2009

Aptamer Selection for Oxidative DNA Lesions

Brandon Lloyd James

APTAMER SELECTION FOR OXIDATIVE DNA LESIONS

By

BRANDON LLOYD JAMES

Honors Bachelors of Science in Chemistry, Oregon State University, Corvallis, OR, 2005
Honors Bachelors of Science in Biochemistry/Biophysics, Oregon State University, Corvallis, OR 2005

Professional Paper

presented in partial fulfillment of the requirements
for the degree of

Master of Science
in Chemistry

The University of Montana
Missoula, MT

July 2009

Approved by:

Perry Brown, Associate Provost for Graduate Education
Graduate School

Dr. Chris Palmer, Chair
Chemistry

Dr. Bruce Bowler
Chemistry

Dr. Stephen Lodmell
Division of Biological Sciences

James, Brandon, M.S., July 2009 Chemistry

Aptamer Selection For Oxidative DNA Lesions

Chairperson: Dr. Chris Palmer

Aptamer science is a growing field of both chemistry and biochemistry. Aptamers bind to specific target molecules, potentially allowing for identification and quantification. The methodologies for selection of aptamers are growing and ever-changing. There is a number of different selection protocols, some specialized and others more general. All have their advantages and limitations. Here I describe a host of these protocols and relate them towards the selection of an aptamer for oxidative DNA lesions, specifically the oxidation products of guanine. Guanine has the lowest reduction potential of the four DNA bases, and as such is the most readily oxidized. The oxidation product 7,8-dihydro-8-oxoguanine (8-oxoG) has been studied extensively over the last decade, but the further oxidation products Spiroiminohydantoin (Sp), Guanidinodihydantoin (Gh), and Iminoallantoin (Ia) are still largely unresearched. Aptamers for these products would prove to be invaluable diagnostic tools for the measurement of oxidative damage to DNA. Attempts to select aptamers toward these compounds are described, and recommendations for further attempts at aptamer selection are provided.

1.0 Introduction

An aptamer is a small, usually single-stranded, DNA or RNA oligonucleotide that preferentially associates with a given target molecule. This association can be used to develop diagnostic tools for these target molecules, including identification and quantification. The term aptamer was coined by the Szostak lab in the early 90s, from the Latin 'aptus', meaning 'to fit'.[1] Aptamer research is a small, yet growing, field that has strong potential for a number of applications. Aptamers are generated from complex synthetic nucleic acid libraries via iterative rounds of selection, partition, and amplification. While the majority of aptamers are man-made molecules, recent research has discovered natural RNA aptamers, termed 'riboswitches', which are used to control gene expression.[2] Aptamers usually range from 30-70 nucleotides, giving them the capacity to form complex three-dimensional structures and the possibility for a variety of binding locations (Figure 1).[3] The aptamer-target binding typically displays a binding constant that is equivalent to most receptor-ligand interactions.[4]

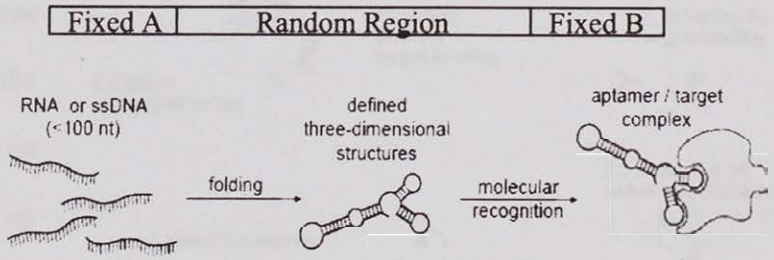

Figure 1: (A) Depiction of a typical ssDNA library. (B) Schematic detailing aptamer-target binding[4]

There is a number of applications for aptamers in therapeutics, biosensing technology, capillary electrochromatography, affinity chromatography, flow cytometry, image analysis, and laser-scanning microscopy.[5,6,7,8,9,10] One of the strengths of the aptamer approach is the ability to select an aptamer for a wide range of targets, from ATP to transcription factors (NF-κB) to

hormones.[11,12,13] A number of aptamer-based biosensors have been developed to date, as well, including biosensors for targets ranging from the nucleoside L-adenosine to cocaine.[14,15]

The most common and widely used selection method for aptamers to date is *in vitro* selection, also termed SELEX (Systematic Evolution of Ligands by Exponential Enrichment). Herein I will discuss the advantages and limitations of various selection methods, including SELEX, CE-SELEX, non-SELEX, Aptamer Selection Express, FluMag-SELEX, Structure-Switching Signaling Aptamer Selection, Tailored-SELEX, and Toggle-SELEX.

1.1 Initial Selection Protocols: *in vitro* Selection

SELEX, also known as *in vitro* selection, has been around for almost 20 years; in August of 1990, the term SELEX was coined and it was used for the first time to select for an aptamer.[16] SELEX relies on variation, selection, and replication (Figure 2) and is limited only by the completeness of the initial library. The idea behind SELEX is fairly simple. A large random DNA or RNA oligonucleotide library is introduced to the required target molecule, at a given concentration. The portions of the library that most strongly associate with the target are isolated, replicated, and reintroduced to the target, potentially at a lower

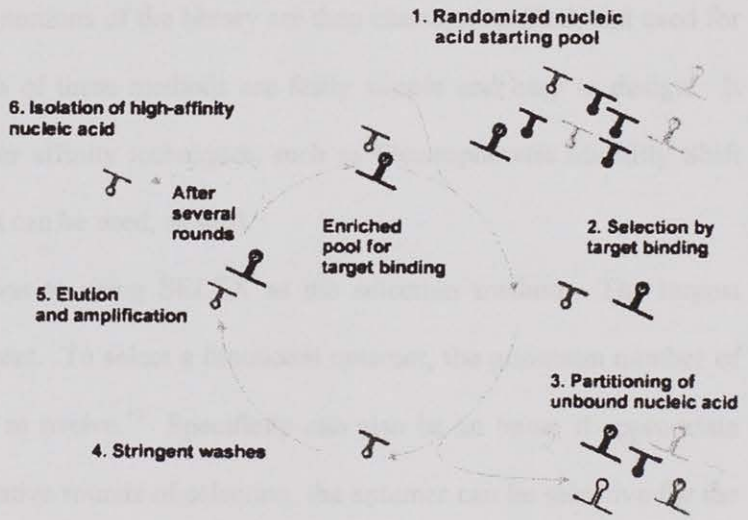

Figure 2: Systematic Evolution of Ligands by Exponential Enrichment (SELEX)[16]

concentration. Once the library has been appropriately reduced, the remaining pool contains potential aptamers. This pool can then be cloned and sequenced to yield the aptamers.

For the selection step, SELEX typically utilizes either affinity chromatography or a filter assay. The filter assay approach is very simple; the target/library solution is passed through a nitrocellulose filter that allows for the non-bound library and target to pass through, while retaining the bound target-library complex, provided that the size difference is sufficient when bound. For the affinity chromatography method, the target molecule is immobilized on an affinity column (typically of sepharose or agarose), the library is passed along this column, and the portion of the library that strongly associates with the target will stick to the column, while the rest washes away. The bound portions of the library are then eluted, amplified, and used for the next round of selection. Both of these methods are fairly simple and easy to design. It should be noted that there are other affinity techniques, such as Electrophoretic Mobility Shift Assay (EMSA) or gel filtration that can be used, as well.

There are several limitations to using SELEX as the selection method. The largest limitation is likely the time constraint. To select a functional aptamer, the minimum number of selection rounds is typically eight to twelve.[17] Specificity can also be an issue; if appropriate steps are not taken to undergo negative rounds of selection, the aptamer can be selective for the wrong target; negative rounds should involve selecting for and discarding aptamer sequences with affinity for similar targets to the one desired, the affinity column itself, or the filter.[18] These portions of the library are thus removed from the main pool before subsequent rounds of selection.

There are additional limitations inherent in the SELEX affinity column approach. One is that the target must be linked to the stationary support. This linkage can block a potential

binding site for the possible aptamer sequences. Also, the strongest binding sequences can pose a problem when it comes to removing them to move on to the next round of selection, due to the strong binding itself. One limitation prevents possible aptamers from finding binding locations, while the other prevents the best aptamers from being selected.

Steps have been taken to overcome the time limitations of traditional SELEX. When the method was originally introduced, each round of selection would take 2-3 days; the entire process could take on the order of months. The Ellington lab at the University of Texas at Austin – one of the first labs to deal with aptamers and SELEX – has managed to improve their protocol with automated steps. They are now able to select an aptamer in a matter of days.[19]

2. Other Selection Techniques

2.1 Capillary Electrophoresis

One way to overcome the time limitations is to utilize different affinity separation techniques for the partitioning of the bound from the non-bound. One such technique is CE-SELEX (capillary electrophoresis SELEX, Figure 3). CE-SELEX is a relatively new method dating back only about five years. The library is incubated in free solution with the target molecule. A small volume of this mixture is introduced to the capillary and separated with an applied potential. Ideally, the sequences of the library that bind to the target will travel at a different velocity

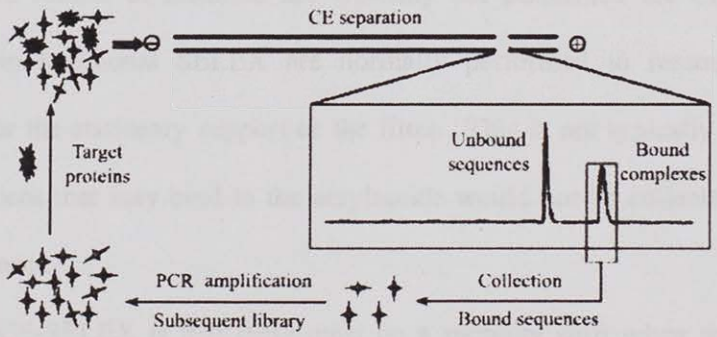

Figure 3: CE-SELEX selection method[20]

through the capillary than those that are not bound. This separates the bound and non-bound quite easily, presuming that there actually is a mobility change for the bound library-target complex. The collected bound sequences are isolated, purified, and PCR amplified before moving on to the next round of selection.[20]

The free solution nature of CE-SELEX prevents two of the previously mentioned limitations of SELEX; there is no linkage to hinder binding site possibilities and strong binding is not a problem because there is no wash step. Both the DNA library and the target are in their normal solution-state structure. There are additional advantages to CE-SELEX, as well. For SELEX, it traditionally takes about six rounds to achieve more than 50% library binding. It has been reported that CE-SELEX can obtain close to 100% library binding within the first two rounds of selection.[21] Rarely is it necessary to perform more than two or three rounds of selection, making CE-SELEX significantly quicker than SELEX. The time factor is a major strength of this method. Negative rounds of selection are typically not performed for CE-SELEX. The negative rounds in traditional SELEX are normally performed to remove sequences that associate with either the stationary support or the filter. This is not typically a concern for CE-SELEX, as sequences that may bind to the acrylamide would not be collected with the bound target/library sequences.

The obvious limitation of CE-SELEX is that there must be a mobility shift when the library sequences are bound to the target. If there is not, it is not possible to resolve the peaks and separate the bound sequences from the nonbinding sequences. The aptamers selected so far using CE-SELEX have been those that exhibit a significant mobility shift. To date, this includes aptamers for the histone H4 peptide, neuropeptide Y, IgE, and protein farnesyltransferase, among others.[22,23,24] These targets range from about 1.5 kDa (a histone H4 peptide) to 200 kDa (IgE).

Targets that would typically allow for an adequate mobility shift would be those that allow for a bound complex with a different net charge than free DNA. For example, protein targets would allow for a mobility shift, whereas DNA base targets would not.

2.2 Non-Selex

One selection method, that utilizes no amplification whatsoever, is referred to as non-SELEX. Non-SELEX selection of aptamers has been around for only a handful of years, as well. The process employs NECEEM (non-equilibrium capillary electrophoresis of equilibrium mixtures) for the partitioning step. The advantage of using NECEEM, as opposed to more classical affinity techniques, is the additional information provided as regards to protein-DNA interactions. The non-equilibrium nature of the electrophoresis promotes the dissociation of the target/library complex. Only the strongest binding sequences with the lowest dissociation constant will elute as the bound complexes. While low-affinity binding has been shown to be a limitation for more traditional CE techniques, NECEEM overcomes this due to the non-equilibrium conditions.[25]

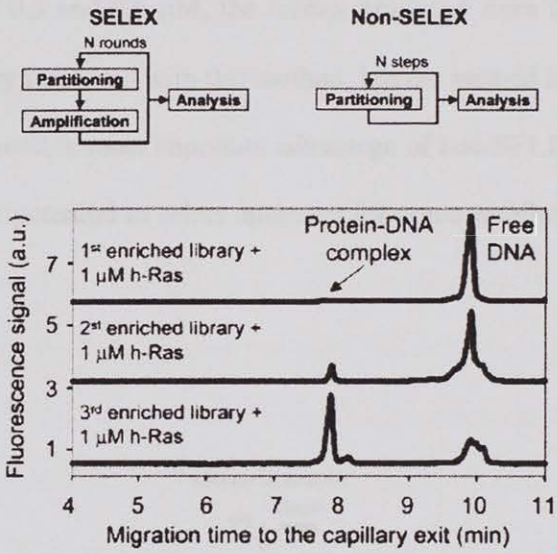

Figure 4: (A) Simple diagram of SELEX vs. Non-SELEX. (B) Example of increased library affinity for the target with subsequent rounds of selection.[26]

Three repetitive partitioning steps are all that is needed for non-SELEX; in one study the K_d improved from 10^4 μM to 0.3 μM after three rounds.[26] Furthermore, the entire process takes close to an hour to complete. This makes the non-SELEX approach far superior when it comes

to time constraints. Non-SELEX is not only fast, it is efficient. Accurate abundances of aptamers can be estimated with this method, as well. However, due to limitations of current commercial CE instruments, the accuracy of the aptamers abundance estimation is limited.

When compared to NECEEM, CE-SELEX – essentially the same as non-SELEX but with the amplification step – non-SELEX compares quite favorably. In one study, the final enriched libraries had comparable affinities of 0.3 and 0.6 μM, the former produced from the non-SELEX method.[26] Not only was the affinity improved with this method, but the method has superior speed and is simpler. The final and possibly most important advantage of non-SELEX over traditional SELEX and CE-SELEX, is the potential to select aptamers for non-amplifiable libraries.

2.3 Aptamer Selection Express

Possibly the newest aptamer selection method is Aptamer Selection Express (ASExp, Figure 5).[27] This method was developed in 2008 at the Air Force Research Laboratories, in an effort to develop a new separation method to combat the cross-reaction (i.e. selectivity) difficulties they were experiencing when attempting to make new DNA capture element (DCE) systems, aka aptamers. With a new method that is more selective

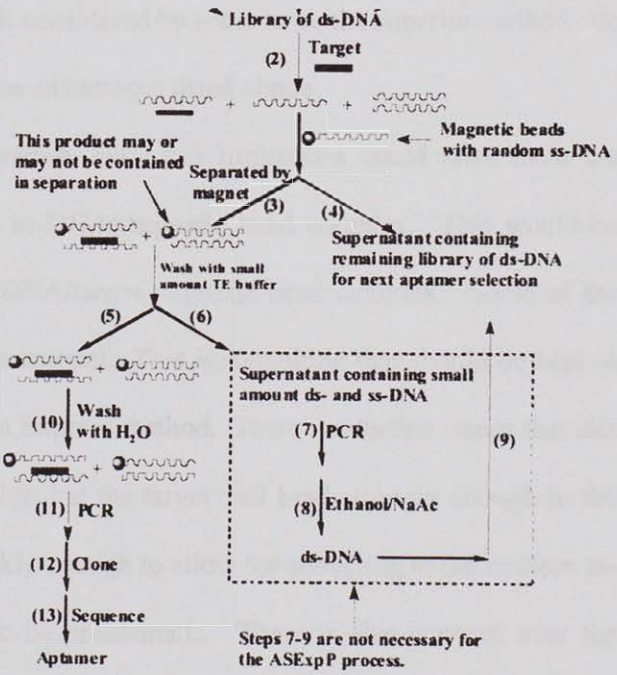

Figure 5: Selection scheme for Aptamer Selection Express.[27]

and specific, the hope was that these selectivity problems would decrease. The ASExp process begins with introducing the target molecule to a ds-DNA library. The amount of target needed can be in the sub-nanogram range. When the target associates with the library, it will form a target/ss-DNA complex. This complex is then separated from the remaining library pool by utilizing magnetic beads that are bound with random ss-DNA. A simple magnet is all that is needed to then separate the bound complex from the unbound library, under the assumption that the target/ss-DNA complex will anneal to the random ss-DNA/magnetic bead pool. The selected library is then washed and the bound aptamers are amplified, cloned and sequenced.

The major stated advantage of this approach is that only a single selection step is necessary. This can drastically reduce the amount of time it takes to select an aptamer. The other major strength of this method is that very little target is actually needed. On the down side, it has been shown that the ASExp method still exhibits the same cross-reaction problem that the traditional SELEX method has. ASExp is still considered by some to be the superior method due to the greatly decreased cost, resulting from the advantages listed above.

It was not stated in the ASExp literature, but other limitations could arise from the resultant unbound ss-DNA binding with the ss-DNA/magnetic bead complex. This would be recognized and amplified along with the ss-DNA/target/magnetic bead complex. Some of the eventual sequenced "aptamers" may not be functional. This is something that should be kept in mind when considering the Aptamer Selection Express method. There are further issues that this author has with this stated approach. The idea that the target will bind strongly enough to the initial library to promote dissociation but weakly enough to allow for annealing to the random ss-DNA/magnetic bead complex would seem to be problematic. There is also concern over the

degree of affinity that would be exhibited by the resultant aptamer, considering that there is only a single step to the reaction.

2.4 FluMag-SELEX

FluMag-SELEX is another modified SELEX protocol, named so because of the fluorescent tags and magnetic beads used in the method (Figure 6). The magnetic beads are used for target partitioning. Targets are immobilized on magnetic beads before introduction to the ssDNA nucleic acid library. Fluorescent labels are used for DNA quantification and are added with the first amplification step with fluorescently-labeled primers. These tags allow for easy determination of the efficiency of the process with each round of selection. FluMag-SELEX was specifically designed to select DNA aptamers for a wide range of targets with different properties and of different sizes. As with Aptamer Selection Express, the magnetic beads provide for fast and efficient separation of bound and non-bound library-target complexes; the placement of the magnetic beads obviously differs between the two methods. Fluorescent labeling is used for

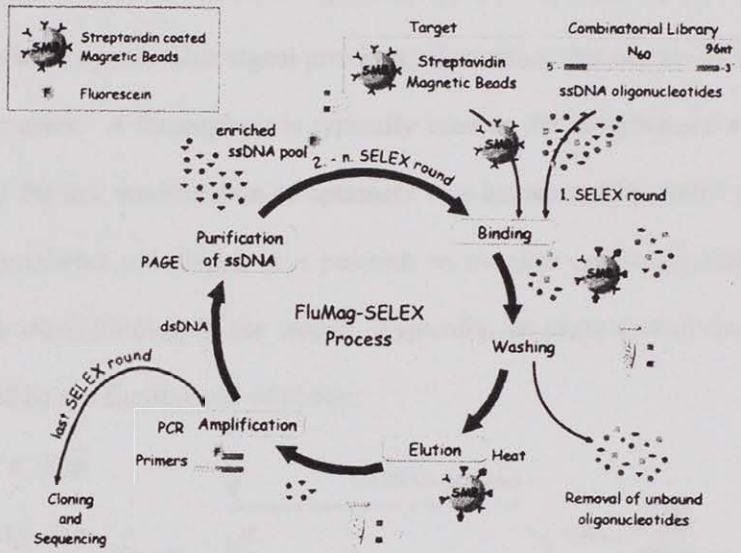

Figure 6: Detailed scheme of the FluMag-SELEX process, specifically for the selection of an aptamer for streptavidin[28]

quantification. This process has been shown to work for a variety of targets and the easy-handling process allows for processing of multiple targets.[28]

2.5 Structure-Switching Signaling Aptamers

Signaling aptamers, like the biosensors mentioned previously, are modified aptamers with the ability to produce a recordable signal. This signal provides information that can be used to quantify the amount of target present. A fluorophore is typically used as the modification to the aptamer of choice. A method for this modification of aptamers was introduced by Andrew Ellington, et al. in 1999.[29] Fluorophores are placed in a position to monitor conformational changes in the aptamer that arise when binding to the target. Typically, residues that do not interact with the target are replaced by the fluorophore of choice.

This method was taken a step further with the idea of using the conformational changes inherent with ligand-binding to produce a stronger fluorescence response than previously by introducing quenchers. This approach is applicable to almost all DNA aptamers, based upon the fact that these aptamers share the trait of forming both a duplex structure with antisense DNA as well as a complex structure with their respective target molecule. The key is to design the antisense DNA in such a way that the interaction with the target molecule allows for the antisense DNA to dissociate; this can involve altering the number

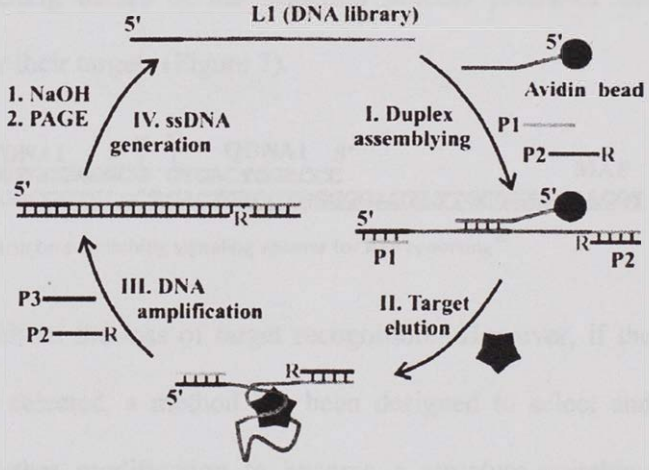

Figure 7: In vitro selection of structure-switching aptamers[30]

of base-pair matches or introducing mismatch pairs. This class of designed aptamers is referred to as structure-switching signaling aptamers.[30]

The selection process itself is a bit different from the other selection techniques. The initial DNA library is bound to a partially complementary sequence, dubbed the capture oligonucleotide. The capture oligonucleotide has a quencher tag adjacent to a fluorophore tag on the library strand. When the target approaches, the portions of the library with the appropriate sequence will adopt a three-dimensional conformation, which ideally will cause dissociation of the library from the capture oligonucleotide, yielding an increase in fluorescence signal. The unbound sequences are partitioned from the bound sequences by spinning the solution down; anything containing a streptavidin bead will sink to the bottom, leaving the bound sequences in the supernatant. The bound sequences are cleaned up, amplified, and moved on to the next round of selection. The structure-switching nature of the selection process promotes the selection of aptamers with high-affinity for their targets (Figure 7).

As mentioned, previously existing aptamers can be modified to become structure-switching signaling aptamers without the loss of target recognition. However, if the desired aptamer has not been previously selected, a method has been designed to select and produce an aptamer which needs no further modification to become a structure-switching signaling aptamer.[31] This method allows for production of ready-made biosensors. This method has produced a number of biosensing aptamers, including one for ATP (Figure 8). The system is designed to place a fluorophore adjacent to the respective quencher. When the system is intact (as in figure 8), the fluorescence signal is quenched. As the binding domain (underlined)

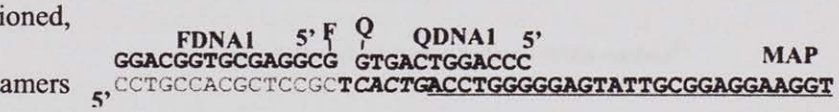

Figure 8: Structure-switching signaling aptmer for ATP reporting[30]

associates with the target, a conformational change will take place leading to the dissociation of the quencher strand. The further the quencher moves from the fluorophore, the stronger the measured fluorescence signal. As such, the measured signal provides a good indication of the amount of target present in the system.

2.6 Tailored-SELEX

Tailored-SELEX was designed with a specific purpose in mind: to rapidly develop and isolate an aptamer sequence with only ten fixed nucleotides in addition to the random region. Typically the required primer sites on either side of the random region require a larger fixed region, usually 15-25 nucleotides. Depending on the length of the random region, the size of the selected aptamer may prove difficult when it comes to chemical synthesis of the developed aptamer. The fixed regions may not be simply omitted because when an aptamer sequence binds to a target it forms a three-dimensional binding structure that may involve the fixed regions. Standard chemical synthesis is most efficient at sizes less than 60 nucleotides. As such, Tailored-SELEX (Figure 9) has

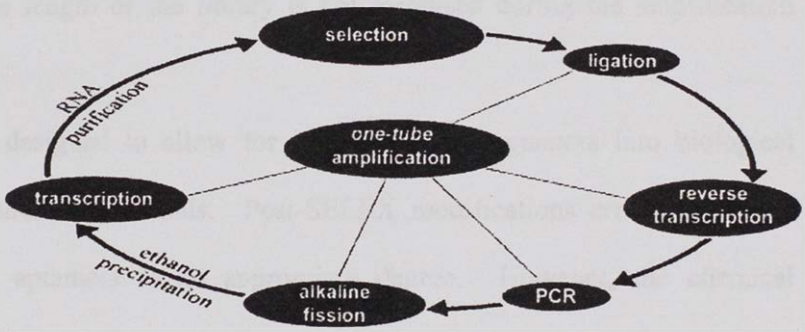

Figure 9: Flowchart for the Tailored-SELEX method[32]

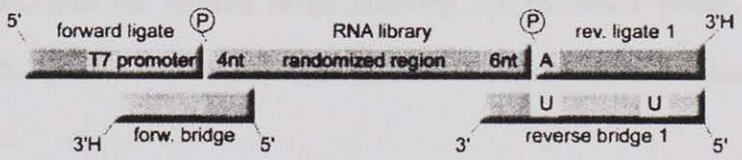

Figure 10: Library design for Tailored-SELEX[32]

developed an aptamer selection methodology involving no more than ten fixed nucleotides. Customized primers are designed for Tailored-SELEX that can be added via ligation before amplification and removed during the amplification process.[32]

The fixed regions are used for the ligation of the primer binding sites. This ligation is carried out with the assistance of the 'bridge' oligonucleotides (Figure 10). The bridges contain complementary sequences to both the fixed regions and the primer binding sites. Once ligation has taken place, amplification can proceed. Reverse transcription takes place under high temperatures to ease the removal of the forward bridge and alkaline fission allows for removal of the reverse bridge. Thus, the length of the library is not increased during the amplification process.

Tailored-SELEX was designed to allow for introduction of aptamers into biological living systems, e.g. cell cultures and animals. Post-SELEX modifications are necessary to increase the stability of the aptamers to an appropriate degree. However, the chemical modifications necessary are not as feasible at the increased size of some aptamer libraries – the cost increases substantially while the yield decreases. Tailored-SELEX is designed to produce aptamers under 60 nucleotides, into the desired range allowing for increased yields and decreased cost. Furthermore, this process can be developed into an automated aptamer selection protocol.

2.7 Toggle-SELEX

The Toggle selection method, developed by the Sullenger Lab at Duke University, is a novel method designed to promote cross-reactivity among species. For example, they developed a family of aptamers that all recognized and bound to both the human and porcine versions of

thrombin. These aptamers with cross-reactivity have a strong potential for gene therapy and other therapeutic applications, due to their ability to recognize variability in targets (e.g., epitopes, homologs, etc). Cross-reactivity is typically desirable when dealing with pre-clinical studies in animal models. To achieve this, specificity of the aptamers must be adequately reduced to allow for the cross-reactivity.[33]

The Toggle-SELEX process is carried about by alternating the target during the selection process (Figure 11). In the aforementioned example of the human-porcine thrombin toggle-aptamer, human thrombin was used to select for the odd rounds and porcine thrombin was used to select for the even rounds. Both human thrombin and porcine thrombin were used as targets in the initial round of selection. By alternating between human and porcine proteins, the aptamer selection tends toward selecting for evolutionarily conserved regions. As with traditional SELEX, once the library was reduced to a significant degree, the remaining pool is cloned and sequenced.

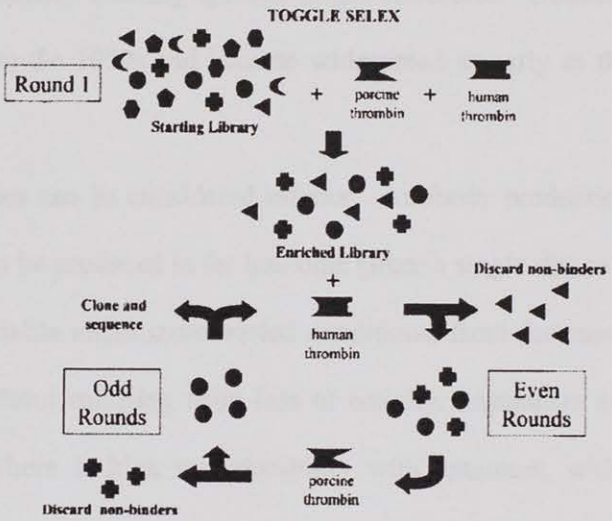

Figure 11: Toggle-SELEX for the human/porcine thrombin aptamer.[33]

3.0 Competing Technologies

Antibodies, aka immunoglobulins (Ig), are proteins found in the body that are utilized by the immune system to find and neutralize foreign agents.[34] The general structure of all

antibodies is quite similar. The difference lies in the hypervariable region, which is a small area on the tip of the protein. It is this region that differentiates antibodies and allows for the binding of an antibody to an antigen.[35] An antigen is a substance that stimulates *anti*body *gen*eration. Once an antibody recognizes and binds to an antigen, it is tagged for response and attack by the immune system. Antibodies are also capable of neutralizing targets. Similar to aptamers, antibodies can be used to recognize and possibly quantify specific target molecules. Utilizing antibodies to detect molecules dates back to the 1950s and became widespread as early as the 1970s.[36]

In comparison to aptamers, antibodies can be considered inferior. Antibody production can take up to 6 months, while aptamers can be produced in far less time (from a single day to 8 weeks). Unlike antibodies, aptamers are stable under more varied conditions, from increased temperature to harsh buffer conditions, without suffering from loss of activity. Aptamers are smaller. They are easier to engineer. There is high reproducibility with aptamers, while antibodies can suffer from batch-to-batch variation in performance. Aptamers are easier to develop into a drug screening program. Aptamers can be chemically synthesized; antibodies require the use of animals. Antibodies have a limited shelf-life and may undergo denaturation.

It has been a slow process, as aptamer science is still in development, but aptamers may replace antibodies in diagnostic applications, identification and quantification.[37] Aptamers have been shown to function more efficiently than antibodies; however, to date, antibodies have been shown to be more versatile for selection. It is uncertain how many different molecules aptamers can be selected for. Furthermore, there is certainly no guarantee that aptamers will always work better than antibodies. Antibodies have been shown to work as well, or better, than aptamers in certain cases. [38] Antibodies are good at what they do, but they can be difficult to work with.

4.0 Selection of Aptamers for Oxidative Lesions

Of the four DNA bases, guanine has the lowest reduction potential. As such, it is the most likely to be oxidized when DNA is subjected to oxidizing agents. Upon oxidation of guanine, 7,8-dihydro-8-oxoguanine – henceforth referred to as 8-oxoG – is formed. 8-oxoG has an even lower reduction potential than guanine. Upon oxidation of 8-oxoG, a number of products are formed, including Spiroiminohydantoin (Sp), Guanidinodihydantoin (Gh), and Iminoallantoin (Ia), the latter two being isomers (Figure 12)[39]. These oxidation products are good indicators of oxidative damage to DNA. A biosensor that can detect and quantify these molecules – possibly in real time and at room temperature – would be an invaluable diagnostic tool. Both Gh and Ia have proven to be rather difficult to recognize and quantify via traditional detection methods. A new detection method, utilizing aptamers was explored. To date, no aptamer for Sp, Gh, or Ia has been selected. However, an RNA aptamer for 8-oxoG was selected by Loeb, et al., at the University of Washington School of Medicine a number of years ago.[40]

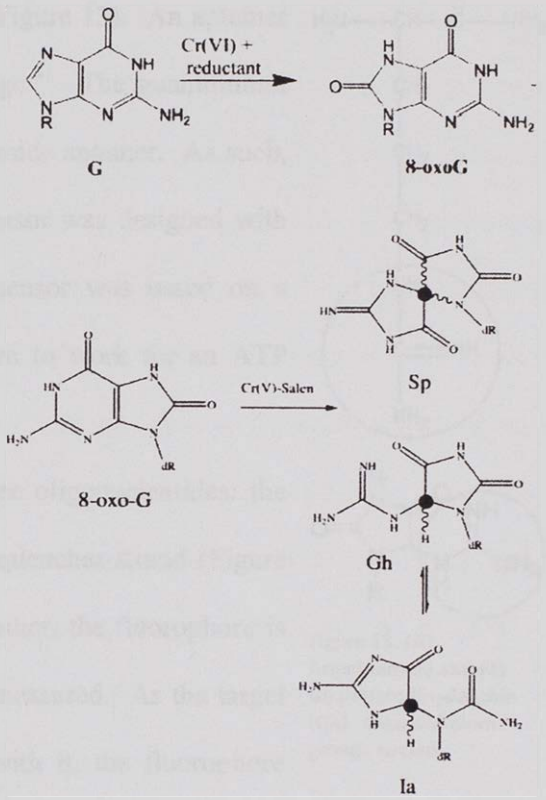

Figure 12: (A) Oxidation of Guanine. (B) Oxidation of 8-oxoG.[39]

4.1 Argininamide Aptamer

Argininamide (R-amide) bears a structural similarity to Gh, in that both contain the guanidinium functional group (Figure 13). An aptamer was selected for R-amide a number of years ago.[41] The guanidinium functional group is the primary target of the R-amide aptamer. As such, this aptamer might also select for Gh. A biosensor was designed with this aptamer and tested against Gh. The biosensor was based on a tripartite system that had previously been shown to work for an ATP aptamer.[42]

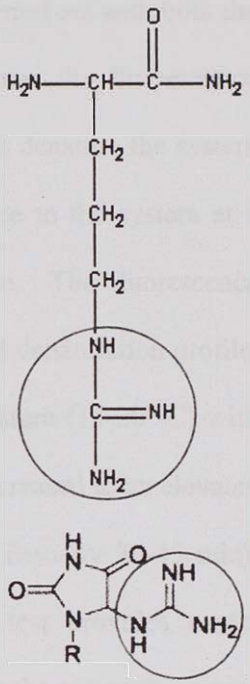

Figure 13: (A) Argininamide and (B) Guanidinodihydantoin (Gh). Guandidinium groups circled.

The tripartite system is composed of three oligonucleotides: the aptamer strand, the fluorophore strand, and the quencher strand (Figure 14). When the system is properly annealed together, the fluorophore is quenched and no fluorescence (or very little) is measured. As the target approaches the aptamer strand and associates with it, the fluorophore strand and the quencher strand dissociate from the aptamer strand. The increased distance between the fluorophore and the quencher leads to an increase in the fluorescence signal, which can be measured and possibly quantified.

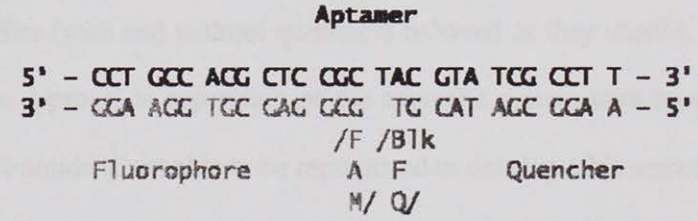

Figure 14: Tripartite aptamer design

The designed system was tested against prepared Gh, as well as various concentrations of R-amide, as a control. Three different tests were performed to test the viability of the system for

both Gh and R-amide. A thermal denaturation profile (from 90-20° C, at 1°/min) was measured to determine at what temperature the system anneals together. This was carried out with both the entire system and without the quencher strand; this information indicates the fluorescence difference maximum, yielding a high end expected signal should the target denature the system at these given temperatures. The second test involves introducing analyte to the system at a specified holding temperature and measuring the change in fluorescence. The fluorescence increase should be roughly equivalent to what is predicted from the thermal denaturation profile. The final test is a temperature jump. The system begins at a low temperature (15-20° C) with analyte present. As quickly as the instrument allows, the temperature is increased to an elevated temperature (45-50° C, typically) and held there for an extended period (usually 30-45 min), after which the temperature is lowered to room temperature. This test provides useful information on how well the target is denaturing the system by increasing the temperature past the annealing point. It is possible that the aptamer strand will not associate strongly enough with the target to induce denaturation and will need assistance.

Ultimately, the system failed to yield positive results for either Gh or R-amide. The acquired thermal denaturation profiles (with and without quencher) behaved as they should, but the analyte testing did not indicate appropriate separation of the tripartite system with analyte present. The aptamer selected for R-amide is not able to be repurposed to develop a biosensor by using this approach. A new approach will be necessary if a biosensor is to be designed for Gh.

4.2 Selection of an 8-oxoG Aptamer

A modification of the structure-switching signaling aptamer selection approach was used to attempt to select an aptamer for 8-oxoG. As mentioned, an aptamer for 8-oxoG has been previously selected by another lab; the ultimate goal here was to select a biosensor for Gh and possibly Sp. It was determined that the method would be used to select an aptamer for 8-oxoG first, as 8-oxoG is more stable than the further oxidation products. Following that, a similar approach would be used to select an aptamer for Gh/Ia and/or Sp.

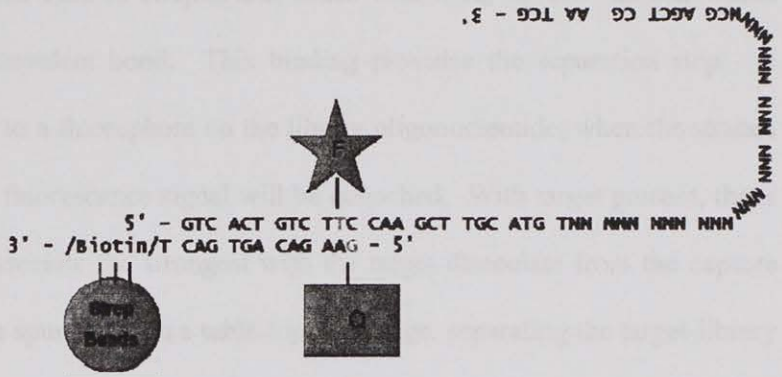

Figure 15: Library oligonucleotide and capture oligonucleotide for the selection of an aptamer for 8-oxoG.

The aptamer selection system was designed using two separate oligonucleotides. The first oligonucleotide, designated as the library oligonucleotide, contained a 24 nucleotide random sequence flanked by primer regions, and a short sequence complementary to the second oligonucleotide. The second oligonucleotide is designated

Round	% Eluted w/ 100 μM G	% Eluted w/ 8oxoG	8oxoG Conc (μM)
1		30	100
2		34	100
3		38	100
4		35	100
5		47	100
6		60	100
7		75	100
8		30	10
9		35	10
10		49	10
11		60	10
12		83	10
Negative	27	37	10
14		43	10

Figure 16: Library affinity for 8-oxoG with increasing rounds of selection.

the capture oligonucleotide (Figure 15). A biotin tag was placed 3' on the capture oligonucleotide. The biotin will bind to streptavidin beads with a K_d of 10^{-15} mol/L, which approaches the strength of a covalent bond. This binding provides the separation step. A quencher is placed 5', adjacent to a fluorophore on the library oligonucleotide; when the strands are appropriately annealed, the fluorescence signal will be quenched. With target present, those sequences in the library that associate the strongest with the target dissociate from the capture oligonucleotide. The solution is spun down in a table-top centrifuge, separating the target-library complex (which remains in the supernatant) from the unbound library-capture complex (which settles at the bottom, along with the unbound capture oligonucleotide). The beads are washed with buffer until the fluorescence of the supernatant reaches background levels. After washing, the remaining library bound to the capture oligonucleotide is eluted with 0.1% NaOH. The fluorescence of this is measured to ascertain the amount of library that did not associate with the target. This provides a useful comparison to the fluorescence measurements of the bound library-target complex.

Fourteen rounds of selection proceeded before the library pool was sufficiently reduced and the affinity for the target was sufficiently high at a concentration of 10 μM 8-oxoG (Figure 16). One round of negative selection with 100 μM Guanine is included in the fourteen rounds.

- **ACCCGGACCACAGGCCAACCCCGC**
- **AGGCGGGGGGACAGAAAGAGGGGG**
- **ACAGGGGGGAGACTTGTCGCAGGC**
- **GGAGGCGGCCGGTGACCTCTGCGG**

Figure 17: Selected Aptamer Sequences for 8-oxoG

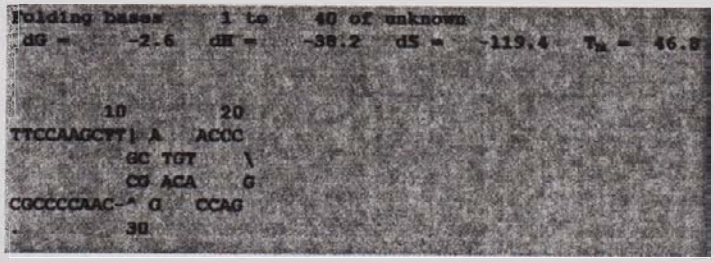

Figure 18: Folding Pattern for Selected 'Aptamer' Sequence

The reduced library pool was cloned with P-GemT-Easy (a TA-based cloning kit) and grown on carbenicillin LB plates. The recovered clones were sequenced to yield potential aptamers. Four different sequences were obtained (Figure 17). Theoretical folding structures were obtained for the four sequences using Mfold (http://mfold.bioinfo.rpi.edu/) and it was determined that the sequence AC CCG GAC CAC AGG CCA ACC CCG C was the most likely aptamer sequence (Figure 18).

A new oligonucleotide was ordered from IDTDNA similar to the original library, with the "aptamer" sequence replacing the random region. This aptamer oligonucleotide was tested against 8-oxoG in a manner similar to the selection. The selection had been designed in such a way that it could easily be turned into a biosensor. The aptamer oligonucleotide was annealed to the capture oligonucleotide and introduced to 8-oxoG. The original library was tested in a similar manner, as a control. The testing did not indicate a strong preference for the 8-oxoG in either the aptamer oligonucleotide or the original library, despite the marked increase in 8-oxoG concentration (>5 mM) (Figure 19). The aptamer oligonucleotide behaved near identically to the original library; a greater affinity was not evident. The indication is that an aptamer was not yielded from the selection.

	Fluorescence Measurements			
Final [8oxoG]	Aptamer		Library	
	w/ 8-oxoG	w/o	w/ 8-oxoG	w/o
	1011	1100	814.1	605.5
5 mM	934.1	1082	679.5	757.5
7 mM	983.1	1128	705	769.4
8 mM	892.5	1104		

Figure 19: 8-oxoG affinity comparisons between the selected 'aptamer' and the initial library.

A second attempt at selecting an aptamer for 8-oxoG proved equally fruitless. The approach was modified slightly in a couple of ways. The complementary bases between the library and the oligonucleotide were adjusted to allow for easier dissociation. The PCR

amplifications were ethanol precipitated to remove the enzymes and purified to remove the primers, to provide for the cleanest possible strands. The amplifications were also run out on a 0.8% agarose gel to ascertain their purity. This attempt was abandoned after the initial rounds indicated neither increased affinity nor much affinity at all.

It is uncertain whether or not it is possible to select an aptamer for 8-oxoG with the method designed in this manner. Furthermore, there has been no evidence to indicate that all molecules are capable of forming aptameric relationships. It is the author's opinion that a different selection method may prove to have more success in the development of an aptamer for this molecule.

4.3 Aptamer Selection Difficulties

The designed method for the selection of the 8-oxoG aptamer as described in section 4.2 was not a strict SELEX-based protocol. Elements from different selection techniques were used to develop a protocol that would potentially yield a ready-to-be-used biosensor. Unlike SELEX, neither an affinity column nor a filter assay was used for the selection step. The selection involved and required the denaturation of a library/capture oligonucleotide complex. This assures that a selected aptamer would have a very high affinity for the target. However, the high affinity that is required is also a deterrent in the selection process itself. The conformational changes that occur in the random region of the library oligonucleotide when bound to target may not be sufficient to dissociate the library from the capture oligonucleotide, despite the relatively small number of complementary base pairs. Possible aptamer sequences can be easily lost in this situation. An aptamer for 8-oxoG may not bind 8-oxoG in the necessary manner for this selection technique to properly work.

The streptavidin bead separation step also yielded a number of problems. The biotin-streptavidin bond never formed as it should. Far too many appropriately-labeled capture oligonucleotide strands either did not attach to the streptavidin beads or the bond broke far too easily – which is highly unlikely, as the streptavidin-biotin bond is among the strongest for any known non-covalent bond.[43] Regardless of the reason why, this step proved to be very limiting; far too much of the library-capture complex was found in the supernatant during the initial wash cycles prior to target introduction. The loss of so much material limits the sequences that are available for aptamer selection.

A different method is necessary for the selection of an aptamer for 8-oxoG. Too many difficulties were found with this method. There are other selection methods that may prove to be more advantageous. A completely different approach would yield additional information as to whether or not an aptamer can actually be selected for this molecule.

4.4 Future Selection Considerations

These initial experiments failed to select or identify a useful aptamer for 8-oxo-G, Sp, Gh, or Ia. However, due to their biological significance and the current analytical difficulties in determining these compounds, they remain important targets for aptamer selection and biosensor development. The question remains, however, what approach is likely to generate a useful aptamer.

A method utilizing CE-SELEX can be a good place to start for the selection of a typical aptamer. It is a fairly straightforward method and results can be seen in a fairly short amount of time. However, in the case of 8-oxoG, this may prove to be difficult. CE-SELEX is known to work for larger molecules and, as yet, is unproven when it comes to molecules the size of 8-

oxoG. It is very likely that no mobility shift will be seen when the library is bound to target. As such, CE-based SELEX would not be a recommended starting place for the selection of this aptamer.

The Aptamer Selection Express method is a protocol that has possibilities for the selection of an 8-oxoG aptamer. Again, the method is fairly straightforward, other than the designing of the random ss-DNA/magnetic bead complex and the random ds-DNA library pool. Ideally, the size of the 8-oxoG target would work well for this method. Hopefully, the 8-oxoG target would associate strongly enough with the appropriate aptameric sequences to allow for the dissociation of that oligonucleotide from its complementary strand, yet weakly enough that the oligonucleotide/target/magnetic bead complex can form. This selection method has been shown to work, but only a handful of times. It is uncertain whether or not this method would work for 8-oxoG. This author has reservations about this method and its design and believes that it bears further investigation.

The Tailored-SELEX approach, while possibly an approach that may work for 8-oxoG, is not a method that this author would recommend for this selection. The method is rather complicated and quite specialized. The considerations that are employed are not a large concern for this selection. Likewise, the Toggle-SELEX method would not be a good method for 8-oxoG. The method is novel and yields highly useful aptamers, but is not applicable to this problem.

The structure-switching signaling aptamer approach is a good approach to take for the selection of this aptamer. The structure-switching signaling approach is valued because of the innate ability to produce ready-made biosensors. As a biosensing 8-oxoG aptamer is the desired product, this would seem to be the natural method to utilize. However, the slightly altered

structure-switching signaling approach used for the attempted selection described in section 4.2 proved unsuccessful. That method employed a target library whose folding structure would alter in the presence of target and produce a signal. Magnetic beads were used for the partitioning step.

Given the lack of success with the attempted selection approach, it may be advisable to apply the structure-switching signaling approach to an aptamer selected by a previous method. A selected aptamer can be modified with fluorophores and quenchers to create a structure-switching signaling aptamer (section 2.5). As mentioned earlier, an RNA aptamer for 8-oxoG was selected a number of years ago.[39] If desired, this sequence could potentially be developed into a biosensing aptamer. Barring that, the approach used to select for this RNA aptamer could be duplicated to select a new aptamer for 8-oxoG or even aptamers for the further oxidation products Sp and Gh.

The FluMag-SELEX approach may prove to be the recommended method for the selection of an aptamer for 8-oxoG, Gh, or Sp. While utilizing streptavidin beads as the partitioning step, this approach removes the biotin-streptavidin binding difficulties. If the target can be directly bound to magnetic streptavidin beads, the partitioning step would be quite simple. FluMag-SELEX has been shown to work for the selection of an aptamer for ethanolamine (Figure 20), which is to date possibly the smallest successful molecular aptamer target.[44] This author feels comfortable in recommending this as the preferred approach.

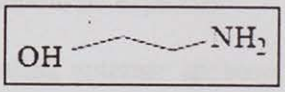

Figure 20: Structure of ethanolamine[44]

5.0 Concluding Remarks

Aptamer selection is a growing field of chemistry and biochemistry. In the almost twenty years since the beginning of aptamer science and the SELEX selection method, a large number of aptamers have been selected for a wide variety of molecules and numerous selection methods have been developed. As an emerging field, aptamers stand poised to replace antibodies in many identification and diagnostic procedures. They offer many advantages over antibodies, while having almost none of the same limitations. The only setback for aptamers in this regard at the moment is the increased number of antibodies that have been found versus the number of aptamers that have been selected.

The SELEX methods that utilize capillary electrophoresis (CE-SELEX and non-SELEX) are among the superior selection methods to date. The time necessary is greatly reduced from traditional SELEX and far fewer selection rounds are needed. The nonspecific binding limitation is also overcome by utilizing capillary electrophoresis. CE-SELEX would be the first step in designing a system to select a new aptamer. However, for 8-oxoG and the further oxidation products, it is more than possible that this approach will not work. A mobility shift will not likely be seen for the bound library-target complex. A different method should be explored.

Had it not already been attempted, the structure-switching signaling aptamer approach might be the recommended method for the selection of aptamers toward oxidative DNA lesions. Another recommended method could be to take a step back and use a simpler SELEX approach, perhaps similar to that which was already used to select the RNA 8-oxoG aptamer to select for Sp and Gh aptamers. The specific attachment of the 8-oxoG to the affinity matrix can not be duplicated for Sp or Gh, but a slightly modified coupling has possibilities. Ideally, an aptamer selected using that method could later be altered and developed into a biosensor. Furthermore,

the previously selected 8-oxoG aptamer can possibly be modified to create a new structure-switching signaling aptamer. There are problems with this method, mainly the time constraints and the need for further modification.

The recommended approach is to use the FluMag-SELEX method. This method has been shown to work for molecules smaller than 8-oxoG, Gh, or Sp. The method is fast and efficient. The previously seen difficulties with streptavidin beads are removed with this method as no biotin-streptavidin binding is necessary. The selected aptamer could later be developed into a biosensor.

---

[1] Ellington AE, Szostak JW. In vitro Selection of RNA Molecules that Bind Specific Ligands. *Nature* 1990; 346: 818-822.

[2] Winkler W, Nahvi A, Breaker RR. Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. *Nature* 2002;419:952-956.

[3] Herman T, Patel DJ. Adaptive Recognition by Nucleic Acid Aptamers. *Science* 2000;287:820-825.

[4] Gold L, Polisky B, Uhlenbeck O, Yarus M. Diversity of Oligonucleotide Functions. *Annu Rev Biochem* 1995;64:763-797.

[5] Mukhopadhyay R. Aptamers Are Ready For The Spotlight. *Anal Chem* 2005; 115-118.

[6] Shamah SM, Healy JM, Cload ST. Complex Target SELEX. *Acc Chem Res* 2008;41(1):130-138.

[7] Rehder MA, McGown LB. Open-tubular capillary electrochromatography of bovine β-lactoglobulin variants A and B using an aptamer stationary phase. *Electrophoresis* 2001;22(17):3759-3764.

[8] Potyrailo RA, Conrad RC, Ellington AD, Hieftje GM. Adapting Selected Nucleic Acid Ligands (Aptamers) to Biosensors. *Anal Chem* 1998;70(16):3419-3425.

[9] Deng Q, German I, Buchanan D, Kennedy RT. Retention and Separation of Adenosine and Analogues by Affinity Chromatography with an Aptamer Stationary Phase. *Anal Chem* 2001;73(22):5415-5421.

[10] Davis KA, Abrams B, Lin Y, Jayasena SD. Use of a high affinity DNA ligand in flow cytometry. *Nucleic Acids Res* 1996;24:702-707.

[11] Nutiu R, Li Y. In Vitro Selection of Structure-Switching Signaling Aptamers. *Angewandte Chemie International Edition* 2005; 44(7):1061-1065.

[12] Cassiday LA, Maher LJ. In Vivo Recognition of an RNA Aptamer by Its Transcription Factor Target. *Biochemistry* 2001;40(8):2433-2438.

[13] Levesque D, Beaudoin JD, Roy S, Perreault, JP. *In vitro* selection and characterization of RNA aptamers binding thyroxine hormone. *Biochem J* 2007;403(1):129-138.

[14] Kleinjung F, Klussman S, Erdmann A, Scheller FW, Furste JP, Bier FF. High-Affinity RNA as a Recognition Element in a Biosensor. *Anal Chem* 1998;70(2):328-331.

[15] Baker BR, et al. An Electronic, Aptamer-Based Small-Molecule Sensor for the Rapid, Label-Free Detection of Cocaine in Adulterated Samples and Biological Fluids. *J Am Chem Soc* 2006;128(10):3138-3139.

[16] Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* 1990;249(2968):505-510.

[17] Rimmele M. Nucleic Acid Aptamers as Tools and Drugs: Recent Developments. *ChemBioChem* 2003;4:963-971.

[18] Jenison RD, Gill SC., Pardi A, Polisky B. High-resolution molecular discrimination by RNA. *Science* 1994;263:1425–1429.

[19] Cox JC, Rajendran M, Riedel T, Davidson EA, Sooter LJ, Bayer TS, Schmitz-Brown M, Ellington AD. Automated acquisition of aptamer sequences. *Comb Chem High Throughput Screen* 2002;5:289–299.

[20] Mendonsa SD, Bowster MT. In Vitro Evolution of Functional DNA Using Capillary Electrophoresis. *J Am Chem Soc* 2004;126:20-21.

[21] Mendonsa SD, Bowser MT. In Vitro Selection of High-Affinity DNA Ligands for Human IgE Using Capillary Electrophoresis. *Anal Chem* 2004;76:5387-5392.

[22] Lin L, Hom D, Lindsay SM, Chaput JC. In Vitro Selection of Histone H4 Aptamers for Recognition Imaging Microscopy. *J Am Chem Soc* 2007;129:14568-14569.

[23] Mendonsa SD, Bowser MT. In Vitro Selection of Aptamers with Affinity for Neuropeptide Y Using Capillary Electrophoresis. *J Am Chem Soc* 2005;127:9382-9383.

[24] Mosing RK, Mendonsa SD, Bowser MT. Capillary Electrophoresis-SELEX Selection of Aptamers with Affinity for HIV-1 Reverse Transcriptase. *Anal Chem* 2005;77(19):6107-6112.

[25] Berezovski M, Krylov SN. Nonequilibrium Capillary Electrophoresis of Equilibrium Mixtures – A Single Experiment Reveals Equilibrium and Kinetic Parameters of Protein-DNA Interactions. *J Am Chem Soc* 2002;124:13674-13675.

[26] Berezovski M, Musheev M, Drabovich A, Krylov SN. Non-SELEX Selection of Aptamers. *J Am Chem Soc.* 2006;128:1410-1411.

[27] Fan M, et al. Aptamer Selection Express: A Novel Method for Rapid Single-Step Selection and Sensing of Aptamers. *Journal of Biomolecular Techniques* 2008;19:311-321

[28] Stoltenburg R, Reinemann C, Strehiltz B. FluMag-SELEX as an advantageous method for DNA aptamer selection. *Anal Bioanal Chem* 2005;383:83-91.

[29] Ellington, et al. Designed Signaling Aptamers that Transduce Molecular Recognition to Changes in Fluorescence Intensity. *J Am Chem Soc* 2000;122:2469-2473.

[30] Nutiu R, Li Y. Aptamers with Fluorescence-signaling properties. *Methods* 2005;37:16-25.

[31] Nutiu R, Li Y. In Vitro Selection of Structure-Switching Signaling Aptamers. *Angew. Chem. Int. Ed.* 2005;44:1061-1065.

[32] Vater A, Jarosch F, Buchner K, Klussman S. Short bioactive Spiegelmers to migraine-associated calcitonin gene-related peptide rapidly identified by a novel approach: Tailored-SELEX. *Nucleic Acids Res* 2003;31(21):130-136.

[33] White R, et al. Generation of Species Cross-reactive Aptamers Using "Toggle" SELEX. *Mol Ther* 2001;4(6):567-573.

[34] Litman GW, et al. Phylogenetic diversification of immunoglobulin genes and the antibody repertoire. *Mol Biol Evol* 1993;10(1):60–72.

[35] Diaz M, Casali P. Somatic immunoglobulin hypermutation. *Curr Opin Immunol* 2002;14(2):235–40.

[36] Yallow RS, Berson SA. Assay of plasma insulin in human subjects by immunological methods. *Nature* 1959;185:1648–9.

[37] Jayasena SD. Aptamers: An Emerging Class of Molecules That Rival Antibodies in Diagnostics. *Clinical Chem* 1999;45(9):1628-1650

[38] Shlecht U, et al. Comparison of antibody and aptamer receptors for the specific detection of thrombin with a nanometer gap-sized impedance biosensor. *Analytica Chimica Acta* 2006;573-574:65-68

[39] Hailer MK, Slade PG, Martin BD, Rosenquist TA, Sugden KD. Recognition of the oxidized lesions spiroiminodihydantoin and guanidinohydantoin in DNA by the mammalian base excision repair glycosylases NEIL1 and NEIL2. *DNA Repair* 2005;4(1):41-50.

[40] Rink SM, Shen JC, Loeb LA. Creation of RNA molecules that recognize the oxidative lesion 7,8-dihydro-8-hydroxy-2*-deoxyguanosine (8-oxodG) in DNA. *Proc Natl Acad Sci* 1998;95:11619-11624.

[41] Robertson SA, Harada K, Frankel AD, Wemmer DE. Structure Determination and Binding Kinetics of a DNA Aptamer-Argininamide Complex. *Biochem* 2000;39:946-954.

[42] Sazani PL, Larralde R, Szostak JW. A Small Aptamer with Strong and Specific Recognition of the Triphosphate of ATP. *J Am Chem Soc* 2004;126:8370-8371.

[43] Gonzalez M, et al. Interaction of Biotin with Streptavidin. *J Biol Chem* 1997;272(17):11288-11294.

[44] Mann D, Reinemann C, Stoltenburg R, Strehlitz B. In vitro selection of DNA aptamers binding ethanolamine. *Biochem. Biophys. Res. Co* 2005;338:1928-1934.