In Vitro Selection of DNA Aptamers Against Prostate Cancer Peptide Biomarkers

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IN VITRO SELECTION OF DNA APTAMERS AGAINST PROSTATE CANCER PEPTIDE BIOMARKERS

by

ELIF KUGUOGLU

A thesis submitted in partial fulfillment of the requirements for the Honors in the Major Program in Molecular and Microbiology in the College of Medicine and in The Burnett Honors College at the University of Central Florida Orlando, Florida

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Thesis Chair: Dr. Dmitry Kolpashchikov
ABSTRACT

This project is aimed toward finding DNA aptamers against prostate cancer peptide antigens. DNA aptamers can function to find and indicate the presence of certain molecules in a specimen. These aptamers will be obtained through the process of evolutionary selection, a specific process called SELEX which stands for Systemic Evolution of Ligands by Experimental Enrichment. By conducting several rounds of SELEX, a DNA aptamer will be selected to bind to a known peptide antigen. A biotinylated column will be utilized to stabilize a random library of DNA aptamers, and those peptides that bind to certain aptamers will cause a conformational change leading to the elution of those specific DNA aptamers. This SELEX process will be conducted again on the eluted aptamers to further select for strong binding DNA aptamers. The DNA aptamers that are obtained can further on be sequenced or used for prostate cancer research studies. Another possible usage of aptamers is to diagnose and determine the stage of various different cancer types. Our prediction is that this research will produce a DNA aptamer that will bind to a specific prostate cancer peptide antigen.
DEDICATION

To all of those who have been affected by cancer.
To my mentor Dr. Kolpashchikov. Thank-you for your patience and guidance, and for making me a better researcher.
To my grandmother Melahat Konyali,
my brother Mehmet Emre Kuguoglu,
and my parents Akin and Latife Kuguoglu.
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INTRODUCTION

The Systematic Evolution of Ligands by Exponential Enrichment (SELEX) technique is more recently being utilized by many researchers in order to discover new diagnostic and therapeutic aptamers. Aptamers can be single stranded oligonucleotides or peptide molecules that bind a specific target molecule. These molecules are selected from a large pool of random sequence molecules, and can be used for clinical or research purposes. In this study DNA aptamers were selected to bind peptide biomarkers. DNA aptamers are useful diagnostic and therapeutic tools mainly because of their predictability, low cost of synthesis, and stability. Aptamers have already made an impact in therapeutics with pegaptanib, a drug that is applied locally and targets the vascular endothelial growth factor. This was the first therapeutic aptamer drug to successfully make it to the market and was designed for patients with macular degeneration [1].

Using the SELEX in-vitro selection technique this study plans to discover DNA aptamers that bind to the prostate cancer peptide biomarker PSA2, where further on these aptamers will be used for further research purposes. The target peptides are nine to twelve amino acids in length, and PSA2 specifically is twelve amino acids in length. The oligonucleotide library will have a random region of 30 nucleotides in length. The agarose bead immobilized DNA library will be incubated with the target peptide, which upon binding to certain members of the library will trigger their conformational change and release from the column. The released DNA will be PCR amplified and used for the next round of in-vitro selection. Finally, the advanced library after
potentially 8-12 rounds of selection will be sequenced. The individual aptamers will be tested in their ability to bind the target peptides.
SYSTEMATIC EVOLUTION OF LIGANDS BY EXPO
ENTIAL ENRICHMENT OR SELEX IS A PROGRESSIVE
EVOLUTIONARY METHOD THAT SELECTS FOR A DNA AP
TER THAT IS OPTIMAL IN THE BINDING OF AN ASSIGNED
TARGET [2]. THIS APTER IS A SINGLE STRANDED
OLIGONUCLEOTIDE THAT IS SELECTED OUT OF A
LARGE POOL OF RANDOM SEQUENCED OLIGONUC
LEOTIDE STRANDS TO BIND TO A SPECIFIC TARGET
MOLECULE. IDEALLY THIS SYSTEM OF SELECTION
UTILIZES A COMBINATORIAL LIBRARY OF OLIGONUCLEOTIDES. SELECTION BY USING THIS
SYSTEM IS FLEXIBLE AND HAS BEEN USED TO SUCCESSFULLY DISCOVER AP\NERS THAT RECOGNIZE CELLS OF
SEVERAL TYPES OF CANCER [3]. THERE ARE VARIOUS STUDIES THAT HAVE UTILIZED THIS SYSTEM TO DISCOVER
APETERS THAT BIND TO TARGET MOLECULES INCLUDING CANCER CELLS [4], STEROIDS [5], IONS [6], AND
PROTEIN MOLECULES [7].

IT IS NOTED BY SEFAH ET AL. THAT DNA APETERS WERE ABLE TO DIFFERENTIATE TWO TYPES OF
CANCERS THAT ARE SIMILAR FROM CLINICAL SAMPLES OF BONE MARROW [4]. THIS INDICATES THE POTENTIAL OF
DNA APETERS TO BE CONVENIENT AND ACCURATE CLINICAL TOOLS IN DIAGNOSIS AND TREATMENT,
PARTICULARLY SINCE DNA IS A FAIRLY STABLE BIOLOGICAL MOLECULE. THESE APETERS CAN FURTHER BE USED
AS THERAPEUTIC DEVICES. WHEN PARTICULARLY COMPARED TO ANTIBODIES, THESE MOLECULES ARE EASIER TO
PRODUCE AND INEXPENSIVE. DNA APETERS CAN ALSO BE CHEMICALLY MODIFIED WITH THE ADDITION OF
DIFFERENT FUNCTIONAL GROUPS. THEIR ABILITY TO RETAIN FUNCTION EVEN WHEN IMP
Mobilized ON A CARRIER OR DELIVERED IN AN ANIMAL HAS MADE THEM THE FOCUS OF INTENSIVE INVESTIGATION AND UTILIZED IN
VARIOUS STUDIES [8]. IN A STUDY DONE BY IRESON ET AL. A GUANOSINE-RICH APETER, AS1411, IS
APPLIED IN CLINICAL TRIALS AS A MOLECULE THAT CAN BE INTERNALIZED AND BIND NUCLEOLIN IN THE TARGET
CELL. THIS IN TURN INHIBITS CELL PROLIFERATION BY INTERFERING WITH THE PATHWAYS IN THE CELL [9]. THE
most significant example is of an aptamer that is already on the market as a drug, pegaptanib which is used to treat macular degeneration [3].

A study done by Mehan et al. introduces another facet of DNA aptamers, where they utilize SOMAmers: DNA aptamers with chemically modified nucleotides that bind to proteins. This group was able to discover DNA aptamers for more than 1,000 proteins by making use of the SELEX method. This discovery provides a useful assay for determining biomarkers and an opportunity for other therapeutic uses [7]. The use of SELEX and DNA aptamers with recognizing different peptides have been evidenced to be successful in this article, and can be applied to diagnostic tests to discover disease biomarkers a use that echoes the focus of this project where a DNA aptamer binding prostate cancer biomarkers is being discovered by in-vitro selection.

In a study done by Rajendran et al. the SELEX in-vitro selection was used to select fluorescent aptamers that indicate the presence of zinc. This study selected for an aptamer that binds zinc and thus causes an increase in fluorescence due to the conformational change in the aptamer which allows it to elute from the quencher that is bound on the column. The participants in this study altered their SELEX procedure to increase selectivity. The group added various metal ions such as cobalt, nickel, and manganese before eluting with zinc. This improved aptamer specificity to the zinc ion. Also the target ion concentration was decreased in order to select for aptamers with higher affinity toward zinc [6]. Similar methods can be planned and utilized in this procedure to improve selectivity.
Steroids were target molecules in another study by Stojanovic et al. In this study DNA aptamers that targeted steroids were selected by use of a streptavidin-agarose column. A bio-capture oligonucleotide molecule is covalently attached to the column where this bio-capture had previously been hybridized to the DNA oligonucleotide library that will be used for selection. As the target molecules are added to the column, a conformational change causes those oligonucleotides to detach from the bio-capture molecule and elute from the column [5]. This method is advantageous over alternative classical selection techniques as it avoids the step of covalent attachment of a target molecule to the resin. The methods that were used in this study reflect some of the main steps in the aforementioned procedure but with alterations to certain steps. The three target peptides in discussion are proteoforms of the prostate-specific antigen. Prostate cancer is the most common cancer that causes death in men over the age of 75. Diagnosis of prostate cancer is better if discovered earlier, but most importantly the stage of the cancer is crucial. It is likely that if not detected, prostate cancer can metastasize and spread throughout the body. Currently prostate-specific antigen or PSA is a biomarker used to help diagnose prostate cancer. The group that this study is collaborating with is currently studying the cleaved forms of the prostate specific antigen. This study aims to discover DNA aptamers that have higher specificity toward PSA2 than PSA1 and PSA3. Thus by using the SELEX in-vitro selection method, this study plans to select for DNA aptamers that bind to the target molecule PSA2.
METHODS

In the SELEX method, DNA aptamers are selected through several rounds of in-vitro selection using a streptavidin-agarose column and polymerase chain reaction, where later on these aptamers can be sequenced, further studied, and utilized for recognition of the presence of cancer biomarkers in biological specimens, in this case specifically for prostate cancer.

The following buffers will be used SELEX buffer: 20 mM Tris pH 7.4, 1 M NaCl, 10 mM MgCl$_2$, washing buffer: 20 mM Tris pH 7.4, 1 M NaCl (no MgCl$_2$), and 2X SELEX reaction buffer: 40 mM Tris pH 7.4, 2 M NaCl, 20 mM MgCl$_2$. The target molecules of this in-vitro selection are short peptide sequences found to be specific biomarkers for prostate cancer. The sequences of these peptides are as follows PSA1: LSEPAELTDAVK, PSA2: HSQPWQVLVASR, and PSA3: IVGGWECEK [12].

The SELEX procedure will be adapted from the earlier published protocol. (Stojanovic) The grand scheme of the in-vitro selection conducted in this study is represented in Figure 1.
Figure 1. Overall methods scheme of in-vitro selection.
1: The DNA library that is hybridized to the bio-capture strand is added to a streptavidin-agarose column, and the biotin on the bio-capture strand attaches to the column. 2: The target peptides are added to the column and cause a conformational change in some of DNA library strands, which causes the release of those specific strands from the column. 3: The oligonucleotides that were collected from the column are then PCR amplified. 4: The in-vitro selection procedure is repeated using the eluted aptamers that were collected and replicated.

Briefly, the first in-vitro selection is conducted as follows. Initially an oligonucleotide mixture is prepared containing 0.1 nmol of the random library and 0.5 nmol of bio-capture prepared in 250 uL SELEX buffer.

Random library: 5'-GTGCACAGTCTTACCGGGCTCTCGGGACGAC (N30)
GTCGTCATAGTGAGTCGTATTAGAATTTCGTC- 3'

Randomized Single Stranded Fragment

Streptavidin Carrying Agarose

Streptavidin Beads

Strand Separation (0.2 M NaOH)

Add:
3 Peptide Biomarkers: PSA1, PSA2, PSA3

Randomized Single Strand undergoes conformational change and elutes from column

PCR Products added to streptavidin column

PCR
Bio-capture: 5’-GTCGTCCCGAGAGCC-bio

The random library and bio-capture mixture is then heated at 95°C for 5 minutes to allow for annealing of the random library to the bio-capture. A biotinylated column is prepared using 250 μL streptavidin agarose resin in a microbiospin chromatography column. The column is washed five times with SELEX reaction buffer. The oligonucleotide mixture is then added to the column, the elution is collected and added to the column again two more times. This ensures that the bio-capture binds to the column well. At this point in time the column resembles the complex shown in Figure 2. The column is then washed ten times with SELEX reaction buffer. The peptide mixture is then prepared, this mixture includes all of the target peptides in the SELEX reaction buffer. For the first round of selection, two out of the three peptides are included in order to narrow the pool of random oligonucleotide library. The 1 μM peptide solution (250 μL) was added to the column three times, and collected in three separate elution samples. During the first 7 rounds the samples were combined and concentrated to 50 μL by centrifugal filtration. At round 8 the samples were used for obtaining the elution profile which will be discussed further on.
Polymerase chain reaction is used to amplify the collected elution products. The cycles of PCR are set as follows: 1. One cycle at 95°C for two minutes 2. One cycle at 95°C for 15 s, one cycle at 57°C for 30 s, one cycle at 68°C for 45 s 3. Final cycle at 68°C for 2 min. The second step of the PCR is set to continue for 15 cycles.
PCR primers:

Forward primer: 5’-GTGCACAGTCTTACCG-3’

Reverse primer: 5’/-5Biosg/ GACGAATTCTAATACGACTCACTAT-3’

In order to isolate the single stranded DNA library for the next round of in vitro selection the reverse primer is biotinylated. The biotinylated PCR amplicon was then bound to streptavidin-modified beads followed by elution of the unbiotinylated DNA library in the binding buffer: 20mM Tris, pH 7.4, 1M NaCl to remove Mg^{2+}. The streptavidin-agarose (0.2mL) column is washed with strand separation buffer (0.2mL of 0.2M NaOH) and the single stranded DNA library is collected, neutralized, and ethanol precipitated. This collected sample is then used for the next round of in-vitro selection after solubilizing the precipitated DNA in reaction buffer.

A further step to confirm the specificity and maturity of the selected DNA aptamer library is a fluorescence assay. For this assay a different bio-capture, one which has a quencher attached to it, will be annealed to the DNA aptamers that have a fluorescent molecule attached to the 5’ end of the forward primer of the aptamer. Figure 3 displays a model representing the format of this fluorescence assay, used to confirm aptamer selectivity, on a molecular level.
In this portion of the experiment the same steps were taken for the PCR amplification, capture, and precipitation of the aptamers. However, in this assay a FAM labeled forward primer was utilized in the preparative PCR before capture and precipitation. Subsequently, the aptamers were precipitated and dissolved in 40 µL of reaction buffer. The concentration of DNA was measured and a dilution was made to 10 nM of DNA aptamers in 50 µL. Following this, the bio-capture sequence that now has a quencher attached to it was added to the 50 µL of DNA and heated at about 95° C for 5 min. After the bio-capture with the quencher was annealed to the...
DNA aptamer library with the FAM molecule the fluorescent assay was conducted and the results were read with a fluorometer at 517 nm. First a 100 nM concentration of non-specific PSA3 was mixed in solution and the fluorescence was measured followed by the target peptide PSA2 with the same 100 nM concentration. The results were graphed in Figure 12 for further analysis.

The cloning and sequencing that followed the confirmation of the maturation of the aptamer library started with the amplification of the final eluate from round 19. This eluate did not contain any biotinylated aptamers, and the DNA aptamers in this final eluate were amplified with a non-biotinylated reverse primer. Following this amplification the concentration of the amplified aptamers was calculated via the absorbance measured by a UV-visual spectrophotometer at 260 nm. In response to the concentration that was observed, a 3:1 ratio and also a 6:1 ratio of the target DNA insert was utilized in the ligation mixtures.

During the ligation step of cloning, the following mixtures were prepared and set overnight to allow for maximum number of ligated aptamers. The standard reaction 1 and 2, positive control, and background control consisted of 5 µL of Rapid Ligation Buffer, 1 µL of the pGEM®-T Easy Vector, and 1 µL of T4 DNA Ligase. In addition to these components, the positive control consisted of 2 µL of the Control Insert DNA and Standard Reactions 1 and 2 consisted of a 3:1 and a 6:1 ratio of insert to vector respectively. These components were mixed as individual ligation reactions and set for incubation at 4°C overnight.

For the transformation step, JM109 High Efficiency Competent cells were utilized in order to ensure high transformation efficiency. Lysogeny Broth Lennox agar was utilized for
growth of cells, of which 8 g was used in order to make 250 mL of agar. The agar was prepared and autoclaved for 15 min at 121°C. Following sterilization, the agar was cooled and ampicillin was added to a concentration of 100μg/ml, IPTG was added to a final concentration of 0.5mM, and X-Gal was added to a final concentration of 80μg/ml. For 2 μL of each ligation reaction that was prepared, 50 μL of JM109 High Efficiency Competent cells was added mixed and placed on ice for 20 min. Along with these samples an extra transformation efficiency control was added with 0.1 ng of uncut plasmid in 100 μL of competent cells. After all samples including the transformation efficiency sample were placed on ice for 20 min, the cells were heat shocked for 50 seconds in a 42°C water bath and then placed back on ice for 2 min. Subsequently 950 μL of SOC medium was added to these tubes, and 900 μL of SOC medium was added to the transformation efficiency tube. All five tubes were then incubated at 37°C for 1.5 hours with 300 rpm shaking. After incubation 100 μL from each tube was plated onto an agar plate containing ampicillin, IPTG, and X-Gal, each sample was plated twice and incubated overnight at 37°C.
RESULTS

In order to obtain DNA aptamers with specificity toward target peptide PSA2, modification of the original SELEX protocol is required in order to increase selection pressure on the aptamer library. Reducing the concentration of the target peptide from 20 µM to 0.2 nM created conditions for isolation of DNA aptamers that will bind to the target peptide with higher specificity.

The initial round of SELEX starts with a 20 µM concentration of peptides. In this case, this study begins with 20 µM of PSA1 and PSA2 which have amino acid sequences of LSEPAELTDAVK and HSQPWQVLSVR. The first step will be the application of an initial pressure on the original random sequence library whose nucleotide sequence is as follows:

5’-GTG CAC AGT CTT ACC GGG CTC TCG GGA CGA C (N30) GTC GTC CCA TAG TGA GTC GTA TTA GAA TTC GTC 3’. The N30 stands for a randomized sequence of 30 nucleotides, a region where the peptide will bind and trigger a conformational change to elute DNA aptamers from the streptavidin agarose column. For each fraction a 100 s time interval was provided, giving a total elution time of 5 min. This time interval seems to be sufficient in that it did allow a fair quantity of aptamers to elute from the column. Since the biotin of the bio-capture is not covalently bound to the agarose beads, after a period of time the aptamers will eventually dissociate from the column. In this case, the main focus is to evolve and optimize specificity of DNA aptamers binding to a target peptide and the concentration of the target peptide. It is important to note that there is no universal protocol for modifying the peptide concentration and SELEX procedure in accordance to what is observed after each round of selection. Thus each
The initial products from the first round may not show significant amplification, and the number of aptamers that will elute from the column is not certain. Thus the PCR conditions following the initial round of SELEX were chosen according to a previous publication by Stojanovic et. al, and our preliminary results. The analysis of the 15 cycle PCR products after the 1st round of selection are in Figure 3.

Figure 4. Native gel analysis of PCR products after 1st round of in vitro selection
M1- 25 Base pair marker; NC- Negative Control; PC- Positive Control; Sa1- 1µL of sample in 25µL of PCR mixture; Sa2- 0.5µL of sample in 25µL of PCR mixture; Sa3- 0.2µL of sample in 25 µL of PCR mixture.
The above figure indicates over-amplification in all three sample lanes. Lanes Sa1, Sa2, and Sa3 contain the PCR products for 1 µL, 0.5 µL, and 0.2 µL of the eluted aptamer sample in 25 µL of PCR reaction mixture respectively. The DNA bands that migrated slower than the 100 base pair marker in the sample lane are considered to be over-amplification products due to a high concentration of DNA. In this situation, instead of limiting the variety of DNA aptamers by performing a PCR, the eluted products were directly used for the next round of SELEX.

In round two of SELEX we decreased the concentration of target peptides from 20 µM to 4 µM. This decrease in peptide concentration subsequently lowered the amount of DNA aptamers eluting from the column. The analysis of the eluted products after a fifteen cycle PCR demonstrates a reduced amount of DNA recovery (Figure 4).
As Figure 5 suggests, the amount of aptamers that have eluted decreased. There are no detectable over-amplification products, and there are no bands that appear in lanes Sa2 or Sa3. This indicates that, in consequence of the decrease in concentration of peptides, less of the aptamers eluted from the column. After decreasing the concentration of peptides the aptamers that still bind and elute from the column are aptamers that bind with higher specificity. By applying pressure on the developing library and amplifying aptamers that elute from the column, each
round of SELEX is subsequently selecting for the DNA aptamers with sequences that bind to the peptide of interest with high specificity. Since the second round of SELEX produced fewer products, in the next round, the same pressure was applied again. The following gel analysis was observed after a 15 cycle PCR.

Figure 6. Native gel analysis of PCR products after 3rd round of in vitro selection
This image is a representation of the PCR products from what eluted in round 3 of SELEX selection. The eluted aptamers were amplified with a 15 cycle PCR. M1- 25 bp marker; NC- Negative Control; PC- Positive Control; Sa1-1µL of sample in 25µL of PCR mixture; Sa2- 0.5µL of sample in 25µL of PCR mixture; Sa3- 0.2µL of sample in 25 µL of PCR mixture.

When compared to the previous round the third round of SELEX selection reveals clearly visible product bands in all three sample lanes, and also over-amplification in the sample lane
with 1 µL of sample. The over-amplification and reappearance of the bands in lanes Sa2 and Sa3 indicates the development of the DNA aptamer library. This image suggests the increase in the number of aptamers that bind to the target peptides. The aptamers bound on the column respond with higher sensitivity and elute more than the previous round. This continued even in the following round when the concentration of peptide eluent was decreased to 1 µM.

Figure 7. Native gel analysis of PCR products after 4th round of in vitro selection
This image is a representation of the PCR products from what eluted in round 4 of SELEX selection. The eluted aptamers were amplified with a 15 cycle PCR.

As can be seen in Figure 7 there is over-amplification in all three sample lanes. The over-amplification indicates a higher concentration of DNA aptamers eluting from the column even
after further pressure is applied on the developed library. This indicates that the peptides can
further be split in order to specify aptamer affinity toward an individual peptide instead of two
peptides. Only one pressure factor is introduced to each round of the SELEX selection process,
this allows a steadier method of selection in which the effect of each pressure can be observed in
subsequent gel analysis of PCR products. It was decided that PSA2 would be the focus of this
study, and that DNA aptamers specific for PSA2 would be collected. Thus in the next round of
SELEX, first the column was washed with 1 µM of PSA1, then washed ten times with reaction
buffer followed by the elution of DNA aptamers with 1 µM of PSA2. Figure 7 consists of the gel
analysis that was obtained after separating the peptides, for this analysis only the eluted aptamers
specific toward PSA2 were collected and PCR amplified for the next round.
As can be seen from this image, washing with PSA1 has produced a higher concentration of aptamers and subsequently more over-amplification in lanes Sa11 through Sa13. Since over-amplification was also observed in the PSA2 lanes, it was decided that the concentration of PSA2 be decreased to 0.2 µM whereas the PSA1 concentration would remain at 1 µM. The over-amplification indicates that the DNA aptamers have been well developed, in that the aptamers with higher specificity are eluting from the column and being amplified again thus increasing the concentration of DNA aptamers that elute from the column in response to the peptides each round.
In round 6 of the selection process, 0.2 μM of PSA2 was utilized in order to elute after a previous wash had been conducted with PSA1. When the concentration was decreased, the gel analysis of products would reflect with less over-amplification or lower band intensity. In round 7, the concentration of PSA1 remained at 1 μM since it is not the target peptide and the concentration of PSA2 decreased to 0.1 μM. This round was followed by an eighth round where an elution profile was developed by collecting the individual washes and eluates. Each 250 μL fraction was collected and 5 μL of each was PCR amplified in a total of 25 μL of PCR reaction mixture.
Figure 9. Native gel analysis of PCR products after 8th round of in vitro selection. SELEX round 8: each sample lane consists of PCR products where 5 µL of sample was added to 25 µL of PCR reaction mixture. Due to the limit of the number of lanes, only the negative control was added. The positive control was later confirmed on a separate gel. W19 and W110 are the last two of ten washes after the aptamer library is loaded onto the column. The PS1 and PS2 lanes are in the order of which the fractions were eluted. Lanes W21-W210 are the washes previous to PS2, and W31 and W32 are the after-washes.

Although the PSA1 lanes show that DNA eluted, it must be noted that the concentration of PSA1 was 1 µM whereas the concentration of PSA2, was only 0.1 µM. Even at these concentrations, PSA2 eluted DNA aptamers in all three lanes whereas PSA1 only eluted in two lanes. This indicates that the specificity of the DNA aptamers is surely developing, but this must be confirmed with another elution profile. Two more rounds were conducted, SELEX 9 and 10,
where in round 9 another elution profile was performed and in round 10 a regular analysis was performed. The elution profile in round 9, where the PSA1 concentration was 1 µM and PSA2 was 0.1 µM, showed some improvement however the bands in both PSA1 and PSA2 lanes seem to have eluted similar amounts. In the analysis of round 10, PSA2 eluate showed over-amplification in the Sa1, 1 µL in 25 µL PCR mixture lane. In this case the higher concentration of eluate in the PSA1 lanes may be due to non-specific binding of the aptamers to for example the peptide bonds of the target molecule instead of a more specific binding. Thus in order to observe whether this type of non-specific binding existed, the third peptide was also added to the elution profile. In Figure 10 the elution profile including PSA3 as an eluent is observed.
Figure 10. Native gel analysis of PCR products after 11th round of in vitro selection
For this round 1 µM of PSA1 and 1µM of PSA3 were first used as washes, followed by elution with PSA2. For PCR, 5 µL from each 250 µL sample fraction was drawn in order to conduct PCR.

As the gel analysis suggests, there seems to be some non-specific binding, thus subsequent rounds were conducted in order to remove aptamers with non-specific binding. After a number of rounds, the following elution profile was obtained.
Figure 11. Native gel analysis of PCR products after 19th round of in vitro selection
For this elution profile, 2 µL of each 250 µL sample fraction was drawn for each 25 µL PCR mixture. PSA1, PSA3, and PSA2 were all separately eluted and each was at a concentration of 0.2 nM. A 15 cycle PCR was conducted to obtain the products seen on the gel.

From the analysis of this final round and elution profile, it can be seen that the eluted product bands in the PSA2 lanes show about three times more intensity in all three lanes when compared to PSA1 and PSA3. Also, the non-specific binding has also decreased significantly where now there is only one clearly visible band in the PSA3 sample lanes. Although there is a light band in the W23 and W34 wash lanes, this is due to some excess peptide in the column and
this can be remedied by increasing the number of washes. Doing so can also decrease the amount of aptamers eluting in the non-target peptide washes in further rounds.

These rounds were followed up by a fluorescence assay using the first method where the quencher is attached to the bio-capture and the fluorescent molecule FAM is attached to the primer sequence on the aptamers. With this method, the resulting readings did not show any change when the peptides were added to solution. All of the readings were around 12 AU, this includes the control with no peptides along with the negative control containing PSA3 and the sample containing PSA2. This lack of change is primarily due to the preliminary nature of this fluorescence assay. The fluorescence molecule is too far from the quencher and thus the bio-capture cannot properly quench the fluorescence molecule on the aptamer. In order obtain a better idea of aptamer specificity, the bio-capture with the quencher was eliminated and just the DNA aptamers with the FAM molecule attached to them was added to the sample. Nucleotides act as weak quenchers, and since the aptamers hybridize to themselves in the presence of the target peptide, this will cause a decrease in the signal of fluorescence after the target peptide is added. The results for this method were as follows.
The result for Control represents the fluorescent reading for DNA aptamers bound with FAM in reaction buffer. PSA3-Negative Control result represents the PSA3 and the FAM labeled aptamers in reaction buffer. The PSA2 result represents PSA2, PSA3 and the FAM labeled DNA aptamers in reaction buffer. The units are arbitrary units.

Following this fluorescence assay, ligation of the DNA aptamers into plasmid vectors followed by their transformation into JM 109 High Efficiency Competent cells was conducted in order to obtain individual aptamers for sequencing. Figure 13 through Figure 17 display the results obtained following overnight incubation of the plates.
Figure 13. Ligated and Transformed Sample Plate 11
The plate in this figure is of the sample with a 3:1 target insert to vector ratio.

Figure 14. Ligated and Transformed Sample Plate 21
This plate is the sample which has a 6:1 target insert to vector ratio.
Figure 15. Ligation and Transformation Postive Control
This control contains the control insert.

Figure 16. Ligation and Transformation Background Control
This plate contains no insert, but it did undergo the same conditions as the sample and positive control during ligation and transformation.
Figure 17. Transformation Efficiency
This plate contains colonies that have the uncut plasmid that only underwent transformation.

The results obtained from these plates are discussed in the Discussion section that follows.
DISCUSSION

The final elution profile suggests that a DNA library that has high specificity toward the target peptide PSA2 has been obtained. At this point further analysis of the library can be conducted by cloning the library and sequencing the DNA aptamers. Sequencing the aptamers will reveal several sequences of DNA. These sequences are normally grouped into families because they are generally similar in sequence and can also be ranked based on specificity. These are further studies that can be conducted on the developed library. It can also be noted that the steps to reach the final developed aptamer library does not have to be quite so gradual. Making decreases in peptide concentration by five times still yielded a good amount of eluted aptamers. Although in a side study it was noted that introducing non-specific peptides too early in the process may decrease the yield of eluted aptamers by a significant amount, and may even lead to situation where there are no aptamers eluting to the target peptide at all. In this study, the pressures applied on the original random DNA library, with a random sequence of 30 nucleotides, yielded an elution profile which indicated that DNA aptamers which bind to the target peptide PSA2 with higher specificity than PSA1 or PSA3 were successfully obtained. In the results of the fluorescence assay, as Figure 11 shows, there is a notable decrease in fluorescence after the addition of PSA2. Unlike PSA3 which showed no significant change, the fluorescence of PSA2 decreased by approximately 15% which suggests that there are aptamers which bind selectively to PSA2. As was discussed in the results section, the format of this fluorescence assay was only preliminary and can be modified so that the signal that results in response to the target peptide PSA2 becomes more distinguishable. Also, the format and method of this fluorescence assay can be further optimized and utilized as a sensor.
Preliminary results for the cloning and sequencing show some white colonies that can be used further on, as seen in Figure 13 and Figure 14. However these white colonies are also present in the background control as can be seen in Figure 16. The white colonies indicate that there is an insert in the plasmid vector which interrupted the sequence that codes for the enzyme which would normally produce the blue color of the colony. Since this is seen in both the sample and the background it may be that two plasmids fused to each other during the overnight ligation period, and this may be what is causing white colonies to appear in the background control. However there is one significant difference between the background control and the sample plates, there are hybrid colonies that are white and blue in only the sample plate. These colonies are present in both sample plates but are more evident in Figure 14, the plate where a higher concentration of insert was used during ligation. Thus these colonies can be those that potentially consist of plasmids which have the target insert. Upon conclusion of this, both the hybrid and the white colonies in the sample plates will be picked up and the plasmids in them will be further purified. Later on, PCR will be conducted on the plasmids that are purified in order to observe whether the target insert is present. If this target insert is present, it will further on be sent off for sequencing. By performing this plasmid purification and PCR on several colonies, several individual aptamer sequences will be obtained for further analysis.
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