SELECTING DNA APTAMERS FOR 17β-ESTRADIOL

BY

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THESIS
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17β-estradiol (E2) is a potent estrogen that has been widely documented in water resources. It falls under the category of endocrine disrupting compounds (EDCs), and hence has been included in the EPA’s Unregulated Contaminant Monitoring Rule (UCM3) that will require the monitoring of this compound. Thus, there is an increasing demand to sensitively and selectively detect E2. Conventional detection methods are time consuming and/or expensive. We explore a promising approach which employs the use of DNA aptamers for the detection of E2. DNA aptamers are single stranded DNA molecules which are capable of binding target molecules with high affinity and selectivity. The aim of this project is to develop DNA aptamers that would function as sensors for the detection of this endocrine disrupting steroid hormone. The first step performed was iterative in vitro selections to identify aptamers from a pool of ~ $10^{14}$ DNA molecules with the application of appropriate selection pressures. This was achieved by passing the pool over a selection column containing the target immobilized on sepharose beads, followed by eluting the sequences bound to the column with the free target. The resulting pool with the desired sequences was enriched by carrying out polymerase chain reaction (PCR). The potential aptamers obtained were cloned and sequenced, following which they were screened for binding activity using a bead binding assay. The dissociation constant for each aptamer was determined by a DMS chemical probing assay. These aptamers can be incorporated into various real-time in-line sensing platforms to generally and selectively monitor the presence of E2 in environmental samples. They represent an improvement over the one existing aptamer for E2, because some are more selective and marginally more sensitive.
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1. INTRODUCTION

Pharmaceuticals and personal care products (PPCPs) have been widely documented in surface and ground waters with other organic wastewater contaminants [1,2]. Among these PPCPs, estrogenic compounds have been detected in sewage treatment plant effluents at ng/L levels [3,4]. The removal efficiency of these compounds by powdered activated carbon (PAC) doses similar to that of a drinking water treatment plant is between 20-80% [5]. These estrogen-rich effluents have been shown to be responsible for sexual disruption and feminization of river fish [3,6,7]. Moreover, estrogens have been hypothesized to reduce sperm count and cause other reproductive disorders in humans [8]. Thus, estrogens fall under the category of endocrine disrupting compounds (EDCs) due to their detrimental effect on the endocrine system. There are multiple sources of EDCs viz. pharmaceutical industries using them in contraceptive pills and hormone replacement therapy, run-off from animal farms and sewage waste.

The influence of one such estrogen called 17β-estradiol (abbreviated as E2 in this document) is important because it is a potent estrogenic compound [9]. For example, E2 at environmentally relevant concentrations was shown to result in the generation of female specific proteins and abnormal gonads in Japanese Medaka [10]. Similar effects were shown for E2 on amphibians [11]. As a result of these adverse effects, the EPA listed E2 in its third contaminant candidate list (CCL3). Following this, E2 was added to the list of contaminants that will require monitoring in wastewater samples from 2013 to 2015 in accordance with the Unregulated Contaminant Monitoring Rule 3 (UCMR3). Therefore, a sensitive and reliable method for detection of E2 in natural water samples is needed.
Analytical methods commonly used to detect E2 in environmental matrices include gas chromatography-mass spectroscopy (GC/MS), liquid chromatography-mass spectroscopy (LC-MS) and liquid chromatography-tandem mass spectroscopy (LC-MS/MS) [3,12]. Despite their low detection limits, these methods are time-consuming and labor-intensive. In order to overcome these disadvantages, a great deal of effort has been devoted to developing fast, inexpensive, and reliable methods based on molecular recognition. One approach is the development of enzyme linked immune sorbent assays (ELISAs) or radio immunoassays (RIAs), which utilize biomolecules like proteins for detection purposes [7,13]. However, ELISAs used to measure estrogens in natural matrices have been found to overestimate concentrations possibly due to interference by other compounds [13]. Hence, there is concern that these assays compromise the selective detection of E2 due to potential cross-reactions of the protein with analogues of E2. This calls for the development of a sensitive and selective method for the detection of E2 which is easy to operate.

One promising technology for E2 detection involves the use of DNA aptamers. A DNA aptamer is a single strand of oligonucleotides that can specifically and selectively bind to a desired target molecule. DNA aptamers have been developed for a variety of compounds ranging from small molecules like ATP [14], cocaine [15] and PCBs [16], to metal ions such as arsenic [17] and large biomolecules such as proteins [18,19]. The dissociation constant (K_d) of the aptamer is a measure of the sensitivity of the aptamer. The aforementioned aptamers have their K_d ranging from pM to µM values for their respective targets. These aptamers have been immobilized on various sensing platforms on such as PEDOTs [20], gold nanoparticles [21], electrochemical
cells [22], and optical fibers [23] in order to develop functional biosensors that have low detection limits.

There has been one aptamer developed for E2 (K\(_d\) = 0.13µM) [Kim et.al, 2007] that has been widely used in aptamer-based biosensors [22,23]. The sensitivity of this aptamers for E2 varies widely depending on the study. Huy et.al [24] found the K\(_d\) of the aptamer to be high (~35µM) compared to a previously noted value of 0.13µM, while Langan et.al measured an even higher K\(_d\) (~70µM) [25]. Despite these discrepancies, this aptamer has been successfully used to develop biosensors that can detect E2 concentrations in the µM [23] to nM range [26,27]. The median environmental concentration of E2 is in the low nM range [1]. Therefore, many of the sensors using this aptamers may not be sensitive enough for E2 detection in natural samples.

The reported E2 aptamer has the ability to bind to other steroid hormones like estrone (E1), ethynyl estradiol (EE) and testosterone. For example, two times more E1 and three times more testosterone than E2 was recovered from solution by binding these EDCs to the aptamer immobilized on magnetic beads [25]. In another study, the E2 aptamer was used on a PEDOT platform doped with gold nanoparticles in order to function as a sensor. The signal recorded only a two-fold difference between E2 and its analogues, EE and E1 [26]. In a similar effort to develop a sensor, a 5-6 fold difference in signal was observed between E2 and other EDCs [23]. The median concentrations of E1, estriol (E3) and EE in environmental samples are higher than that of E2 by a factor of 2-10. Hence, interference by analogues of E2 poses a potential problem in natural systems since most of the analogues are more frequently detected than E2 [1]. This
may give rise to false positives, erroneous signals and overestimations during detection as the aptamer is not adequately selective.

The goal of this study is to develop a new set of aptamers that can be used to selectively and sensitively detect E2 in the presence of analogues and natural water constituents. The DNA aptamers are developed using the Systematic Evolution of Ligands by EXponential enrichment (SELEX) method and characterized using dimethyl sulfate (DMS) probing in order to estimate the $K_d$ of the aptamer. These aptamers could potentially be immobilized on any platform as the sensing element of the biosensor.
2. METHODS

2.1 Materials
A random pool of over $10^{14}$ unique DNA sequences was used for DNA aptamer selection. Each DNA sequence contained a 40 nucleotide random region bound at the 5’ end by a 16 nucleotide constant region, and on the 3’ end by a 29 nucleotide constant region (5’-CGAAGCGCTAGAACAT–N40–AGTACATGAGACTTAGCTGATCCTGATGG–3’). A 16 nucleotide forward primer (5’-CGAAGCGCTAGAACAT-3’) and a 29 nucleotide reverse primer (5’-XCCATCAGGATCAGCTAAGTACTCATGTACT-3’, where X = Glen spacer 18) were used in the polymerase chain reaction (PCR) step of the selections. The random DNA pool and the DNA primers were purchased from Integrated DNA Technologies Inc. (USA).

Epoxy-activated Sepharose 6B beads for derivatization were purchased from GE Healthcare (USA). The EDCs E1 (>99%), E2 (>98%), E3 (>97%) and EE (>98%) were purchased from Sigma Aldrich (USA). DMS (>99%) was obtained from Acros Organics (Fischer, USA). A Beckman LS 6500 scintillation counter was used for monitoring the radioactivity through selections and assays. The Thermo Scientific NanoDrop 2000 UV-Vis spectrophotometer was used to monitor the amount of EDCs on the beads and in solutions.

A coupling buffer used during bead derivatization contained 100mM Na$_2$CO$_3$ and 50% isopropanol at pH 13. The composition of selection buffer used through the selections was 50mM Tris, 300mM NaCl, 5mM MgCl$_2$ at pH 7.5. Wash buffer used during the selections was the selection buffer containing 20% ethanol.
2.2 Bead derivatization
E2 was immobilized on epoxy-activated sepharose beads by reacting the phenolic hydroxyl group of E2 with the epoxy group on the beads at pH 13. The beads were first suspended in distilled water and allowed to swell, and then washed on a sintered glass filter for 3 hours (200 mL water for every gram of freeze dried beads). The beads were then rinsed three times with an equal volume of coupling buffer. The coupling reaction was carried out between equal volumes of the beads and the coupling buffer, containing 20mM of E2, for a period of 7.5 hours at 37°C. Following this, the unreacted epoxy groups were capped by exposure to an equal volume of 1M ethanolamine solution for 20 hours at room temperature. The degree of bead derivatization with the target compound was quantified using UV-Vis spectrophotometry by measuring the absorbance of the beads suspended in 50% polyethylene glycol in a 1:2 ratio at 280 nm.

2.3 Selections
The most widely used approach to identify DNA aptamers is in vitro selection, also known as “Systematic Evolution of Ligands by EXponential enrichment”, or SELEX [28,29]. In vitro selection is an iterative method that typically involves first immobilizing the target analyte on a solid support, such as an agarose column [14,17,28]. An oligonucleotide pool (containing $10^{14}$ - $10^{15}$ sequences) is then generated and exposed to the target on the solid support. The sequences that bind to the target are retained on the column, and then eluted by a buffer containing a denaturant [30] or the unbound target analyte [14]. The eluted product is amplified using PCR. The enriched pool at the end of the selection round is used to initiate another round, and so on, until DNA sequences with the desired binding affinity to the target is obtained.
In vitro selections were initiated from the DNA library with ~10^{14} unique sequences. The scheme of the selections is depicted in Fig. 1B. In the first round of selections, 1% of the pool was 5'-^32P labeled to monitor the binding activity. The sequences contained in 400 µL of selection buffer were first heated to 95°C for 5 minutes, followed by cooling at room temperature for 30 minutes. The sequences were then incubated with a pre-selection column containing 50 µL of ethanolamine derivatized beads for 10 minutes, to discard sequences that bind to the support. Subsequently, the flowthrough from the pre-selection column was incubated with the selection column for 1 hour, which contained 200µL of beads derivatized with E2. The selection column was then washed 10 times with 200 µL of wash buffer. Following this, the selection column was eluted two times with 300 µL of 200µM E2 in the wash buffer for 1 hour each. The binding activity of the pool radiolabeled with phosphorous (^32P) was monitored by the liquid scintillation counter. The sequences from the elution underwent two amplification steps by PCR. In the first
step, a 10-cycle PCR was performed on the sequences obtained at the end of a round of selection. In the second step, a 30-cycle PCR was performed which made use of 1μL of the 10-cycle PCR product along with α-32P dCTP in order to incorporate 32P in the pool. This was followed by purification on an 8% polyacrylamide gel by electrophoresis (PAGE), extraction and ethanol precipitation. These sequences were then carried forward to the next round.

During later stages in the selections, more stringent selection pressures were applied in an attempt to obtain better binding aptamers. For example, the concentration of E2 in the elution step was reduced to select for more sensitive aptamers. In a parallel effort, the E2 elution was carried out for a total of 6 hours instead of 2 hours in order to get the sequences that were tightly bound to the bead-bound E2.

2.4 Cloning and sequencing
For cloning the sequences of interest, the 30-cycle PCR was performed with the following changes: 0.5 μL of the 10-cycle PCR product was used; the α-32P-dCTP was omitted; the forward and reverse primers (without Glen spacer 18) containing stop codons were used. The stop codons serve the purpose of disrupting of the β-galactosidase gene, which reduces the number of false negative E. coli colonies. The double-stranded DNA product was isolated by 2% agarose gel (QIAquick extraction kit, Qiagen). Cloning of aptamers was done using an Invitrogen TOPO TA kit. Miniprep DNA (Fermentas) of individual clones was prepared, and the presence of the insert of the appropriate length was confirmed by EcoRI digestion followed by 2% agarose gel. Automated sequencing was performed at the Biotechnology center at UIUC.
2.5 Bead binding assay
In order to conduct a preliminary screening of the clones obtained from the selections, a bead binding assay was performed. The individual aptamers were first incubated for 1 hour with the selection column containing beads derivatized with E2, as described in the previous section. Following this, the flowthrough from the selection column was saved for scintillation counting and the selection column was rinsed with the wash buffer as described earlier. Then, the selection column was put through a series of 300 µL elutions containing increasing concentrations of the E2 in order to assess the sensitivity of the aptamers. The more sensitive aptamers were eluted from the selection column at lower concentrations of E2, whereas the less sensitive aptamers were eluted at higher concentrations of E2. The sequences that were found to be “active” were carried forward for DMS probing described in the following section.

2.6 DMS probing
DMS chemical probing is a method to determine the binding constant (K_d) of the aptamer to the target in solution phase. DMS is a chemical reagent that methylates the N7 position of a guanine base if it is accessible. The guanines interacting with the E2 are thought to be protected from methylation by DMS, and can be used as a measure of K_d. About 0.2 pmol of 5’-32P labeled aptamer is first allowed to bind a known concentration of E2 in 15µL of selection buffer containing 2.5% ethanol (final concentration) for 1 hour. Following this, the aptamers were exposed to 10 µL of 1.2% DMS (initial concentration) for 30 minutes for methylation. This was followed by ethanol precipitation of the aptamers. Subsequently, the methylated aptamers were allowed to undergo depurination in the presence of 50 µL of 10% piperidine (by volume) for 30 minutes at 95°C, leading to strand cleavage at the methylated sites. The sample was dried under vacuum and then run on a 12% PAGE. This test is carried out over a range of concentrations of
the target and the variation of the relative intensity of a band specific to certain guanines can be plotted against the concentration of the target. This plot can be fit to a curve given by

\[
I_{\text{obs}} = \frac{(I_{\text{low}} + I_{\text{high}} \cdot K_d \cdot \text{[target]}^n)}{(1 + K_d \cdot \text{[target]}^n)}
\]

(1)
to obtain a \(K_d\) for the specific aptamer, where \(I_{\text{low}}\) and \(I_{\text{high}}\) are the relative intensities at the low and high target concentrations respectively and \(n\) is the hill coefficient. In most cases, the relative intensity \(I_{\text{obs}}\) of the bands decreases with increasing concentration of the EDC, as a result of the protection of the guanines on binding to the target.

In order to assess the selectivity of the aptamer towards E2 over its analogues, DMS probing was used to measure the \(K_d\) of the obtained aptamer for all four EDCs viz. E1, E2, E3 and EE. The ratio of the analogue-\(K_d\) to the E2-\(K_d\) for each aptamer was indicative of its selectivity.
3. RESULTS AND DISCUSSION

3.1 E2 coupling to beads
Three sets of beads were derivatized, as depicted by the absorbance plot in Figure 2. The pre-selection/control beads were derivatized with ethanolamine only, while the low and the high derivatized beads were coupled with E2 followed by ethanolamine. The coupling reaction was carried out for 7.5 hours in the case of the low derivatized beads and 72 hours for the high derivatized beads. The wavelength of maximum absorption ($\lambda_{\text{max}}$) for E2 is 280 nm. As seen in Figure 2, the absorbance peak at 280 nm indicates that the derivatization was successful. The concentration of E2 on the low and high derivatized beads was 1mM and 4mM respectively.

![Absorbance plot of E2 derivatized beads](image)

**Figure 2. Absorbance plot of E2 derivatized beads**

3.2 Selections progress
Three sets of selections were carried out: one set was the negative control which made use of the pre-selection beads in the selection column, the second set used the high derivatized E2 beads in the selection column and the third set used the low derivatized E2 beads in the selection column. Results are shown in Figure 3. Binding activity (~25%) of the DNA pool was observed during
round 7 of the selections that incorporated the low derivatized beads and this activity was sustained in round 8.

![Selections progress graph](image)

Figure 3. Selections progress of aptamer selections

Following round 8, more stringent selection pressures were applied on the selected pool obtained with low derivatized beads. For part of the selection, we applied a longer elution time (ET) pressure to improve sensitivity. For another part, we applied lower E2 concentration pressure during the elution, also to improve the sensitivity. A control was run using the unmodified selection pressure. The results are shown in Figure 4. There was no significant change in activity through the three rounds that followed, as seen from Figure 4. At this stage, we cloned the pool obtained from Round 10 of the selections that made use of a long ET.

![Selection Pressures graph](image)

Figure 4. Selection progress on imposing stringent selection pressures for rounds 9 through 11
3.3 Activity screening and sequencing
36 clones from the selection effort were screened for binding activity using the bead binding assay. Also screened was an E2 aptamer identified in the literature [22]. Representative data is shown in Figure 5 for three aptamers, 10EstGd8, 10EstGd17 and 10EstGd18. Of the 36 clones, 33 were found to be active through the bead binding assay. Some aptamers were eluted at low E2 concentrations, whereas others were eluted at relatively higher E2 concentrations. This reflects the affinity of the aptamers between bead-bound E2 versus free E2 in solution. The identified aptamers covered a range of E2 concentrations (between 1 and 10µM) at which they were eluted from the column. However, the E2 aptamer from the literature did not show any binding activity based on this assay.

![Figure 5. Plot of cumulative elution of aptamers with increasing concentration of E2 in the bead binding assay](image)

All of the 33 active clones were sequenced and 16 unique sequences were identified. The sequence information of the random region of these unique sequences was fed into CLC sequence viewer in order to inspect any recurring motifs in the E2 aptamers. These are shown in Figure 6.
A dot in the lower sequences indicates that the position is occupied by the same nucleotide as the one in the first sequence of the list. Therefore, all sequences are quite different from each other as there are no recurring segments across the identified sequences. The sequences obtained from this selection effort are also different from the literature E2 aptamer (see Figure 7).
Of the 16 sequences that were identified to be active based on the bead binding assay, 8 were characterized by DMS probing for their dissociation constants. These 8 sequences (from Figure 5) are 10EstGd1, 10EstGd2, 10EstGd6, 10EstGd7, 10EstGd8, 10EstGd9, 10EstGd20 and 10EstGd32. The 8 sequences were chosen based on the concentration at which approximately half of the aptamers were eluted from the column in the bead binding assay.

### 3.4 Dissociation constants

DMS probing was used to measure the solution phase dissociation constant of the aptamers. Results for 10EstGd8 are shown in Figure 8. Only certain bands, corresponding to certain guanine positions, showed a change in intensity with increasing concentration of E2 due to change of the accessibility of those guanines on binding to E2. Typically, the accessibility of the guanines decreased, as seen by the decreasing intensity of the bands on increasing the concentration of E2 (e.g., see bands G50/49, G45, G40-G38, G30-G28 in Figure 8). However, some guanines of certain aptamers behaved contrarily (data not shown).

The intensity of bands that showed changes was normalized with the intensity of a band that did not change with E2 concentration, for each lane in the gel image. In addition to this, a background correction was made to the intensities. The change in relative intensity corresponding to each guanine is plotted against the concentration of E2 to obtain a $K_d$ by fitting the data points to the equation (1). On fitting of the data for the aptamer 10EstGd8, we obtained a $K_d$ of 80 nM.
Figure 8. (A) PAGE of 10EstGd8 for DMS probing. The different guanine positions are marked by cleavage of the strand at those positions in the presence of TE (Tris-EDTA) buffer and at zero E2 concentration. Bands specific to certain guanines show change in intensity with respect to concentration of E2 (B) Plot of observed relative intensity of bands versus the concentration of E2 (C) Sequence information of random region of 10EstGd8 with protected guanines indicated in red

Not all the aptamers exhibited a DMS probing pattern. Two aptamers, 10EstGd1 and 10EstGd6, did not show any guanines that reflected change in their accessibility. Thus, only six of the eight aptamers could be characterized by a dissociation constant, as shown in Figure 9. Since the literature aptamer did not show a DMS probing pattern either, the value in the plot corresponds to that reported by the authors [22]. As seen from the plot below, two aptamers (10EstGd2 and 10EstGd8) identified from this selection effort are marginally better than the literature aptamer in terms of the sensitivity, due to their lower $K_d$. 

5' - GGCACGCGCGAGGGCGTAGCTGGGAAGAAGGGCGCCAG - 3'

(c)
3.5 Selectivity

The selectivity tests were conducted using the same DMS probing method used for E2, except that incubations were performed over a range of concentrations for the three analogue estrogens i.e., E1, E3 and EE. The data pertaining to the relative band intensities obtained from the PAGE images was plotted as before, and the $K_d$ values of the aptamer – target complex were obtained. The ratio of the $K_{d,\text{analogue}}$ to $K_{d,E2}$ gives us a measure of the selectivity of the aptamer towards E2 over the analogues.

The PAGE image of the aptamer 10EstGd9 in the presence of the four estrogens is shown in Figure 10. There is some degree of selectivity towards E2 over the analogues since the intensity of the bands (marked G46, G45, G31 to G27) changes to a greater extent at a lower concentration of E2 compared to the analogues.
The selectivity is better illustrated by plotting the relative intensities of the bands against the concentration of the target. Figure 11 illustrates that the intensity of the bands is relatively lower for E2 as opposed to the analogues at the same concentration. This indicates that interaction of the aptamer with E2 is stronger than that with the analogues at the same concentration. Therefore, the aptamer is more selective towards E2 over the analogues. The EE-specific points have not been plotted since the data did not fit the curve, implying that the aptamer did not interact with EE (PAGE shows no change in band intensity).
The ratio of $K_{d,\text{analogue}}$ to $K_{d,E2}$, is plotted in Figure 12 for the five aptamers. The aptamers are marginally selective towards E2 over E1 (within a factor of 8 to 12) except in the case of 10EstGd9, where the aptamer appears almost 40 times more selective. Also, the aptamers are more selective towards E2 over E3 (by a factor of 30 to 300).
The selectivity of the identified aptamers is an improvement over the selectivity of the literature aptamer which is only about 2 times more selective for E2 over E1 and EE [26] and approximately 5-6 times more selective towards E2 over E3 [23].

The structures of E1, E2, E3, and EE are shown in Figure 13. The E2 derivatized beads used in the selections are immobilized through the phenolic group. Therefore, the pool is exposed to the ring that carries the functional group specific to E2 (OH group) throughout the selections, necessitating the presence of that group for binding. The pool shows selectivity for E2 over E1 or E3 because of changes in functional groups on the pentane ring, i.e., a switch from OH to =O for E1, and a switch of one OH to two OHs for E3. The pool likely does not show sensitivity to EE because of the relatively larger functional group on the pentane ring C≡CH.

![Figure 13. E2 and its analogues, E1, E3 and EE](image)

Overall, the E2 aptamers identified are capable of binding to bead bound and free E2 satisfactorily well. They exhibit a degree of selectivity towards E2 over its analogues, especially E3 and EE, and this bodes well for their use in biosensors.
4. CONCLUSION

The E2 aptamer selections were performed using the SELEX approach and a set of 16 unique aptamers were identified. Some of them were characterized by their dissociation constants which ranged from 60 nM to 700 nM. This is marginally better than the sensitivity of the aptamer reported in literature. The aptamers were also found to exhibit some selectivity towards E2 over its analogues. This selectivity towards E2 ranges from a factor of 8-40 over E1, and from 30-300 over E3. The aptamers do not bind to EE based on the DMS probing patterns. The selectivity of these aptamers is an improvement over the speculated selectivity of the literature aptamer which varies from a factor of 2 in favor of E2 over E1 and EE [26], and a factor of 5-6 over E3 [23].

For future work, we can improve the selectivity of these aptamers further by conducting negative selections against the E2 analogues. This could be done by eliminating sequences that bind to the analogues followed by amplifying only those sequences that bind to E2 alone.

We could also aim to develop aptamers for the range of estrogens i.e. E1, E2, E3 and EE, so that they could function as a system of aptamers to individually detect estrogens and in combination measure the total estrogen level in the natural samples.

The ultimate goal of this effort is to develop a biosensor that can be used to detect and monitor E2 in natural water samples. This can be achieved by immobilizing the obtained aptamer as a biosensing element onto various sensing platforms. One such platform is a gold electrode onto which we could immobilize thiol-modified aptamers to function as an electrochemical biosensor.
REFERENCES


