REAL-TIME APTAPCR: A NOVEL APPROACH EXPLOITING NUCLEIC ACID APTAMERS FOR ULTRASENSITIVE DETECTION OF ANALYTES FOR CLINICAL DIAGNOSTIC AND IN FOOD ANALYSIS

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Dipòsit Legal: T. 288-2012

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DOCTORAL THESIS

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Tarragona,2012

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I, Ciara K.O'Sullivan,

CERTIFY:

That the present study, entitled "*Real-Time aptaPCR, a novel approach exploiting nucleic acid aptamers for ultrasensitive detection of analytes in clinical diagnostics and in food analysis"*, presented by Alessandro Pinto 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 European Doctorate.

Tarragona,

ACKNOWLEDGEMENTS

"O frati [...]che per cento milia
perigli siete giunti a l'occidente,
a questa tanto piccola vigilia
d'i nostri sensi ch'è del rimanente
non vogliate negar l'esperïenza,
di retro al sol, del mondo sanza gente.
Considerate la vostra semenza:
Fatti non foste a viver come bruti
ma per seguir virtute e canoscenza"

Dante Alighieri – Divina Commedia, Inferno, Canto XXVI

When everything was about to begin, the day before I left for Tarragona, I received an e-mail from my future advisor who wished me *Buen Viaje – Bon Voyage.* Definitely, it has been an extraordinary journey, where I grew up as a man and as a scientist. Now, I do understand that it would not been otherwise, because she showed me the way. Because of this and all the rest, I would like to express my deepest gratitude to her, my advisor: Professor Ciara K. O'Sullivan.

In these years, in anything I have done, in anything I looked for or I found, I was not alone, always surrounded by amazing people and scientists. Absolutely, I owe $10^{13\text{-}15}$ thanks to Dr Cengiz V. Özalp, a mentor for me, who has been looking after me when I landed in Tarragona and later on. Likewise, my infinite gratitude is for Professor Günter Mayer who welcomed me in his fabulous group for a short stay, giving me a priceless opportunity. I was not alone and my most sincere thankfulness are for the awesome members (and former members) of the NBG and the BBG research groups: M.Carmen, Jos, Mary Luz, Diego, Valerio, Pablo, Mayreli, Alex, Tete, Oli, Magda, Carlos, Viji, Hany, Laia, Hossam, Marketa, Tesfaye, Archan, Güray, Thomas S., Thomas H., Noe etc. Besides, I would like to thanks Barbara and Sira from ATIC, and Nuria from the DEQ. And of course I would like to thanks Nuria & Lorena: I owe them much more than I can write in a single thesis…

In these years, I had the chance to met people like *mi hermano* Dr. Frank Jayson Hernandez, Pedro Nadal, *il dottor* Salvatore Cito, Nuria Rovira, *"Edus"* Edoardo Cito, Monsant, *el malaka* Xristos, Jordi Ribè, Ramon, Magda…and I could counted on Paolo, Roberto*, il giaguaro* Gian…I owe to all of them much more than I can express:

friendship is not just a word, and my thesis is also because of you. Besides, a special thanks goes to Francesco, Maria and Marco, because they welcomed me in their family as well as they offer me their unconditioned support.

Above anyone, my gratitude is for my fiancé Serena. She took care of me when I was lost, she kicked me when I was wrong and she gave love me without restriction. She is my present, my future, my everything.

Finally I would like to thank my family. My mom and her strength, my sister Daniela and her love, my brother Antonino and his patience. And of course my father and his courage. I was not there when they had needed me, but they are always there for me when I am in troubles.

This thesis is for them, dedicated to my mother Anna and in loving memory of my father, Giuseppe Pinto. Proud to be their son.

Tarragona, 16/01/2012

Alessandro Pinto

SUMMARY

The thesis aimed to develop and characterize a novel detection approach, which we termed aptaPCR exploiting nucleic acid aptamers as combined recognition and reporter biocomponents for the ultrasensitive detection of analytes.

Nucleic acid aptamers are synthetic ligands selected from vast combinatorial libraries through a process referred to as SELEX – Systematic Evolution of Ligand By Exponential Enrichment. As compared to other natural and synthetic receptor, aptamers possess unique chemical and biochemical characteristics, such as: a well known chemistry, remarkable stability, an ability to be selected against virtually any target even in non-physiological conditions.

Exploiting the unique characteristics of aptamers, we aimed: I) To combine the specificity of a generic aptamer with the sensitivity of nucleic acid amplification for ultrasensitive detection (aptaPCR) II) To apply the developed aptaPCR to the functional detection of proteins, without affecting their physical proprieties using stimuli-responsive aptamer referred to as *caged aptamers* III) To select a DNA aptamer against the food allergen gluten to demonstrate the functionality of the aptamer using the developed aptaPCR method.

Specifically outlining the work achieved in this PhD thesis, the work is organized into individual chapters.

Chapter I: Statement of the objectives and introduction to the state of the art.

Chapter II: A real-time apta-PCR for the ultrasensitive detection of thrombin is reported, where the thrombin aptamer acts not only as a biomolecular recognition element, but also as a reported molecule for amplification via real-time PCR. Aptamers can be easily converted to a reporter agent for detection by real-time PCR, simply via flanking of the aptamer's recognition moiety with primer sequences. Here, we use a sandwich format, where two existing thrombin binding aptamers with distinct binding epitopes were used to capture and detect thrombin in a streptavidin-coated microtiter plate. The amount of thrombin was calculated from real-time PCR analysis of the eluted captured reporter aptamer. The technique can also be used for aptamer–antibody sandwiches, or simply with single aptamers. A greater than 20 000-fold increase in

sensitivity was achieved, highlighting the potential of this approach for the detection of very low levels of target analytes. The use of the aptamer itself as the reporter molecule eliminates the necessity of laborious enzyme/DNA labelling, facilitating a significantly more straightforward assay with a vastly enhanced sensitivity, and is a technique generally applicable to all aptamers.

Chapter III: While many diagnostic assay platforms enable the measurement of analytes with high sensitivity, most of them result in a disruption of the analyte's native structure and, thus, in loss of function. Consequently, the analyte can be used neither for further analytical assessment nor functional analysis. Herein we report the use of "*caged aptamers*" as templates during apta-PCR analysis of targets. "*Caged aptamers*" refer to variants that bear one or more photolabile groups at strategic positions. The activity of caged aptamers can thus be turned on or off by light irradiation. The latter allows the mild elution of target-bound aptamers while the target's native structure and function remain intact. We demonstrate that this approach allows the quantitative analysis functional assessment of analytes. Since caged aptamers can be generated emanating from virtually every available aptamer, the described approach can be generalized and adopted to any target–aptamer pair and, thus, have a broad applicability in proteomics and clinical diagnostics.

Chapter IV: Celiac disease (CD) is an immune mediated enteropathy triggered by ingestion of gluten. So far, the only possible treatment is a lifelong gluten-free diet, which implies the need for detection methods of gluten in food. Herein we describe the selection of the first DNA anti-gliadin aptamer reported to date, and its use for the detection of gliadin in foodstuff. The aptamer selection was achieved by using the Systematic Evolution of Ligand by EXponential enrichment (SELEX) process, which permitted the screening of a vast library of a synthetic single stranded DNA library (10^{13} members) through the repetition of different rounds of amplification and selection. After eight rounds of selection the enriched library was cloned and sequenced, identifying a high conserved sequence. This sequence was characterized for its specificity and was used to set in competitive aptaPCR assay for the detection of gliadin. Here different concentrations of the analytes free in solution compete for the selected aptamer with a standard concentration of the target adsorbed on a microtiter plate. Upon its elution from the plate, the aptamer served as template for subsequent qPCR and quantification.

Chapter V: Overall conclusions.

LIST OF PUBLICATIONS

Research articles:

Frank Jeyson Hernandez; Srujan Kumar Dondapati; Veli Cengiz Özalp; Alessandro Pinto; Ciara K. O'Sullivan; Thomas A. Klar, Ioannis Katakis; *Label free optical sensor for Avidin based on single gold nanoparticles functionalized with aptamers*; Journal of Biophotonics;2:227-231; 2009

Alessandro Pinto, Maria del Carmen Bermudo Redondo, Veli Cengiz Özalp and Ciara K.O'Sullivan; *Real-time apta-PCR for 20 000-fold improvement in detection limit*; Molecular Biosystems, 5: 548-553; 2009

Alessandro Pinto, Sabine Lennarz, Alexander Rodrigues-Correia, Alexander Heckel, Ciara K.O'Sullivan and Günter Mayer; *Functional detection of proteins by caged aptamers*; ACS Chemical Biology; 7: 360-366; 2012

Pedro Nadal, Alessandro Pinto, Marketa Svobodova, Nuria Canela and Ciara K. O'Sullivan; *DNA aptamers against the Lup an 1 food allergen; PLoS ONE (accepted March 2012)*

Book chapters:

Pedro Nadal, Alessandro Pinto, Marketa Svobodova and Ciara K.O'Sullivan; *Aptamers for Analysis: nucleic acids ligands in the post genomic era***;** Molecular Analysis and Genome Discovery, Second Edition; John Wiley & Sons, Ltd, 2011

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LIST OF ABBREVIATIONS USED

Ab	Antibody
AFM	Atomic Force Microscopy
ALP	Alkaline Phosphatase
Au	Gold
AuNP	Gold NanoParticle
ATP	Adenosine-5'-triphosphate
BSA	Bovine Serum Albumin
DPV	Defferential Pulse Voltametry
dsDNA	Double stranded DNA
EIS	Electrochemical Impedance Spetroscopy
ELISA	Enzyme Linked ImmunoSorbent Assay
ELONA	Enzyme Linked OligoNucleotide Assay
FITC	Fluorescein IsoThioCyanate
FMN	Flavin MonoNucleotide
FRET	Fluorescence Resonance Energy Transfer
ISFET	Ion-Selective Field-Effect Transistor
HD1	Thrombin Binding Aptamer (TBA) 15 mer
HD2	Thrombin Binding Aptamer (TBA) 29 mer
HIV	Human Immunodeficiency Virus

HRP	HorseRadish Peroxidase
K_D	Dissociation Constant
LoD	Limit of Detection
Mb	Magnetic beads
OTA	OchraToxin A
PAGE	PolyAcrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived growth factor
PEG	PolyEthyleneGlycol
PQQGDH	Pyrrole Quinoline Quinone Glucose Dehydrogenase
QCM	Quarz Crystal Microbalance
QD	Quantum Dot nanoparticle
qPCR	Quantitative real time PCR
RBP	Retinol Binding Protein
RCA	Rolling Cycle Amplification
RP-HPLC	Reverse Phase - High Performance Liquid Chromatography
RT-PCR	Real time-PCR
RLAA	Reporter Linked Aptamer Assay
SAW	Surface Acoustic Wave
SELEX	Systematic Evolution of Ligands by Exponential enrichment
SPR	Surface Plasmon Resonance

TBA	Thrombin Binding Aptamer
TBA15	Thrombin Binding Aptamer fifteen mer
TBA29	Thrombin Binding Aptamer twenty-nine mer
qPCR	Quantitative PCR
VEGF	Vesicular Endothelial Growth Factor

Chapter I | INTRODUCTION

I.I | A NOVEL ULTRASENSITIVE DETECTION ASSAY

The constant pursuit for a higher analytical sensitivity is reflective of the raising needs of our society. Nowadays, monitoring of health, foods, drinks, pollution and even security at airport relies on the detection of molecular indicators. In analysis, sensitivity is defined by IUPAC as "*the slope of the calibration curve"*[1], i.e. the correlation between analyte concentration and response signal[2].

Immunoassays, first described in the late 1950s, rely on the formation of specific antibody-analyte complexes. The first assays used radioactive label receptors to generate detectable output signal coming from the sandwich type complexes formed with surface immobilized and reporter antibodies "sandwiching" the capture antigen[3]. About ten years later, the undesirable radioactive label was replaced by the introduction of a novel approach named Enzyme Linked ImmunoSorbent Assay –ELISA[4, 5]. Here, the complex formation is monitored thanks to the fluorogenic-or chromogenic- activity of an enzyme conjugated to the reporter antibody. When a single analyte molecule recruits an enzyme -as a part of the antibody-antigen complex-, it will generate a multitude of reporter molecules, which produce a detectable signal measured using fluorescence -or UV/Vis- spectroscopy. The result is an assay that is safe and easy to perform, where the sensitivity is comparable to that attained using radioactivity. Nonetheless, such sensitivity is still far from the ultralow detection levels sought.

The detection of such levels became possible in the middle of the 80s, when K. Mullis first described a breakthrough technique for the amplification of nucleic acids: the polymerase chain reaction (PCR)[6]. In PCR a thermophile enzyme is used facilitating an exponential rather than a linear amplification and thus allowing detection of a few nucleic acid molecules. Exploiting the advantages of PCR in the early 1990s Cantor's group modified the ELISA format to Immuno-PCR[7] approach, where the reporter enzyme is replaced by a DNA tag, which serves as a template for PCR, enhancing the sensitivity of a common immunoassay by up to 100000-fold [8-11].

Whilst Immuno-PCR offers great sensitivity, the technique does suffer from some important drawbacks, such as difficulties in labelling the antibody with nucleic acids and, furthermore, this linkage, either directly to antibodies or via biotin-streptavidin linker bridges is prone to a lack of precision often resulting in uneven numbers of oligos

per antibody, resulting in high rates of error and affecting sensitivity [12, 13]. Additionally, following the immunorecognition step, the DNA needs to be separated from the antibody for subsequent amplification.

Taking advantage of the aforementioned considerations, we wished to explore the opportunity of further improvements in the field of ultrasensitive detection, pursuing an user-friendly, inexpensive but ultrasensitive assay; replacing the cumbersome antibody conjugated to a DNA tag with a DNA aptamer. Indeed, aptamers do not require to be conjugated to a label as they can inherently act both as detecting and reporting molecule, overcoming the problems with immuno-PCR. Once we had demonstrated the proof of concept of "*aptaPCR*", we applied the approach to the functional detection of a food allergen using an aptamer selected against gliadin.

I.II | APTAMERS

I.II.I. IN VITRO EVOLUTION

Aptamers are artificial receptors that bind to their cognate target with high affinity and specificity and are selected through a Darwinian-like evolution method. Aptamers are generally single stranded nucleic acid molecules such as RNA[14, 15], single stranded DNA (ssDNA)[16, 17] or their unnatural analogues[18, 19]; but dsDNA[20, 21] and peptide aptamers[22, 23] have also been described.

First described in 1990, aptamers came from advances in medicinal chemistry and molecular biology achieved over the previous 30 years. In the 1960s along with extraordinary progress in the chemical synthesis of artificial nucleic acids[24] and the understanding of the genetic code[25, 26], there was an increasing awareness of the role of nucleic acids. Molecular biologists understood that oligonucleotide could represent something more than mere storage information (or structural) biopolymer, playing a crucial role in the evolutionary path, from macromolecule to life[27-29]. Indeed, since nucleic acids bear the blueprint for their own synthesis, they can be selected for their inherent attributes through Darwinian evolution, or as Leslie Orgel wrote "*...polynucleotide chains could make primitive selection among organic molecules (...) by forming stereospecific complexes stabilized by hydrogen-bonding"*[29].

Experimentally, in the mid 1960s Spiegelman and co-workers provided the first evidence of the evolution of nucleic acids. The group reported the "*extracellular"* evolution of RNA molecules, as a result of the amplification of bacteriophage genomes through a species-specific RNA replicase[30]. They selected short RNA molecules - characterized by a high amplification rate- starting from genomic RNA through serial transfer experiments - where the product of one replication was used as a template for subsequent replication.

Spiegelman's work probably represented the main breakthrough towards the discovery of aptamers, as Darwinian evolution was finally elevated to a chemical process[31]. Nonetheless, the Spiegelman's *"extracellular"* evolution presented a fundamental bias: the replicase used for the replication was extremely specific, so that the process could not produce other than high affinity ligands for replicase itself. In the

middle of the 1980s, the introduction of the PCR – which allows the replication of all oligonucleotides - overcomes such bias. Updating the Spiegelman approach using PCR, in March 1990 Robertson and Joyce published the *direct* selection of a RNA ribozyme that cleaves ssDNA[32]. Here, after incubation with ssDNA to cleave, the collection of *Thetrahymena* ribozyme's derivatives was amplified through RT-PCR, and the two steps were repeated. Similar to Spiegelman, also in this case selection occurred at the amplification stage where the cleavage, mediated by the RNA, caused the ligation of part of the ssDNA to the RNA itself, creating a new region necessary for the successful annealing of a primer.

In August 1990, the report from Robertson and Joyce was followed by the publication of two more articles, independently reporting examples of *in vitro* evolution of nucleic acids. Remarkably, these new reports described a process further modified and -more interesting- used to screen vast combinatorial RNA libraries for the identification of target-specific RNA ligands.

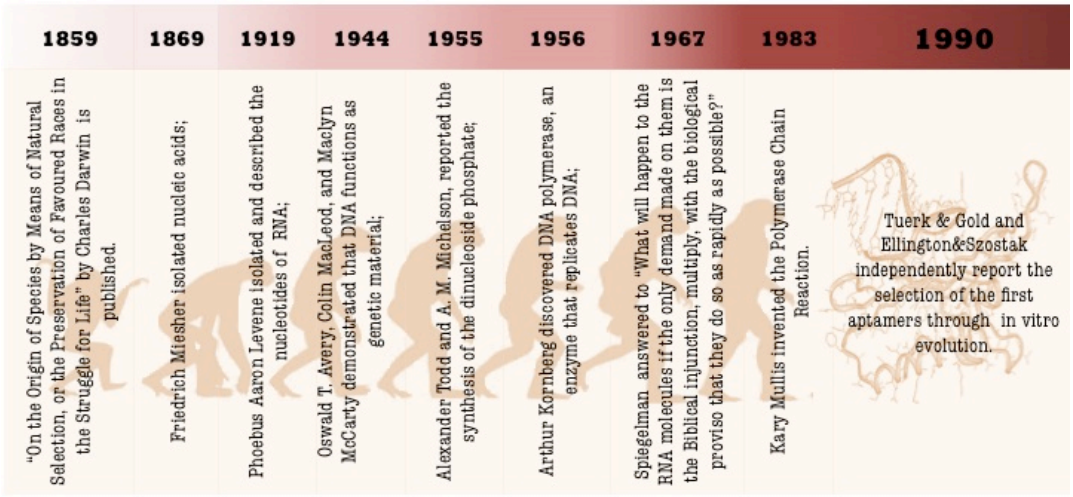

Figure I-1. BREAKTHROUGHS FROM DARWIN'S "ON THE ORIGIN OF SPECIES" PUBLICATION TOWARDS THE IN VITRO SELECTION OF APTAMERS.

Tuerk and Gold from the University of Colorado described the selection of RNA molecules that bind to the bacteriophage T4 DNA polymerase[33]. The two scientists created a library of about 65536 different RNA molecules, based on the natural T4 polymerase target – a hairpin structure- randomizing eight positions of a template contains regions for the amplification, and used it in a novel *in vitro* evolution process, referred as SELEX - Systematic Evolution of Ligands by Exponetial enrichment. Unlike the process used by Joyce and Spiegelman, in SELEX the selection occurs before

amplification, through the physical separation of the bound sequences from the un-bound ones. Not surprisingly, thanks to its simplicity, the SELEX process patented by its creator more than 20 years ago is still the main strategy for the identification of artificial nucleic acid ligands.

Using a similar approach – just about twenty days after the publication of Tuerk and Gold- Ellington and Szostak from the Massachusetts General Hospital reported the selection of RNA molecules that bind to small organic dyes [34]. In this case no natural RNA ligands for the target were known, furthermore the selection started from a combinatorial library of RNA molecules containing a random region of hundred random nucleotides flanked by regions for amplification, representing a pool of more than 10^{13} different molecules. Using six different dyes immobilized in agarose columns as potential targets, the duo isolated individual RNA sequences that specifically bound to some of the organic dyes used. The group coined the term aptamer from the Latin word *aptus* means to fit.

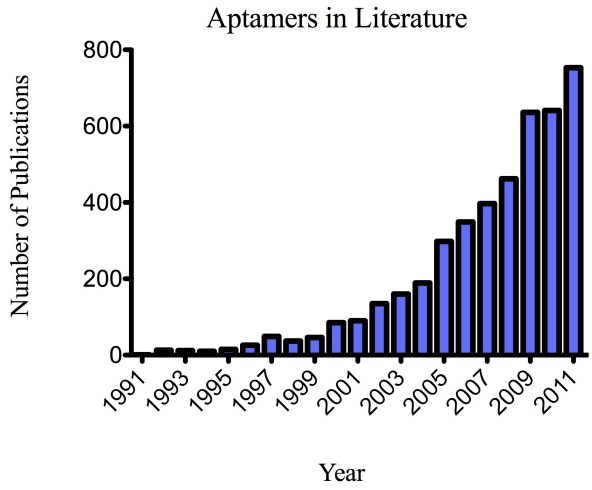

Figure I-2 APTAMERS IN LITERATURE, FROM 1991 TO DECEMBER 2011. Results obtained seeking the word "aptamer" in title, abstract and key words of articles indexed by Scopus.

Since their first appearance, aptamers have garnered constantly increasing attention, reflected in the growing number of publications [35]- Table 1, which encompass the development of diagnostic reagents and therapeutic lead compounds.

Aptamers possess unique chemical and biochemical characteristics, such as a very well known chemistry, remarkable stability and attractive pharmacokinetic and

pharmacodynamics features; moreover aptamers can be selected against virtually any target and in non-physiological condition, as no animal host is required.

I.II.II. OVERVIEW OF THE SELECTION PROCESS

The SELEX procedure developed by Tuerk and Gold is routinely employed in the selection of aptamers. The process starts with the synthesis of a ssDNA library containing a random region flanked at both ends by fixed sequences -necessary for annealing of the primers for amplification. Upon amplification of the pool, RNAs or ssDNAs are generated (conditioning), and the single stranded nucleic acid pool is incubated with the desired target and subsequently the bound nucleic acids are recovered (partitioning). Finally the enriched pool is amplified to start a new round of selection. Typically, a SELEX starts with 10^{13} - 10^{15} different molecules and requires 8-15 rounds to obtain a pool of aptamer candidates with a high binding affinity for the target. The number of rounds depends on different parameters, such as design of the oligonucleotide library, type of target, oligonucleotide to target ratio, selection conditions, and the efficiency of the partitioning methodology.

To improve the possibilities of selecting high affinity aptamers, the stringency during the selection process can be increased by reducing the target concentration or changing the binding and washing conditions [36]. Additionally, to reduce the chances of evolving non-specific binders, those sequences of the library that bind to the support material (e.g. affinity chromatography column, magnetic beads, nitrocellulose filters etc.) can be excluded, performing a pre-incubation without the target before each round, a process termed as negative selection [37]. In a similar manner, to reduce the cross-reactivity of the selected aptamer, a pre-incubation step in the presence of potentially interfering molecules, that are structurally similar to the target, can be carried out in an additional step referred to as counter selection [38, 39].

The evolution of the pool affinity along with the selection process can be monitored, and well-timed changes in the procedure can be introduced accordingly. The technique most commonly used for monitoring of evolution uses radioactively labelled nucleic acids [40-42], but the use of fluorescent labels [43] or dye-linked aptamer [44] have also been

reported as have the use of capillary electrophoresis, affinity chromatography and surface plasma resonance (SPR) [45-48].

When the enriched library shows no more affinity enhancement, the pool is cloned and sequenced. The identification of common sequences as well as homologous regions in the pool and the comparative analysis of the sequences are used to determine the *consensus motif*: the region playing the main role in the specific binding of the target. The selected aptamers are subsequently characterized by means of binding studies to determine both their specificity and affinity. For this purpose, techniques like SPR, enzyme linked oligonucleotide assays (ELONA) and fluorescent or radioactive binding assays are often used.

Despite the great potential, the rate of success of SELEX has been reported to be ~50%[49]. To improve the likelihood of success, during the last twenty years the patented Gold's procedure has been modified: instead of sepharose or agarose columns [50-53], magnetic beads have been used as solid support for the target immobilization or alternatively ultrafiltration[38, 54-56] without target immobilization can be used. As alternatives, the use of flow cytometry [57-59], Surface Plasmon Resonance (SPR)[46], Atomic force microscopy (AFM)[60], electrophoretic separation [61-64] and centrifugation [65, 66] have also been described.

I.II.III. APTAMER FEATURES

From 1990, nucleic acid aptamers binding hundreds of different targets have been reported – from small inorganic molecules to complex targets like target mixtures or whole cells (Table 2). Aptamers – ranging in size from 6 to 40 KDa - bind to their cognate target through the formation of specific complexes stabilized by non-covalent interactions: which are a combination of Van der Waals forces, hydrogen bonds and electrostatic interactions. As reviewed by Patel *et al.,* the binding triggers an adaptive folding in which the target promotes and stabilizes the secondary and tertiary structures of aptamers[67, 68]. The dynamic conformations of free aptamers[69] – consisting of labile knots, loops and stems, become stable architectures in the aptamer-target complexes where non-canonical base-pairing (Hoogsteen pairing to form triplex, Wobble base pairing, g-quadruplex structure etc.) along with the inter molecular interactions create "*(…) unique ligand-binding pockets*"[70].

As a result, aptamers bind to their cognate with high affinity as well as specificity. Remarkably, the affinity achieved by aptamers is similar - sometimes even better- than that achieved by antibodies[71]. In terms of dissociation constant (Kd), the affinity of aptamers range from sub-pM to μM, with low nM Kd being common for protein targets.

The specificity of aptamers is not less appreciable than their affinity. A classic example of this being the anti-theophylline aptamer[72], which is able to discriminate between theophylline and caffeine: two molecules that only differ structurally by a methyl group on the imidazole ring and the affinity of the selected aptamer for theophylline is about 11000 times higher than its affinity for caffeine. Remarkably, aptamers are also capable of enantiomer discrimination as described by Geiger *et al*. who successfully selected an anti- L-arginine aptamer that showed an affinity for the L-arginine 12000 times higher than its affinity for the D- form of the same aminoacid[73].Furthermore aptamers have been selected that distinguish between related macromolecules (e.g. ADP from ATP[74]) or protein family members- such as the Keratinocyte growth factor and other fibroblast growth factors[75].

Asides from their noteworthy affinity and specificity, aptamers are exceptionally stable, and may undergo denaturation, but the process is totally reversible within minutes. Hence, temperature changes or long-term storage do not affect their functionality.

Natural nucleic acids aptamers –RNA or DNA - are generally sensitive to the hydrolytic action of nucleases universally present in biological samples. RNA aptamers are slightly less stable as the hydroxyl group at 2'-position on the ribose promotes phosphodiester bond hydrolysis, particularly at high pH or in the presence of metal ions. To overcome these shortcomings chemical modification can be used. Indeed, compared to other receptors, aptamers can be easily modified by the introduction of new functional groups, which can confer higher stability and nuclease resistance, bring novel physical/chemical proprieties and even improving the overall affinity for the target.

New functional groups can be introduced *a priori* -within the SELEX process- using modified nucleic acid libraries, or *a posteriori* modifying a selected DNA/RNA through chemical synthesis [86]. While the first approach relies on the compatibility of the modification adopted with the enzymatic amplification necessary for selection; the latter

approach is streamlined by detailed knowledge of the structure of both the aptamer and the aptamer-target complex.

Target	Molecular weight (Da)	Class	RNA/DNA	Kd	Ref.
Ethanolamine	61	Small organic compound	DNA	6-19 nM	76
Zinc	65	Metal ion	RNA	100-400 μM	77
Cocaine	303	Drug	DNA	0.4-10 μM	78
Tetracycline	444	Antibiotic	DNA	63-483 nM	79
ATP	507	Coenzyme	RNA	450 nM	80
Sulforhodamine B	558	Organic dye	DNA	190 ± 20 nM	81
Sialyllactose	665	Carbohydrate	Modified DNA	4.9 μM	82
Peptide of REV protein of HIV	2106	Peptide	DNA	19-36 nM	83
Oligo $(dT)_{25}$	7543	Oligonucleotide	DNA	.	84
Keratinocyte growth factor	19000	Protein	Modified RNA	0.3pM	75
Burkitt's lymphoma cell	-	Whole Cell	DNA	49.6±5.5 nM	85

Table I-1 EXAMPLES OF APTAMERS DESCRIBED IN LITERATURE.

To improve the stability of aptamers, modified RNA pools are commonly employed: in particular by using 2′-amino or 2′-fluoropyrimidine derivatives both of which are suitable for enzymatic amplification [75, 87-91]. The use of phosphorothioate modified RNA[92] and DNA[93-95] aptamers have also been described. Aiming to improve both the affinity and the specificity of the aptamers, RNA pools containing 5-iodouridine instead of uridine, and DNA pools containing 5-bromo-2′-deoxyuridine instead of thymine have been used in an updated SELEX protocol named photo-SELEX. As an example of addition of functional groups *a priori,* in an attempt to increase the chemical diversity of the natural nucleic acid, an anti-thrombin modified

DNA aptamer was obtained from a library of modified DNA containing 5-pentinyldeoxyuridine instead of thymidine[96]. Likewise, an ATP-binding aptamer was selected from a cationic-modified DNA pool containing 5-amino-propynyl deoxyuridine[97], and a library of modified DNA containing 5-(2-(6-aminohexylamino)-2-oxoethyldeoxyuridine was successfully employed in the selection of a modified DNA aptamer specific to the thalidomide derivative[98].

Post-selection modifications are extensively used. Indeed, aptamers are commonly conjugated to reporter moieties [99]. Aptamers can be easily linked to biotin, thiols, amines, phosphates, and cholesterol to protect them from exonuclease[100-102] and to allow their immobilization and conjugation to enzymes[103] or nanoparticles[104, 105].

Improving the overall stability of a previously identified HIV-1 reverse transcriptase (RT) aptamer[106] and vascular endothelial growth factor (VEGF) binding aptamer, Green et al. studied the effect of appending methoxyl groups to the 2'-position of the ribose. Locked Nucleic acids (LNA) – which contains a 2'-O,4'-C-methylene bridge - were used to modify the anti- HIV-1 trans-activation responsive (TAR) RNA aptamer [107] and the avidin-binding DNA aptamer[108].

Chimeras formed by the conjugation of the aptamer with other functional macromolecules have been described. For example the VEGF aptamer was linked to distearyl glycerol through phosphoramidite chemistry, for the subsequent anchoring of the VEGF ligands in liposomes[109]. As a result this lipid-modified aptamer exhibited a dramatic improvement in its anti-VEGF activity. Constructing a chimera by conjugating a small tetrapeptide competitor (*N*-methoxysuccinyl–Ala–Ala–Pro–Val cloromethylketone) to the 3′-sulfhydryl modified end of the aptamer, Lin *et al.* were able to improve the inhibitory function of a previously developed human neutrophil elastase binding aptamer[110]. Furthermore, chimeras obtained by the fusion of two different aptamers have been described: either to create bi-functional molecules [111, 112] or to improve the overall affinity for the target[113].

Using analogues of natural nucleotides that bear photolabile groups at strategic positions, known as "*cages*", the thrombin binding aptamer[114, 115] as well as the cytohesin binding aptamer[116] were modified. As a result the activity of such variants - referred to as caged aptamer- can be turned on or off by light irradiation.

To summarize, aptamers can be tailored for many potential applications, in ways unavailable for other receptor molecule (e.g. antibody). Other advantages include the lack of batch-to-batch variation, their small size and compatibility with platform developed for genomics (e.g. DNA microarray) and finally the production cost of aptamers is about the 90% less than that of an antibody[117].

I.II.IV. APTAMERS IN ANALYSIS

Affinity Assay

Since their appearance, aptamers have shown great promise as biocomponents for analysis, not only due to their high affinity and specificity, but also due to their increased stability, flexibility and versatility as compared to antibodies. Techniques involving antibodies or phage displayed antibody fragments are increasingly being replaced by aptamers in different configurations, taking advantage of the unique properties of aptamers.

Ellington was the first to exploit aptamers for the quantification of a specific protein in a cell extract using radio-labelled aptamers in a filter binding assay [118]. By means of previously selected RNA aptamers immobilized on a nitrocellulose filter, βII isozyme of rat protein kinase C was quantified with high reproducibility and specificity in the presence of rat brain extract. While Ellington was demonstrating the concept, at Larry Gold's NeXus Pharmaceutical Inc., Drolet et al. reported the first use of aptamers in an enzyme-linked immuno-sorbent assay (ELISA)-like assay, referred to as ELONA – Enzyme-Linked Oligo-Nucleotide Assay - or more correctly RLAA – Reporter Linker Aptamer Assay. In this first reported RLAA [119] the reporting antibody of a sandwich ELISA was substituted by a fluorescein-tagged RNA aptamer to detect the vesicular endothelial growth factor (VEGF) in serum. A monoclonal antibody specific for VEGF was immobilized and used to capture the target, followed by incubation of a VEGF-binding fluorescein-labelled RNA aptamer with the detection facilitated by an enzyme-labelled anti-fluorescein antibody. This cumbersome assay yielded results very similar to those obtained in typical ELISA, being able to detect concentrations down to 1 pM and without showing any cross-reactivity towards other cytokines. Although this work did not exploit specific properties of aptamers, it did highlight the possibility of

aptamers to compete with, and complete the use of, antibodies in bioanalysis, paving the way for a new approach for detection.

Since its conceptualization in 1971, ELISA [120] has been extensively used in diagnostics as well as in research, due to the robustness, suitability to automation and relative simplicity of the method. Nevertheless, the emerging needs of ever decreasing detection limits, necessitates the search for new assays and methodologies - such as immuno-PCR - that can meet these demands and keep ELISAs at the cutting edge. As such, RLAAs represent a natural development of ELISAs, exploiting the unique flexibility of aptamers to achieve reliable and consistent detection of very low levels of target molecules, but still using a microtitre plate format.

In simple formats, aptamers have been exploited in RLAA in much the same way as antibodies in ELISA [121]. Amongst others, Vivekananda and Keil reported a RLAA format that employs aptamers both as capturing and reporting elements [122]. Aiming to detect an antigen associated with Francisella tularenis japonica - a bacterium that causes the infectious endemic disease tularemia, the group selected different DNA aptamers against the target to be used in the RLAA, where the reporting oligonucleotide was labelled with biotin, recognised in turn by streptavidin-HRP. The results obtained demonstrate that in terms of detection, the RLAA sandwich is superior to ELISA, lowering the Limit of Detection (LoD) by about 3 times - from 6.9 to 1.7 x 10^3 bacteria/mL, whilst also improving the specificity. An exhaustive work regarding the different RLAA sandwich formats was accomplished by Baldrich *et al.*, where different RLAA and mixed antibody/aptamer formats using the Thrombin Binding Aptamer (TBA) were studied, elucidating the critical parameters for optimal aptamer performance, highlighting that the conditions for each aptamer assay must be individually optimized and unlike ELISA, that no universal optimal operating parameters exist [123]. The authors went on to detail the first report of an aptamer-based displacement assay. Based on the observation that the TBA had a lower affinity for a modified form of thrombin than for the native form, microtitre plates were coated with the aptamer to retain horse peroxidise (HRP) modified thrombin. Following the addition of the native thrombin, HRP-thrombin was displaced, achieving a LoD of below 10 nM. Although, displacement assays with antibodies have been a long-time goal, very few reports of successful assays have appeared [124-128], and this first report of an aptamer in a displacement assay not only highlighted the flexibility of aptamers to different assay

formats, but also demonstrated the long-term stability of immobilized aptamers. Demonstrating significant advantages of aptamers over their antibody counterparts, Cruz-Aguado and Penner also demonstrated the displacement assay is possible with other aptamers, obtaining nanomolar detection limits of ochratoxin A (OTA) through a displacement assay by means of anti-OTA aptamer and a complementary fluorescein labelled oligo with fluorescent polarization detection [129]. The group of O' Sullivan (oral communication and manuscript in preparation) has also demonstrated the format with an in house selected avidin aptamer, where SPR has been used to evaluate the Kd of the aptamer for avidin, streptavidin and streptavidin-HRP, observing an one order of magnitude lower Kd for the latter two. The group exploited this in a displacement assay where immobilized avidin aptamer was incubated with the streptavidin-HRP followed by a 15-minute incubation with the target avidin, achieving nanomolar detection limits.

Magnetic beads (Mb) have also been exploited as supports for RLAA formats, using either immobilized or labelled aptamers as capture or detection reagents. Bruno and Kiel used magnetic beads to develop a RLAA sandwich able to detect non-pathogenic Sterne strain Bacillus anthracis spores [130], cholera whole toxin and Staphylococcal enterotoxin B at nanogram to low picogram levels [131]. The approach used relies on a selected pool of DNA aptamer immobilized on tosyl-activated magnetic beads used to capture the analyte in solution. The detection was accomplished by exposing the beads to a second biotinylated aptamer pool followed by addition of either streptavidin-conjugated ruthenium trisbypyridine or avidin-HRP conjugate. Rye and Nustad developed a hybrid immuno-beads assay based on 5'-biotinylated DNA thrombin aptamer and anti-thrombin antibody [132]. In this case, sheep anti-mouse IgG was conjugated to magnetic beads and used to bind the IgG anti-thrombin monoclonal antibody. The modified beads were then exposed to a pre-incubated mixture of thrombin and biotinylated thrombin aptamer. Finally, europium (Eu)-labelled streptavidin was added for detection. The results revealed the TBA could bind the target under both stringent conditions and physiological concentrations, again highlighting the enhanced flexibility of aptamers for analytical applications.

Demonstrating the impressive robustness of their use, aptamers have been tested in depth in many affinity assay formats: besides RLAAs, aptamers have been exploited in flow cytometry, affinity chromatography and capillary electrophoresis, amongst others. In flow cytometry analysis, labelled aptamers were used for detecting (and isolating)

analytes immobilized on beads [99], as well as biomarkers expressed on cell surfaces [133-135]. Fluorescent labelled aptamers have also been used in capillary electrophoresis to detect IgE in buffer and serum samples [136, 137] or the reverse transcriptase of the Human Immunodeficiency Virus type 1 (HIV-1) [138, 139]. Furthermore, the high affinity, stability and the small size of the aptamers have facilitated their use in affinity chromatography. Aptamers immobilized in the stationary phase were demonstrated to obtain a very high selectivity in the retention of targets, as demonstrated by the efficient separation of arginine enantiomers [73], the purification of fusion protein from cell lysate [140] or the separation of adenosine at different phosphorylation levels [141].

Aptasensors

According to the IUPAC definition [142] "*A biosensor is a self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element.*" The analytical power of biosensors relies on the molecular recognition element used. Aptamers offer many advantages as biocomponents, such as their size, ease of modification, facile immobilization compatible with developed microarray technologies, as well as flexible detection formats. Furthermore, aptamers offer unique chemical and physical proprieties for the construction of re-usable biosensors.

Optical aptasensors

One of the first examples of aptasensors was reported by Kleinjung et al. [143] for the detection of L-adenosine through the cognate RNA aptamer immobilized over optical fibres via a biotin-streptavidin bridge, where total internal reflection fluorescence from the fluorescein isothiocyanate (FITC) labelled L-adenosine in competition with the unlabelled L- and D- enantiomers was used. In parallel, Ellington's group designed an aptasensor in which a FITC modified thrombin binding aptamer (TBA) was used as a signalling probe for thrombin detection [144], monitoring the evanescent-wave-induced fluorescence anisotropy of a microscope slide-immobilized aptamer, detecting subnanomolar levels of analyte in a few picoliters in less than five minutes. Using the conformational switch properties of aptamers, Jahaveri et al. exploited the unique properties of aptamers to achieve detection introducing fluorescent labels on the aptamer at positions where the fluorescent moiety enhanced the intensity of the

emission upon target binding [145]. The authors described the successful modification of both RNA and DNA ATP binding aptamers by using a moiety of acridine phosphoramidite, thus positioning the fluorophore outwards following target binding. Using the same principle Katilius et al. developed DNA aptamer detection systems for thrombin, IgE and platelet-derived growth factor using fluorescent nucleotide analogues [146]. The advantage of this last approach is that little or no decrease in binding affinity is expected due to the introduction of the fluorescent moiety, but it is still critical to correctly identify the optimal position to introduce the reporter moiety.

The use of a reporter pair rather than one single molecule has been intensively investigated to detect the conformational changes induced by the target recognition. The first report of this type of strategy was from Tyagi and Kramer who detailed a nucleic acid molecular beacon for the detection of specific nucleic acid sequences [147]. Here single stranded nucleic acid probes form a characteristic hairpin structure with each terminal labelled with a fluorophore and a quencher, respectively, which, in close proximity result in fluorescent quenching. Following hybridization with its complementary sequences, the hairpin structure undergoes a spontaneous conformational change, breaking apart the hairpin structure, and thus generating the signal. Due to the nucleic acid nature of aptamers, they can easily be formatted into a molecular beacon structure, again demonstrating their superior flexibility to alternate assay formats for analytical applications, as compared with antibodies.

Anti-cocaine [78] and anti-PDGF [148] aptamers were engineered to form an aptabeacon involving the 3'- and 5'- ends when bound to the analyte. Using a fluorophore at one extremity and a quencher on the opposite one, the binding produced a measurable 'switch-off' of the signal. The anti-thrombin aptamer (TBA) was used in a 'off-on' mechanism [149], where the original DNA aptamer was appended with a sequence to form a hairpin structure that was destabilized by target binding, which inherently promoted the formation of the particular G-quadruplex structure of the TBA thus distancing the quencher from fluorophore, with a concomitant increase in fluorescence signal. A similar approach was used by Tang et al. who modified the aptamer with a polyethyleneglycol (PEG) spacer followed by a short antisense oligonucleotide modified with a quencher in the 3'-terminus [150], demonstrating the viability of the method with TBA and the anti-ATP aptamer, achieving 90% of signal intensity in just five seconds.

Nutiu and Li have exploited the intermolecular displacement of an oligonucleotide from a complementary sequence. The authors used a fluorophore-labeled DNA aptamer and an antisense small oligonucleotide conjugated to a quenching moiety [151], and was facilitated by the signal generated due to displacement of the antisense oligonucleotide, where an aptamer/antisense-oligonucleotide duplex was used with the antisense sequence being released following recognition of the target, and the fluorophore thus able to generate signal. Similarly, Li and Ho described the detection of ATP using a switch-off mechanism taking advantage of a duplex formed by the anti-ATP aptamer and the antisense oligonucleotide labelled at the extremities with a fluorophore-quenching pair. The signal is quenched when the analyte promotes the displacement of the antisense strand, which is free to form a beacon structure[152]. Avoiding direct conjugation of the aptamer with reporter moieties, a chimera comprised of two active aptamers fused together, has been detailed by Stojanovic and Kolpashchilov. The authors used ATP, theophylline or flavin mononucleotide (FMN) binding aptamers merged with the anti-malachite green aptamer, forming a two domain chimera [153]. When the analyte domain was bound to the target, an allosteric activation occurs allowing the binding of the malachite green to the second domain, promoting the enhancement of the dye fluorescence.

As well as organic dyes, metal complexes [154] and water-soluble cationic polymers have been used as reporters [155]. An easy and universally applicable set up was described by Ho and Leclerc to detect thrombin using polythiophene, a cationic water soluble polymer that binds to the negatively charged backbone of the oligonucleotide [156]. Upon binding to thrombin the TBA undergoes a conformational change, inducing the polymer to wrap the folded G-quadruplex structure rather than form a planar and highly conjugated structure with unfolded ssDNA, producing a colorimetric signal used to detect as low as 2×10^{-15} mol of human thrombin.

Gold nanoparticle (AuNPs) have also been used as reporters, exploiting their unique property to change color upon aggregation [157]. This property has been used by Mirkin et al. to develop oligonucleotide-modified AuNP probes [158], where hybridization with the promoted nanoparticle aggregation inducing a color change. Using a similar principle Liu and Lu designed ATP and cocaine biosensors, by means of two oligonucleotides each conjugated to a AuNP and complementary to two different regions of the anti-ATP aptamer; in the absence of analyte a duplex is formed, crosslinking the nanoparticle and

resulting in aggregation. However, when the aptamer folds up, binding the analyte, the duplex is broken causing disaggregation [159]. In a similar approach, Zhao et al. used an anti-ATP aptamer, which was hybridized to a short oligo attached to a AuNP, where the presence of the duplex prevented aggregation due to the repulsion of the negative charges of the aptamer backbone, with duplex destabilization due to the presence of the target resulting in aggregation [160].

Quantum dot nanoparticles (QDs), nanocrystals offering greater photostability, longer fluorescence lifetime and sharper emission bands than traditional dyes, have also been used as reporters. Levy et al. reported on a Fluorescence Resonance Energy Transfer (FRET)-based aptasensor where a QD nanoparticle was conjugated to TBA [161], which formed a duplex with an antisense oligonucleotide labeled with quencher, dampening the QD signal. Following addition of thrombin the aptamer underwent a conformational change, releasing the complementary antisense and the QD signal was restored.

Surface plasmon resonance (SPR) has also been used to monitor aptamer-target binding, for example for detection of the retinol-binding protein 4 (RBP4) in serum [162], as well as the detection of the 2'-5' oligoadenylate synthase [144] and others [163-167]. In a further report exploiting surface plasmon phenomena, the use of nanoparticle plasmon resonance (NPPR) has also been recently reported, where Hernandez et al. detailed an aptananosensor comprised of a noble metal nanoparticle coated with an anti-avidin aptamer used to detect as low as 20nM avidin. [168]

Mass aptasensors

Piezoelectric transduction via a quartz crystal microbalance (QCM) has been used to detect thrombin and the HIV-1 Tat protein with low detection limits [169]. In addition, the catalytic activity of immobilized aptazymes has been monitored in real time looking at the changes in QCM frequency, where Knudsen et al. adapted the catalytic ligation activity of the anti-HIV-1 Rev peptide aptazyme to detect the HIV-1 Rev peptide as well as the cleavage activity of the theophylline aptazyme for the detection of theophylline [170]. Another example of mass based aptasensing exploits the use of a microcantilever functionalized with aptamers to detect Taq DNA polymerase and the hepatitis C virus [171, 172]. Furthermore, Schlensong et al. immobilized aptamers on a

surface acoustic waves (SAW) device to detect thrombin and HIV-1 Rev peptide [173], using the changes in the propagation of the acoustic waves for transduction.

Electrochemical aptasensors

The first example of an electrochemical aptasensor was reported by Ikebukuro et al., who reported on a sandwich assay to detect thrombin using chronoamperometry [164], where the TBA was immobilized via a thiol terminal on a gold electrode to capture the target analyte. Following exposure to the analyte, the electrode was exposed to a second aptamer against a different epitope of the thrombin and conjugated to pyrrole quinoline quinone glucose dehydrogenase (PQQGDH), which generated an electrochemical signal using glucose substrate and methoxyphenemethosulfonate mediator, achieving a LoD of 10nM. A similar detection limit was obtained by Bang et al. who used a thrombin aptabeacon immobilized over the electrode surface with the redox indicator methylene blue [174], which was released when the stem loop was opened upon binding with thrombin, with a concomitant decrease in electrochemical signal. A similar format was reported by Xiao et al. who conjugated the methylene blue moiety directly to an extremity of the TBA and attaching this electrochemical aptabeacon to an electrode surface, achieving a detection limit of 10 nM LoD and applying the sensor to the analysis of thrombin in real serum samples [175]. In this case, the authors used the longer TBA and in the absence of target the aptabeacon has enough elasticity to contact the electrode surface, but upon binding the quadruplex is induced, forming a rigid structure, with the MB label located far from the electrode surface in a "switch-off" mechanism. In order to achieve a "signal-on" mechanism, Xiao et al. immobilized the TBA aptamer on a gold electrode surface via an oligonucleotide linker, and then hybridized the probe to an oligonucleotide conjugated at one extremity with the methylene blue. Upon the addition of the analyte, the aptamer favoured target binding with the thrombin and dehybridized from its' complementary, rendering flexibility to the probe and facilitating accessibility of the methylene blue moiety to the electrode surface [176].

Using an alternative format, where the folding of the TBA into a G-quadruplex structure upon the binding with the thrombin was exploited, Radi et al reported an electrochemical aptabeacon with a "switch-on" mechanism [177]. The TBA was immobilized on a gold electrode via one terminus and ferrocene labelled at the other terminus, which, in the absence of the target, was located too far from the electrode surface to facilitate electron transfer. However, the conformational change of the

aptamer from a random coil to a G-quadruplex upon target binding brought the ferrocene label in close proximity to the gold surface, facilitating electron transfer and signal generation with a sub nanomolar detection limit. Further reports of electrochemical aptabeacons also exploited the conformational changes undergone by the aptamer upon binding, for example the use of methylene blue to monitor the formation of the thrombin-TBA complex via chronoamperometry and DPV with a LoD of 10 nM [178] as well as the cocaine-cognate aptamer complex via DPV with a LoD of 500μM [179].

In another report, an ion-selective field-effect transistor (ISFET) was used to detect adenosine in a particular displacement method where no label was required [180]. An anti-adenosine aptamer was immobilized on an electrode surface and hybridized to a shorter complementary oligonucleotide, and when adenosine interacted with the aptamer, it displaced the shorter oligonucleotide, upon which a change of charge was detected achieving a detection limit of a few μM. This detection limit has been improved to a few hundred nM by Shen et al. [181] who implemented the system described using $[Ru(NH_3)_6]^{3+}$, which bound electrostatically to the DNA and when one strand is displaced the signal decreases. Similar results have been obtained by Li et al., [182], who modified the original set up, by immobilizing a probe complementary to the aptamer on the surface and upon addition of the target, the aptamer is liberated from the surface.

For labeless detection, impedance is one of the most commonly reported transduction techniques for electrochemical aptasensors, as detection can be achieved simply upon target binding, which changes the impedance. The transduction principle is generally based on monitoring the electron-transfer of the electrochemical redox marker $[Fe(CN)_6]^{4-/3-}$, whose access to the surface is impeded or enhanced, depending on the specific experimental set-up. Furthermore, as the aptamer is negatively charged and is also relatively small as compared to an antibody, one can expect much better sensitivity as compared to immunosensors exploiting impedance transduction. Xu and colleagues [183] first reported the detection of human IgE using Electrochemical Impedance Spectroscopy (EIS) with the cognate aptamer as recognition element, immobilizing the aptamer on a gold film electrode via self-assembly, achieving a detection limit of 0.1 nM. With an analogous set up, Pan et al. [184] described the detection of a few thousand cells/mL of the T-leukemia cell line CCRF-CEM, whilst Radi at al. achieved a detection limit of 2 nM thrombin exploiting the 15 mer TBA [185]. Interestingly, using the same

approach but replacing the gold electrode with a microfabricated gold thin film, Cai and co-workers further decreased the detection limits of thrombin by about 20 times [186] and Lee et al. achieved a detection limit of as low as 0.5nM thrombin with pyrolyzed carbon film [187].

Similarly, but using the opposite format, Rodriguez et al. immobilized the biotinylated anti-lysozyme aptamer onto a streptavidin-modified indium tin oxide surface, exploiting changes of the surface modified charges upon the binding event through Faradaic impedance spectroscopy, achieving a detection limit of 10 nM of lysozyme [188]. Before the binding event, the negatively charged aptamer on the surface creates an electrostatic barrier to the electron transfer of the negatively charged ferricyanide couple and this barrier is disrupted when the aptamer binds the positively charged cognate target. To enhance the detection limit of a system relying on impedance spectroscopy transduction, Xu and co-workers realized an interesting approach, where following binding with its cognate aptamer immobilized on a gold surface, thrombin was denatured by the addition of the chaotropic agent guanidine hydrochloride, resulting in an increase in impedance, achieving a dramatically reduced detection limit of just 10fM [189].

Electrochemical stripping analysis has also been used in aptasensors. Hansen et al. [190] demonstrated a multi-displacement assay of thrombin and lysozyme labelled with quantum dots. Following deposition of thiolated anti-thrombin and anti-lysozyme aptamers on gold electrode surfaces, quantum dot labelled thrombin and lysozyme were captured by the aptamer and electrochemically stripped at a coated glassy carbon electrode. The sensitivity and selectivity of the system was tested with BSA and IgG as controls, giving a detection limit of 0.5 pM for thrombin in a signal displacement set-up.

Magnetic beads have also been used in combination with electrochemical detection. One example cites the use of an anti-lysozyme aptamer labelled with magnetic beads for the separation, concentration and detection of lysozyme [191], where the captured protein was released from the aptamer for its electrochemical detection by chronopotentiometric stripping, achieving a detection limit of 7 nM.

An indirect square wave voltammetry detection method of aptamer–thrombin interaction was reported by Le Foch et al. [192] using the biochemical properties of the nucleic acid aptamers. This strategy was based on the specific enzymatic hydrolysis of

single stranded DNA molecules by a nuclease, which did not degrade aptamer attached to the target. Thus following enzymatic degradation, thrombin was denatured to release the aptamer, which was then hybridized with a DNA capture probe for detection of the aptamer achieving a detection limit of 75nM.

An alternative approach was based on the direct adsorption of thrombin on a modified gold electrode surface, with subsequent specific interaction with anti-thrombin labelled aptamer [103] detected using chronoamperometry with a detection limit of 3 nM. The same group also reported another thrombin aptasensor, based on the thrombin based catalysis of the chromogenic substrate, β-Ala–Gly–Arg–p-nitroaniline, producing p-nitroaniline, where the rate of p-nitroaniline formation was followed by UV adsorption at 405 nm, or electrochemically by the reduction of its nitro group. Electrochemical detection was carried out by DPV.

Finally carbon nanotubes have been exploited as elements in an electrochemical aptasensor architecture, where a single-walled carbon nanotube field-effect transistor was reported for thrombin sensing [193]. The change of the electrical double layer before and after interaction with the target was detected and the system achieved a detection limit of 10 nM, with a linear range of 0–100 nM. Furthermore, Maehashi et al. [194] produced label-free protein biosensors based on aptamer-modified carbon nanotube field-effect transistors for the real-time detection of IgE, demonstrating a better performance than monoclonal antibodies under the same assay conditions.

In conclusion, a wide range of aptasensor formats has been reported. Exploiting formats not possible with immunosensors, impressive detection limits have been achieved with facile to use formats, which can be easily applied to mass-producible sensors. There has also been a move away from the 'model system' of the thrombin binding aptamer, demonstrating the flexibility and potential widespread application of aptasensors for highly sensitive, specific and rapid detection of targets, potentially at the site of analysis, such as at the point-of-care, for example at a physician's office.

I.III | OBJECTIVES AND STRUCTURE OF THE THESIS

Nucleic acid aptamers are naturally predisposed to an assay format that combines the selectivity of the aptamers with the efficiency of nucleic acid amplification techniques, producing an impressive signal enhancement with considerably lower detection limits. As highlighted before, the concept of integrating the sensitivity of nucleic acid amplification with an immunoassay, in a technique known as Immuno-Polymerase Chain Reaction (Immuno-PCR), precedes the exploitation of aptamers in bioanalysis[7].

In the 2002 Friedriksson *et al* [195] first reported a detection assay exploiting aptamer amplification for detecting zeptomoles of the cytokine platelet-derived growth factor (PDGF). The approach used, referred as "Proximity Ligation Assay" (PLA), does not rely on the immobilization of either the analyte or the probe: two aptamers that bind two closely located binding sites are ligated, via a template probe and a ligase enzyme to form a unique amplicon to be detected via qPCR. Following this, the same group went on to use the technique to detect microbial pathogens [196] and the prostate specific membrane agent (PMSA) [197]. In a similar format, Yang and Ellington developed a set-up exploiting the conformation switching of the TBA [198], achieving detection limits in the picomolar range. Here taking advantage of the secondary structures assumed by the aptamers following target binding, with the conformational change the aptamer undergoes upon binding, promoting ligation within a primer region for subsequent real time amplification. Later on, this concept was used by the same group to detect PDGF in the nanomolar range using rolling cycling amplification, where the conformation-switching aptamer was circularized upon interaction with its target [199]. In an alternative approach, Wang *et al.* [200] described a novel approach where an exonuclease is added to a solution containing thrombin and its cognate aptamers, to digest all the oligonucleotides free in solution whilst not digesting those that form a complex with the analyte. Subsequent amplification of the surviving TBA molecules facilitated a detection limit of just a few hundred molecules of thrombin, demonstrating the power of this technique for highly sensitive analyte detection.

Simplifying the PLA approach, avoiding the use of ligases or nucleases, Zhang *et al.* reported on the use of capillary electrophoresis (CE) for the separation of the aptamer-target complex followed by downstream quantitative PCR, achieving the detection of

just 180 molecules of HIV type 1 reverse transcriptase [201]. Following this, Fisher *et al.* demonstrated detection of thrombin via Rolling Cycle Amplification (RCA) and real time quantitative PCR (qPCR) [202]. By means of different concentrations of thrombin modified magnetic micro-particles to bind the TBA flanked by two primer regions for amplification, the group achieved a 2nM detection limit using the Rolling Cycle Amplification (RCA) and as low as a few hundred fM using qPCR.

In this context, we aimed to integrate detection mediated by aptamer amplification within a sandwich assay format. Exploiting two different thrombin binding aptamers (TBAs), we detailed a sandwich format where qPCR was used. Here the biotinylated TBA for the heparin-binding site was immobilized onto a streptavidin-coated plate, which was used to capture the analyte in solution. Subsequently the TBA-thrombin complex was exposed to the TBA specific for the fibrinogen-binding site flanked by two primer regions, which was finally eluted and detected using qPCR, achieving a femtomolar detection limit, as detailed in chapter 2.

Subsequently we expanded our approach using a caged aptamer as reporter, the affinity of which was totally inactivated upon irradiation. Always using the same thrombin binding aptamers, we introduce chemical modifications in the reporter aptamer, obtaining a novel ligand that loses its activity upon the UV exposure. As a result, by using the light to inactivate the reporter aptamer we developed a detection approach that was not only ultrasensitive but also capable of maintaining unaffected the protein of interest, as detailed in the chapter 3.

Finally, we selected a DNA aptamer against the food allergen gliadin through the SELEX process and we demonstrated its functionality for the detection of gliadin using aptaPCR. A competitive assay was set up, where different concentrations of analytes free in solution competes for the aptamer with a standard concentration of gliadin adsorbed on a microtiter plate. Upon its elution from the plate, the aptamer was used as template for a qPCR as detailed in chapter 4.

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Chapter II | REAL-TIME APTAPCR FOR 20 000-FOLD IMPROVEMENT IN DETECTION LIMIT

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Molecular BioSystem, 2009, vol. 5, pag. 548-553.

DOI: 10.1039/b814398f

II.I | ABSTRACT

A real-time apta-PCR for the ultrasensitive detection of thrombin is reported, where the thrombin aptamer acts not only as a biomolecular recognition element, but also as a label for amplification via real-time PCR. Aptamers can be easily converted to a reporter agent for detection by real-time PCR, simply via flanking of the aptamer's recognition moiety with primer sequences. The reported technique has the advantage of the ultrasensitivity achievable with immuno-PCR, but without the complications of addition of a DNA label, and is a technique generically applicable to all aptamers. Here, we use a sandwich format, where two existing thrombin binding aptamers with distinct binding epitopes have been utilised to capture and detect thrombin in a streptavidin-coated microtiter plate. The amount of thrombin is calculated from real-time PCR analysis of eluted captured reporter aptamer. However, the technique can also be used for aptamer–antibody sandwiches, or simply with single aptamers. A greater than 20 000-fold increase in sensitivity is achieved, highlighting the potential of this approach for the detection of very low levels of target analytes. The use of the aptamer itself as the reporter molecule eliminates the necessity of laborious enzyme/DNA labelling, facilitating a significantly more straightforward assay with a vastly enhanced sensitivity.

II.II | INTRODUCTION

Biomolecular recognition elements have been exploited in detection systems for a wide variety of applications ranging from food quality control to clinical diagnostics[1]. Antibodies have been widely used as selective and specific biocomponents, being routinely used in enzyme linked immunosorbent assays (ELISA), easy-to-use, sensitive assays applicable to the detection of a vast number of analytes. Despite the enormously advantageous properties of antibodies, they do suffer from some drawbacks.

Aptamers are artificial nucleic acid ligands, specifically generated against certain targets, such as amino acids, drugs, proteins or other molecules. For the generation of artificial ligands, they are isolated from combinatorial libraries of synthetic nucleic acid by exponential enrichment, via an in vitro iterative process of adsorption, recovery and re- amplification known as SELEX (Systematic Evolution of Ligands by EXponential enrichment)[2]. Indeed, the number of reports of the use of aptamers for analytical applications has risen exponentially over the last 15 years, and there are many promises that aptamers will rival antibodies for use in simple, rapid and sensitive assay systems [2-4]. Aptamers have several advantages over antibodies as recognition elements— firstly, SELEX is carried out in vitro, avoiding the need for animals, and aptamers can also be selected against non-immunogenic and toxic targets, as the process does not rely on the induction of an animal immune system, as in the case of antibody generation. Moreover, SELEX can be manipulated to produce aptamers to a specific region of the target, which is sometimes not possible with antibodies, as the animal immune system inherently hones the epitopes on the target molecule. SELEX can be carried out under non-physiological conditions, providing aptamers that will function under these conditions (e.g. in the presence of solvent, low pH), and aptamers can recover their native conformation after denaturation. Aptamers with different binding domains or with binding characteristics that can specifically recognize an aptamer target complex can be specifically designed. Demonstrating their increased flexibility as compared to antibodies, aptamers can be formatted into molecular beacon structures[4-6] and one of the major advantages of aptamers is their flexibility to adapt to different assay formats.

The immuno-polymerase chain reaction (immuno-PCR), first reported in 1992, is a method for the ultrasensitive detection of analytes, which combines the selectivity of immunoassay techniques with the extremely high efficiency of nucleic acid amplification techniques and highly sensitive approaches to detect the amplified material[7]. In immuno- PCR, the reporting antibody is labelled with DNA, either directly, or via a biotin–streptavidin bridge. A study has been carried out to compare the different assemblies, concluding that antibodies directly labelled with DNA offer better sensitivity and reproducibility than those labelled via the biotin–streptavidin bridge[8]. This combination of PCR can lead to an increase in sensitivity of up to five orders of magnitude greater than those previously reported through the use of standard detection systems[9]. There have been many developments in immuno-PCR since its first report, and these developments have been extensively reviewed elsewhere[10-12]. In summary, the developments have concretely demonstrated that the initial report of 1992 is applicable to the ever-increasing demands of routine diagnostics and several commercial immuno-PCR kits are now available. Many of the reported developments have led to significant increases in assay sensitivity, with some reports of dramatic improvements of a greater than 100 000-fold enhancement in detection limit compared to conventional ELISA[13-15]. One of the more interesting of these developments has been the use of real-time PCR for combined amplification and highly sensitive detection[16].

Despite the demonstration of immuno-PCR in various applications, there are some drawbacks with this approach, namely the requirement to link nucleic acids directly to antibodies, or via biotin–streptavidin linker bridges, as well as the potentially high background due to non-specific binding of the nucleic acid–antibody conjugates to solid phases[17, 18]. Some alternative approaches have been reported, where labelling of the antibody with DNA is not required, such as that of Banin et al., where double-stranded 5'-phosphorylated DNA is used as a substrate for an alkaline phosphatase (ALP) label, which takes advantage of the substrate specificity of an exonuclease for this form of DNA. The ALP label removes the 5'-phosphate groups, and the DNA is consequently not degraded by subsequent exonuclease treatment. Any nucleic acid amplification technique can then be used to amplify and detect this non-degraded, ''protected'' DNA[19].

In this study, as a model, we report a real-time apta-PCR assay system based on the double aptamer recognition of thrombin. The apta-PCR is a further advancement and

simplification of immuno-PCR, where a single aptamer molecule has the dual function of being both a biorecognition and reporter molecule. A sandwich assay was developed with a biotinylated 29-mer aptamer immobilised on streptavidin- coated microtiter plates to capture the target thrombin, and a second 15-mer reporter aptamer, which was extended at both flanking sites to include primer sequences for PCR amplification.

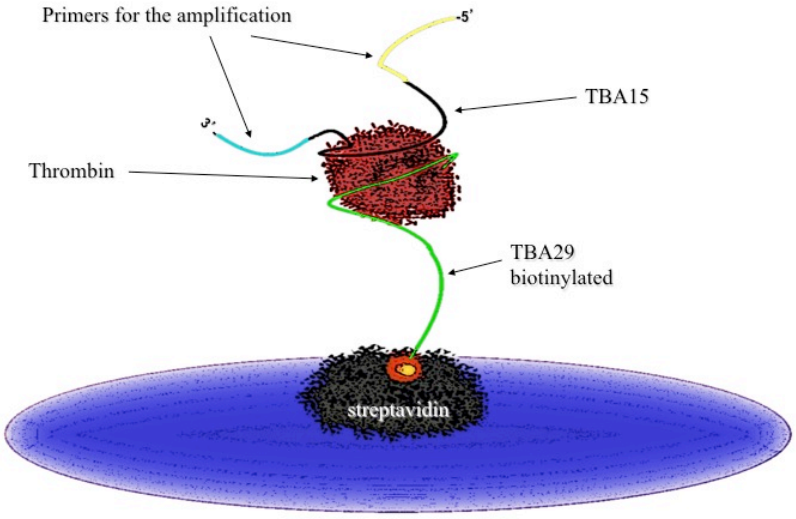

Figure II-1 A SCHEMATIC REPRESENTATION OF REAL-TIME APTA-PCR. This assay is similar to a sandwich ELISA where the antibodies are replaced by aptamers one aptamer (TBA29) is immobilized via biotin onto a streptavidin-coated microtiter plate to capture the target, while the other aptamer (TBA15), flanked by primer sequences for amplification, is used as a reporter probe. Detection is then achieved by eluting the secondary aptamer for subsequent amplification through real-time PCR.

There have been several reports of the use of double aptamer recognition as a sandwich assay for the detection of thrombin. The advantage of using sandwich assays is the combination of sensitivity and selectivity in the same assay format. Two different aptamers were used, one 15-mer aptamer having the sequence, 5'-GGTTGGTGTGGTTGG-3', and the other one a 29-mer aptamer, 5'-AGTCCGTGG-TAGGGCAGGTTGGGGTGACT-3'[20, 21]. In all of the reports detailed below, the 15-mer aptamer was used to capture the target thrombin and the 29-mer aptamer was conjugated to different labels as the reporter aptamer.

Ikebukuro *et al.* first reported the double sandwich aptamer assay using electrochemical detection and a pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenase label, achieving a detection limit of 1 mM[22], and the team subsequently

improved the detection limit to 10 nM[23]. Mir *et al*. reported the electrochemical detection of an enzyme-labelled sandwich format[24]. In another report using the double aptamer sandwich format, the enzyme label was replaced with cadmium sulfide (CdS) quantum dots, and using potentio- metric detection, an improved detection limit of 0.14 nM was obtained[25]. A further improvement in the detection limit to 0.02 nM was achieved using gold nanoparticles as the label, with impedimetric detection[26]. In a more recent report, an electrochemical magnetic bead-based assay using the double aptamer sandwich and a streptavidin–ALP conjugate of the reporting aptamer, achieving a detection limit of 450 pM[27]. A significant improvement in detection limit to 1 fM, was achieved using tris(2,2'-bipyridyl) ruthenium(II)-doped silica nanoparticles and electrochemiluminescence detection[28]. Despite the admirable detection limits that have been achieved with these reported approaches, they all have a definitive drawback in that it is necessary to prepare conjugates of oligomers to the different labels and this can pose some difficult problems in purification of the conjugates, as well as difficulties in upscaling to mass production.

Fischer *et al*.[29] recently reported a sandwich assay system based on detection of the reporter aptamer using real-time PCR or rolling-circle amplification (RCA). The reported approach was first demonstrated using a biotinylated target analyte, which is linked to streptavidin-coated magnetic beads and then exposed to a thrombin-binding aptamer, which, following magnetic separation, is amplified. They further extend the method to the use of streptavidin-coated magnetic beads linked to a biotinylated anti-thrombin antibody, which captures the target thrombin, and the subsequently bound thrombin binding aptamer is isolated via magnetic separation and detected via real-time PCR/RCA, achieving a detection limit of 10 pM.

In the approach we report here, we use a sandwich assay with the 29-mer aptamer immobilised as a capture biorecognition element and the 15-mer aptamer flanked by primer- specific sequences as a reporter aptamer, which is directly used as the label. This is facilely provided via synthesis, and thus completely avoids any problems encountered during conjugation and subsequent purification, without sacrificing specificity or sensitivity. An 20000-fold enhancement in sensitivity is achieved, with a demonstrated detection limit of 450 fM achieved using the apta-PCR, as compared to a detection limit of 5 nM attained with thrombin detection using the sandwich format and an enzyme label[22, 30]. The reported approach is applicable to all aptamers, independent of

whether there exists double aptamers against the target, and the technique can thus be applied to the detection of very low concentrations of target analytes, which is garnering ever increasing importance in pre- emptive medicine and the early diagnosis of disease.

II.III | MATHERIALS AND METHODS

II.III.I. MATERIALS AND INSTRUMENTATION

Biotinylated and non-modified oligonucleotides were synthesized by MWG-Biotech. Thrombin, human serum albumin (HSA) and Tris-buffered saline–Tween 20 (TBS-T) were purchased from Sigma. Streptavidin-coated 96 well titer plates were obtained from Thermo Scientific (Fisher Scientific). Real-time PCR analyses were performed on an Applied Biosystems HT-7900 using SYBR green labelling procedure with a power sybr kit (Applied Biosystems). The PCR was performed on a Biorad Icycler thermal cycler and agarose gel images were captured in a Biorad gel imaging system. Taq polymerase was obtained from Invitrogen. A Biacore 3000 was used for surface plasmon resonance (SPR) analysis. The CM5 sensor chip was a carboxymethylated dextran surface covalently bound to gold surface.

II.III.II. OLIGOMER SEQUENCES

The sequences of oligomers used are listed in Table 1. TBA29, referred to as 'capture aptamer', represents the 29-mer thrombin-binding aptamer reported by Tasset *et al.*[21] and TBA15M, referred to as 'reporter aptamer' is the thrombin- binding 15-mer aptamer reported by Bock *et al.*,[20] flanked by primer sequences for amplification at the 50 and 30 ends.

II.III.III. SURFACE PLASMON RESONANCE BINDING ANALYSIS

The binding affinities of the aptamers were analysed by SPR at 25ºC by a Biacore 3000 system using CM5 chips (Biacore). Biotin-modified TBA15 and TBA29 aptamers were immobilized on streptavidin-covered CM5 chips (Biacore) by injection at a 5 mM concentration in TBST buffer (pH = 7.5), to achieve an increase of approximately 1000 RU. Binding analysis was performed by primarily flowing 100 nM thrombin, over the sensor chip at 15 μL min^{-1} rate for 180 s. This was followed by an injection of 100 nM of the reporter aptamer, again applied for 180 s at a flow rate of 15 μL min^{-1}. The running and binding buffers were 100 mM NaCl, 5 mM MgCl2, 5 mM KCl, 0.01%

Tween 20, 10 mM Tris (pH 7.4). The chips were regenerated by 50 mM NaOH. A channel without an immobilized capture aptamer was used as a control.

II.III.I. APTA-PCR ASSAY

TBS-T buffer was used for all binding and washing steps of the assay, and all incubations were carried out at 37ºC with agitation. 50 μL of biotinylated TBA29 was added to each well of a Nunc streptavidin-coated microtiter plate for 1 h. The plate was then washed three times with the same buffer and incubated in a thrombin or HSA solution in buffer, as detailed above, for 1 h. The reporter aptamer was then added to each well of the washed plate and incubated for another hour. Following thorough washing to remove any non-specifically bound reporter aptamer, the bound reporter aptamer was then eluted via incubation for 15 min in 50 μL of 200 mM NaOH, followed by neutralization with acetic acid. 5 μL of the elution was directly used in the PCR/real-time PCR reactions.

Name	Sequence (from 5'- to -3')	Mer
Bio-TBA15	Biotin-C6-GGTTGGTGTGGTTGG	15
Bio-TBA29	Biotin-C6-AGTCCGTGGTAGGGCAGGTTGGGGTGACT	29
TBA 15M	AGCTGACACAGCAGGTTGGTTGTGGTTGGCACGACACGAGTCGAGCAATCTCGAAAT	56
Forward Primer	AGCTGACACAGCAGGTTGGTG	21
Reverse primer	ATTTCGAGATTGCTCGACTCGTG	23

Table II-1 SEQUENCES OF APTAMERS USED IN AFFINITY STUDIES AND APTA-PCR.

II.III.II. STANDARD PCR ANALYSIS

Standard PCR reaction conditions used 50 μL volume and the amplicons were analyzed in 3% w/v agarose gels.

II.III.III. REAL-TIME PCR ANALYSIS

Real-time PCR reactions were performed in 20 μL volume in an Applied Biosystems 7900-HT Fast real-time PCR system with Power SYBR Green kit (Applied Biosystems). The results were analyzed by Applied Biosystem SDS 2.2.2 Analysis software to calculate the amount of reporter aptamer present in the samples. The PCR cycle consisted of an initial denaturation step at 95 1C for 2 min followed by 35 cycles of 95 ºC for 30 s, 58 ºC for 30 s and 72 ºC for 30 s.

II.IV | RESULTS AND DISCUSSION

Aptamers were first used in combination with antibodies for a sandwich assay by Drolet *et al.*[31] in which VEGF was detected in the serum by capturing the target on immobilized antibodies and using a VEGF-aptamer conjugated to fluorescein as detecting element. This study did not exploit the potential of aptamers in sandwich assays, but showed the possibilities of implementing aptamers in microtiter assays. Since then, there have been many reports of aptamer- incorporated assays in similar systems using fluorescent, electrochemical, redox agents or PCR and rolling circle detection systems. Among them, Ikebukuro *et al.*[22] developed a double aptamer sandwich assay by conjugating glucose dehydrogenase to TBA29 with an electrochemical detection system to achieve a 1 μM detection limit, and as outlined in the introduction, several approaches exploiting different labels have improved on this detection limit using PCR. An approach for the ultrasensitive detection of thrombin using PCR/RCA was recently reported, but this approach used a capture antibody, which is required to be functionalised with a magnetic particle to facilitate efficient separation of bound and unbound reporter aptamer[29].

Here, we report on the ultrasensitive detection of thrombin by exploiting a new method of real-time apta-PCR, which is applicable to all aptamers for the detection of very low levels of target molecules. To demonstrate the concept, we modified the flanking regions of TBA15 to include PCR primers for the simultaneous recognition and quantification of thrombin molecules captured on solid surfaces. As shown in Fig. 1, the approach exploits an immobilised thrombin-binding aptamer (TBA29), the capture aptamer, on a streptavidin- coated microtiter plate. Consequently the captured thrombin molecules were incubated with a second thrombin-binding aptamer (TBA15M), the reporter aptamer, which is known to be directed against a different area of the thrombin target. Following incubation of the sandwich, the reporter aptamer was eluted, and detected qualitatively by traditional PCR and electrophoresis, and quantitatively using real-time PCR.

II.IV.I. *ASSAY DESIGN*

In order to elucidate the optimum format for the assay, SPR analysis was carried out to determine the kinetic constants of TBA15 and TBA29 for the target thrombin. We wished to determine the respective KD and k_{on}/k_{off} values in order to evaluate which aptamer should be used as a capture aptamer according to lower potential detectability and lower k_{off}. Using a site-directed mutagenesis approach, it has been demon- strated that TBA15 binds to the fibrinogen recognition site, which is determined to be exosite I (Asp21)[32]. This binding configuration has been confirmed by NMR[33]. TBA29 was shown to bind to heparin-binding exosite (exosite II) by photocrosslinking and competition experiments[21], and the core binding site of the aptamer has a very similar sequence to TBA15. However, TBA29 has been reported to have 20 to 50 times better binding affinity to thrombin as compared to TBA15. Recently, Tang *et al.*[34] studied the binding of both aptamers in detail using SPR analysis, confirming separate binding sites of two aptamers by simultaneous binding in real- time. They reported a lower affinity of immobilised TBA29, as compared to immobilised TBA15, but postulated this to be due to the lack of a spacer. In a similar approach, we tested the design of the double aptamer sandwich assay using SPR analysis of simultaneous binding of both aptamers in order to determine the optimal configuration for the apta-PCR, and the affinity and kinetic constants for each immobilised aptamer were determined (Fig. 2).

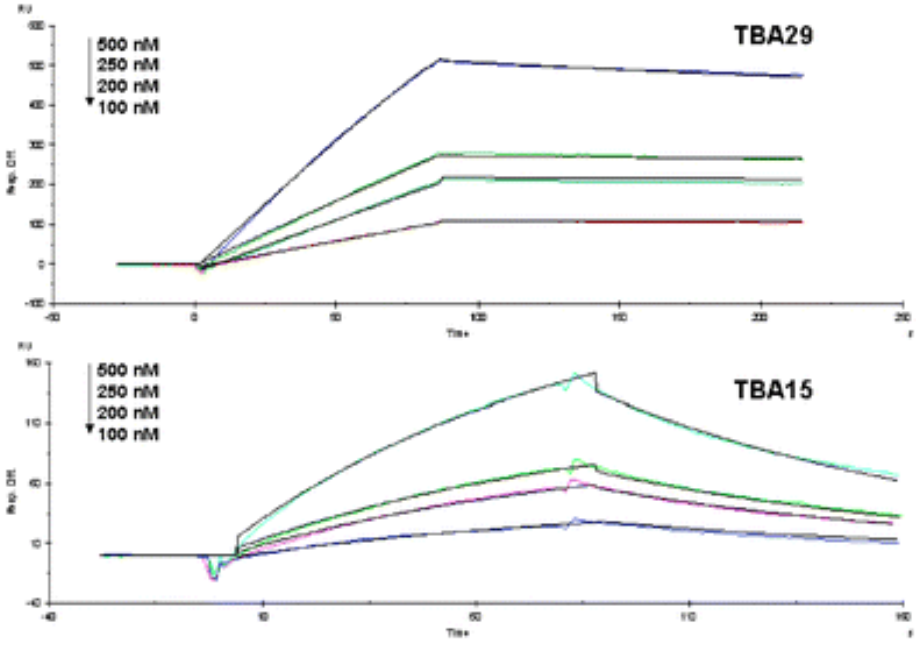

Figure II-2 SENSORGRAMS OF IMMOBILISED TBA15 AND TBA29 WITH VARIOUS CONCENTRATIONS OF THROMBIN. Data was used to calculate affinity and kinetic constants

Table 2 shows that TBA15 has an affinity constant of 421 nM and TBA29 a constant of 4.91 nM when they are immobilized on a streptavidin-coated surface of a Biacore chip. These KD values are far higher than those obtained by Bock *et al.*[20] and Tasset *et al.*[21], of 26 and 0.5 nM for TBA15 and TBA29, respectively. This, however, is to be expected, as these KD values were obtained for the aptamers in solution, whereas the KD values obtained here are for the immobilised aptamer, which would be expected to have limited spatial flexibility, and consequent a lower affinity. The far lower KD value obtained here with immobilised TBA29 would indicate it is the better of the two to have as the capture aptamer. In this work, we have incorporated a C6 spacer to facilitate the minimum possible steric hindrance of the immobilised aptamer, and it is presumably the presence of this spacer that obviates the decreased affinity of immobilised TBA29 compared to immobilised TBA15, as reported by Tang *et al.*[34]

	K_D [nM]	K_{on} [$\times 10^4 \bullet M^{-1} \bullet s^{-1}$]	K_{off} [$\times 10^{-3} \bullet s^{-1}$]
TBA15	421	2.27	9.54
TBA29	4,91	58,2	2,86

Table II-2 AFFINITY AND KINETIC CONSTANTS FOR IMMOBILISED BIOTINYLATED TBA15 AND TBA29.

Moreover, importantly, there is a clear difference in the dissociation kinetics of the two aptamers, with TBA29 having almost three times lower k_{off} and 25 times higher k_{on} values, thus demonstrating that TBA29 resulted in a more stable complex with thrombin than TBA15, further supporting that TBA29 is preferable as the capture aptamer. In order to confirm that the double aptamers could simultaneously bind to thrombin, and that the modification of TBA15 with the flanking primers did not affect its ability to bind to thrombin, a sequential immobilization of TBA29 and capture of thrombin, followed by TBA15M, was detected using SPR (Fig. 3). Thrombin and the corresponding modified detection aptamer were injected over immobilized aptamers at 100 nM concentrations, and the corresponding molecular interactions were detected and quantified. The SPR analysis showed that modified aptamer (TBA15M) and TBA29 could bind simultaneously to thrombin with comparable KD values to original TBA aptamers. Additionally, analysis was carried out to ensure that there was no binding between the two aptamers in the absence of thrombin. Biotin-labelled TBA29 was

immobilized on sensor chips and modified reporter aptamer TBA15M was injected over the sensor chip, but no interaction was observed.

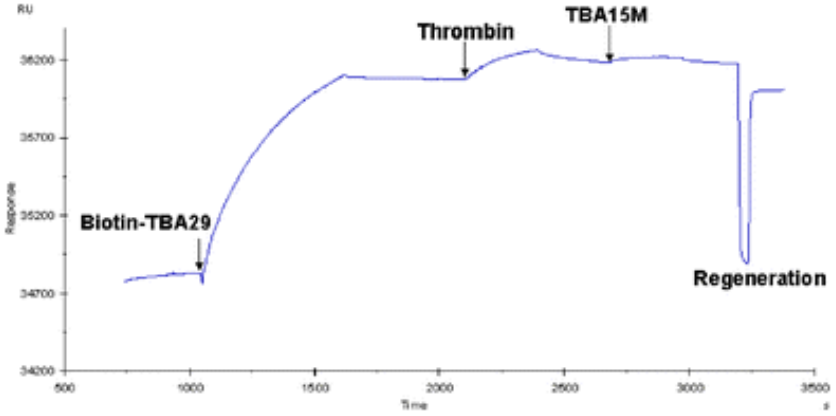

Figure II-3 BINDING ASSAY BETWEEN TBA29, TBA15M AND THROMBIN BY SPR. Biotinylated TBA29 was immobilized on a CM5 chip and 100 nM thrombin and TBA15M were injected at the indicated points. The formation of the aptacomplex of TBA29-thrombin-TBA15M could be followed in real-time.

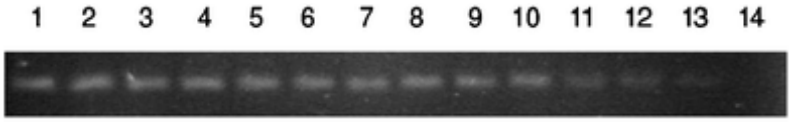

Figure II-4 THE DETECTION OF ELUTED REPORTER APTAMER TBA15M BY STANDARD PCR. Lanes 1–12 are the PCR results of samples prepared in microtiter plates, as described in the materials and methods section, with a serial dilution starting from 100 nM thrombin sample to 0.1 nM. Lane 13 is the control in the absence of thrombin, with 500 nM TBA15M, and lane 14 is the control with 50 nM TBA15M.

II.IV.II. OPTIMIZATION OF ASSAY CONDITIONS FOR PCR DETECTION

A range of concentrations of biotinylated TBA29 (from 1 μM to 10 nM), TBA15M (from 10 to 500 nM) and target thrombin (100 nM) were tested in a microtiter assay to determine the optimum conditions of the assay. The optimised concentrations of capture and reporter aptamer were then applied to determine the detection limit by standard PCR detection. The presence of amplicons was analyzed by 3% w/v agarose gel electrophoresis. An example of the analysis is shown in Fig. 4. A specific band at 56 bp was used as evidence of the detection aptamer captured on thrombin and compared to a control sample without thrombin. A decrease in band intensity was a qualitative indication for the range of capture and detection aptamer concentrations to be used in

the set-up of the real-time apta-PCR. The results showed that the concentration of capture aptamer at concentrations higher than 50 nM was enough to detect the presence of reporter aptamer by standard PCR. Concentrations of reporter aptamer over 200 nM resulted in unspecific bands in control conditions. Thus, the optimum concentrations of TBA15M and TBA29 to be used in a standard apta-PCR procedure were 100 nM and 500 nM, respectively. However, as can be seen in lane 13 in Fig. 4, which represents a control where no thrombin is present, this high concentration of reporter aptamer results in a low level of non-specific binding/adsorption of the reporter aptamer. However, when a lower reporter aptamer concentration of 50 nM was used (see lane 14 in Fig. 4), detection of thrombin concentrations as low as 1 nM was possible, with no non-specific binding/adsorption observed, and this concentration was thus applied in the real-time apta-PCR. The minimum detectable amount with standard PCR was deter- mined to be 0.1–1 nM.

II.IV.III. DETERMINATION OF THE DETECTION LIMIT BY REAL-TIME PCR

In real-time assays, a detection aptamer at a concentration of 50 nM was used to decrease the unspecific background. An example of real-time analysis results is shown in Fig. 5 and the eluted TBA15M was directly used in real-time PCR quantification.

There were clear differences in the Ct values for thrombin concentrations of 1 nM, 100 pM and 500 fM. 150 fM thrombin was indistinguishable from the control samples in the absence of thrombin and TBA29. This could be due to a low level of unspecific binding of the reporter aptamer in microtiter wells after washing. Fig. 5 shows how Ct values form separate groups by increasing the thrombin amount in assay procedures for fM, pM and nM concentration samples. A graph of thrombin concentration vs. Ct values was used to determine the LOD value of the real-time apta-PCR as 450 fM, representing an impressive enhancement in sensitivity of 20 000 fold as compared to a conventional enzyme linked aptamer assay (ELAA)[30].

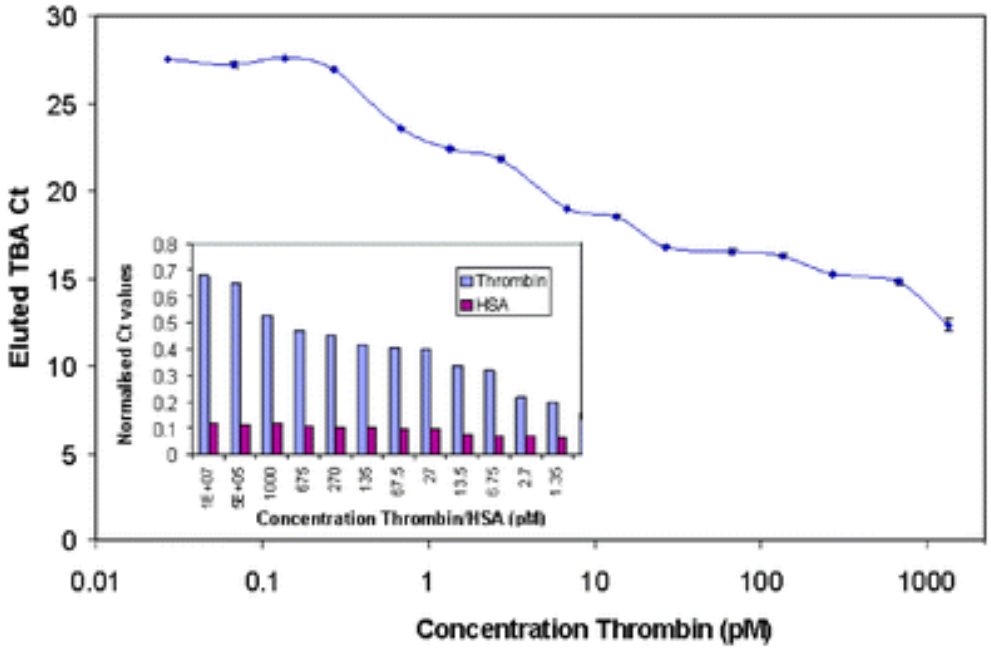

Figure II-5 REAL-TIME PCR ANALYSIS OF ELUTED DETECTION APTAMER AFTER THE DOUBLE APTAMER SANDWICH ASSAY. The error bars represent the standard deviation of 3 independent experiments. The inset represents the normalised Ct values *i.e.* 1-(Ct sample/Ct no template control), of the values obtained for a range of thrombin and HSA concentrations.

II.V | CONCLUSIONS

We report here a real-time apta-PCR for the ultrasensitive detection of proteins, using thrombin as a model target analyte. In this approach, we use a sandwich assay using an immobilised 29-mer aptamer, which interacts with the heparin-binding exosite of thrombin as a capture biorecognition element, and a 15-mer aptamer, which interacts with the fibrinogen recognition site of the thrombin, flanked by primer specific sequences as a reporter aptamer. This reporter aptamer is directly used as the label, which is facilely provided *via* synthesis, and thus evades any problems encountered during conjugation and subsequent purification, without sacrificing specificity or sensitivity. An 20000-fold enhance- ment in sensitivity is achieved with a demonstrated detection limit of 450 fM using the apta-PCR, as compared to a detection limit of 5 nM attained with thrombin detection using the sandwich format and an enzyme label[22].

The reported approach is applicable to all aptamers, independent of whether there exists double aptamers against the target, and the technique can this be applied to the detection of very low concentrations of target analytes, which is garnering ever increasing importance in pre-emptive medicine and the early diagnosis of disease. Ongoing work is focused on the application of this technique to single aptamers, to demon- strate the flexibility of the reported real-time apta-PCR for the ultrasensitive detection of proteins.

II.VI | ACKNOWLEDGEMENTS

V. O. is financed by the Marie Curie Grant FP7-PEOPLE- 2007-2-221310-GLUSENS. A. P. wishes to thank the Ministerio de Educacion y Ciencia, Spain for the financing of his doctoral fellowship. This work has been carried out as part of the Plan Nacional GLUTACATCH and was partly financed by the Grup Emergente INTERFIBIO, 2005SGR00851.

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Chapter III | FUNCTIONAL DETECTION OF PROTEINS BY CAGED APTAMERS

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DOI: 10.1021/cb2003835

III.I | ABSTRACT

While many diagnostic assay platforms enable the measurement of analytes with high sensitivity, most of them result in a disruption of the analyte's native structure and, thus, in loss of function. Consequently, the analyte can be used neither for further analytical assessment nor functional analysis. Herein we report the use of caged aptamers as templates during apta-PCR analysis of targets. Aptamers are short nucleic acids that fold into a well-defined three-dimensional structure in which they interact with target molecules with high affinity and specificity. Nucleic acid aptamers can also serve as templates for qPCR approaches and, thus, have been used as high affinity ligands to bind to target molecules and subsequently for quantification by qPCR, an assay format coined apta-PCR. Caged aptamers in turn refer to variants that bear one or more photolabile groups at strategic positions. The activity of caged aptamers can thus be turned on or off by light irradiation. The latter allows the mild elution of target-bound aptamers while the target's native structure and function remain intact. We demonstrate that this approach allows the quantitative and subsequently the functional assessment of analytes. Since caged aptamers can be generated emanating from virtually every available aptamer, the described approach can be generalized and adopted to any target–aptamer pair and, thus, have a broad applicability in proteomics and clinical diagnostics.

Introduction

Immuno-polymerase chain reaction (immuno-PCR) enables the ultrasensitive detection of analytes via the combination of the selectivity of antibodies with the high efficiency and sensitivity of nucleic acid amplification methods.[1] Immuno-PCR utilizes a reporter antibody equipped with a short DNA strand, which is employed as a template for quantitative PCR (qPCR). Recent developments in the field have demonstrated that immuno-PCR addresses the increasing demands of diagnostic assays achieving a significant increase in sensitivity, culminating in a more than 100,000-fold enhancement of detection limits as compared to conventional ELISA. [2-6] However, immuno-PCR relies on the availability of high affinity antibodies and requires chemical conjugation of the antibody to reporter oligonucleotide tags. In the past twenty years, nucleic acid aptamers have emerged as powerful rivals to antibodies for use as diagnostic reagents. Aptamers are short nucleic acids that fold into a well-defined 3D-structure upon which they interact with target molecules with high affinity and selectivity.[7] Aptamers can be identified by an in vitro selection approach, termed SELEX (Systematic Evolution of Ligands by EXponential enrichment),[8] and have been reported to interact with a diverse set of target molecules, such as peptides, proteins, drugs, organic and inorganic molecules or even whole cells[9-13]. Many of the aptamers reported to date reveal affinities for their target molecules, comparable to, if not better than respective monoclonal antibodies, with picomolar K_d-values.[13, 14] Furthermore, the specificity of aptamer recognition has been observed, facilitating a 10,000-fold to 12,000-fold[13, 15] discrimination of cognate target molecules and related structures. The synthetic nature of aptamers enables their site-directed modification, which clearly sets them apart from antibodies. For example, aptamers can be generated as molecular beacon structures or equipped with functional tags for rapid adaptation to diverse assay formats without loss of aptamer activity. Consequently, due to their nucleic acid nature an approach termed aptaPCR has been developed. AptaPCR employs the dual function of aptamers acting both as a selective ligand for target molecules and as a template for qPCR. Aptamers are exceptionally well suited for this approach and virtually every aptamer identified to date can be used without laborious optimization procedures. Most importantly, this approach bypasses the mandatory conjugation of antibodies with DNA-reporter tags exploited in immuno-PCR, which can interfere with the recognition properties of antibodies.

Several reports have been described that use the template and recognition properties

of aptamers, while different separation methods to enrich for the bound target have been employed.

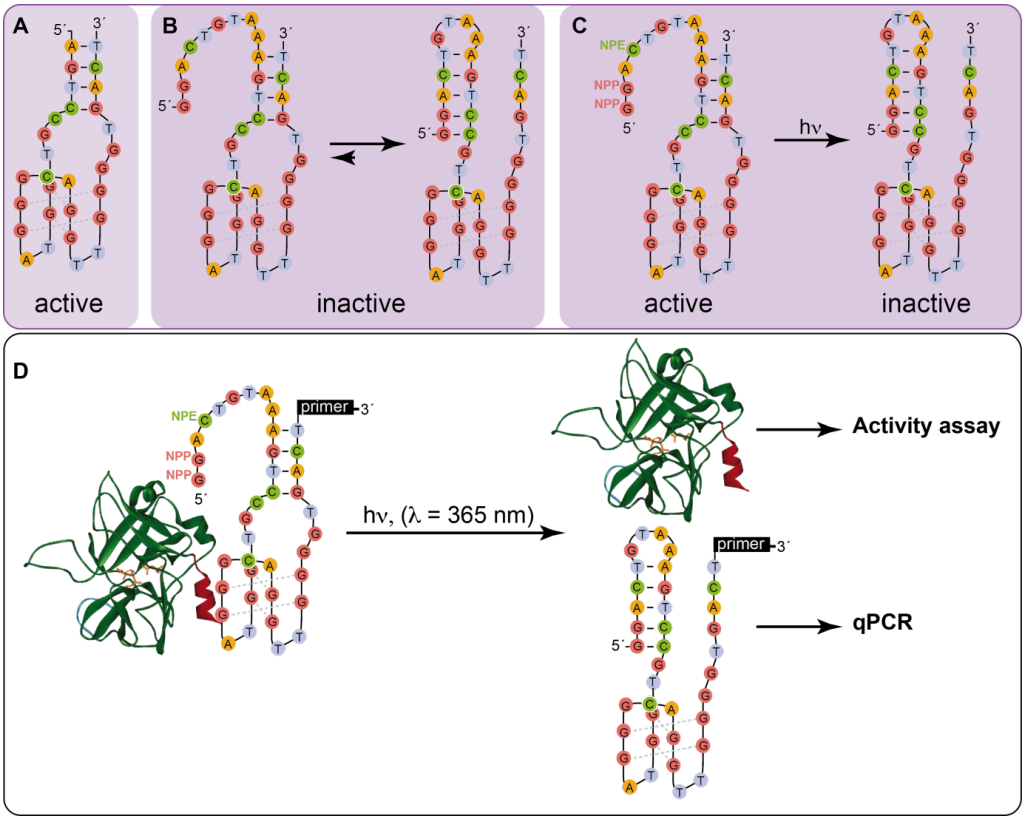

Figure III-1 **A)** PROPOSED SECONDARY STRUCTURE OF THE HD22 APTAMER, WHICH BINDS (ACTIVE) THROMBIN VIA EXOSITE II. **B)** EXTENSION OF THE APTAMER WITH A GNRA-TETRALOOP SEQUENCE (GTAA) AND FURTHER COMPLEMENTARY NUCLEOTIDES INDUCE HAIRPIN FORMATION AND, THUS, UNFOLDING OF THE APTAMER (INACTIVE). **C)** ADDITION OF PHOTO-LABILE GROUPS (*O*-NITROPHENLYPROPYL – NPP AND *O*-NITROPHENYLETHYL –NPE) AT DEFINED POSITIONS (G1, G2, AND C4) PREVENTS HAIRPIN FORMATION AND KEEPS THE APTAMER ACTIVE. Inactivation is achieved through irradiation with light (λ = 365 nm), whereas the photo-labile groups are removed and hairpin formation is induced. **D)** After recognition of thrombin the caged aptamer is irradiated and thus will be release for qPCR analysis whereas the captured thrombin can be further assessed for specific activity.

Most of the described assays take advantage of the unique amplification properties of aptamers, whereas the captured aptamer were amplified using qPCR or rolling circle amplification[16-20] as well as proximity ligation assays.[21-23] We have recently employed two well-known thrombin binding aptamers and extended aptaPCR towards a sandwich-like assay format.[24] Most of the described assays for analyte detection make use of denaturing conditions to elute the bound aptamers from their target molecule, thereby potentially leading to target inactivation. Consequently, the target cannot be applied for further analyses, and to ensure that the functional integrity is maintained, the implementation of mild elution methods that allow an efficient disruption of aptamer-

target interactions for the liberation of the aptamer for qPCR and the native target for further functional investigations, is required. One approach makes use of antisense molecules that hybridize with the aptamer, thereby disrupting its active conformation and another reported alternative employs so-called caged aptamers. Caged aptamers bear photolabile groups at strategic positions and, thus, their target recognition properties can be controlled by light irradiation.[25-28] In this way, we hypothesize that caged aptamers can be designed to recognize and bind a target molecule and can consequently be liberated upon irradiation. The released aptamer can subsequently be used as template for aptaPCR and the remaining captured target analyte for functional analysis (Scheme 1). In the present work we describe the entire route to accomplish the aforementioned strategy. Starting from the well-known aptamer HD22 that recognizes thrombin's exosite II, we illustrate the generation of a caged aptamer-variant, which binds thrombin with high affinity, but can be released by light irradiation. Using this molecule we established a 'direct' and 'sandwich'-type kind of aptaPCR assay. Furthermore, we demonstrate that after light irradiation the target protein's enzyme activity remains functional. This model system can be generalized and adopted toward the detection of a series of analytes for which quantitative, qualitative and functional analysis in one assay format is applicable and desirable.

III.II | EXPERIMENTAL SECTION

III.II.I. *REAGENTS*

Unmodified oligonucleotides, PCR primers (forward: 5'-AAAGTCCGTGGTAGGGCA-3'; reverse: 5'-TCTCTTCGAGCAATCCTCAC-3') and biotinylated HD1 aptamer (5'-GGTTGGTGTGGTTGG- biotin-3') were synthesized and HPLC purified (Ella Biotech, Martinsried, Germany). Human α -thrombin was purchased from Cell Systems Biotechnologie Vertrieb GmbH (Troisdorf, Germany). Thrombin and Bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Germany). Fluorogenic peptide substrate Pefafluor TH (H-D-CHA-Ala-Arg-AMC) was purchased from Loxo (Dossenheim, Germany). Streptavidin was purchased from Applichem (Darmstadt, Germany). E. *coli* tRNA was purchased from Roche Diagnostics (Germany). Taq DNA polymerase and PCR reagents was purchased by Invitrogen (Germany). B&W buffer (binding and washing buffer) consists of 137

mmol/L NaCl, 2.7 mmol/L KCl, 9.6 mmol/L Na_2HPO_4, 1.5 mmol/L KH_2PO_4 and 3mM magnesium chloride at pH 7,4.

III.II.II. FILTER RETENTION ANALYSIS

The sequence of interest was radiolabelled at the 5'-end with γ^{32}-ATP using T4 polynucleotide kinase (New England Biolabs GmbH, Germany). After one hour incubation at 37°C, unreacted γ^{32}-ATPs were removed using G25 microspin columns (GE healthcare, Germany) following the manufacturer's instructions, and the radioactively labeled aptamer was subsequently analyzed via electrophoresis on a 12% polyacrylamide denaturing gel. To determine the dissociation constant of the sequences studied, 0,4 nM of radiolabeled nucleic acids were mixed with increasing concentrations (0 to 1μM) of human α-thrombin and incubated at 37°C for 30 minutes in B&W buffer in the presence of 1mg/mL of BSA and 10μM of tRNA. After incubation the mixture was filtered thought a 0.45μM nitrocellulose membrane (Whatman, Germany), previously activated with 0.4 M of potassium hydroxide and rinsed with 600μL B&W buffer. Finally, the nitrocellulose membrane was washed with 800μL of B&W buffer, dried and exposed on a storage phosphor screen. Following overnight exposure, the screen was analyzed using a FUJIFILM FLA-3000 with AIDA Image software (Fujifilm, Germany). To characterize the affinity of the HD22 derivatives, 0.4 nM of radioactively labeled HD22 was incubated with 10nM of thrombin in the presence of an increasing amount (0-1 μM) of each of the unlabelled derivatives. Following a 30 minute incubation at 37°C in B&W buffer in the presence of 1mg/mL of BSA and 10 μM of tRNA, the mixture was filtered and analysis carried out as described above. Two independent measurements were performed for each derivative.

III.III | SYNTHESIS OF THE CAGED OLIGONUCLEOTIDES

The phosphoramidite building blocks for the introduction of a caged dG^{NPP} [29] residue and a dC^{NPE} [25] residue were synthesized according to established literature procedures. Caged oligonucleotides were synthesized on an ABI-392 synthesizer using standard coupling protocols. For the cleavage, aqueous ammonia (65 °C, 4 h) was used. The resulting crude product was purified by RP-HPLC (Nucleosil 100-5 C18, 0.1 M triethylammonium acetate pH 7, acetonitrile), detritylated, and again purified by RP-

HPLC (same protocol). The identity of the oligonucleotides was established by ESI-MS: 5'-AAGCAGTGGTAAGTAGGTTGATT-$G^{NPP}G^{NPP}AC^{NPE}$TGTAA-**HD22**-TCTCTTCGAGCAATCCTCAC-3': 25,681.0 Da (calc. 25,680.61), 5'-$G^{NPP}G^{NPP}AC^{NPE}$TGTAA-**HD22**-TCTCTTCGAGCAATCCTCAC-3': 15,548.9 Da (calc. 15,549.14)

III.IV | APTAPCR

Direct aptaPCR

A range of human α -thrombin concentrations (0 to 10nM in 0.05 M carbonate/bicarbonate buffer pH 9.6) were incubated on the wells of a microtiter plate (50µL/wells) for 30 minutes at 37°C. Following incubation, the plate was washed three times with B&W buffer (200 µL/wells each wash), and the surface was blocked by incubating the plate for 30 minutes at 37°C in the presence of Tween-20. Subsequent to a further washing step, 5.3amp1 was added to each well and incubated at 37°C for 30 minutes (50 µL/well, 250 pM final concentration) in the presence of 10µM of tRNA, followed by a final wash with B&W buffer (800 µL/well). Finally, following the addition of fresh B&W buffer (50µL/well), the reporter aptamer was eluted. Elution was carried out in three different ways: a) incubation for 10 minutes at room temperature in B&W buffer b) incubation for 5 minutes at 95°C and c) irradiation with UV light (λ = 365 nm) for 5 minutes followed by 5 minutes incubation at room temperature. Following elution, 1 µL of the eluted supernatant was used for successive amplification using qPCR.

Sandwich aptaPCR

Firstly, streptavidin (10µg/mL) in carbonate/bicarbonate buffer (0,05M, pH 9.6) was added to the microtiterplate (100µL/well). Following an overnight incubation at +4°C, the plate was washed with B&W (600µL/ well), and after a one hour incubation at 37°C in the presence of 1mg/mL of BSA in B&W buffer the 96-well plate was further rinsed with B&W buffer (600µL/well). Finally, the buffer was removed and the functionalized microtiter plate, stored at +4°C, was used within two days. Biotinylated HD1 aptamer was dissolved in B&W buffer to a final concentration of 500 nM and added to the streptavidin-coated wells of microtiterplates (100µL/well). Following one hour incubation at 37°C, the wells were washed with B&W buffer (600µL/well).

Subsequently, a range of concentrations of human α-thrombin (0 to 10nM) were added (50µL/well) and incubated for one hour at 37°C. After washing step, 5.3amp1 (2.5nM) was added (50µL/well) and incubated for one hour at 37°C. After thorough washing (800 µL/well) the caged aptamer was eluted as described for the direct assay.

qPCR

1µL of the elution fraction was added to 19 µL of a PCR master mix, which consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3mM $MgCl_2$ 100nM of forward and reverse primer, 1x final concentration SYBR Green I (Sigma-Aldrich, Germany), 10nM FAM (Bio-Rad, Germany) and 0.25 units of Taq DNA Polymerase. The amplification was performed in an iCycler thermal cycler upgraded with the iQ5 real-time PCR detection system (Biorad, Germany) programmed according to the following protocol: 1 minute at 95°C, followed by 40 repetitions of 30 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C. The LoD was calculated from two independent experiments in which each sample was analysed in duplicate, where the LoD was calculated as the background value plus three times the standard deviation of the background.

Measure of the functional integrity via thrombin activity

1 pmol of thrombin was either captured by immobilized HD1-aptamer (sandwich assay) or physically adsorbed (direct assay) on the microtitre plate surface. Following incubation with the caged aptamer and subsequent elution, Pefafluor TH (50 µL/well, 100 µmol/L of in TBS, pH 8.5) was added to each well of the microtiter plate and the change in fluorescence (λ_{Abs}= 342nm, λ_{Em}=440nm) after 30 minutes was taken as a measure of the functional active thrombin.

III.V | RESULTS AND DISCUSSION

III.V.I. CONSTRUCTION OF A LIGHT-RESPONSIVE VARIANT OF APTAMER HD22

We previously described the synthesis and characterization of a variant of the 15mer thrombin's exosite I recognizing aptamer HD1, which can be switched off upon irradiation with light.[26] However, due to its short length it would be necessary to add primer-binding sequences to modify HD1 to render it a suitable template for qPCR. However, we recently discovered that extensions at the 5'-end result in a loss of HD1 activity.[25]

NAME	MODIFICATION
3.1	5'-**HD22**-GTAAAGTCACCCC-3'
3.2	5'-**HD22**-GTAAAGTCACC-3'
3.3	5'-**HD22**-GTAAAGTCA-3'
3.4	5'-**HD22**-GTAAAGT-3'
3.5	5'-**HD22**-GTAA-3'
5.1	5'-CCACGGACTGTAA-**HD22**-3'
5.2	5'-ACGGACTGTAA-**HD22**-3'
5.3	5'-GGACTGTAA-**HD22**-3'
5.4	5'-ACTGTAA-**HD22**-3'
5.5	5'-GTAA-**HD22**-3'
5.3amp	5'-AAGCAGTGGTAAGTAGGTTGATT-GGACTGTAA-**HD22**-TCTCTTCGAGCAATCCTCAC-3'
5.3amp1	5'- GGACTGTAA-**HD22**-TCTCTTCGAGCAATCCTCAC-3'

Table III-1 : LIST OF OLIGODEOXYNUCLEOTIDES USED IN THIS STUDY.

Consequently, we thus decided to generate variants of the aptamer HD22 whose activity can also be switched off by irradiation with light. Emanating from our previous studies we synthesized variants of HD22 that bear extensions at either its 5′- or its 3′-end, which can base pair with neighbouring nucleotides of HD22 and, thus, disrupt the active aptamer structure. Herein, the aptamer was extended by the means of a GNRA-tetraloop followed by an antisense sequence of up to 9 complementary nucleotides (Table 1). As shown previously, this design facilitated the formation of a hairpin structure, which alters the conformation of HD22 and, thus, its recognition properties.[25, 26] We evaluated the recognition behaviour of the extended HD22 variants by competitive binding experiments, where we incubated radioactively labeled HD22 with a constant amount of thrombin and increasing concentrations of extended HD22 variants. Those variants that still interact with thrombin will reduce the HD22 signal whereas those that fail, most likely due to hairpin formation, will have no effect on HD22 binding. As shown in Figure 1, the extended HD22 versions differ significantly with respect to their ability to compete with HD22 thrombin recognition. While extensions at the 3'-end have less influence on thrombin recognition, the aptamer HD22 is sensitive toward extensions at the 5'-end.

Addition of 5 complementary nucleotides (variant 5.3) abolishes thrombin recognition almost completely. In contrast, a similar extension located at the 3'-end (variant 3.3) still recognizes thrombin and even the addition of 9 complementary nucleotides at the 3'-end does not completely inactivate HD22. These data are in accordance with previous findings where both the length and position of an antisense oligonucleotide region are important factors for the perturbation of aptamer function, with the effect of the length and position being difficult to predict. The control variants, which were solely extended with the GNRA-tetraloop sequence (variants 5.5 and 3.5) still revealed high affinity binding to thrombin exemplified through a strong competition activity, which in fact, seemed to be even more efficient when compared with the wild type aptamer HD22. Having identified the HD22 variant 5.3 as non-active we next started to adopt it for a photo-elution aptaPCR setup. Therefore, we added primer-binding sites at either the 3′-end (5.3amp1) or at both the 3′- and 5′-end (5.3amp) of HD22 to enable amplification by qPCR (Table 1). These sequences were chosen to be non-complementary and the primer-tethered HD22 still interacted with thrombin (Supporting Information). We then synthesized the caged variants of 5.3amp1

and 5.3amp (Scheme 1 and Table 1). To achieve this we introduced 3 photo-labile groups at positions G1, G2 and C4 of the extension, functioning as a temporary block of hairpin formation, resulting in an active HD22 variant that can be inactivated upon light-irradiation. Using filter retention analysis, we analyzed whether the caged variants functioned as predicted (Figure 2). As expected, the caged variants bind to thrombin with high affinity and interestingly, compared to the wild type aptamer HD22 slightly increased K_D–values of the modified aptamers (90 nM, 5.3amp1, and 9 nM, 5.3amp), were oserved. Light irradiation (λ = 365 nm), however, induces a loss of the caged aptamers' recognition activity and more importantly this can be also observed in the case of the already formed thrombin-caged aptamer complex.

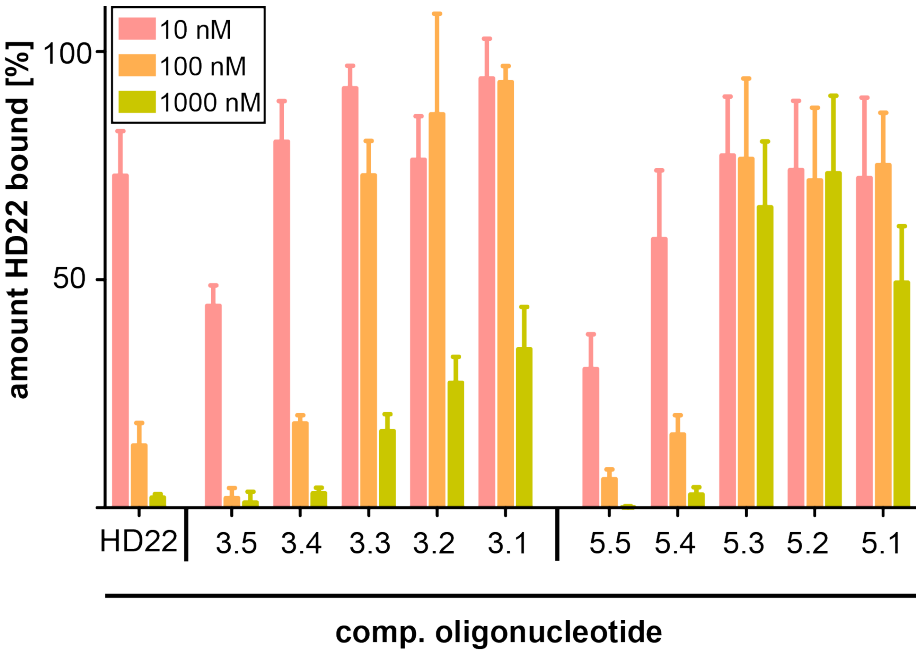

Figure III-2 COMPETITION OF HD22-THROMBIN INTERACTION BY HD22-VARIANTS. To evaluate the impact of the 5´- and 3'-extensions filter retention analysis were done by incubating radioactively labeled HD22 with thrombin [10 nM] in the presence of increasing concentrations [10 nM, 100nM, and 1000 nM] of each variant. After passing through nitrocellulose [0.45 µm] the retained amount of labeled HD22 was determined by phosphorimager analysis and quantified using ImageQuant software.

However, the variant with extensions on both ends (5.3amp) does not react in the predicted way. It reveals recognition properties even in the irradiated version (Figure 2B), which might be explained by competitive base pairing options of the 5´-extension with the uncaged sequence (Table 1, underlined), leading to an intact HD22 structure and, thus, preserved recognition properties. Due to these findings we focused on 5.3amp1 for the further development of the photo-apta-PCR assay.

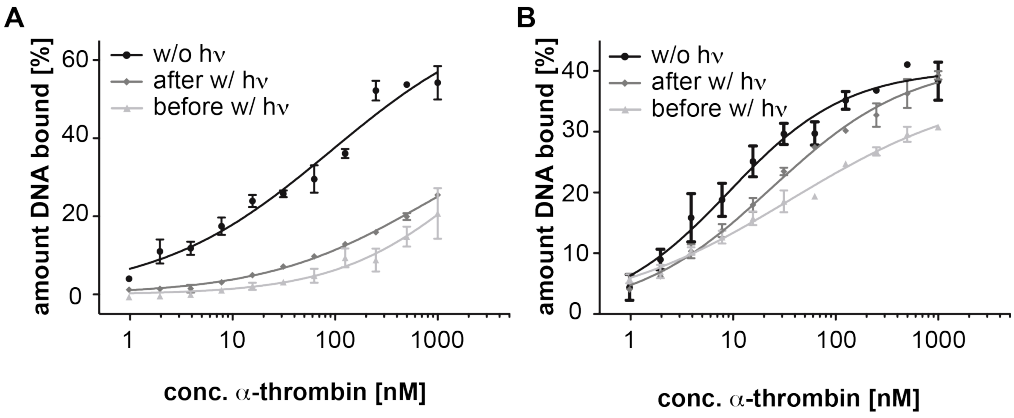

Figure III-3 LIGHT-DEPENDENT INTERACTION OF THE CAGED APTAMERS 5.3AMP1 AND 5.3AMP WITH THROMBIN. A) Filter retention analysis of 5.3amp1 with increasing concentrations of thrombin without (w/o hν) and after (after w/ hν and before w/ hν) irradiation (λ = 365 nm). Irradiation was performed either before (before w/ hν) of after (after w/ hν) incubation with thrombin. B) Filter retention analysis of 5.3amp with increasing concentrations of thrombin without (w/o hν) and after (after w/ hν and before w/ hν) irradiation (λ = 365 nm). Irradiation was performed either before (before w/ hν) of after (after w/ hν) incubation with thrombin. After passing through nitrocellulose [0.45 µm] the retained amount of labeled HD22 was determined by phosphorimager analysis and quantified using ImageQuant software.

III.V.II. DIRECT AND SANDWICH APTA-PCR

In a first step, we established a direct assay format, in which thrombin was directly physically adsorbed on the surface of microtiter plate wells. Thrombin-coated wells were subsequently incubated with increasing concentrations [0.025 nM to 500 nM] of 5.3amp, washed and the bound 5.3amp1 was then eluted by UV-exposure (λ = 365 nm). The supernatant was removed and directly assayed by qPCR. The optimized concentration of caged 5.3amp1 was determined to be 250pM. At this concentration, the maximum difference between controls (absence of thrombin) and the lowest detectable concentration of thrombin was achieved, whilst the threshold cycle (Ct) of the control equaled that of the non-template control (NTC). A detection limit of 6 femtomoles thrombin (120 pM) was achieved (Figure 3A).

Secondly, the sandwich variant of the apta-PCR was employed. This makes use of a 3'-biotinylated HD1 aptamer, immobilized on streptavidin coated microtitre plate wells. These were exposed to increasing concentrations of thrombin [0 – 10 nM] and after incubation and washing, the 5.3amp1 was added. The optimal concentrations of both aptamers were determined through investigating different concentrations of one aptamer while the other was kept constant. Interestingly, the optimal concentration of 5.3amp1

was found to be tenfold higher in the sandwich-assay compared to the direct assay approach, which can be attributed to the fact that lower levels of thrombin had been captured by the immobilized aptamer HD1. Concentrations of 5.3amp1 concentrations lower 2.5 nM result in an increase of the Ct-value comparable to those obtained from controls. After thorough washing, the bound 5.3amp1 was photo-eluted and directly used as a template for qPCR. The detection limit was determined to be 2 femtomoles (40 pM), slightly improved as compared with the direct assay format (Figure 3B). Both assays revealed specific thrombin based detection, since control experiments using BSA or activated protein C (APC) instead of thrombin resulted in no signals (Figure 3C). To evaluate the elution efficiency of the photo-elution approach, from both the direct and the sandwich apta-PCR, the 5.3amp1 aptamer recovered from 10pM of thrombin through different elution methods was quantified via standard qPCR. Interesting, the results suggest than in terms of efficiency photo-elution was comparable to the thermal elution, being about four times more efficient then the elution at room temperature (Figure 3D).

Having demonstrated the photo-aptaPCR formats we analyzed whether this approach allows the quantification of thrombin and its successive qualitative and functional assessment. To this end, the activity of thrombin in both aptaPCR assays was monitored before and after elution through cleavage analysis of PEFALFUOR-TH (H-D-cyclohexylalanyl-alanine-arginine-7-amino-4-methylcoumarin), a fluorogenic thrombin substrate (Figure 4).[30] Using this approach we demonstrated that the photo-elution approach facilitates the quantitative detection of thrombin and that photo-elution of the caged aptamer does not interfere with the structural and functional integrity of thrombin, whilst thermal elution results in complete thrombin inactivation. The use of caged aptamers and, thus, a photo-elution step for aptaPCR is very promising and avoids the disadvantages encountered through chemical or thermal release procedures.

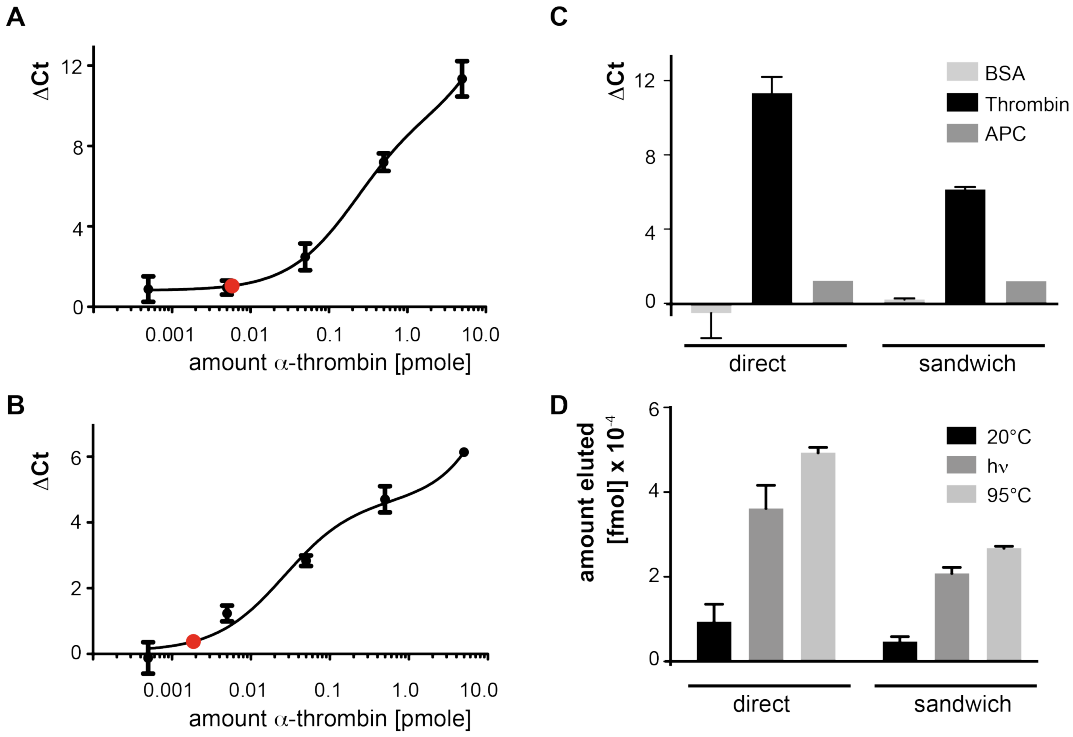

Figure III-4 THE PHOTO-APTAPCR ASSAY. Determination of the limit of detection (LoD) indicated by the red dots of the direct (A) and sandwich-type (B) photo-apta-PCR assay. ΔCt-values in dependence of increasing amounts of thrombin are given. C) ΔCt-values determined using 5 pmoles of BSA (light grey bars), thrombin (black bars), or APC (activated protein C, dark grey bars) as analytes in the direct (left) and sandwich-type photo-apta-PCR assay. D) Determination of the amount of 5.3amp1 detected in dependence of different elution methods.

Furthermore the use of chemical release, for example by employing chaotroping reagents such as urea, requires their subsequent removal or neutralization prior to qPCR analysis, which in turn results in dilution of the eluted aptamer and often requires precipitation of the template prior to further analysis. Thermal release generally causes protein denaturation and degradation and, thus, protein fragments are frequently present in the template fraction, thus also necessitating precipitation of the eluted DNA template prior to further analysis. Photo-elution, however, requires only a short UV-exposure and no further sample treatment is required. The possibility of maintaining structural and functional integrity following analysis may enable many applications in diagnostic and proteomic approaches, where small amounts of isolated proteins have to be detected and quantified and then further investigated regarding functional integrity.

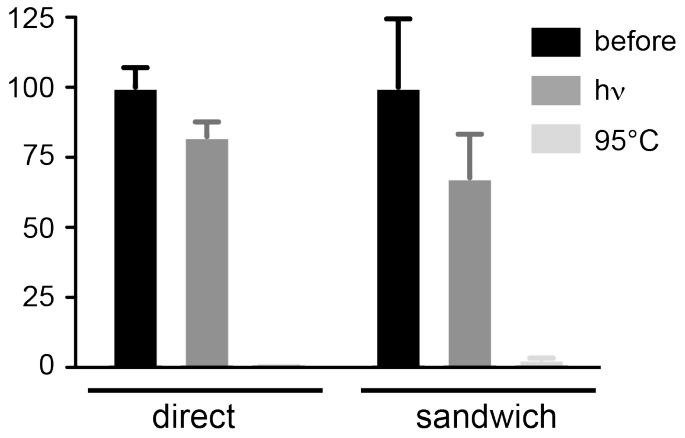

Figure III-5 THROMBIN ACTIVITY IN DEPENDENCE OF DIFFERENT ELUTION METHODS. Thrombin protease activity was measured by means of the cleavage of a fluorogenic peptide substrate after thermal elution (95°C, light grey bars) and after photo-elution (hn, dark grey bars) in either the direct (left) or sandwich-type (right) assay. The values obtained prior elution were normalized to 100% (before, black bars).

III.VI | CONCLUSIONS

In summary we have demonstrated a novel application of caged aptamers as light-dependent templates for apta-PCR. Caged aptamers enable the disruption of analyte and aptamer complexes by irradiation with light (λ = 365 nm). The advantages of this approach, compared to conventional apta-PCR assays, are given through a straightforward qPCR format, where no purification steps of the eluted aptamer is necessary and, thus, there is minimal loss of the reporter aptamer prior to quantification. Moreover, the elution by irradiation maintains the analyte in its native state allowing it to be used for further testing. This has been exemplified in our study, where we investigated the functional integrity of thrombin before and after irradiation and elution of the aptamer for qPCR amplification. The approach introduced in this study can be applied to virtually every aptamer-target pair and, thus, it can be generalized to measuring a diverse set of clinically relevant proteins and biomarkers. This approach may be also applicable to analyze proteomes with spatiotemporal resolution.

III.VII | ACKNOWLEDGEMENTS

AP acknowledges financial support by Ministerio de Educacion y Ciencia, Spain for an FPI doctoral fellowship (BES-2007-16431). The work was supported by grants from

the German research Council (DFG, Ma 3442/1-1 and Ma 3442/1-2) to GM and (EXC 115 and HE 4597/3-1) to AH. The authors thank Jens Müller for technical support and provision of the fluorogenic peptide substrate Pefafluor TH.

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III.IX | SUPPLEMENTARY INFORMATION

1. Competition assays

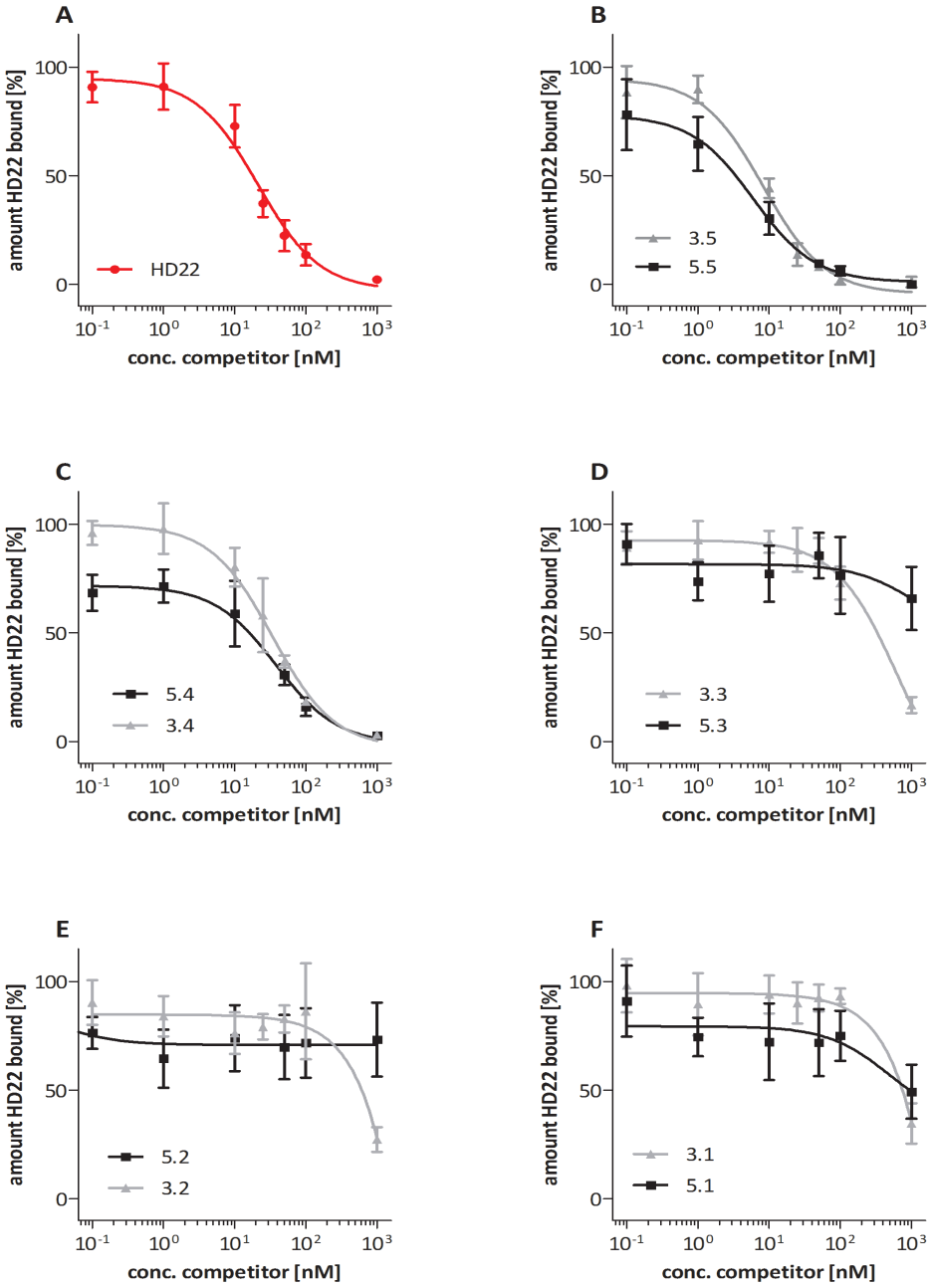

Figure III-6 Supplementary Figure 1: COMPETITION OF HD22-THROMBIN INTERACTION BY HD22-VARIANTS: DOSE-RESPONSE CRUVES. To evaluate the impact of the 5′- (black) and 3'- (grey) extensions filter retention analysis was performed by incubating radioactively labeled HD22 with thrombin [10 nM] in the presence of increasing concentrations [from 0 to 1000 nM] of each variant. After passing through nitrocellulose [0.45 µm] the retained amount of labeled HD22 was determined by phosphorimager analysis and quantified using ImageQuant software.

2. Competition curve of HD22amp

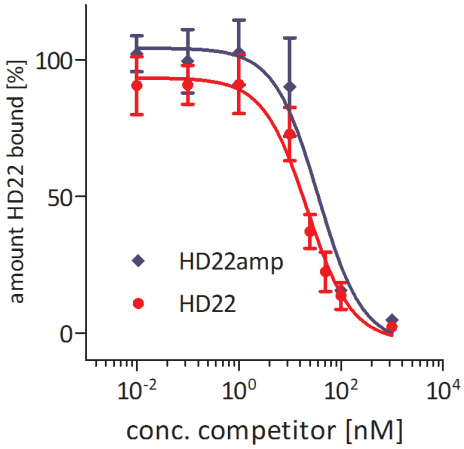

Figure III-7 Supplementary Figure 2: COMPETITION OF HD22-THROMBIN INTERACTION BY HD22AMP OR UNLABELLED HD22: DOSE-RESPONSE CURVE. To evaluate the effects of both primer regions – at 5' and 3'end-, filter retention analysis was performed by incubating radioactively labeled HD22 with thrombin [10 nM] in the presence of increasing concentrations [from 0 to 1000 nM] of HD22amp variant (dark blue line) or unlabeled HD22 wild type (red). After passing through nitrocellulose [0.45 µm] the retained amount of labeled HD22 was determined by phosphorimager analysis and quantified using ImageQuant software.

3. Binding isotherm of 5.3 and 3.3 compared to HD22

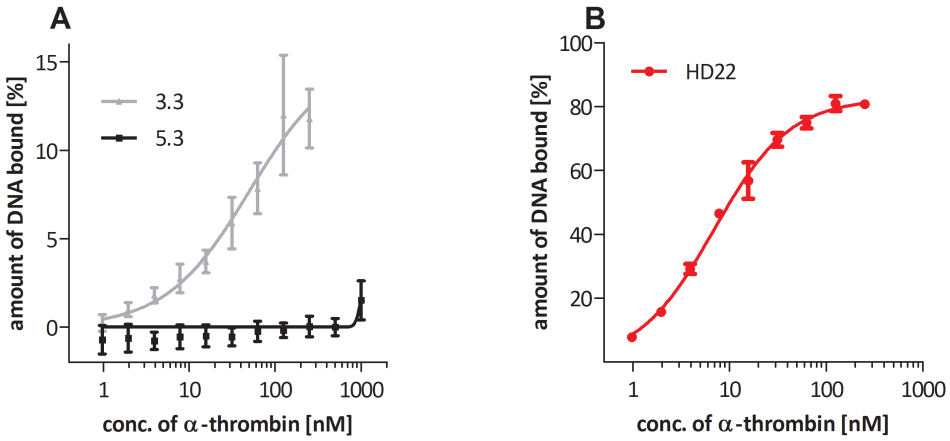

Figure III-8 Supplementary Figure 3: BINDING CURVES OF THE 3.3 AND 5.3 VARIANT IN COMPARISON WITH THE WILD TYPE HD22. A) Filter retention analysis of the 5.3 (black) and the 3.3(light grey) with increasing concentrations of thrombin (from 0 to 1000 nM). B) Filter retention analysis of the wild type HD22 aptamer with increasing concentrations of thrombin (from 0 to 1000 nM). After passing through nitrocellulose [0.45 µm] the retained amount of labeled aptamer was determined by phosphorimager analysis and quantified using ImageQuant software.

Chapter IV | SELECTION OF APTAMER AGAINST GLIADIN AND DETECTION MEDIATED BY APTAPCR

Alessandro Pinto, Pedro Nadal Polo, Marketa Svobodova, Maria del Carmen Bermudo Redondo, Ciara K. O'Sullivan; *to be submitted to ACS Analytical Chemistry*

IV.I | ABSTRACT

Celiac disease (CD) is an immune mediated enteropathy triggered by the dietary gluten ingestion. So far, the only possible treatment is a lifelong gluten-free diet, which implies the need for detection methods of gluten in food. Herein we described the selection of the first DNA anti-gliadin aptamer reported to date, and its use for the detection of gliadin in foodstuff. Aptamers are short nucleic acids that bind to target molecules with high affinity and specificity. The aptamer selection was achieved by using the Systematic Evolution of Ligand by EXponential enrichment (SELEX) process, which permitted the screening of a vast library of a synthetic single stranded DNA library (10^{13} members) through the repetition of different rounds of amplification and selection. After eight rounds of selection the enriched library was cloned and sequenced identifying a high conserved sequence. This sequence was characterized for its specificity and used to set up a competitive aptaPCR assay. Here different concentrations of the analytes free in solution compete for the selected aptamer with a standard concentration of the target adsorbed on a microtiter plate. Upon its elution from the plate, the aptamer served as template for qPCR, achieving a detection limit of 100ng/mL.

IV.II | INTRODUCTION

Celiac disease (CD) is an immune mediated enteropathy triggered by the ingestion of dietary gluten ingestion. Characterized by symptoms that can range from nausea, diarrhoea and weight loss to growth problems, celiac disease affects at least the 1% of worldwide population[1]. To date, Celiac disease has no cure and the only possible treatment is a lifelong gluten-free diet. Gluten-free foods are classified in two different categories: the first comprise those foods that naturally do not contains gluten, but could contain gluten due to accidental contamination in the production-process; the second comprise those foods that are rendered gluten-free. According to the Codex Alimentarius, the threshold of gluten in naturally gluten free-foods is 20mg/Kg dry mass, while the maximum content of gluten for foods rendered gluten-free is 200mg/K[2]. In both cases it is evident that gluten-free foods should be carefully verified through sensitive and reliable methods for the quantification of any gluten present. Hitherto, various Enzyme Linked ImmunoSorbent Assay (ELISA) approaches have been developed and are recommended due to their specificity and sensitivity[3].

ELISA relies on antibodies, which while being highly specific and having high affinity they require the sacrifice of host animals and are relatively expensive. Furthermore, they suffer from interference from reducing agents routinely used to extract protein from foodstuffs.

In the early 2000s an alternative analytical approach was proposed, which relies on the quantification of DNA biomarkers through the polymerase chain reaction (PCR). The method exploits the high specificity and sensitivity of PCR to detect specific DNA biomarkers in gluten-rich plant cells. Despite the potential of the PCR, the method fails to determine the total gluten content, allowing the identification and the quantification of plants cells used in the production instead.

Herein we propose the use of nucleic acid aptamers for the detection of gluten in foodstuffs. In the last two decades, nucleic acid aptamers have emerged as powerful competitors to antibodies for use as diagnostic reagents. Aptamers are short nucleic acids that interact with target molecules with high affinity and selectivity[4], usually identified by an in vitro selection approach, termed SELEX (Systematic Evolution of Ligands by EXponential enrichment)[5]. Aptamers were reported to interact with a

diverse set of target molecules, such as peptides, proteins, drugs, organic and inorganic molecules or even whole cells[6-10]. Many of the aptamers detailed to date reveal affinities for their target molecules, comparable to, if not better, than respective monoclonal antibodies with picomolar Kd-values[10, 11]. Furthermore, the specificity of aptamer recognition has been observed, which allows 10,000-fold to 12,000-fold[10, 12] discrimination of cognate target molecules and related structures. The synthetic chemical access of aptamers enables their site-directed modification, which clearly sets them apart from antibodies.

Due to their nucleic acid nature an approach termed aptaPCR has recently been developed. AptaPCR employs the dual function of aptamers acting both as a selective ligand for target molecules and as a template for qPCR thus acting both as binding and reporter molecule. Aptamers are exceptionally well suited for this approach and virtually every aptamer identified to date can be applied to aptaPCR without laborious optimization procedures. Most importantly, this approach bypasses the mandatory conjugation of antibodies with DNA-reporter tags exploited in immuno-PCR, which can interfere with the recognition properties of antibodies.

With the aim of achieving the cost effective and sensitive detection of gluten, herein we describe the first example of specific gluten detection mediated by nucleic acid aptamers.

IV.III | EXPERIMENTAL SECTION

IV.III.I. *REAGENTS*

PAGE purified DNA pool and HPLC purified primers were purchased from Biomers.net GmbH (Germany). Individual DNA sequences were synthesized and HPLC purified (Ella Biotech, Martinsried, Germany). Whole Gliadin, bovine serum albumin (BSA) and rabbit anti-wheat gliadin polyclonal antibody developed in rabbit were purchased from Sigma-Aldrich (Spain); streptavidin modified magnetic beads (streptavidin-Mb) were from Chemicell GmbH (Berlin, Germany) beads, E. *coli* tRNA was purchased from Roche Diagnostics (Germany). Taq DNA polymerase and all PCR reagents were purchased by Invitrogen (Madrid, Spain), B&W buffer (binding and washing buffer) consists of 10 mM Tris–HCl (pH7.5),1mM EDTA, 2M NaCl; Selection Buffer (SLX buffer) consist of 0.01 M phosphate buffered saline buffer pH 7.4 (PBS) purchased from Sigma-Aldrich (Spain) supplied with 3mmol/L $MgCl_2$.

IV.III.II. *APTAMER SELECTION*

Plate preparation

Before each round of selection, 1 mg gliadin powder was dissolved in 1 mL PBS, stirred at RT for 30 minutes and centrifuged at 6000g for 10 minutes and the pellets re-suspended in 60% (v/v) ethanol. Following incubation for 20 minutes under stirring conditions, the solution was further centrifuged. Afterwards the supernatant was diluted in carbonate/bicarbonate buffer (0,05M, pH 9.6) to a final concentration of 10 µg/mL, and 200 µL of this solution were incubated in each well of a NUNC Maxisorp microtiter plate (200µL) overnight at 37°C. After three washing steps with selection buffer, the well was dried.

SELEX

In the first round of selection, 5 µL – 500 pmol- of the S1 pool (5'-GCCTGTTGTGAGCCTCCTAAC-Nx49-CATGCTTATTCTTGTCTCCC-3') were heated to 95ºC for 3 minutes and subsequently cooled on ice for 10 minutes. The aliquot was then diluted in SLX buffer supplied with 1mg/mL of BSA and 10µM of tRNA. The

pool was incubated for 30 minutes at room temperature in the presence of the target previously immobilized on the microtiter plate. Following incubation the supernatant was carefully removed and the well washed three times with 200 µL of selection buffer. Finally, the bound DNA was eluted in water via the addiction of 50µL of Milli-Q water to each well and the plate was heated to 95ºC for 3 minutes. Upon collection of the supernatant, the elution was repeated. Finally the eluted DNA (100µL) was used as a template for PCR amplification in the next round of SELEX. In order to increase the selection stringency, in the second round a one-minute incubation in milli-Q water was introduced. In the third round three further washing steps with selection buffer were performed and in the fourth round a further incubation with milli-Q water was added. In this way, in the eighth round the microtiter plate was washed 24 times in selection buffer plus 4 incubations in milli-Q water (1 every 3rd washing).

PCR amplification

Following each cycle, the eluted DNA was used as a template for a 1000 µL final volume PCR reaction. The PCR mixture contains 0.35 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM forward primer (5'-GCCTGTTGTGAGCCTCCTAAC-3'), 0.15 µM biotinylated revers primer (5'biotin-GGGAGACAAGAATAAGCTG-3'), 0.5 mg/ml BSA and 10 Units of Taq polymerase. The amplification was performed in an iCycler thermal cycler (Biorad, Spain) programmed according to the following protocol: 2 minute at 95°C, followed by N repetitions of 30 seconds at 95°C, 30 seconds at 54°C and 30 seconds at 72°C. In the first cycle N=18, but starting from the second round a pilot PCR was performed in order to titrate the optimal number of repetition. In the pilot PCR 10 µL of the eluted DNA was added to 100µL final volume PCR reaction. Thus every 4 cycles of PCR an aliquot of the reaction was taken. Finally the quantity and quality of the PCR product was evaluated using gel electrophoresis.

Strand Displacement

2.5 mg of streptavidin-Mb were taken from the stock and the storage buffer was removed via magnetic separation (Dynal Sample Mixer, Invitrogen, Madrid, Spain). Subsequently the Mbs were washed three times with 1x B&W buffer (250 µL each wash) and re-suspended in 500 µl of PCR-product and 500 µL of 2x B&W buffer. Upon incubation for 30 minutes at room temperature under moderate shaking, the sample was briefly centrifuged and the supernatant was removed using magnetic separation. After

three washes in B&W buffer, the dried Mbs were re-suspended in 100 µL of 0,1M NaOH and incubated for 3 minutes under gentle shaking conditions. The supernatant was finally recovered, titrated with 0.5M HCl and ethanol-precipitated, by the addition of three volumes of 99% v/v ethanol and 1/10 volume of sodium acetate 5M, and incubation for 2 hours at -20ºC, followed by 15 minutes centrifugation at 15000 rpm, drying and resuspension in 500 µL of 70% v/v ethanol and again centrifuged for 5 minutes. The resultant pellet was finally re-suspended in the desired buffer.

Monitoring of SELEX Evolution through Surface Plasmon Resonance (SPR) analysis

BIAcore 3000 (GE Healthcare) with the BIAevalution software was used for SPR experiments. Proteins of interest were immobilized, via amine coupling, on separate channels of a CM5 sensor chip. Fist the chip was activated by EDC/NHS followed by an injection of the protein to be immobilized (5 µl/min for 10 min). After immobilization of the protein, unreacted NHS esters were deactivated by injecting an excess of ethanolamine hydrochloride. Unspecifically bound proteins were then washed from the surface with 100 mM NaOH. The single stranded DNA pool from each cycle of SELEX was diluted in selection buffer, to a final concentration of 50nM, and injected for 6 minutes at a flow rate of 10 µl/minutes followed by 3 minutes stabilization time and 2 minutes dissociation time. The binding of DNA was analyzed through corresponding changes in the refractive index of optical signals, and expressed as resonance units (RU). All reagents and buffers were prepared in milliQ water and were previously filtered.

Cloning and Sequencing

DNA was cloned into the plasmid pCR2.1 using the TOPO TA Cloning® kit (Invitrogen, Spain) according to the manufacturer's instructions. Colonies were subsequently selected and grown overnight in a culture of 5 ml LB medium under vigorous shaking. Plasmid clones were purified with a QIAprep Spin Miniprep kit (Qiagen, Spain). Purified plasmid DNA were sequenced through the GenomeLab DTCS Quick Start Kit (Beckman Coulter) according to the manufacturer's instructions, and analyzed in a CEQ8000 Beckman Coulter instrument. The sequences derived from SELEX procedure were aligned using Clustal (http://www.clustal.org) and T Coffee (http://tcoffee.crg.cat/) softwares.

IV.IV | APTAPCR

Characterization of the candidates

A range of gliadin concentrations (0 to 5 µg/mL in 0.05 M carbonate/bicarbonate buffer pH 9.6) were incubated on the wells of a microtiter plate (50µL/wells) for two hours at 37°C. Following incubation, the plate was washed three times with the selection buffer (200 µL/wells each wash), and the surface was blocked by incubating the plate for 1 hour at 37°C in the presence of BSA. Subsequent to a further washing step, the selected sequences were added to each well and incubated at RT for 30 minutes (50 µL/well, 500 nM final concentration) in the presence of 10µM of tRNA, followed by a final wash with the selection buffer (800 µL/well). Finally, following the addition of milliQ water (50µL/well), the reporter aptamer was heat-eluted.

Development of the competition assay

10µg/mL whole Gliadin (in 0.05 M carbonate/bicarbonate buffer pH 9.6) was incubated on the wells of a microtiter plate (50µL/wells) for two hours at 37°C. Following incubation, the plate was washed three times with the selection buffer (200 µL/wells each wash), and the surface was blocked by incubating the plate for 1 hour at 37°C in the presence of BSA and 0.05% v/v Tween-20. Subsequent to a further washing step, while the plate was incubated in presence of tRNA for 1 hour at 37ºC, off plate 100nM G33 were incubated with different concentration of gliadins (from 0 to 50µg/mL). Following the washing of the plate, the G33 pre-incubated with gliadin free in solution was incubated on the plate, at RT for 30 minutes (50 µL/well) in the presence of 10µM of tRNA. Following a final wash with the selection buffer (800 µL/well), milliQ water (50µL/well) is added, and the reporter aptamer heat-eluted.

qPCR

1µL of the elution fraction was added to 19 µL of a PCR master mix, which consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3mM $MgCl_2$ 100nM of forward and reverse primer, 1x final concentration SYBR Green I (Sigma-Aldrich,Spain), 10nM FAM (Bio-Rad, Spain) and 0.25 units of Taq DNA Polymerase. The amplification was performed in a HD 7900 thermal cycler thermal (Applied Biosystems, Spain)

programmed according to the following protocol: 1 minute at 95°C, followed by N repetitions of 30 seconds at 95°C, 30 seconds at 54°C and 30 seconds at 72°C.

IV.IV.I. EXTRACTION OF PROLAMINS

Each flour sample (Corn, Oats and Wheat) was weighed out in 100 mg aliquots. Flour samples were washed twice with 0.067 M phosphate buffer (in 0.4 M NaCl, pH = 7.6) to remove the albumins and globulins and phosphate buffer was then added to flour aliquots to a final concentration of 100 mg/ml. The suspension was then vortexed for 2 min at RT, followed by magnetic stirring for 15 min and spun down at 6000 g for 20 min. Prolamins were extracted twice with 60% ethanol via addition of 1 ml of extractant to the flour pellets. The suspension was then vortexed vigorously at room temperature over a period of 30 min and spun down at 6000 g for 20 min.

IV.V | RESULTS AND DISCUSSION

IV.V.I. *SELEX AGAINST WHOLE GLIADIN*

A SELEX process can be described as the repetition of two different steps: partitioning and amplification. Partitioning is the core of the whole process: here the pool of nucleic acid is selected according to its binding to target and thus it is crucial to separate the bound sequences from the unbound ones. Generally, such separation occurs by using two different phases: a solid phase where the target-candidate aptamer is retained and a liquid phase where the unbound sequence is contained to be easily eluted and discharged. As gliadin is highly hydrophobic in nature, microtiter plates were used as the solid phase to immobilize the target: gliadins assume a well-ordered orientation when adsorbed onto a hydrophobic surface [13], preferable to the random orientation otherwise assumed, e.g. when covalently attached to a solid support.

In the selection method used, the DNA pool was added to each wells of gliadin immobilized microtiter plate. The starting pool consisted of 500 pmol of a PAGE purified ssDNA pool- with an estimated complexity of 10^{14} different molecules-. In each selection round prior to incubation with the target, the pool underwent heating followed by rapid cooling, to unfold any pre-existing structures, formed due to intra- and inter- molecular pairing. The pool was diluted in the selection buffer with the tRNA and the BSA blocking agents and incubated with the immobilized gliadin for 30 minutes at room temperature. After which the unbound sequences were removed in the supernatant, and following multiple washing steps, the sequences bound to the immobilized gliadin were eluted in 95°C hot water and amplified. In order to increase the stringency of the selection, the washing conditions became more extensive round by round: starting with three washings in binding buffer and ended by washing the plate twenty-four times in the binding buffer plus four additional times in milliQ water, to reduce weakly or unspecifically bound sequences[14]. Following its elution, the selected pool was amplified using PCR. While in the first cycle 18 cycles of amplification were used, in the following rounds the number of cycles was experimentally titrated, and as the process advanced the concentration of eluted DNA increased until only 9 amplification cycles were required in the eighth round. To titrate the number of amplification cycles pilot PCR was performed, were a small aliquot of the eluted pool

was amplified in a range of 5 to 20 PCR cycles in order to decide the desired conditions of amplification to maintain the same amount of DNA for each cycle of SELEX. Following the final PCR, the resulting dsDNA pool was divided into two aliquots, one used in the subsequent round of selection, following generation of single stranded DNA and the other stored for the evolutionary studies. After eight SELEX rounds, the binding proprieties of the DNA pools from each round were evaluated using Surface Plasmon Resonance (SPR). Gliadin, anti-gliadin polyclonal antibody and streptavidin were each immobilized on different channels of a BIAcore CM5 chip, which consists of a four channels chip with a carboxy-methylated dextran modified gold surface. A reference channel containing only ethanolamine, used as blocking agent, was also prepared. The DNA pool from the last five selection rounds was amplified and 100nM ssDNA of each round was purified and injected into the four channels of the CM5 chip. The signals for all the three unspecific channels were similar and lower than the specific signal in gliadin channel, which was observed to increase after each round of SELEX (Fig 1).

Once evolution had been established, the last round was cloned and sequenced. In the enriched library a small family of three members from 25 total sequences obtained was identified and referred to as G3, characterized by a high homology of the members (Fig.2).

IV.V.II. DEVELOPMENT OF THE APTAPCR ASSAY

The sequences obtained were tested for their ability to bind to gliadin adsorbed over a plate surface. Three different concentration of gliadin were immobilized on the microtiter plate. Subsequently 500nM of candidate aptamers were incubated for 30 minutes. Following three subsequent washings, the bound DNA was heat-eluted and used as a template for the qPCR reaction.

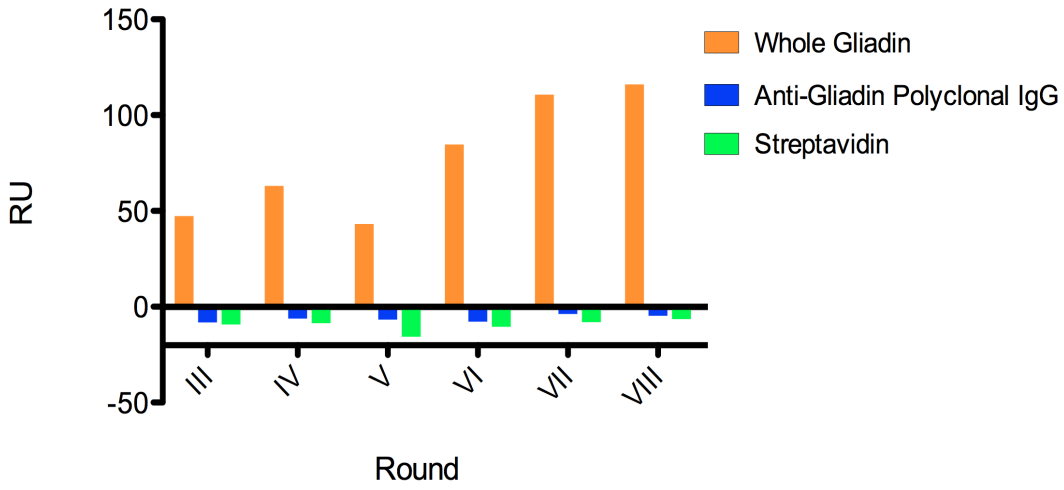

Figure IV-1 EVALUATION OF THE BINDING PROPERTIES OF THE SSDNA POOLS AFTER EACH ROUND OF SELEX. RU reached at the plateau of the association curve for all different channels of the Biacore CM5 chip with DNA isolated from various rounds of SELEX (from the third to the eighth) using channels coated with whole gliadin, anti-gliadin Polyclonal IgG and streptavidin. Reference and buffer responses were subtracted.

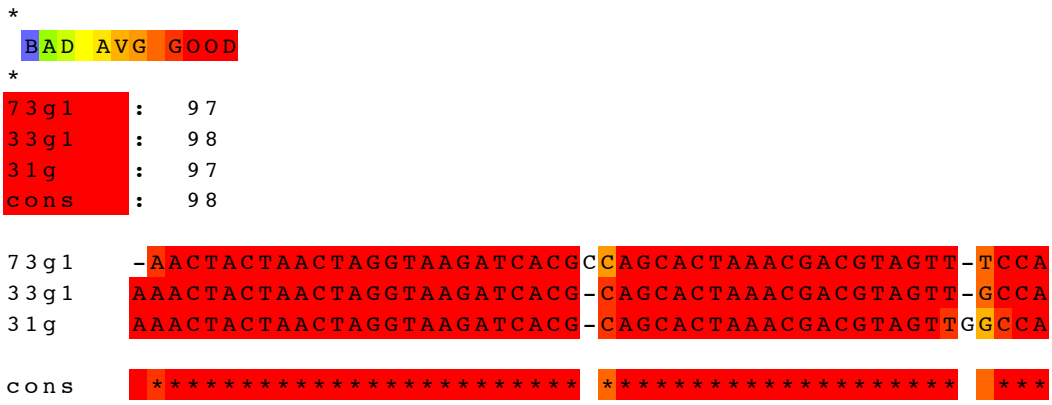

Figure IV-2 ALIGNMENT OF THE G3 SEQUENCES, PERFORMED USING TCOFFEE.

The sequence G33 belonging to the family G3, was observed to be the best ligand, and was also observed to be able to discriminate gliadin from BSA and anti-gliadin IgG (Fig.3a and b).

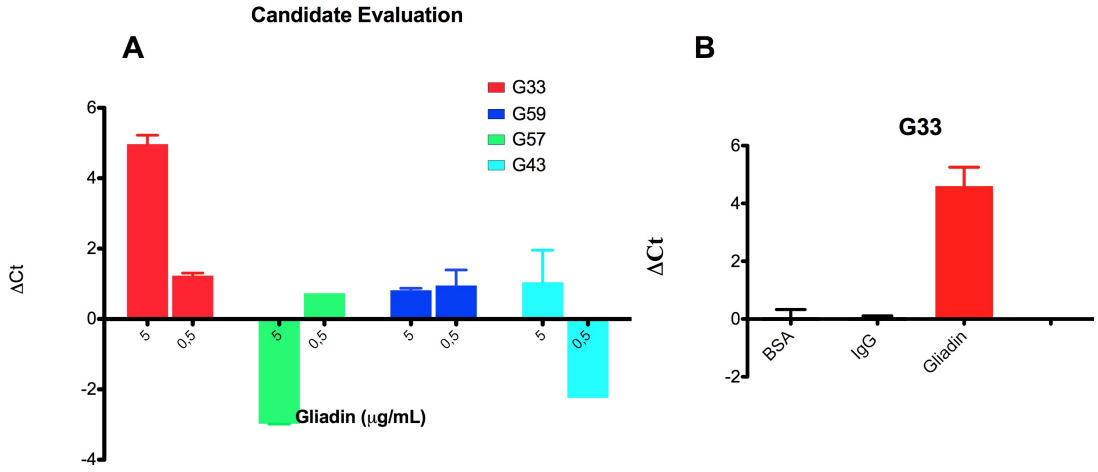

Figure IV-3 Candidates evaluation using aptaPCR. (A) ΔCt-values determined using four different candidates incubated with three different concentration of gliadin, from 5 to 0 μg/mL.(B) ΔCt values determined using 5μg/mL of BSA, antiglaiding polyclonal antibody (IgG) and Glaidin incubated with G33.

With the aim of optimizing the assay, the concentration of the G33 was titrated (Supplementary Figure 2) and subsequently a competition assay was developed. Following the immobilization of 10μg/mL gliadin, the plate was blocked with 0.1% w/v of BSA, then thoroughly washed, followed by addiction of tRNA to minimize the unspecific adsorption of G33. Meanwhile, off plate 100nM G33 was incubated with different concentrations of gliadin (50 to 0 μg/mL) in the selection buffer and then added to each wells of the microtiter plate, where the gliadin in solution competes with the gliadin adsorbed on the plate for the binding of the aptamer. Finally the plate was washed and the eluted aptamer used as a template in qPCR. The limit of detection achieved was ≈ 100 ng/mL (Fig.4.A). As can be seen in figure 4B using either BSA or the albumin/globulin fraction extracted from the wheat flour or the prolamins extracted from corn and oats flours no cross-rectivity was observed as a solution of 30μg/mL of these proteins did not compete with the immobilized gliadin for the aptamer (Fig.4.B), highlighting the specificity of the selected G33 aptamer.

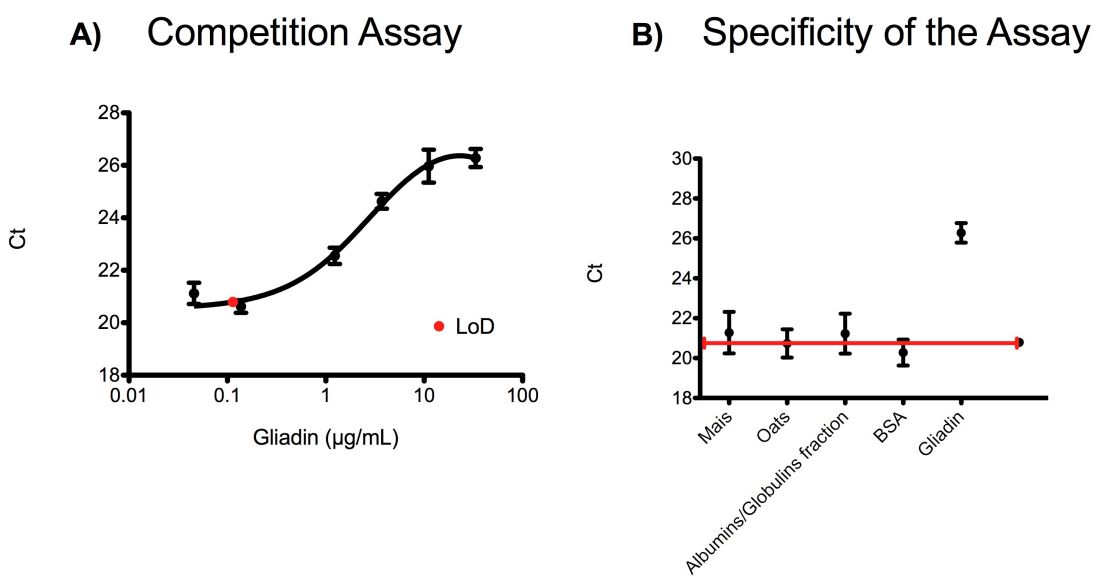

Figure IV-4 Gliadin detection through competitive assay. A) Determination of the Limit of the detection (LoD) indicated by the red dot. B) Ct value determined using 30 µg/mL of different proteins. The red line indicates the Limit of Detection calculated from two independent experiments.

IV.VI | CONCLUSIONS

Herein, we described the selection of an aptamer against food allergen gliadin - one of the first examples of an aptamer selected against a food allergen. Gliadin represents a particularly challenging target for aptamer selection due to its highly hydrophobic nature. However, this hydrophobic nature was exploited for its immobilization and a highly specific apatmer was successfully selected. The selected aptamer was applied to the detection of gliadin. Using aptaPCR, where the reporter aptamer was amplified using real time PCR, a detection limit of as low as 100ng/mL was achieved, and the aptamer showed negligible cross-reactivity to potential interferents. This is the first example of the use of aptamer for food safety in the apta-PCR methodology and further demonstrates the wide potential use of the approach in diverse areas such as clinical diagnostics, food quality control and environmental monitoring

IV.VII | ACKNOWLEDGEMENTS

AP acknowledges financial support by Ministerio de Educacion y Ciencia, Spain for an FPI doctoral fellowship (BES-2007-16431). The authors thanks the Servei de

Recursos Científics i Tècnics of the Universitat Rovira I Virgili and Dr Julia Ellis for their support.

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IV.IX | SUPPLEMENTARY INFORMATION

1. Sequences obtained from the sequencing

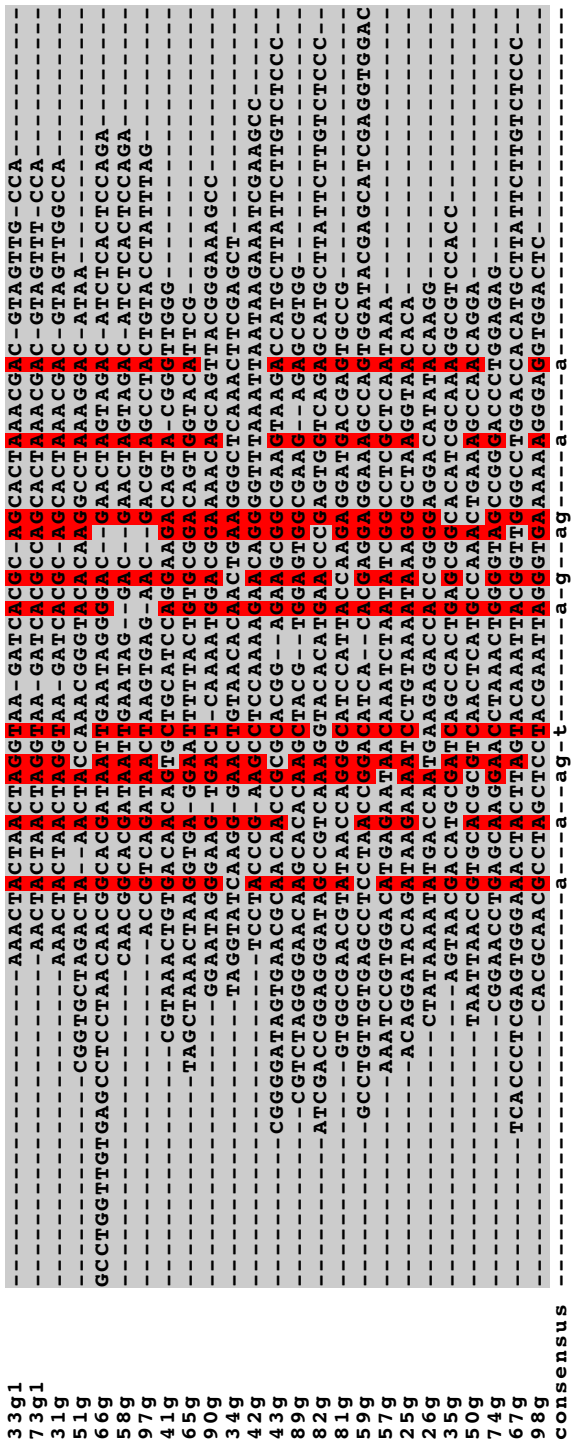

Figure IV-5 Supplementary Figure 1. SEQUENCES OBTAINED FROM THE SEQUENCING OF THE EIGHTH ROUND OF SELEX, ALIGNED THROUGH CLUSTAL X.

2. Tritation of the G33 aptamer to be used in the competition assay

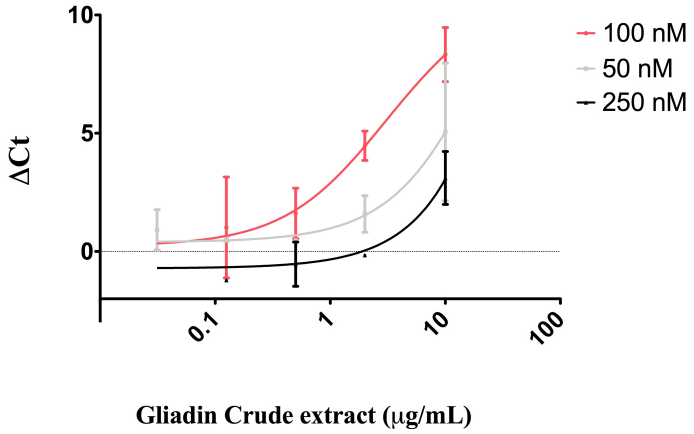

Figure IV-6 Supplementary Figure 2. TITRATION OF THE .G33 APTAMER TO BE USED IN THE COMPETITION ASSAY. To find the experimental conditions to be used in the competitive assay, different concentration of gliadins (from 0 to 100μg/mL) where immobilized on a microtiter plate and incubated with different concentrations of G33 aptamer.

Chapter V | CONCLUSIONS AND FUTURE WORK

The thesis introduces and characterizes a novel ultrasensitive detection approach referred to as aptaPCR. The assay relies on the use of nucleic acid aptamers as combined binding reporter elements, a solution that combines the specificity of a generic aptamer with the sensitivity of nucleic acid amplification avoiding the need for any kind of labelling.

In contrast to previously reported methods, the aptaPCR does not rely on the use of nucleases or ligases[1-6] enzyme, facilitating a generally applicable assay for any aptamer-target complex since there are no limitations imposed by the activity of an enzyme different from the polymerase. Likewise, the aptaPCR does not required the use of sophisticated equipment such as the capillary electrophoresis) [7]. Our work can be considered as a crucial improvement of the work developed by of Fisher *et al.*[8], as we merge the detection and the amplification propriety of a generic aptamer in a technique that can be applied to any type of ELISA format. In this thesis we used two different thrombin binding aptamers and their cognate target in a sandwich-type assay, where one aptamer was immobilized on a surface and captured the protein of interest which then exposed to a second aptamer (the reporter). Following elution of this reporter, it acts as a template for subsequent amplification, facilitating its detection. It was demonstrated that the novel approach yields a massive improvement (20000-fold enhancement) in terms of limit of detection, when compared with methods previously described. The reported approach is applicable to all aptamers, independent of whether there exists double aptamers against the target, and the technique can thus be applied to the detection of very low concentrations of target analytes, which is garnering ever-increasing importance in pre-emptive medicine and the early diagnosis of disease, as well as in food analysis control and environmental monitoring.

Subsequently it was demonstrated that the developed approach could be successfully used for the detection of ultralow levels of proteins that are not inactivated or disrupted by the detection itself. Further improving on the assay previously developed, we demonstrated a novel application of caged aptamers as light-dependent templates for aptaPCR. Caged aptamers enable the disruption of analyte and aptamer complexes by irradiation with light (λ = 365 nm). This approach, compared to other reported aptaPCR assays, is a far more straightforward qPCR format, where no purification of the eluted aptamer is necessary and, thus, there is minimal loss of the reporter aptamer prior to quantification. Moreover, the elution by irradiation maintains

the analyte in its native state allowing it to be used for further testing. This has been exemplified in our study, where we investigated the functional integrity of thrombin before and after irradiation and elution of the aptamer for qPCR amplification. The approach introduced in this study can be applied to virtually every aptamer-target pair and, thus, it can be generalized to measure a diverse set of clinically relevant proteins and biomarkers. This approach may be also applicable to analyze proteomes with spatiotemporal resolution.

Finally, further extending the applicability of the aptaPCR established, the detection of the food allergen gluten was described. The first DNA anti-Gliadin aptamer so far described was selected and subsequently used in the detection of its cognate target through the aptaPCR approach. The aptamer selection was achieved using the Systematic Evolution of Ligand by EXponential enrichment (SELEX) process, which permitted the screening of a vast library of synthetic single stranded DNA library (10^{13} members) through the repetition of different rounds of amplification and selection. After eight rounds of selection the enriched library was cloned and sequenced identifying and a highly conserved sequence. This sequence was characterized for its specificity and used to set up a competitive aptaPCR assay. Here different concentrations of the analytes free in solution compete for the selected aptamer with a standard concentration of the target adsorbed on a microtiter plate. Upon its elution from the plate, the aptamer served as a template for the subsequent qPCR, permitting to achieve a limit of detection of hundreds ng/mL of analyte.

Overall, this thesis has contributed significantly to the development of assays using aptamer as a recognition element (apta-assay). We pioneered the development of a novel assay format, termed aptaPCR, which exploits the unique biochemical and chemical proprieties of the aptamers. Our assay mimics the commonly used immunoassay and can be widely used with any aptamer – target combination.

To extend the application of the developed aptaPCR, it's robustness and wide applicability should be addressed by application to the detection of thrombin in real samples, and extending to new aptamer-target combinations including other food allergens and clinical biomarkers. Furthermore, the combination of aptaPCR with caged aptamers could lead to a novel ultrasensitive detection assay for the capture of disseminated whole cancer cells, whilst keeping them intact for further analysis.

Concluding this dissertation, sometimes before the start of this work, in 2007 C.Stein, PhD and MD at Albert Einstein College of Medicine in New York, talking about aptamers declared: "*Aptamers ... are going to find their own niche, which I think could be very substantial.*"[9]. I belive that this thesis demonstrated that the aptaPCR assay will find such a niche for aptamers in analysis.

V.I | REFERENCES

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