SELECTION OF APTAMERS AGAINST PROSTATE SPECIFIC ANTIGEN FOR DIAGNOSTIC AND THERAPEUTIC APPLICATIONS

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DOCTORAL THESIS

UNIVERSITAT ROVIRA I VIRGILI

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Supervised by Dr. Ciara K. O´Sullivan

Department of Chemical Engineering

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CERTIFY:

That the present study, entitled "Selection of aptamers against prostate specific antigen for diagnostic and therapeutic applications" presented by Markéta Svobodová for the award of the degree of Doctor, has been carried out under my supervision at the Chemical Engineering Department of the University Rovira i Virgili, and that it fulfills the requirements to obtain the Doctor European Mention.

Tarragona, 16th Abril 2012,

Dr. Ciara K. O´Sullivan

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Summary

Cancer is one of the leading causes of death worldwide. Prostate cancer (PC) is the most common male cancer in Western Countries, and second most common cause of cancer death in men. The survival rate of cancer patients is substantially increased when they are diagnosed at an early stage. There are several prostate tumor markers such as human prostatic acid phosphatase, glutathione S-transferase Π, or α-methylacyl coenzyme A racemase. However, prostate specific antigen (PSA) is currently considered the best cancer marker available in clinical medicine. PSA is protein produced by the cells of prostate gland, is found in the blood of healthy men in very low quantities, but the levels of PSA are elevated in the presence of prostate cancer or other prostate disorders. For the detection of higher PSA levels, antibodies are routinely used. However, the production of antibodies requires animals, which are subjected to a number of invasive procedures. On the other hand chemically synthesized aptamers with specificity and affinity equal to those of antibodies can be an alternative approach how to detect PSA. Aptamers offers several advantages over antibodies, including more flexibility to different assay formats, which allows them to address the ever-increasing requirements of analytical applications. Several facets of aptamers such as their stability, ease of immobilization and labelling contribute to this enhanced flexibility and present them as attractive candidates for diagnostic and therapeutic applications.

The main objective of the work described in this thesis is the development of aptamers against PSA for their potential applications in diagnostics and therapeutics. To achieve this objective, several sub objectives such as the characterization of PSA, evaluation of different immobilization strategies, preparation of oligonucleotide library and single-stranded DNA (ssDNA) were performed.

This thesis is divided in seven separate chapters. A general introduction covering the different topics of the thesis is presented in Chapter 1. Chapter 2 details the characterization of PSA and the evaluation of different immobilization strategies, whilst Chapter 3 discusses the preparation of the oligonucleotide library and Chapter 4 compares different methods for generation of ssDNA for use in SELEX. Chapters 5 and 6 comprise the studies carried out for the development of DNA and RNA based aptamers against PSA.

In more detail, Chapter 1 provides a general introduction to the state of the art of the research area. This chapter sets out the objectives of the thesis and lists the main contributions obtained with the achievement of objectives.

The selection of aptamers depends highly on the target properties, therefore, in Chapter 2, the characterization of PSA obtained from seminal fluid was performed using techniques such as SDS-PAGE and Mass spectrometry. Target immobilization is one of the efficient partitioning methods used for the separation of target bound and non-bound nucleic acids in SELEX process. Different immobilization strategies were thus investigated in order to determine the best one for immobilization of PSA. Two promising strategies including (i) amine coupling using carboxyl modified beads and (ii) the use of the bioaffinity biotin – streptavidin system were demonstrated to be optimal for PSA immobilization.

Chapter 3 focuses on the preparation of an oligonucleotide random library. As the oligonucleotide random library consists of a multitude of ssDNA, this heterogeneity can cause non-uniform amplification during the polymerase chain reaction (PCR). Therefore, a correct choice of library and efficient PCR amplification of this selected library can contribute to the success of aptamer selection. PCR optimisation of three different libraries was carried out to ensure high purity and yield of the amplified libraries. The composition of random libraries changes during the SELEX process, and another PCR optimisation of the enriched libraries was thus carried out, and it was observed that the addition of several additives (dimethyl sulphoxide (DMSO), Betaine, Single Stranded Binding

protein) with optimised PCR protocols could prevent the production of undesired nonspecific products.

In Chapter 4, the comparison of different techniques generally used in the preparation of ssDNA was carried out. The generation of ss-DNA is a critical step in SELEX as the purity and yield can have a significant impact on the successful evolution throughout the SELEX process. Several techniques for the generation of ss-DNA for SELEX were compared, including the previously reported methods of asymmetric PCR, lambda exonuclease digestion and magnetic separation, as well as new methods such as the use of T7 Gene 6 exonuclease and the combination of asymmetric PCR and exonuclease digestion. The efficiency of each system for the generation of ss-DNA was quantitatively evaluated using an enzyme linked oligonucleotide assay (ELONA) and the quality of the PCR product was assessed using agarose gel electrophoresis. According to the results obtained, all reported methods can be used for the generation of ss-DNA, once their disadvantages are clearly taken into consideration. Asymmetric PCR followed by T7 Exonuclease digestion was clearly demonstrated to have the highest efficiency, achieving a final yield of ss-DNA around 85%. Furthermore, we have demonstrated that the yield of ss-DNA obtained from unpurified PCR product is comparable to that obtained from purified PCR product, overcoming a loss of ds-DNA during the purification step, and thus decreasing the time required for each SELEX cycle.

In Chapter 5, the selection process of DNA based aptamers against PSA is described. Variations of protocols were used to identify DNA aptamers, differing in the type of partitioning methodology, generation of ss-DNA, starting random library and buffer compositions. Several aptamer candidates were selected and analysed for their ability to bind to PSA. Two DNA aptamers (I1, I4) with dissociation constants in the μM range showed the lowest cross reactivity with control proteins such as BSA or streptavidin. For the evaluation of the functionality of aptamer I1, competitive Reporter Linked Aptamer Assay (RLAA) with different concentrations of PSA was used, achieving the limit of detection 268 nM.

Nevertheless, the low specificity of selected aptamer does not permit its use for clinical diagnostics.

Another possibility to select an aptamer against PSA was the selection of an RNA aptamer. The variety of conformations achieved by RNA molecules is theoretically more diverse, thus increasing the probability of finding an aptamer against PSA. RNA aptamers with a dissociation constant in the nM range were selected using an automated SELEX process. Moreover, the RNA aptamers were demonstrated to be specific and selective. The potential use of the selected aptamers for diagnostics was studied using apta-PCR, achieving a limit of detection of 11 nM for aptamer S2, demonstrating an improvement on the limit of detection achieved by the previously published DNA aptamer for PSA. For the future use of selected RNA aptamers in therapeutics, the stability of selected aptamers as well as potential effect on the activity of PSA was studied, indicating a high stability and moderate inhibitory effect of selected 2´F modified RNA aptamers.

Chapter 7 presents the main conclusions of the thesis, the principal contributions and some future research lines for the continuation of this research.

Resumen

El cáncer es una de las principales causas de muerte en todo el mundo. El cáncer de próstata (PC) es el cáncer masculino más común en los países occidentales, y la segunda causa de muerte por cáncer en los hombres. La tasa de supervivencia de pacientes con cáncer aumenta sustancialmente cuando se diagnostica en una etapa temprana. Hay varios marcadores tumorales de próstata tales como la fosfatasa ácida prostática humana, glutatión S-transferasa Π, o α-metilacil coenzima A racemasa, entre otros. Sin embargo, el antígeno prostático específico (PSA) es actualmente considerado el mejor marcador del cáncer disponible en medicina clínica. PSA es una proteína producida por las células de la glándula de la próstata que se encuentra en la sangre de los hombres sanos en cantidades muy bajas, pero los niveles se elevan en presencia de cáncer de próstata u otros trastornos de la próstata. Para la detección de niveles de PSA se utilizan habitualmente anticuerpos. Sin embargo, la producción de anticuerpos requiere animales, que están sometidos a una serie de procedimientos invasivos. Por otro lado, los aptámeros sintetizados químicamente con especificidad y afinidad iguales a los de los anticuerpos pueden ser la alternativa para la detección de PSA. Varias características de los aptámeros, tales como la estabilidad, facilidad de inmovilización y marcaje, hacen de ellos candidatos atractivos para aplicaciones con fines diagnósticos y terapéuticos. Además, su gran flexibilidad les permite su uso en diferentes formatos de ensayo para aplicaciones analíticas.

El objetivo principal de este trabajo es el desarrollo de aptámeros contra PSA para sus posibles aplicaciones en el área terapéutica y de diagnóstico precoz. Para completar estos objetivos, varios subobjetivos, como la caracterización del PSA, la evaluación de diferentes estrategias de inmovilización, la optimización del proceso de amplificación de ADN aleatorio y la comparación de diferentes técnicas para la preparación de la cadena sencilla de ADN se han propuesto.

Esta tesis se divide en siete capítulos. Una introducción general sobre los diferentes temas de la tesis se presenta en el Capítulo 1. El Capítulo 2 está relacionado con la caracterización de PSA y la evaluación de diferentes estrategias para su inmovilización, mientras que el Capítulo 3 trata de la optimización de la amplificación de ADN aleatorio. En el Capítulo 4, se presenta una comparación de diversas técnicas para la generación de ADN de cadena sencilla (csADN) para su posterior uso en SELEX. Finalmente, los Capítulos 5 y 6 versan sobre la selección de aptámeros de ADN y ARN contra el PSA. En último lugar se presentan las conclusiones generales.

El en Capítulo 1 se presenta introducción general, en el que se acotan, tanto el ámbito como el alcance del campo de investigación. En este capítulo, se plantean los objetivos de la tesis y se enumeran las principales aportaciones obtenidos con la consecución de los objetivos.

La selección de aptámeros depende en gran medida de las propiedades de la diana molecular, por lo tanto en el Capítulo 2, se ha realizado la caracterización del PSA utilizando técnicas como la electroforesis en gel de poliacrilamida con dodecilsulfato sódico (SDS-PAGE) y la espectrometría de masas. La separación de los ácidos nucleicos afines con la diana del resto de la librería es uno de los puntos críticos durante la selección *in vitro* de los aptámeros. En este contexto, la inmovilización de dicha diana es una de las metodologías de partición más eficaces y generalmente utilizadas. La extrapolación de las estrategias de inmovilización es compleja y por lo general resulta ineficaz principalmente debido a las características y propiedades específicas de cada diana. Por lo tanto, diferentes estrategias de inmovilización han sido evaluadas con el fin de determinar la mejor para la inmovilización del PSA. Dos estrategias prometedoras incluyendo (i) el acoplamiento peptídico con partículas magnéticas modificadas con carbodiimida (ii) y la técnica basada en el uso de la diana biotinilada con las partículas magnéticas modificadas con estreptavidina mostraron ser óptimas para la inmovilización de PSA.

El Capítulo 3 se centra en la preparación de la librería aleatoria para su posterior uso en el SELEX. Las librerías aleatorias constan de una multitud de oligonucleotidos, y esta heterogeneidad puede causar una amplificación no uniforme durante la reacción en cadena de la polimerasa (Polymerase Chain Reaction - PCR). Por lo tanto, una elección correcta de la librería y su posterior optimización de las condiciones de amplificación puede contribuir al éxito de la selección del aptámero. Se determinaron las condiciones óptimas para la amplificación de tres librerías diferentes, garantizando la pureza de las librerías amplificadas. La composición de las librerías cambia durante el proceso de SELEX, con lo cual suele ser necesario una optimización adicional de la PCR de las librerías enriquecidas. La adición de varias sustancias (dimethyl sulfoxido (DMSO), betaina, proteína unida a simple cadena de ADN (SSB) con los protocolos optimizados disminuyó la producción de productos no deseados.

En el Capítulo 4, se lleva a cabo la comparación de diferentes técnicas más utilizadas para la preparación de ADN de cadena sencilla. La generación de csADN es un paso crítico en SELEX porque la pureza y el rendimiento de diferentes técnicas pueden tener un impacto significativo para el éxito del proceso. Varias técnicas previamente usadas durante la selección *in vitro*, incluyendo PCR asimétrica, el uso de lambda exonucleasa y separación magnética, así como nuevos métodos tales como el uso de T7 Gene 6 exonucleasa y la combinación de la PCR asimétrica con la digestión del enzima han sido evaluados. La eficacia de cada sistema de preparación de csADN fue determinada cuantitativamente utilizando un ensayo especifíco para los oligonucleótidos (Enzyme Linked OligoNucleotide Assay - ELONA) y la calidad del csADN obtenido se evaluó mediante electroforesis en gel de agarosa. Según los resultados obtenidos, todos los métodos pueden ser utilizados para la generación de csADN, una vez tenidos en cuenta sus pros y contras. La PCR asimétrica combinada con la digestión con T7 Gene 6 exonucleasa demostró tener la mayor eficiencia, logrando rendimientos alrededor del 85%.

En el Capítulo 5 se describe el proceso de selección de aptámeros de ADN contra PSA. Se utilizaron varios protocolos para identificar dichos aptámeros ,

usando diferentes técnicas de partición, de generación de ADN de cadena sencilla, diferentes librerías y composición del tampón. Varios candidatos de aptámeros fueron seleccionados y analizados para determinar su afinidad a PSA. Dos aptámeros de ADN (I1, I4) con constantes de disociación en el rango μM, mostraron la menor interferencia con las proteínas que sirvieron como control (BSA y estreptavidina). La funcionalidad del aptamero I1 fue evaluada mediante un ensayo aptamérico específico (Reporter Linked Aptamer Assay - RLAA), obteniendo un límite de detección de 268 nM, Sin embargo, el aptámero I1 con baja afinidad y especificidad no cumple los requisitos para el uso como elemento de reconocimiento molecular en la diagnóstica clínica.

Otra posibilidad para seleccionar el aptámero contra el PSA consiste en la selección de aptámeros de ARN y se describe en el Capítulo 6. La variedad de conformaciones alcanzadas por las moléculas de ARN es mayor que las de ADN, por lo que existe una probabilidad más alta de seleccionar un aptámero de ARN contra el PSA. Diferentes aptámeros de ARN con una constante de disociación en el rango nM fueron seleccionados mediante un proceso automatizado de SELEX. Además, los aptámeros finales fueron capaces de distinguir entre otras proteínas, tales como estreptavidina o BSA. La utilidad de los aptámeros seleccionados de ARN en contra de PSA se validó también para su uso potencial en aplicaciones diagnósticas utilizando apta-PCR, obteniendo un límite de detección de alrededor de 11 nM para el aptámero S2, mejorando el límite de detección alcanzado por el aptámero de ADN previamente publicado. Para su uso potencial en la terapéutica, la estabilidad de los aptámeros seleccionados, así como los posibles efectos sobre la actividad de PSA se han estudiado, indicando una alta estabilidad y un efecto ligeramente inhibidor de los aptámeros de ARN modificados.

En el Capítulo 7 se presentan las conclusiones principales de la tesis doctoral, las aportaciones realizadas y algunas líneas de investigación futuras para la continuación de este trabajo de investigación.

List of publications

Articles

Pedro Nadal Polo, Alessandro Pinto, Markéta Svobodová, Nuria Canela and Ciara K.O'Sullivan, *DNA aptamers against the Lup an 1 food allergen*; PLOS ONE 7 (4):e35253.

Markéta Svobodová, Alessandro Pinto, Pedro Nadal Polo, and Ciara K. O´Sullivan; *Comparison of different methods for generation of single stranded DNA for SELEX*, accepted in Analytical and Bioanalytical Chemistry.

Maréta Svobodová, David Bunka, Peter Stockley, and Ciara K. O´Sullivan, *Selection of a 2´F modified RNA aptamer against Prostate Specific Antigen for diagnostic and therapeutic applications*, to be submitted.

Alessandro Pinto, Pedro Nadal Polo, Markéta Svobodová, Maria del Carmen Bermudo Redondo, Ciara K. O'Sullivan; *Whole Gliadin detection mediated by Apta-PCR,* to be submitted.

Hamdi Joda, Valerio Beni, Markéta Svobodová, Andreas Willems, Rainer Frank, Ioanis Katakis, Ciara K. O. Sullivan. *Modified primers for direct detection of double stranded PCR product*, to be submitted.

Book Chapter

Pedro Nadal, Alessandro Pinto, Markéta Svobodová 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.

Abbreviations

Ab	Antibody
AMBs	Amine modified magnetic beads
AMD	Age-related macular degeneration
A-PCR	Asymmetric PCR
APS	Ammonium persulfate
Asp	Aspartic Acid
ATP	Adenosine-5'-triphosphate
BCA	Bicinchoninic acid
bFGF155	Basic fibroblast growth factor 155
BSA	Bovine Serum Albumin
CE-SELEX	Capillary electrophoresis SELEX
CMBs	Carboxylated magnetic beads
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
DTT	Dithiothreitol
ECEEM	Equilibrium capillary electrophoresis of equilibrium mixtures
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
ELISA	Enzyme Linked ImmunoSorbent Assay
ELONA	Enzyme Linked OligoNucleotide Assay
EMBs	Epoxy activated magnetic beads
FDA	Food and Drug Administration
FRET	Fluorescence Resonance Energy Transfer
Glu	Glutamic Acid
HMBs	Hydrazide activated magnetic beads
HRP	HorseRadish Peroxidase
K_D	Dissociation Constant
LOD	Limit of Detection
Lys	Lysine
Mb	Magnetic beads
NECEEM	Non-equilibrium capillary electrophoresis of equilibrium mixtures
NHS	N-Hydroxysuccinimide
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEG	PolyethyleneGlycol
Pmf	Peptide mass fingerprinting
PSA	Prostate Specific Antigen

qPCR	Quantitative real time PCR
RLAA	Reporter Linked Aptamer Assay
RNA	Ribonucleic acid
RU	Resonance units
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SELEX	Systematic Evolution of Ligands by EXponential enrichment
Ser	Serine
SPR	Surface Plasmon Resonance
SSB	Single-strand binding protein
ssDNA	Single Stranded DNA
T7	T7 Enzyme 6 Gene Exonuclease
TBS	Tris Buffered Saline
TEMED	Tetramethylethylenediamine
Thr	Threonine
TMB	3,3',5,5'-Tetramethylbenzidine substrate
ΔG	Free energy

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Chapter 1

1.1 Introduction

1.1.1 Aptamers

At the beginning of the nineties, three different laboratories [1-3] reported their results on the development of an *in vitro* selection and amplification technique for the isolation of specific nucleic acids able to bind to target molecules with high affinity and specificity. The technique was coined SELEX (Systematic Evolution of Ligands by EXponentional enrichment) and the resulting oligonucleotides were named aptamers, taken from the latin "aptus", to fit. Aptamers are typically composed of RNA (RNA aptamers), single-stranded DNA (DNA aptamers) or a combination of both with unnatural nucleotides, and range in size from approximately 6 to 40 kDa. Due to their three-dimensional structure, characterized

by stems, loops, hairpins, bulges, triplexes and quadruplexes, aptamers can bind to a wide variety of targets from single molecules to complex target mixtures or a whole organism. They bind selectively to their target due to structural compatibility, electrostatic and van der Waals interactions, hydrogen bonding, or a combination of these effects [4], with dissociation constants (Kd) typically in the low nanomolar range, comparable to those observed for monoclonal antibodies. Aptamers are more flexible to different assay formats than their antibody counterparts, allowing them to address the ever-increasing requirements of analytical applications. Several facets of aptamers such as stability, ease of immobilization and labelling contribute to this enhanced flexibility and present them as very attractive candidates for diagnostic and therapeutic applications. However, aptamers do have drawbacks - each aptamer behaves differently and requires a specific set of conditions for optimal operation. Furthermore, there are a limited number of aptamers available, although an increasing number of aptamers are being reported in the literature.

1.1.2 SELEX process

A typical SELEX procedure begins with a nucleic acid library, generally consisting of about 10^{13} - 10^{15} different sequences [5], containing a random region flanked by two primers used for amplification (Figure 1.1). A randomized single stranded DNA (ss-DNA) or RNA pool is incubated directly with the target molecule, which is immobilized on a matrix material or is free in solution. Following incubation, the bound complexes are separated from the unbound and weakly bound oligonucleotides. Target bound oligonucleotides are eluted and amplified by polymerase chain reaction (PCR) in the case of DNA or reverse transcription PCR (RT-PCR) in the case of RNA. From the resulting double stranded DNA (ds-DNA), ss-DNA or transcribed RNA is generated, and this

enriched pool of selected oligonucleotides is used in the next SELEX round. Generally, 8-15 rounds of SELEX are required in order to obtain a pool of aptamer candidates with the highest binding affinity for the target. When affinity saturation of the enriched pool of oligonucleotides is achieved, aptamer candidates are cloned and sequenced, and individual sequences investigated for their ability to bind to the target.

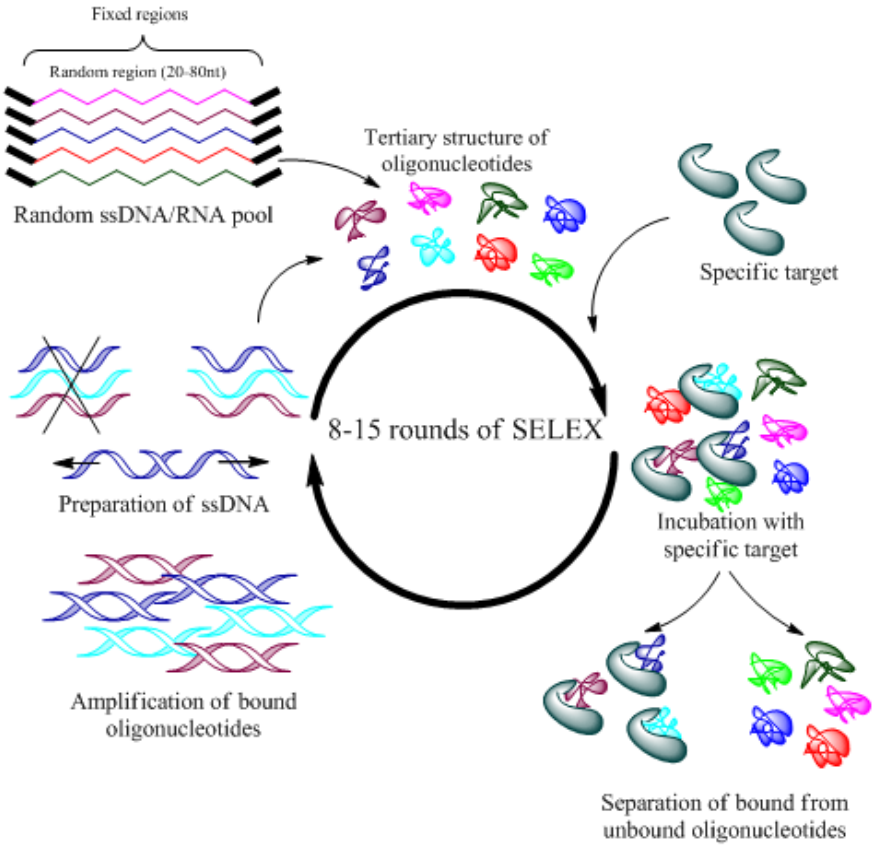

Figure 1.1. Schematic process of DNA/RNA SELEX.

1.1.3 Libraries

A typical SELEX library consists of a multitude of oligonucleotides comprising a central random region of typically 30-60 nt flanked by primer sequences of around 20 nt. In classical SELEX experiments DNA or RNA libraries are usually used. DNA has no 2´hydroxyl group, which is highly reactive, and accordingly is much more stable to hydrolysis under conditions of higher than neutral pH and elevated temperature [6-7]. DNA libraries are thus often used for applications requiring increased aptamer stability whilst RNA libraries yield aptamers with higher binding affinities due to the ability of RNA to take on a wider variety of conformations than DNA [8], but this is not universally true and indeed there is evidence that ssDNA aptamers do not differ in affinity and specificity from RNA aptamers [9].

With the goal of increasing the stability of oligonucleotides, modified libraries are often used in the selection process. Modifications are introduced into the 2´position of the pyrimidines, using 2´-aminopyrimidines [10-11], 2´fluoropyrimidines [12-13], 2´O-methyl nucleotides [14-15], position 5 of pyrimidines [16-17], and 4 of pyrimidines using thio UTP and CTP [18], amongst others. A detailed description of the different modifications can be found in several reviews [7, 19-21]. In some experiments structurally constrained libraries are used in selection. In this case, the random region is flanked by fixed sequences able to form a particular secondary structure such as a hairpin, G-quartet or pseudoknot [1, 22-24].

The libraries in genomic SELEX are derived from the genome of the organism of interest. To create such libraries, genomic DNA is excised into short fragments and appended with the fixed sequences. In all other aspects, *genomic SELEX* is similar to conventional SELEX. This variant of SELEX is used for the

identification of important nucleic-acid-protein interactions within any organism [25-26].

A variation of the SELEX protocol called *tailored-SELEX* allows isolation of aptamers free of fixed regions with desired properties [27]. In this case, a library with 4 or 6 nucleotides on both sides of the random region is used in the SELEX process. After each selection round, the primer binding sequences are ligated to the library using special adapters and removed before the next round. A very similar technique called *primer-free genomic SELEX* can also avoid the primers' influence on aptamer selection. This method is used to solve a specific problem with genomic SELEX, where fixed regions of the library often form complexes with the central genomic fragments. Using this method, fixed regions are removed from the genomic library before incubation with the target and are subsequently regenerated to allow amplification of the selected genomic fragments. An important point in the regeneration of primer-annealing sequences is to use thermal cycles of hybridization-extension, using sequences from unselected pools as templates [28]. More recently, there have been reports of a *minimal primer* and *primer-free* SELEX protocols for screening of aptamers from random DNA libraries [29-30].

1.1.4 Target molecules

The evidence that aptamers can be generated against small ions, such as Zn^{2+} [31], Ni^{2+} [32], to nucleotides such as ATP [33-34], oligopeptides [35], and large glycoproteins such CD4 [36], with the size range 65Da – 150kDa, demonstrates their tremendous flexibility. The smallest molecular target to date is ethanolamine [37], and furthermore, the classes of targets are diverse, including organic dyes [3, 38], neutral disaccharides [39], antibiotics [40], neurotransmitters [41], pigments [42] and vitamins [43].

However, SELEX has been most often exploited for the generation of aptamers against proteins [44]. When the aptamer-binding sites (aptatopes) on large molecules are mapped, it is usually found that they are coincident, even if the aptamers fall into unrelated sequence families, signifying that only a small proportion of the surface of the macromolecule is accessible to aptamers - most probably regions of high solvent exposure where positively charged residues provide a degree of electrostatic steerage towards the aptatope [5].

SELEX has also been applied towards complex cellular targets – cell SELEX [45-51]. In the case of cancer cells, aptamers are able to penetrate tumours rapidly and can be cleared quickly from the blood due to their small size (10-25kDa), making them an effective tool for molecular medicine, biomarker discovery and cancer biology. However, due to the abundance of targets on cell membranes, it is often impossible to know the precise target of an aptamer prior to its´ selection and often aptamers are selected against purified cell surface markers [47, 52]. Moreover in every cell preparation dead cells are also present [53], which cannot be removed by traditional methods like washing or centrifugation and thus may contribute to an inefficient selection process [54]. The use of FACS (fluorescence-activated cell sorting) in the selection step facilitates selection of aptamers with the simultaneous removal of selection-hampering dead cells [55].

The diversity of targets that have been already used in SELEX indicates that aptamers can be selected for virtually any target. However, there are some general prerequisites for a potential target to successfully select aptamers of high affinity. Selected target molecules should be present in sufficient amount and be of high purity. The purity of protein is usually checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Mass Spectrometry, which measures the mass to charge ratio of charged particles, and is generally used to determine the elemental composition of a protein sample.

Target immobilization is one of the efficient partitioning methods used for separation of target bound and non-bound nucleic acids in SELEX, and can be accomplished by a number of different approaches including physical, covalent, and bioaffinity immobilization [56]. A commonly used method is affinity chromatography with immobilization of the target on a sepharose or agarose column [31, 35, 57-58]. The use of magnetic beads methodology revolutionized separation methodologies in the 1980s and different types of magnetic beads have used in SELEX, selecting aptamers with high specificity and affinity [59-61].

1.1.5 Preparation of single-stranded DNA

The preparation of single-stranded DNA is one of the critical steps during DNA based SELEX process. One of the most widely used methods is magnetic separation with streptavidin-coated beads and biotinylated forward or reverse primers [62-64]. In this technique, immobilization of biotinylated dsDNA on the surface of streptavidin coated beads, followed by denaturation of the dsDNA by alkaline treatment or high temperature is used. An alternative method used for the generation of ssDNA exploits a urea-polyacrylamide gel, where PCR is performed with a specific long primer to produce strands of different lengths, followed by strand separation with denaturing gel electrophoresis [37, 61, 65-67]. Another possibility is to perform asymmetric PCR, which is used to amplify one strand of the original DNA more than the other by using an unequal molar ratio of forward and reverse primers. After asymmetric PCR, the PCR products are separated by gel electrophoresis and the ssDNA extracted and purified, representing a lengthy procedure, which is also known to suffer in terms of efficiency [68-69]. Another option for the preparation of single stranded DNA is the use of enzymes such as T7 Gene 6 exonuclease [70-71] and Lambda exonuclease via modified primers [72-

75]. T7 Gene 6 exonuclease hydrolyzes one strand of DNA non-processively in the 5´ to 3´ direction, while the second strand is protected against the hydrolytic activity of the enzyme with several phosphorothioates at its 5´end, whilst the lambda exonuclease selectively digests the phosphorylated strand of double stranded DNA and has greatly reduced activity on single stranded DNA and non-phosphorylated DNA.

1.1.6 Alternative SELEX strategies

There are a variety of techniques available for the isolation of aptamers and the choice of technique is largely dependent on the target molecule and the application and there is no definitive pattern to predict which method is preferred, but it is clear that more efficient methods of partitioning target bound nucleic acids are being reported, leading to higher affinity aptamers.

1.1.6.1 Photo SELEX

In photochemical SELEX (Photo-SELEX), modified oligonucleotides are capable of photocross-linking the target molecule. The method is based on the incorporation of a modified nucleotide that can be activated by absorption of light, in place of a native base in RNA/DNA libraries. This method is based on the strength of covalent bonding rather than affinity, therefore the specificity of the aptamer can be higher, but the false positives obtained with this method is also higher, requiring the cross-linking conditions to be optimized to ensure the screening validity. An example of the type of modified oligonucleotide used is that of 5-bromo-2´-deoxyuridine (5-Br-dUTP) or 5-iodouracil (5-IU) used as a

substituent for TTP [76]. The modified oligonucleotide absorbs UV light in the 310 nm range, where nucleic acids and proteins absorb very weakly, resulting in an excited singlet that specifically cross-links with the target's aromatic or sulphur-containing amino acids, when in close proximity [77-79]. This technique was first used in 1995 by Jensen [80], who obtained RNA aptamers for human immunodeficiency virus type 1 Rev protein using 5-IU. Later, with a random 61-mer oligonucleotide library, in which 5-Br-dUTP replaced thymidine, aptamers against the recombinant human basic fibroblast growth factor 155 (bFGF155) were selected [81]. The kinetic analysis of photoaptamer-protein photocrosslinking reactions was studied by Koch and a mathematical model for this was presented [82]. The model is based on the hypothesis that specific binding of aptamer and target followed by laser excitation can lead to either an aptamer/target complex formed with covalent bonds or irreversible photo damage to the aptamer. This model was used to characterize the photocross-linking between three photoaptamers and their targets and the results obtained from cross-linking data were confirmed by other independent measurements [83]. PhotoSELEX results in high affinity and highly specific aptamers by improving the separation method; however, a major limitation is that low molecular mass molecules that do not have functional groups to crosslink cannot be used as targets for the photo SELEX procedure.

1.1.6.2 Toggle SELEX

Toggle-SELEX allows isolation of aptamers with a wide range of specificities by selecting against related targets in alternating cycles [84]. Alternation of the target between homologous proteins of different species ensures that aptamers will bind to both proteins, most likely to domains conserved between the two proteins

[85]. Aptamers that bind both human and porcine thrombin were selected with this method [86]. The same method with another name „target switching" was used in order to obtain aptamers called „oligobodies" against ERK2 [87] and protein phosphatase 2A [88].

1.1.6.3 Capillary electrophoresis based SELEX

Methods for aptamer selection that exploit capillary electrophoresis report a considerable improvement in the separation of bound and unbound nucleic acids, only requiring a few cycles for the isolation of very high affinity aptamers. Capillary electrophoresis SELEX (CE-SELEX) involves the selection of binding oligonucleotides based on a mobility shift due to complex formation. Sequences bound to the target move through the capillary electrophoresis at a velocity different from those not bound to the target, allowing separation of complex bound sequences from the rest of the library. The main advantages of CE-SELEX are the ability to perform the selection in free solution, reduction of non-specific binders, and increased capability of separation. Using CE-SELEX, high affinity aptamers against IgE and neuropeptide Y were selected in just a few selection rounds [89-91]. Two aptamers with the same sequence were selected against cytotoxin ricin using both affinity chromatography SELEX and CE-SELEX, but with markedly shortened selection time in the case of the latter [92]. The determination of kinetic and thermodynamic constants can be achieved using an efficient selection process based on equilibrium capillary electrophoresis of equilibrium mixtures (ECEEM). Using this method an aptamer binding MutS protein was selected with a K_d of 15 nM in just three rounds of selection [93]. Capillary electrophoresis has also been used in a format called non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM). NECEEM also

enables the "selection" of aptamers with nanomolar K_d values in a few rounds of selection [94-96], and has the same advantages as ECEEM for the selection of aptamers with pre-defined kinetic parameters [8].

1.1.6.4 Non – SELEX

Non – SELEX is another method that has been used for aptamer screening. This method is based on a process that involves repetitive steps of partitioning with no amplification step. A high affinity aptamer for h-ras was obtained using Non-SELEX with NECEEM as a separation step [97]. The advantage of this method is its potential applicability to non-amplifiable libraries, such as those of DNA tagged small molecules obtained by DNA-template synthesis.

1.1.6.5 MonoLEX

The MonoLex selection protocol demonstrated that a one-cycle approach can isolate ligands with high affinity [98]. The aptamers are isolated in one single cycle by exponential amplification after the separation of bound oligonucleotides by affinity chromatography. To obtain aptamers with high affinity, desorption of oligonucleotides from individual column segments derived from a physical disintegration of the affinity column is performed. During the selection process the target bound oligonucleotides are sorted along the column with respect to their affinity, while low binders are eluted from the column during thorough washing. Due to the use of affinity sorting, aptamers amplified from a part of a single column show a similar affinity but differ in the binding domain of the target molecule. These pools are very similar to those of polyclonal antiserum.

1.1.7 Advantages of Aptamers

Aptamers are identified through an *in vitro* process of selection that does not depend on animals, and thus toxins as well as molecules that do not elicit a good immune response, can be used to generate high-affinity aptamers. Selection conditions and kinetic parameters can be manipulated and as a result the properties of aptamers can be changed on demand. Aptamers are produced by chemical synthesis with high accuracy and reproducibility and of high purity with little or no batch-to batch- variations expected. Reporter molecules such as a fluorescein, biotin, etc. can be attached to aptamers at precise locations without loss of binding affinities. Aptamers can undergo denaturation, but the process is reversible, and aptamers can be regenerated easily within minutes, and can also be easily transported at ambient temperature and are inherently stable for long-term storage [99]. Aptamers are highly selective in nature and designed to have high affinity for a particular target molecule or specific isoform of a target protein and can even distinguish between different conformational states of the same protein [100-101]. One of the not-yet-understood features of non-natural aptamers is their apparent lack of immunogenicity [102], which makes them a favorable therapeutic choice [103-104].

1.1.8 Aptamers as diagnostic and therapeutic agents

1.1.8.1 Diagnostics

Antibodies contribute to a wide range of applications based on molecular recognition, and are widely used in a range of different clinical diagnostic assays and techniques. However, there are limitations associated with antibodies. The production of antibodies exploits the use of animals via induction of an immune response, and thus there can be difficulties in obtaining antibodies against targets that are similar in structure to endogenous proteins, or, alternatively, against compounds that are toxic for the animal host. The generation of polyclonal antibodies tends to vary from batch to batch and while monoclonal antibodies overcome this problem, they are expensive and laborious to produce. Additionally, antibodies can be sensitive to non-physiological conditions and harsh storage environments, and, furthermore, antibody affinity can be affected upon conjugation to reporter molecules and the kinetics of antibody-antigen interaction cannot be changed on demand [99].

The application of aptamers as biocomponents in biosensors offers a multitude of advantages over their antibody counterparts. Due to their nucleic acid nature, aptamers can take advantage of a plethora of surface chemistries, assay formats, technology platforms (e.g. microarrays) and linkage chemistries that have been developed for genosensors, highlighting their enhanced flexibility, as they can effectively perform as both antibodies and nucleic acids. A considerable number of comprehensive reviews regarding the application of aptamers in diagnostics are available [58, 99, 105-107].

The first use of aptamers in an enzyme-linked immunosorbent assay (ELISA)-like assay, referred to as ELONA (enzyme-linked oligonucleotide assay),

or more correctly RLAA (reporter-linked aptamer assay) was reported by Drolet [108]. This work highlighted the possibility of aptamers to compete with antibodies in bioanalysis, paving the way for a new approach for detection. In simple formats, aptamers have been exploited in RLAA in much the same way as antibodies in ELISA [109]. Different RLAA sandwich formats were studied [50, 110], elucidating the critical parameters for optimal aptamer performance and highlighting that the conditions for each aptamer assay must be individually optimized and, unlike ELISA, that no universal optimal operating parameters exist. The applicability of aptamers in displacement assay highlighted the flexibility to different assay formats of aptamers, and has been exploited in a aplethora of formats since the first report [111].

The concept of integrating the sensitivity of nuleic acid amplification with the selectivity of aptamers (apta-PCR) has been described, where different sandwich formats and amplification methods have been reported producing an impressive signal enhancement with considerably lower detection limits [112-115].

An assay format that exhibits the unique properties possessed by aptamers is that of the molecular aptamer beacon, where a plethora of formats in immobilized and solution forms using optical and electrochemical detection have been reported [116-120], extrapolating from the original concept reported by Tyagi and Kramer for the detection of DNA [121]. The beauty of the molecular aptamer beacon is that the only required end-user intervention is sample addition, with a quantitative response being available in a matter of seconds to minutes. Many of the reported molecular aptamer beacons exploit the conformational changes the aptamers undergo upon interacting with their target [122-126], whilst others have been engineerd into beacon structures [117, 127-129].

1.1.8.2 Therapeutics

The concept of using aptamers as therapeutic agents is more than 20 years old, based on the specific interaction with the target molecule which can modulate its function in a competitive (and antagonistic) way if associated aptatopes are involved in the binding [48-49, 130-134]. Aptamers can directly influence metabolic processes, they can act by mimicking the target sequence of proteins [135], and have been identified as powerful antagonists of protein interactions related to several diseases [136], offering the possibility for pharmaceutical applications. For example aptamers selected against anti-reverse transcriptase of HIV-1 protein have been shown to form a pseudoknot structure that tightly interacts with the polymerase active site [137]. However, these aptamers not only did inhibit viral replication, but can also be co-packaged into virions, thereby crippling progeny virions [138-139].

A rapid blood clearance due to their small size (10 – 30 kD) in comparison to antibodies (150 kD) can be overcome by pegylation (polyethylene glycol), by embedding aptamers in liposomes [130, 140-141], or by 3´biotinylation [142].

The stability in serum of a 2´aminopyrimidine modified anti-bFGF aptamer was increased at least 1000-fold relative to un-substituted RNAs [10], while a 2´aminopyrimidine with 2´O-methylpurine modified anti-VEGF aptamer could survive for up to 17 hours in urine [143]. After significant efforts, stabilized aptamers have been developed for use as intravenous or subcutaneous injectables, with blood half lives in the 6-12 hours range [144-145]. New oligonucleotide stabilization chemistries continue to be developed, so the possibility of injectable aptamer formulations with longer (days or even week time scale) blood half-lives are feasible. Moreover, there is evidence of using an unmodified DNA aptamers in serum for up to 6 days, more than enough for in vivo and *in vitro* applications [98].

RNA and DNA aptamers intended for clinical applications are usually modified in order to optimize their pharmacodynamic and pharmacokinetic profiles and to promote their safety [146]. Studies of the possible toxic effects of aptamers in clinical applications were negative in all cases, and based on no-adverse-effect levels it has been concluded that the expected pharmacologically applicable doses in animals and humans are safe [135]. Moreover, there are no evidence for aptamers being immunogenic or activating complement or anticoagulation, showing the possibility of repeated administration of doses to the same individual [135-136]. By addition of a second, complementary oligonucleotide, the function of the aptamer drug can be deactivated. This aptamer antidote principle was first demonstrated for the coagulation factor IX [13, 147]. Recently, universal sequence independent antidotes were developed for extracellular oligonucleotide-based drugs in animals and humans [148].

Recent developments in aptamer-based therapeutics have been rewarded in the form of the first aptamer-based drug approved by the U.S. Food and Drug Administration (FDA) for the treatment of age-related macular degeneration (AMD), called Pegaptanib (brand name Macugen). In addition to the RNA-aptamer Macugen, a DNA-aptamer directed against the platelet-derived growth factor-B has been proposed for exudative AMD treatment [149]. A guanosine-rich oligonucleotide aptamer (AS-1411) selected against nucleolin is the first oligodeoxynucleotide aptamer to reach phase I and II clinical trials for the potential treatment of cancers [150]. Among aptamers currently in clinical trials, some are directed against blood clotting factors. For example, a DNA-aptamer called ARC1779 is directed against the A1 domain of von Willebrand factor (vWF) and is able to inhibit its binding to the glycoprotein Ib receptors on platelets, resulting in an antithrombotic effect [151]. Another aptamer that prevents blood clotting is Nu172, a 26-mer DNA aptamer, directed against thrombin with no chemical modifications in its molecular structure. Two spiegelmers, NOX-A12 and NOX-

E36, for the treatment of hematologic tumors and complications of type 2 diabetes are also in clinical trials [152].

1.1.9 Prostate Cancer

Prostate Cancer is one the most common male cancers in Western Countries, mostly in Europe and North America. It has been estimated to be the second leading cause of male cancer death in United States (Figure 1.3) and the third in the European Union in years 2008-2011, after lung and colorectal carcinomas [153-155]. The incidence of prostate cancer increases with age and about 80% cases are in men over 65. The early diagnosis of prostate cancer is of major importance, as when prostate cancer is detected early it can be easily treated, resulting in a 5-year relative survival rate of 100%. On the other hand, when prostate cancer is diagnosed at advanced stages, the 5-year relative survival rate drops to 30% [153].

Estimated New Cases*

Males			Females		
Prostate	240,890	29%	Breast	230,480	30%
Lung & bronchus	115,060	14%	Lung & bronchus	106,070	14%
Colon & rectum	71,850	9%	Colon & rectum	69,360	9%
Urinary bladder	52,020	6%	Uterine corpus	46,470	6%
Melanoma of the skin	40,010	5%	Thyroid	36,550	5%
Kidney & renal pelvis	37,120	5%	Non-Hodgkin lymphoma	30,300	4%
Non-Hodgkin lymphoma	36,060	4%	Melanoma of the skin	30,220	4%
Oral cavity & pharynx	27,710	3%	Kidney & renal pelvis	23,800	3%
Leukemia	25,320	3%	Ovary	21,990	3%
Pancreas	22,050	3%	Pancreas	21,980	3%
All Sites	**822,300**	**100%**	**All Sites**	**774,370**	**100%**

Estimated Deaths

Males			Females		
Lung & bronchus	85,600	28%	Lung & bronchus	71,340	26%
Prostate	33,720	11%	Breast	39,520	15%
Colon & rectum	25,250	8%	Colon & rectum	24,130	9%
Pancreas	19,360	6%	Pancreas	18,300	7%
Liver & intrahepatic bile duct	13,260	4%	Ovary	15,460	6%
Leukemia	12,740	4%	Non-Hodgkin lymphoma	9,570	4%
Esophagus	11,910	4%	Leukemia	9,040	3%
Urinary bladder	10,670	4%	Uterine Corpus	8,120	3%
Non-Hodgkin lymphoma	9,750	3%	Liver & intrahepatic bile duct	6,330	2%
Kidney & renal pelvis	8,270	3%	Brain & other nervous system	5,670	2%
All Sites	**300,430**	**100%**	**All Sites**	**271,520**	**100%**

Figure 1.2. Ten leading cancer types for estimated new cancer cases and deaths, by Sex, United States 2011. **Estimates are rounded to the nearest 10 and exclude basal and squamous cell skin cancers and in situ carcinoma except urinary bladder. Extracted from Siegel at al. 2011 [155].*

1.1.9.1 Tumor markers

The identification of tumor markers is a powerful tool in cancer diagnostics and therapeutics. Tumor markers are substances that can be found in blood, urine or body tissues when cancer is present. They can be products of the cancer cells themselves or of the body in response to cancer or other conditions. Most tumor markers are proteins, antigens, or hormones. Cancer marker tests are not used alone in diagnosis because most markers can be found in elevated levels in

people who have benign conditions, and because no tumor marker is specific to a particular cancer. Also not every tumor will cause an elevation in the cancer marker test, especially in the early stages of tumor and certain tumor markers are more accurate than others in their sensitivity to detection of cancer. The more sensitive they are, the earlier it is possible to diagnose. In any case, markers can help determine if a cancer is likely, they can help diagnose the source of widespread cancer in a patient when the origin of the cancer is unknown, and they also help to determine the prognosis of certain cancers and effectiveness of cancer treatment. A list of the commonly used tumor markers for certain types of cancer is presented below (Table 1.1).

Table 1.1. List of the commonly used tumor markers for certain types of cancer.

CANCER MARKER	TYPE OF CANCER	NORMAL VALUES
Alpha-fetoprotein (AFP)	liver cancer	≤ 0 - 6.4 U/ml in men and non-pregnant women
Beta-2-microglobulin (B2M)	multiple myeloma, chronic lymphocytic leukemia	≤ 2.5 μg/ml
Bladder tumor antigen (BTA)	bladder cancer	≤ 14 U/ml
CA 15-3/MUC1	breast cancer	≤ 25 - 31 U/ml
CA 27-29	breast cancer	≤ 3 - 40 U/ml
CA 125	epithelial ovarian cancer	≤ 33 -35 U/ml
CA 72-4	ovarian and pancreatic cancer, cancer of digestive tract	≤ 4 U/ml
CA 19-9	panreactic and colorectal cancer, digestive tract cancer	≤ 33 -37 U/ml
Calcitonin	medullary thyroid carcinoma	≤ 0.155 ng/ml for men ≤ 0.105 ng/ml for women
Carcinoembryonic antigen (CEA)	colorectal, lung and breast cancer	≤ 5 ng/ml in smokers ≤ 3 ng/ml in non-smokers
Chromogranin A (CgA)	neuroendocrine tumor	≤ 76 ng/ml for men ≤ 51 ng/ml for women

Estrogen receptors/ Progesterone receptors	breast cancer	ER, PR negative
Human epidermal growth factor HER2, 3	breast cancer	The test is to determine if the tissue over expresses HER 2.
Nueron-specific enolase (NSE)	neuroendocrine tumors	≤ 13 ng/ml
NMP 22	bladder cancer	≤ 10 U/ml
Postate Specific Antigen (PSA)	prostate cancer	≤ 4 ng/ml
Prostatic acid phosphatase (PAP)	prostate cancer	≤ 0,5 - 1,9 U/ml
Prostate-specific membrane antigen (PSMA)	prostate cancer	≤ 273 ng/ml in men **<**50 ≤ 359 ng/ml in men **>**50

Normal levels differ between people and between laboratories. Most labs list their own „ reference ranges"of cancer markers. The values listed on the chart are the normals established at The University of Iowa Hospitals and Clinics, and on the web page of American Cancer Society.

1.1.9.2 Tumor markers in prostate cancer

There are several tissue prostate cancer markers such as for example Glutathione S-transferase π (GSTP1) and α-methylacyl coenzyme A racemase (AMARC) [156]. Prostate Specific Membrane Antigen (PSMA) is another target for the diagnosis and treatment of cancer prostate, and is unregulated in the vasculature of most solid tumors, and can be a potential target for the generation of novel anti-angiogenesis agents [157].

The earlist reported serum marker for prostate cancer was human prostatic acid phosphatase (PAP) [158]. Recently, early prostate cancer antigen -2 (EPCA-2) detection in serum has shown some promising results as it was able to differentiate between men with and without prostate cancer with 92% specificity (measures the proportion of negatives which are correctly identified) and 94% sensitivity (measures the proportion of actual positives which are correctly identified) [159].

Prostate-specific antigen serum level is currently considered the best tumor marker available in clinical medicine. Protate Specific Antigen (PSA), a serine

protease, is a glycoprotein produced by prostate epithelium [160]. PSA is secreted as propeptide, which is inactive, with removal of the first seven aminoacids yielding the mature, active PSA (Figure 1.4).

Figure 1.3. Structure of PSA forms. *The leader sequence on preproPSA is cleaved to generate proPSA (inactive form). Cleavage of the propeptide by human Kallikrein 2 generates the active mature PSA. Truncated inactive form of proPSA can also be generated by cleavage within the propeptide, including (-2) proPSA and (-5) proPSA. Active PSA in the lumen of prostate ducts can be further cleaved at the indicated positions to generate inactive PSA. Active PSA that enters the bloodstream is rapidly bound to protease inhibitors, while all other forms circulate as free PSA. Extracted from Balk, Ko & Bubley 2003 [161].*

Seventy percent of PSA in the seminal fluid is enzymatically active, and the rest is in an inactive form. On the other hand the predominant molecular PSA form present in blood is an inactive 80-90 kDa complex of PSA with alfa-1-antichymotrypsin (ACT), alfa-2-macroglobulin, and alfa-1-antitrypsin. These fractions are called complexed PSA (cPSA). Free active PSA (fPSA) represents a small but variable proportion of the total PSA serum concentration, containing a mixture of different inactive PSA forms: nicked PSA (mature forms with different broken peptide bonds) and pro PSA forms with the N-terminal propeptide truncated at different positions (-2), and (-5) and (-7) pro PSAs [162].

PSA is found in the blood of healthy patients at very low concentrations. However, prostate cancer patients show a characteristic loss of the basal cell layer of prostate glands which allows PSA contained in the prostatic glands access to the peripheral circulation and thus to be found in blood in higher quantities. The cut-off value of PSA concentration of around 4 ng/ml indicates a risk and recurrence of prostate disease; however PSA is not fully specific for prostate cancer as other prostatic pathologies such as benign prostate hyperplasia (BPH) and prostatitis can show serum PSA elevations. Men, who have 4 to 10 ng/ml of serum PSA are often subjected to repeated screening and sometimes to biopsy tests. [163]. The variation of PSA measurement has been considered due to the different forms of PSA [164], and the PSA index (ratio free to total PSA) has been approved by the FDA (Food and Drug Administration) to differentiate between prostate cancer and BPH patients in the 4 -10 ng/ml range. In spite of its limitations, PSA is considered a useful marker of prostate pathologies, including prostate cancer, because PSA serum levels provide an early warning of the development and presence of prostate cancer.

The main function of PSA is the liquefaction of the seminal fluid by the degradation of semenogelin I, semenogelin II and fibronectin. PSA also cleaves a variety of proteins including insulin-like growth factor binding protein-3 (IGFBP-3), and IGFBP-4, releasing active insulin-like growth factor 1 (IGF-1) that can enhance tumor growth factor [165-166]. Moreover, PSA also cleaves laminin [167] and activates latent TGF-beta [168], which is involved in tumor progression and metastasis. The discovery of the involvement of PSA in several events leading to the development of malignant tumors has made it a target for prevention and intervention [169], and has also prompted some interest in the use of serine protease inhibitors and their modulators as useful agents for blocking the progression of some preinvasive to invasive carcinomas [170]. However not all serine protease inhibitors are candidates for PSA modulation, but it has been

reported that PSA can be also inhibited by Zn^{2+} [171] or boronic acid compounds [169].

Apart from the ability of PSA to cleave several substrates, PSA may also have other functions relevant to tumor growth [172-174]. A high expression of PSA in prostate cancer tissue has been associated with low vasculature density and angiogenic potential [175-176]. High PSA concentrations in the tumor environment may contribute to the slow growth of prostate cancer, since PSA has been shown to exert antiogenic activity [177-178]. Thus, is possible that PSA plays a dual role in cancer progression, first favoring tumorigenesis by activation or release of growth factors and degradation of extracellular matrix components, while at later stages PSA may slow down tumor growth through its antiangiogenic activity [179].

1.2 Objectives

Currently, PSA is considered the most sensitive marker available for prostate cancer detection and for monitoring its progression. To date monoclonal/polyclonal antibodies have been used to detect PSA due to their high specificity and sensitivity. However, the production of antibodies is time-consuming and expensive. Moreover the production of antibodies requires a substantial number of animals, and the animals are subjected to a number of invasive procedures such as antigen injection and blood collection. On the other hand, aptamers with specificity and affinity equal to those of antibodies could be an alternative way for the detection of PSA. To date there are several reports detailing DNA/RNA aptamers selected against cancer markers with affinity in the low nanomolar to picomolar range. Both DNA and RNA aptamers can be used in diagnostic applications, but DNA aptamers have several advantages over RNA in this regard. The selection process for DNA aptamers is simpler and faster and in addition DNA aptamers can be easily modified and are less expensive than modified RNA aptamers, making them more suitable for diagnostic applications. Therefore, a PSA detection method using DNA aptamers (molecular aptamer beacon, AptaPCR) would be a comfortable and low cost procedure, and could be used to screen for a risk and recurrence of prostate cancer.

In addition to its importance as a tumor marker, PSA has biological activity with regard to cancer growth and proliferation. Therefore the inhibition or activation of its biological activity can be used in prostate cancer therapy. Innovative targeted therapeutic strategies aim at developing new molecules with high affinity and specificity with suitable pharmacokinetic properties for *in vivo* applications. In this perspective aptamers are able to bind with high affinity to a

specific protein by folding into complex tertiary structures with the possibility to modulate the function of the target. To date, nine RNA and DNA aptamers have entered the clinical development pipeline for treating a number of diseases in different pathologic states[180].

This PhD work focused on the selection of both DNA/RNA aptamers against Prostate Specific Antigen and their use in diagnostic and therapeutic applications. The objectives of this work can be divided in several points; (i) selection and amplification of a random library, its optimization and use in SELEX process, (ii) characterization of PSA and evaluation of different immobilization strategies, (iii) comparison of different methods used for preparation of single stranded DNA and their application in SELEX process, and finally (iv) selection of DNA and RNA aptamers against PSA and study of their potential use in diagnostic and therapeutic applications.

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Chapter 2

2.1 Introduction

The diversity of targets that have been used in SELEX indicates that aptamers can be selected for virtually any target. Peptides, proteins, carbohydrates, inorganic or small organic molecules, as well as complex targets like target mixtures or whole cells and organisms have been used as targets in aptamer selection. However, there are some general prerequisites for a potential target to successfully select aptamers of high affinity. Selected target molecules should be present in sufficient amount and of high purity. This helps to minimize the enrichment of un-specifically binding nucleic acids and to increase the specificity of the selection. Some target characteristics such as positively charged groups (primary amino groups), the presence of hydrogen donors and acceptors, or planarity (aromatic compounds) can facilitate aptamer selection[1]. On the other hand aptamer selection can be more difficult for targets with hydrophobic character and negatively charged molecules (containing phosphate groups) [2-3]. Specific target

features have an important role not only in the binding step during selection but also in the immobilization of the target molecule on a particular matrix surface. Target immobilization is one of the efficient partitioning methods used for separation of target bound and non-bound nucleic acids in SELEX, and the target should be immobilized onto the surface with a high density and nonspecific adsorption should be avoided or at least minimized in an optimal selection process [4].

2.1.1 Characteristics of PSA

PSA also known as kallikrein-3 (KLK3), seminin, semenogelase, γ-seminoprotein or P-30 antigen is a glycoprotein encoded by the KLK3 gene located on the nineteenth chromosome (19q13) in humans. PSA is a chymotrypsin like serine protease produced by the epithelial cells of the prostate. It consists of 237 amino acids with molecular weight ranging from 30 to 36 kDa [5]. The molecule is 8% carbohydrate comprised of three O- and one N-linked oligosaccharide chains [6]. There is considerable microheterogeneity in PSA glycosylation, resulting in isoelectric points ranging from 6.8 to 8.0 [7]. Moreover, differences in sugar composition between normal PSA and PSA from tumoral origin have been observed [8-10]. As mentioned before, PSA can be found in different molecular forms including intact PSA, precursor PSA (pro PSA), and nicked PSA. Enzymatically highly active intact PSA is a serine protease cleaved at the NH_2 - terminal end of inactive pro PSA by trypsin or hK2 [11-12]. Different pro-PSA forms (zymogen forms) with 1-7 amino acids in the precursor part have been identified and characterized [11-14]. Nicked PSA is an inactive form resulting from a nick in the PSA sequence, probably at the lysine at position 145 and/or 182 [15].

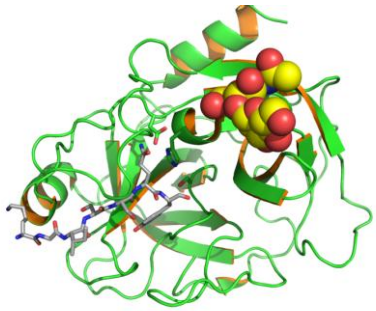


Figure 2.1 Crystal structure of human prostate specific antigen *(green/gold cartoon) covalently attached to an substrate acyl intermediate (multicolor sticks, carbon: white, nitrogen: blue, oxygen: red) and an glucosamine-manose disaccharide (spheres, carbon: yellow and oxygen: red.[16].*

2.1.2 Immobilization of target on the surface of magnetic beads

An immobilization process can be defined as the attachment of molecules to a surface which leads to a decrease or loss of mobility. In some cases, immobilization may lead to partial or complete loss of protein activity, due to random orientation and structural deformation. In order to retain biological activity, proteins can be attached onto surfaces without affecting conformation and function. The choice of a suitable immobilization strategy is usually determined by the chemical and physicochemical properties of both the surface and protein. Immobilization techniques are mainly based on the following three mechanisms: physical, covalent, and bioaffinity immobilization and frequently magnetic beads with coatings suitable for these types of immobilization are used [17-20].

2.1.3 Physical Immobilization

When physical immobilization is used, proteins can be absorbed on surfaces via intermolecular forces, such as ionic, hydrophobic and polar interactions, with the type of intermolecular forces taking part in the interaction

being dependent on the particular protein and surface involved. The magnetic beads are generally non porous, although the potential of porous magnetic beads as "microreservoirs" for enzyme entrapment has also been studied [21]. The disadvantages of the adsorption mechanism includes random orientation and weak attachment of the protein, as proteins immobilized in this way can be removed by some buffers or detergents used in the SELEX process.

2.1.4 Covalent Immobilization

Proteins can be covalently bound to an immobilization support through functional groups of exposed amino acids. Covalent bonds are generally formed between functional groups exposed on the side-chains of proteins with suitable modified surfaces, resulting in irreversible binding. The functional groups on the support are produced by chemical treatment, and several pretreated magnetic beads are commercially available (Table 2.1).

Table 2.1 The most common commercially available modified magnetic beads with corresponding aminoacids containing functional groups.

Type of magnetic beads	Aminoacids	Side groups
Carboxylic acid	Lys	- NH_2
Amine	Asp, Glu	- COOH
Bromacetyl	Cys	- SH
Epoxy	Ser, Thr	- OH
	Lys	- NH_2
	Cys	- SH

Magnetic beads modified with carboxylic acids (Figure 2.2 a) are the most commonly utilized in SELEX as lysine is typically present on protein exteriors. The other large fraction of surface groups present on proteins are aspartic and glutamic acid, which can be immobilized on amine modified magnetic beads (Figure 2.2 b).

Carboimidine chemistry is the most commonly used method for coupling with amine groups, forming stable amide bonds.

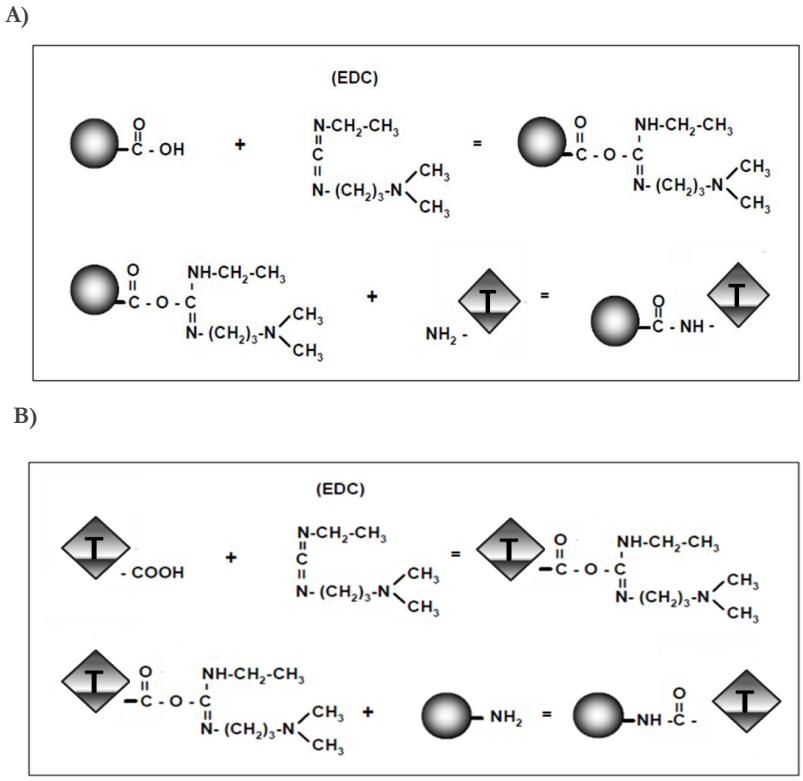


Figure 2.2 Covalent coupling with carbodiimide method using carboxyl and amine modified beads. *In carboxyl modified beads (a) activation of carboxyl groups on the surface of the beads by EDC is followed by formation of reactive O-acylisourea complex. Covalent binding is performed via amine groups of target. In the case of amine modified beads (b) activation of carboxyl groups is done on the surface of target. After activiation with EDC and formation of O-acylisourea complex, covalent binding is performed in the presence of amine modified beads.*

Thiol chemistry is based on linking to the cysteine to amino acid which contains a thiol group, and generally forms internal disulfide bonds, that contribute to the stability of the protein. The coupling reaction between bromacetyl activated beads and proteins containing sulfhydryl groups is very fast and the coupling product offers extremely stable thioether bonds between the modified bead and

protein (Figure 2.3). Nevertheless this type of bead is not very common in SELEX process since cysteines are not as abundant or accessible as the above mentioned amino acids.

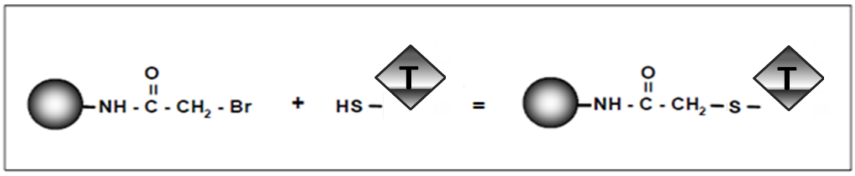


Figure 2.3 Covalent binding on autoreactive bromacetyl groups.

Epoxide chemistry is another widely used system, due to its stability at neutral pH values, wet conditions, and reactivity with several functional groups to form strong bonds with minimal chemical modification of the protein. The epoxide chemistry can be used to link to ligands containing amino, thiol and hydroxyl functional groups (Figure 2.4).

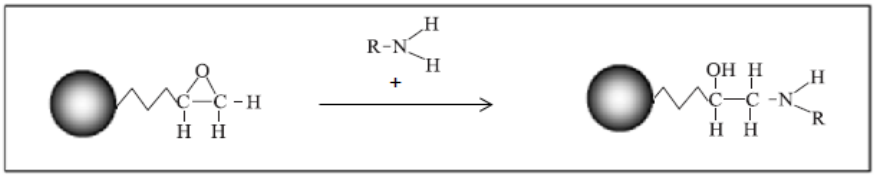


Figure 2.4 Example of epoxide chemistry.

There is a wide range of special types of covalent bindings available. Different terminal reactive groups allow the convenient and efficient covalent binding of various biomolecules. Magnetic beads can be modified with cyanuric, hydroxyl, polyglutaraldehyde, chitosan, thiol, and many other reactive groups, including hydrazide modified beads, which link with ligands containing aldehyde and ketone groups forming stable hydrazone bonds (Figure 2.5).

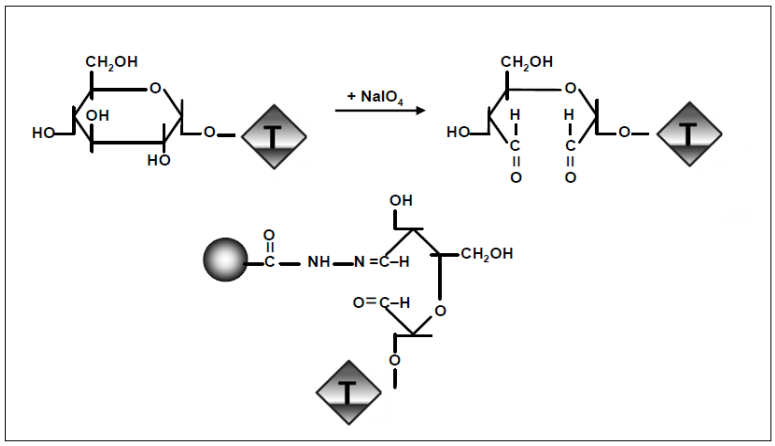


Figure 2.5 Site–directed immobilization through carbohydrates with hydrazide modified beads. *Oxidation of carbohydrate residues is followed by fformation of aldehyde groups which react with hydrazide modified beads.*

2.1.5 Bioaffinity immobilization

Biochemical affinity immobilization offers an oriented attachment of proteins, and the most common system takes advantage of the avidin-biotin interaction (Figure 2.6). This approach is based on one of the strongest non-covalent bonds ($K_d = 10^{15}$ M^{-1}) ever known and the interaction is very specific and permits uniformly oriented protein immobilization. An alternative bioaffinity system is used with recombinant proteins, which are produced by genetic engineering and can be expressed with affinity tags [19] that are placed at defined positions, in order to achieve optimal accessibility of the protein. Histidine is the most popular tag due to the advantages of small size, compatibility with organic solvents, low immunogenicity, and effective purification under native and denaturing conditions.

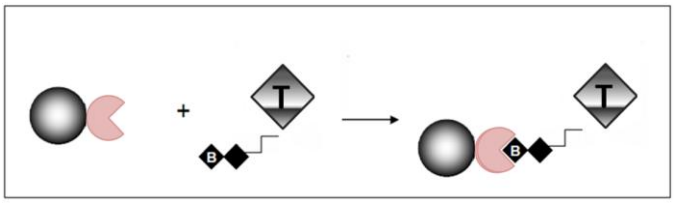


Figure 2.6 Biotinylated target is attached to the streptavidin coated magnetic beads.

2.2 Objectives

The choice of a suitable immobilization strategy is determined by the physicochemical properties of the target. The extrapolation of immobilization strategies is difficult and mostly unsuccessful due to the wide subset of characteristics and functional properties of proteins. Therefore, the question of which strategy is best should be studied for each specific target. This chapter can be divided in three parts. The first part is dedicated to methods used for the characterization and quality control of PSA, the second part deals with different types of immobilization processes, including covalent, and bio-affinity immobilization techniques, and finally the last part of this section details the characterization of PSA-coupled beads.

2.3 Methodology

2.3.1 Reagents

Prostate Specific Antigen (>96% pure) purified from seminal fluids was purchased from SCIPAC (Kent, UK), and fluorogenic PSA substrate from Calbiochem/Bionova Scientifica (Madrid, Spain). Monoclonal antibody Anti-PSA 10 was obtained from Fujirebio (Barcelona, Spain). All reagents used for polyacrylamide gel were supplied from Biorad (Madrid, Spain). Simag carboxyl, amine and hydrazide activated beads were purchased from Chemicell (Berlin, Germany), and M 270 epoxy and My One T1 streptavidin coated beads from Invitrogen (Madrid, Spain). EZ-Linker sulfo-NHS-biotin, EZ-Link Amine PEG_n-Biotin, and Slide-A-Lyzer Dialysis cassettes were from Fisher scientific (Madrid, Spain). Polyvinylchloride (PVC) microtiter plate, TMB substrate, EDC/NHS, and enzyme linked secondary antibody (AP 9044) were purchased from Sigma (Madrid, Spain). Amicon spin columns, 10 K device were from Milipore Iberica S.A.U (Madrid, Spain).

2.3.2 Quality control of protein

The protein concentration was determined by measuring the OD_{280}, using the extinction coefficient for PSA (1.84 mL/mg/cm). The quality of the protein was checked by 15% (w/v) polyacrylamide gel. The gel consisted of two sections: a *resolving gel* (12.5 ml 30% (w/v) acrylamide (37:5:1 acrylamide: bisacrylamide); 6.3 ml 1.5 M Tris HCl pH 8.8, 5.7 ml di-distilled water (dd-water), 250 µl 10% (w/v) SDS, 250 µl 10% (w/v) APS, 15 µl TEMED) and *stacking gel* (1.3 ml 30% (w/v) acrylamide (37:5:1 acrylamide:bisacrylamide), 1.9 ml 1M Tris HCl pH 6.8, 80 µl

10% (w/v) SDS, 80 µl 10% (w/v) APS, 10 µl TEMED). The resolving gel was poured first, overlaid with butanol (to form a straight, level interface) and allowed to polymerise. After removal of the butanol (and rising with dd-water) the stacking gel was poured and allowed to polymerise. Samples were mixed with an equal volume of SDS/loading buffer (50 mM Tris HCl pH 6.8, 100 mM DTT, 2% (w/v) SDS, 0.1% (w/v) bromphenol blue, 10% (v/v) glycerol) heated to 95°C for 3 minutes before loading. The gels were run in SDS/running buffer (25 mM Tris, 250 mM glycine pH 8.3, 0.1% (w/v) SDS) at 120 V for 90 minutes. The gel was stained for approximately 1 hour in Coomasie Blue, and then placed in destaining solution for approximately 12 hours. Sample of PSA for mass spectrometry were prepared in 5 mM ammonium acetate. The composition of different SELEX binding buffers is detailed in Table 5.1.

2.3.3 Immobilization of protein on the surface of magnetic beads

2.3.3.1 Amine chemistry using carboxylic acid modified magnetic beads

Ten milligrams (200 µl, 50 mg/ml) of carboxylated magnetic beads (CMBs) were washed twice with 500 µl of 100 mM MES, pH 5, for ten minutes with good mixing to remove preservatives. Washed CMBs were activated with 150 µl of 80 mg/ml of EDC and NHS in cold 100 mM MES, pH 5 and incubated for 20 minutes at room temperature under shaking conditions. Following incubation, the EDC/NHS solution was removed and activated CMBs were washed twice with 500 µl of 100 mM MES pH 5 to ensure elimination of free EDC/NHS. After activation with EDC/NHS 150 µg of PSA in 100 mM MES, pH 5 (total volume 300 µl) was added to activated beads and incubated overnight at room temperature with slow tilt rotation. Following incubation, the mixture of CMBs with PSA was placed in contact with a magnet for 4 minutes and any unbound target was removed. PSA coated beads were washed three times with 500 µl of PBS buffer and un-reacted

carboxylic groups on the CMB surface were blocked with 300 µl of 50 mM ethanolamine in PBS, pH 8 for 1 hour under shaking conditions. After blocking, the beads were again washed, separated and rinsed in 200 µl of SELEX binding buffer.

2.3.3.2 Carboxyl chemistry using amine modified magnetic beads

Ten milligrams (200 µl, 50 mg/ml) of amine modified magnetic beads (AMBs) were washed twice with 500 µl of 100 mM MES, pH 6, for ten minutes with good mixing to remove preservatives. Washed AMBs were resuspended in 150 µl of 80 mg/ml of EDC and NHS in cold 100 mM MES, pH 6 and mixed at room temperature. PSA (150 µg) was added to the solution and mixed on a shaker. After incubation, the mixture of AMBs with PSA was placed in contact with a magnet for 4 minutes and any unbound target was removed. Target coated beads were washed three times with 500 µl of PBS buffer and rinsed in 200 µl of SELEX binding buffer.

2.3.3.3 Epoxide chemistry

Ten milligrams (200 µl, 50 mg/ml) of epoxy activated magnetic beads (EMBs) were resuspended by vortexing for 1-2 minutes and transferred to a new tube. After washing, 150 µg of PSA in 0.1 M phosphate buffer and ammonium sulfate was added to the solution. The final concentration of ammonium sulfate was 1 M. The target was incubated with EMBs for 24 hours at 37°C with slow tilt rotation. After incubation, the coated beads were washed four times with 500 µl of PBS and rinsed in 200 µl of SELEX binding buffer.

2.3.3.4 Hydrazide

Glycons in the PSA target were oxidized by dissolving 0.5 mg of PSA in 100 µl 0.1 M sodium phosphate buffer, pH 7. This solution was added to an opaque vial containing 0.5 mg oxidation reagent ($NaIO_4$) and swirled gently to dissolve the oxidizing agent, and then incubated in the dark at room temperature for 30 minutes. Unreacted oxidation reagents were removed and buffer was changed by using spin columns. Ten milligrams (200 µl, 50 mg/ml) of hydrazide activated magnetic beads (HMBs) were washed with phosphate buffer using the magnetic separator. After the washing step HMBs were resuspended in 200 µl of coupling buffer containing 150 µg of PSA, and the mixture was incubated for 12 hours at room temperature. After incubation, the PSA coated beads were washed and rinsed in 200 µl in SELEX binding buffer.

2.3.3.5 Immobilization of biotinylated target on streptavidin coated magnetic beads

Biotinylation of PSA using EZ-Linker sulfo-NHS-biotin

One milligram of EZ-Linker sulfo-NHS-biotin was dissolved in 1 ml of RNAse free water reaching 1 mg/ml concentration. 50 µl of prepared solution was added to 200 µl of 1 mg/ml of PSA and incubated together on ice for 15 minutes. After incubation, 100 µl of 1M Tris HCl, pH 7.2 was added to inactivate the reaction. The non-reacted biotin was removed by dialysis with 2 mM Tris HCl, pH 7.2 overnight at 4°C.

Biotinylation of PSA using EZ-Link Amine PEGn-Biotin

Alternatively, one milligram of EZ-Link Amine PEGn-Biotin was dissolved in 50 µl of RNAse free water reaching 50 mM solution in 0.1 M MES pH 4.7 buffer. Immediately before use, 100 mM solution of EDC in MES buffer was prepared and 2 µl of this solution was added to the 50 mM biotin solution. Finally 200 ul of 1 mg/ml of PSA in MES buffer was added to the solution and incubated together for 2 hours at room temperature with mixing. Non-reacted biotinylation reagents and EDC by-products were removed by desalting using spin columns.

Coupling of biotinylated PSA to streptavidin beads

Two milligrams (200 µl, 10 mg/ml) of streptavidin beads were rinsed in 1 ml of 20 mM Tris HCl pH7, 200 mM NaCl to remove preservatives. The prepared beads were then suspended in 200 µl of 20 mM Tris HCl pH7, 1mM EDTA, 1M NaCl containing 150 µg of biotinylated PSA, and the beads were incubated for 30 minutes at RT, on an orbital shaker (200 r.p.m.) to prevent the beads settling out. Following incubation, the PSA-functionalised streptavidin coated beads were separated and rinsed in 200 µl SELEX binding buffer.

2.3.4 Characterization of target coupled beads

2.3.4.1 Competitive ELISA bead assay

Competitive ELISA bead assay was performed using a PVC microtiter plate. The plate was coated with 50 µl of 5 µg/ml of PSA in carbonate buffer and blocked with 200 µl PBS tween. PSA of different concentrations (0 – 60 µg/ml) and PSA coupled magnetic beads were incubated off-plate with anti PSA 10

antibody (9.6 μg/ml) and enzyme–linked secondary antibody (1/10 000 dilution). The appropriate concentration of antibody and enzyme linked secondary antibody was determined using a checkerboard titration. After this off-plate incubation step, the mixture of PSA, PSA beads and antibodies were added to the plate and incubated together. For each step, plates were incubated for 1 hour at 37°C on an orbital shaker (150 r.p.m.) before being washed three times with PBS-tween buffer. Finally 50 μl of TMB substrate was added to each well and incubated for 15 minutes at room temperature. Following addition of 50 μl of 0.1 M sulfuric acid, plates were analyzed using a Spectra max 340 PC plate readers (BioNova cientifica, Barcelona, Spain) at 450 nm.

2.3.4.2 Peptide Mass Fingerprinting

Digestion of PSA immobilized on the surface of beads

Ten microliters of PSA-functionalised magnetic beads were washed twice with 50 μl ammonium bicarbonate 25 mM (pH 8) for 2 minutes incubation followed by removal of supernatant using magnetic separation. After washing, the beads were resuspended in 10 μl of 20 mM DTT in 50 mM ammonium bicarbonate and incubated for 1 hour at 56ºC. The supernatant was removed and 10 μL of 50 mM iodoacetamide in 50 mM ammonium bicarbonate was added to the beads and incubated for another 20 minutes at RT, protected from light. Following reduction and alkylation, the PSA on the bead surface was digested by adding trypsin (14 ng/μl) in 25 mM ammonium bicarbonate, in a protein/trypsin ratio (w/w) of 1/50, for 16 hours at 37ºC. To allow complete peptide elution, the resulting digestion was sonicated for 10 min at 4º C and the supernatant containing peptides was collected and stored for further analysis.

MALDI-TOF Mass Spectrometry

One microliter of extracted peptides was spotted onto a MALDI plate and let to dry, followed by addition of 1 µl of the matrix (3 mg/ml α-cyano- 4-hydroxycinnamic acid matrix in 50% acetonitrile, 0.1% trifluoroacetic acid). Peptides were selected in the mass range of 750–3500 Da, and acquired in a positive reflector mode. All mass spectra were externally calibrated with the Sequazyme peptide mass standards kit and internally with trypsin autolysis peaks. The spectra obtained were processed using Data Explorer Software and MASCOT for matching the spectra profile obtained with the NCBI/ UniProtKB/ TrEMBL data-base.

2.3.4.3 PSA stability assay

PSA coupled beads were stored at 4°C for 4 weeks and the amount of PSA was studied using the ELISA bead assay (2.3.4.1).

2.3.4.4 Measurement of PSA activity

PSA activity was compared using 25 µg/ml of free PSA and PSA immobilized on the surface of the beads. PSA substrate ($C_{44}H_{60}N_{12}O_{13}F_3 \cdot 2HOAc$) was diluted to a final concentration of 400 µmol/L. The hydrolysis of the substrate by PSA was measured with a spectrofluorimeter (Cary Eclipse, Varian), using an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

2.4 Results and Discussion

2.4.1 Physicochemical characterization of PSA

Two different lots of PSA sample were studied in order to allow comparison of lot-to-lot variations. The purity and homogeneity of PSA obtained from seminal fluids was analyzed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE, Figure 2.7). The bands of major intensity migrated to the position of about 25 kDa under reduced conditions and 31-33 kDa under non-reduced conditions. It should be noted that SDS-PAGE tends to overestimate the molecular weight of glycoproteins. On reducing SDS-PAGE gel electrophoresis, fragments of lower intensity with different molecular weights were observed, which could be due to the nicked form of PSA or different forms of pro-PSA. The majority of the nicked PSA remains intact in vivo because of the presence of multiple disulfide bonds. Therefore under non-denaturing conditions, the nicked PSA co-migrates with the intact PSA, while on reducing SDS-PAGE, the PSA fractions separate, and molecules of lower molecular weight are revealed [22]. Fragments of different molecular weights observed on native PAGE can also indicate that PSA samples are composed of multiple forms of pro PSA varying in sugar moieties, which would affect the isoelectric point and resulting in heterogeneous pattern on the gel [23].

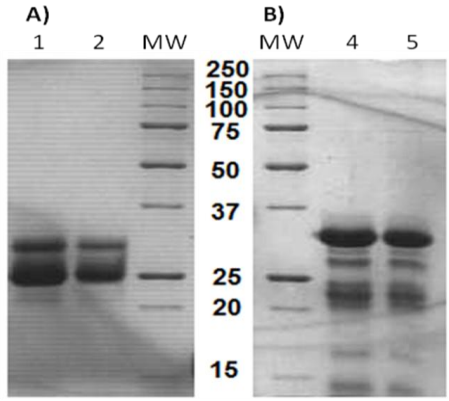


Figure 2.7 SDS-PAGE gel with two different lots of PSA sample obtained from seminal fluid. *A) under non denaturating conditions B) under reduced conditions, 1, 4 PSA sample with with lot number, 2, 5 PSA sample with another lot number.*

Mass spectrometry (MS) ionizes chemical compounds to generate charged molecules or molecule fragments and measures their mass-to-charge ratio [24]. The presence of different forms of PSA in our sample was also confirmed by MS (Figure 2.8). The predominant PSA molecular species (B bubble) with M_r 28 433 is very close to the calculated molecular mass of the intact PSA molecule (M_r 28 431) that contains a sequence of PSA (M_r 26 079.5) with a carbohydrate residue (M_r 2351.8). It must be noted that the original stock of PSA was dissolved in a potassium phosphate buffer, which can explain the formation of the potassium adduct at M_r 28 474 (C bubble). The other peaks in the spectrum observed at M_r 28 286 and 28 324 were assigned to the major glycoform of PSA minus fucose (M_r 146.1) and its potassium adduct (M_r 39.1), respectively as described in Belenger 1995 [25]. The two different lots of PSA sample used for the analysis showed almost identical spectra, indicative of a high consistency in the proportion of PSA isoforms in seminal fluids.

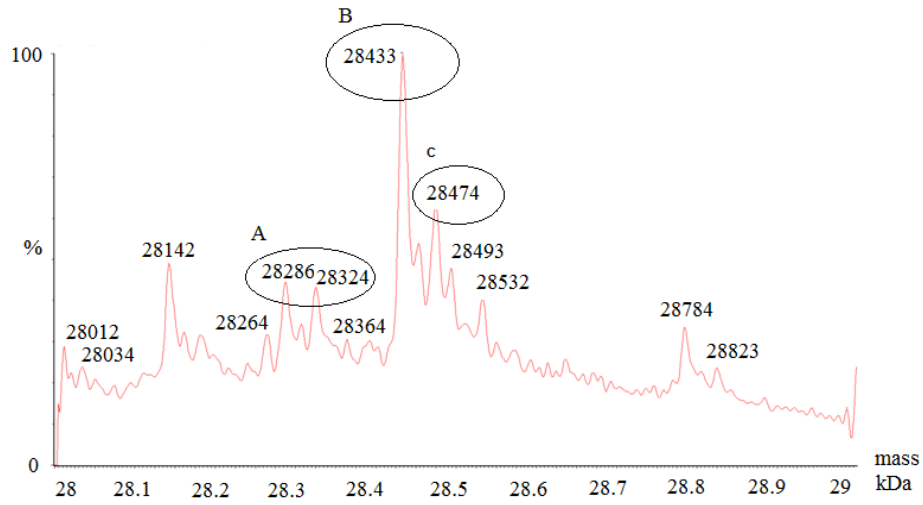


Figure 2.8 Spectrum of molecular weight for the PSA protein as obtained by TOF MS ES+ and reconstructed mathematically from m/z to molecular scale.

2.4.2 Immobilization of PSA on the surface of the beads

Taking into account specific features of PSA such as the composition of exposed amino acids, hydrophobicity, size of molecule, and the presence of carbohydrate chains, several immobilization strategies were evaluated. PSA contains primary amines in the side chain of 12 lysine (Lys) residues and the N-terminus of each polypeptide that are available for linkage. There are also 23 aminoacids containing carboxyl groups (Asp, Glu) and the carboxyl groups on C-terminus. Taking into consideration these factors carboxyl, amine, and epoxy modified magnetic beads were selected for the binding of ligands containing amine and carboxyl functional groups. PSA also contains 10 cysteine residues whose sulphur-containing side chains participate in stabilizing tertiary and quaternary structure through the formation of disulfide bonds. These disulfide bonds can be reduced to expose free sulfhydryls, but this can significantly interfere with required protein function or structure, therefore thiol chemistry was excluded from these studies. The presence of carbohydrate chains in the PSA structure provided another type of

site-directed immobilization using hydrazide modified beads. The bioaffinity biotin-avidin system was also evaluated.

2.4.3 Characterization of PSA-coupled beads

The degree of magnetic particle surface coated with PSA was studied using different techniques. Enzyme-Linked ImmunoSorbent Assay (ELISA), is a biochemical technique used to detect the presence of a target analyte in a sample, and we developed a competitive ELISA to quantify the amount of PSA immobilized on the beads. (Figure 2.9). Peptide mass fingerprinting was another method used to confirm the presence of PSA on the surface of the beads. In this method, protein immobilized on the beads is digested and the masses of released peptides are measured with mass spectrometry, and provides qualitative information.

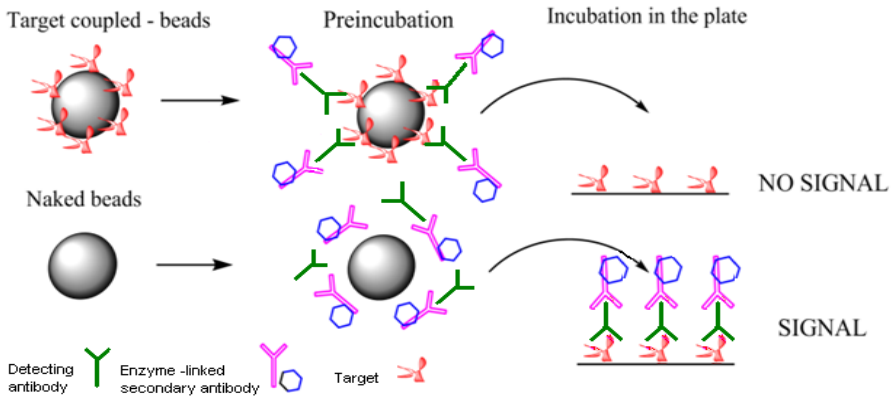


Figure 2.9 Scheme of competitive ELISA - bead assay. *Target coupled and naked beads are preincubated with detecting antibody and enzyme-linked secondary antibody. This mixture is then added to target coated well and incubated together. The plate is washed, and unbound antibodies removed. The more target in the preincubation solution, the less antibody will be able to bind to the target in the well, hence "competition". Finally substrate is added, and remaining enzymes elicit a signal. Naked beads are used in order to substract the background, which can be caused due to the presence of magnetic beads.*

2.4.3.1 Peptide Mass Fingerprinting

The attachment of PSA was firstly studied using peptide mass fingerprinting (p.m.f.). The resulting mass spectrometric data was processed using Data Explorer Software (Figure 2.10) and the Swiss Prot.2008.06.10 database (Table 2.2). Clear spectra were obtained indicating immobilization of pro-PSA for all the beads studied (the example given in Figure 2.10 is for carboxyl magnetic beads). This technique provided qualitative confirmation of the immobilization of the PSA target, but does not give quantitative information as to which of the beads provides the best surface for a high loading of PSA. However, in the case of targets for which no antibody exists and thus no ELISA can be developed to quantify the amount of immobilized target, this is a very useful technique to confirm target immobilization.

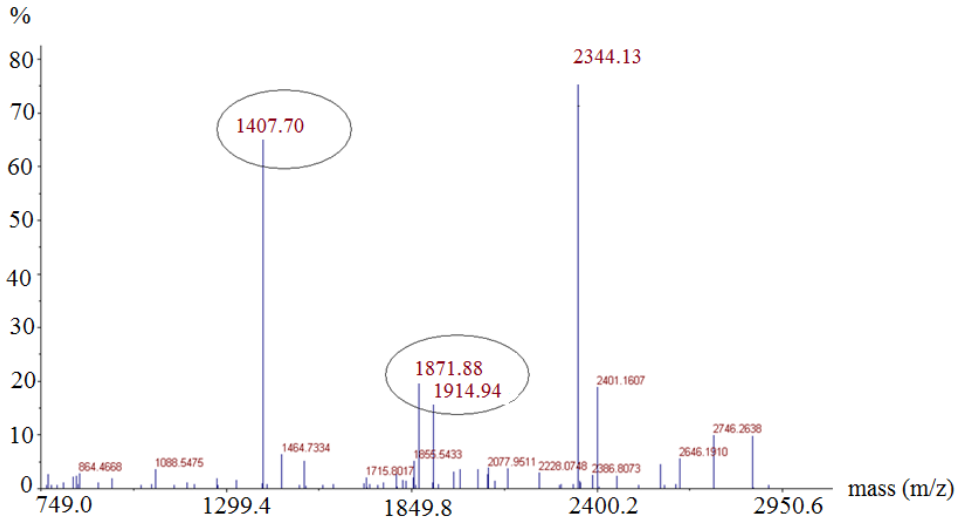


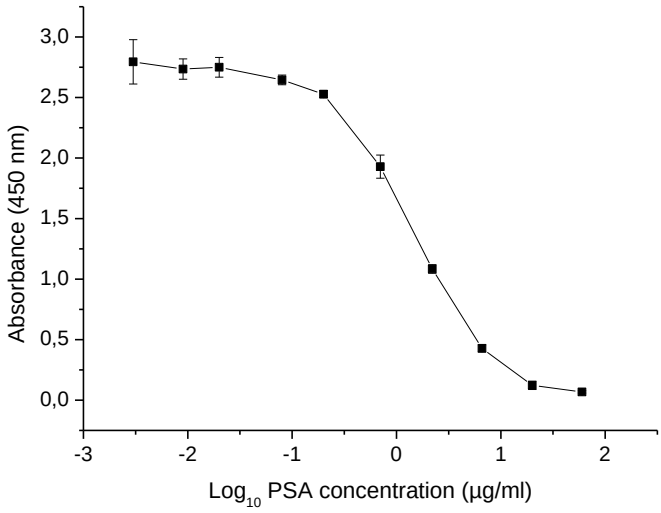
Figure 2.10 Mass spectrometric data obtained from Data Explorer Program. In bubbles there are peaks that correspond to pro-PSA.

Table 2.2 Peptide mass fingerprinting of protein immobilized on the beads obtained from Swiss Prot database.

Hit Number	Protein name	MOWSE	Protein MW	pI	Accession	Species
1	Prostate Specific Antigen precursor	2.96 + 6	28 742	7.6	P07288	Human

2.4.3.2 Quantification of PSA coupled beads

An ELISA bead assay, based on competition between PSA immobilized on the plate and PSA coupled to the surface of beads was used for the quantification of immobilized PSA. A calibration curve was constructed in the range of 0 to 60 μg/ml and a sigmoidal relationship was obtained ($R^2 = 0.999$), with a detection limit of 0.023 μg/ml (Figure 2.11). The calibration curve was used to quantify samples of PSA coupled beads prepared by different methodologies. Ten milligrams (200 μl, 50 mg/ml) of amine, carboxyl, epoxy and hydrazide functionalized beads and two milligrams (200 μl, 10 mg/ml) of streptavidin beads were used for these studies. The different bead concentrations were chosen due to the theoretical different binding capacities of the selected beads according to the manufacturer's instructions.



FIT VALUES	
LOD	0,023 µg/ml
EC50	1,468 µg/ml
R^2	0,999
Replicates	3

Figure 2.11 Standard curve for competitive ELISA bead assay and the best fit values.

The amount of PSA bound on the surface of amine, carboxyl, and epoxide functionalized beads is depicted in Figure 2.12. Similar amounts of PSA was observed using carboxyl (204 ± 12 µg/ml, n=5) and epoxy modified beads (218 ± 25 µg/ml, n=5), whilst a lower amount was found in the case of amine functionalized magnetic beads (107 ± 16, n=5). With respect to control (naked) beads, a high background was observed in the case of amine modified beads (31 ± 12 µg/ml, n=5), while in the other cases, a low (epoxide beads 7 ± 3 µg/ml, n=5) or no background (carboxyl beads) was observed. This background is probably due to the presence of magnetic beads in the assay, which could be eliminated using more washing steps.

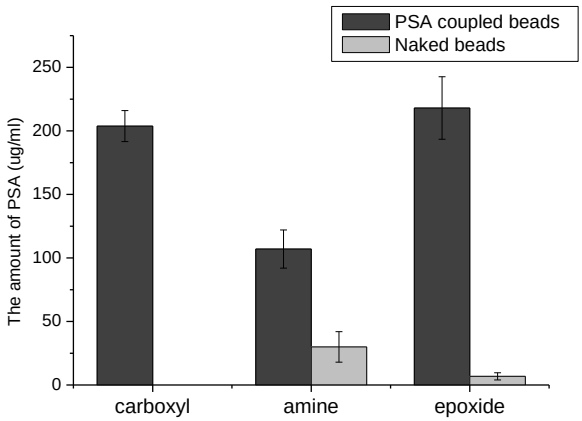


Figure 2.12 The degree of PSA on the surface of PSA coupled and control (naked) beads determined by ELISA bead competitive assay, (n=5).

In the case of the hydrazide modified magnetic beads, no consistent results were obtained (data not shown), which can be attributed to large losses of target during the oxidation process and a very low amount of PSA immobilized on the surface of the beads.

For the biotin-avidin system, two different linkers were used for biotinylation of PSA. Sulfo-NHS-Biotin enabled simple and efficient biotin labeling of any primary amine on the PSA, while Amine-PEG-Biotin linked to carboxyl groups on the PSA. The biotinylation of the protein was confirmed by Mass Spectra (Figure 2.13), where we could see additions of biotin xn (Mr 452.75) to the un-derivatized PSA (Mr 28 433).

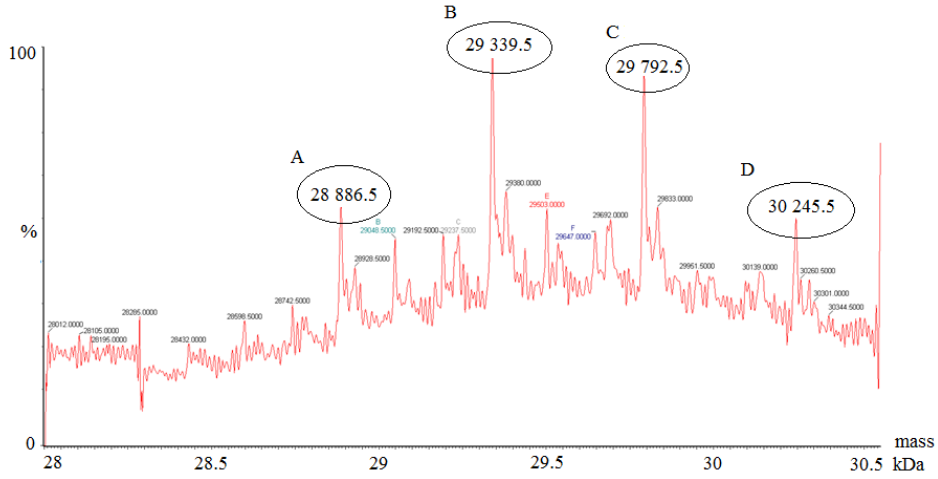


Figure 2.13 Spectrum of molecular weight for the biotinylated PSA with Sulfo-NHS-Biotin as obtained by TOF MS ES+ and reconstructed mathematically from m/z to molecular scale. *In the spectrum additions of 1 (A), 2 (B), 3 (C), and 4 (D) biotins can be observed.*

The amount of biotinylated PSA immobilized on the surface of streptavidin coated beads was estimated by measuring the OD_{280} of pre and post-bead binding solutions (Table 2.3).μ

Table 2.3 The amount of PSA immobilized on the surface of streptavidin coated beads determined by the measuring the OD_{280} *(N = 5).*

Type of biotinylation	PSA bound to the beads (μg/ml)	Total PSA for one mg of beads (in μg)	Total PSA for one mg of beads (in pmol)
Sulfo-NHS-Biotin	107 ± 12	11 ± 1	356 ± 41
Amine-PEG-Biotin	79 ± 11	8 ± 1	263 ± 37

These results are in good correlation with those obtained from ELISA bead assay (Figure 2.14), where the amount of immobilized PSA was 98 ± 10 μg/ml (n=5) in the case of Sulfo-NHS-Biotin, and 67 ± 12 μg/ml (n=5) in the case of Amine-PEG-Biotin. The slightly higher values obtained with absorbance

measurements may be due to non-specific absorption of biotinylated PSA to the tube. A higher amount of immobilized PSA was observed when Sulfo-NHS-Biotin was used for biotinylation of PSA. This is in agreement with the high levels obtained with the carboxyl coated beads and it appears that linking through the amine groups on PSA achieves a higher loading even though a lower number of lysine residues are available as compared to carboxyls.

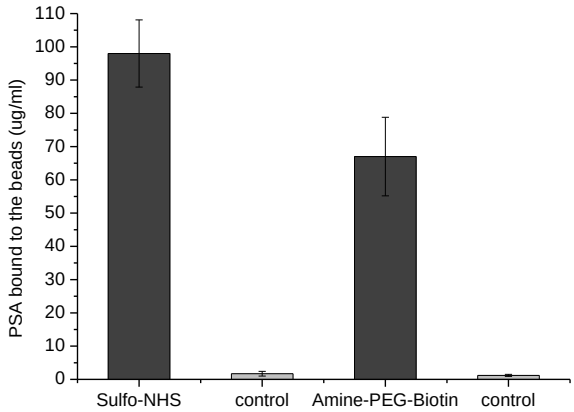


Figure 2.14 The degree of PSA on the surface of streptavidin coated beads determined by ELISA bead competitive assay, (n=5).

It is not only the amount of immobilized PSA but also the amount of magnetic beads in solution that is important during SELEX. A higher amount of beads represents more surface, which can result in higher nonspecific binding of oligonucleotides. For this reason we calculated the amount of immobilized PSA per milligram of modified beads (Figure 2.15), obtaining 134 ± 8 pmol (n=5) on carboxyl, 145 ± 16 pmol (n=5) on epoxide, and 71 ± 10 pmol, (n=5) on amine modified beads. In the case of the streptavidin beads, as expected due to the different binding capacity, higher values were observed per milligram of beads, with values differing according to the linkers used for biotinylation with 223 ± 39 pmol (n=5) for the Amine-PEG-Biotin linker and 326 ± 33 pmol (n=5) for the Sulfo-

NHS-Biotin linker showing the highest values of PSA immobilized on the surface of the beads, the latter thus having the highest loading of PSA/mg beads.

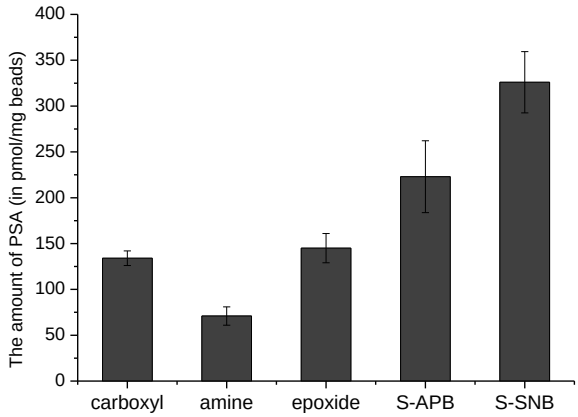


Figure 2.15 The amount of PSA (in pmol) calculated for one mg of beads (n=5)*. S-APB - streptavidin beads with Amine-PEG-Biotin linkedr, S-SNB - streptavidin beads with Sulfo NHS linker.*

2.4.3.3 Stability of immobilized PSA

The stability of PSA coupled beads was also evaluated. PSA coupled beads prepared as described previously were stored at 4° C for 4 weeks. After this period of time, the amount of remaining PSA was studied using the ELISA bead assay. The amount of PSA after 4 weeks storage was 117 ± 15 pmol (n=5) on carboxyl, 64 ± 10 pmol (n=5) on amine, and 83 ±13 pmol (n=5) on epoxy modified beads, whilst for the streptavidin beads, the amount of PSA was 325 ± 33 pmol (n=5) when Sulfo-NHS-Biotin linker was used, while 223 ± 39 pmol (n=5) was found in case of Amine-PEG-Biotin linker. In most of cases, ≥ 80 % of the amount of PSA was obtained, indicating that the PSA coupled beads are relatively resistant to storage conditions. Only PSA immobilized on the epoxy beads showed a severe reduction of PSA to 57% (Figure 2.16).

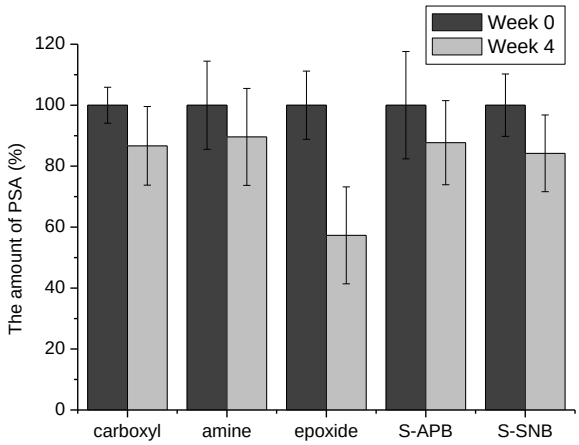


Figure 2.16 The amount of PSA converted into %. *100 % represent the amount of PSA obtained for each methodology in first ELISA beads Assay (week 0). S-APB - streptavidin beads with Amine-PEG-Biotin linkedr, S-SNB - streptavidin beads with Sulfo NHS linker.*

2.4.3.4 Activity of immobilized PSA

The influence of the different types of immobilization process on the activity of PSA bound to the beads was evaluated by using fluorogenic PSA substrate. To this end, the activity of PSA was monitored before, immediately after, and 4 weeks after the immobilization process (Figure 2.17). Results indicate, that none of the various types of immobilization process used in this work interfere with the structural and functional integrity of PSA.

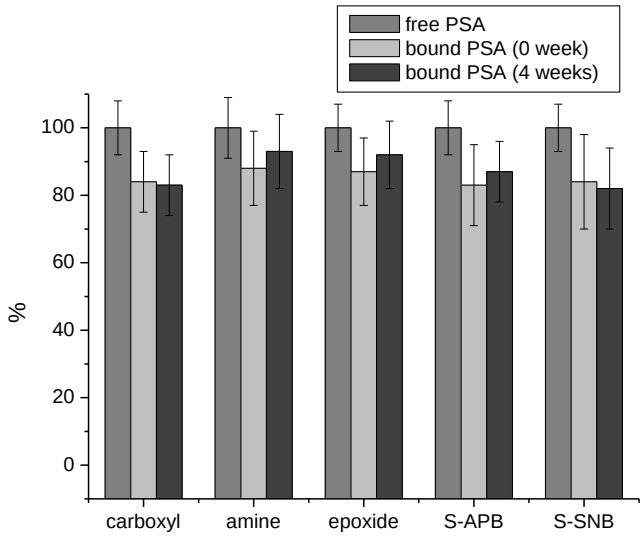


Figure 2.17 PSA activity before and after the immobilization process. *PSA protease activity was measured by means of the cleavage of a fluorogenic peptide substrate before (free PSA), immediately after (bound PSA 0), and 4 weeks (bound PSA 4) after the immobilization process. The values obtained prior to the immobilization were normalized to 100%. S-APB - streptavidin beads with Amine-PEG-Biotin linkedr, S-SNB - streptavidin beads with Sulfo NHS linker.*

2.5 Conclusions

The characterization of PSA samples obtained from seminal fluids was performed using different techniques. The purity and homogeneity of the samples were analyzed on SDS-PAGE. Fragments of different molecular weights observed on the gel indicated that the PSA samples are composed of different forms of PSA resulting in heterogeneous pattern on the gel. This heterogeneity of PSA samples was also confirmed by Mass spectrometry. Two different lots of PSA samples used in these studies showed identical MS spectra and a very similar pattern on SDS-PAGE gel electrophoresis, indicating a high consistency in the proportion of PSA isoforms in seminal fluids in the sample tested. Different immobilization strategies were investigated in order to determine the best strategy for PSA immobilization. Two promising strategies have been obtained from these studies: (i) the amine coupling using carboxyl modified beads and (ii) the use of bioaffinity technique with the biotin – streptavidin system. The use of carboxyl modified beads represents a covalent type of immobilization. This type of target binding can sometimes lead to the changes in protein structure or enzymatic activity, nevertheless in the case of PSA no changes in enzymatic activity were observed. In addition generally low non-specific binding of oligonucleotides is observed with this type of bead. On the other hand, the use of streptavidin coated beads seemed to be the best choice in terms of the amount of immobilized PSA. In comparison to other methods a lower amount of streptavidin beads needs to be used for the same amount of immobilized PSA. However the presence of streptavidin can be problematic during SELEX, as it can present an additional target for aptamer selection. Hitherto, several aptamers against streptavidin have been selected which is favoured as streptavidin appears to be an ideal target for aptamer selection [20, 26-27]. However, this can be solved using a negative selection step prior to the positive selection in order to remove oligonucleotides that bind to streptavidin.

2.6 References

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Chapter 3

3.1 Introduction

The starting point of a SELEX process is a chemically synthesized random library. This library consists of a multitude of single stranded DNA (ssDNA) fragments (~10^{15} molecules) comprising a central random region flanked by two fixed sequences, which function as primer binding sites in the PCR amplification (Figure 3.1). For the selection of RNA aptamers, this library needs to be transcribed into a RNA library. In the case of a DNA based SELEX process, this library can be used directly in the first round of selection. Amplification of the oligonucleotide random library at the beginning and throughout the SELEX is of considerable importance, as the correct choice of library and efficient PCR amplification of the random library contribute to the success of aptamer selection.

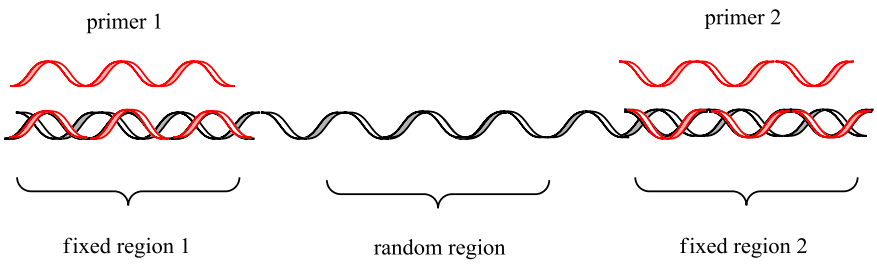

Figure 3.1 Scheme of oligonucleotide library. *Random region is flanked by two fixed regions which serve for annealing of primers during PCR amplification.*

3.1.1 Design of primers and oligonucleotide library

The design of the oligonucleotide library such as the size of the randomized region and primer-binding sequences flanking the randomized region plays an important role for the amplification of DNA. The size of the random region varies from 20 to 80 nucleotides. Although any length of randomized region may be used to provide 4^n theoretical random sequences, the maximum number of molecules that can be manipulated in standard molecular biology laboratories is in the order of 10^{15}, and errors of synthesis and workup have been estimated to reduce this diversity to 10^{13} - 10^{14} [1]. This number corresponds to the total number of different sequence variants for oligonucleotides of 25 nucleotides in length ($4^{25} \approx 10^{15}$). Libraries with the random region longer than 25 nt are thus under represented and contain the same number of different variants as shorter libraries [2]. An aptamer for the human thrombin was selected by Bock [3] from a DNA library of 96-mer oligonucleotides containing a random sequence with 50 nucleotides. The aptamer was truncated to the minimal size required for binding of the thrombin target and a consensus sequence with 15 nucleotides was obtained,

suggesting that libraries with short randomized regions are sufficient for aptamer selection. On the other hand longer randomized regions of libraries can lead to greater structural complexity and provide more opportunities for the selection of aptamers [4]. It has been demonstrated with selection of aptamers to isoleucine that both short (16nt) and the long (90nt) randomized regions complicate aptamer selection [5]. In practice, therefore, randomized regions of 30-60 nucleotides are most common. The primer binding regions should be long enough (20 - 23 nt) to ensure stable and specific hybridization of primers at the required temperature. These regions should not contain regions of internal complementary sequences so as to avoid hairpin structures. In addition, regions of sequence overlap with another primer should be avoided to circumvent primer-dimer formation [6]. A non-observance of these points may lead to the generation of longer or smaller size PCR products.

3.1.2 PCR amplification of a random library

An efficient SELEX procedure has to be accompanied by efficient PCR amplification to obtain one defined length of double stranded DNA (dsDNA). The specificity of PCR amplification is subject to variation depending on a fine balance of target concentration, primer specificity and amplification conditions [7]. Within the cocktail of components the concentration of magnesium, primer concentration and DNA polymerase activity may all influence the efficiency of the PCR reaction [8]. In addition, temperature, time, and number of PCR cycles must be optimized for the selected library and amplification conditions.

3.1.3 PCR optimization of a random library during SELEX process

In PCR for the selection of aptamers, the random DNA or RNA is used as the PCR template, and the heterogeneity of template sequences with different GC contents can cause non-uniform amplification during the PCR due to the stable stem-loop secondary formation of GC-regions. These structures promote polymerase jumping during PCR amplification, which yields PCR products of smaller sizes missing the stem-loop region of the template [9]. This non-uniform amplification then biases the composition of DNA sequences in the amplified library and consequently will affect the selected sequences. In addition, this will cause a loss of sequences that form stable secondary structures, which are usually found in ss-DNA or RNA aptamers. For this reason, it is critical to retain those sequences that form stable secondary structures during the iterative cycles of amplification during the selection process in order to obtain high-affinity aptamers [10].

3.1.3.1 Additives in PCR amplification

One of the common methods to improve yield and specificity of PCR amplification is the addition of certain organic additives, such as DMSO, betaine, polyethylene glycol, glycerol, formamide, 7-deaza-dGTP, or dITP to the reaction mixture [11-18]. Specifically, the effect of DMSO in the PCR amplification of some GC-rich sequences is the most widely studied [12-14]. There are also reports demonstrating that the addition of betaine also improves the amplification of GC-rich sequences [15-17]. Both additives can also act simultaneously and improve PCR amplification by increasing the ratio of full-length products to shortened products [10]. The presence of single-strand binding protein (SSB) in a PCR mixture, which is commonly used to prevent premature annealing, can also improve PCR efficiency [19]. The use of these additives for amplification of a

random library during SELEX process, will provide a greater chance to isolate aptamers, which have stable secondary structures and, therefore, high affinities to target molecules.

3.2 Objectives

The main objectives of this chapter were the analysis and optimisation of PCR amplification of three selected random libraries. The optimisation of PCR conditions was performed particularly for SELEX. More specifically, this encompassed 1) selection of 3 different random libraries and their specific primers, 2) analysis and optimisation of PCR amplification of the initial pool of random libraries, 3) optimisation of PCR amplification of random libraries during the SELEX process.

3.3 Methodology

3.3.1 Oligonucleotide libraries

Three different libraries were used in this work. All libraries contain a random region of the sequence flanked by fixed primer hybridization regions. Library I (5'-AGC TGA CAC AGC AGG TTG GTG - N(49) - CA CGA GTC GAG CAA TCT CGA AAT-3') was amplified using a forward primer F_1 (5´-AGC TGA CAC AGC AGG TTG GTG-3´), and reverse primer R_1 (5´-ATT TCG AGA TTG CTC GAC TCG TG-3´). Library II (5´-GCC TGT TGT GAG CCT CCT AAC – N(49) – CA TGC TTA TTC TTG TCT CCC-3´) was amplified using a forward primer F_2 (5´- GCC TGT TGT GAG CCT CCT AAC-3´), and reverse primer R_2 (5´-GG GAG ACA AGA ATA AGC ATG-3´). Library III (5´-AGC TCC AGA AGA TAA ATT ACA GG – N(53) – CAA CTA GGA TAC TAT GAC CCC-3´) was amplified by forward primer F_3 (5´- AGC TCC AGA AGA TAA ATT ACA GG-3´), F_3 primer containing T7 promotor sequence F_3_T7 (5´- CCC AAG CTT AAT ACG ACT CAC TAT AGG GAG CTC CAG AAG ATA AAT TAC AGG-3´), and reverse primer R_3 (5´-GGG GTC ATA GTA TCC TAG TTG-3´). The first two random libraries and their primers were synthesized, purified, lyophilized and purchased from BIOMERS (Ulm, Germany). Library III was supplied by Eurofins MWG Operon (Ebersberg, Germany).

3.3.2 Amplification of a random libraries

3.3.2.1 Library I

The amplification of library I was performed in 100 µl reactions with 200 nM of primers (R_1, F_1), 3 mM of $MgCl_2$, 0.2 mM of dNTP, 1x Taq buffer (200 mM Tris pH 8.4, 500 mM KCl), and 2.5 units of Taq polymerase. Four PCR reactions containing different concentrations of initial template (100 pM, 200 pM, 300 pM, 400 pM,) with one PCR reaction without DNA template as the negative control were heated at 95°C for 2 minutes, followed by 15 rounds of PCR, with a denaturing step at 95°C for 30 seconds, annealing step at 60°C for 30 seconds, and an elongation step at 72°C for 30 seconds. A final extension step was performed at 72°C for 5 minutes. PCR amplification was carried out in a DNA Engine Dyad® Peltier Thermal cycler. All PCR reagents were obtained from Invitrogen (Barcelona, Spain). Double stranded PCR products were checked by electrophoresis. For each sample, 10 µl was run on the gel with 4 µl of loading buffer (Invitrogen, Madrid, Spain) on a 4% w/v agarose gel stained with GelRed nucleic acid stain (Biotium, Hayward, USA) and visualized with a UV lamp (Biorad, Madrid, Spain). Certified Low Range Ultra Agarose was supplied by Biorad (Madrid, Spain). Fragment sizes were determined by comparison with a 10 bp molecular weight standard (Invitrogen, Madrid, Spain) and analyzed using Image-J software.

3.3.2.2 Library II

The amplification of library II was performed in 100 µl of PCR reactions with 1.1 µM forward primer (F_2), 0.9 µM reverse primer (R_2), 3.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µg/µl BSA, 1x Taq PCR buffer, 2.5 units of Taq polymerase, 1 µl of template and water to final volume. Five PCR reactions containing different

concentrations of initial template (100 pM, 200 pM, 300 pM, 400 pM) or water in place of DNA in the case of the negative control were heated at 95°C for 2 minutes, followed by settings: 1 min 95°C, 1 min 54°C, 1.5 min 72°C for 15 times. The final extension was performed at 72°C for 5 minutes. The gel electrophoresis and analysis of amplified product was carried out as described above (section 3.3.2.1).

3.3.2.3 Library III

The initial pool Library III was generated by amplification using 50 µl of PCR reactions. Six PCR reactions containing different concentrations of starting library (0.4 µM, 40 nM, 4 nM, 0.2 nM, 20 pM) were used. Each reaction contained 0.2 mM dNTPs, 0.5 µM of both primers (R_3, F_3_T7) 1 x KAPA 2G Buffer (containing 1,5 mM $MgCl_2$), 1 x KAPA enhancer, 2 Units of 2G robust enzyme, 1 µl of template, and nuclease free water to 50 µl of final volume. This was heated for 2 minutes at 95°C, followed by 10 cycles of 30 seconds at 95°C, 60°C, and 72°C. After PCR, amplified product was checked on agarose gel as described above (section 3.3.2.1.).

3.4 Results and Discussion

3.4.1 Selection of random libraries

There are a lot of commercially available oligonucleotide libraries which have been successfully used to select aptamers with high affinities for their targets. These random libraries are reliable and the affinity characteristics of the selected oligonucleotides are generally very good. Three different libraries were used in this work. Library I was previously used by Bock in 1992 for the selection of the thrombin binding aptamer [3]. Library II was previously used for the selection of different biotinylated targets bound to the streptavidin beads [20]. The last library (III) was used in the selection of 2´F modified RNA aptamers in the group of Prof. Peter Stockley (University of Leeds, England). All these three libraries contained 49-50 randomized regions, which give the libraries a greater structural complexity. This is particularly important for challenging targets. Therefore, a longer random sequence pool may provide better opportunities for the indentification of aptamers [4].

3.4.2 PCR analysis and optimisation

3.4.2.1 Library I

Library I was primarily amplified using standard PCR conditions. In the case of primers > 20 nt it is recommended to use an annealing temperature about 3°C higher than the lowest T_m of the pair of primers used in the PCR reaction [21-22], therefore the annealing temperature of about 60°C (T_a) was selected for the first amplifications of Library I. The template concentration was used in the range 100 – 400 pM. In the preliminary assessment (Figure 3.2.), a PCR product of desired size was amplified, but also significant non-specific amplification (by-product) was observed. It was deemed that this could be overcome with further optimisation of the PCR reaction.

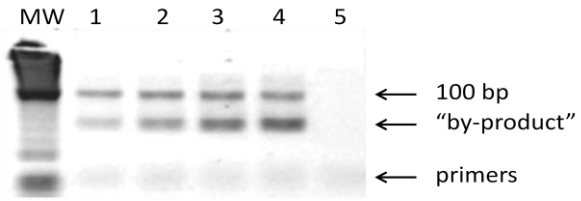

Figure 3.2 Preliminary PCR amplification of library I with different concentration of template *(1: 100 pM, 2: 200 pM, 3: 300pM, 4: 400 pM, 5: Non Template Control (NTC)), MW – 10 bp molecular weight marker.*

Optimisation of $MgCl_2$ concentration using two different DNA polymerases

The most suitable polymerase for SELEX process was evaluated by using *Taq* polymerase, and *Tfi* polymerase. These polymerases were selected as they are readily available and are cost effective in comparison to more specialised

polymerases on the market. The $MgCl_2$ concentration was optimised for each enzyme by amplifying 300 pM of synthetic library in PCR cocktails containing one of the two polymerases and using between 3 and 7 mM of $MgCl_2$ (Figure 3.3). In the case of *Taq* polymerase the $MgCl_2$ concentration associated with a higher yield of desired DNA band was 5 mM and above. However, with increasing concentrations of $MgCl_2$ non-specific products were also observed. *Tfi* polymerase showed the optimum concentration of $MgCl_2$ to be 5 mM. At higher concentrations of $MgCl_2$ the amount of specific product decreased, which can be due to the inhibition of DNA polymerases by high concentrations of $MgCl_2$. Taking into consideration the amount of amplified specific band and the amount of non-specific product, *Tfi* polymerase with 5 mM of $MgCl_2$ was selected for the subsequent SELEX process.

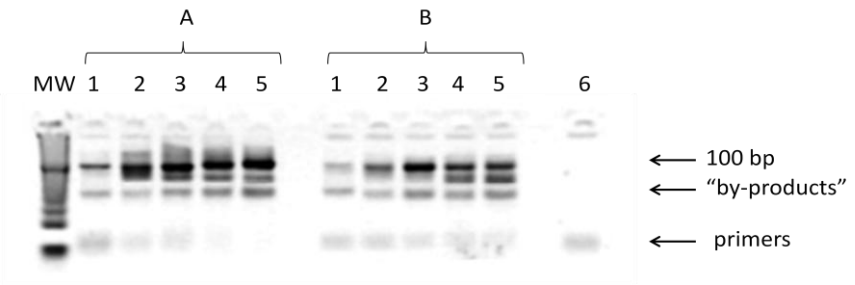

Figure 3.3 Optimisation of concentration of $MgCl_2$ for DNA polymerases. *Amplification was carried out with each polymerase (A – Taq polymerase, B – Tfi polymerase) and optimised with $MgCl_2$ concentration ranging from 3 to 7 mM (1: 3mM, 2: 4mM, 3: 5mM, 4: 6mM, 5: 7mM). Negative controls were run for each sample, on the gel the NTC is shown (6) for Tfi polymerase with 5 mM concentration of $MgCl_2$.*

Optimisation of annealing temperature, dNTP´s, primer and polymeras concentrations, and number of PCR cycles.

Having selected the DNA polymerase, $MgCl_2$ and template concentration, the remaining components of the PCR reaction were optimised with the aim of

improving PCR specificity. The primer, dNTPs and polymerase concentrations, along with the amplification temperature and number of PCR cycles were optimised in a series of multi-factorial experiments. The specificity and the amount of yield of PCR product was not improved by changing the primer, dNTPs or polymerase concentrations. On the other hand, a considerable improvement in the specificity of the PCR amplification was observed by decreasing the annealing temperature from 60° C to 58° C (Figure 3.4).

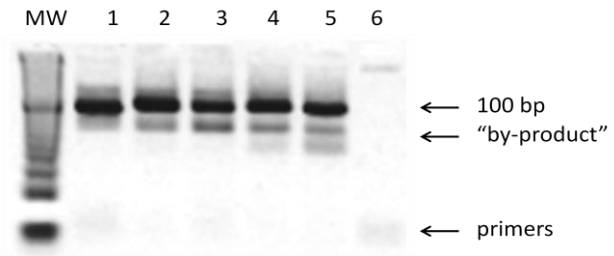

Figure 3.4 Effect of annealing temperature on the specificity of PCR product. *The optimisation was carried out ranging from 58° C to 60° C (1: 58° C, 2: 58.5° C, 3: 59° C, 4: 59.5° C, 5: 60° C). MW – 10 bp molecular weight marker, 6 – NTC.*

This was further improved by decreasing the number of PCR cycles, resulting in specific amplification without any non-specific product (Figure 3.5).

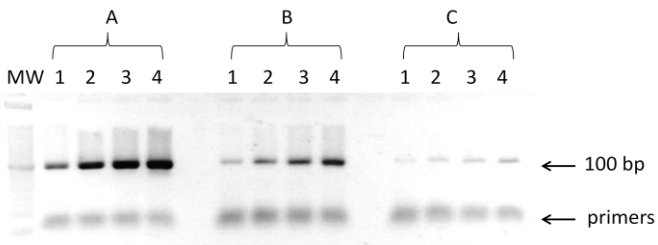

Figure 3.5 Different template concentration using 15 (A), 10 (B), 5 (C) numbers of PCR cycles. *MW – 10 bp molecular weight marker. Different template concentration (1: 100 pM, 2: 200 pM, 3: 300 pM, 4: 400 pM).*

We have also found that when more than 15-20 PCR cycles was used, non-specific amplification occurred. As PCR amplification in this work involves libraries of random DNA sequences, it is in essence different from PCR amplification of homogenous DNA templates. Product formation for a homogenous DNA template progresses until primers are depleted, however for a random DNA library, synthesis stops even when PCR primers are still in abundance. The products then promptly transmute into by-products. Moreover, over-amplification may also cause a complete loss of products. The optimal conditions elucidated for the amplification of library I was 200 nM primers, 200 μM dNTP, 2.5 units of *Tfi* polymerase, 5 mM $MgCl_2$ with an annealing temperature of 58°C and the number of PCR cycles not exceeding more than 15 cycles. This optimized protocol was subsequently adopted for SELEX.

3.4.2.2 Library II

The amplification of library II was achieved using a previously described protocol [20]. No PCR product of desired size was present in any sample, while very significant amounts of non-specific "by products" were observed (Figure 3.6). Non-specific amplification was also found in the non-template control indicating a problem due to the high concentration of primers.

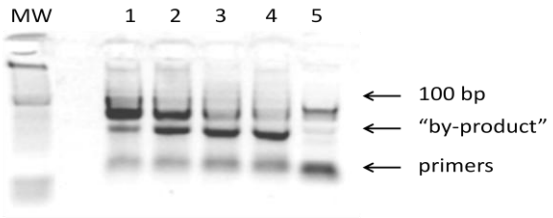

Figure 3.6 Preliminary PCR amplification of library II with different concentrations of template *(1: 100 pM, 2: 200 pM, 3: 300 pM, 4: 400 pM, 5: NTC), MW – 10 bp molecular weight marker.*

A series of multi-factorial experiments were carried out using different PCR conditions such as amount of template, primer, dNTPs, DNA polymerase, and $MgCl_2$ concentrations with changes in the number of PCR cycles, time and temperature. Following optimisation, a new protocol containing 200 nM primers, 200 μM dNTP, 2.5 units of *Tfi* polymerase, 5 mM $MgCl_2$, 0.5 μg/μl BSA with a reduced cycle length (30 sec 95°C, 30 sec 54°C, 45 sec 72°C) resulted in a considerable improvement in the specificity of PCR product (Figure 3.7).

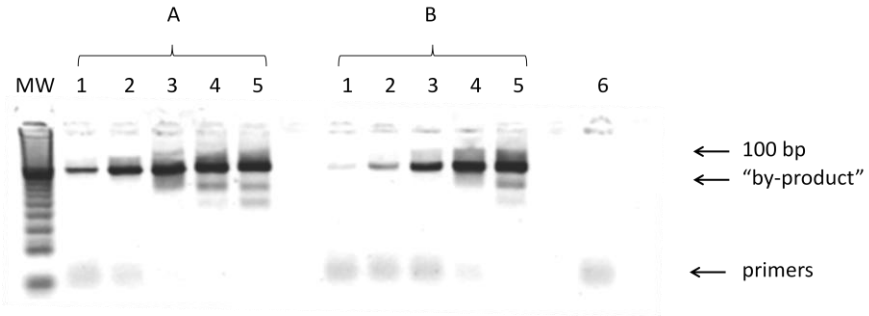

Figure 3.7 Different number of PCR cycles using two different template concentrations *(A – 300 pM, B – 100 pM) using new optimised protocol. MW – 10 bp molecular weight marker. Different number of PCR cycles (1: 5, 2: 10, 3: 15, 4: 20, 5: 25), NTC (6).*

3.4.2.3 Library III

Library III was used for the preparation of a RNA library, and thus a specific forward primer containing T7 promotor sequence (F_3_T7) was used for PCR analysis. The amplification of this library was performed using serial dilutions of starting library, and the optimum concentration of the initial pool was found to be 4 nM (Figure 3.8) where a discrete and specific band was observed without any non-specific products.

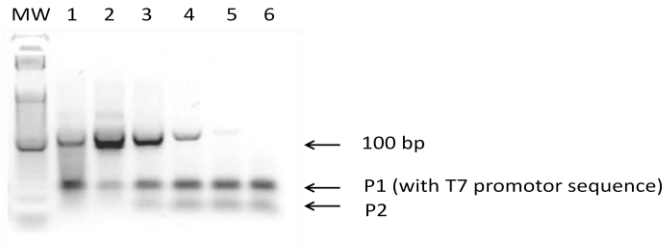

Figure 3.8 Preliminary PCR amplification of library III with different concentrations of template *(1: 0.4 uM, 2: 40 nM, 3: 4 nM, 4: 0.2 nM, 5: 20 pM, 6: NTC), MW – 10 bp molecular weight marker.*

3.4.3 Amplification of random libraries during SELEX process

During several selection processes problems with the amplification of the random library were observed. The most evident was that an initial nucleic acid population that appeared as a discrete band on a separating agarose gel after three or four rounds of the SELEX process smeared into a multi-sized population (Figure 3.9).

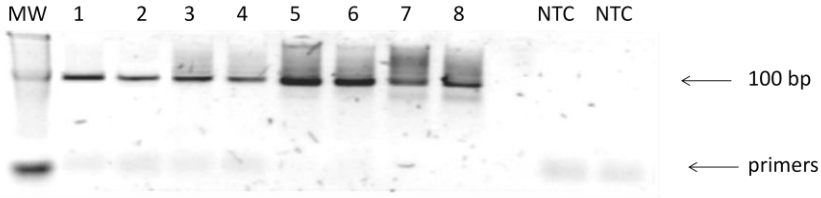

Figure 3.9 PCR product from different rounds of SELEX. *MW - 10 bp molecular weight marker, 1-8 selection rounds. From the third round of SELEX we can observe smearing into multisized population.*

The most likely explanation is that there is mispriming of the 3´ends of DNA in the random regions of other molecules in the population. This smeared band affected subsequent generation of ssDNA, resulting in a lower yield. To solve

this problem, different methods such as emulsion PCR [23], or addition of different reagents which are potentially effective for amplification of random libraries (see section 2.1.3.1) were used. The addition of single stranded binding protein (SSB), and optimization of PCR conditions after each round of SELEX with shorter annealing times, higher concentrations of template and a low number of PCR cycles were found to prevent such smearing (Figure 3.10).

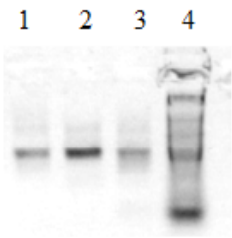

Figure 3.10 Double stranded DNA product of enriched library using optimized PCR conditions. *Enriched library was amplified using optimized conditions with 0,1 μg/ml SSB (1), x 0,5 μg/ml SSB (2), or without SSB , 4 - 10 bp molecular weight marker.*

In another case of selection, after several rounds of SELEX, PCR products of smaller sizes appeared. This problem seemed to be related to the selection of sequences with high GC contents as mentioned above. Periodic gel band purification and addition of different concentrations of certain organic additives, such as DMSO, betaine, polyethylene glycol, glycerol and formamide, to the reaction mixture were used to resolve this problem. Figure 3.10 depicts one of the selection processes with this specific problem, where a smaller band appears in the second selection round, and with increasing intensity in further selection rounds. For this reason, the purification of the 100 bp band was performed after the 4th and 6th round of SELEX. Nevertheless, this band repeatedly appeared during the selection process. The best conditions to decrease the amount of these undesired products were obtained by performing PCR amplification with 3% DMSO and 500 mM Betaine.

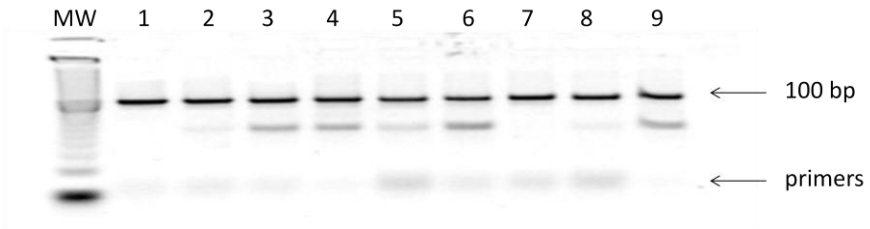

Figure 3.11 PCR product from different rounds of SELEX. *MW – 10 bp molecular weight marker, 1-9 different SELEX rounds. Lower band is evident from third round. After 4th and 6th round purification of 100bp band from agarose gel was performed.*

3.5 Conclusions

The selection of three different libraries with their specific features was performed, and the analysis of amplified products and the optimization of PCR conditions for all libraries were carried out to ensure a high purity of the amplified libraries. At the end of PCR optimisation new protocols specific for each library were determined for use in SELEX (Chapters 5, 6). The composition of random libraries changes during the SELEX process and optimisation of enriched libraries is often required. We have found that the addition of SSB protein with optimized PCR conditions prevented smearing of the band into a multi-sized population. When products of smaller sizes appeared during SELEX process, PCR amplification performed with 3% of DMSO and 500 mM of Betaine helped to decrease the amount of these undesired products.

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Chapter 4

4.1 Introduction

Single stranded DNA (ssDNA) generation is a crucial step in several molecular biology applications, such as sequencing or DNA chip and microarray technology. Molecules of ssDNA also play a key role in the selection of ssDNA aptamers through SELEX. The generation of the ssDNA is a critical step as the purity and the amount of ssDNA can have a significant impact on the successful evolution throughout the SELEX process.

Various methods have been reported for the generation of ssDNA, and one of the most widely used methods is magnetic separation using streptavidin coated beads [1-2]. In this technique, a biotinylated primer is used during amplification, resulting in a biotinylated double-stranded (ds) DNA duplex, which is then captured by the strepatavidin coated magnetic beads, and subjected to chemical/thermal denaturation, where the non-biotinylated DNA strand is released

into a supernatant and eluted. An alternative method used for the generation of ssDNA exploits a urea-polyacrylamide gel [3-5] where PCR is performed with a specific long primer to produce strands of different lengths, which are separated using denaturing gel electrophoresis and the separated strands are identified by UV shadowing or fluorescence and the desired strand is excised from the gel. In another approach termed asymmetric PCR, one strand of the template DNA is amplified more than the other by using an unequal molar ratio of forward and reverse primers, and when the primer present at a markedly lower concentration becomes exhausted, single stranded DNA is thus produced in each PCR cycle [6-7]. Alternatively, exonucleases such as the T7 Gene 6 exonuclease [8-9] and Lambda exonuclease [10-12] can be used to produce ssDNA, via modification of the primers used in amplification. The T7 Gene 6 exonuclease hydrolyses one strand of DNA non-processively in the 5' to 3' direction, while the second strand is protected against the hydrolytic activity of the enzyme with several phosphotorothioates at its 5'end. In the case of the lambda exonuclease, phosphorylated strands of DNA are selectively digested by the enzyme, which has greatly reduced activity on ssDNA and non-phosphorylated DNA. Finally, using denaturing high performance liquid chromatography, a biotinylated primer is used for amplification and, following denaturation, the strands are separated based on differences in retention time [13].

Generation of ssDNA is not only important in SELEX, but also for other applications such as Pyrosequencing [14-15], solid phase DNA sequencing [16] DNA single strand conformation polymorphism (SSCP) analysis [17], nuclease S1 mapping [18], probe preparation and labelling [19], subtractive hybridization [20], as well as for DNA chips and microarrays [21-22].

Specifically related to SELEX, several studies comparing different methods for the generation of ssDNA have been reported [17, 22]. Recently, detailed review about DNA aptamers achieved by different type of ssDNA generation methods was described [23], showing that aptamers with similar characteristics have been selected by different methods, and suggesting that success of DNA SELEX is not principally influenced by the type of methods employed in generating ssDNA. The

most critical points are the quality of ssDNA and the amount of obtained ssDNA. Final ssDNA should be free from impurities such as remaining dsDNA, proteins and enzymes which can be present together with ssDNA depending on the technique used for ssDNA generation. And no less important is the amount of ssDNA which should be controlled in each selection round for successful selection of highly affine aptamers. All generally used methods required many parallel reactions in order to prepare sufficient amount of ssDNA which can be used for one selection round.

Moreover, generation of ssDNA is very time-consuming in methods such as enzyme digestion and magnetic separation due to the several purification steps, including purification of PCR product in order to use buffers specific for each technique and purification of ssDNA at the end of process which is absolutely necessary to remove any possible impurities which remain in the solution. In this work we compare the yield obtained from purified and unpurified PCR product showing the possibility of ssDNA generation directly from PCR product without the need of extra purification step.

In addition, we present a new technique which combines the high efficient asymmetric PCR with enzyme digestion resulting in minimum remaining impurities and absolutely lowest cost required for ssDNA preparation.

4.2 Objectives

The main objective of this part was to compare techniques generally used for the generation of ssDNA, including the previously reported methods of asymmetric PCR, lambda exonuclease digestion and magnetic separation, as well as new combined method mentioned above. The efficiency of each system for the generation of ssDNA was quantitatively evaluated using an enzyme linked oligonucleotide assay (ELONA) which specifically quantifies ss DNA and the quality of ssDNA was assessed using agarose gel electrophoresis. Finally, all studied methods were compared in terms of time and cost needed for preparation of 50 pmol of ssDNA generally used for one selection round of SELEX.

4.3 Methodology

4.3.1 Reagents

A single stranded DNA library containing a 49-mer sequence flanked by fixed primer hybridization regions (SL-lib_ 5'-AGC TGA CAC AGC AGG TTG GTG - N(49) - CA CGA GTC GAG CAA TCT CGA AAT-3') was used. This library was amplified using a forward primer (F_ 5´-AGC TGA CAC AGC AGG TTG GTG-3´), and reverse primer (R _ 5´-ATT TCG AGA TTG CTC GAC TCG TG-3´). Modifications of the reverse primers with biotin (R_bio), phosphate (R_pho), and modifications of the forward primer with phosphorothioate (F_pto) were used depending on the method of ssDNA generation. Horseradish peroxidase labelled probe (HRP probe_ 5´-CAC CAA CCT GCT GTG TCA GCT-3´) was used in ELONA studies. The random library, all primers, and HRP labelled probe were HPLC-purified, lyophilised and purchased from BIOMERS (Ulm, Germany). Taq polymerase and dNTP Set were supplied from Invitrogen (Madrid, Spain). Certified Low Range Ultra Agarose was purchased from Biorad (Madrid,Spain), and T7 Gene 6 Exonuclease with Lambda Exonuclease from Affymetrix, Wycombe Lane, United Kingdom.

4.3.2 Amplification of the random library

Amplification of the random library was performed with 300 pM of initial template. All PCR reactions were performed in a total volume of 100 µl with 200 nM of primers, 5 mM of $MgCl_2$, 200 µM of dNTP, 1x Taq buffer (200 mM Tris pH 8.4, 500 mM KCl) and 2.5 units of Taq polymerase. The PCR reactions were heated at 95°C for 2 minutes in order to completely denature the template,

followed by 10 rounds of PCR, with 30 seconds of denaturation at 95°C, 30 seconds of annealing at 58°C, and 30 seconds of elongation at 72°C. A final extension step was performed at 72°C for 5 minutes. Double stranded PCR products were checked using electrophoresis. For each sample, 10 μl was run with 4 μl of loading buffer (Invitrogen, Madrid, Spain) on a 4% w/v agarose gel stained with GelRed nucleic acid stain (Biotium, Hayward, USA) and imaged with UV lamp (λ 254 nm). When purified dsDNA was used, 100 μl of PCR product was purified using Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen, Madrid, Spain) according to the manufacturer's instructions. Purified dsDNA was diluted in 100 μl of 10 mM Tris-HCl, pH 8.5 and checked using electrophoresis. For the semi-quantitative determination of the concentration of the purified and unpurified PCR products, 10 μl of each sample were compared with 10 μl of dsDNA standards of known concentration (50 nM, 60 nM, 70 nM, 80 nM, 90 nM) using agarose gel electrophoresis. All standards were prepared Electrophoretic analysis of the band intensities was performed using Image J program.

4.3.3 Generation of ssDNA

4.3.3.1 Magnetic separation with streptavidin coated beads

The random library was amplified using R_Bio modified reverse primer and unmodified forward primer. A half miligram of streptavidin beads (Chemicell, Berlin, Germany) was washed twice with 200 μl of 1x B & W buffer (150 mM NaCl, 15 mM trisodium citrate, pH 7.0) and re-suspended in 100 μl of 2 x B&W. For each reaction, 100 μl of biotinylated PCR product was incubated for 20 minutes with pre-washed magnetic beads on a Dynal Sample Mixer (Invitrogen, Barcelona, Spain). After incubation, the beads were washed with 1x B&W buffer, and subsequently an alkaline denaturation with 50 μl of 150 mM NaOH for 3 minutes was performed. The eluted non-biotinylated ssDNA was purified using the

QIAEX II Gel Extraction kit, which desalts and concentrates DNA (Qiagen Iberia, Madrid, Spain) and was diluted in 100 µl of 10 mM Tris-HCl, pH 8.5.

4.3.3.2 Enzyme digestion

The random library was amplified using R_pho or F_pto modified primer with the corresponding un-modified primer. For each reaction, 100 µl of PCR product modified with F_pto primer was incubated with 40 U (40U/µl) of T7 Gene 6 Exonuclease in a total reaction volume of 101 µl at 37°C. Similarly, 100 µl of PCR product modified with R_pho was incubated with 10 U (10U/µl) of Lambda Exonuclease. Digestion times of 10, 30, 45 and 60 minutes were evaluated for both enzymes. All reactions were stopped by 10 minutes incubation at 80°C. Samples were purified and diluted in 100 µl of 10 mM Tris-Cl, pH 8.5.

4.3.3.3 Asymmetric PCR with combination of enzyme digestion

The random library was first amplified as described above using primers depending on the method used for elimination of remaining dsDNA. For Lambda digestion modified reverse primer (R_pho) was required, whilst for T 7 Gene 6 exonuclease and for extraction of ssDNA from agarose gel, modified F_pto was used. Asymmetric PCR (A-PCR) was then performed using an optimised protocol as described previously [24]. All A-PCR reactions were performed in a total volume of 120 µl, with 100 µl of master mix (5 mM of $MgCl_2$, 200 µM of dNTP, 1x Taq buffer (200 mM Tris pH 8.4, 500 mM KCl), and 2.5 U of Taq polymerase) with 0.4 µM of a single primer (R_pho or F_pto) and direct addition of 20 µl of the amplified random library (ds-PCR). The thermocycling protocol established for amplification of the random library was modified by increasing the extension time from 30 seconds to 2 minutes. The optimum number of A-PCR cycles was 12 for

both single primers. Following A-PCR amplification, the product was divided into two aliquots. One aliquot was purified and diluted in 100 µl of 10 mM Tris-HCl, pH 8.5, whilst the other aliquot was treated with the appropriate exonuclease enzyme as described above. When extraction of ssDNA from agarose gel was used, fifty microliters of A-PCR product was run through the gel. The ssDNA band was subsequently excised from the gel, and purified using the QUAEX II Gel Extraction kit for purification of DNA fragments from gels and solutions according to the manufacturer's instructions (Qiagen Iberia, Madrid, Spain) and diluted in 100 µl of 10 mM Tris-HCl, pH 8.5.

4.3.4 Qualitative evaluation of ssDNA by electrophoretic analysis

For all the studied methods for ssDNA generation, 10 µl of the ssDNA generated were run on an agarose gel with 4 µl of loading buffer and 2 µl of 150 mM NaOH**.**

4.3.5 Quantitative evaluation of the ssDNA by ELONA

ELONA experiments were carried out on streptavidin-coated plates (Sigma, Barcelona, Spain). Biotinylated reverse primer (20 nM in 50 mM PBS, pH 7.4) was added to each well of the SA plates and incubated for 1 hour at 37°C. The plates were then washed with the Hydroflex automatic plate washer (Tecan, Barcelona, Spain) three times with 200 µl of PBS. A dilution series of different concentrations (0.1 to 100 nM) of synthetic ssDNA of the same length as the random library (93 bases) were applied to the immobilized R-bio primer and incubated for 1 hour at 37°C. Following thorough washing, 1 nM of HRP probe

was added to the plate for one hour incubation at 37°C. The presence of the HRP enzyme label was detected using TMB substrate (Sigma, Madrid, Spain), and monitoring of colour development was carried out at 650 nm and stopped after 20 minutes with 0.1 M H_2SO_4, and measured at 450 nm using a Spectra max 340 PC plate reader (BioNova cientifica, Barcelona, Spain). The ssDNA generated using the different techniques were diluted using different dilution factors, and quantified by interpolation of the constructed calibration curve.

4.4 Results and Discussion

4.4.1 Concentration determination of the starting dsDNA

Single stranded DNA generation is the most time-consuming step of the DNA-SELEX process, normally requiring purification of dsDNA from the PCR product. Not only is the purification a time-intensive and expensive process, but it also results in loss of the dsDNA product which can varied from 10 to 40% depending on the purification methods used. To this end, the different methods studied were carried out on purified and unpurified/purified dsDNA. In order to calculate the efficiency of the generation of the ssDNA, it is necessary to know the starting concentration of dsDNA. However, in the case of the unpurified dsDNA, it is not possible to use UV-Vis spectrometry to measure the DNA concentration at λ= 260 nm, as the primers, smaller/larger side-products of the PCR as well as the Taq polymerase would interfere and contribute to the absorbance reading. Therefore, the concentration of the dsDNA was determined via electrophoretic gel analysis using the Image J software to measure the intensity of bands of dsDNA standards of known concentration, which were used to construct a calibration curve (R^2 = 0.9934), which was used to quantify the purified and unpurified dsDNA. For the unpurified dsDNA, concentrations of 71 ± 4 nM (n=3), 72 ± 3 nM (n=3), 70 ± 3 nM (n=3), were obtained for each of the pto, pho and bio modified PCR products, respectively, whilst for the purified concentrations of 50 ± 3 nM (n=3), 49 ± 3 nM (n=3) and 52 ± 3 nM (n=3) were obtained for the pto, pho and bio modified PCR products. The concentration of both type of PCR products was confirmed by 2100 Bioanalyzer (Agilent) using DNA 1000 chip and excellent correlation was obtained, highlighting the reliability of the electrophoretic gel analysis methods. It is interesting to note that in this case 30 % of dsDNA was lost during the purification process.

4.4.2 Qualitative and quantitative analysis of ssDNA

In order to check the quality of ssDNA, samples obtained from different methodologies were analyzed by electrophoretic analysis. Standards of ssDNA of known concentration were run in agarose gel together with other samples in order to roughly quantify the yield of ssDNA using Image J software. The accurate quantification of ssDNA was carried out using ELONA (Figure 4.1). Biotinylated probe (R-bio) was immobilized on a streptavidin coated plate, followed by addition of ssDNA, which hybridises to the immobilised probe. After incubation, complementary probe labelled with HRP was added and detected using TMB.

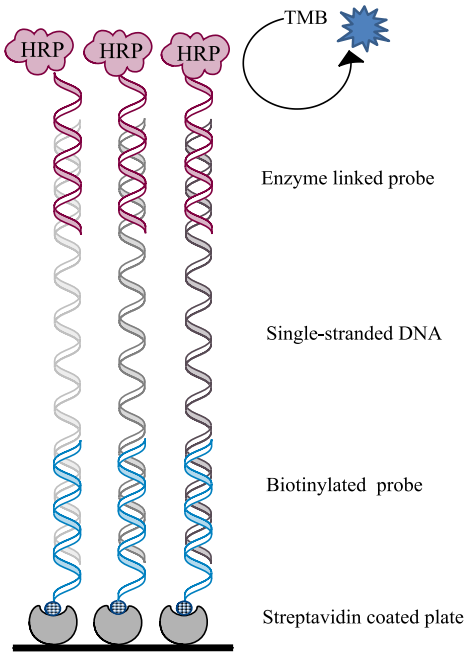


Figure 4.1 Scheme of the Enzyme Linked OligoNucleotide Assay (ELONA). *Biotinylated probe (R_bio) is immobilized on a streptavidin coated plate, followed by addition of ssDNA which binds to the biotinylated probe via base pairing. In the final step another complementary probe modified with HRP is added and detected using TMB substrate.*

4.4.3 Enzyme digestion and magnetic beads based methods

The quality of the single stranded DNA obtained from purified and unpurified PCR products was checked by agarose gel electrophoresis. As can be seen in Figure 4.2 a, there is a clear and well-defined band that corresponds to ssDNA. For the enzyme digestion methods, some remaining dsDNA was observed and, as expected, when unpurified dsDNA was used, there were bands corresponding to the primers, whilst only the ssDNA band was observed for the magnetic bead method. Electrophoretic analysis of the DNA bands in the agarose gel showed similar yields of ssDNA obtained from both purified and unpurified PCR products indicating the possibility of generation of ssDNA directly from unpurified PCR product, which was confirmed by the quantification of ssDNA using ELONA. The theoretical maximum yield of ssDNA was derived from the initial dsDNA concentration (i.e. ~ 70 nM for unpurified PCR product, and ~ 50 nM for purified PCR product). The percentage recovery yields of ssDNA obtained using the different methods for both purified and unpurified PCR products is depicted in Figure 4.2 b. A calibration curve was constructed in the range 0.1 to 100 nM and a sigmoidal relationship was obtained (R^2= 0.9976), with a detection limit of 0.19 nM. The yield of ssDNA obtained by T 7 enzyme digestion was 58 ± 10 % (n=5) for purified PCR product, 63 ± 9 % (n=5) for unpurified product. For the magnetic beads based method the yield of ssDNA was 58 ± 8 % (n=5) for purified PCR product and approximately 5 % less for unpurified PCR product, 52 ± 8 % (n=5), and in the case of Lambda exonuclease digestion the yield of ssDNA differed 15 % dependent on the purity of the dsDNA (69 ± 7 % (n=5) for purified PCR product, and 52 ± 7 % (n=5) for unpurified PCR product). The total amount of ssDNA obtained from unpurified PCR products is higher in all cases taking into account the loss of dsDNA in purification step (Figure 4.2 c).

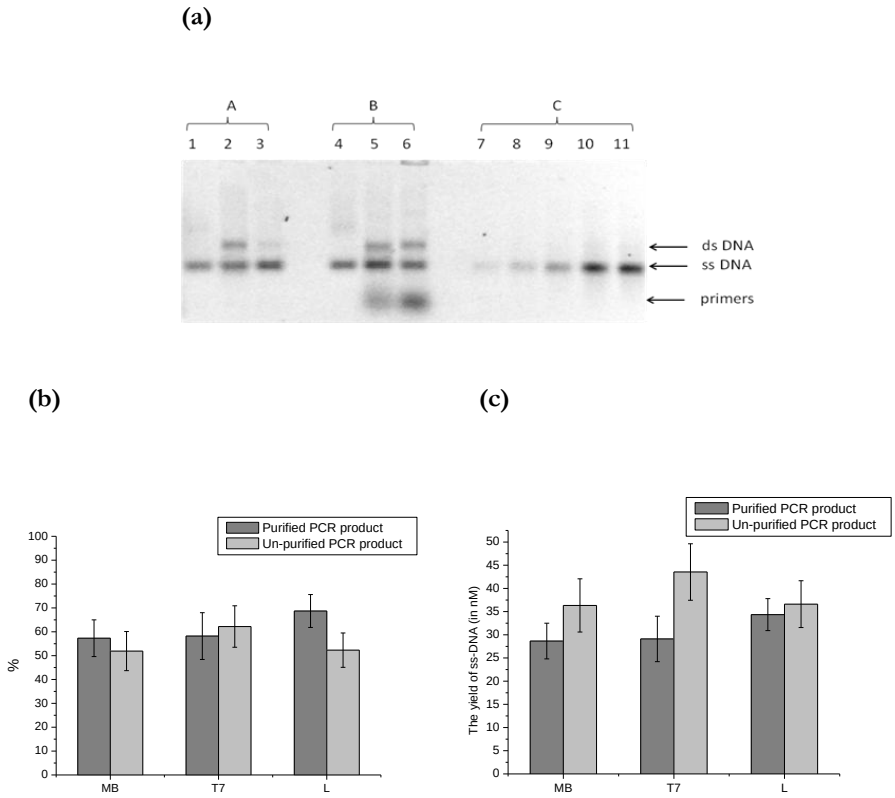


Figure 4.2 Analysis of ssDNA obtained from purified and unpurified PCR product by magnetic beads (MB), T7 gene 6 exonuclease (T7), and Lambda exonuclease (L) *(a) 4% w/v agarose gel analysis; A –purified PCR product; B – unpurified PCR product; (1, 4) - ssDNA from MB; (2, 5) – ssDNA from T7; (3, 6) – ssDNA from L; C - ssDNA standards (7: 10 nM, 8: 20 nM, 9: 30 nM, 10: 50 nM, 11: 70 nM). (b) Recovery of ssDNA in % obtained from MB, T7, and L by ELONA. (c) The final concentration of ssDNA (in nM) obtained from purified / unpurified PCR products.*

As we mentioned previously the magnetic beads based method is one of the most widely used in SELEX. However, previous studies have demonstrated the possible dissociation of streptavidin due to high temperature or alkaline treatment [25-26] and the presence of this streptavidin during the incubation step of SELEX can cause selection of an aptamer against this additional target, which is favoured as streptavidin appears to be an ideal target for aptamer selection[3, 27-28]. This is a

major problem for SELEX where the target is not immobilised and is in its' preferred native state (e.g. use of nitrocellulose membrane for partitioning of bound and unbound DNA). The presence of streptavidin can be eliminated by phenol/chloroform extraction and subsequent ethanol precipitation, which is however, associated with a loss of approximately 30% of DNA. This loss of DNA could be overcome by the use of specific purification kits, which minimise the loss of DNA, but are relatively expensive. Alternatively, aptamer selection against dissociated streptavidin can also be avoided by employing an appropriate counter selection step, but this is time-intensive, and increases the complexity of the SELEX. Furthermore, and affecting all types of SELEX independently of whether the target is immobilised, any biotinylated capture DNA linked to the dissociated streptavidin can re-anneal to the complementary strands of the random pool, and thus cause loss of tertiary structure, which is important for target binding [10].

In the case of the exonuclease digestion, Lambda exonuclease has a reduced activity on ssDNA but after a certain period of time it can start to digest un-phosphorylated ssDNA. A study was thus carried out to optimise the time for maximal ssDNA generation and minimal ssDNA digestion. As can be seen in Figure 4.3, a decreasing intensity of the dsDNA band was observed as digestion time increased, reaching a plateau at 45 minutes. However, still after 1 hour there was a visible weak band which corresponds to dsDNA. Nevertheless, this band is not necessarily composed from complete dsDNA product, as sometimes ssDNA alone or bound to the primer leads to the similar migration behaviour as the dsDNA [10]. The optimal time of incubation for the efficient generation of ssDNA was thus estimated to be 45 minutes for both enzymes, as the longer digestion time did not result in increase of the yield of ssDNA.

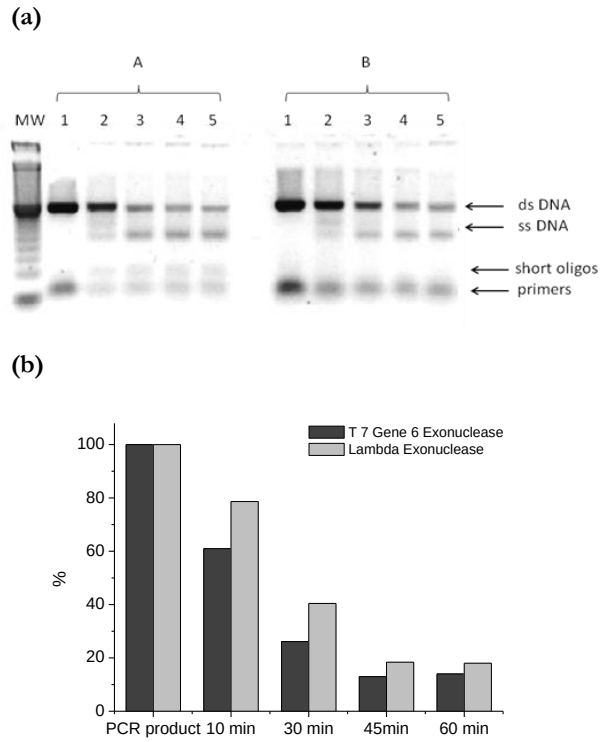


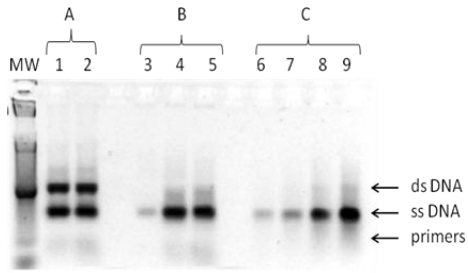
Figure 4.3 Evaluation of the effect of time on dsDNA digestion. *(a) 4% Agarose gel analysis. Samples for T7 (A) and Lambda (B) enzyme with starting ds-PCR product (1), after 10 min (2), 30 min (3), 45 min (4), and 60 minutes of digestion (5). (b) Graphic analysis of remaining dsDNA during different time of digestion using electrophoretic analysis with Image J program.*

4.4.4 Asymmetric PCR with combination of enzyme digestión

An asymmetric PCR generates one of the strands by linear amplification. In comparison to others method, A-PCR produces new molecules of ssDNA, resulting in a final yield of ssDNA that can be much higher than initial concentration of dsDNA template. The diversity of ssDNA, which is also important for the SELEX process, depends on the concentration of initial dsDNA template. However, the error prone nature of the polymerases used in PCR leads to the introduction of mutations that effectively result in a greater diversity [29].

Products from Asymmetric PCR (A-PCR) contain both ssDNA and dsDNA, which can interfere in the binding step during the selection process, and to eliminate this dsDNA a time-consuming purification step, extracting from polyacrylamide or agarose gel has been reported [7, 30-31]. However this purification step based on the extraction of band from agarose or polyacrylamide gel results in a poor yield of ssDNA (29 ± 10 %, n=5) that can affect the selection process due to the large loss of aptamer candidates, and thus diversity, which is particularly important in the first cycles of SELEX where the amplification step and preparation of ssDNA is critical. We thus explored the possibility of combining A-PCR with the exonuclease digestion to improve the yield of ssDNA. As can be seen in Figure 4.4 a, using agarose gel electrophoresis the band of dsDNA is not visible indicating that most of the remaining dsDNA had been converted into ssDNA. The ssDNA samples generated were then quantified using ELONA (Figure 4.4 b). For asymmetric PCR, the theoretical maximum yield of ssDNA (366 nM) was calculated by single primer concentration and dsDNA template used in A-PCR reaction. The yield of ssDNA obtained by A-PCR was 72 ± 5 % with modified forward primer (F-pto) and 67 ± 5 % with modified reverse primer (R-pho), demonstrating a marked improvement in the yield of ssDNA, but is still contaminated with dsDNA. However, after enzyme digestion of A-PCR product the yield increased to 84 ± 6 % with T7 enzyme and 75 ± 6 % due to elimination of the remaining dsDNA.

(a)



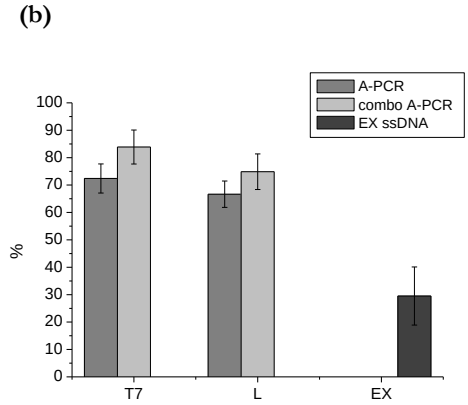


***Figure 4.4 Analysis of ssDNA obtained from Asymmetric PCR (A-PCR)**, A-PCR and enzyme digestion (combo A-PCR), and extraction of ssDNA band from agarose gel (EX-ssDNA). (a) 4% w/v agarose gel analysis; A – A-PCR with modified primers (1 – F_pto for T7 enzyme digestion, 2 – R_pho for lambda digestion); B – Single stranded DNA obtained from A-PCR with combination of T 7 enzyme (4), or lambda digestion (5), and 2 x diluted sample of extraction of ssDNA band (3); C – standards of ssDNA (6: 50 nM, 7: 100 nM, 8: 200 nM, 9: 400 nM) (b) The recovery of ssDNA obtained from A-PCR, combo A-PCR (T7 or L), and EX-ssDNA by ELONA.*

Other factors that should be considered in selecting the preferred method for SELEX are the time required for generation of ssDNA and the cost. Table 1 summarizes the percentage recovery yield with the final amount of ssDNA obtained for each of the approaches studied, as well as the time required for each technique and finally an estimation of the costs associated with the preparation of 50 pmol of ssDNA. Asymmetric PCR followed by enzyme digestions produces the highest amount of ssDNA with low cost in comparison to other methodologies reported in this study. The highest cost is associated with the generation of ssDNA from purified PCR products, which is 10-15 times more expensive than asymmetric PCR followed by enzyme digestion. Asymmetric PCR and both enzyme digestions require ca. 1 hour, whilst the rest of methods can be performed in less than 2 hours.

Table 4.1 Comparison of all methodologies in terms of ssDNA recovery, the amount of ssDNA obtained, time needed for its preparation and estimated costs calculated for 50 pmol of ssDNA (n=5).

Method used for ssDNA generation	Percentage Recovery ± RSD%, (n=5)	Amount of ssDNA (nM)	Estimated time (hours)	Estimated costs for 50 pmol of ssDNA (€)
Magnetic beads*	57.3 ± 7.7	28,6 ± 3,9	1.5-2.5	68
T7 Gene 6 Exonuclease*	58.2 ± 9.8	29,1 ±4,9	1.5-2	52
Lambda Exonuclease*	68.7 ± 6.9	34,6 ± 3,5	1.5-2	45
Magnetic beads	51.9 ± 8.2	36,3 ± 5,7	1-2	30
T 7 Gene 6 Exonuclease	62.6 ± 8.7	43,5 ± 6	1-1.25	15
Lambda Exonuclease	52.3 ±7.2	36,6 ± 5	1-1.25	14
A-PCR $_{\text{with F\_pto primer}}$	72.4 ± 5.3	265 ± 19	0.75-1	2
A-PCR $_{\text{with R\_pho primer}}$	66.7 ± 4.8	244 ± 19,5	0.75-1	2
A-PCR with T7 enzyme	83.9 ± 6.2	307 ± 22	1.5-2	4
A-PCR with Lambda	74.9 ± 6.5	274 ± 23,8	1.5-2	3
A-PCR (extraction of ssDNA)	29.1 ± 10.6	106 ± 39	2-2.5	14

***** *Single stranded DNA generated from purified PCR product*

4.5 Conclusions

The preparation of high-quality ssDNA is of paramount importance to ensure the success of SELEX and numerous other molecular biology procedures. In this work we have performed comparison studies of different methods that can be used for the generation of ssDNA for consequent use in SELEX. The quality of ssDNA compared using electrophoretic analysis showed a clear and well-defined band, which corresponds to ssDNA, for all the studied methods. The quantity of ssDNA was determined using ELONA, which permitted quantification of the different yields of ssDNA, showing high efficiency and good reproducibility for all studied methods confirming that all the reported methods can be used for the generation of ssDNA, once their disadvantages are clearly taken into consideration and the SELEX adapted accordingly. We have demonstrated that the purification of PCR product is not necessary as the yield of ssDNA obtained directly from PCR product is comparable to that obtained from purified PCR product, thus decreasing the time required for ssDNA preparation. In comparison to others methods, asymmetric PCR followed by enzyme digestion was clearly demonstrated to have the highest efficiency, producing the highest amount of ssDNA.

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Chapter 5

5.1 Introduction

The selection of an aptamer depends greatly on the properties of the target, its´ availability and purity. Moreover, there is no a well established and generic methodology for aptamer selection and in many cases, the success of SELEX depends on the experimental conditions used during the selection process [1]. Futhermore, the pI value and hydrophobicity of the target molecule can influence the capacity of negatively charged oligonucleotides to bind to the target, and conditions for the final use of the aptamer also should be taken into consideration [2]. To date, a large number of aptamers have been selected against various targets, with several examples of both RNA and DNA aptamers being selected against the same target, showing similar affinities and specificities [3]. Compared with RNA aptamers, DNA aptamers have certain advantages, such as a simpler and faster selection process, greater stability than unmodified

RNA aptamers, ease of labelling, as well as being less expensive than RNA or modified RNA. Consequently, DNA aptamers have become more widespread during recent years and seem to be more suitable as a molecular recognition element in biosensors.

5.1.1 DNA based SELEX process

A typical DNA based SELEX round involves five basic steps, namely, (i) incubation of single stranded DNA (ssDNA) oligonucleotides with the target of interest; (ii) partitioning of target bound oligonucleotides from the rest of the library; (iii) elution of bound ssDNA from ssDNA/target complex; (iv) amplification of eluted oligonucleotides, and (iv) preparation of ssDNA oligonucleotides for use as template in the next SELEX round (Figure 5.1). This selection and amplification process is repeated (usually 8 to 15 rounds) and the ligands with the highest affinity for the targets are then isolated, cloned and sequenced.

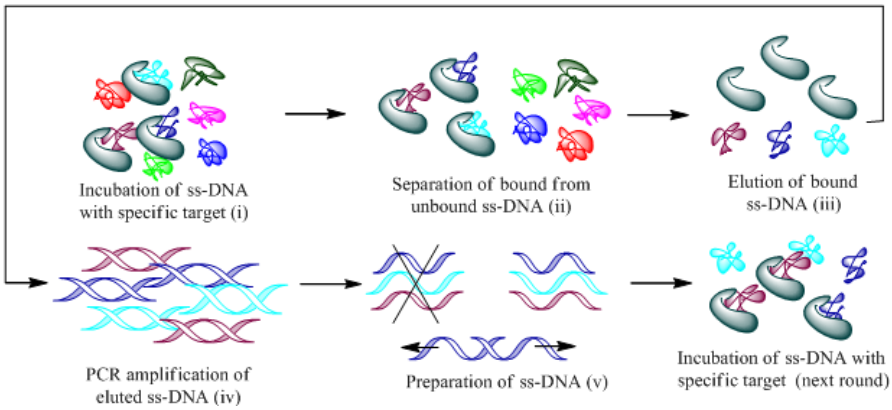


Figure 5.1. Schematic of DNA based SELEX.

5.1.1.1 Incubation of oligonucleotide pool with target molecule

The binding properties of aptamers depend on the folding of nucleic acid sequences and the docking with its target, which is very sensitive to environmental conditions. Changes in the environment can affect the conformation and hence binding properties of aptamers. Buffer composition also plays an important role, as well as the net negative charge on all nucleic acids which make their conformation and interaction with other molecules extremely sensitive to salt concentrations [4]. The best buffer to use in selection experiments is therefore one as close as possible to the conditions in which the aptamer will be applied and used.

Another important parameter is the length incubation of the library with the target, which should allow an efficient interaction between the different species. Incubation for too short a period of time can result in the selection of an aptamer with rapid association rates but not necessarily high affinity. A good rule of thumb is that competition between species with nanomolar dissociation constants (K_D) requires minutes, and picomolar requires hours [5]. In order to avoid incubation periods of several hours, one can simply increase the competition between species by adjusting the pool:target ratio. A low ratio will allow species with low binding affinities to remain in the pool whereas high ratios will favor competition between aptamers and therefore generate a population with a higher binding affinity. In most cases a pool:target ratio between 10:1 and 100:1 is optimal [5-6].

5.1.1.2 Partitioning methodology

After binding, partitioning of the oligonucleotide-target complex from oligonucleotides not bound to the target can be achieved by various techniques.

A commonly used method is affinity chromatography with immobilization of the target on a sepharose or agarose column [7-10]. The use of magnetic beads is also widely used for the separation of target and nucleic acids [11-13], as this method only requires small amounts of target and is simple to handle. Whilst the immobilization of the target on a specific matrix allows an effective separation, protein immobilization can destroy key features of some aptatopes and reduce the mobility of the protein; to overcome this, an alternative is to use ultrafiltration without target immobilization [14-17]. Alternative partitioning strategies have been reported, based on the use of flow cytometry [18-20], and surface plasmon resonance (SPR), which provide binding efficiency information and on-line evaluation during the selection process [21]. Atomic force microscopy (AFM) can dynamically detect the adhesion or affinity force between a sample surface and a cantilever, a feature that is useful for the selection of high affinity aptamers, but is inherently expensive [22]. Electrophoretic separation [23-26] or centrifugation [27-28] are further examples of tools for partitioning.

5.1.1.3 Elution of ssDNA from the ssDNA/target complex

Following removal of the unbound part of the library, bound oligonucleotides are generally eluted from the ssDNA/target complex. Methods used for elution include denaturing methods such as heat treatment [29] or addition of denaturing substances like urea, SDS, EDTA or NaOH [14]. Other approaches used are affinity elution [30] or photoelution by application of a photocleavable linker [31].

5.1.1.4 PCR Amplification

For the first round of DNA selection, a chemically synthesized ssDNA library can be used directly without PCR amplification. Some researchers perform amplification of the starting random DNA library before SELEX in order to eliminate damaged DNA synthesis products, which cannot be amplified by PCR [32]. This preliminary step can lead to the loss some of the target-binding sequences after the first selection round due to the different efficiency of DNA polymerase to amplify chemically synthesized DNA [3]. Whilst large-scale PCR is a time and material consuming process, this initial PCR step provides several copies of the original library which can bind to the target and thus increases the probability of selection of the desired sequence.

The high degree of diversity of the initial oligonucleotide library results in a very small percentage of the library bound to the target at the end of the first selection step, which consequently requires to be amplified. Single stranded DNA aptamers are amplified by straightforward PCR, and it is possible to attach modifications *via* special primers during the amplification step, which can be used to monitor a specific oligonucleotide enrichment during the selection process or for the preparation of single stranded DNA [3].

5.1.1.5 Preparation of single stranded DNA

The generation of ssDNA and their application in DNA based SELEX process was previously discussed in detail in Chapter 4.

5.1.2 Regulation of aptamer specificity

The aptamer specificity to a target can be regulated during selection. Thus, to obtain an aptamer with high specificity, it is necessary to select only sequences that bind the specific target but do not interact with the matrix, or other molecules structurally similar. On the other hand, if it is necessary to obtain an aptamer to recognize several related molecules, then sequences are selected with the ability to broadly bind several targets [33].

5.1.2.1 Negative SELEX

To exclude oligonucleotides absorbed by the support matrix material (e.g. affinity chromatography column, magnetic beads, nitrocellulose filters etc.), negative SELEX step is performed with the aim of selecting aptamers that bind only to the specific target. During negative selection, oligonucleotides are incubated with the matrix in order to eliminate the absorbed sequences. The unbound part is subsequently incubated with targets to select the desired aptamers. It has been demonstrated that the use of negative selection during SELEX process reduces the possibility of evolving non-specific binders [34].

5.1.2.2 Counter SELEX

The purpose of counter selection is to produce highly selective aptamers. The aptamer´s selectivity is improved by excluding oligonucleotide molecules with affinity to molecules similar in structure to the target [15, 35]. An example of the importance of incorporating a counter SELEX step is shown in case of the the RNA aptamer selected against theophylline, where caffeine was used as the counter target and resulted in a discrimination factor of 10,000 of the

aptamer for theophylline over caffeine, despite the fact that their structures only differ by a methyl group [35]. Another excellent example is the highly selective aptamer for ADP, which was selected incorporating a counter selection step against ATP [36].

5.1.3 Monitoring of evolution during SELEX

Another key aspect for the success of SELEX is the efficient monitoring of the evolution of the selection process, which allows control and adjustment of the selection stringency to achieve the desired properties of the selected aptamers. The most commonly used technique for monitoring the evolution uses radioactively labelled nucleic acids [37-39], which is a very sensitive method that enables detection of nucleic acids at low concentrations. However, the use of radioactivity is not possible in every laboratory and strict safety precautions have to be taken. Fluorescence, with fluorescent labels inserted *via* PCR, allows rapid and non-radioactive monitoring of the selection progress [29]. This method is rather costly and might also interfere with the binding properties of the nucleic acid [40]. The fluorescent dye-linked aptamer assay (OliGreen) is an alternative approach used for the monitoring of evolution [41]. The fluorescent dye OliGreen is specific for single-stranded DNA and enables the quantification of nucleic acid concentrations around 100 pg/ml with a standard spectrofluorimeter. Denaturing high-performance liquid chromatography [42], a technique based on the fact that the diversity of a nucleic acid library is dramatically reduced during the selection process has also been reported. Other methods used for the monitoring of aptamer selection include capillary electrophoresis, affinity chromatography, Reporter Linked Aptamer Assay (RLAA) and SPR [21, 43-45].

5.1.4 Cloning and Sequencing

When the affinity saturation of an enriched library is achieved, the final oligonucleotide pool is cloned into a bacterial vector and individual colonies are sequenced. Typically 50 - 100 clones are sequenced and analyzed by sequence analysis. Sequence alignments are used to identify aptamers with homologous sequences and a comparative analysis of the aligned sequences is performed to determine the consensus motif. Indeed, these conserved regions are crucial for the specific target binding of the aptamers.

5.1.5 Determination of secondary structure

Analysis of the conserved regions is used for the prediction of the secondary structure of aptamer candidates using specialized programs such as M-fold [46]. This program calculates the possible configuration of single stranded nucleic acids considering stems, loops and bulges by energy minimization methods. The determined consensus motifs are often located in stem-loop structures, pseudoknot formations, or G-quadruplexes. Knowing the secondary structure of aptamers, truncation studies of selected aptamers can be performed to increase their specificity and affinity, as well as to reduce the length and inherently the cost of the aptamer.

5.1.6 Binding studies

Binding experiments are used to study the specificity and affinity of selected aptamers, and the correct estimation of these parameters is essential for the successful application of the selected aptamers. In this context, the determination of the dissociation constant (Kd) is fundamental in order to

estimate the affinity of an aptamer for its corresponding target (i.e. a small K_D value designates high affinity for the target). Techniques generally used for binding studies are SPR, RLAA, fluorescent and radioactive binding assays.

5.2 Objectives

The main objective of this chapter was the selection of a DNA aptamer against PSA. As there is no standard SELEX protocol suitable for all targets, the conditions of an *in vitro* selection need to be optimized for PSA taking into consideration the potential applications of the desired aptamer. The subobjectives of this chapter can be divided in; (i) selection of DNA aptamers against PSA using two different partitioning methods for the separation of the oligonucleotide/PSA complex from the rest of the library, (ii) monitoring of the evolution of the aptamer selection using different techniques, (iii) cloning and sequencing of the enriched oligonucleotide pool, (iv) prediction of secondary structures of aptamer candidates and (v) characterization of selected aptamer candidates and evaluation studies for their potential use in further applications.

5.3 Methodology

5.3.1 Reagents

A filter holder 13 mm in diameter was purchased from GE Osmotics Labstore (Spain). Nitrocellulose filter with pore size 0,45 mm, Bovine serum albumin (BSA), Tris Buffered Saline (TBS), Phosphate Buffered Saline (PBS), and NUNC Maxisorp plates were obtained from Sigma (Spain). Binding buffer A consists of TBS at pH 7.9 with 1,5 mM of $MgCl_2$, Binding buffer B consists of PBS at pH 7.4 with 3 mM of $MgCl_2$, and Binding buffer C has the same composition as buffer B with addition of 1 mg/ml of BSA, washing buffers (A, B, C) have the same composition as binding buffers with addition of Tween. TOPO TA Cloning® kit was obtained from Invitrogen (Spain), QIAprep Spin Miniprep kit from Qiagen (Spain), and GenomeLab DTCS Quick Start Kit from Beckman Coulter (Spain).

5.3.2 *In vitro* selection process using nitrocellulose filter

Before the incubation step of the random library with target, a negative selection was performed in order to eliminate potential binders to the nitrocellulose filter. The binding buffer (500 µl) containing 500 pmol of the starting library was heated to 95°C for 5 min and then gradually cooled to 25° C over a period of 30 min. This solution was incubated with a pre-wetted nitrocellulose filter for 1 hour at 25°C. After incubation, the binding buffer containing a random library was centrifuged at maximum speed through the nitrocellulose filter, collecting the elute. The unbound random library was then incubated with 50 pmol of target for 1 hour at 25°C with slight tilt rotation. Followed incubation, the solution was centrifuged as previously described and

filter containing bound oligonucleotides was thoroughly washed several times with 500 µl of washing buffer. Bound oligonucleotides were eluted from the filter by denaturation at 95° C for 5 minutes, precipitated and resuspended in 100 µl of MilliQ water. A sample of eluted DNA was amplified using pilot PCR where different numbers of rounds of PCR were used in order to elucidate the optimal number of rounds for amplification of the remaining DNA. Ten microliters of DNA were amplified in 100 µl of Master Mix using protocols previously optimized for each library (see section 3.3.2). The resulting dsDNA was used for the preparation of ssDNA which was then used for the next SELEX round. For the generation of ssDNA T7 gene 6 enzyme digestion and magnetic separation with streptavidin beads were used. Both methods were used as described in Chapter 4 (see section 4.3.3).

5.3.3 *In vitro* selection process using magnetic separation with functionalized beads

In the initial round of selection, the random library was incubated with 50 pmol of PSA immobilized on the surface of the beads using a 10-fold molar excess of ssDNA in selection buffer. Half nanomole of library was diluted into 100 µl of binding buffer in a PCR tube, heated to 95°C for 5 min and immediately cooled at 4° C. PSA coupled beads were then added to this mixture and incubated under shaking conditions for 1 hour at 25° C. Following incubation, the beads were washed several times with 500 µl of washing buffer. DNA bound to the beads was eluted twice with 50 µl MilliQ water by denaturation at 95° C for 5 minutes, and the eluted DNA was subsequently amplified by PCR as described above. After the first round, a negative selection step was introduced prior to the positive selection step, in order to remove non-specific binders. Selected DNA was incubated with control (naked) beads in binding buffer for 1 hour at 25° C and unbound DNA was used for the positive

selection step. Different approaches used for the preparation of ssDNA such as T7 enzyme 6 gene exonuclease, magnetic separation with streptavidin beads or a combination of asymmetric PCR with T7 Gene 6 enzyme digestion were used in these selection processes. All methods were used as described in Chapter 4 (section 4.3.3).

5.3.4 Monitoring of SELEX evolution

5.3.4.1 PCR studies

After the amplification step at the end of each selection round, nucleic acids that had bound to the matrix (pre-selection step) and nucleic acids that bound specifically to PSA were amplified using pilot PCR. In pilot PCR a small aliquot of the SELEX pool was amplified in ranges from 4 to 16 PCR cycles in order to elucidate the optimal number of PCR cycles. Following PCR, the amplified products were checked on agarose gel and analyzed as described previously (Chapter 3, section 3.3.2).

5.3.4.2 Direct Reporter-Linked Aptamer Assay (Direct -RLAA)

Ten μg/ml (330 nM) of PSA was immobilized on plates in 50 mM carbonate buffer, pH 9.6 for 1 hour at 25° C, followed by a blocking step with PBStween (10 mM pH 7.4, 0.05% v/v Tween 20) for 1 hour. The wells were then washed three times with 200 μl of PBS-Tween. After the protein immobilization step, normalized aliquots of the biotinylated ssDNA obtained from different SELEX cycles were diluted in binding buffer, heated to 95°C and gradually cooled to 25° C. A 50 μl aliquot of different ssDNA samples were

incubated with proteins on the plate for 1 hour at 25°C, shaking gently. The plates were then washed 5 times with 200 µl of PBS-tween for 5 minutes each on a plate vortex, followed by the addition of streptavidin-HRP (0.02 µg/ml) in 50 µl of PBS. The wells were again washed as described above, and 50 µl of TMB substrate were then added to each well. After 20 minutes the reaction was stopped with 1 M H_2SO_4 and measured at 450 nm using a Spectramax 340PC^{384} plate reader.

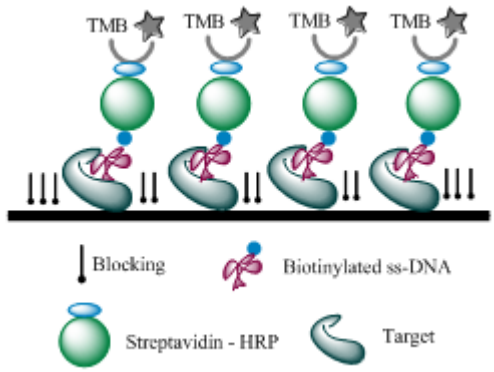


Figure 5.2. Scheme of Direct RLAA assay.

5.3.5 Cloning and Sequencing

The enriched library was cloned into the plasmid pCR2.1 using the TOPO TA Cloning® kit according to the manufacturer's instructions. Colonies were subsequently selected and grown overnight in a culture of 5 ml LB medium under vigorous shaking conditions. Plasmid clones were purified with a QIAprep Spin Miniprep kit. Purified plasmid DNA were sequenced by the GenomeLab DTCS Quick Start Kit according to the manufacturer's instructions, and analyzed in a CEQ8000 Beckman Coulter instrument. The sequences derived from SELEX were analyzed using the GeneBee service

(http://www.genebee.msu.su/), SDSS Biology Workbench (http://workbench.sdsc.edu/), and CLC Main workbench version 6.

5.3.6 Secondary structure prediction

The secondary structure model of the sequences obtained was predicted using M-fold program by fixing parameters such as temperature (25ºC) and concentration of Na^+ (0.138 M). The concentration of magnesium differed with respect to the binding buffer used in selection process (A – 1,5 mM, B, C - 3 mM).

5.3.7 Binding studies

5.3.7.1 Direct RLAA assays

Direct RLAA assay was used for screening of binding capacities of all aptamer candidates. This assay was used as previously described (see section 3.3.4.2), with aptamer candidates at concentrations of 1 μM each.

5.3.7.2 Competitive RLAA assay

PSA (10 μg/ml) was immobilized on microtiter plate in 50 mM carbonate buffer, pH 9.6 for 1 hour at 37°C, followed by 1 hour blocking with PBS-Tween. The plates were then manually washed three times with PBS-Tween. In individual eppendorf tubes, serial dilutions of PSA ranging from 0-33 μM (0-1 mg/ml) were incubated with the selected biotinylated aptamer

candidate I1 (200 nM) in binding buffer for 30 minutes at 25°C. The plates were washed three times, followed by addition of streptavidin–HRP (0.02 μg/ml) and incubation for 30 minutes. The wells were again washed as described above, and TMB substrate was added to each well. The reaction was stopped after 20 minutes with 1 M H_2SO_4 and the colour measured at 450 nm using a Spectramax 340PC^{384} plate reader.

5.3.7.3 SPR experiments

Surface plasmon resonance (SPR) was performed with a BIAcore 3000 (Biacore Inc.). Proteins of interest (PSA, and control proteins such as streptavidin, and BSA) were immobilized on separate channels of a CM 5 sensor chip activated with EDC/NHS (30 μl of a 1:1 mixture of EDC (400 mM) and NHS (100 mM)) followed by injection of 200 μg/ml (6,7 μM) protein at a flow rate of 5 μl/min. After immobilization of the proteins, unreacted NHS esters were deactivated *via* injection of an excess of ethanolamine hydrochloride (1M). Unbound proteins were then washed from the surface with 75 mM NaOH. The aptamer candidates were diluted in binding buffer to a final concentration of 1 μM and injected during 6 min at a flow rate of 5 μl/min followed by a 3 min of stabilization time and 10 min of dissociation time. The binding of DNA was analyzed through the corresponding changes in the refractive index of the optical signal, and expressed as resonance units (RU). All reagents and buffers were previously filtered and dissolved in MilliQ water.

5.4 Results and Discussion

5.4.1 *In vitro* selection process

Variations of SELEX protocols were performed for the selection of a DNA aptamer against PSA. Protocols differed in the method used for partitioning bound nucleic acids and the generation of ssDNA, as well as in the type of library and binding buffer used in the selection process.

Two types of partitioning including ultrafiltration and magnetic beads methodology were used. For ultrafiltration, nitrocellulose filters with 0,45 μm pore size were selected. Unbound ssDNA (~28 kDa) and free PSA (~30 kDa) passed through the filter, whilst the complex (~60 kDa) remained on the filter. The problem with use of nitrocellulose filters is the high level of non-specific binding of ssDNA to the filters. The negative-selection step before each round of selection decreased the non-specific binding but did not eliminate it completely. The immobilization of PSA on the surface of magnetic beads allowed an effective separation with minimum losses of target bound sequences. Different immobilization protocols have been studied previously (Chapter 2), and two strategies were found to be optimal for PSA immobilization and their use in SELEX. These strategies include covalent immobilization by amine-coupling with carboxyl modified beads and bioaffinity immobilization with streptavidin beads and biotinylated PSA.

Single-stranded DNA preparation is the most time consuming step in the standard SELEX procedure. The comparison of different methods used for ssDNA generation was studied previously (see Chapter 4). According to the results obtained from these experiments, several methods were selected and used for the selection of DNA aptamers, including magnetic separation with streptavidin beads, enzyme digestion with T7 gene 6 exonuclease, and

asymmetric PCR with a combination of enzyme digestion with T7 gene 6 exonuclease. The preparation of ssDNA was directly performed from PCR-product as it was observed that the yield of ssDNA obtained from unpurified PCR product was comparable to that obtained from purified PCR product, overcoming a loss of dsDNA during the purification step, and thus decreasing the time required for SELEX.

Two binding buffers - Tris-buffered Saline (TBS) and Phosphate-buffered saline (PBS) were used (Table 5.1). These buffers were chosen for SELEX experiments, due to their osmolarity and similar ion concentrations to that found in human serum. PBS contains sodium chloride, sodium phosphate, potassium chloride and potassium phosphate, and the phosphate ions help to maintain a constant pH (7.4). TBS also maintains the pH in a relatively narrow range (7-9.2) and has a slightly alkaline buffering capacity which is influenced by the temperature of the solution. Tween 20 was added to both buffers (TBS-Tween, PBS-Tween) for use as a washing buffer in the selection process and as a blocking buffer in binding assays. Divalent cations are generally required for high-affinity aptamers [47] and therefore its presence in the binding buffer is desirable. One of the sources of divalent cations is magnesium chloride and as the binding ability of aptamers has to be functional in physiological conditions, concentrations between 1-3 mM were chosen as optimal for binding buffer solutions. Bovine Serum Albumin (BSA) was another component of these binding buffers, as the presence of BSA helps to prevent nonspecific protein adhesion to surfaces.

Table 5.1. Different buffers used in DNA based SELEX process.

BB		**WB**	
A	TBS + 1,5 mM $MgCl_2$	**A**	TBS-Tween + 1,5 mM $MgCl_2$
B	PBS + 3 mM $MgCl_2$	**B**	PBS-Tween + 3 mM $MgCl_2$
C	PBS + 3 mM $MgCl_2$ + BSA	**C**	PBS-Tween + 3 mM $MgCl_2$ + BSA

BB – binding buffer, WB – washing buffer.

Two different libraries (I, II) were used for the selection of DNA aptamers against PSA. Both libraries had been previously optimized to ensure a high purity of the amplified product (see Chapter 3). In some cases (SELEX A, B, C, D, H) large-scale PCR amplification of the random library was carried out before initiating the selection process in order to eliminate damaged DNA synthesis products, which cannot be amplified by PCR. However, as mentioned in the introduction of this chapter, it is possible to lose some of the target binding sequences from the original library. This library was checked using denaturing polyacrylamide gel, showing the intact length of the majority of the oligonucleotides. Thus, in other cases (SELEX E, F, G, I, J), 500 pmol library (about 3 x 10^{12} different sequences) was used directly in the first round of the selection process. For the following rounds of selection, 100 pmol of ssDNA were employed when magnetic beads and enzyme digestion were used for the generation of ssDNA, while 250 pmol of ssDNA were used in the case of asymmetric PCR combined with enzyme digestion due to the higher efficiency for the ssDNA production.

Taking into account the concentration of the random library in every selection cycle, the amount of protein was reduced from the intitial 50 pmol to 10 pmol (rounds 4-6), and subsequently 5 pmol (from 7 round onwards) in the case of magnetic beads and enzyme digestion ssDNA generation methods and 25 pmol (rounds 4-6) and 10 pmol (from 7 round onwards) in the case of asymmetric PCR with a combination of enzyme digestion in order to maintain the optimum library:target ratio between 10:1 to 100:1.

All protocols used are summarized in Table 5.2 according to the type of partitioning, method for generation of ssDNA, the random library and buffer used in each selection process. In Table 5.2, it is also indicated when large-scale PCR amplification was used and which of the selection processes led to the cloning and sequencing of enriched libraries.

Table 5.2. Variations of SELEX protocols.

SELEX	Partitioning	ssDNA	Library	B&WB	L-S PCR	C/S
A	F	MS	I	A	X	
B	F	T7	I	A	X	X
C	CMB	MS	I	B	X	X
D	CMB	T7	I	B	X	
E	CMB	T7	I	B		
F	CMB	A-T7	I	B		X
G	StMB	MS	II	C		
H	StMB	T7	II	C	X	X
I	StMB	T7	II	C		X
J	StMB	A-T7	II	C		X

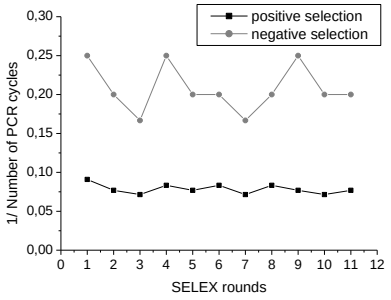
F – nitrocellulose filter, CMB – carboxyl modified magnetic beads, StMB – Streptavidin modified magnetic beads, MS – magnetic separation, T7 – T7 Gene 6 Exonuclease, A-T7 – Asymmetric PCR with T7 enzyme digestion, Library I, II, B&WB – binding and washing buffer, L-S PCR – Large -Scale PCR amplification, C/S – Cloning/Sequencing.

5.4.2 Monitoring of *in vitro* selection

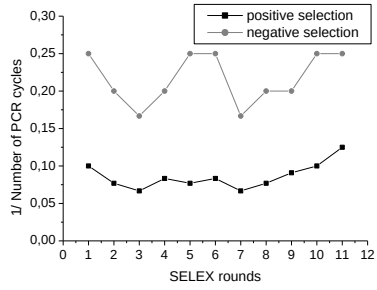
The first and simplest manner to monitor the evolution of a selection process is to check the amount of ssDNA at the end of each selection round. Hence, the amount of ssDNA bound to the target as well as the amount of ssDNA bound to the matrix (nitrocellulose filter or naked magnetic beads) was measured throughout whole selection process. The amount of ssDNA was represented by the number of PCR cycles needed for optimal PCR amplification. A higher amount of bound ssDNA required less PCR cycles and *vice versa*. An increasing amount of ssDNA bound in positive selection was observed in SELEX B, C, F, I and J indicating that the ssDNA pool was evolving to be more selective towards the PSA target, whilst in SELEX A, D, E, G, H no evolution was observed (Figure 5.3). Usually, when nitrocellulose filters are used, a high amount of ssDNA binds to the matrix (Figure 5.3,

SELEX A, B), probably due to the large matrix surface. In the case of magnetic beads nonspecific ssDNA binding was less pronounced, and a slight decrease in the amount of ssDNA bound to the matrix was obtained during the selection process with magnetic beads. In the majority of cases a decreasing amount of ssDNA (around 2-3 PCR cycles) was observed in the second round of SELEX. This jump in PCR cycles was due to the use of a lower concentration of ssDNA after the first round of SELEX, as well as the introduction of negative selection (in the case of magnetic beads). Other smaller shifts can be observed in the 4th and 7th selection round when smaller concentrations of target were introduced to the selection process.

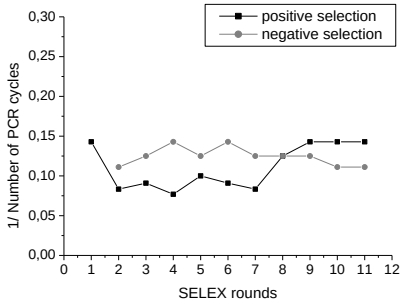
SELEX A



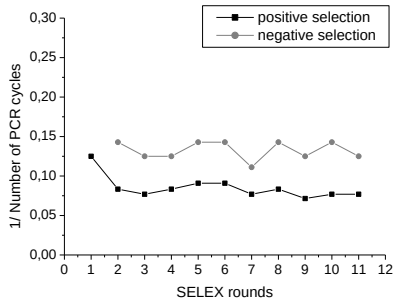
SELEX B



SELEX C



SELEX D



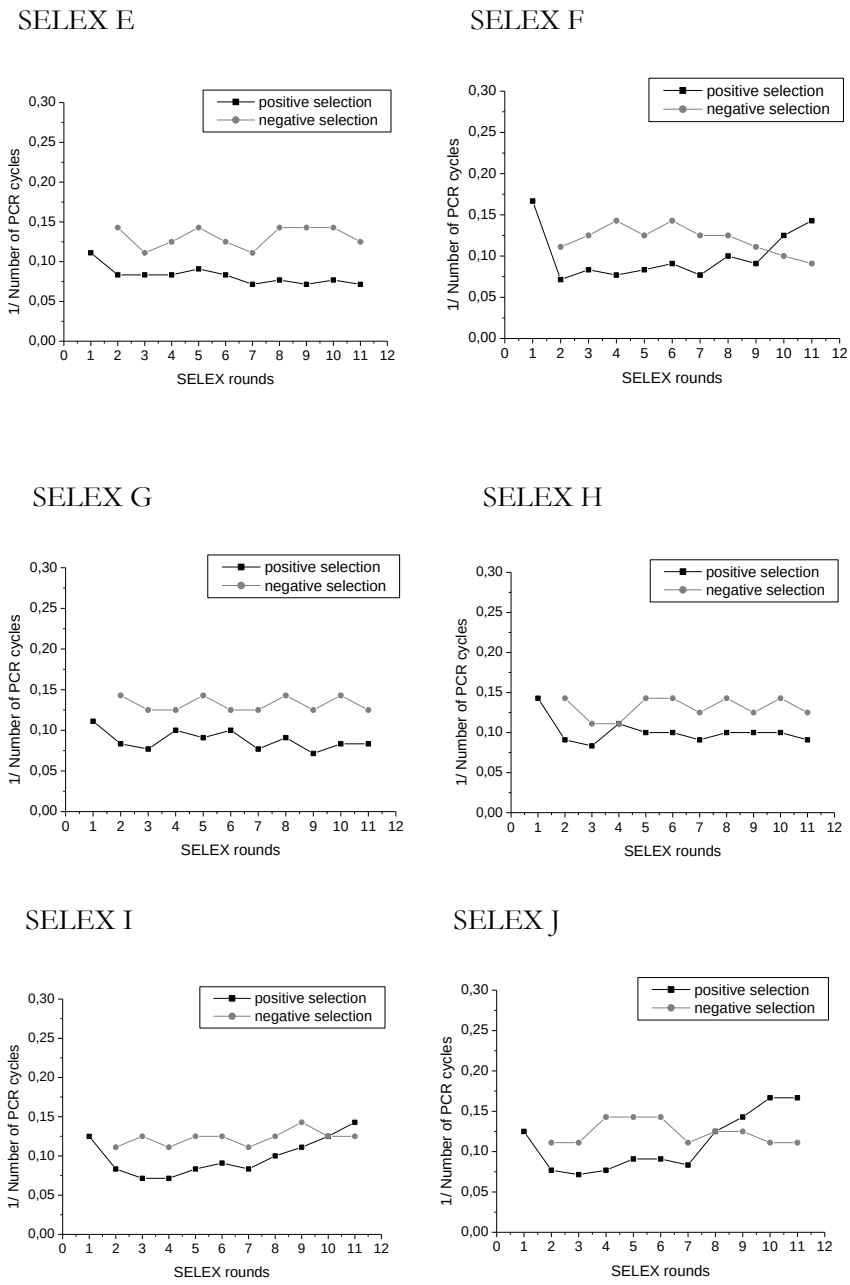
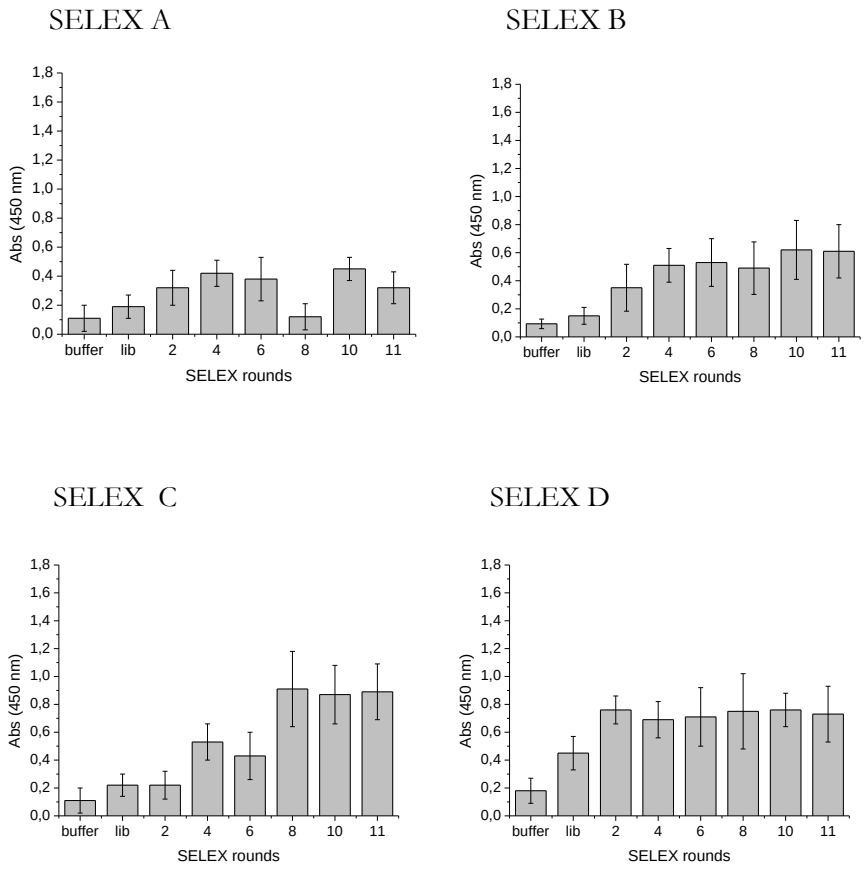


Figure 5.3. Monitoring of evolution with PCR studies.

To monitor evolution, RLAA was also carried out. As shown in Figure 5.4, with an increasing the number of SELEX rounds in SELEX B, C, F, J and I, the binding affinity for PSA increased, again indicating evolution. In SELEX A, D, E, G, and H no evolution was observed.



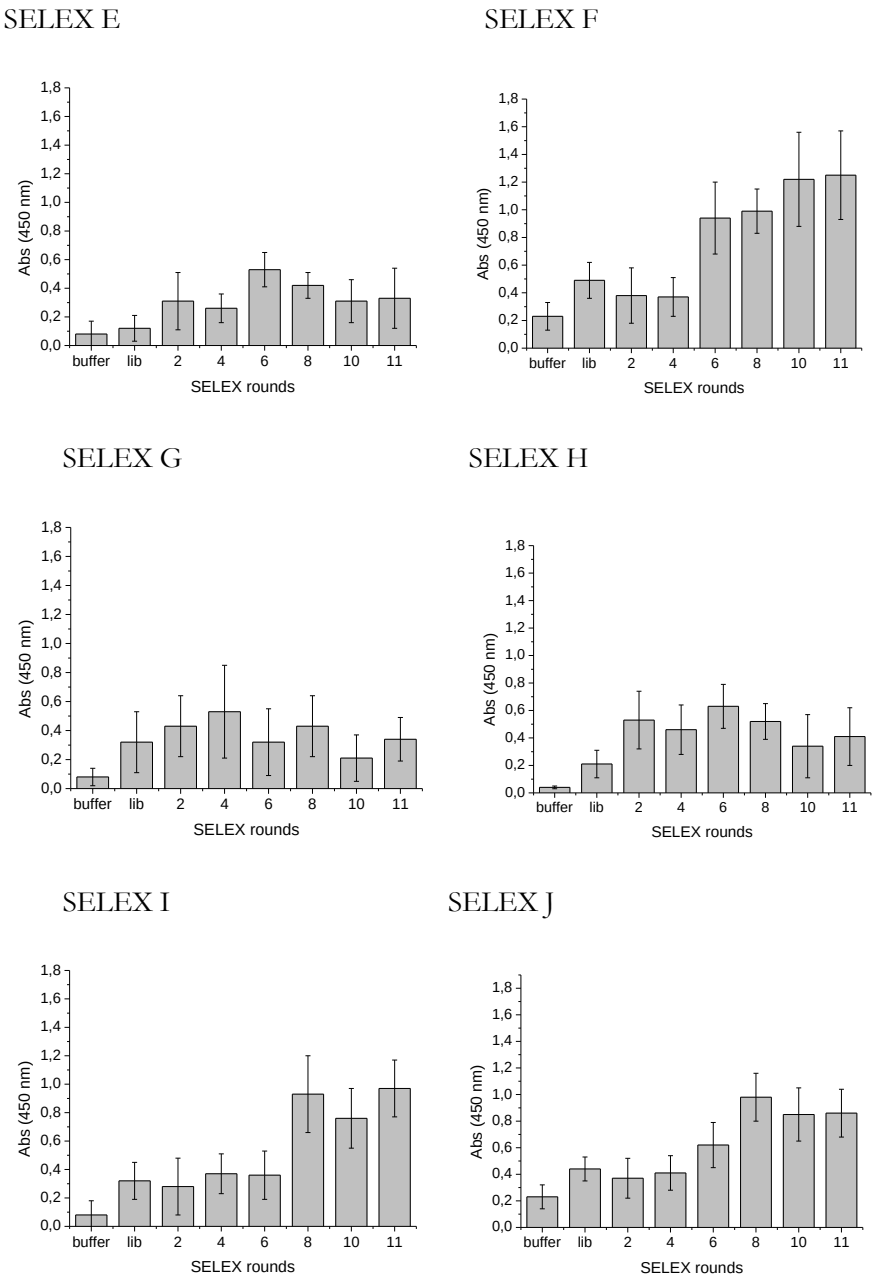


Figure 5.4. RLAA type assay. *All data was corrected for nonspecific binding by subtracting the signals of binding nucleic acids to PSA no coated wells, (N=3).*

5.4.3 Cloning and Sequencing

According to results from PCR and RLAA monitoring studies, SELEX B, C, F, I, and J were selected to be cloned and sequenced. At the end of the cloning process, plasmids were extracted from individual clones and amplified to ensure the insertion of the aptamer sequence. Plasmids containing the desired aptamer sequence were selected and used for sequencing. Sixteen sequences were obtained for SELEX B, 19 for SELEX C, 16 for SELEX F, 20 for SELEX I, and 20 for SELEX J. The aptamer sequence can be inserted in the plasmid vector in either orientation, thus the complementary sequence can be determined if it is needed by using a sequence manipulation software (CLC Main Workbench), to ensure that all the data will be analyzed in the correct orientation. The modified sequence data were searched to locate the inserted sequences using (SDSS Biology Workbench). Altogether, 91 individual sequences were characterized and revealed 75 different variants; from which 11 sequences were represented twice and 5 sequences three times (Table 5.3). Some of the sequences had a slightly shorter or longer length than expected. This often happens due to errors in replicating sequences in the random region of the aptamer pool. In some cases (aptamer candidates B12-14) the random region was considerably longer than 50 nt (around 60-70 nt). This can be explained due to the fact that non-proof reading DNA polymerases, such as *Tfi* polymerase, often ¨slip¨ when amplifying multiple short-sequence repeats, leading to elongation of the amplified region [6].

Table 5.3 Sequence frequency of selected aptamers.

SELEX	Aptamers	Number of clones	Length (nt)	Frequency (%)
SELEX B	B01-B02	2	93	13%
	B03-B11	1	92-95	6%
	B12-B14	1	105-115	6%
SELEX C	C01 - C03	2	93-94	11%

	C04 - C16	1	91-95	5%
SELEX F	F01-F02	3	93	19%
	F03-F05	2	91-93	13%
	F06-F09	1	92-94	6%
SELEX I	I01-I02	2	90	10%
	I03-I18	1	89-91	5%
SELEX J	J01-J03	3	90-91	15%
	J04	2	90	10%
	J05-J13	1	89-91	5%

Once the random regions had been identified, alignments were attempted using the GeneBee program. This program identifies local sequence motifs by searching for a conserved motif present in more than one sequence, and an alignment of aptamer candidates revealed several distinct regions of conservation (Figure 5.5). Furthermore, alignments of all sequences together trying to identify families of aptamers with conserved motif were carried out. However, no obvious pattern was observed, apart from the fact that the majority of aptamer candidates appear to be very rich in A residues.

(i) SELEX B

```
                ....++++.++..+ .  ......
B01        (2) ggatCGGACCGGcactctatttaa
B02        (2) cgatCGGACCGGcacatgatgtat
B09        (1) ----CGGAGCGAAAtatcgaaaga
B10        (1) -tgaCGGACCGGAAgtaaaataga

                   +      ++ +++++++ +++     +   +
B07        (1) cacgtagtatAGAAAAATAGTTAAaacaccacaa
B10        (1) acggaccggaAGTAAAATAGATAActgtgcttga

                        +++++++
B05        (1) cgccgcgcaaCCGTAAAGCCA----------
B06        (1) ggcgagaggttaGTAAAGCCAagggaggcgg
B14        (1) tctgtgtaatCCGTAAAGCtttgcaaaa---
```

(ii) SELEX C

```
                  ....... ...+ ..+....  ..+ .+ .+.+  ...  .. ....  . ....
C03        (2) -----------ACAAAAGAATAGC-A-AAAAGAACC--ATCCGAGTCTAAggacacgtaa
C06        (1) ---gttgAATGACAACGAAAACAA-GGACAACAAAC--CGAATAACATAGAcagcca---
C13        (1) ----------ccatACGAAAAAAAcTGATTATCAAC--ATATTAAAACAAAaccctatcg
C14        (1) taagtaaatgaACTAAAGACTCGT-A-AGTAAAAACgtATCCGGGGCTAA----------
```

```
C16        (1) ---caaaAATGACTATAAAATAAC-AGAAAAGGACC--AGAAAAAgccttcgca------

                        .   .++....+.+.. +..... ...+ + ..  + .+.+..+......
C08        (1) gatccggcgtcAATAGAACTCATACATAAGAGAAGAAG--GAATATAAGCCa-----
C13        (1) ccatacgaaaAAAACTGATTATCAA-------CATATTAAAACAAAACCCTATCGC-
C16        (1) ---------cAAAAATGACTATAAAATAACAGAAAAGGACCAGAAAAAGCCTTCGCa

                    .  ....   ++.. +++... .... .....  ..+. .   +..... .  .  ...
C08        (1) atccggcgtcAATAGAACTCAT-----ACATAAGAGAAGA---AGgaatataagcca---
C10        (1) -------gaaAAAGTAACA-AA-----TAGCACGGAAACG---AGCATgaagtcgtaaat
C12        (1) ------------ACAACAGTCTATCCACATTTACAAtactatAGCCTTCactcagaaag
C13        (1) ---ccatacgAAAAAAACTGATTATCAACATATTAAAACA---Aaaccctatcgc-----
C16        (1) aaatgactatAAAATAACAGAA-----AAGGACCAGAAAA---AGCCTTCgca-------
```

(iii) SELEX F

```
                      .     . .+++      +....+..+ + ++..+ +.++.+..+.+       .
F04        (2) --ggagcaacaGGC------ACAGGCGGACAGGA-GC-ACCGCTAAATAgcttccggtc
F05        (2) cggcgtatagGGGC------AGAACCGGAGAAGAATC-AACGGTATAGA----------
F08        (1) ------ggtaGGGCagatcaAGCGCCAAAAACGAATCtAACGGTGTAGAcagaagg---

                         +  ++ +++++++++        +
F02        (3) ---atatggtAACACAGCAGACgacaagccat
F07        (1) cttgctggacAAGACAGCAGACagacgtgaaa

                          + ++++ +++++++       +
F03        (2) aggataccgCTAGGGGGGACCcactccaaag
F07        (1) --------caCTAGAGGGGACCttgctggaca
```

(iv) SELEX I

```
                    .    . .  ...++.+++..   ..++ +..+..++. . .  .
I03        (1) ----------tgACCGATCT---CCGAAGCGAAGGAagcaggtatg
I10        (1) gctagcagttAACACCGATCT---GTGAGGCAAAAGAgcccgtaaaa
I11        (1) ggactcggcaAACACTGATtcgcaGTGACGGAATAGAa---------

                      ..   .  ..++.+++.++.++ +.  ..    .
I02        (2) aaacgcaggagaAACGATAACAGAAAGa---------
I16        (1) --------tgCTAAAGATAACAGAtacgacactacgt
I17        (1) tcactaggctCTAAAGATTACTGAGAGttcaaacaga
```

(v) SELEX J

```
                        ++++   +   +++++ +++  ++      +++ + +++ +++ +
J9         (1) cgtgAGTAGACCAA-CCCTTGAAAaTACtgtctgGTGTGGTGCTTCCttt-------
J12        (1) ---tAGTACCTCCGgCCCTTTAAA-GAC----gtGTGAGATGCCTCCatatctgcgg

                           ++ + ++  +++++ +++  ++++  +
J2         (3) tccaagcgacAGTCTGATTGGCGTcATTACGGAAcgat------
J6         (1) --------ggAGACGGAAGGGCGT-ATTCAGGAAtaaatcatgc

                        ++ ++++  + + ++++ ++++ +  +
J1         (3) aagcGACGACCCAGCGAGGCATCCAGCTtataccacgg
J7         (1) ----GAGGACCACGTGGGGCAGCCAGGTatttgtggct
```

(vi) All aptamer candidates

```
                     .  . .. . .  .    .+.+  .++.+.+ ..+.....+
B08        (1) ----------------------ATAACgcacaTACGCACCATAA
B11        (1) ---------------------GATAACgcacaTACGCACCATAA
B13        (1) ---ttagggccattgatgagAGATACTttataTTACGAACGTCA
```

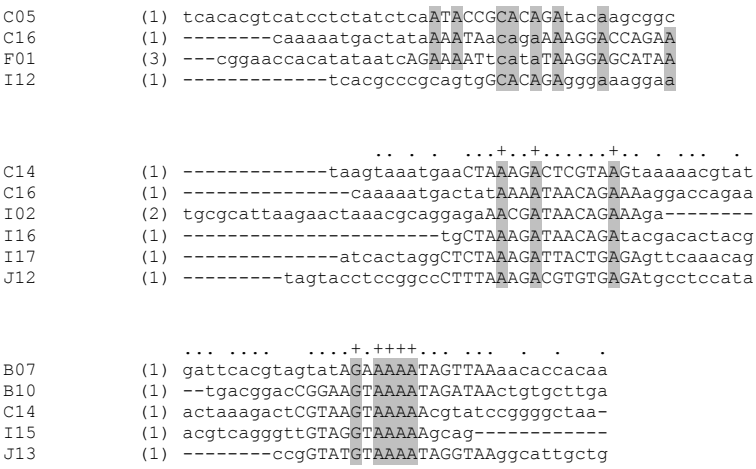
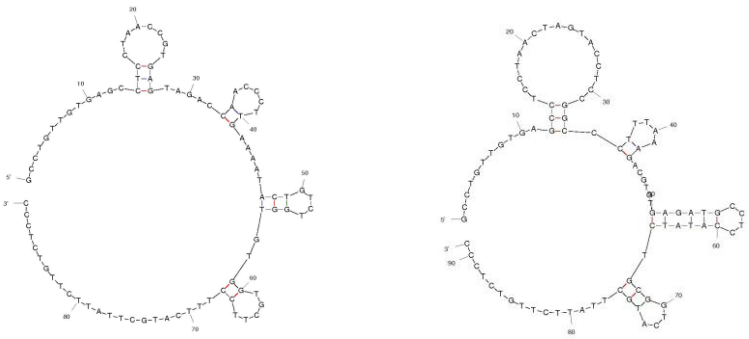


Figure 5.5. Local super-motifs identified within the random regions of sequenced aptamer candidates selected against PSA. *Conserved motifs are shown in grey colour. Perfect matches are indicated by "+", strong bias towards one base are indicated by "." The frequency of individual sequence is written in parentheses. Supermotifs were found in SELEX B (i), SELEX C (ii), SELEX F (iii), SELEX J (iv), SELEX I (v), and in all aptamer candidates (vi).*

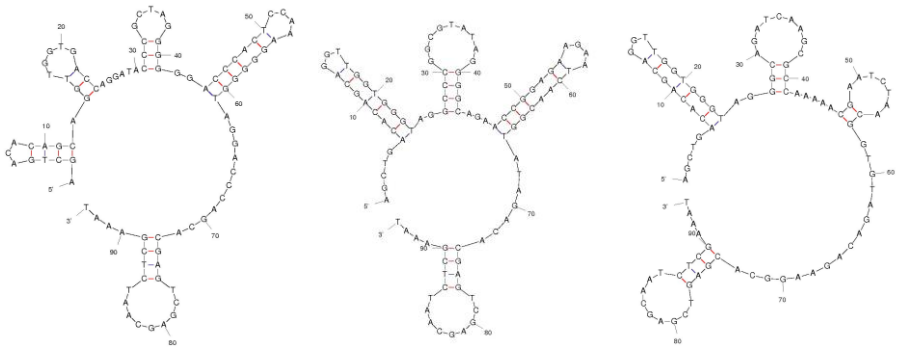
5.4.4 Secondary structure of aptamer candidates

An alternative method of identifying aptamer families is to compare the secondary structures and thus, identify common elements. Theoretical aptamer secondary structures were predicted by the Zuker algorithm [48], using the M-fold program (version 3.2) with conditions set up as those used in the selection process. The majority of selected sequences were folded into different structures and no common secondary structure elements could be assigned to these variants. The similar secondary structures were found comparing sequences J 9 and J 12 with large central loop and 3 smaller loops on the side of the central loop (Figure 5.6), and between sequences F 3, F5 and F8 with big central loop and 4 or 5 hairpin loops on the side of the central loop (Figure 5.7).



J 9 ΔG = -3,4 J 12 ΔG = -4,3

Figue 5.6. Predicted secondary structure for aptamer candidate J9 and J12, ⊿G indicates the predicted free energy of secondary structure formation given in kcal/mol.



F 3 ΔG =-18,4 F 5 ΔG = -17,03 F 8 ΔG = -11,61

Figue 5.7 Predicted secondary structure for aptamer candidate F3, F5 and F8, ⊿G indicates the predicted free energy of secondary structure formation given in kcal/mol.

5.4.5 Binding studies

The binding capacities of all unique aptamer candidates for PSA were examined in a screening manner by using a RLAA. The 13 different sequences displayed some affinities to the PSA compared with the random ssDNA library and the background control (Figure 5.8). The rest of the sequences showed lower or the same affinities to PSA as the starting random ssDNA library.

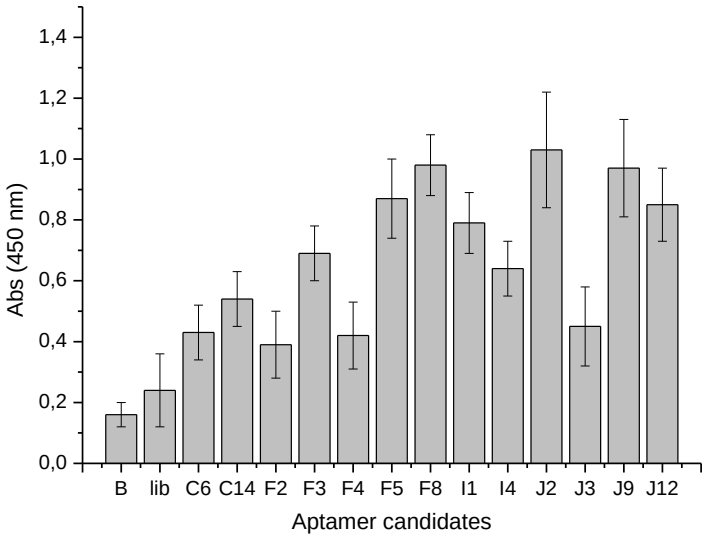


Figure 5.8 Binding test of aptamer candidates. *The x axis shows the names of different sequences, B indicates binding buffer used as background control, L indicates starting random ssDNA pool, (n = 3).*

To characterize the binding of the selected aptamer candidates to PSA, and to determine their ability to discriminate between different targets such as BSA or Streptavidin, SPR was used (Figure 5.9). The binding capacities of selected aptamer candidates to PSA obtained from SPR were similar to those obtained from RLAA. In terms of specificity, higher affinities to BSA were observed in SELEX F, where BSA was present in the binding buffer, and

similarly in SELEX J, where higher affinities to Streptavidin were observed. It should be noted that sequences with similar secondary structures showed similar affinities and specificity to BSA (F3, F5, F8) or to Streptavidin (J9, J12). It may simply be a coincidence that several aptamer candidates with comparable specificities and affinities have similar 2D structures. Moreover, aptamers often contain non-canonical base pairing which is not accounted for in 2D structure prediction programs such as M-fold [6]. The possible selection of aptamers against BSA or Streptavidin is not so surprising, as both proteins seem to be ideal targets for aptamer selection [29, 49-51]. The aptamer candidates with the highest specificity to PSA and the lowest nonspecific binding to other proteins were sequences obtained from SELEX I (I1, I4).

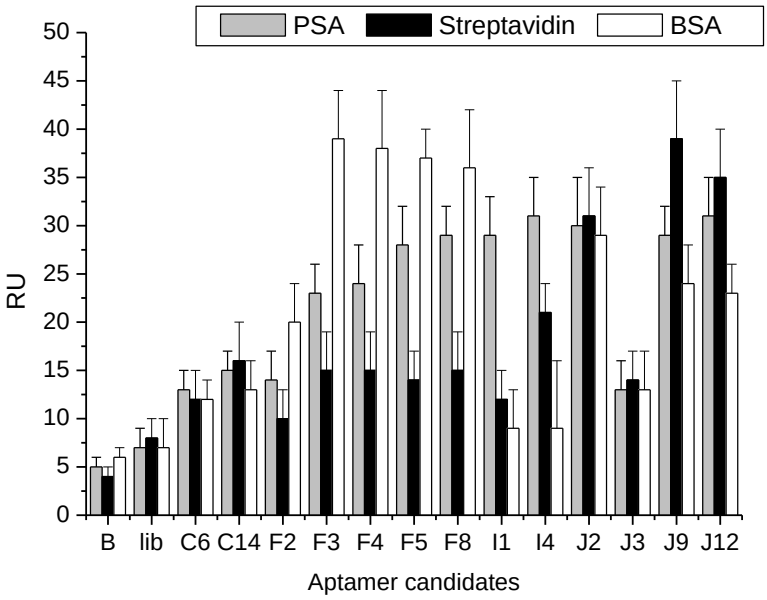
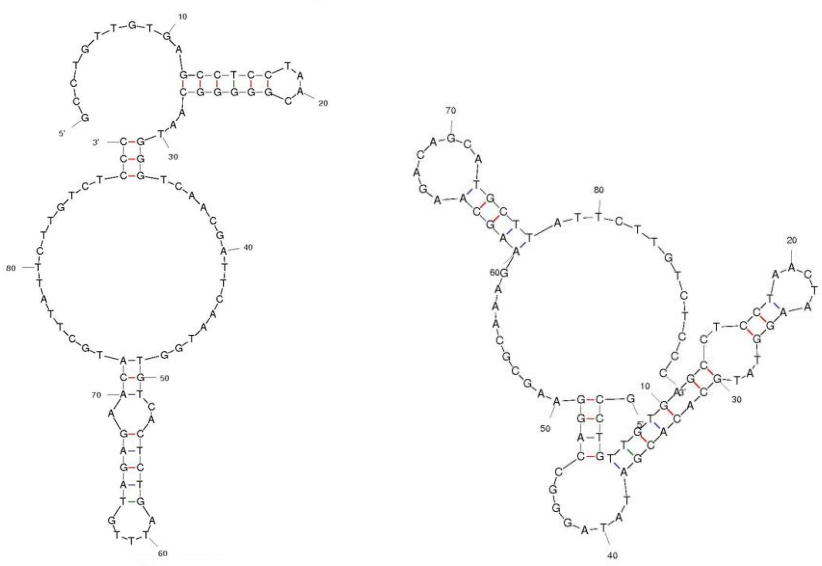


Figure 5.9 SPR experiments showing the specificity and cross-reactivity of selected aptamer candidates, n = 2.

Aptamer truncation was carried out to define the part of the sequence that is essential for binding as well as to obtain shorter and better binders for possible future applications. Since sample I1 and I4 (Figure 5.10) showed the lowest unspecific binding, these aptamer candidates were truncated by removing fixed primer regions (Table 5.4).



I 1 ΔG = -7,8 I 4 ΔG = -9,5

Figure 5.10 Predicted secondary structure for aptamer candidates I1 and I4 ΔG indicates the predicted free energy of secondary structure formation given in kcal/mol.

Table 5.4 Sequences I1 and I4 and truncated variants with the appropriate length and the predicted free energy of secondary structure formation (ΔG)

	Sequence (5´-3´)	Length	ΔG
I1	**GCCTGTTGTGAGCCTCCTAAC**GGGGGCAATGGGTCAACGATTCAATGGTGTC ACTCTGATTTGTAGAGAA**CATGCTTATTCTTGTCTCCC**	90	-7,8
I1a	GGGGGCAATGGGTCAACGATTCAATGGTGTCACTCTGATTTGTAGAGAA**CAT GCTTATTCTTGTCTCCC**	69	-3,1
I1b	**GCCTGTTGTGAGCCTCCTAAC**GGGGGCAATGGGTCAACGATTCAATGGTGTC ACTCTGATTTGTAGAGAA	70	-7,7

I1c	GGGGGCAATGGGTCAACGATTCAATGGTGTCACTCTGATTTGTAGAGAA	49	-2,9
I4	**GCCTGTTGTGAGCCTCCTAAC**TAAGGTATGCACACGATATAGGGCCAGGAAG CGCAAAGAAGCAAGACAG**CATGCTTATTCTTGTCTCCC**	90	-9,5
I4a	TAAGGTATGCACACGATATAGGGCCAGGAAGCGCAAAGAAGCAAGACAG**CAT GCTTATTCTTGTCTCCC**	69	-4,8
I4b	**GCCTGTTGTGAGCCTCCTAAC**TAAGGTATGCACACGATATAGGGCCAGGAAG CGCAAAGAAGCAAGACAG	70	-6,5
I4c	TAAGGTATGCACACGATATAGGGCCAGGAAGCGCAAAGAAGCAAGACAG	49	-1,3

However, the predicted free energy of secondary formation of truncated sequences indicated a lower stability as compared to the full length I1 and I4, and as expected the ability of short variants to bind to PSA was lower (Figure 5.11).

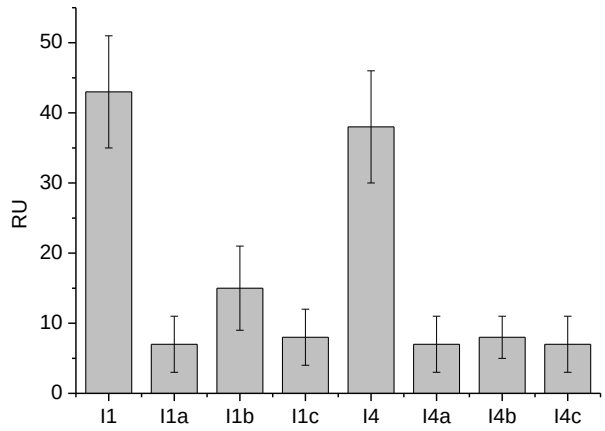


Figure 5.11 Binding test of all short variants using SPR with PSA immobilized on the surface of the chip, (n = 2).

The K_D values of full length aptamer candidates, I1 and I4, were estimated by analyzing the binding of a range of concentrations (0.1 μM to 10 μM) with PSA, using a one to one Langmuir binding model (Figure 5.12). The resulting K_D were 5.8 x 10^{-6} M and 6.3 x 10^{-6} M for aptamer candidate I1 and I4,

respectively (Table 5.5). In many other cases, DNA aptamers selected by conventional SELEX strategy achieved similar affinities [52-59]. However, since the dissociation constant of the antibody used for the detection of PSA, had been reported to be 1 x 10^{-7} to 1 x 10^{-10} M, the DNA aptamers with affinities in a K_D 1 x 10^{-6} M order are markedly weaker than that of antibody to antigen [60-64].

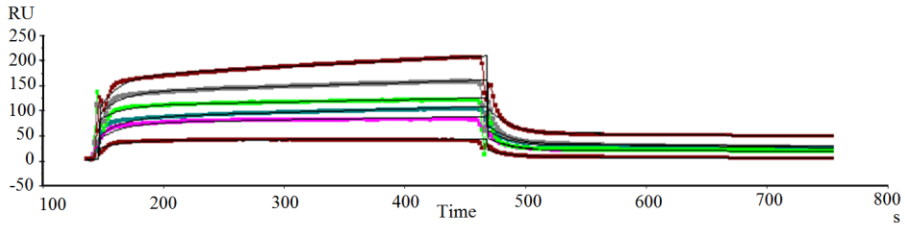


Figure 5.12 SPR Sensogram for aptamer candidate I1 using range of concentrations from 0,1 µM to 10 µM.

Table 5.5 Dissociation constants obtained in Biacore with relevant statistics of the aptamer candidates obtained.

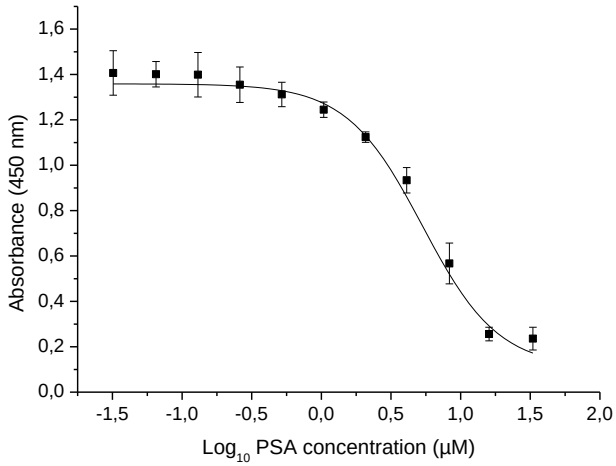
Aptamer	Model	K_D (µM)	χ^{2*}
I1	Langmuir Binding	5.8	1.145
I4	Langmuir Binding	6.3	1.265

*The Chi^2 value is a standard statistical measure of the closeness of fit of data to the model used for elucidation of the K_D, where for good fitting to ideal data, χ^2 is of the same order of magnitude as the noise in RU, typically <2.

Aptamer I1 was subsequently used in competitive RLAA assay for the quantitative detection of PSA in order to evaluate the functionality of aptamer candidate I1. PSA was immobilised on a microtiter plate competing with PSA analyte in solution for binding to the aptamer candidate I1, where similarly to a competitive ELISA format, in the presence of higher concentrations of PSA analyte, less aptamer bound to the immobilised PSA, resulting in a lower signal.

A calibration curve between 32 nM and 33 μM (0,001 – 1mg/ml) of PSA was obtained (Figure 5.13), with r^2 0.993, EC_{50} 5.6 μM and LOD of 268 nM demonstrating the possible functionality of the aptamer candidate I1.

Nevertheless, similar response was observed also in case of control proteins (BSA, Streptavidin) which were used at the same concentration range (Figure 5.14). Results obtained from RLAA assay showed that the low specificity which aptamer I1 exhibit does not meet the requirement for its use as molecular recognition element.



FIT VALUES	
LOD	268 nM (8,6 μg/ml)
EC50	5,6 μM (168 μg/ml)
R²	0,993
Replicates	3

Figure 5.13 Detection of PSA by competitive RLAA assay using aptamer candidate I1, with relevant statistics obtained, *(n=3).*

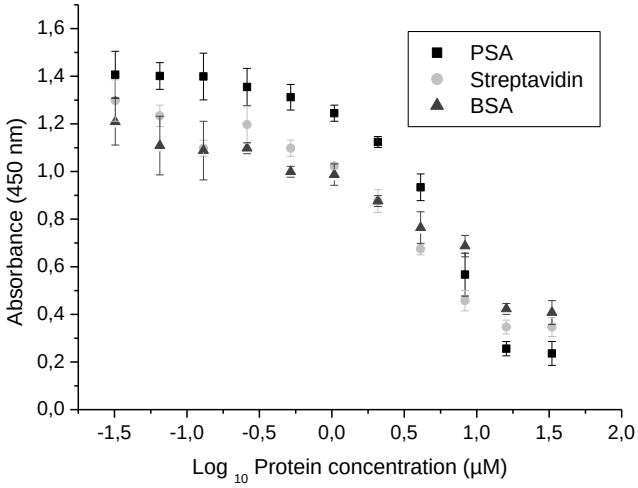


Figure 5.14 RLAA assay with PSA and control proteins (streptavidin, BSA), n=3.

5.5 Conclusions

Variations of protocols used to select DNA aptamers differed in the partitioning methodology, generation of ssDNA, starting random library and buffer compositions. Several aptamer candidates that exhibited ability to bind to PSA were identified, however all aptamer candidates showed high cross-reactivity with control proteins (BSA, streptavidin). Aptamer I1 and I4, with dissociation constant in low μM range, showed the lowest cross-reactivity with control proteins. For the evaluation of the functionality of aptamer I1, a competitive RLAA with different concentrations of PSA was used achieving the limit of detection 268 nM. However, the low specificity of selected aptamer does not permit its use as molecular recognition element.

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Chapter 6

6.1 Introduction

Despite a chemical structure that is similar to DNA, RNA adopts two- and three-dimensional conformations that are more complex than its DNA counterpart offering the possibility to select an RNA aptamer when DNA SELEX has failed. In terms of affinity and specificity, there is no difference between RNA and DNA aptamers, but DNA aptamers are inherently more stable. Nevertheless the stability problem of RNA can be overcome by specific modifications that greatly increase the stability of RNA allowing them comparable, or even improved stability as compared to DNA aptamers.

6.1.1 RNA based SELEX

For the selection for RNA aptamers, the random DNA library has to be transformed into a RNA library before starting the first round of the selection process. A special forward primer with an extension at the 5´end containing a T7 promoter sequence and normal reverse primer are necessary to convert the ss-DNA library into a ds-DNA library by PCR. The ds-DNA is then *in vitro* transcribed by the T7 RNA polymerase resulting in a randomized RNA library, which can be used to start RNA SELEX. During the SELEX process, a randomized RNA pool is incubated with the target of interest (i), RNA bound to the target is separated from the rest of the library (ii) and eluted (iii). Eluted RNA has to be reverse transcribed (iv) and amplified with special forward primer as described above (v). The new RNA pool is then generated by *in vitro* transcription (vi), remaining DNA is hydrolyzed using DNAse (vii), resulting RNA is purified (viii) and can be used for the next round of the selection process (Figure 6.1).

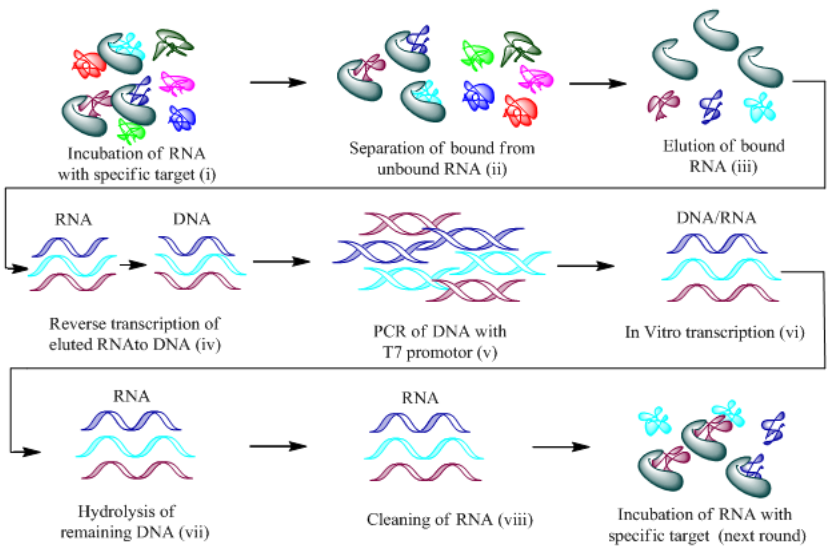


Figure 6.1 Schematic of RNA based selection process.

6.1.2 Modifications of RNA aptamers

The susceptibility of RNA to enzymatic degradation can be overcome by modifying the 2' position of the ribose ring in the RNA backbone. The most used modified nucleotides use 2'-amino and 2'-fluoro or 2-O alkyl groups [1]. However, the incorporation of modified nucleotides may render them not to be recognized and processed by the T7 RNA polymerase. This can be solved by using 2- amino or 2- fluoro modified CTP and UTP, as it has been shown that modified pyrimidines can be incorporated by a special type of T7 RNA polymerase [2-4].

Alternatively, it is possible to introduce similar chemical modifications at the end of SELEX process. Moreover, modifications added at 3´ and 5´ ends of oligonucleotides such as amine, phosphate, phosphotiothate, cholesterol, fatty acids etc., protect oligonucleotides from exonucleases [5-7]. However, these modifications may result in a change of aptamer affinity and specificity.

Furthermore, aptamers can be easily modified for desired applications to increase the stability or to regulate their *in vivo* function. Using analogues of natural nucleotides that bear photolabile groups, aptamer can be modified and activity of the aptamer can be regulated by light irradiation [8-9].

Spiegelmers, whose name was coined from German word for mirror, *spiegel*, offer an interesting alternative route to produce nuclease-resistant aptamers. Spiegelmers are mirror-images of aptamers, composed of L-ribose or L-2´deoxyribose, that bind specifically to a target, but are not recognized by ribonucleases, the chiral inversion leading to high biological stability and long lifetime [10-11]. Spiegelmers are produced using a mirror-image SELEX procedure. Since RNA and DNA polymerases are not able to carry out polymerization of L-nucleotides, selection is performed using original D-oligonucleotides that are selected against synthetic enantiomers of a chosen target. It is accepted that spiegelmers do not hybridize with nucleic acids of natural configuration and it has been shown that spiegelmers show the same high affinity for targets as their aptamer analogues [12]. These favourable properties led to the identification of

several spiegelmers that bind to various target molecules [12-17]. Moreover, as they are chemically and structurally similar to D-oligonucleotides, they induce minimal immunogenic response [18], making spiegelmers ideal candidates for *in vitro* and *in vivo* diagnostics as well as their application as *in vivo* imaging agents [19], and for therapeutics.

6.1.3 Automated SELEX

The *in vitro* selection process in practice is repetitive, time-consuming and not always applicable for high-throughput selections. To overcome these limitations the SELEX process has been automated, handling multiple targets efficiently since they are processed in parallel [20]. The first robotic work station was a modified Beckam Biomek 2000 pipetting robot in which a PCR thermal cycler, a magnetic bead separator, reagent trays and a pipette tip station were integrated [21]. The automated selection was later further optimized, substituting the magnetic separator by more efficient vacuum-filtering [22]. The new protocol was applied to the generation of anti-protein aptamers in a matter of days, and the automated selection was demonstrated through the selection of an anti-lysosyme aptamer that functions as an efficient inhibitor of cell lysis. This automated selection was successful in developing aptamers towards proteins such as CYT-18, MEK1 and Rho with dissociation constants in the pico- to nanomolar range [23], against proteins that had been transcribed and translated directly on the robotic workstation [24], and against the mirror image configuration (Spiegelmer) of substance P [20]. The prototype of a microfluidic, microline-based assembly that uses Labview-controlled actuable valves and a PCR machine has also been reported for the selection and synthesis of an anti-lysozyme aptamer [25].

6.1.4 Next Generation Sequencing

Next generation sequencing (NexGen sequencing) technologies provide possibilities for global investigation of multiple genomes and transcriptomes in very short time frames and with much lower cost than Sanger-based sequencing methods. These technologies have already been used in different technologies including; polymorphism discovery [26], non-coding RNA discovery [27-30], large-scale chromatin immunoprecipitation [31], gene-expression profiling [32], mutation mapping and whole transcriptome analysis. Reports that demonstrate the usefulness of next generation sequencing in the SELEX process are also reported [33-34]. Currently three commercially available next-generation sequencing platforms exist at the forefront of the sequencing industry, the original Roche 454 platform and the Illumina, and SOLID sequencing technologies.

6.2 Objectives

The main objective of this chapter was to perform 2´F modified RNA aptamer against Prostate Specific Antigen within the laboratory of Prof. Peter Stockley in the University of Leeds, UK. The chapter can be divided in four parts. The first part is dedicated to *in vitro* selection process performed on a Biomek 2000 laboratory automation workstation. The second part deals with sequencing and characterization of selected aptamer candidates, and the third part details binding studies of the aptamer candidates and finally the last part outlines the potential use of the selected aptamers in diagnostic and therapeutic applications.

6.3 Methodology

6.3.1 Reagents

2´F modified CTP and UTP were supplied from TRI-LINK BioTechnologies (USA). TEMED, APS, spermidine were purchased from Sigma-Aldrich (Spain). QIAgen PCR up kit was obtained from Qiagen (Spain). RNA 3´end Biotinylation Kit, and Sulfo LC Biotin linker from Thermofisher (Spain). PSA substrate was supplied from Bionova (Spain), and RNA Clean from IZASA (Spain). Binding and Washing buffer (B&W) is composed of PBS buffer containning 3 mM $MgCl_2$.

6.3.2 *In Vitro* Selection

6.3.2.1 Generation of starting RNA pool

A synthetic ssDNA library (Library III) was used for generating dsDNA pool using reverse primers (R_3), and forward primer containing T7 promotor sequence (F_3_T7) as described previously (Chapter 3.3.2.3). The efficiency of PCR was assessed on a 10 % (w/v) DNA polyacrylamide gel. The gel was composed from 35 ml of 10 % (w/v) acrylamide (29:1 acrylamide: biscarylamide) in 1 X TBE buffer with 500 µl 10 % (w/v) APS, and 50 µl TEMED. The samples were thoroughly mixed with 6 x loading buffer and loaded into separate wells. A 10 bp DNA ladder was used as a size marker. The gel was run in 1 x TBE at 300 V for 45 minutes. The gel was stained with ethidium bromide 1 µg/ml in 1 x TBE buffer for 5 minutes and then viewed and photographed under UV light. *In vitro* transcription reactions were carried out using the starting ds-DNA pool as a

template. Each reaction was set up by mixing 5 µl of 10 x transcription buffer (400 mM Tris HCl pH7.9, 260 mM $MgCl_2$, 0.1% (v/v) Tritor X-100, 25 mM spermidine), 2,5 µl 100 mM 2´F modified NTP mix, 0,05 Units YIP, 2 µl 100 mM DTT, 100 Units T7 RNA polymerase, 10 µl template DNA and R- water (to 50 µl final volume). The samples were thoroughly mixed and incubated at 37° C for 2 hours. Transcripts were treated with DNase (5 units DNse, 5 µl of 10 X buffer, and 45 µl of template) and purified with RNA clean kit according to the manufacturer´s instructions. A sample of the clean transcript was analysed on a 10% denaturing polyacrylamide gel. In this case, 35 ml of 10 % (w/v) acrylamide (19:1 acrylamide:bisacrylamide) in 1 x TBE with 7.5 M urea, with 200 µl 10 % (w/v) APS, and 20 µl TEMED were used to analyze RNA samples. A prepared gel was run in 1 x TBE at 15 watts for 30 minutes before loading to allow the gel temperature to reach 40° C. Samples were thoroughly mixed with an equal volume of formamide loading buffer and heated to 95°C for 3 minutes before loading. A 10 bp DNA ladder was used as a size marker. The gel was then run for 45 minutes, and then stained and analyzed as described above.

6.3.2.2 Automated *in vitro* selection

In vitro selection was performed using a Biomek 2000 workstation, using magnetic beads as the partitioning method. PSA was biotinylated using EZ-Linker sulfo-NHS-biotin linker and immobilized on the surface of streptavidin coated magnetic beads as described before (Chapter 2.3.3.5). Twenty microliters of PSA bound streptavidin coated beads (approximately 65 pmol of PSA) were added to 80 µl of B&W buffer and mixed. Fifty microliters of the starting RNA pool (around 750 pmol) which had been preincubated with the magnetic beads (bare), in order to remove nonspecific binders, was then added and incubated at 37° C for 30 minutes. Mixing was repeated every 5 minutes to prevent the beads settling out. Following the incubation, the beads were magnetically separated. Buffer and unbound RNA

species were removed by slow aspiration from the bottom of the eppendorf. The magnetic beads were washed several times in 1 ml of B&W buffer. Finally bound RNA species were resuspended in 30 µl nuclease free water, incubated at 95°C for 15 minutes, and eluted. The eluted RNA was reverse transcribed by adding 10 µl RT buffer, 1 µl of 100 µM reverse primer, 2 µl 10 mM dNTP mix, 10 units of reverse transcriptase and water to a final volume of 50 µl. This was thoroughly mixed and incubated at 52°C for 2 hours. The resulting DNA template was amplified and analyzed as described previously (Chapter 3.3.2), and used as the template for the subsequent round. PAGE was used to assess the success of the RT-PCR at the end of each selection round.

6.3.2.3 Pull down assays

In the pull-down assay (Figure 6.2), free biotinylated PSA (30 pmol) was incubated with RNA pool (300 pmol) in 100 µl of binding buffer at 37° C for 30 minutes. Following incubation, the RNA-PSA complex was immobilized on the surface of magnetic beads. Following the immobilization, the RNA-PSA bead complex was washed several times in 1 ml of B&W buffer and unbound RNA was removed. RNA bound to PSA was eluted, amplified, and analysed by PAGE as described above. Negative selection with naked beads was performed prior to the incubation of RNA with biotinylated PSA, in order to remove surface binders.

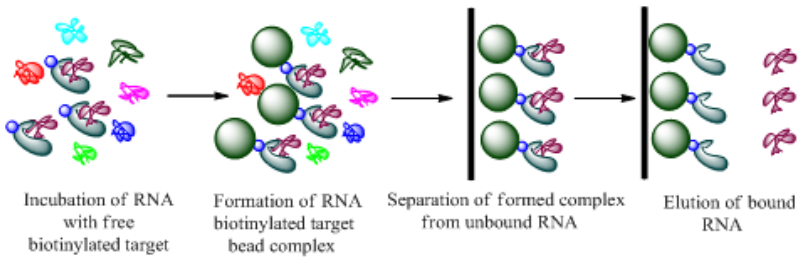


Figure 6.2 Schematic of pull down assay.

6.3.3 Cloning & Sequencing

Samples for NexGen sequencing were prepared by taking the enriched pool obtained at the end of the SELEX process. The enriched pool was PCR amplified using short forward primer (which lacks the T7 promoter) and the reverse primer. The resulting PCR product was purified using the QIAgen PCR up kit, checked on Bioanalyzer and used for sequencing. For standard cloning and sequencing processes protocols were used as described previously in Chapter 5 (section 5.3.5).

6.3.4 Binding studies

Surface plasmon resonance (SPR) binding studies were performed with a BIAcore 3000 (Biacore Inc.). The protein of interest (PSA, and control proteins such as streptavidin, and BSA) were immobilized on separate channels of a CM 5 sensor chip activated with EDC/NHS (30 μl of a 1:1 mixture of EDC (400 mM) and NHS (100 mM) followed by injection of 200 μg/ml (6,7 μM) protein at a flow rate of 5 μl/min. After immobilization of the proteins, unreacted NHS esters were deactivated via injection of an excess of ethanolamine hydrochloride (1M). Unbound proteins were then washed from the surface with 100 mM NaOH. The aptamer candidates were diluted to a final concentration of 1 μM in binding buffer and injected during 6 minutes at a flow rate of 5 μl/min followed by 3 min stabilization time and 10 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 previously filtered and dissolved in MilliQ water.

6.3.5 Apta PCR

A range of PSA concentrations (1, 5, 10 μg/ml) diluted in 50 mM carbonate buffer, pH 9.6 were incubated on a microtiter plate for 1 hour, followed by 1 hour blocking with PBS-Tween. After the washing step, selected aptamer candidates (200 nM) were added to each well and incubated for 30 minutes at 25°C in the presence of 3 μg/ml of salmon sperm. Finally, after the last washing step, MilliQ water was added to heat eluted aptamer.

6.3.6 Competitive Apta PCR

PSA (10 μg/ml) was immobilized on a microtiter plate in 50 mM carbonate buffer, pH 9.6 for 1 hour at 37°C, followed by 1 hour blocking with PBS-Tween. The plates were then manually washed three times with PBS-Tween. In individual eppendorf tubes, serial dilutions of PSA ranging from 2.6 pM – 8.3 μM (80 pg/ml - 250 μg/ml) were incubated with 100 nM of aptamer candidate S2 in binding buffer for 30 minutes at 25°C in the presence of 3 μg/ml salmon sperm. Following the final washing step performed with binding buffer, MilliQ water was added, and the aptamer heat-eluted. Eluted RNA molecules were reverse transcribed and amplified using a 1-step protocol with Power Sybr Green RNA-to-Ct (Applied Biosystems, Spain) according to the manufacturer's instructions.

6.3.7 Stability studies of selected aptamers

Aptamers at a concentration of 500 nM were incubated in 80% human serum at 37°C for periods of up to 48 hours. Following incubation, all samples were diluted in PBS buffer and purified with the RNA clean kit according to the manufacturer´s instructions. Purified aptamers were run on 4% (w/v) agarose gel and analysed with Image J software.

6.3.8 PSA activity assays

PSA was incubated with aptamer candidates at equal concentrations (1μM) in selection buffer for 2 hours prior to addition of fluorogenic substrate. Substrates were diluted to a final concentration of 400 μM. The hydrolysis of the substrate by PSA was measured with a spectrofluorimeter (Cary Eclipse, Varian), using an excitation wavelength of 400 nm and an emission wavelength of 505 nm.

6.4 Results and Discussion

6.4.1 *In vitro* selection process

A DNA library III with a random sequence of 50 nt was used for *in vitro* transcription by amplifying it with a long forward primer bearing T7 promotor and reverse primer (Figure 6.3). Modification of RNA library was performed by introducing 2´F modified CTP and UTP into the *in vitro* transcription reaction.

Figure 6.3 Diagramatic representation of the 3 oligos used to generate the dsDNA pool of library III. *The sequence shown in red indicates the T7RNA polymerase binding site.*

The *in vitro* selection process was performed utilizing a Biomek 2000 automated workstation (Beckman Coulter) based upon protocols previously described by Cox [24] and optimized by Bunka [35]. This automated system was then used with PSA as the target in 10 rounds of *in vitro* selection with 2´F modified RNA library. Selection was carried out using buffer conditions and temperature appropriate for PSA activity and taking into account the possible application of RNA aptamers. The negative selection was carried out by incubating 2´F modified RNA library with underivatized bare streptavidin coated beads in the absence of PSA. Samples from the reverse transcriptase - PCR products were taken at the end of each round and analyzed by native PAGE to confirm the isolation of products for the next round of selection (Figure 6.4).

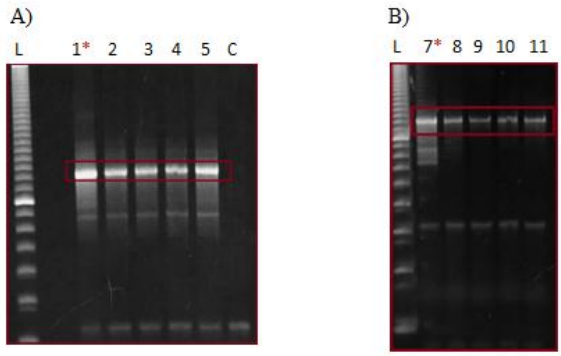


Figure 6.4 PAGE analysis of RT-PCR products from 10 selection rounds performed on automated workstation. *L – 10bp ladder, 1-11 selection rounds (round 6 was perdformed manually using pull down assays), C – non template control. *The intensity of band obtained from first round during automated SELEX is more intense due to higher initial concentration.*

6.4.2 Pull down assays

The *in vitro* selection process was separated in two parts, and the automated part of the selection was performed using the Biomek Workstation 2000 and the pull down assays were performed manually with the aim of increasing selection stringency. In the pull down assays, 2´F modified RNA pool was incubated with free biotinylated PSA in solution before the capture and immobilization of formed complex onto streptavidin coated beads. The advantage of this method is the incubation of RNA with PSA free in solution, overcoming problems due to protein immobilization, which can destroy key features of some aptatopes as well as reduce the mobility of the PSA. The 2´F modified RNA pool obtained at the end of 5th round of SELEX was compared with the starting pool of 2´F modified RNA (Figure 6.5).

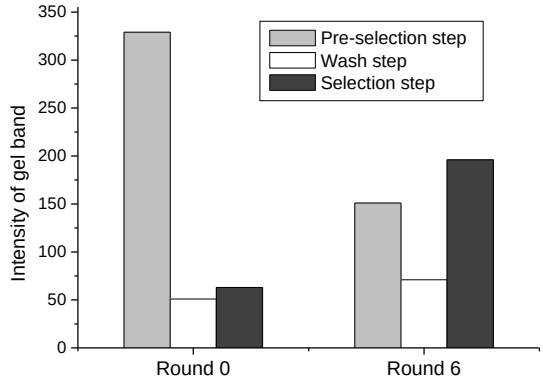


Figure 6.5 Fraction of 2´F modified RNA obtained using starting random pool (Round 0) and 5th round (Round 6) of SELEX in pull down assay. *The columns indicate the amount of RNA eluted and amplified in the negative selection step in the absence of PSA, after washing steps, and in the selection step with PSA. The amount of RNA is represented by the intensity of ds-DNA bands obtained by native PAGE gel.*

The analysis of band intensities from the native PAGE gel clearly shows the difference between the starting RNA pool and the enriched pool obtained at the end of the 6th round of SELEX performed by pull down assay. In the starting RNA pool a higher amount of RNA bound to the streptavidin beads and lower amount in the selection step with PSA coupled beads is observed, whilst in the 6th round, more RNA is bound in the selection step. Similarly, pull down assays were performed in the 11th, 12th, and 13th rounds of SELEX.

6.4.3 Monitoring of evolution using pull down assays

The amount of RNA obtained in both the pre-selection and selection procedures using pull down assays in round 0, 6, 11, 12, and 13 were used to monitor the evolution of the SELEX process, and Figure 6.6 shows the increasing amount of RNA bound to PSA. Since there was no significant increase in cycle 13,

the SELEX was assumed to have reached completion. There was no obvious increase of RNA bound in the negative selection step.

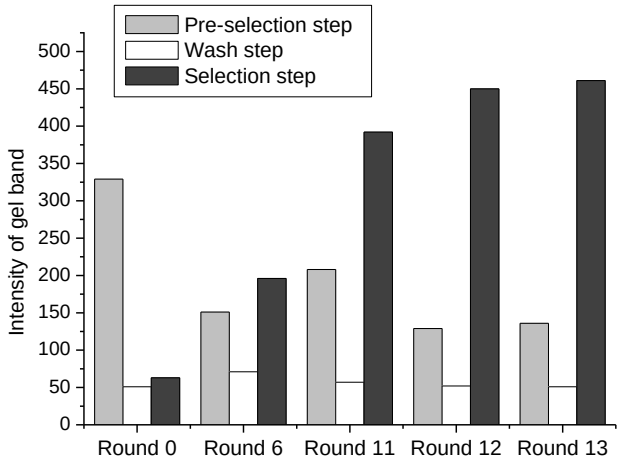


Figure 6.6 Fraction of 2´F modified RNA obtained in different rounds of SELEX*. The columns indicate the amount of RNA eluted and amplified in the pre-selection step in absence of PSA, after washing steps, and in selection step with PSA. The amount of RNA is represented by the intensity of ds-DNA bands obtained by native PAGE gel.*

6.4.4 Cloning and Sequencing process

The enriched pool of 2´F modified RNA library obtained from round 13 was used for NexGen sequencing. Unfortunately, the major fraction of sequences obtained from NexGen sequencing was composed from parts of primers, indicating some problems in the preparation of the sample or during the sequencing process (Figure 6.7).

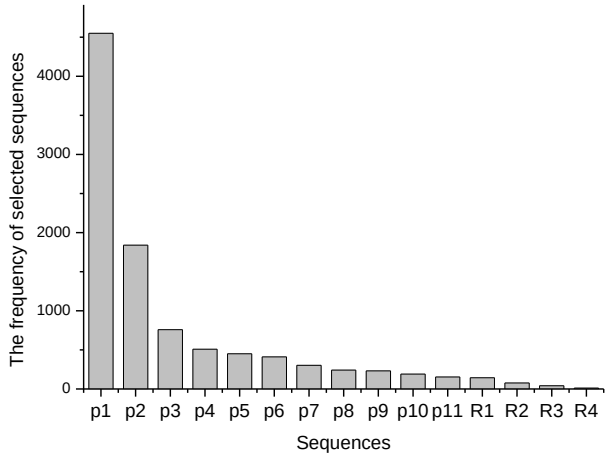


Figure 6.7 The frequency of selected sequences obtained from NexGen sequencing. *P1-P11 present sequences composed from parts of primers with different length, R1-R4 sequences containing random region.*

Nevertheless, from the analysis of all sequences, 4 different variants containing a random region from 37 to 40 nt was found. In Table 6.1 the final length of aptamer candidates which differ from the original 94 nt pool RNAs and the frequency of individual variants, is summarized.

Table 6.1 Sequences obtained from NexGen sequencing.

Aptamers	Number of clones	Lenght (nt)
R1	144	81
R2	77	81
R3	41	84
R4	11	81

Based on the sequence analysis, it was found that aptamer candidate R1 differed from R4 only in one base (Figure 6.8), while aptamer candidates R2 and R3 were unique sequences.

R1 (144) CGAGUUUUGAACCGCGUAAUAAAACAUUCCUCAUUUG
R4 (11) CGAGUUCUGAACCGCGUAAUAAAACAUUCCUCAUUUG

Figure 6.8 Sequences of aptamer candidates R1 and R4. The frequency of individual sequence is written in parentheses.

Due to the problems in NexGen sequencing, the enriched library was cloned and sequenced again as described previously in Chapter 5. 48 individual clones were obtained, which revealed 22 different sequences (Table 6.2). The lengths of the majority of sequences were the same as the original 94 nt pool of RNAs, however some of the sequences were shorter, as had been observed with the NexGen sequencing.

Table 6.2 Sequence obtained from Sanger sequencing.

Aptamers	Number of clones	Length (nt)
S1	7	84
S2	6	84
S3	3	94
S4-S6	2	93-94
S7 – S22	1	91-95

Eight sequences were represented more than once. Moreover, sequence S1 which appeared 7 times in second sequencing process was identical to sequence R3 (Figure 6.9). From the sequence analysis of all aptamer candidates no obvious pattern was obtained.

R3 (41) UCCAGGCGCGUUAGCAAAACCGCGGAUCAAACUUAGUUGA
S1 (7) UCCAGGCGCGUUAGCAAAACCGCGGAUCAAACUUAGUUGA

Figure 6.9 Sequences of aptamer candidates R3 and S1. *The frequency of individual sequence is written in parentheses.*

6.4.5 Binding analysis

The binding capacities of all unique aptamer candidates for PSA were examined in a screening manner using SPR and BIACORE (Figure 6.10). Ten different sequences displayed higher binding abilities to PSA compared to the starting library and the background control. Significant binding was observed for sequences R1, R2, R3, and S2.

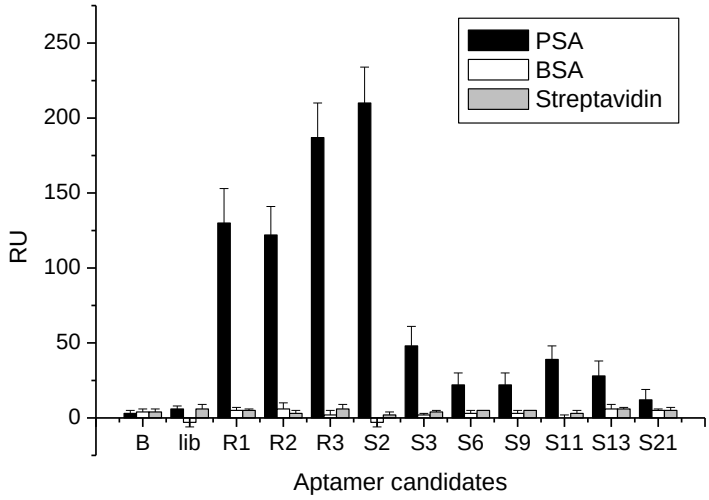


Figure 6.10 SPR experiments showing the specificity and cross-reactivity of selected aptamer candidates (1 μM), n = 2.

The signal observed in the control channels with immobilized BSA and streptavidin was negligible for all selected aptamers, demonstrating the high specificity of these aptamer candidates. Aptamer candidate S2 was passed through the channels at 10 μM in order to confirm the specificity of aptamer in higher concentrations (Figure 6.11)

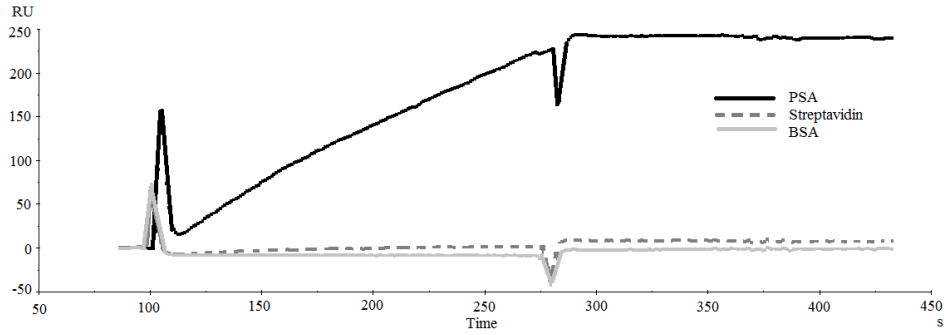
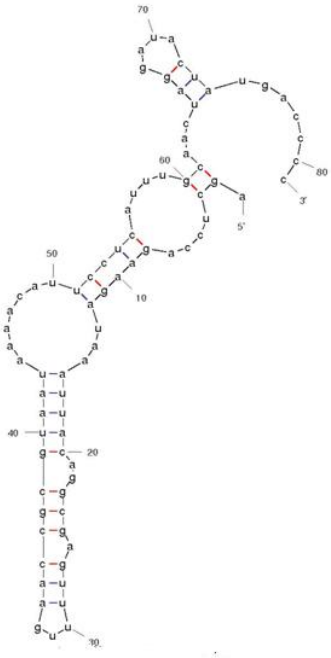


Figure 6.11 SPR Sensogram obtained for aptamer candidate S2 at 10 µM passed through the CM 5. *Biacore chip surface showing the interaction with the immobilized target (Channel 2: PSA, Channel 3: BSA, Channel 3: Streptavidin) All sensograms were corrected for nonspecific binding and refractive index changes by subtracting the signals of an equivalent aptamer injection across the underivatized flow cell (Channel 1).*

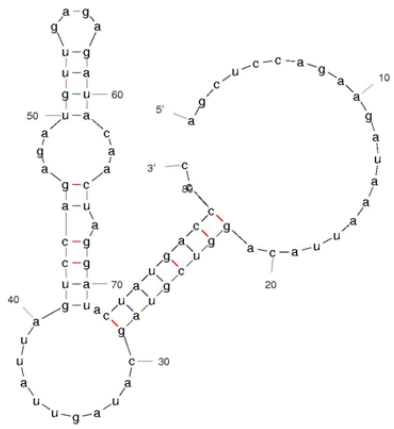
The secondary structure of the selected aptamer candidates R1, R2, R3 and S2 (Table 6.3) was predicted using the online program M-fold for RNA folding (Figure 6.12). According to the proposed secondary structures, all four variants could be folded into similar loop-stem structures. The predicted free energy of secondary structure formation of aptamer candidates R2, R3 and S2 indicated a higher stability of these sequences as compared to the DNA aptamers reported in Chapter 5.

Table 6.3 Sequences R1, R2, R3, and S2 with the appropriate length and the predicted free energy of secondary structure formation (ΔG).

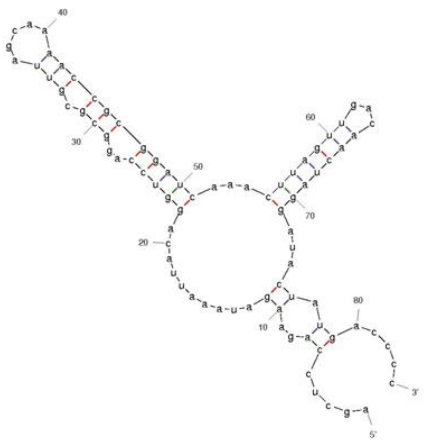
	Sequence (5´-3´)	Length	ΔG
R1	agcuccagaagauaaaauuacaggcgaguuuugaaccgcguaauaaaacauu ccucauuugcaacuaggauacuaugacccc	81	-7,5
R2	agcuccagaagauaaaauuacaggucguagcauaguuauuaguccagagau guugagagauacaacuaggauacuagacccc	81	-14,9
R3	agcuccagaagauaaaauuacagguccaggcgcguuagcaaaaccgcggauc aaacuuaguugacaacuaggauacuaugacccc	84	-15,7
S2	agcuccagaagauaaaauuacagguacgguucacgccugucucaugcugac uaagaaaguuuagcaacuaggauacuaugacccc	84	-15,2



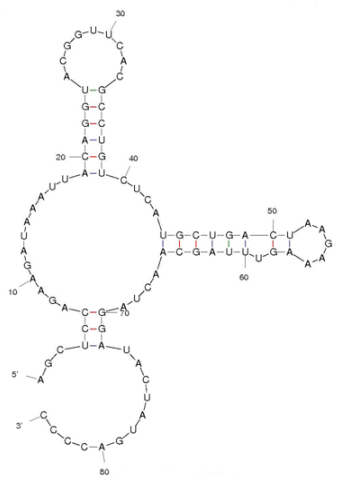
R1 ΔG = -7,5



R2 ΔG = -14,9



R3 ΔG = -15,7



S2 ΔG = -15,2

Figure 6.12 Predicted secondary structure for aptamer candidates R1, R2, R3, and S2, ΔG indicates the predicted free energy of secondary structure formation given in kcal/mol.

The dissociation constant (K_D) of aptamer candidate R3 and S2 was estimated by analyzing the binding of a range of concentrations (50 nM to 1 μM) with PSA, using a one to one Langmuir binding model (Figure 6.13). The resulting K_D were 8,7 x 10^{-7} M, and 6,3 x 10^{-7} M for aptamer candidates R3 and S2, respectively, and a good fit to the model was obtained (Table 6.4).

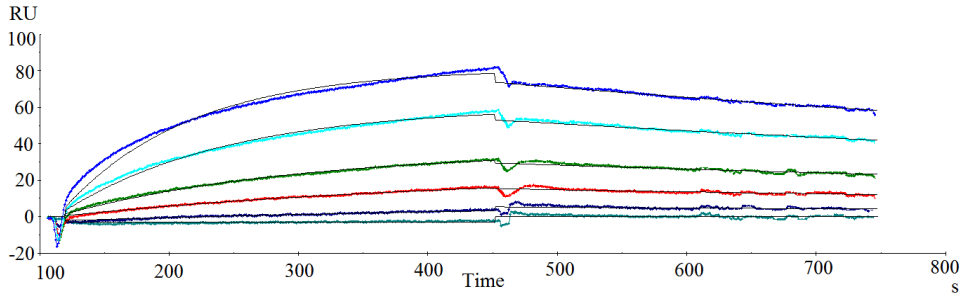


Figure 6.13 SPR sensogram for aptamer candidate S2 using range of concentrations (50 nM - 1 μM).

Table 6.4 Dissociation constants obtained in Biacore with relevant statistics of the aptamer candidates obtained.

Aptamer	Model	K_D (nM)	χ^{2*}
R3	Langmuir Binding	870	0,776
S2	Langmuir Binding	630	0,943

*The Chi^2 value is a standard statistical measure of the closeness of fit of data to the model used for elucidation of the K_D, where for good fitting to ideal data, χ^2 is of the same order of magnitude as the noise in RU, typically <2.

6.4.6 Development of apta-PCR assay

A development of real-time apta-PCR for the ultrasensitive detection of thrombin has been reported previously [36]. In apta-PCR the aptamer acts not only as a biomolecular recognition element, but also as a label for amplification *via* real-time PCR. The use of the aptamer itself as the reporter molecule eliminates the

necessity of laborious labeling, which is particularly atractive in the case of modified RNA aptamers.

The aptamer candidates R3 and S2 were tested for their ability to bind to PSA adsorbed on a microtiter plate. Three different concentrations of PSA were immobilized on the microtiter plate and incubated with aptamer candidate S2 or R3, and the aptamer S2 was observed to be the ligand with the highest affinity and specificity (Figure 6.14).

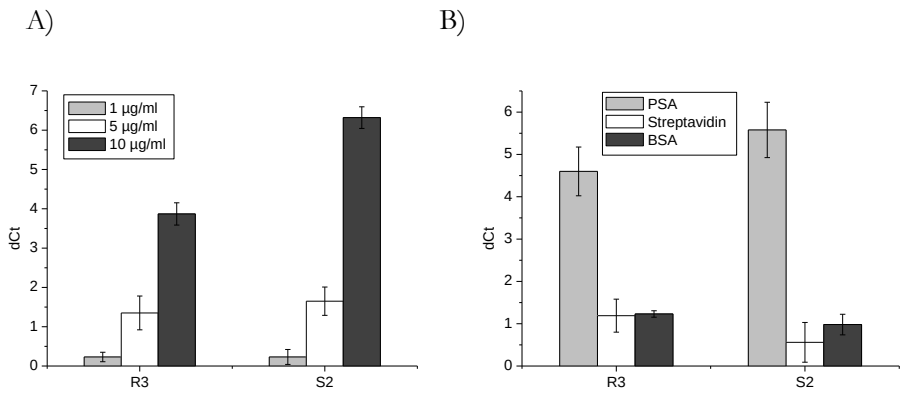
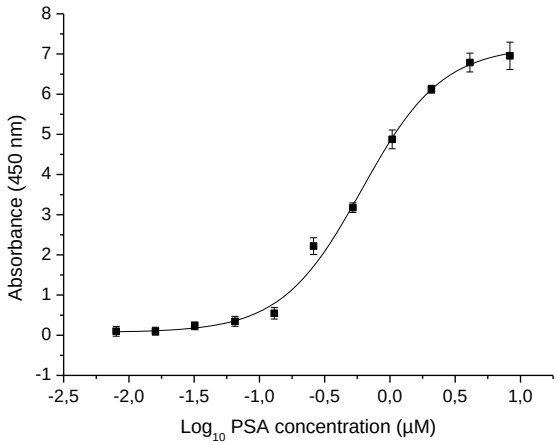


Figure 6.14 Evaluation of binding affinities and specificities of selected aptamers in Apta PCR assay.

Aptamer candidate S2 was thus used to develop a competitive apta-PCR assay. This assay is based on immobilization of PSA on the microtiter plate, which competes with PSA free in solution for the binding to the aptamer. After the incubation step, the captured RNA aptamer is reverse transcribed and amplified using qPCR. A calibration curve between 2.6 pM to 8.3 µM (80 pg/ml – 250 µg/ml) was obtained, with r^2 0.994 and EC_{50} value of 596 nM (Figure 6.15). The limit of detection (LOD) 11 nM (0,329 µg/ml) obtained from the apta-PCR assay is lower in comparison to the previously published DNA aptamer selected against PSA (pDNA aptamer) [37], which had a LOD above 40 nM (1.23 µg/ml). In comparison to anti PSA antibodies which can achieve LOD around 1-500 pg/ml

[38-40], the LOD of aptamer S2 is several order of magnitude higher and does not meet the requirement for diagnosing prostate cancer.



FIT VALUES	
LOD	11 nM (0,329 µg/ml)
EC50	596 nM (17.88 µg/ml)
R^2	0,994
Replicates	3

Figure 6.15 Detection of PSA by apta-PCR assay using aptamer candidate S2, with relevant statistics obtained (n=3).

6.4.7 Clinical potential of 2´F modified RNA aptamers

6.4.7.1 Stability of aptamers S2 and R3 in human serum

A prerequisite for a potential therapeutic use of the aptamers is a reasonable stability in human serum. In contrast to unmodified RNA, which is easily degraded in biological fluids, 2´F modified RNA is much more stable. To test the stability of aptamer S2 in human serum, stability assays were carried out incubating the

aptamer S2 and R3 in human serum for up to 48 hours (Figure 6.16, a, b). The starting 2´F modified RNA pool used for the SELEX was also tested (Figure 6.16, c) as well as pDNA aptamer (Figure 6.16, d) and unmodified RNA aptamer (pRNA aptamer) selected against active PSA [41] (Figure 6.16, e).

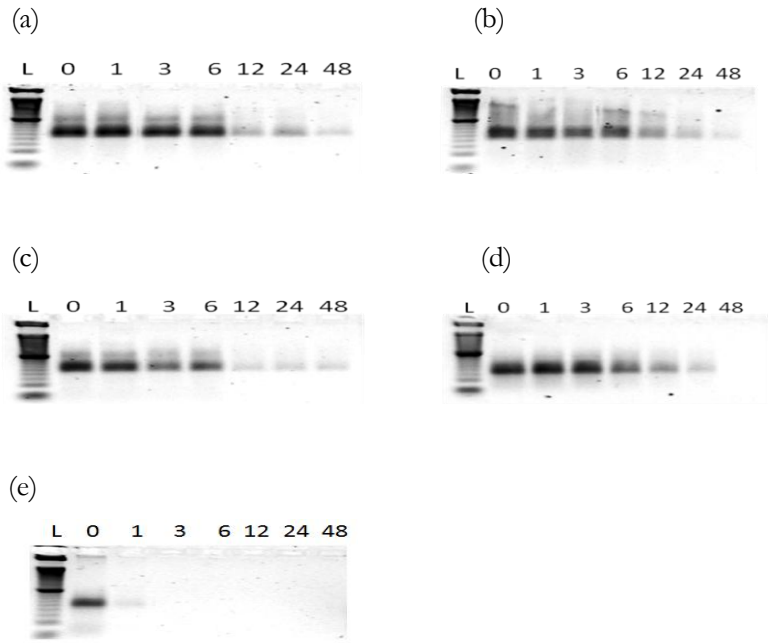


Figure 6.16 Stability of aptamer S2 (a), R3 (b), starting pool of 2´F modified library (c), pDNA aptamer (d), pRNA aptamer (e) in human serum.

The incubation of aptamer S2 and R3 in human serum showed no evidence of degradation after 6 hours of incubation. Only partial degradation was observed after 12 and 24 hours. Even after 48 hours of incubation, the aptamer could still be detected on the gel. Similarly, the starting pool of 2´F modified library showed comparable stability. The stability of pDNA aptamer was not as high as that of the 2´F modified RNA aptamers, but was much higher than that of pRNA aptamer, which degraded rapidly in human serum.

6.4.7.2 Aptamers as modulators of PSA activity

The effect of aptamers R3 and S2 and previously published PSA aptamers (pDNA[37], pRNA[41]) on the activity of PSA was studied using a synthetic PSA substrate (Figure 6.17). The activity of PSA was moderated in the presence of the selected RNA aptamers R3 and S2, whereas the starting RNA pool, pDNA, and pRNA had almost no effect at same concentrations. To analyze the potential of aptamer S2 to moderate the PSA activity, the activity of PSA was studied in the presence of increasing concentrations of aptamer S2 (Figure 6.18). PSA activity was moderated by aptamer S2 achieving 60% of the initial activity in high concentrations.

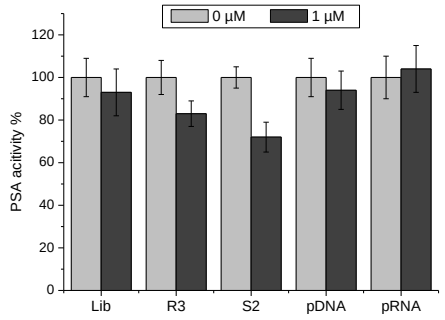


Figure 6.17 Inhibition of PSA activity by aptamer candidates S2, R3 starting random RNA pool, and published PSA aptamers (pDNA, pRNA), n = 3.

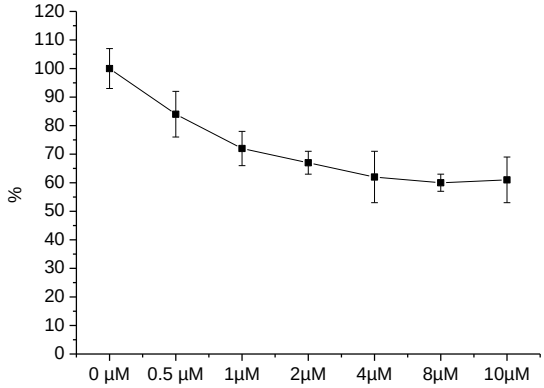


Figure 6.18 Concentration dependency of inhibition of PSA activity by S2.

6.5 Conclusions

RNA based SELEX for a PSA target was successfully performed on an automated Biomek 2000 workstation, achieving aptamers with dissociation constants in the nM range. Moreover, the final aptamers could distinguish PSA from other proteins, such as streptavidin or BSA highlighting their high specificity. The potential use of the selected aptamers in diagnostic and therapeutic applications was studied. In order to demonstrate the functionality of the selected 2´F modified aptamers, apta-PCR was developed for the quantitative detection of PSA. The limit of detection of aptamer S2 was found to be 11 nM, improving the limit of detection achieved by previously published DNA aptamer, but still several orders of magnitude higher than in case of anti PSA antibodies. Nevertheless, we have demonstrated the possibility of PSA detection using selected S2 2´F modified aptamer and with further investigation, the aptamer S2 may be applied to clinical diagnostics. An alternative application is for therapeutics, to this end stability of selected aptamers S2 and R3 was carried out incubating aptamers in human serum up to 48 hours. The results demonstrated that 2´F modified aptamers are significantly more stable in human serum than the previously published unmodified RNA aptamer and slightly more stable than the previously published DNA aptamer. The potential use of RNA aptamers S2 and R3 in therapeutics was validated in PSA activity assays, where the activity of PSA was moderated in the presence of selected aptamers R3 and S2, whereas the starting RNA pool, pDNA, and pRNA had almost no effect at the same concentrations. Although the effect of the aptamer S2 on the activity of PSA was moderate, it may be significant for potential treatment of prostate cancer, as prostate cancer generally grows very slowly, and even a modest reduction in tumor growth rate can play some role in the prevention of the development of prostate cancer in diagnosed patients.

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Chapter 7

7.1 Overall conclusions and outlook

This work overviews the selection of aptamers against prostate specific antigen (PSA). Fundamental aspects such as the characterization of PSA, evaluation of immobilization strategies, the preparation of the oligonucleotide library and single stranded DNA (ssDNA) have been evaluated. Finally, the selection of DNA and RNA based aptamers against PSA and their potential use in diagnostic and therapeutic applications have been described.

The selection of aptamers depends highly on the target properties, therefore the characterization of PSA obtained from seminal fluid was performed using sodium dodecyl sulphate-polyacrylmide gel electrophoresis (SDS-PAGE) and Mass spectrometry (MS). A high heterogeneity of PSA was found, indicating the presence of different PSA isoforms comprising proPSA, intact PSA and nicked

form of PSA as described previously [1]. Moreover, these multiple forms of PSA can vary more, due to differences in carbohydrate structure [2]. The complex distribution of PSA leads to uncertainties in the immunological measurement of PSA [3] and can also cause difficulties during the selection process. A comparison of two different lots of PSA showed a high consistency in the proportion of PSA isoforms in both samples, confirming previously published results [2].

In order to determine the best strategy for PSA immobilization, different techniques were evaluated, including covalent immobilization using amine, carboxyl, and epoxy modified beads, as well as bioaffinity immobilization with avidin-biotin interaction. These were studied using competitive enzyme-linked immunosorbent assay (ELISA) and peptide mass fingerprinting (p.m.f). The amount of immobilized PSA per miligram of beads was found to be similar in the cases of carboxyl and epoxy modified beads. Although a higher number of aminoacids containing carboxyl groups are available as compared to aminoacids containing lysines, the lowest amount of immobilized PSA was found on amine modified beads, probably due to the lower exposure on the surface of PSA. Bioaffinity immobilization with avidin-biotin was absolutely unrivaled in terms of the amount of immobilized PSA, achieving a two times higher PSA immobilization. The stability of immobilized PSA on the surface of magnetic beads was studied after 4 weeks of storage. In most cases, ≥ 80 % of the amount of PSA was obtained, indicating that the PSA coupled beads are relatively resistant to storage conditions. Only PSA immobilized on the epoxy beads showed a severe reduction of PSA to 57%. After considering all the pros and cons of the methods studied, two strategies including (i) amine coupling using carboxyl modified beads and (ii) the use of bioaffinity technique with biotin – streptavidin interaction were selected for their use in SELEX. Moreover, both methods had been previously used for immobilization of other proteins in SELEX achieving high affinity aptamers [4-6].

Another critical point of SELEX is an oligonucleotide random library consisting of a multitude of ssDNA. The heterogeneity of the random library can cause non-uniform amplification during the PCR amplification [7], therefore the

choice of library, and efficient PCR amplification of the selected library can contribute to the success of aptamer selection. The optimisation of three different libraries with specific features was carried out to ensure high purity and yield of amplified libraries. The composition of random libraries changes during SELEX, therefore optimisation of the enriched libraries was also required. It was found that the addition of single stranded binding protein (SSB protein) to the PCR master mix with optimized PCR conditions could prevent smearing into a multi-sized population. The addition of SSB protein was previously used to increase amplification efficiency with a number of diverse targets achieving comparable results [8]. In the case of production of smaller bands that can appear during the SELEX process, PCR amplification performed with 3% of DMSO and 500 mM of Betaine helped to decrease the amount of these undesired products. These two substances are often used to improve PCR amplification and our results are consistent with those previously published [9-10].

Aptamers can be selected from single stranded DNA (ss-DNA) or RNA libraries and in the case of DNA based SELEX, each round is completed by the generation of single stranded DNA, available for target binding in the subsequent round. The generation of the ss-DNA is a critical step in SELEX as the purity and yield can have a significant impact on the successful evolution throughout the SELEX process. We compared several techniques generally used for ssDNA generation including asymmetric PCR, lambda exonuclease digestion and magnetic separation, as well as a new technique which combines the highly efficient asymmetric PCR with enzyme digestion. Moreover, generation of ssDNA is very time-consuming in methods such as enzyme digestion and magnetic separation due to the several purification steps, including purification of PCR product and purification of ssDNA at the end of process. We hypothesized that yield obtained from unpurified PCR product should be similar to those obtained from purified PCR product. The efficiency of each system for the generation of ss-DNA was quantitatively evaluated using an enzyme linked oligonucleotide assay (ELONA) and the quality of the PCR product was assessed using agarose gel electrophoresis.

According to the results obtained from our comparison studies, all reported methods can be used for the generation of ss-DNA, once their disadvantages are clearly taken into consideration. Asymmetric PCR followed by T7 Exonuclease digestion was demonstrated to have the highest efficiency, achieving a final yield of ss-DNA around 85%. Furthermore, we have demonstrated that in methods such as enzyme digestion and magnetic separation with streptavidin beads when purification of PCR product is usually used, the yield of ssDNA obtained from unpurified PCR product is comparable to those obtained from purified PCR product, overcoming a loss of dsDNA during purification step, and thus decreasing the time required for each SELEX cycle.

Having all fundamental aspects of SELEX evaluated and optimised, in vitro selection of DNA aptamers against PSA was performed. Variations of SELEX protocols were used to identify aptamers against this enzyme, as aptamer selection against PSA presented significant difficulties. Partitioning of bound nucleic acids from the unbound ones was one of these problems. Two methods, one focused on the use of nitrocellulose filters with the target free in solution, and another focused on target immobilization with modified beads were used. The latter one was observed to be more effective with minimum losses of target bound sequences. Another problem found during the selection process was associated with the amplification of random libraries, and as we discussed in Chapter 3, optimization of enriched library with the use of specific additives could prevent incorrect amplification. Diverse methods of single stranded DNA generation and selected buffers were not observed to be critical for aptamer selection. Finally, several aptamer candidates that exhibited an ability to bind to PSA were identified. However, all the aptamer candidates showed high cross-reactivity with control proteins (BSA, streptavidin). Aptamer I1 and I4, with a dissociation constant in the low μM range, showed the lowest cross reactivity with control proteins. For the evaluation of the functionality of aptamer I1, a competitive RLAA with different concentrations of PSA was used achieving a limit of detection of 268 nM. Nevertheless, the low specificity of selected aptamer does not permit its use for

analysis. Despite the great number of publications related to the selection of aptamers, showing that aptamers can be routinely obtained for almost every desired target [11], one of the true problems of SELEX is the uncertainty of the success of the selection, as it impossible to judge the ability of a target molecule to be suitable for the selection process [12], with the success rate for homogenous targets being around 50% [13].

Finally, selection of an RNA based aptamer against PSA using an automated SELEX process was carried out. Aptamers with dissociation constants in the nM range with high specificity were developed. The potential use of the selected aptamers for diagnostics was studied using apta-PCR, achieving a limit of detection of around 11 nM for aptamer S2, improving the limit of detection achieved by the previously published DNA aptamer for PSA, but still several orders of magnitude higher than the anti PSA antibodies generally used in diagnostics. Although the limit of detection does not meet the requirement for the use of selected aptamers for PSA detection in diagnostics, we have demonstrated the functionality of the selected aptamers. Another promising application of selected aptamer in diagnosis could be in vivo imaging of prostate cancer, due to the discrimination and targeting capacities of aptamers making them suitable imaging agents for non-invasive diagnostic procedures [14].

One major hindrance to the clinical use of aptamers is their instability in body fluids due to the high levels of nuclease activity. However, the selected 2´F modified aptamers remained stable in human serum for dozens of hours, indicating their high resistance to nucleases.

Finally, the potential use of the selected RNA aptamers in therapeutics was evaluated in activity assays, demonstrating an inhibiting effect on the PSA activity. Due to the inhibition of PSA activity, the amount of several substrates related to cancer cell invasion and tumor growth which are cleaved by PSA could be limited in the early development of cancer. Recently, peptides that bind specifically to enzymatic active PSA and stimulate its activity was reported [15]. The stimulation

of PSA activity by these peptides reduced angiogenesis and could be potentially used at later stages of cancer therapy.

In addition, anti PSA aptamers could be used in targeted therapy. Aptamers are suitable to serve as escort ligands in which the aptamer may be used to deliver an active drug, radionucline, toxin, or cytotoxic agent to the desired sites for targeted therapies [16-17]. In respect to prostate cancer, there is another well established and characterized aptamer A 10 selected against the prostate-specific membrane antigen (PSMA) [18], which is already used as a drug-delivery vehicle with an efficient targeting of solid tumors, leading to the reduction in the size of tumor and significant reduction of prostate tumor proliferation [19-22]. Small interfering RNA (siRNA) has also been used to conjugate with aptamer A10 obtaining cell specific siRNA-mediated reduction of the corresponding mRNA and protein levels [12, 23-24].

To conclude, since the first description of aptamers in 1990, considerable progress has been made in the field of aptamer technology. The characteristics of aptamers make them promising tools for diagnostic applications, where aptamers could be an alternative to antibodies. Recently, new technology platform using SOMAmers (Slow Off-rate Modified Aptamers) to accurately screen dozens of patient samples simultaneously has been reported [29], allowing quantification of many specific proteins in a multiplexed, high-throughput manner. The company SomaLogic has thus far validated SOMAmers to a broad array of over 1000 different protein targets for use in biomarker discovery, diagnostics, and drug discovery and development. In therapeutics, there are many aptamers selected against a large variety of targets that play crucial roles in several diseases showing the potential use of aptamers in therapy. Aptamers have been used to deliver therapeutic agents to cells or tissues, showing their inmense potential as they are relatively small and can easily penetrate tissues [25]. However, the in case of intracellular target proteins, the application of aptamers is limited due to their negative charge that will not easily cross cellular membranes [11]. However, recent

developments have demonstrated the possibility to transfer the functional properties of aptamers to small molecules [26-27].

7.2 Outlook

For the potential use of the selected aptamers in further applications, there are still several parameters and issues that need to be investigated, starting with the elucidation of the two and three-dimensional structures of the selected RNA aptamers, and truncation studies, which often leads to an improvement in detection limits. A selection experiment employing a partially randomized pool based on the already selected aptamers could also be used in order to improve the affinity of aptamers towards the low nanomolar or even picomolar range [28]. Another possibility would be use of new alternative SELEX strategies with higher partitioning efficiency which could lead to higher affinity aptamers. In case of a complicated target, where selection of "normal" aptamers is not successful, there exists the possibility to use libraries which bear dU residues that are uniformly functionalized at the 5-position with moieties (e.g. benzyl, 2-napthyl, or 3-indolyl-carboxamide) and can participate in interactions with target molecules, as well as form novel secondary and tertiary motifs [29]. Selection of aptamers immobilizing the PSA via amine groups could be another option to see whether the orientation of immobilized PSA can affect the selection of aptamers.

The evaluation of external factors that can affect aptamer-PSA interaction should be assessed to see if the limit of detection could be improved as well as the use of aptamers in different assay formats to test the flexibility, adaptability and limitations of the selected aptamers. Among other factors, the effect of changes in temperature, pH, and composition of ions as well as immobilisation/modification strategy and binding efficiency should be studied. This information will be of immense importance in the development of for example, a molecular beacon format.

The specificity of the DNA aptamers could be improved by incorporating counter SELEX, which is established in order to obtain aptamers with stronger specificity. The aptamers´ selectivity could be improved by excluding ssDNA with

coaffinity to BSA, Streptavidin. In the case of the selected 2´F modified RNA aptamers, the specificity of selected aptamers should be further investigated using proteins with higher similarities to PSA such as human kallikrein 2 (hK2) or proteins present in human serum to be sure that the selected aptamers do not interact with other serum proteins. Human kallikrein 2 with approximately 80% of sequence homology is particularly important as hK2 activates and regulates the activity of PSA.

Moreover, the effect of 2´F modified RNA aptamers on the activity of PSA should be investigated in more detail using different PSA substrates and combination of selected aptamers, or a combination of anti-PSA aptamers with anti-PSA antibodies to see if the inhibitory effect can be improved. The effect of aptamers on the PSA activity will be also studied using human serum as the inhibition effect that the selected aptamers exhibit may be different in other buffer conditions.

It would be also interesting to study which specific sites on the target surface (aptatopes) are recognized by the selected aptamers. The similar secondary structures of the 2´F modified aptamers S2 and R3 suggest that both aptamers bind to one single aptatope on PSA. Analysis of numerous protein SELEX experiments shows that during the selection, one aptatope of a protein is dominant even in the presence of many other potential sites for aptamer binding [30]. However, the existence of a dominant aptatope of a protein target does not exclude selection of ligands that bind to other sites [31]. Competition assays with different types of anti-PSA antibodies that recognize different epitopes on the surface of PSA could be used to determine binding site specific for selected aptamers.

To conclude, the main contribution of this thesis is the optimization of several critical points in the aptamer selection process. The comparison between different methods showing the benefits as well as limitations of many protocols described in the literature will serve to guide future research that would improve the knowledge gained in the field of aptamer selection.

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