Selection of aptamers for a non-DNA binding protein in the context of cell lysate

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ABSTRACT

Aptamer-facilitated Protein Isolation from Cells (AptaPIC) is a recently introduced method that allows, in particular, generation of aptamers for a protein target in a context of a crude cell lysate. The approach enables efficient, tag-free, affinity purification of target proteins which are not available in a pure form *a priori*, and for which no affinity ligands are available. In the proof-of-principle work, AptaPIC was used to develop aptamers for and purify MutS, a DNA mismatch repair protein. The DNA-binding nature of MutS raised concerns that AptaPIC was not a generic technique and could be inapplicable to protein targets that do not possess native nucleic acid-binding properties. Here we prove that these concerns are invalid. We used AptaPIC to generate pools of aptamers for human Platelet-Derived Growth Factor chain B (PDGF-B) protein, a non-DNA binding protein, in the context of a bacterial cell lysate, and subsequently purify it from the same lysate. Within a small number of rounds, the efficiencies of aptamer selection were similar in conventional Systematic Evolution of Ligands by Exponential Enrichment (SELEX) for pure protein and in AptaPIC for protein in the cell lysate. The conventional selection approach resulted in an aptamer pool with an EC_{50} value of $2.0 \pm 0.1\,\mu$M, while the AptaPIC selection approach resulted in a pool with an EC_{50} value of $3.9 \pm 0.4\,\mu$M. Our results clearly demonstrate that selection of aptamers for proteins in the cell lysate is not only realistic but also efficient.

1. Introduction

Affinity purification remains the most efficient protein isolation approach, but it is often hindered by a lack of target protein-specific affinity ligands. While genetically fused affinity tags address this issue, their use might not always be favored; in some cases they can introduce problems, such as changes in target protein conformation, decreased protein expression levels, and toxicity to host cells [1]. Moreover, it is often difficult to efficiently cleave off an affinity tag without affecting the target protein. While the use of target-specific affinity ligands, such as antibodies or aptamers, is preferential, their development requires the target protein in a pure form. This requirement creates a problem for newly discovered proteins, as the use of multiple non-specific purification steps becomes necessary, before a specific purification approach can be developed. In the process of characterization of novel proteins, such detours may become lengthy and hindering. Recently, we have introduced a technique that allows the development of affinity ligands to a non-purified target protein. Aptamer-facilitated Protein Isolation from Cells (AptaPIC) is an integrated set of methods that allows for: (i) the development of an aptamer pool for a single protein target in the context of a crude cell lysate, and (ii) the subsequent use of the developed aptamer pool in affinity purification of the target protein [2]. This approach was designed to facilitate affinity purification of over-expressed recombinant proteins for which affinity ligands are not available beforehand.

Aptamers are DNA or RNA-based affinity ligands that display strong binding capabilities and specificities towards their molecular targets [3,4]. DNA aptamers are obtained *in vitro* from diverse combinatorial libraries of DNA molecules through a procedure of Systematic Evolution of Ligands by Exponential Enrichment (SELEX) [5]. Briefly, the molecular target of aptamer selection is first incubated with a naive DNA library (a pool of DNA molecules of random individual nucleotide sequences) to allow for a chemical equilibrium to be established; this is followed by a separation of target-bound DNA molecules from those that remain unbound; DNA molecules that display affinity to the target are then isolated and amplified by PCR; this procedure is repeated in a number of rounds to obtain an aptamer-enriched DNA pool. Aptamers are often compared to antibodies for their ability to bind a wide range of targets with high affinity and specificity. Due to a number of significant advantages [6], DNA aptamers have been successfully employed in applications that would be difficult, if not impossible,

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to perform with antibodies. Some examples of such applications include the development of aptamers with pre-determined binding parameters [7] and selection of aptamers to complex targets, such as whole live cells [8,9]. AptaPIC is an additional example of such an application, and it takes advantage of the versatility of the aptamer-generation process in order to develop aptamers to a specific target in the context of a complex mixture.

To make the generation of aptamers possible in the context of a cell lysate, AptaPIC integrates methods that reduce protease- and nuclease-facilitated degradation of the target protein and the DNA ligands, respectively; reduce the negative effects of DNA-binding proteins on the efficiency of aptamer generation; and increase specificity of aptamer generation to the target protein of choice [2]. The latter is achieved by carrying out the aptamer selection in two steps – the positive and negative selections. In positive selection, the DNA library is exposed to the target-containing cell lysate (e.g. the lysate of cells in which the expression of the recombinant gene has been induced) and the bound fraction of DNA is collected and retained for further processing. This step results in the enrichment of the DNA pool with aptamers for both the target protein and for the non-target components of the cell lysate. To eliminate these non-target-specific aptamers, the step of negative selection is then introduced. In this step, the enriched DNA pool obtained in the positive aptamer selection step is exposed to the target-free lysate (e.g. the lysate of the cells in which expression of the recombinant protein is not induced), but in contrast to the positive selection step, the non-bound fraction of DNA is collected. Interestingly, we have discovered that if the target protein is present in the cell lysate at the levels of modest to high over-expression, the target-specific aptamers could be obtained without having to resort to the negative selection steps. While this option may not be applicable for some protein targets, it provides an opportunity to significantly reduce the time required for the procedure with suitable targets. Similarly to the conventional SELEX, the collected DNA fractions are PCR-amplified between each of the aptamer selection rounds, and such rounds are repeated until the aptamer-enriched DNA pool displays the desired affinity and specificity characteristics. Once such target-specific DNA pool is obtained, it can be used in affinity purification of the target protein form the cell lysate. In analogy with polyclonal antibodies, AptaPIC uses a pool of aptamers, rather than individual aptamers, to facilitate the purification in shortest possible time.

AptaPIC utilizes capillary electrophoresis (CE) as the partitioning method for DNA sequences with and without affinity to the target protein. Due to its high resolving power, the use of CE significantly decreases the time of aptamer generation while maintaining its stringency [10,11]. CE can also be used to make quantitative measurements of equilibrium and rate constants, allowing for convenient affinity characterizations of obtained aptamer-enriched pools and individual aptamers. To characterize affinity of resultant aptamer pools we have used Non-equilibrium Capillary Electrophoresis of Equilibrium Mixtures (NECEEM) mode of CE, as described elsewhere [12]. In addition, the availability of various modifications of the CE method, such as gel capillary electrophoresis (Gel CE), make it possible to perform other measurements, such as estimations of an extent of DNA degradation, using the same instrument [13].

In our previous report, we have described the development of the AptaPIC technique using a model system, in which MutS protein from *T. aquaticus* was used as the molecular target present in a crude *E. coli* cell lysate. Using this model system, we have shown that high affinity and specificity aptamers can be obtained for MutS protein in the context of the cell lysate, and subsequently used in affinity purification of the target protein from cell lysate. MutS is a DNA-binding protein that plays a key role in DNA mismatch repair pathways [14]. After the publication of the initial results, the nature of the chosen model has raised some concerns about

the versatility of AptaPIC: it has been suggested that the technique would not be applicable to protein targets that do not possess inherent DNA-binding properties. It seems that there is a persisting notion within the community that DNA-binding proteins are more prone to aptamer generation than those that do not display such properties. While a detailed literature review, as reported in the supporting information for the original article, did not yield any experimental support to this notion, we felt that it was still important to address these concerns and to test whether the developed aptamer selection approach is effective with a non-DNA binding protein as the target. In this work we demonstrate that AptaPIC technique can indeed be used to generate aptamers for a target protein that does not have intrinsic DNA-binding characteristics.

We have used human Platelet-derived Growth Factor chain B (PDGF-B), supplemented into *E. coli* cell lysate at the level of 10% of total lysate protein, as the target for aptamer development. PDGF-B is an extracellular signaling molecule that functions by binding cell surface receptors [15]. No inherent nucleic acid-binding properties were reported for the protein in the two decades since its initial characterization. Aptamers have been selected for purified PDGF-B in the past [16]. The choice of this target for the study, thus, ensured that if negative results were observed, it was due to an inherent flaw in the method, and not due to the nature of the target protein. Using the AptaPIC procedure, we were able to obtain an aptamer-enriched DNA pool with high specificity for PDGF-B. To assess the efficiency of aptamer generation through the AptaPIC technique, the generated aptamer pools were compared to those generated through the conventional selection approach, in which purified PDGF-B served as the selection target. The aptamer-enriched DNA pools obtained through AptaPIC technique were comparable in affinity and specificity to aptamer-enriched pools obtained through the conventional approach.

2. Experimental

2.1. Chemicals and materials

Human mature peptide of Platelet Derived Growth Factor B isoform (PDGF-B) was purchased from R&D BioSystems (NJ, USA). Fused-silica capillaries were purchased from Polymicro (Phoenix, AZ). All solutions were prepared using the Milli-Q-quality deionized water and filtered through a 0.22 μm filter (Millipore, Nepean, ON, Canada). Recombinant *Taq* DNA polymerase, buffer components and all other chemicals were from Sigma–Aldrich (Oakville, ON, Canada) unless otherwise stated.

The naive DNA library contained a central randomized sequence of 40 nucleotides (nt) flanked by 20-nt primer hybridization sites (5′-/FAM/-CTC CTC TGA CTG TAA CCA CG-(N)$_{40}$-GCA TAG GTA GTC CAG AAG CC-3′). A 6-carboxyfluorescein-labeled 5′-primer (5′-/FAM/-CTC CTC TGA CTG TAA CCA CG-3′) and a biotinylated 3′-primer (5′-/Bio/-GGC TTC TGG ACT ACC TAT GC-3′) were used in PCR reactions for the synthesis of double-labeled double-stranded DNA molecules. A biotinylated 5′-primer (5′-/Bio/-CTC CTC TGA CTG TAA CCA CG-3′) and non-labeled 3′-primer (5′-GGC TTC TGG ACT ACC TAT GC-3′) were used to generate aptamer pools for the aptamer-facilitated protein purification experiments. The DNA library, PCR primers, and single stranded masking DNA (5′-CAA AAA ATG AGT CAT CCG GA-3′) were custom-synthesized by Integrated DNA Technologies (Coraville, IA).

2.2. Preparation of cell lysate

E. coli BL21(DE3) were grown at 37 °C to a density corresponding to an absorbance of 1.5–1.6 at 600 nm measured in a cuvette with an optical path-length of 1 cm. Cells were harvested by centrifugation

at $5000 \times g$ for 10 min at 4 °C. Pelleted cells were resuspended in the sonication buffer: 50 mM Tris–HCl, 2.5 mM MgCl$_2$, 5 mM KCl at pH 8.3 containing the protease inhibitors (PI) cocktail as instructed by the manufacturer (Sigma Aldrich, Oakville, ON, Canada). Bacterial lysates were prepared by sonication on ice with 5 second "on"/15 s "off" intervals for a total of 10 min. Cell debris was pelleted by centrifugation at $15,000 \times g$ for 20 min at 4 °C and removed. Cell lysates were aliquoted and stored at −80 °C. The concentration of "total" protein in the cell lysate was measured by bicinchoninic acid (BCA) assay, using bovine serum albumin as a standard.

2.3. NECEEM

All NECEEM procedures [11] were performed using the following instrumental setup. CE was carried out with a P/ACE MDQ apparatus (Beckman Coulter, Mississauga, ON, Canada) equipped with a fluorescence detector; a 488-nm line of continuous Wave Solid-State laser (JDSU, Santa Rosa, CA) was utilized to excite fluorescence. Uncoated fused-silica capillaries with an inner diameter of 75 μm and outer diameter of 360 μm were used. For the aptamer selection, an 80-cm-long (70 cm to the detection window) capillary was utilized. NECEEM-facilitated analysis of enriched DNA libraries was carried out in a 50-cm-long (40 cm to the detection window) capillary.

Both the inlet and the outlet reservoirs contained the electrophoresis run buffer (50 mM Tris–acetate at pH 8.3). The samples were injected into the capillary, pre-filled with the run buffer, by a pressure pulse of 16 s at 1 psi (13.4 kPa) and 5 s at 0.5 psi (3.4 kPa) for 80-cm-long and 50-cm-long capillaries, respectively. The length and the volume of the corresponding sample plugs were 23 mm and 101 nL for 80-cm-long capillary and 6 mm and 26 nL for 50-cm-long capillary, respectively. Electrophoresis was carried out with a positive electrode at the injection end of the capillary; the direction of the electroosmotic flow was from the inlet to the outlet reservoir. Separation in the 80-cm-long capillary was carried out by an electric field of 250 V cm^{-1}; and in the 50-cm-long capillary at 400 V cm^{-1}. The temperature of the capillary during the separation was maintained at 15 °C. When needed, fractions were collected in an automated mode by replacing the regular outlet reservoir with a fraction collection vial containing 5 μL of deionized water.

The capillary was rinsed with the run buffer solution for 2 min prior to each run. At the end of each run, the capillary was rinsed with 100 mM HCl for 2 min and 100 mM NaOH for 2 min, followed by a rinse with deionized water for 2 min. After the fraction collection step, the capillary was rinsed with RNase-away solution for 1 min to remove any remnants of DNA from the capillary.

2.4. Confirmation of the nature of protein–DNA complex

In order to confirm the identity of the peak suspected to represent the complex of PDGF-B with DNA, enriched DNA library obtained after three rounds of aptamer enrichment against pure PDGF-B was titrated with the following concentrations of PDGF-B: 0, 0.3, 0.5, 0.8, and 1.6 μM. The prepared mixtures containing the variable amounts of the protein and 100 nM of the DNA were incubated for 20 min at 25 °C and analyzed by NECEEM using 50 mM Tris–acetate pH 8.3 as the run buffer.

2.5. Selection of aptamers for PDGF-B in cell lysate

The equilibrium mixture for NECEEM-based selection of aptamers was prepared in the incubation buffer (50 mM Tris–HCl, 2.5 mM MgCl$_2$, 5 mM KCl, and 5 mM EGTA at pH 8.3) using the following two-step procedure. First, a DNA library (a 100 μM solution in the selection buffer) was denatured by heating at 99 °C for 5 min and immediately transferred on ice. Second, 10 μM naive

DNA library was mixed with 40 μM single stranded masking DNA, 100 nM fluorescein, E. coli cell lysate (containing 235 μg mL^{-1} of the total protein) and 0.5 μM of PDFG-B. The addition of the masking DNA plays a dual role: to decrease the nuclease-facilitated degradation of DNA aptamers and to decrease the effects of non-specific binding of the components of the cell lysate to DNA. Finally, the mixture was incubated at 25 °C for 20 min to obtain the equilibrium mixture.

A 23-mm-long (101 nL) plug of the equilibrium mixture was injected into the capillary pre-filled with the run buffer; the plug contained approximately 5×10^{12} molecules of DNA. The injected equilibrium mixture was subjected to NECEEM. In the beginning of NECEEM, the electrophoresis was carried out with the outlet reservoir containing 5 μL of deionized water to collect bound fraction of DNA. Fluorescein was used as a marker of the right-hand side boundary of the fraction collection window; the outlet reservoir was changed back to the run buffer as soon as fluorescein eluted from the capillary. A fraction of DNA of approximately 4 μL was collected and amplified by PCR. In addition to the collected DNA template, the PCR mixtures contained 50 mM KCl, 10 mM Tris–HCl (at pH 8.6), 2.5 mM MgCl$_2$, all four dNTPs (200 μM each), primers (300 nM each), and 0.05 unit μL^{-1} Taq DNA polymerase. The total volume of the PCR reaction mixture was 50 μL. The optimum number of cycles for amplification was determined by real-time PCR, as described elsewhere [17]. The optimum number of PCR cycles (11 cycles for the first round of aptamer selection) was conducted, with every cycle consisting of melting at 94 °C for 30 s, annealing at 56 °C for 15 s, and extension at 72 °C for 15 s. The product of symmetric PCR was used as a template in asymmetric PCR to produce single stranded DNA. The asymmetric PCR mixture contained 50 mM KCl, 10 mM Tris–HCl (at pH 8.6), 2.5 mM MgCl$_2$, all four dNTPs (200 μM each), 50 nM biotinylated reverse primer, 1 μM fluorescently labeled forward primer, 0.05 unit/μL Taq DNA polymerase, and 3 μL of the symmetric PCR product. The total volume of the PCR reaction mixture was 50 μL. 15 cycles of PCR were carried out following the same temperature protocol as for symmetric PCR. Double stranded DNA produced in asymmetric PCR was removed using streptavidin-coated magnetic beads (Pierce, Rockford, IL). After pulling-down the magnetic beads, the supernatant was transferred onto a 30-kDa molecular cut-off filter (Millipore) to separate the excess of primers from the PCR product. The obtained enriched DNA libraries were subjected to NECEEM-facilitated evaluation of their affinity towards PDGF-free cell lysate and PDGF-B.

The subsequent rounds of aptamer selection were carried out following the same procedure as described in this section, but with the following modifications: 1 μL of enriched DNA library (concentration ~200 nM) was used instead of the naive DNA library; the concentration of the masking DNA was lowered to 300 nM (instead of 4 μM) in the equilibrium mixture. A total of three rounds of aptamer selection were carried out.

Selection of aptamers for pure PDGF-B was carried out essentially as described above for PDGF-B in the cell lysate with the only exception that the equilibrium mixtures used for the aptamer selection did not contain cell lysate, protease inhibitors and masking DNA.

2.6. Analysis of aptamer-enriched DNA pools

Progression of aptamer selection was monitored by NECEEM-facilitated analysis of affinities of the enriched DNA libraries for PDGF-B or PDGF-free cell lysate. One μL of the obtained enriched DNA library (at a concentration of approximately 200 nM) was mixed with, 1 μM PDGF-B or 300 nM mDNA and cell lysate (containing 235 μg mL^{-1} of the total protein) in a total volume of 5 μL. The equilibrium mixtures were incubated for 20 min at 25 °C. A 5-mm-long (26 nL) plug of the equilibrium mixture was injected by

pressure into the capillary pre-filled with the run buffer and was subject to electrophoresis at 400 V cm^{-1}.

2.7. Gel CE analysis of DNA degradation

The extent of DNA degradation was analyzed through gel capillary electrophoresis (Gel CE) with the use of the OLIGEL SC Kit from Advanced Analytical Technologies (Ames, IA). Loading of the gel in to a capillary, sample preparation and CE-based separation were carried out according to manufacturer's recommendations.

2.8. Protein isolation from cell lysate

The obtained aptamer pools were used to purify PDGF-B protein from the cell lysate. Each aptamer pool was biotinylated in asymmetric PCR amplification in a mixture similar to that indicated above, but containing 1 μM biotinylated forward primers and 50 nM non-labeled reverse primers. To each 150 μL of PCR mixture, 9 μL of the aptamer pool was added; 15 cycles of amplification were carried out as described above. For the control experiments, biotinylated naive DNA library, as well as biotinylated aptamer pool specific to MutS protein, generated in a similar manner as described earlier, were prepared by amplification of 9 μL of 50 nM initial naive library and 9 μL of 50 nM MutS aptamer pool, respectively, in the same procedure. Products of asymmetric PCR were purified from primers on a 30 kDa molecular weight cut-off filters. Due to the fact that the double-stranded product of PCR was also biotinylated, we could not purify the biotinylated ssDNA product from dsDNA.

The entire product of PCR was mixed with 40 μM single-stranded masking DNA, 5 μM double-stranded masking DNA, cell lysate (containing 70 μg mL^{-1} of the total protein), and 7 μg mL^{-1} PDGF-B in the incubation buffer. The mixture was incubated for 15 min at 25 °C. Thirty μL of 5 mg mL^{-1} Streptavidin-coupled magnetic beads (Pierce, Rockford, IL) were added to each of the samples and the mixture was incubated for another 10 min at 25 °C. The magnetic beads with the attached aptamers and proteins were pulled down using a magnet and washed 3 times with the incubation buffer. To elute the bound protein from aptamers, the bound fraction was re-suspended in the SDS-PAGE loading buffer and incubated 10 min at 99 °C. After the incubation, magnetic beads were pulled down by a magnet and supernatants of bound fractions were collected and analyzed on a 17% SDS-PAGE with Coomasie Blue staining. The cell lysate sample, containing PDGF-B at 10% of total protein concentration, was diluted 3 times before it was loaded on to the gel, to reduce band intensity and allow for a more convenient comparison between the lanes.

3. Results and discussion

### 3.1. Selection of aptamer pools and peak identification

To assess the efficiency of aptamer selection for a non-DNA-binding protein, PDGF-B, in the context of a bacterial cell lysate (the AptaPIC selection mode) we concurrently performed three rounds of aptamer selection for a purified sample of the protein to act as a positive control (the conventional selection mode). In order to confirm our previous observations, that high affinity aptamers for a target in the context of a cell lysate can be generated with the omission of the negative selection steps, only the positive selection steps were performed for each selection round. It should be noted, that the omission of the negative selection step would not be applicable for some protein targets and protein expression systems, as it may result in decreased specificity of the resultant aptamer pools. However, in systems which yield high levels of protein over-expression, this loss of aptamer pool specificity
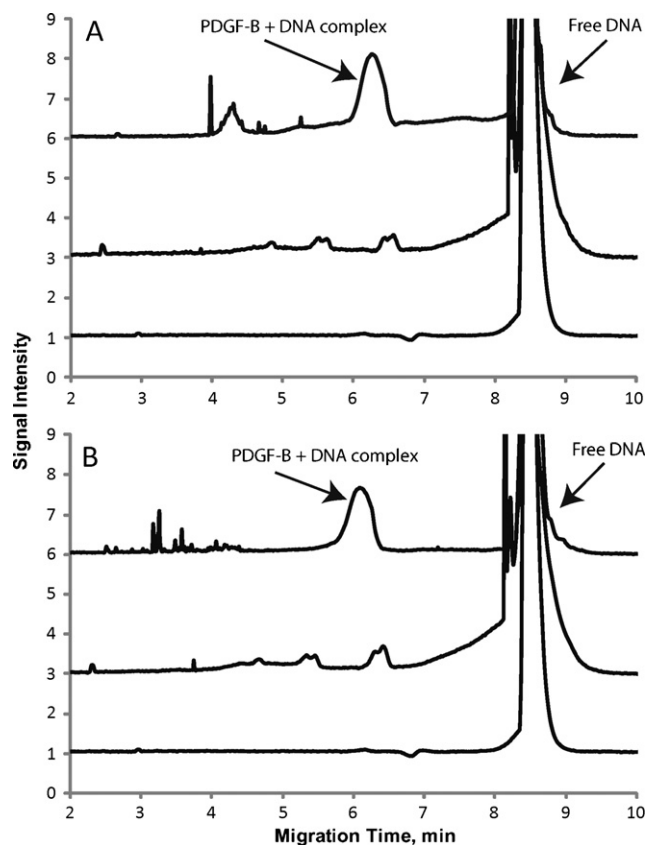
Fig. 1. NECEEM-facilitated affinity analysis of aptamer-enriched DNA pools obtained through: (A) conventional SELEX for pure PDGF-B, and (B) through AptaPIC for PDGF-B in the cell lysate. Traces from bottom to top are: (i) equilibrium mixture of PDGF-B and the naive DNA library, (ii) equilibrium mixture of bacterial lysate not containing PDGF-B and the aptamer-enriched DNA pool after three rounds of selection, (iii) equilibrium mixture of PDGF-B and the aptamer-enriched DNA pool after three rounds of selection. Aptamer-enriched pools obtained through the two modes of aptamer selection show similar affinities for PDGF-B target protein, as well as similarly low affinity for non-target components of the bacterial cell lysate. Equilibrium mixtures contained PDGF-B at concentration of 1 μM and DNA pools at the concentration of 200 nM. For middle traces, cell lysate was included at a final concentration of 235 μg mL^{-1} of total protein. Only the zoomed-in areas of the original electropherograms are presented.

might be less significant when compared to the decrease in the time required for the procedure. Rather than directly expressing PDGF-B protein in a bacterial expression system, purified PDGF-B protein obtained from a commercial source, was added to a bacterial cell lysate at predetermined concentrations. Extrinsic addition of the purified PDGF-B to the cell lysate ensured that there was no difference in protein folding or post-translational processing between the purified and the lysate-containing samples, which, otherwise, may have confounded the comparison of the two methods of aptamer selection. To simulate modest levels of protein over-expression in a bacterial culture [18], equilibrium mixtures for AptaPIC aptamer selection approach contained PDGF-B protein at the concentration of 10% of total protein in cell lysate. In addition to the target protein, DNA library and the bacterial cell lysate, AptaPIC aptamer selection mixtures also contained protease inhibitors, to minimize protein degradation, and non-labeled masking DNA, to minimize nuclease activity upon potential aptamer sequences and to suppress any non-specific binding of non-aptamer sequences [2].

After performing the aptamer-enrichment steps for both modes of aptamer selection, the obtained DNA pools were subjected to affinity analysis by NECEEM (Fig. 1). As evident from the affinity analysis (Fig. 1, bottom traces), the naive DNA library used in this
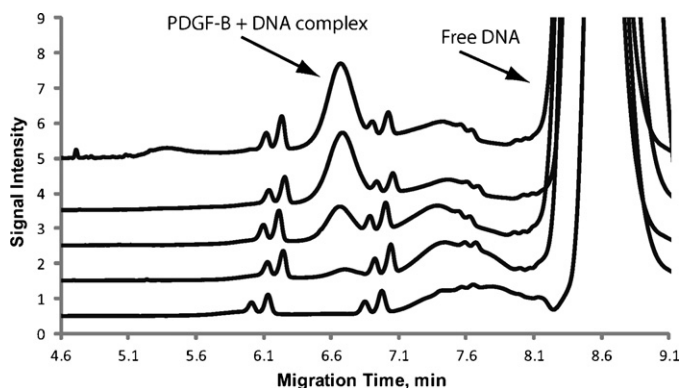
Fig. 2. Titration with PDGF-B of an aptamers-enriched DNA library obtained after three rounds of selection by AptaPIC with PDGF-B as the target. The concentration of the enriched DNA library was 100 nM everywhere while the concentration of PDGF-B varied from bottom to top: 0, 0.3, 0.5, 0.8, and 1.6 μM. Only the zoomed-in areas of the original electropherograms are presented.

Table 1
Comparison of EC_{50} values for aptamer-enriched DNA pools for PDGF-B protein. EC_{50} values were calculated for a total PDGF-B concentration of 1 μM and DNA pools at the concentration of 200 nM. Each value is a mean of 3 measurements.

| DNA pool	Conventional enrichment	AptaPIC enrichment
Naive library	$\sim$450 μM	$\sim$450 μM
Round 1 pool	2.0 $\pm$ 0.2 μM	3.9 $\pm$ 0.6 μM
Round 2 pool	2.1 $\pm$ 0.2 μM	4.2 $\pm$ 0.5 μM
Round 3 pool	2.0 $\pm$ 0.1 μM	3.9 $\pm$ 0.4 μM

work had no significant affinity towards PDGF-B. After three rounds of aptamer selection both of the employed modes – AptaPIC and conventional aptamer selection modes – resulted in a significant improvement of the affinities of the pools to PDGF-B (Fig. 1, top traces). Additionally, neither of the modes resulted in improved affinity towards non-target components of the cell lysate (Fig. 1, middle traces), proving our hypothesis that the negative selection step can be omitted in cases when concentration of the target protein is sufficiently high compared to other components of the cell lysate. The results of affinity analyses between the obtained pools and PDGF-B have revealed the presence of two peaks (migration times 4.5 and 6.5 min) in addition to that of the unbound DNA (migration time 9 min). We have hypothesized that the peak with the migration time of 6.5 min corresponds to the affinity complex between PDGF-B and the aptamers in the enriched DNA libraries. Alternatively, such peak could arise as a result of DNA degradation, in which case shortened DNA molecule fragments, with electrophoretic mobilities different from those of the unbound intact strands would be produced.

To eliminate the second possibility, gel capillary electrophoresis (Gel CE) of the same affinity analysis reaction mixtures were performed. The denaturing conditions of Gel CE analysis cause the dissociation of any non-covalent complexes, and as a result, can only differentiate between the degraded DNA fragments and the intact DNA ligands. The comparison of the Gel CE and NECEEM affinity analysis data of the same reaction mixtures support our hypothesis. The total amount of DNA degradation in the samples was determined to constitute only 7 $\pm$ 3% of the total fluorescence signal. This number is lower than the percent area of the peak hypothesized to correspond to PDGF-B and DNA affinity complexes, which constitutes 26 $\pm$ 3% of the total fluorescence signal. Thus, it is clear that the peak observed in the affinity analysis mixtures cannot fully correspond to DNA degradation products. To further verify the identity of the suspected PDGF-B and DNA complex peak, the enriched DNA library was titrated with PDGF-B in the range of concentration between 0 and 1.6 μM and analyzed by NECEEM (Fig. 2). Signal intensity of the suspected peak increased with concentration of PDGF-B, further suggesting that our assignment of the peak identity was correct.

In addition to the suspected PDGF-B and DNA complex peak with the migration time of 6.5 min, a smaller potential complex peak was observed with the migration time of 4.5 min (Fig. 1, top traces). Due to the fact that PDGF-B can form homodimers [15], we believe that the two observed peaks correspond to 1:1 and 2:1 PDGF-B to DNA stoichiometry binding complexes. A possible explanation for the absence of this peak in traces for AptaPIC-resultant pools is that

PDGF-B may have a smaller tendency to dimerize when in the context of cell lysate. Thus, aptamer pools selected for a pure target would possess more sequences that are specific to the protein in the dimeric form. However, as the contribution of the smaller peak to the total signal area was lower than the contribution of DNA degradation in the samples, the identification of the nature of the smaller peak was not pursued further. As the development of aptamers to dimeric PDGF-B is not a priority in this study, it was not included in the calculations of the EC_{50} values. This omission can only result in an underestimation of the affinity of the DNA pools to the protein target.

3.2. Affinity characterization of aptamer pools

To quantitatively assess the affinity of a particular ligand–target pair, the equilibrium dissociation constant value (K_d) is typically used. Aptamer-enriched DNA pools, however, consist of multiple ligands, making K_d values inappropriate in description of the pool's binding capabilities. Instead, an EC_{50} value can be employed to quantitatively compare affinities of various heterogeneous DNA pools to a target molecule. Here, EC_{50} is defined as the concentration of the target protein at which a half of the ligands from a heterogeneous pool, are bound to the target protein, with the concentration of the ligands in the heterogeneous pool kept constant.

Affinity analysis of the aptamer-enriched DNA library, obtained after the first round of AptaPIC mode of selection, has revealed that its binding affinity to PDGF-B has improved by more than two orders of magnitude when compared to the affinity of the naive DNA library (Table 1). A very similar trend has been observed in the analysis of the DNA libraries enriched through the conventional approach of aptamer selection. In addition, affinity of the AptaPIC-generated aptamer pool showed similar levels of cross-reactivity with non-target components of the cell lysate to those of the pool generated through the conventional approach (Fig. 1, middle traces). This similarity suggests that selection of aptamer pools to a target in the context of the cell lysate did not result in a significant decrease of specificity of the pool to PDGF-B, as it might have been expected. Consequent rounds of aptamer selection did not yield significant improvements in the affinities of the aptamer-enriched DNA pools to PDGF-B, for both modes of selection. While further improvements to affinity and specificity of resultant pools could have been obtained with additional rounds of selection, this approach was not pursued. The generation of aptamers with exceptional affinity and specificity characteristics was not within the objectives of the study. AptaPIC aims at efficient protein purification, where time considerations are often critical. Instead of continuing to refine affinity characteristics of the obtained pools, we opted to test the ability of pools obtained with a small number of selection rounds to efficiently purify PDGF-B. Remarkably, our results indicate that aptamers obtained through AptaPIC mode of selection have a comparable affinity for the PDGF-B as aptamers obtained by conventional method. This, in turn, suggests that when protein is present in cell lysate at a relatively high concentration, and if suitable conditions are used, AptaPIC can be as efficient in aptamer selection as conventional SELEX.
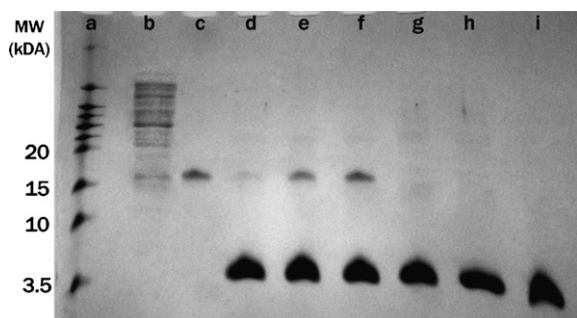
Fig. 3. SDS-PAGE analysis of products of affinity-based protein isolation. Samples in lanes from left to right are: (a) molecular weight markers, (b) bacterial lysate containing PDGF-B before the affinity isolation procedure, (c) sample of purified PDGF-B, (d) products of affinity isolation procedure, performed with the naive DNA library, (e) products of affinity isolation procedure performed with the DNA pool obtained through the AptaPIC mode of aptamer selection, (f) products of affinity isolation procedure, performed with the DNA pool obtained through the conventional SELEX mode of aptamer selection, (g) products of affinity isolation procedure, performed using a negative aptamer control, (h) products of affinity isolation procedure, performed with naked magnetic beads, and (i) boiled magnetic beads.

3.3. Aptamer-facilitated protein isolation from cell lysate

To further test and compare the affinity and specificity characteristics of the two aptamer-enriched DNA pools, obtained through the AptaPIC and the conventional modes of selection, they were used to perform aptamer-facilitated protein isolation from cell lysate. Briefly, the naive DNA library (negative control), an aptamer pool obtained for a protein that is not present in *E. coli* cell lysate (negative aptamer control), and the aptamer pools generated for pure PDGF-B and PDGF-B in cell lysate were biotinylated in asymmetric PCR amplification. These samples were then individually mixed with a bacterial cell lysate to which PDGF-B protein was added at 10% of total protein concentration. The mixtures were allowed to equilibrate, and streptavidin-coated magnetic beads were then added to each of the samples. The magnetic beads, bound to potential aptamer sequences and their respective protein targets, were pulled down using a magnet and washed to eliminate any non-specifically bound contaminants. The fraction of the lysate bound to the beads was next eluted, the magnetic beads pulled down by a magnet, and the supernatants of bound fractions were collected and analyzed by SDS-PAGE with Coomassie Blue staining (Fig. 3).

As negative controls, we showed that PDGF-B was not isolated from the cell lysate in significant amounts, when the naive DNA library, the negative aptamer control, or magnetic beads without any DNA ligands attached to them were used in affinity protein isolation procedure (Fig. 3, lanes d, g and h). In contrast, when aptamer-enriched DNA pools have been used in affinity isolation of PDGF-B from cell lysate, SDS-PAGE analysis of the resultant products revealed the presence of distinct bands that co-migrated with the purified PDGF-B control (Fig. 3, lanes c, e, and f). This demonstrates that high affinity aptamers for PDGF-B were indeed developed through both modes of selection. The absence of the bands that correspond to any of the other components of the cell lysate suggests that the developed aptamers were highly specific for PDGF-B. DNA libraries enriched through the AptaPIC selection mode displayed similar binding affinity and specificity to PDGF-B protein as the DNA libraries enriched through the conventional aptamer selection mode, suggesting that the aptamer selection for PDGF-B in the cell lysate was as successful as the one for the pure target. To explain the presence of a prominent band that co-migrates with 3.5 kDa molecular standard, a sample of naked magnetic beads was exposed to the conditions at which the proteins were eluted from the beads in previous samples (Fig. 3, lane

i). The presence of the 3.5 kDa band in this sample suggests that it represents the dissociation of bead-conjugated groups and not a contaminant from the bacterial cell lysate. The use of the obtained aptamer-enriched pools with higher yield purification methods, such as aptamer-facilitated affinity chromatography, should not encounter similar issues [19]. It should be noted, that as no purification method is perfectly efficient, AptaPIC-mediated purification should be followed by refining purification steps, if very high levels of purification are desired. In such cases, a more sensitive method for purity estimation, such as gel silver staining, should also be used.

The success of the aptamer-mediated protein isolation procedure suggests that even an aptamer pool, developed within a small number of selection rounds and displaying a relatively low bulk affinity value towards it target protein, may possess sufficient specificity to allow for efficient purification of the target protein from cell lysate. While the aptamer pools obtained through AptaPIC may not possess sufficient affinity and specificity characteristics for analytical applications, they can be used as a starting point for subsequent aptamer selection steps for the pure target.

4. Conclusions

Combined, the results of our previous works and results presented here demonstrate that AptaPIC is a reliable method, which can be successfully applied to both DNA-binding proteins and to proteins that do not have an inherent DNA binding function. In addition, this work re-emphasizes the fact that the omission of the negative selection step is a viable option, in cases where protein over-expression is achieved at high levels. AptaPIC approach has the potential to become a valuable tool for purification of newly discovered proteins, for which no established isolation protocols are available.

Acknowledgments

References

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