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Emulsion PCR Significantly Improves Nonequilibrium Capillary Electrophoresis of Equilibrium Mixtures-Based Aptamer Selection: Allowing for Efficient and Rapid Selection of Aptamer to Unmodified ABH2 Protein

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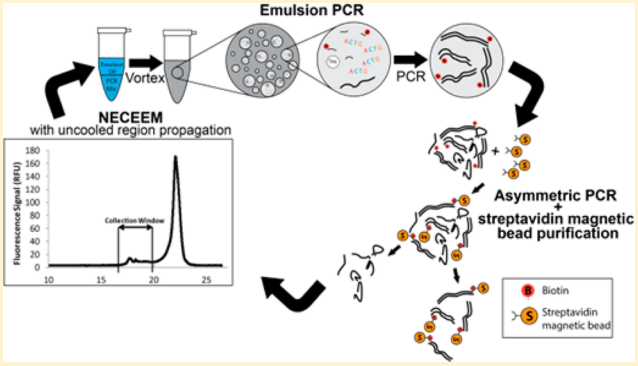
ABSTRACT: Nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM), a homogeneous approach to select DNA aptamers, is among the most efficient partitioning techniques. In contrast with surface-based systematic evolution of ligands by exponential enrichment (SELEX) approaches, the ability of NECEEM to select aptamers to unmodified proteins in solution is preferable for identifying aptamers for eventual *in vivo* use. The high stringency and low sample volumes of NECEEM, although generally beneficial, can result in binding of very few aptamers, requiring highly efficient amplification to propagate them. When amplified with standard PCR, detectable library enrichment can fail due to the fast conversion of the aptamers into byproducts and preferential amplification of nonbinders. As an alternative, we proposed the use of emulsion PCR (ePCR), which is known to reduce byproduct formation, as a PCR mode for coupling with NECEEM partitioning. For the first time, we tested the advantages of ePCR in NECEEM-based aptamer selection to a medically relevant DNA repair enzyme, ABH2. We report that the combination of ePCR with NECEEM allowed for the selection of aptamers in the first three rounds of SELEX, while SELEX with conventional PCR failed in a number of attempts. Selected aptamers to an unmodified ABH2 protein have potential use in diagnostics and as leads for anticancer cotherapies, used as enhancements of alkylating agents in chemotherapy.



Single-stranded oligonucleotides, including proteins, that can bind molecular targets with high affinity and specificity are DNA aptamers.[1,2] Aptamers are promising affinity ligands with a potential to challenge antibodies as diagnostic, analytical, and therapeutic reagents.[3,4] Despite the promise of aptamers and significant efforts in their development over 25 years, useful aptamers have been obtained for a much smaller number of protein targets than antibodies.[5] The relatively slow progress is in part due to the limitations of the technologies used for aptamer development. Aptamers are typically obtained by a process termed SELEX (Systematic Evolution of Ligands by EXponential enrichment), which involves multiple rounds of two alternating processes: (1) partitioning of protein-bound DNA from free DNA and (2) PCR amplification of the collected target-bound DNA (Figure 1A). Figure 1B classifies the major partitioning and PCR-amplification approaches used in SELEX for protein targets. Partitioning has been typically done by heterogeneous methods: either filter-based separation, in which filters retain protein−DNA complexes better than free DNA, or affinity chromatography, in which the protein is immobilized on a surface and retains protein-bound DNA.[6] These surface-based techniques suffer from nonspecific adsorption of free DNA to the surface of the filters or stationary phase. The nonspecific binding reduces the efficiency of partitioning and leads to the necessity of multiple rounds of partitioning/amplification as well as negative selection steps, typically requiring between 10 and 30 rounds of SELEX.[7−9] Ideally, partitioning is to be done in a homogeneous environment thereby avoiding nonspecific binding and the adverse effects associated with it.

Homogeneous methods are highly desirable for the selection of aptamers to protein targets in cases where the aptamers are to be used in therapeutic applications. The immobilization process used in heterogeneous partitioning techniques can affect protein structure, so potentially reducing biological relevance.[6] Aptamers selected to a protein modified by immobilization may be unable to bind the protein in its native form. Therefore, additional confirmation of their affinity to the

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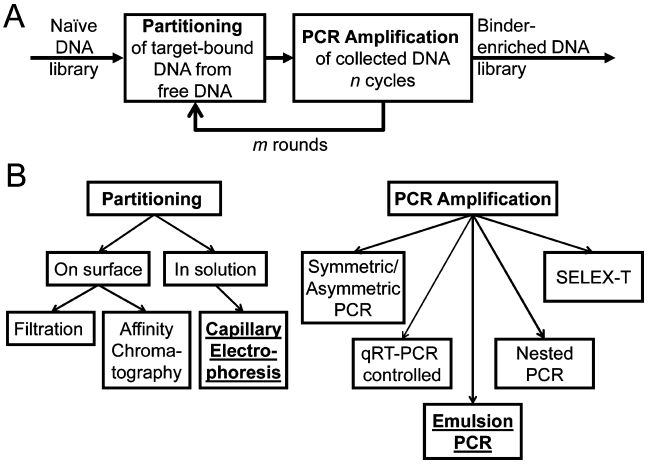

Figure 1. Concept of aptamer selection: major steps (A) and (B) major approaches for partitioning and PCR amplification. See details in the text.

native structure of the protein using solution-based approaches is required. In contrast, aptamers selected to unmodified proteins *in vitro* using homogeneous techniques are more likely to function similarly *in vivo*. Methods employing kinetic capillary electrophoresis (KCE) have been suggested as an alternative to the surface-based methods. KCE methods are homogeneous and, therefore, do not suffer from nonspecific binding in the way surface-based methods do. KCE methods can drastically increase the efficiency of partitioning and facilitate selection of aptamers with desirable binding parameters.[7] Additionally, KCE methods can facilitate measurements of equilibrium and rate constants of aptamer-protein binding allowing for easy assessment of selection progress from round to round.[10] Nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) is the most widely used KCE method for the highly efficient selection of aptamers to unmodified protein targets.[10−16] The efficiency of partitioning by NECEEM is at least 2 orders of magnitude higher than those of conventional surface-based selection techniques.[11] As a result, library enrichment can be completed in as few as 2−4 rounds of NECEEM-based SELEX, in contrast to the 10−30 rounds required in SELEX based on heterogeneous partitioning.[7−9,11]

An important intrinsic property of NECEEM is that the number of identified aptamers is very small which creates a significant challenge for the amplification step. To successfully amplify a small number of aptamers, the PCR must be highly efficient and specific, i.e., a high yield of a single amplicon product generated per corresponding intended template sequence, as opposed to byproducts generated due to nonspecific primer binding and amplification of template heterodimers. As PCR is the step following partitioning (Figure 1A), inefficient PCR can drastically affect the overall performance of SELEX. Despite this, relatively little attention has been paid to improving PCR in SELEX. For a number of years, the classical PCR amplification protocol was used, the one that is used for homogeneous DNA templates. Such conventional PCR amplification was found to quickly degrade the products and rapidly form byproducts through the formation of product heterodimers.[17] Additionally, DNA amplification by conventional PCR is susceptible to nonspecific primer hybridization, primer dimers, amplification bias,[18] and potential aptamer loss due to their adhesion on walls of PCR tubes.[19] These issues are particularly detrimental to NECEEM-based aptamer selection and must be addressed for this highly efficient partitioning method to be used successfully.

A number of approaches have been attempted over the years to mend the aforementioned inefficiencies of PCR amplification in SELEX. To this end, quantitative real-time PCR (qRT-PCR) was used to determine the optimal number of PCR cycles by gauging the cycle at which 50% of maximal yield is reached (usually 11−13 cycles), thus reducing byproduct formation by preventing overamplification. Once the cycle number was determined, symmetrical PCR (same concentrations of forward and reverse primers) was combined with linear asymmetric PCR (excess of forward primer) to decrease byproduct formation and increase product yield.[20] Although effective in its goal, this method does not address amplification bias, byproduct formation due to primer-dimers, nonspecific primer binding in early amplification cycles, or DNA loss due to adsorption. More recently, Nested-PCR, a method which uses a series of two or more sets of flanking primers to amplify a shorter sequence in a master template, was introduced to increase amplification specificity.[21] This approach, however, would exacerbate the bias and product-heterodimer-induced byproducts due to the increased size of the intermediate amplicon and the increased number of required PCR reactions. For instance, a 64-bp sequence was required to amplify a 16-bp random aptamer sequence using 6 PCR reactions per selection round.[21] The bias issue was tackled specifically in SELEX-T.[22,23] This method first generates cDNA from RNA aptamers, followed by T7 RNA polymerase transcription to linearly amplify the RNA aptamers. Using excessive primers and low number of PCR cycles, the bias due to heteroduplex formation is prevented by amplifying the cDNA template solely and avoiding the RNA product. This approach, however, only applies to RNA aptamers and introduces additional purification and bias-prone enzymatic reaction to the selection process.

Here we describe the combination of NECEEM partitioning with a highly efficient amplification procedure termed Emulsion PCR (ePCR). ePCR is a method to compartmentalize and miniaturize the PCR reaction *in vitro*.[24,25] Microreactors enclosing PCR components and (ideally) a single template are created by vigorously mixing mineral oil and surfactants with a conventional PCR mix.[24] In effect, a local homogeneous-like PCR amplification environment is created.[26] These conditions prevent the formation of the highly detrimental product−product hybrids (the main source of byproducts).[17] They allow for the increase of the effective local concentration of the template DNA in each micelle thus reducing primer-dimer formation and nonspecific primer hybridization (the major cause of PCR inefficiencies in early amplification cycles).[24] They also increase product yield due to the close proximity of the template to primers and polymerase and, importantly, reduce amplification bias.[24,25] Furthermore, the hydrophobic properties of the oil does not allow for the DNA template to adsorb to the reactor tube wall,[19] a significant issue in alternative single molecule amplification methods.[24] We believe that the efficient amplification of aptamers through their isolation in micelles, as provided by ePCR, will significantly reduce potential aptamer loss due to stresses introduces by NECEEM, allowing it to function as an efficient homogeneous partitioning method in the context of aptamer selection.

The utility of ePCR was previously suggested for the amplification of aptamers.[25] A recent semiautomated aptamer selection method, dubbed "just in time-selection", uses

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magnetic beads and emulsion PCR to amplify aptamers.[27] The method, however, relies on a heterogeneous separation approach and requires elaborate washing and negative selection steps. This results in the need for 15 rounds of SELEX. In addition, this approach requires a separate method (e.g., surface plasmon resonance) for affinity measurements after aptamer synthesis. To our knowledge, ePCR has never been used in conjunction with homogeneous separation techniques to select aptamers for difficult protein targets.

To test our approach, we selected aptamers to ABH2 (AlkB homologue 2), a DNA damage repair enzyme. This human protein is a challenging target for aptamer selection as it is relatively unstable and, therefore, requires very careful handling. It is also a difficult target for CE as the protein is positively charged at neutral pH and, thus, can potentially adhere to negatively charged capillary walls during separation. The adsorption can further reduce the amount of collected aptamers and, therefore, applies additional stress on PCR. ABH2 is a 2-oxoglutarate (2OG)- and iron(II)-dependent oxygenase able to repair toxic 1-meA as well as 3-meC damage in double stranded DNA (dsDNA) in mammalian cells.[28,29] Normally, ABH2 acts as one of the main alkylation housekeeping enzymes, repairing alkylation that can result in the disruption of replication and transcription, triggering of the cell-cycle checkpoints, induction of apoptosis or, in some cases, cancerous growth.[30] Alkylating agents, termed alkylating antineoplastic agents, have been successfully used as anticancer chemotherapy drugs since the conception of chemotherapeutics.[31,32] These drugs damage dsDNA in such a way that the strands cannot uncoil and, therefore, suppress the cell division of cancerous cells.[33] Many cancers, however, develop resistance to chemotherapeutic alkylation by taking advantage of the cell's innate natural DNA damage defenses, which include repair enzymes such as ABH2 and related enzymes. Using these DNA repair enzymes, cancer cells can efficiently dealkylate damaged DNA, thus requiring higher doses of the drug to inhibit their growth and as a result increasing the chance for lethal side effects or rendering the drug ineffective altogether.[34−36] ABH2 expression has been directly implicated in a variety of cancers as well as neurological and developmental disorders.[29,37] Aptamers specifically binding ABH2 can potentially be used to detect tumors, deliver drugs to the therapeutic target (thus lowering the chance for potentially lethal side effect), or act as drugs through binding ABH2 (or other DNA repair enzymes) and inhibiting its enzymatic activity.

A number of attempts to select aptamers for the ABH2 protein by NECEEM partitioning with conventional PCR (combination of symmetric and asymmetric PCR) were unsuccessful; they showed no detectable library enrichment in five rounds of SELEX. The very first attempt of aptamer selection using the combination of NECEEM and ePCR was successful, thus revealing that ePCR is a viable approach to improve NECEEM-based aptamer selection for challenging protein targets.

RESULTS AND DISCUSSION

Unsuccessful Attempts to Select Aptamers for ABH2 by NECEEM with Conventional PCR. As described in the introduction, aptamers able to inhibit ABH2 enzymatic activity can be potentially used to improve the effect of chemotherapies. Maintaining the ABH2 protein unmodified during the selection procedure should help in finding inhibitors binding to the protein in its native structure. Thus, NECEEM, a homogeneous partitioning approach, is desirable. In previous studies, we were able to select aptamers for a bacterial homologue of ABH2, AlkB, using a SELEX protocol based on partitioning by NECEEM and amplification using a combination of symmetric and asymmetric PCR.[4] We demonstrated that AlkB aptamers are able to inhibit DNA demethylation 1000 times more efficiently than the best reported small-molecule inhibitors.[38] This success was the major motivation for attempt to select aptamers for human ABH2 using the same selection approach.

The ABH2 aptamer selection was performed using an 80-nt long ssDNA library containing 20-nt long primer regions and 40-nt random-sequence region (the library was identical to the one used in aptamer selection for AlkB). DNA has the advantage of being more stable and easier to process than RNA as it does not require a transcription step. Additionally, DNA aptamers, in general, were found to have similar binding affinities to their RNA counterparts.[39]

Three attempts with five rounds of SELEX in each were conducted with monitoring library enrichment after every round. No detectable enrichment was observed indicating that SELEX was failing despite the known high efficiency of NECEEM partitioning and the previous success with AlkB. Analysis of ABH2 crystal structures reveals that it is a larger, more complex protein than its bacterial homologue,[40] and thus, it may behave differently in selection.[40] ABH2 contains a short loop of positively charged arginine and lysine residues and an additional relatively long, flexible dsDNA-binding loop containing arginine, glycine, and lysine residues.[41] The positive charge imposed by these residues likely increases the propensity of ABH2 to interact with the negatively charged capillary walls, thus, potentially retarding the aptamer−ABH2 complexes. In addition, ABH2 requires a variety of cofactors for activity and has been observed to be less stable than AlkB. The activity of ABH2 has been found to decrease within hours of thawing its frozen solution. As such, the protein may be partially degraded by the time of selection, further decreasing the amount of the aptamer−protein complex. All these factors could contribute to the potential reduction of the number of DNA templates bound to the protein target during NECEEM, thus challenging the efficiency of PCR and resulting in the observed failure of SELEX. This failure motivated us to study in detail its potential reasons and search for a solution that could rescue the failing SELEX.

Why Can SELEX Fail? First, we will demonstrate the role of PCR in SELEX by developing simple algebra of SELEX exemplified by the schematic in Figure 2. For our simple consideration, we assume that the library contains two classes of DNA molecules: binders (B) and nonbinders (N). The number of binders and nonbinders that enter partitioning are B_{in} and N_{in}, respectively (typically, $B_{in} \ll N_{in}$). The collection coefficients for binders and nonbinders are k_B and k_N, respectively; in ideal partitioning, $k_B = 1$ and $k_N = 0$. The number of binders and nonbinders at the exit of partitioning are $B_{in}k_B$ and $N_{in}k_{NB}$, and this is exactly what enters the PCR amplification step. The PCR amplification efficiency may be different for binders and nonbinders, for example, because binders are structured DNA molecules with hindered access of the polymerase enzyme; nonbinders are typically amplified preferentially.[42,43] Polymerases are also sequence-sensitive and binders may have sequences that are more difficult to amplify. The difference in amplification between binders and nonbinders can be expressed by different bases of the exponent

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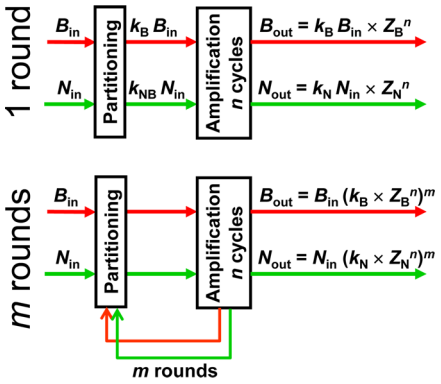

Figure 2. "Algebraic" model of SELEX for a single round (top) and m rounds (bottom).

describing amplification: Z_B and Z_N for binders and nonbinders, respectively. In ideal amplification two copies of DNA are produced from a single template copy, that is $Z = 2$. In unbiased PCR amplification, $Z_B = Z_N$, but for reasons including those described above it is likely that $Z_B < Z_N$. At the exit of n cycles of PCR, the number of binders, B_{out}, and nonbinders, N_{out}, will be

$$B_{out} = B_{in} k_B Z_B^{\,n}, \quad N_{out} = N_{in} k_N Z_N^{\,n} \tag{1}$$

For SELEX to enrich the binders the following inequality must be satisfied:

$$B_{out}/N_{out} > B_{in}/N_{in} \tag{2}$$

By using eq 1, we can rewrite eq 2 as

$$k_B/k_N > (Z_N/Z_B)^n \tag{3}$$

If the inequality in eq 3 is not satisfied then SELEX fails unconditionally. The increasing PCR bias toward amplification of nonbinders (growing Z_N/Z_B) leads to exponentially increasing minimally required efficiency of partitioning (k_B/k_N). For example, if Z_N is 1% greater than Z_B and there are 40 cycles of PCR, the minimum required efficiency of partitioning is ~1.5, which is easily achieved even with surface-based partitioning approaches. However, if Z_N is 10% greater than Z_B, k_B/k_N must exceed ~50 and this is hardly achieved with any surfaced-based technique. In other words, if the efficiency of preferential partitioning of binders is less than the efficiency of preferential amplification of nonbinders, then SELEX will be selecting not binders but nonbinders. For SELEX to work very efficiently, the strong inequality must be satisfied:

$$k_B/k_N \gg (Z_N/Z_B)^n \tag{4}$$

Our simple algebra of SELEX also allows us to illustrate how PCR bias can affect the number of rounds, m, required for SELEX completion. We define that SELEX is complete when $B_{out}/N_{out} > 1$ is reached. By using eq 1, this condition can be rewritten as

$$B_{in}(k_B Z_B^{\,n})^m > N_{in}(k_N Z_N^{\,n})^m \tag{5}$$

which can be expressed for m as

$$m > \frac{\log(B_{in}/N_{in})}{\log(k_N/k_B(Z_N/Z_B)^n)} \tag{6}$$

It is instructive to estimate m required for SELEX completion for two different values of PCR bias Z_N/Z_B. We will assume reasonable values for the initial aptamer abundance, the efficiency of partitioning, and the number of PCR cycles in a single round: $B_{in}/N_{in} = 10^{-8}$, $k_N/k_B = 0.01$, and $n = 40$. If the PCR is unbiased ($Z_N/Z_B = 1$), then from the inequality in eq 4, we get that the minimum number of rounds for SELEX should be 4. If, however, there is a 10% PCR bias toward amplification of nonbinders, then the minimum number of required rounds is 23. Thus, the number of rounds required for SELEX completion greatly depends on PCR bias. In the presence of such a bias, the number of required rounds increases with increasing number of PCR cycles in a single round.

Options for Improving Efficiency of PCR. NECEEM's advantages, however, come at a cost by imposing challenges on

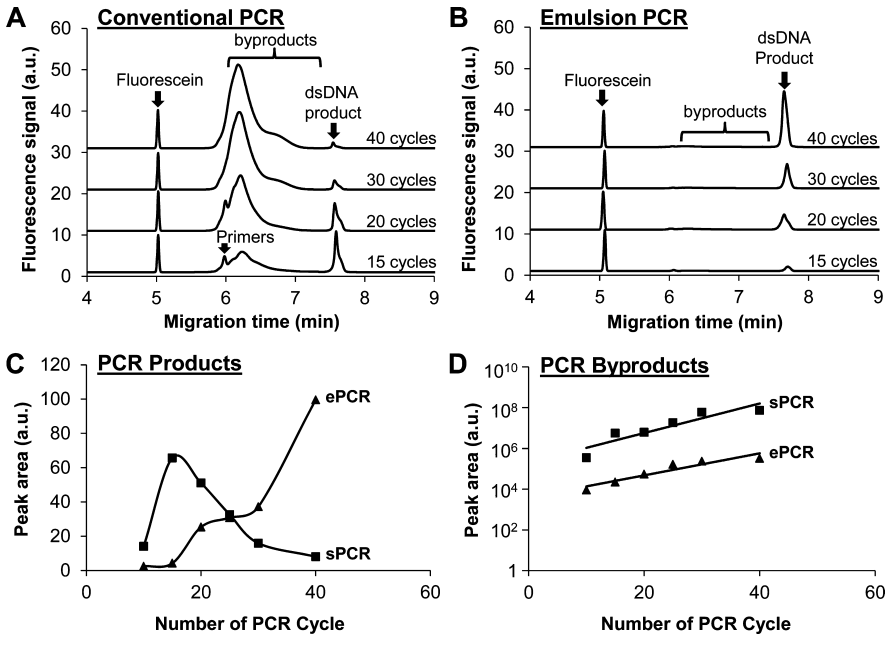

Figure 3. Comparison of product loss due to byproduct formation in conventional PCR and emulsion PCR.

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the amplification part of the selection process. First, whereas conventional partitioning techniques use the entire aptamer pool, NECEEM uses nanoliter volumes for analysis and separation. Although this has the benefit of conserving reagents and costly protein targets, it also means that during the first round of selection only a small fraction of the available sequences in the initial library are used in partitioning. In subsequent rounds, if the aptamers are not present in sufficient quantities (e.g., due to biased amplification) they may be lost in following partitioning rounds due to the small injection volume. Lastly, the unmatched stringency of NECEEM, although enabling isolation of high affinity aptamers, greatly reduces the number of aptamers which are present in the final fractioned pool. For these reasons, NECEEM imposes a requirement of highly efficient amplification that will preserve, and sufficiently amplify, the relatively small number of aptamers between the partitioning rounds.

The major problem of PCR amplification is rapid conversion of products into byproducts and potential bias toward the amplification of nonbinders. Many methods attempting to solve this issue only address one of the problems, while ignoring or, in some cases, aggravating the other. As mentioned above, qRT-PCR-controlled amplification reduces overamplification of byproducts but does not address the primer dimerization or bias issue.[20] Nested-PCR increases amplification specificity[21] but worsens the bias due to the increased size of the intermediate amplicon. Other methods, such as SELEX-T,[22] reduce bias and byproduct formation but only apply to RNA aptamers. Emulsion PCR, which is carried out in microdroplets can help alleviate both product conversion to byproducts and PCR bias. For these reasons, emulsion PCR is commonly used in next-generation sequencing, including the 454, the Polonator, and SOLiD platforms.[44]

We first validated the efficiency of ePCR as compared with conventional symmetric PCR (sPCR) with regards to product yield and product-to-byproduct conversion. Conventional symmetric amplification of a heterogeneous ssDNA library produces a significant number of byproducts by the 10th cycle of amplification and, more importantly, the rate of product loss due to the conversion to byproducts surpassed product generation by as early as the 15th cycle (Figure 3A,C,D). By the 30th cycle, practically all the products have been converted to byproducts. These byproducts have been previously found to arise from the formation of primer dimers and nonspecific primer binding in early PCR cycles when primer concentration is high and, more significantly, from formation of product−product hybrids later in the PCR process when the template concentration is high.[17] In contrast, ePCR produced virtually no byproducts in comparison to sPCR and, as a result, no clear product loss was observed (Figure 3B,C). Conventional PCR product yield peaked at cycle 15 whereas an exponential increase in product was observed in ePCR well into the completion of the procedure. By the 40th cycle, over 10 times greater amount of products and 2 orders of magnitude lower amount of byproducts have been observed in ePCR as compared to conventional symmetric PCR (Figure 3C). This is consistent with a previous study that found that conventional PCR produced byproducts as early as the 15th cycle and significant product loss by the 20th round.[17]

PCR product loss, such as that occurring due to their conversion to byproducts, is highly detrimental to the aptamer selection process, particularly when using NECEEM for partitioning. During the first round of selection, high affinity aptamers are present in single copies in the partitioned aptamer pool. The stringency and low volumes used by NECEEM reduce the quantity of aptamers further. Any loss of these sequences permanently removes them from the pool and may introduce a significant bottleneck to the aptamer selection process.

ePCR has been found to possess a Poission distribution of templates with most micelles containing a single template.[24] A small fraction of microreactors may have a larger number of templates or no templates at all.[24] In these cases, primer dimers, nonspecific primer binding, and product−product hybrids may form. Nevertheless, ePCR prevents any such byproducts, or other contaminations, from propagating throughout the PCR reaction thus limiting the amplification of byproducts to negligible amounts. In contrast, byproducts formed during conventional PCR are able to propagate throughout the entire process, increase in numbers exponentially, and in addition to converting products to byproducts, limit the resources available for aptamer amplification. This allows the byproducts to outnumber the aptamers rapidly, significantly decreasing the chance of aptamers to be injected into the capillary in the following NECEEM partitioning round.

The remaining aptamers, which were not converted to byproducts in conventional PCR, are subjected to amplification bias. The variety of aptamer sequences present have different amplification efficiencies owing to factors including steric hindrance imposed by the secondary structure and polymerase sequence preference.[18,45] Aptamer sequences that are amplified more efficiently are overrepresented in the final pool as opposed to the desired high-affinity aptamers. This bias is bypassed in ePCR as the polymerase is exposed to a single template and the template concentration is effectively increased in their respective micelles, thus eliminating competition. To summarize, our results showed that ePCR leads to a greater amount of products and 100-times lower amount of byproducts formed.

Aptamer Selection for ABH2 Based on NECEEM-ePCR.

The previous successful use of ePCR in aptamer selection by heterogeneous methods and our own results showing significantly reduced product loss (see previous section) prompted us to test the combination of NECEEM and ePCR as applied to ABH2 as a challenging target for aptamer selection.

The selection process proceeded as follows: first, the library is allowed to equilibrate with the target ABH2 protein and the mixture is injected into a capillary (time t_1 in Figure 4). High affinity oligonucleotides (aptamers) bound to the target are partitioned from the unbound or weakly bound and dissociated sequences using an electric field of 400 V/cm (time t_2 in Figure 4). This causes the mixture components to separate into distinct regions, or zones, determined by charge to friction-coefficient ratios of the analytes.[46] Fluorescent tagging of the DNA enables visualization of the unbound DNA peak as well as the complex of DNA with ABH2 and the so-called dissociation region. A fraction-collection window is determined to include the complex as well as a portion of the DNA pool that dissociated from the protein−DNA complex (Figure 4). Essentially, the closer the right boundary of the window to the ligand peak, the weaker ligands (higher rate constants of complex dissociation, k_{off}) will be collected.[47] The boundary is generally chosen toward the middle of the dissociation region with a bias toward the complex peak. This allows for sufficient distance as to not contaminate the high affinity aptamers with

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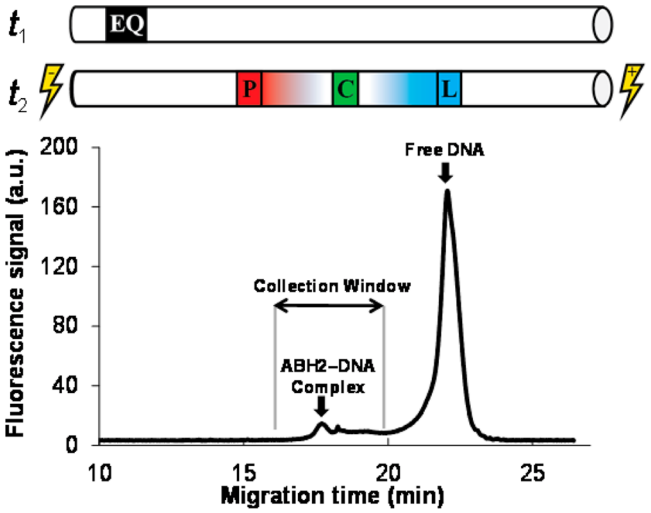

Figure 4. Determination of the aptamer-collection window in NECEEM for ABB2 as a target. In NECEEM, an equilibrium mixture containing the protein and DNA aptamer library is injected (t_1) into the capillary and an electric field is applied to separate the complex from the protein and ligand (t_2). The electropherogram contains peaks of the complex and ligand due to the florescent tag on the DNA. A window is chosen to capture the intact complex and a part of the dissociation region.

nonbinding or low affinity aptamers so ensuring that the entire complex peak is collected. This classic NECEEM experiment allows for the estimation of EC_{50} (analog of K_d used for a pool of different aptamers, EC_{50} is defined as the concentration of target at which 50% of aptamers in the pool are bound) and k_{off} constants during the fraction-collection stage for future reference and monitoring of the selection progression.[48]

Once obtained, the enriched DNA aptamer pool is amplified using ePCR followed by the generation of an enriched single stranded pool using asymmetric PCR,[49] completing the selection round. The next round is then initiated by incubating the enriched pool with ABH2 and subjecting the mixture to NECEEM partitioning. This classical NECEEM experiment allows for the determination of the binding constants of the new enriched pool of DNA and for the selection of a new fraction at the same time. The rounds of alternating partitioning and amplification rounds are then repeated until no further enrichment is observed.

After amplification, the ePCR emulsion can be easily broken using organic solvents. Originally, the highly volatile and flammable organic solvent diethyl ether was used followed by a precipitation of the amplified DNA. Here, we follow Schütze's work to simplify the process and replace diethyl ether with isobutanol. We also replace the emulsification method with vortexing as opposed to the original contamination prone magnetic stirring method.[26]

To regenerate the ssDNA library, asymmetric PCR (aPCR) was utilized at a 20:1 forward to biotinylated reverse primer ratio.[49] The residual dsDNA remaining after aPCR amplification was removed using streptavidin magnetic beads and biotinylated reverse primers. The resulting amplified DNA was purified from other components of the solution (primers, nucleotides, and enzyme) by using the MinElute purification method (QIAGEN, Mississauga, ON, Canada). This combination of ePCR and aPCR is a very simple, rapid, and efficient way to regenerate an enriched aptamer pool.

The affinity of the enriched pools, as assessed through EC_{50} values, was measured using NECEEM immediately following aPCR purification and a new fraction was collected at the same time.[48] Obtaining binding constants prior to sequencing is of substantial benefit for aptamer selection. By following the progression of enrichment, one can halt selection as soon as no further enrichment is observed so as to avoid extra selection steps and labor intensive affinity measurements as required in other selection methods. An EC_{50} value of 600 nM was reached by the fourth round of SELEX (Figure 5). The fifth and sixth

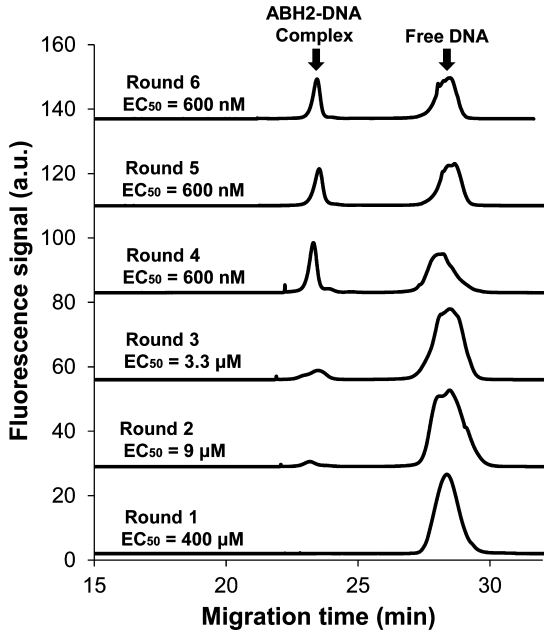

Figure 5. Progress of library enrichment with number of SELEX rounds. NECEEM-based aptamer selection combined with ePCR was used to select aptamers for ABH2 from an unbiased ssDNA library.

selection rounds showed no further improvement and the selection procedure was stopped. A common trend for EC_{50} enrichment, particularly in CE selection techniques, is for affinity to reach a maximum, followed by a worsening in EC_{50} following each successive round.[50,51] Although the reason for this phenomenon is not well understood, we believe that it occurs, at least partly, due to the limitations imposed by PCR. It is likely that at the point of loss of enrichment, the rate of PCR-related aptamer loss surpasses the rate of enrichment. Here, we did not observe this pattern. Although enrichment was not observed past the fourth round, no loss in enrichment was observed. It is likely that this occurs due to the improvements provided by ePCR which curbs PCR related aptamer loss.

Pools displaying the lowest EC_{50} values were cloned and eight aptamers with the lowest K_d values were sequenced and synthesized. Although 2OG was not present during the selection process, binding measurements of the synthetic C10 aptamer without 2OG had a K_d of 1 μM whereas the addition of 2OG increased the strength of binding by ~5-fold, decreasing the K_d to 200 nM (not shown). This is explained by the ordered sequential mechanism of 2OG oxygenases, including ABH2, in which 2OG binding precedes that of substrate; 2OG coordinates to the active site Fe(II), which induces substantial structural changes in ABH2 that preorganizes the active site for substrate binding.[52,53] Selecting aptamers in the presence of cofactors and inhibitors will be the

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subject of future work. The synthetic aptamers obtained from the forth round of selection were found to be characterized by K_d values ranging from 38 nM to 1 μM (Figure 6, Table 1).

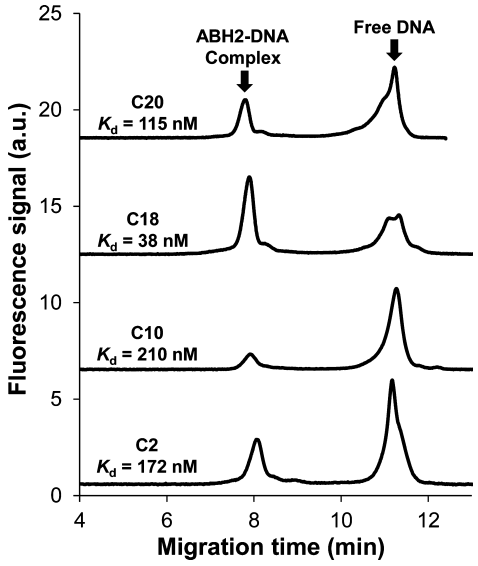

Figure 6. Aptamer binding studied by NECEEM: 200 nM aptamer was mixed with 200 nM of ABH2 in the presence of 160 μM 2-OG.

The identified ABH2 aptamers were tested for cross-reactivity with the homologous 2OG oxygenases, AlkB and ABH3, and found to successfully discriminate between close human and bacterial homologues. The binding assay did not reveal any aptamer−protein complex formation when 100 nM C2 or C10 aptamers were incubated with 400 nM ABH3 or AlkB.

MATERIALS AND METHODS

Materials. Fused silica capillary, 75 μm inner diameter, 365 μm outer diameter, was purchased from Polymicro Technologies (Phoenix, AZ). ABH2 was produced and purified as described elsewhere[54] and stored in 50 mM Tris-HCl, 500 mM NaCl, 1 mM DTT pH 7.5 buffer at −80 °C. A ssDNA library, labeled at the 5′ end with fluorescein (F488) for detection, 80 bp in length, including two 20-bp primer regions, was synthesized and HPLC-purified by Integrated DNA Technologies (IDT, Mississauga, ON, Canada) and stored at −20 °C. F488-labeled as well as unlabeled ssDNA forward primers, as well as 3′ biotinylated and unbiotinylated reverse primers, 20 bp in length, were synthesized and purified with HPLC by IDT (Mississauga, ON, Canada) and stored at −20 °C. Single stranded, 5′ F488-labeled DNA aptamers (80 bp in length, containing 20-bp primer regions) were synthesized by IDT (Mississauga, ON, Canada). Streptavidin-labeled magnetic beads as well as all other chemical reagents and buffers were obtained from Sigma-Aldrich (Toronto, ON, Canada). Solutions were prepared using double distilled deionized water and filtered through a 0.22 μm filter (Millipore, Nepean, ON, Canada).

Instrumentation. Experiments were performed using a P/ACE MDQ instrument from Beckman Coulter (Fullerton, CA) utilizing laser-induced fluorescence (LIF) detection with excitation at 488 nm and emission at 520 nm. Uncoated fused silica capillaries were used with total lengths of 50 cm for K_d and EC_{50} measurements and 80 cm for aptamer partitioning; the distances to the detection window were 40 and 70 cm, respectively.

Brand new capillaries were preconditioned using a wash with 2 capillary volumes of methanol, followed by 5 capillary volumes of sequential washes with 100 mM HCl, 100 mM NaOH, deionized water and, finally, equilibrated with the running buffer for 10 min.

The sample (either the equilibrium mixture of aptamers and protein or aptamers alone) was introduced into the capillary by a pressure at 1 psi (6.89 kPa) for 28 s and was propagated past the uncooled region of the capillary when the protein was present (see Propagation Past Uncooled Region). The capillary temperature was kept at 15 °C. Electrophoresis was carried out at electric field of 400 V/cm. PCR amplification was performed with a Bio-Rad iCycler (Mississauga, ON, Canada).

Determination of the Aptamer-Collection Window. To determine bulk affinity (EC_{50}) prior to the first selection round, a plug of 200 nM DNA library incubated with 1.6 μM ABH2 for 15 min was injected into a 80 cm-long capillary by a 10 s × 0.5 psi (3.45 kPa) pressure pulse followed by buffer propagation by 0.3 psi pressure for 135 s. In the first selection round, a plug of 25 μM DNA library incubated with 500 nM ABH2 for 15 min was used for injection. In all subsequent rounds, a 100 nM aptamer pool and 400 nM ABH2 were used. The components were separated by electrophoresis at 400 V/cm. Once the protein−aptamer complex was observed, an aptamer-collection window was determined to include the complex as well as a part of the region corresponding to aptamers dissociated from the complex during separation. Care was taken to maintain a 0.5−1 min gap between the collection window boundary and the beginning of the peak of unbound DNA. The following formula was used to calculate elution time ($t_{elution}$) of the selected fraction based on the observed time of

Table 1. Binding Constants and Sequences of Synthesized ABH2 Aptamers

ID	K_d (nM)[a]	k_{off} (s^{-1})	k_{on} (M^{-1} s^{-1})	core sequence (5′→3′)
C1	220	2.2×10^{-3}	1.0×10^{4}	AAGCTATTACCCGCGTTCATAGCTTTGCGTCAGAGCCTCA
C2	150	5.1×10^{-4}	3.4×10^{3}	CTGGAAGGGTTCAGAGGTAAGACTACGATCTTGGCGGGCC
C10	180	2.2×10^{-4}	1.2×10^{3}	GGTTCGGAGGAATCCATGTTGCGAACGACCGCTGCTAGCA
C13	240	1.9×10^{-3}	7.9×10^{3}	AAGTGATGGGGCCAGGTTCAGAATGAGGACATACGGGAGG
C15	390	1.8×10^{-3}	4.6×10^{3}	ATACCAGGGCTCAGAGGATCTGGGGGCCGATGCGTTGGAT
C17	630	9.3×10^{-4}	1.5×10^{3}	AGCGGGGTTGCGGAGTTCTGAGGTCGGCCACTCATGGTGC
C18	35	2.4×10^{-4}	6.9×10^{3}	GCATTACTGGTTCTGAGGAAAGTGCGACTCAGCCGTACGC
C20	85	9.3×10^{-4}	1.1×10^{4}	TGACCGGTTCAGATGGATGGGTCCTTCGCATAGACCTACA

[a]Primer sequences: forward, CTCCTCTGACTGTAACCACG; reverse, GCATAGGTAGTCCAGAAGCC. Experiments were done in duplicates, and the results of a single set of experiments are shown in the table.

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travel to detector ($t_{detector}$), the total length of the capillary (L_{total}), and distance from the inlet to the detector ($L_{detector}$):

$$t_{elution} = t_{detector}\frac{L_{total}}{L_{detector}}$$

The aptamer collection was repeated three separate times per pool with slightly adjusted right boundaries. qRT-PCR was used to confirm a successful collection by comparing the collected fraction to a negative collection (without the presence of the protein target).

Aptamer Selection. For the first round of selection, 25 μM ssDNA library was mixed with 500 nM ABH2 in 50 mM HEPES buffer at pH 7.5. The binding reaction was allowed for 15 min to approach equilibrium, and the DNA−ABH2 complex was separated from the unbound DNA by using NECEEM in an electric field of 400 V/cm. The 50 mM Tris-acetate pH 8.2 was used as a separation buffer. The complex was collected by using a method described in in Determination of the Aptamer-Collection Window, and the amount of DNA was quantified by qRT-PCR using iQ SYBR Green Supermix Bio-Rad iCycler (Mississauga, ON, Canada).

Propagation Past Uncooled Region. In order to move the sample past the uncooled region[55] of the capillary, the pressure-injected plug was further pressure propagated 5 cm into the cooled region of the capillary. The required pressure pulses were 0.3 psi (2.07 kPa) × 2.25 min for an 80 cm long capillary and 0.3 psi (2.07 kPa) × 1 min for a 50 cm long capillary.

Emulsion PCR. Emulsion PCR oil was prepared by combining three emulsion oil components provided by the Micellula DNA Emulsion and Purification Kit (EURx, Poland) in a 73:7:20 ratio as recommended by the manufacturer. A conventional 50 μL PCR mixture with the addition of BSA was added to 300 μL of the oil phase and contained the following: 10× PCR buffer (New England BioLabs, Toronto, ON, Canada), 400 nM forward and reverse primers, 200 nM dNTP mix (each, IDT, Mississauga, Canada), 5 U/μL Taq (New England BioLabs, Toronto, ON, Canada), 0.75 mg/mL BSA (New England BioLabs, Toronto, ON, Canada), nuclease free H_2O (IDT, Mississagua, Canada), as well as 10−20 pM of the collected aptamers. Once combined, an emulsion was formed by vortexing the mixture at a maximum speed for 5 min on ice. After amplification (15 cycles), the contents of PCR tubes were pooled and the emulsion was broken through the addition of 1 mL of isobutanol. The PCR mixture was then purified using the Micellula DNA Emulsion and Purification Kit (EURx, Poland) as per manufacturer's instructions. The purified amplified PCR reaction was eluted with 20 μL of provided elution buffer (EURx, Poland) incubated for 2 min followed by a 1 min centrifugation at 1.3 × 10^4 rpm into a collection tube.

Regeneration of Enriched Pool. The purified emulsion PCR product was amplified asymmetrically in triplicate with a 20:1 ratio of F488-labeled forward primer to biotinylated reverse primer to generate ssDNA product. A volume of 20 μL of streptavidin magnetic beads (Sigma-Aldrich, Oakville, ON, Canada) was magnetized and resuspended in 10 mM Tris-HCl buffer containing 10 mM NaCl and 1 mM EDTA at pH 8.0. Once amplified, the triplicate PCR reactions were combined and incubated with the streptavidin magnetic beads for 30 min at room temperature. The beads were magnetized, discarded and the PCR product was purified using a MinElute purification kit (QIAGEN, Mississauga, ON, Canada) as per manufacturer's recommendation. DNA was then eluted using 10 μL of 50 mM HEPES pH 7.5.

Affinity Analysis. K_d and k_{off} values were determined from NECEEM electropherograms as described elsewhere;[10,56] k_{on} values were calculated as k_{off}/K_d. The NECEEM experiments were performed as follows. A 50-nL plug of the equilibrium mixture containing 100 nM aptamer and 400 nM protein in 50 mM Tris-HCl pH 7.5 was injected into a 50 cm-long capillary by a 0.5 psi (3.45 kPa) × 10 s pressure pulse. An electric field of 400 V/cm was applied to the capillary with a positive electrode at the inlet. The electrophoresis run buffer was 50 mM Tris-acetate, pH 8.2. The sample was detected by LIF detection with excitation at 488 nm and emission at 520 nm.

Cloning. Aptamer pools were amplified using symmetric PCR and unlabeled primers. The formation of dsDNA was assessed using a 4% agarose gel, and the appropriate band was extracted and purified using the QIAquick Gel Extraction Kit (QIAGEN, Mississauga, ON, Canada). The concentration of the DNA was quantified using qRT-PCR. Cloning was performed using the pT7 Blue-3 Perfectly Blunt cloning kit (VWR, Mississauga, ON, Canada) as follows. The eluted DNA was ensured to have blunt ends by using an End-Conversion Mix (provided with the cloning kit). The mix is then ligated into a Blunt vector using T4 DNA Ligase (VWR, Mississauga, ON, Canada). Finally, NovaBlue Singles Competent Cells (VWR, Mississauga, ON, Canada) were transfected with the ligation mixture prepared above. SOC medium (VWR, Mississauga, ON, Canada) was added to the cells, and the mixture was plated on ampicillin agar plates. The cultures were allowed to grow in an incubator at 37 °C overnight. Twenty four colonies were picked and resuspended in 200 μL of H_2O. The resuspended colonies were then placed in a boiling water-bath for 5 min followed by centrifugation at 1.3 × 10^4 rpm for 10 min to pellet the bacterial debris. The supernatant containing the DNA was then amplified in 30 cycles of symmetric PCR as described above using F488-labeled forward primers followed by 15 cycles of asymmetric PCR as described in Regeneration of Enriched Pool. The K_d value was measured for each clone prior to sequencing. Plasmids for sequencing were extracted using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, Toronto, ON, Canada). Once extracted, the plasmids were sent to TCAG DNA Sequencing facilities (The Hospital for Sick Children, Toronto, ON, Canada).

CONCLUSIONS

In conclusion, the procedure for efficient and rapid aptamer selection by NECEEM has been significantly improved. By creating a homogeneous-like amplification environment using ePCR, aptamers with K_d values ranging from 38 nM to 1 μM were selected in as few as four rounds whereas the same method using conventional PCR failed to show any detectable enrichment.

The work leading to the selection of the ABH2 aptamers reported here will serve as a basis in the development of efficient ABH2 and other DNA repair enzyme inhibitors for their potential use in combination with current alkylating drugs.[45,46] This approach could greatly reduce the chemotherapy resistance developed by many neoplastic cells. Such aptamers also have potential to be used as delivery vehicles for cytotoxic drugs to cancer cells.[57] Because of their high affinity and relatively small size, aptamers exhibit durable target tissue retention as well as rapid blood clearance, essential features for

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their use in *in vivo* imaging and drug delivery.[57] The newly developed method can be employed for the rapid discovery of aptamers with potential therapeutic use for other unstable proteins.

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Notes

The authors declare no competing financial interest.

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