Selective Aptamers for Detection of Estradiol and Ethynylestradiol in Natural Waters

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Supplemental Experimental Section

Materials. The DNA oligonucleotides (random pools) used for the E2 and EE aptamer selections were, respectively, 5′-CGAAGCGCTAGAACAT-N_{40}-AGTACATGAGACTTAGCTGATCCTGATGG-3′ and 5′-CGAA-GTCGCCATCTCTTC-N_{40}-ATAGTGAGTCGTATTAAGCTGATCCTGATGG-3′, where N_{40} denotes 40 consecutive random nucleotides. In the PCR step of each selection, the primers were E2 forward 5′-CGAAGCGCTAGAACAT-3′, E2 reverse 5′-$(AAC)_4$XCCATCAGGATCAGCTAAGTCTCATGTACT-3′, EE forward 5′-CGAAGTCGCCATCTCTTC-3′, and EE reverse 5′-$(AAC)_4$XCCATCAGGATCAGCT-3′, where X = HEG spacer to stop Taq polymerase (thereby allowing separation of the two DNA single strands). For cloning the E2 selection, the forward and reverse primers were respectively modified to 5′-TAATTAATTAATTACGAAGCG-CTAGAACAT-3′ and 5′-TAATTAATTAATTACCCATCAGGATCAGCT-3′, each containing stop codons in all three reading frames. For cloning of the EE selection, only the reverse primer was modified by inclusion of stop codons.

Immobilization of E2 and EE on Agarose Support. For immobilization, approximately 25 mL of distilled water was added to 6 g of epoxy-activated agarose support in a 50 mL plastic (polyethylene) tube until the combined volume of swelled support and water was 33 mL (~15–20 min). The suspension was transferred to a 60 mL medium-porosity sintered glass funnel and washed by suction with 1200 mL of water in 30 mL portions with manual agitation over 3 h. The support (~15 mL volume) was transferred to a 50 mL plastic tube and washed with 3 × 15 mL of immobilization buffer [100 mM Na_2CO_3, 50% (v/v) isopropyl alcohol, pH 13], centrifuging at 250 × *g* for 5 min after each addition. The immobilization reaction was performed in a 15 mL plastic tube using 5 mL of support and 5 mL of immobilization buffer containing 20 mM E2 or EE for 8 h (E2) or 26 h (EE) at 37 °C; the EE immobilization was found empirically to require the longer time. The sample was centrifuged as above, and the immobilization buffer was decanted. The support was washed with 3 × 5 mL of immobilization buffer. The unreacted epoxy groups were capped by incubation with 5 mL of 1 M ethanolamine for 20 h at 23 °C, followed by centrifugation, decanting, and washing with three complete cycles of 5 mL of 0.1 M sodium acetate, pH 4.0, 5 mL of 0.1 M Tris, pH 8.0, and 5 mL of water. For preparation of the pre-selection support with only immobilized ethanolamine, the support was directly derivatized via the capping procedure. The stock of derivatized support was stored in 5 mL of 20% (v/v) ethanol at 4 °C.

To obtain a portion of derivatized support, the stock sample was vortexed, and a 400 μL aliquot of the suspension was removed to a 1.7 mL tube. The sample was centrifuged at 2000 × *g* for 10 s and the supernatant was removed by pipet, providing 200 μL of derivatized support.

The extent of derivatization of the support was quantified by UV-visible spectroscopy, by measuring the absorbance at 280 nm (A280) of 75 μL of the support suspended in 225 μL of 50% (w/v) poly(ethylene glycol) (PEG 4000, Fluka). A calibration plot was constructed by measuring the A_{280} values of a series of suspensions of 75 μL of unmodified support (Sepharose 6B, Sigma, catalog number 6B100) in 222 μL of 50% (w/v) PEG 4000 containing 60–300 nmol of aqueous phase E2 or EE, along with 3 μL of ethanol for solubility. The calibration plot indicated that typically 50–100 nmol of E2 or EE was immobilized on 75 μL of support, equivalent to 133–267 nmol of E2 or EE on 200 μL of support, which was the amount used in each selection round.

In vitro Selection Procedure. The in vitro selection procedure was performed as follows.

Procedure for Initiating Selection (Round 1). The selection strategy for DNA aptamers is shown in Figure 1B. In the first selection round, 50 μL of pre-selection support was transferred to a Micro Bio-Spin chromatography column (Bio-Rad) and washed with 5 × 250 μL of binding buffer (50 mM Tris, pH 7.5, 5 mM $MgCl_2$, and 300 mM NaCl), each time centrifuging at 3000 × *g* for 30 s. To suppress nonspecific binding of DNA to the support, a "blocking" DNA oligonucleotide, $(AAC)_{20}$, was used. Approximately 100 pmol of the blocking oligonucleotide in 400 μL of binding buffer was added to the washed support in the pre-selection column. The suspension was transferred to a 2 mL tube sealed with an O-ring and incubated on a nutator at 23 °C for 10 min. The suspension was returned to the pre-selection column, which was centrifuged, and the filtrate was discarded. In a separate 1.7 mL tube, 500 pmol of a random DNA pool (~3 × 10^{14} unique sequences, of which 5 pmol was 5′-^{32}P-radiolabeled to enable monitoring of binding activity) in 400 μL of binding buffer was annealed by heating at 95 °C for 5 min and cooling at 23 °C for 30 min. The annealed DNA pool was added to the washed support containing the blocking oligonucleotide. The suspension was transferred to the 2 mL O-ring tube, incubated on the nutator for 10 min, returned to the pre-selection column, and centrifuged. The pre-selection support was suspended in 400 μL of binding buffer, and the suspension was transferred to a 7 mL scintillation vial (sample A). The flow-through from the pre-selection column was retained for incubation with the selection support.

Into a separate column was placed 200 μL of E2/EE-derivatized selection support, which was washed with 10 × 500 μL of binding buffer, centrifuging each time. The support was treated with 400 pmol of $(AAC)_{20}$ blocking oligonucleotide as described above. The retained flow-through from the pre-selection column was added to the blocking oligonucleotide-treated selection support. This suspension was transferred to a 2 mL O-ring tube, incubated on the nutator for 1 h, returned to the selection column, and centrifuged. The flow-through was transferred to a scintillation vial (sample B), and the selection support was retained for subsequent washes.

The selection support was washed with 10 × 200 μL of wash buffer, which was the binding buffer containing 20% (v/v) ethanol for E2 support or 2% (v/v) ethanol for EE support. For each wash, the buffer was incubated in the column for 3 min followed by centrifugation. Two consecutive washes were combined and transferred to a scintillation vial (sample C). The selection support was eluted twice with free E2/EE in solution. For both elutions, 300 μL of the appropriate wash buffer containing 200 μM E2 or 20 μM EE was transferred to the selection column. The suspension was transferred to a 2 mL O-ring tube, incubated on the nutator for 2 h, returned to the selection column, and centrifuged. The two elutions were combined and transferred to a scintillation vial (sample D). A suspension of the E2/EE support in 400 μL of binding buffer was transferred to a scintillation vial (sample E).

A scintillation counter (Beckman Coulter LS 6500) was used to quantify the amount of ^{32}P in each of the five samples A–E. To track the selection progression (Figure S1), the fraction of total counts (samples A–E) that bound to the selection column and subsequently were eluted specifically with free E2/EE (sample D only) was used as quantification of the pool binding activity.

After counting, sample D was divided between two 1.7 mL tubes and precipitated with ethanol. Two PCR reactions were performed, i.e., 10-cycle PCR followed by 30-cycle PCR. First, a 100 μL sample was prepared containing the ethanol-precipitated product, 200 pmol of forward primer, 50 pmol of reverse primer, 20 nmol of each dNTP, and 10 μL of 10× Taq polymerase buffer (1× = 20 mM Tris-HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, and 0.1% Triton X-100). This sample was cycled 10 times according to the following PCR program: 94 °C for 2 min, 10× (94 °C for 30 s, 47 °C for 30 s, 72 °C for 30 s), 72 °C for 5 min. Taq polymerase was removed by phenol/chloroform extraction. Second, a 50 μL sample was prepared containing 1 μL of the 10-cycle PCR product, 100 pmol of forward primer, 25 pmol of reverse primer, 10 nmol of each dNTP, 20 μCi of α-^{32}P-dCTP (800 Ci/mmol), and 5 μL of 10× Taq polymerase buffer. This sample was cycled 30 times according to the following PCR program: 94 °C for 2 min, 30× (94 °C for 30 s, 47 °C for 30 s, 72 °C for 30 s), 72 °C for 5 min. The sample was separated by 8% PAGE, extracted with TEN buffer, and precipitated with ethanol.

Procedure for Subsequent Selection Rounds 2+. Each subsequent selection round was performed as described for Round 1, with the following modifications. The pre-selection support was incubated simultaneously with annealed 30-cycle PCR product (~5-10 pmol) and 100 pmol of blocking oligonucleotide in 400 μL of binding buffer. 400 pmol of blocking oligonucleotide in 5 μL of binding buffer was added to the flow-through from the pre-selection column, before the sample was incubated with the selection support. After the selection support was washed with the appropriate wash buffer, the two elution steps were each performed for 30 min (1 h total). For rounds 9+ of the E2 selection, a longer elution time of 3 h for each elution step (6 h total) was used to promote elution of tighter-binding sequences.

Cloning and Sequencing. After 10 rounds for both the E2 and EE selections, the 30-cycle PCR was performed as described above, with the following modifications: 1 μL of 1/1000 dilution of the 10-cycle PCR product was used; α-^{32}P-dCTP was omitted; one or two cloning primers incorporating stop codons were used (see Materials); and 25 pmol of each cloning primer was used. The resulting 114 bp (from E2 selection) and 104 bp (from EE selection) double-stranded DNA product with single adenosine overhangs as added by Taq polymerase was isolated by 2% agarose gel (Fermentas gel extraction kit). Individual aptamers were cloned using a TOPO TA kit (Life Technologies). Miniprep DNA (Fermentas) was prepared, and the presence of the expected aptamer insert was confirmed by EcoRI digestion and 2% agarose gel electrophoresis. Sequencing was performed at the UIUC Biotechnology Center. Aptamers for preliminary binding studies were prepared by 30-cycle PCR directly from the miniprep DNA. For subsequent studies, aptamers were prepared by solid-phase synthesis (IDT). Aptamer sequences that were studied in detail are shown in Figure S2.

Preliminary Assays for Binding of Aptamers to Immobilized E2/EE. As a preliminary screen of individual clones, binding assays were performed. The pre-selection step was omitted; the E2/EE-derivatized support was incubated with an individual clone (obtained by 30 cycles of PCR using the miniprep DNA as template) followed by washing with the appropriate wash buffer as described above in the selection procedure. The column was eluted with 300 μL portions of appropriate wash buffer containing increasing concentrations of E2/EE, ranging from 0.1 to 300 μM. Each elution was collected separately for scintillation counting. Individual clones were characterized further when they showed substantial binding activity, as assessed by an increase in the amount of material eluted with increasing concentration of E2/EE.

Equilibrium Filtration Assay. The rationale for arriving at equation (4) in the main text is described here. Because of the molecular weight cutoff membrane, the retentate contains both A•L and free L, whereas the filtrate has only free L. The two experimentally measureable concentrations of L are total L in the retentate and free L in the filtrate, noting that there is no bound L in the filtrate (which has no aptamer). Moreover, the concentration of free L is the same in the retentate and filtrate, because the EDC passes freely through the cutoff filter. The scintillation counting data were correlated to molar concentrations by $(c_R + c_F)/(v_R + v_F) = c$ (= 0.5 μM). From the experimental values of c_F/v_F, the concentration of free L was calculated and used to determine the concentration of A•L in the retentate. Finally, noting that the concentration, y, of A•L in the full sample is defined in reference to the total volume of the full sample $(v_R + v_F)$, the value of y was calculated and plotted according to equation (3), as shown in Figure 2A.

DMS Probing. Each aptamer was probed using dimethyl sulfate (DMS) in the presence of varying concentrations of E2 or EE. In a 1.7 mL tube, 0.4 pmol of 5′-^{32}P-radiolabeled aptamer in 10 μL of modified binding buffer (50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, and 300 mM NaCl, where HEPES replaces Tris in the original binding buffer because Tris can be methylated by DMS) was annealed by heating at 95 °C for 5 min followed by cooling at 23 °C for 1.5 h. To this sample was added 10 μL of modified binding buffer containing 5% (v/v) of ethanol and a particular concentration of E2/EE (final concentration 1 nM to 100 μM). As a standard, a sample of 0.4 pmol of 5′-^{32}P-radiolabeled aptamer in 20 μL of TE (10 mM Tris, pH 8.0, and 1 mM EDTA) without E2/EE was prepared and annealed. To 15 μL of the aptamer sample was added 10 μL of methylation buffer [50 mM sodium cacodylate, pH 8.0, 300 mM NaCl, 2.43% (v/v) ethanol, 1.25 % (v/v) DMSO, and 0.075% (v/v) Triton X-100] containing 0.27% (v.v) DMS. The 25 μL sample was incubated at 23 °C for 30 min and precipitated with ethanol. The product was dissolved in 50 μL of 10% (v/v) piperidine and heated at 95 °C for 30 min followed by cooling on dry ice for 5 min. The samples were dried under vacuum overnight and separated by 12% PAGE, with imaging by PhosphorImager and quantification by ImageQuant (GE Healthcare).

Selection progressions

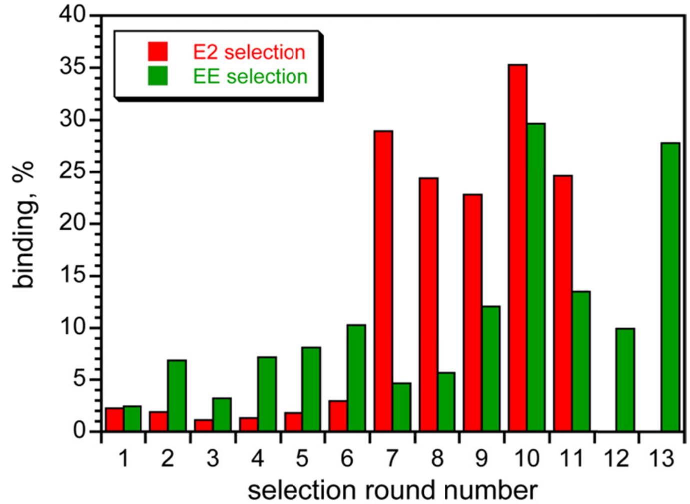

Figure S1. Selection progressions for E2 and EE selections. Long elution time pressure was applied from Round 9 for E2 selections only. Individual aptamers were cloned from the Round 10 pools in both selections once sustained stable activity was observed.

Sequences of individual deoxyribozymes

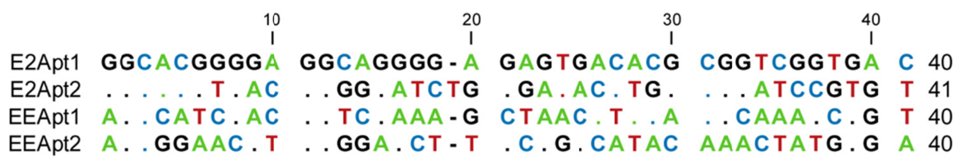

Figure S2. Sequences of E2 and EE aptamers that were characterized by equilibrium filtration and DMS probing assays. Only the initially random (N_{40}) sequences are shown. All aptamers were used as 5′-CGAAGCGCTAGAACAT-N_{40}-AGTACATGAGACTTAGCTGATCCTGATGG-3′ (E2Apt1, E2Apt2) and 5′-CGAAGTCGCCATCTCTTC-N_{40}-ATAGTGAGTC-GTATTAAGCTGATCCTGATGG-3′ (EEApt1, EEApt2). Identical residues in comparison to E2Apt1 are represented as dots. Gaps are depicted as dashes. The E2Apt2 sequence includes one additional nucleotide (41 nt), apparently due to a spontaneous insertion by Taq polymerase during an unknown selection round.

Equilibrium filtration assay data

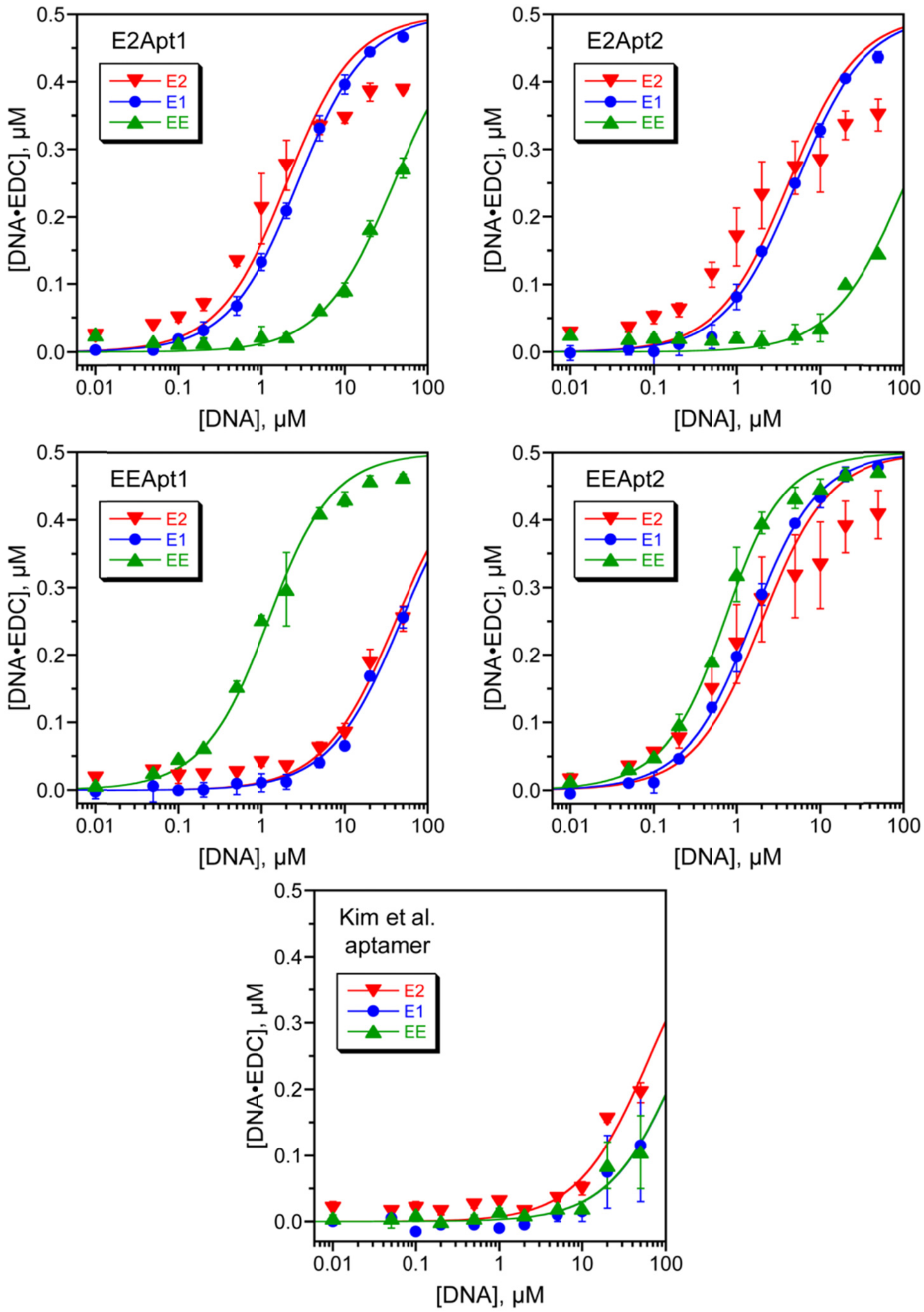

Figure S3. Equilibrium filtration assay data for the E2Apt1, E2Apt2, EEApt1, and EEApt2 aptamers as well as the E2 aptamer identified by Kim et al.[1] These data are fit using the model that assumes Hill coefficient $n = 1$. K_d values are tabulated in Table 1. See Figure S4 for alternate fitting approach with Hill coefficient $n \neq 1$ for the E2 data.

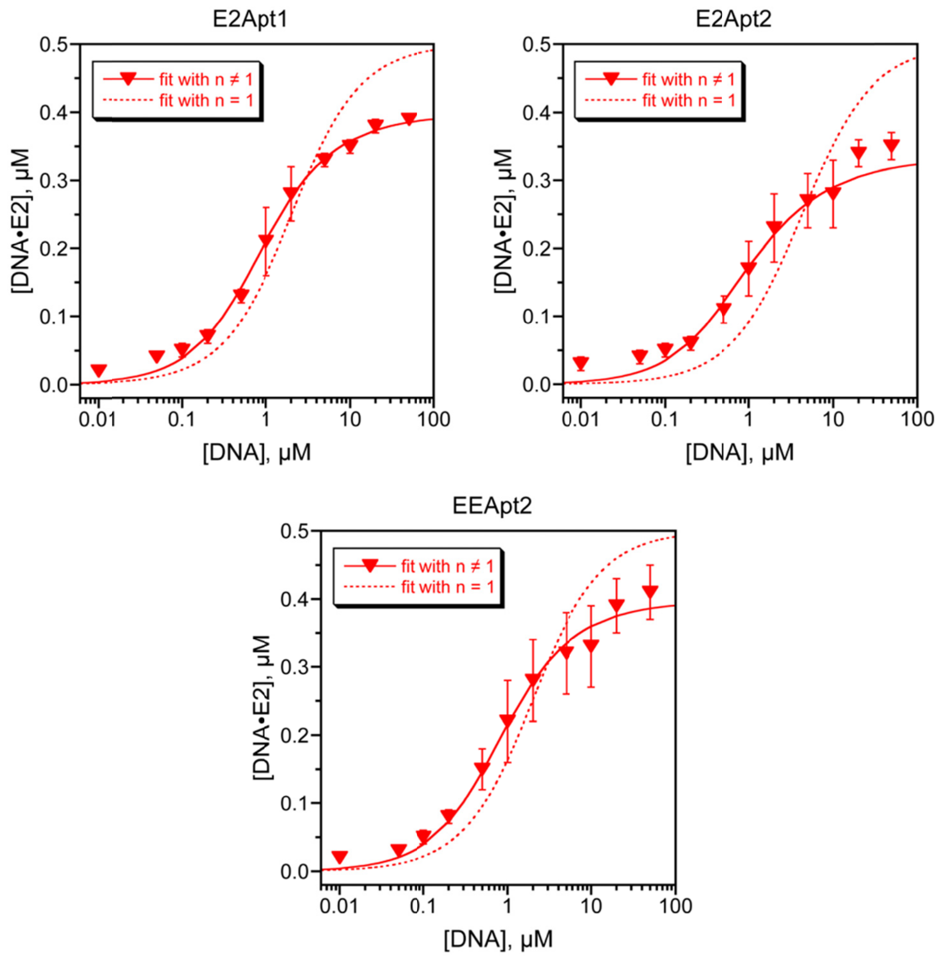

Figure S4. Equilibrium filtration assay data for the E2Apt1, E2Apt2 and EEApt2 aptamers binding to E2, fit using the model with Hill coefficient $n \neq 1$. K_d and n values are tabulated in Table 1. Fits to the same data with $n = 1$ from Figure S3 are shown for comparison. There is no plot for EEApt1 because this aptamer did not bind appreciably to E2.

Errors for equilibrium filtration assay parameters

Best-fit parameter values (K_d, n) were obtained using equation (7) and minimizing the root mean square error (RMSE) given by

$$RMSE = \sqrt{\frac{\sum \left(y_{\text{expt}} - y_{pred} \right)^2}{N}}$$

where N is the number of data points ($N = 11$), y_{expt} is the experimentally obtained value of A•L, and y_{pred} is the value of A•L computed using specific values of K_d and n. In order to obtain error bars (standard errors) for the best-fit values of parameters, K_d and n, we first calculated the covariance matrix V. V is computed using variance (σ^2) and the first derivative matrix X for each data point, where

$$V = (\mathrm{X}^T . \mathrm{X})^{-1} \sigma^2$$

$$\sigma^2 = \frac{RMSE^2 \times N}{N - p}$$

$$X_{i1} = \frac{\partial y_{i,pred}}{\partial K_d} = \frac{y_{pred}(K_d + \Delta K_d) - y_{pred}(K_d)}{\Delta K_d}$$

$$X_{i2} = \frac{\partial y_{i,pred}}{\partial n} = \frac{y_{pred}(n + \Delta n) - y_{pred}(n)}{\Delta n}$$

where p is the number of parameters ($p = 2$ in our case), and the parameters are increased by 15% to compute the first derivatives, e.g., $\Delta K_d = 0.15 * K_d$. The standard error for K_d is given by $\sqrt{V_{11}}$ and that for n is given by $\sqrt{V_{22}}$.

DMS probing data

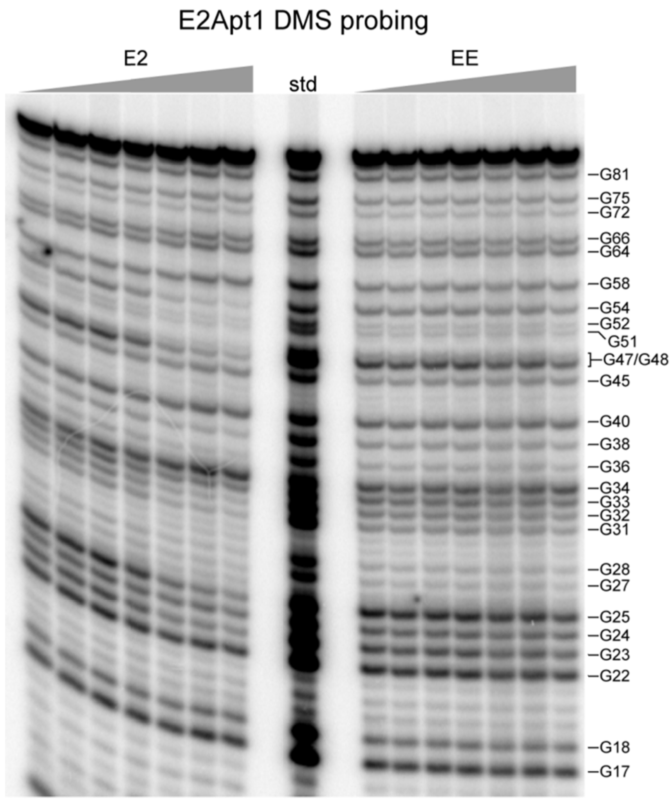

Figure S5. Full PAGE image for the experiment shown in Figure 4B. For each set of lanes, E2/EE concentrations are (left to right) 0, 0.001, 0.01, 0.1, 1, 10, and 100 µM.

Table S1. K_d values determined from DMS probing data for individual guanosine nucleotides of E2Apt1, E2Apt2, and EEApt1 with E2, E2, and EE, respectively. Values were derived from data such as that shown in Figure 4C. Error bars are from the fits to equation (6) as described in the Experimental Section. For nucleotide G64 of EEApt1, the normalized band intensity was observed to increase two-fold as the EE concentration was increased; this was the only nucleotide in any of these aptamers for which the normalized band intensity increased rather than decreased.

E2Apt1 nt	E2 K_d, µM	E2Apt2 nt	E2 K_d, µM	EEApt1 nt	EE K_d, µM
G54	0.24 ± 0.08	G54	0.65 ± 0.21	G64	1.5 ± 0.5
G47/G48	0.23 ± 0.07	G48	0.75 ± 0.35	G62	3.7 ± 1.0
G32	0.17 ± 0.04	G46	0.77 ± 0.14	G37	4.0 ± 1.9
G31	0.47 ± 0.13	G45	0.80 ± 0.10	G30	1.3 ± 0.5
G25	0.31 ± 0.07	G31	0.52 ± 0.12		
		G30	0.80 ± 0.16		
		G29	0.65 ± 0.15		
		G28	0.70 ± 0.19		
		G27	0.78 ± 0.15		
		G24	0.63 ± 0.28		

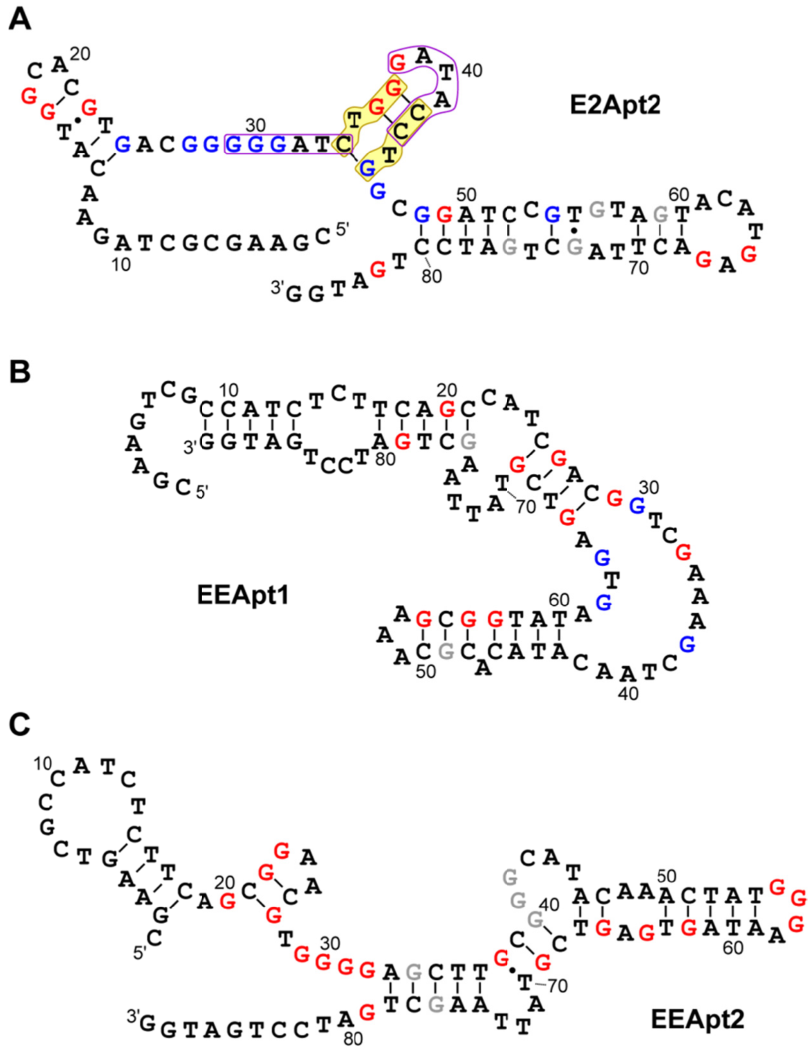

Figure S6. mfold-predicted secondary structures for E2Apt2, EEApt1, and EEApt2, similar to Figure 4D for E2Apt1. For each aptamer, only the lowest-energy structure is illustrated. Blue G nucleotides = substantial concentration-dependent change in DMS accessibility with E2 (E2Apt2) or EE (EEApt1 and EEApt2); red = no change in DMS accessibility; grey = band intensity too low to quantify; black = non-G nucleotides and G nucleotides at the 5′- and 3′-ends. For E2Apt2, the structure with the purple instead of yellow stem is 0.5 kcal/mol higher in free energy.

Lake and tap water characteristics

Table S2. Characteristics of the lake and tap waters used in the equilibrium filtration assay. The water samples were characterized by the Illinois State Water Survey lab facility in Champaign. nd = not detected.

Component	Units	Lake	Tap
pH		8.29	8.47
alkalinity	mg/L as $CaCO_3$	141	135
B	mg/L	0.11	0.36
Ba	mg/L	0.06	0.08
Ca	mg/L	47.4	13.8
Cu	mg/L	nd	0.08
K	mg/L	3.40	2.58
Mg	mg/L	15.9	12.5
Mn	mg/L	0.003	nd
Na	mg/L	113	32
S	mg/L	5.38	nd
Si	mg/L	0.99	3.63
Sr	mg/L	0.10	0.15
Tl	mg/L	0.02	nd
F	mg/L	0.18	0.86
Cl	mg/L	182.1	13.2
NO_3 (as N)	mg/L	nd	0.05
SO_4	mg/L	14.69	0.32
TDS	mg/L	477	166
dissolved TOC	mg/L	4.43	1.25

Relationship between K_d and LOD

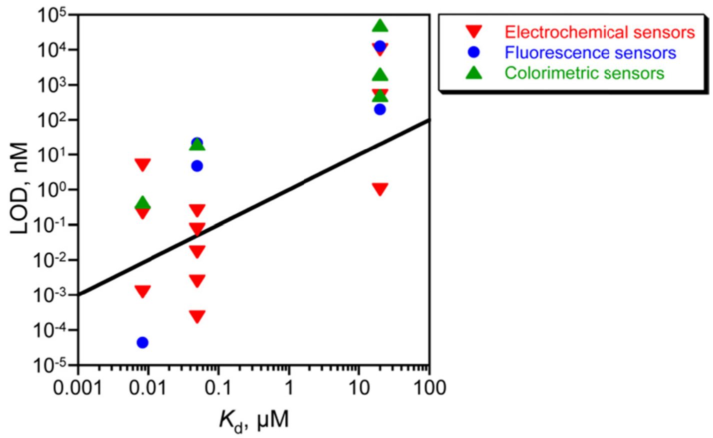

Figure S7. LOD versus K_d for three broad categories of platforms: electrochemical sensors, fluorescence sensors, and colorimetric sensors. The cited studies involve aptasensors developed for three small molecules: bisphenol A (K_d = 8.3 nM),[2-6] ochratoxin A (K_d = 50 nM),[7-13] and cocaine (K_d = 20 μM).[14-22] The slope of the straight line is 1.

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