinylated-SA-HRP aptamer (i.e. the labelled aptamer not bound to the β-conglutin AND the labelled aptamer bound to β-conglutin) was observed to bind to the immobilised complementary sequence indicative that any bound labelled aptamer was effectively displaced from β-conglutin. To confirm the observation two additional controls were also carried out. In the first, only biotinylated aptamer-SA-HRP was added to each blocked well to demonstrate that neither the probe nor the HRP is no binding non-specifically, and in the second control, the complementary DNA sequence was not immobilised for each

concentration of β-conglutin, demonstrating that neither β-conglutin or aptamer binds non-specifically (Figure 6.3b). In both cases, no significant signal was observed. Furthermore, simply to ensure that the assay was correctly mimicking the lateral flow assay, a competitive assay was carried out by immobilizing the β-conglutin and performing a pre-incubation of a range of concentrations of β-conglutin with a constant concentration of pre-mixed biotinylated aptamer and SA-HRP, prior to addition of the complex to the plate (Figure 6.3c). As can be seen in Figure 6.3d, a trend indicative of a competitive type assay is clearly observed, demonstrating that the assay is truly performing as the lateral flow assay does.

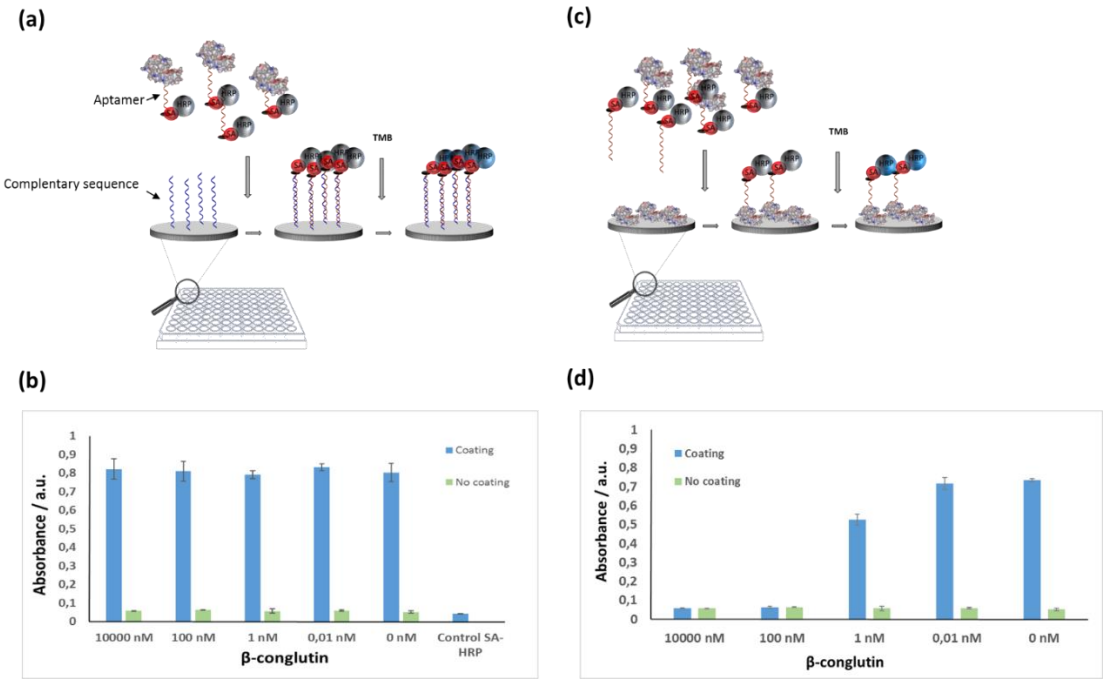

Figure 6.3: Control Displacement Experiment 1: (a) schematic representation of displacement assay; (b) results of displacement assay; (c) schematic representation of competitive ELONA assay; (d) results of competitive assay.

In a second control experiment to demonstrate the displacement hypothesis, thiolated DNA sequence complementary to the β-CBA II aptamer was immobilised on maleimide plates. β-conglutin coupled to carboxylated magnetic beads was pre-incubated with a constant concentration of pre-mixed biotinylated aptamer and SA-HRP. Three different strategies were evaluated to probe the displacement phenomena between the solution-phase complex aptamer-β-conglutin and the immobilised complementary sequence to the aptamer. In the first case, following pre-incubation, all the solution containing free β-conglutin, β-conglutin immobilized on the surface of magnetic beads, biotinylated aptamer - streptavidin-HRP (bound to both to free (target) and immobilised β-conglutin), was added directly to the wells of the microtiter plate (Figure 6.4a). In the second case, the beads (functionalised with β-conglutin and complexed with varying concentrations of biotinylated aptamer-SA-HRP following competition) were magnetically isolated and only the supernatant was added to the

wells of the microtiter plates (Figure 6.4c). Finally, these magnetically isolated beads were re-suspended in binding buffer and added to the well plate (Figure 6.4b). In all the cases, controls with no immobilised complementary DNA sequence was carried out for all the concentrations in order to check for any non-specific binding. Moreover, the direct addition of streptavidin-HRP to a blocked plate was also checked as a control. The results indicated that when all the solution is added directly to the plate, always there is the same absorbance, independent of the concentration of β-conglutin suggesting that all labelled aptamer is binding to its' complementary sequence i.e. labelled aptamer not bound to β-conglutin, as well as labelled aptamer bound to β-conglutin, that is displaced by the interaction between aptamer and its' complementary sequence. This phenomenon was confirmed with the results achieved with the supernatant or the beads, with increasing amounts of immobilised β-conglutin resulting in increasing amounts of labelled aptamer bound to β-conglutin (beads) and decreasing amounts of free labelled aptamer in the solution (supernatant), thus confirming the hypothesis of displacement of aptamer-AuNP for the control line to indicate successful migration through the lateral flow strip. Additionally, these assays were carried out with three different concentrations of aptamer – 100 nM was observed to be too high a concentration of aptamer, with aptamer being available for binding both to target and immobilsed and β-conglutin, and both 10 and 50nM aptamer were in the required limiting amounts, with 50 nM providing high signal and a clearer trend (Figure 6.5).

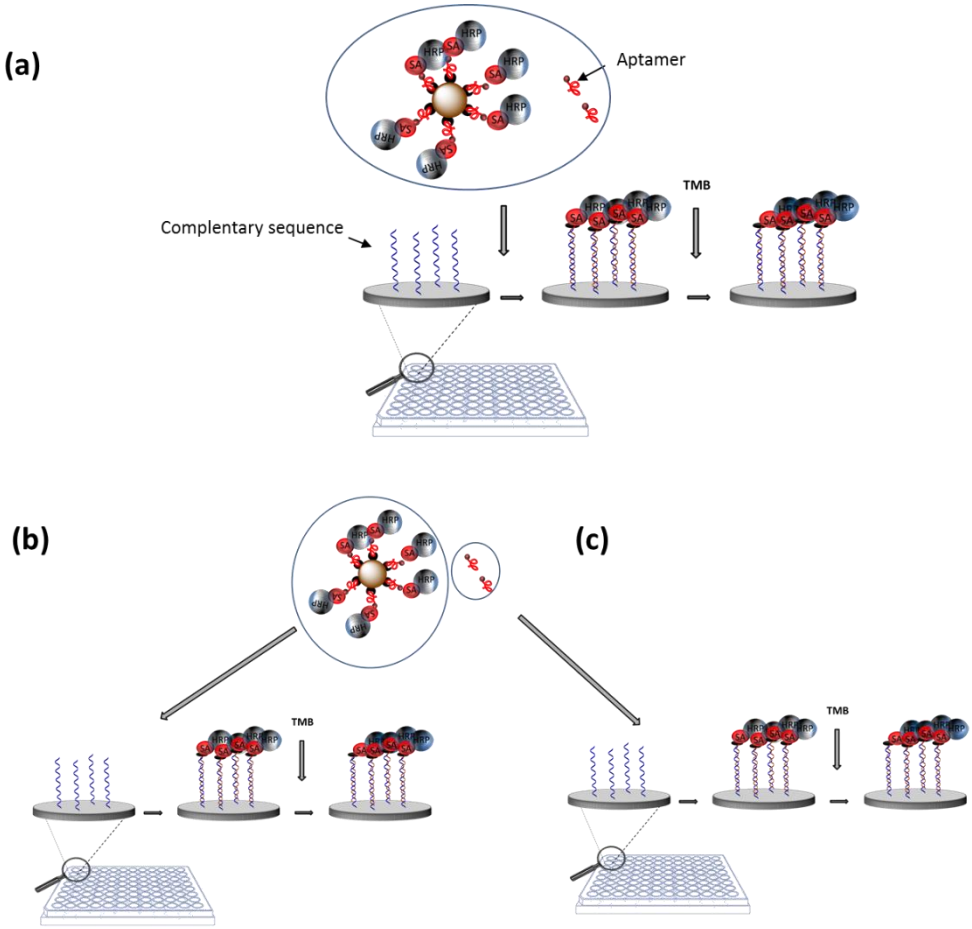

Figure 6.4: Schematic representation of Control Displacement Experiment 2: (a) all solution, containing beads and supernatant is added to the wells of microtiter plate – thus containing all the labelled aptamer; (b) only isolated beads – thus only containing labelled aptamer bound to immobilised β-conglutin – increasing amounts of β-conglutin functionalised beads resulting in increasing concentrations of bound labelled aptamer; (c) only supernatant – thus only containing free labelled aptamer in solution – increasing concentrations of amounts of β-conglutin functionalised beads resulting in decreasing concentrations of free labelled aptamer in solution.

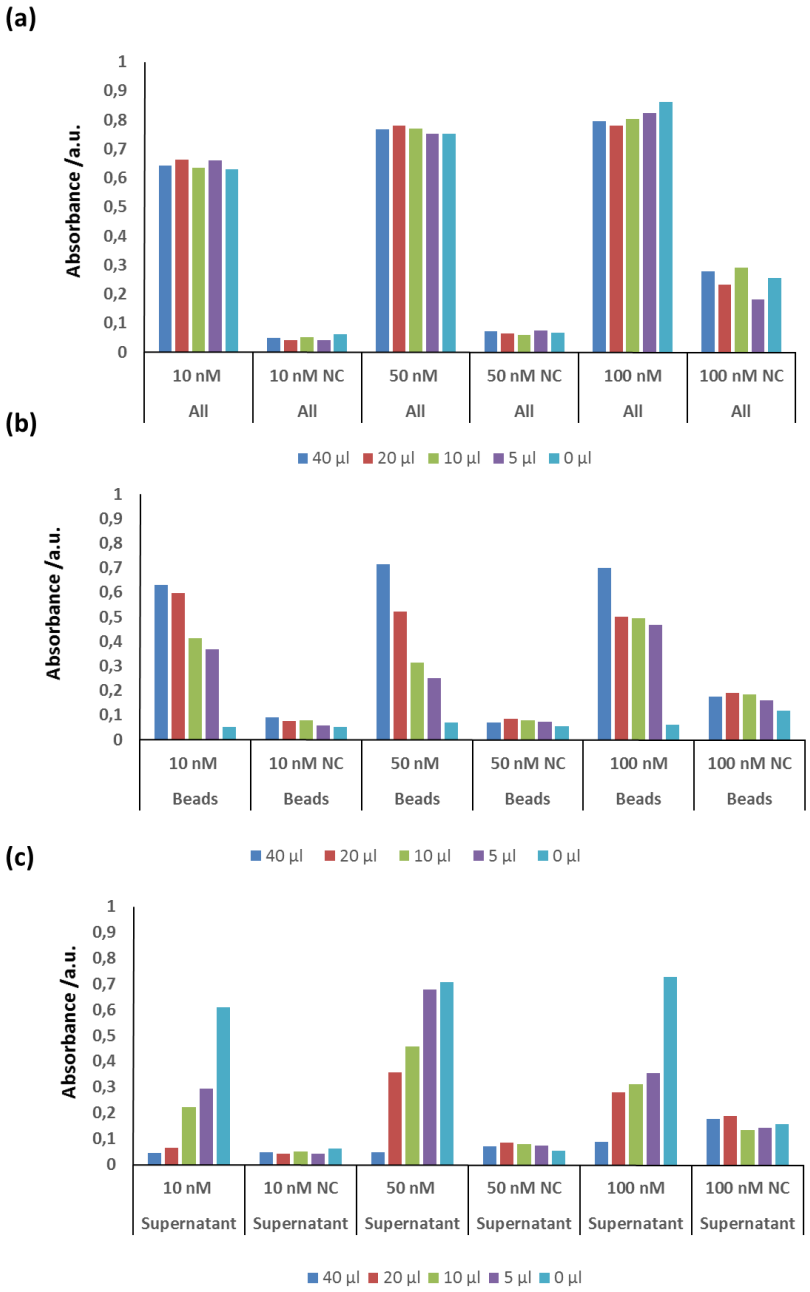

Figure 6.5: Results Control Displacement Experiment 2 (a) all solution, containing beads and supernatant is added to the wells of microtiter plate – thus containing all the labelled aptamer; (b) only isolated beads – thus only containing labelled aptamer bound to immobilised β-conglutin – increasing amounts of β-conglutin functionalised beads resulting in increasing concentrations of bound labelled aptamer; (c) only supernatant – thus only containing free labelled aptamer in solution – increasing concentrations of

amounts of β-conglutin functionalised beads resulting in decreasing concentrations of bound labelled aptamer. NC corresponds to no coating control.

To test the specificity of the assay, two non-specific proteins known to cross-react with a large number of reported aptamers [68, 69], bovine serum albumin (BSA) and streptavidin (SA) were prepared at 2 µM and incubated for 2 min with the aptamer-AuNPs conjugate prior to addition to the strip. Non-specific binding was not observed, demonstrating that the aptamer is highly specific to β-conglutin and that the membrane is correctly blocked (Figure 6.7a).

A Smartphone inbuilt camera was used to take an image of the strip followed by analysis using Image J software to calculate the intensity of the bands, and these values were then were plotted using GraphPad Prism software in order to obtain the limit of detection of the assay (LOD). The values were normalized using the highest value corresponding the control of the blank, 0 nM β-conglutin. The LOD achieved was 5.5 x 10^{-11} M, or 1.1 fmoles β-conglutin (Figure 6.7b). These results were compared with the use of a free mobile application termed IJ_mobile based on Image J software and the same results were obtained (data not shown). The entire assay was complete in just 5 minutes, 2 minutes pre-incubation of sample with AuNP labelled aptamer and 1 minutes migration on the lateral flow, followed by 2 minutes colour development.

6.4.2 Combined competitive assay with recombinase polymerase amplification and lateral flow detection

The competition assay was carried out as described above. Following competition, bound aptamer (94 nucleotides in length) was eluted via sonication and used as a template for amplification by recombinase polymerase amplification (RPA), using tailed primers resulting in amplicons of double stranded DNA with two single stranded DNA tails on each end. The RPA product was mixed with the reporter probe conjugated with gold nanoparticles (AuNPs) and added to the lateral flow strip. In this case the lateral flow strip is based on the immobilization of two biotinylated capture probes (pre-mixed with streptavidin), composing the test line and the control line. In the

test line the probe is complementary to the tail in 5' region of the amplified DNA, meanwhile in the control line, the probe is complementary to the reporter probe conjugated with AuNPs. The reporter probe conjugated with AuNPs is also complementary to the other tail in 3' end from the amplified DNA. Thus, the reporter probe conjugated with gold nanoparticles will bind to the amplified DNA forming a sandwich on the test line and with the capture probe in the control line, generating in both cases a red line visible to the naked eye (Figure 6.6).

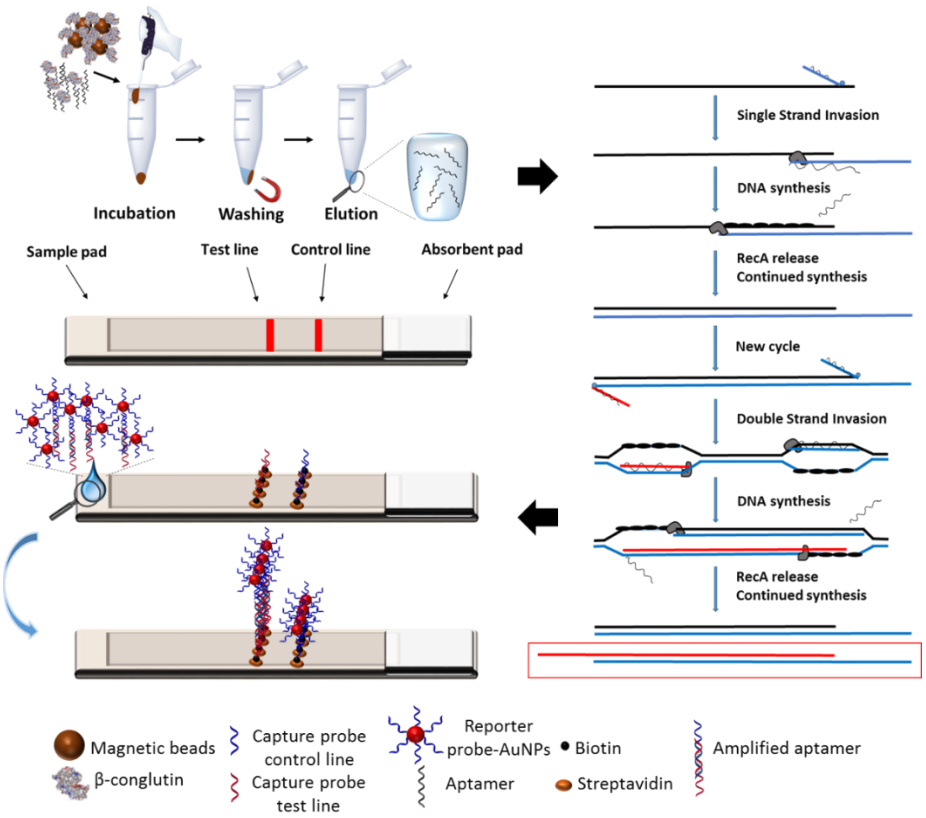

Figure 6.6: Schematic representation of β-conglutin detection via combined competitive assay, recombinase polymerase amplification with tailed primers and lateral flow detection.

A range of concentrations of amplified DNA were tested to evaluate the sensitivity of the assay, and a LOD of 9 x 10^{-15} M achieved. The specificity of the assay was demonstrated by incubating a non-specific DNA sequence (the β-CBA I aptamer) with the conjugate prior to addition to the lateral strip, and only a band in the control line was observed demonstrating the specificity of the assay (Figure 6.7c).

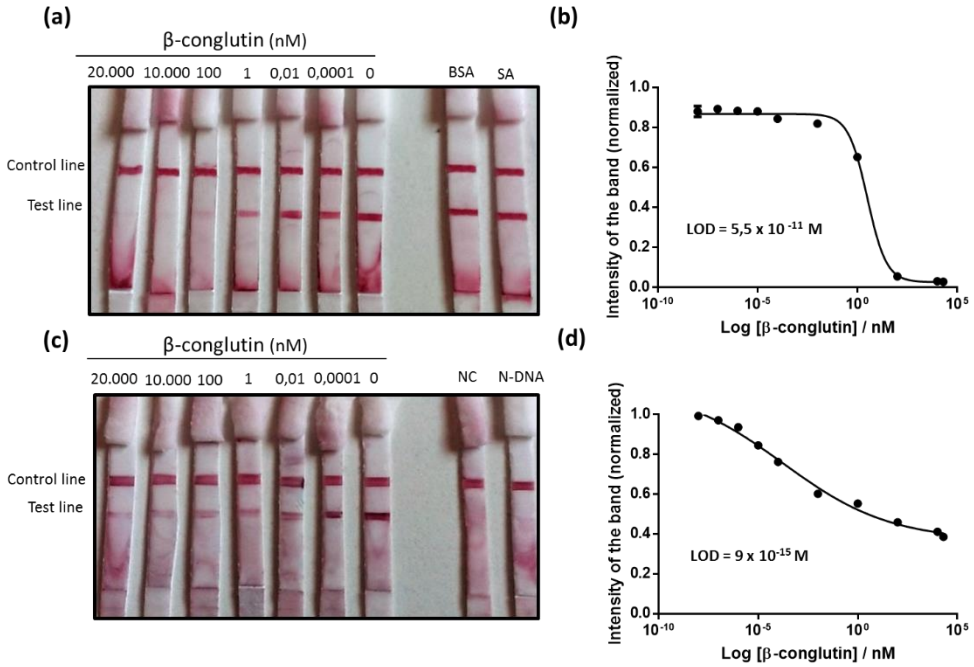

Figure 6. 7: Comparison of both methods: (a) competitive β-conglutin on the lateral flow strip, Control proteins: BSA (bovine serum albumin), SA (streptavidin); (b) calibration curve and LOD of Competitive β-conglutin on the lateral flowstrip; (c) β-conglutin detection using combination of competitive assay, recombinase polymerase amplification with tailed primers and lateral flow detection, NC (negative control), N-DNA (non-specific DNA); (d) calibration curve and LOD of β-conglutin detection using combination of competitive assay, recombinase polymerase amplification with tailed primers and lateral flow detection.

6.5 CONCLUSIONS

We described two different generic methodologies for lateral flow aptamers assays, which can be applied to any aptamer-target, using the detection of the anaphylactic β-conglutin allergen as a model.

Direct competition on the lateral flow strip facilitated an extremely rapid test, with the entire assay being completed in less than 5 minutes, with a 2-minute pre-incubation of aptamer-AuNP and sample, and one-minute migration and two minute colour development. The control line was achieved via binding of unbound AuNP-aptamer to its' complementary sequence (no or low concentrations of target) or via displacement from the β-conglutin, demonstrating a generic methodology for creation of the control line. Previous reports of competitive lateral flow aptamer assays have exploited the use of aptamers modified with a poly A tail, with the control line being functionalised with poly T, which will bind to both complexed and non-complexed aptamer [55], or alternatively, an additional AuNP labelled with another DNA sequence was used, with its' complementary immobilised on the control line, or the use of an antibody to detect a dual labelled aptamer [47]. However, whilst these approaches are functional, the use of additional DNA-AuNP or antibodies adds both to the complexity and cost of the assay, whilst the use of a poly A tail can interfere with aptamer functionality in some cases. Whilst the detection limit achieved using our competitive lateral flow assay, of just 55 pM (1.1 fmol), is impressive, there are cases when better sensitivity is required. To address these requirements, again using the β-conglutin as a model target, the competitive assay was modified to incorporate an amplification step, where competition was executed off-strip using functionalised magnetic beads off-strip, following by aptamer elution and use as a template in isothermal recombinase amplification using tailed primers. Following RPA the amplicons of duplex DNA flanked by single stranded DNA tails were directly wicked onto the lateral flow strip, achieving a LOD of 9 x 10^{-15} M (0.17 amol), with the entire assay only requiring 48 minutes, comprising 30 minutes competition, 15 minutes amplification, 1 minute lateral flow migration and 2 minutes colour development. These generic platforms, combining

aptamer with lateral flow, can be applied to a huge plethora of targets, including other food allergens, pathogens, cancer cells, foetal cells, where ultrasensitive detection is critical. Ongoing work is expanding the demonstrated platforms to multiplex detection.

6.6 ACKNOWLEDGEMENTS

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Chapter 7

General conclusions and future perspectives

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Chapter 7: General conclusions and future perspectives

7.1 GENERAL CONCLUSIONS

The overall objective of this thesis was to select a second aptamer against the anaphylactic allergen β-conglutin for use in various dual aptamer configurations. Fundamental aspects such as the selection of a second aptamer (β-CBA II) against the β-conglutin and the evaluation of its' affinity and specificity were described. The selected β-CBA II aptamer was then exploited in diverse applications to detect β-conglutin using sensitive, rapid and user-friendly platforms, which can be easily adapted as point-of-care tests.

In more detail, the first part of thesis details the selection and characterization of a second aptamer (β-CBA II) against a second aptatope on the β-conglutin target, for use in dual aptamer formats. The rationale for the selection of a second aptamer was that the simultaneous recognition of a target by two aptamers provides higher binding affinity, avidity and specificity compared to the use of an individual aptamer alone. The aptamer was selected using SELEX based on magnetic bead separation and after 7 selection rounds aptamer candidates were sequenced and analysed using Ion Torrent sequencing. Ion Torrent sequencing is a highly flexible platform and in terms of sequence coverage alone, this ultra-high-throughput technology offers a dramatic advance over capillary-based sequencing. From 150000 sequences obtained, following sequence analysis, five aptamer candidates were tested for their affinity and specificity, and sequence 3 (now termed β-CBA II) was observed to have significant affinity for β-conglutin, with a dissociation constant (K_D) of 2.4 x 10^{-8} M determined using surface plasmon resonance (SPR). To corroborate these results two independent laboratories evaluated the binding capacities of β-CBA II aptamer. The affinity constants observed by microscale thermophoresis (MST) and radiolabelling filter binding assay (RFBA) were in the same range (1.2 x 10^{-8} M and 1.81 x 10^{-9} M respectively) and confirmed the results obtained by SPR. Despite a high number of articles reporting new aptamers selected

against diverse molecules, there is relatively low number of "robust aptamers" which work in different laboratories, in the hands of several researchers. Therefore, the evaluation of the affinity of the β-CBA II aptamer was performed in three independent laboratories, in hands of at least 4 researchers and using 4 different techniques (SPR, SPRi, MST, RFBA).

The selected aptamer was then truncated in a variety of manners, with the aim of increasing its' affinity. It is always desirable to truncate the aptamer to eliminate the non-essential nucleotides after the upstream aptamer selection process. However, truncation studies of β-CBA II aptamer did not lead to better results and indeed seemed to suggest that both termini of the aptamer were somehow involved in the binding process.

Following characterization of the β-CBA II aptamer, it was used in combination with the previously selected β-CBA I and the shortened version of β-CBA I (11-mer) in a dual–aptamer approach. The specific and simultaneous binding of the dual aptamer to different aptatopes on β-conglutin was tested using MST and SPR. Competition assays monitored using MST demonstrated that the previously selected and truncated β-CBA I (11-mer) and the newly selected β-CBA II bind to different aptatopes on the protein target. These results were confirmed by SPR where β-CBA II served as the primary capturing aptamer and full length β-CBA I or 11-mer as the secondary signalling aptamer, demonstrating the formation of sandwich aptacomplexes. With the aim of determining the preferred format of the sandwich aptacomplex, real-time label-free surface plasmon resonance imaging (SPRi), a powerful technique that can be used to monitor molecular interactions, was used. The preferred approach for formation of a sandwich aptacomplex was when β-CBA II was used as the immobilised coating aptamer and short 11-mer aptamer as the reporter aptamer, with a pre-incubation of the β-conglutin with the 11-mer prior to addition to the surface-tethered aptamer.

The second part of this thesis details different applications with specific methodologies which can be used to detect β-conglutin allergen. The sensitive and specific detection of allergenic lupin proteins, particularly those subunits that induce a toxic response, is a critical task for producers of raw materials, and food manufacturers

due to the risk of inadvertent contamination and consumer safety concerns. Rapid detection methods with low limits of detections should offer defined advantages to traditionally used methods such as enzyme linked immunoassay assay (ELISA). 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, facilitating ultrasensitive detection in an assay format termed Apta-PCR, was achieved. The Apta PCR assay is composed of a competitive plate assay and the amplification of eluted aptamer which corresponds to the amount of detected target molecule. Apta-PCR for the β-conglutin allergen using β-CBA II achieved a limit of detection of 2 x 10^{-10} M with negligible cross-reactivity to non-specific targets. This LOD is a 170-fold improvement of the LOD obtained by the competitive enzyme linked oligonucleotide assay (ELONA). The main difference between these two assays is the initial concentration of aptamer used (1 nM in Apta-PCR; 150 nM in ELONA) and the last step where instead of adding the probe with horseradish peroxidase the aptamer is eluted and amplified using real time PCR. The main drawback of Apta-PCR is the need of expensive equipment such as thermocyclers. To avoid this, as well as reducing assay time and decreasing costs, recombinase polymerase amplification (RPA), an isothermal amplification, was used to replace PCR. This first report of Apta-RPA, achieved an even lower LOD (8.1 x 10^{-11} M) in comparison to Apta-PCR. Finally, the microtiter plate was substituted by the use of magnetic beads, further improving the simplicity of the assay and demonstrating that this new Beads Apta-RPA method is robust, selective, isothermal and rapid (the whole assay can be completed in 50 minutes) with an improved LOD of 9.9 x 10^{-11}M.

In order to achieve the final objective of this thesis, the development of highly specific, sensitive, rapid, portable, low-cost and user-friendly platforms, lateral flow assays have been explored to be used for the facile and rapid detection of β-conglutin for point of care diagnostics. First of all, the proof of concept of a highly specific and sensitive lateral flow assay based on nucleic acid detection was developed. In this novel strategy, β-CBA II aptamer was used as a template for RPA amplification “off strip” using special tailed primers. As a result, double stranded DNA with single stranded DNA tails on each extreme is performed and solid-phase hybridisation of the amplicon with

complementary probes on the membrane facilitated a very low LOD of 1 x 10^{-11} M. This is the first example of lateral flow detection of DNA, combining isothermal amplication with tailed primers for binding of the amplicon and gold nanoparticle labelled DNA, thus avoiding the use of antibodies or fluorescently labelled probes and presenting an inexpensive and stable assay for point-of-care diagnostics.

Finally, two competitive lateral flow assays for the ultrasensitive and inexpensive detection of β-conglutin were developed and compared. Both assays were demonstrated to be highly specific and each assay had its' own specific advantages. Direct competitive β-conglutin assay on the strip is rapid, only requiring 10 minutes and is very attractive for applications where a rapid point of care diagnostic is required. On the other hand, beads Apta-RPA combined with RPA amplification using tailed primers achieved an impressive LOD of 9 x 10^{-15} M and can be used in applications requiring very low detections limits. In both cases, there is no requirement for any equipment, and when quantitative detection is required Smartphone with specific applications can be used.

To conclude, the main contribution of this thesis to the state-of-the-art is the development of a second aptamer (β-CBA II) against the β-conglutin which binds to a different aptatope of the target. The development of 5 different applications for the selected aptamer and a comparison of the different methods highlighted their limitations and benefits. This thesis will serve to guide future research that would improve the knowledge gained in the field of aptamers and application of aptamers in point of care diagnostics.

7.2 FUTURE PERSPECTIVES

Future perspectives will be addressed to the development of the dual aptamer aptasensor platform based on lateral flow strip assay for the detection of the β-conglutin allergen. In addition, aptasensors will be applied for real sample analysis and cross-reactivity with other food allergens will be tested. Finally, the crystallization of the β-conglutin as well as the β-conglutin with its aptamer will be achieved.

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