Biophysical Characterization of DNA Aptamer Interactions with Vascular Endothelial Growth Factor

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ABSTRACT:
The binding of a DNA aptamer (5'-CCGTCTTCAGACAAGAGTGCAGGG-3') to recombinant human vascular endothelial growth factor (VEGF165) was characterized using surface plasmon resonance (SPR), fluorescence anisotropy and isothermal titration calorimetry (ITC). Results from both fluorescence anisotropy and ITC indicated that a single aptamer molecule binds to each VEGF homodimer, unlike other VEGF inhibitors that exhibit 2(ligand):1(VEGF homodimer) stoichiometry. In addition, ITC revealed that the association of the aptamer to VEGF at 20°C is enthalpically driven, with an unfavorable entropy contribution. SPR kinetic studies, with careful control of possible mass transfer effects, demonstrated that the aptamer binds to VEGF with an association rate constant $k_{on} = 4.79 \pm 0.03 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ and a dissociation rate constant $k_{off} = 5.21 \pm 0.02 \times 10^{-4} \text{ s}^{-1}$ at 25°C. Key recognition hot-spots were determined by a combination of aptamer sequence substitutions, truncations, and extensions. Most single-nucleotide substitutions, particularly within an mfold-predicted stem, suppress binding, whereas those within a predicted loop have a minimal effect. The 5'-end of the aptamer plays a key role in VEGF recognition, as a single-nucleotide truncation abolished VEGF binding. Conversely, an 11-fold increase in the association rate (and affinity) is observed with a single cytosine nucleotide extension, due to pairing of the 3'-GGG with 5'-CCC in the extended aptamer. Our approach effectively maps the secondary structural elements in the free aptamer, which present the unpaired interface for high affinity VEGF recognition. These data demonstrate that a directed binding analysis can be used in concert with library screening to characterize and improve aptamer/ligand recognition. © 2008 Wiley Periodicals, Inc. Biopolymers 91: 145–156, 2009.

Keywords: Biacore; kinetics; SPR; equilibrium binding; DNA secondary structure; aptamer modification

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INTRODUCTION

Aptamers are RNA or DNA molecules selected in vitro against specific targets which can recognize their binding partners with an affinity and specificity comparable with those of antibodies.1–5 Aptamers have been developed to recognize a variety of targets, including many of medical significance.6–9 In this work, we studied the binding of recombinant human vascular endothelial growth factor (VEGF165) to a 25-nucleotide truncated version of a DNA aptamer identified by Gold and Janjic10 using surface plasmon resonance (SPR), fluorescence anisotropy, and isothermal titration calorimetry (ITC).

VEGF is an essential growth factor and a primary regulator of angiogenesis through its interaction with two tyrosine receptor kinases Flk-1 and Flt-1.11,12 VEGF plays a key role in pathological processes, such as tumor growth,13,14 rheumatoid arthritis, and age-related macular degeneration (AMD).15 As a result, it is a key pharmaceutical target: the first anti-VEGF drug, bevacizumab (Avastin, a humanized monoclonal antibody) was approved by the Food and Drug Administration (FDA) in 2004 for the treatment of colorectal cancer. The structure of VEGF is that of an antiparallel homodimer,16 and the extracellular region of VEGF binds its tyrosine kinase receptors and other peptide inhibitors to form a 2(ligand):1(VEGF-homodimer) complex.12,17

Pegaptanib (Macugen), the first aptamer-based therapeutic, was approved by the FDA for the treatment of AMD in 2004. Pegaptanib is a modified 27-nucleotide RNA aptamer that inhibits angiogenesis by specifically targeting the heparin-binding domain of VEGF165.18,19 Pegaptanib has a dissociation constant (Kd) of 49 pM and its clinical version with a 5′-polyethylene glycol group has a Kd of 200 pM.20 Protein/DNA interactions play an important role in gene expression and other physiological processes, but for most of these interactions the DNA molecule is linear and double-stranded. Moreover, natural interactions have coevolved such that the contact interface may vary on both the DNA and protein sides to optimize binding. Aptamers, by contrast, are single-stranded RNA or DNA molecules with a three-dimensional structure that are selected against their binding partners with an affinity and specificity comparable with those of antibodies.21–25 There has been limited work on the biophysical aspects of aptamer–protein interactions.26–30 Here, we demonstrate by fluorescence anisotropy and ITC that the VEGF binding stoichiometry of a truncated form of the Gold and Janjic aptamer is 1(aptamer):1(VEGF-homodimer). Additionally, we report the kinetics of anti-VEGF aptamer/VEGF interaction determined using SPR experiments controlled for possible mass-transfer effects.

EXPERIMENTAL

Materials

The 25-mer anti-VEGF DNA aptamer (5′-CCGTCTTCCAGACAA GAGTGCAGGG-3′), its derivatives, and other oligonucleotides were purchased from Operon Technologies (Huntsville, AL), MWG Biotech (High Point, NC), or Sigma-Genosys (The Woodlands, TX).5′-Fluorescein labeled aptamer was purchased from MWG Biotech. Fluorescein was attached via custom phosphoramidite synthesis and the labeled oligonucleotide was purified by RP-HPLC after synthesis. VEGF165 (MW 37.8 kDa, pI = 8.5) was expressed in E. coli and purified as described by Zhang et al.2 Carboxymethylated dextran (CM5) Biacore™ SPR chips were obtained from GE Healthcare, Piscataway, NJ (previously Biacore). Failsafe Enzyme mix, Failsafe PCR Optimization Kit, AmpliScribe T7-Flash Transcription Kit, and Failsafe buffers were purchased from Epicentre Biotechnologies (Madison, WI). Ultra-pure water (conductivity 18 mΩ) from a Barnstead Nanopure Diamond water purification system was used in all experiments. All other reagents were purchased from Sigma-Aldrich, Co. (St. Louis, MO).

Fluorescence Anisotropy

A SPEX Fluorolog 212 fluorometer (Horiba Jobin Yvon, Edison, NJ) with two emission channels (one with a grating monochromator and one with a 520 nm long-pass filter) in T-format equipped with Glan-Thompson polarizers was used for the fluorescence anisotropy experiments. Excitation was at 496 nm and emission was at 518 nm with a 9.0 nm bandpass. A 100 μl quartz cuvette (Starna cells, Atascadero, CA) was used in all experiments. The G factor (ratio of IHH to IHH; where IHH and IHH are the observed intensities at horizontal or vertical orientations of the polarizers; excitation position is denoted by the first subscript) was determined before each experiment. For the settings above, the G factor ranged from 10 to 11 in T-format because of differences in channel sensitivity. During fluorescence anisotropy measurements, loss of the fluorescent label to photobleaching was minimized by performing all titrations within 15–20 min (found in control experiments to limit photobleaching to less than 5%). Also, a high concentration of VEGF (10 μM) was used for each titration to minimize dilution effects (the final volume after titration was 1.13 times the initial volume).

Equilibrium binding isotherms were constructed by titrating 100 nM of the fluorescein-labeled aptamer with increasing concentrations of VEGF in 20 mM Tris, pH 7.4 + NaCl (0, 50, 100, 150, or 200 mM NaCl), PBS (to match the SPR experimental conditions), and PBS + 5 mM MgCl2 at 25°C. The temperature in the cuvette was controlled to within ±0.8°C with a circulating water bath (IsoTemp 3016; Fisher Scientific, Pittsburg, PA). Before each experiment, the aptamer was heated at 80°C for 2 min and then slowly cooled to room temperature to facilitate folding.
Fluorescence Anisotropy Data Analysis

An approach by Titolo et al. was used to determine the equilibrium dissociation constant, $K_d$. The aptamer fraction bound ($f_b$) was determined using the following equation:

$$f_b = \frac{(A - A_t)}{(A_t - A_l)q + (A - A_t)}$$  (1)

where $q$ is the normalized intensity [see Eq. (2)] and $A$, $A_t$, and $A_l$ are the anisotropies of the sample, free aptamer, and aptamer saturated with protein, respectively. The normalized intensity was calculated using:

$$q = \frac{(I_{VV} + 2I_{VH})_{sample}}{(I_{VV} + 2I_{VH})_{free}}$$  (2)

where $I_{VV}$ and $I_{VH}$ are the observed intensities at different orientations of the polarizers as described earlier. The $q$ value ranged from 0.7 to 1.0. Binding was assumed to be described by a 1:1 binding model: $L + P \rightarrow LP$ where $L$, $P$, and $LP$ are free aptamer, VEGF-homodimer, and aptamer/VEGF complex, respectively. The fraction of aptamer bound is given by:

$$f_b = \frac{(L_l + P_l + K_d)}{2L_l}$$  (3)

where $L_l$ and $P_l$ are the total aptamer and VEGF concentrations, respectively. Using the experimentally determined values of $A$, $A_t$, and $q$, the anisotropy data were fitted by nonlinear least squares regression to Eq. (3) with $K_d$ and $A_l$ as parameters using Igor Pro 4.04 (WaveMetrics, Lake Oswego, OR).

Isothermal Titration Calorimetry

ITC measurements were performed at 20°C with a VP-ITC calorimeter (MicroCal, Northampton, MA). Ten milliliter each of 2 μM VEGF and 20 μM DNA aptamer were coidalyzed overnight at 4°C in 2 l of PBS buffer. The sample cell contained 1.495 ml aptamer in PBS buffer. The injection syringe contained 300 μl aptamer in the same coidalyzed buffer. Typically, a first injection of 2 μl preceded 30 injections of 10 μl. An injection speed of 0.5 μl s$^{-1}$ was used with a syringe rotational speed of 290 rpm. The time interval between injections was 300 s. The results were analyzed using Origin 7.0 (OriginLab Corporation, Northampton, MA).

SPR Chip Surface Preparation

SPR experiments were performed at 25°C in 10 mM phosphate buffered saline; 138 mM NaCl; 2.7 mM KCl; pH 7.4 with 0.005% Tween 20 (buffer A) using a Biacore 2000 instrument. CMS sensor chips were used for all binding analyses. Before aptamer immobilization, the chips were preconditioned in buffer A at a flow rate of 100 μl min$^{-1}$ by treating the unmodified surfaces separately with 50 mM sodium hydroxide, 33 mM hydrochloric acid, 3.47 mM sodium decyl sulfate, and 17 mM phosphoric acid, each applied as two consecutive 12-s pulses. Streptavidin (Sigma-Aldrich) was immobilized on the chip as described later, at a flow rate of 5 μl/min. Freshly prepared 400 mM N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC) and 100 mM N-hydroxysulfosuccinimide (sulfo-NHS) were mixed in a 1:1 ratio and passed over the sensor chip for 7 min followed by 200 μg ml$^{-1}$ streptavidin in 10 mM sodium acetate, pH 6.5 for 7 min. Unreacted carboxyl groups were then quenched with 1 M ethanolamine, pH 8.5 for 7 min. The 3'-biotin-modified DNA aptamer was immobilized on selected flow cells by passing 12.5 nM aptamer for 2 min at 5 μl min$^{-1}$. A low loading of 20–50 response units (RU) was used to minimize mass transport limitations. Biacore sensor chips were also prepared by immobilizing VEGF (using EDC/NHS coupling chemistry). However, the RU on flowing aptamer through VEGF-immobilized flow cells was negligible even at high VEGF loadings (200 RU) and aptamer concentrations (1 μM), suggesting that VEGF immobilization hinders sites on VEGF essential for aptamer binding.

DNA Aptamer-VEGF Binding Kinetics

VEGF at various concentrations (6.25, 12.5, 25, 50, and 100 nM in buffer A) was passed through the cell at 50 μl min$^{-1}$ to minimize mass transport limitations (see later). Association and dissociation profiles were monitored for 5 and 10 min, respectively. Regeneration was achieved by treating the surface with 9.5 mM sodium phosphate, 156 mM NaCl, 2.5 mM NaOH, pH 10.5 (50 mM NaOH + 500 mM NaCl diluted 20-fold with buffer A) for a 10-s pulse at 40 μl min$^{-1}$. Inclusion of 1 μM aptamer in the dissociation buffer did not alter observed dissociation rates, suggesting negligible rebinding of VEGF to unoccupied aptamer binding sites at the low loading densities used. All sensorgrams were double-referenced before data analysis. First, the response from the reference flow cell (without the immobilized aptamer; typically 10–15% of signal from the aptamer-loaded cell) was subtracted. Second, the response from an average of two-blank injections of buffer A was subtracted to account for any artifacts between the flow cells. The sensorgrams (duplicates for each concentration) were globally fit with parameters $k_{on}$ (association constant; M$^{-1}$ s$^{-1}$), $k_{off}$ (dissociation constant; s$^{-1}$), and $J_{max}$ (the theoretical maximum amount of VEGF that can bind to the aptamer; RU) using Scrubber 2.0 (Center for Biomolecular Interaction Analysis, University of Utah, UT). The data were also fit to test any effect of mass transfer by considering an additional parameter, $k_{int}$, which is a function of flow rate, diffusion coefficient, and flow cell dimensions. The following flow cell dimensions were used to calculate $k_{int}$: length = 2.4 mm; width = 0.5 mm; height = 0.05 mm. The diffusion coefficient of VEGF was calculated using the correlation $D = 3.6 \times 10^{-7}$ (MW)$^{-0.34}$ m$^2$ s$^{-1}$ (where $D$ is the diffusion coefficient in free solution at 23°C) and was adjusted to 25°C using the Stokes-Einstein relation. Using these values, $k_{int}$ was calculated to be $7.1 \times 10^8$ RU M$^{-1}$ s$^{-1}$ for a flow rate of 50 μl min$^{-1}$.

Matlab Simulations

The effects of mass transfer can result in erroneous SPR measurement of kinetic constants if not carefully addressed. To control possible mass transfer effects, a diffusion-reaction model proposed by Sigmundsson et al. was used to simulate the binding process. These authors proposed an analytical solution to this initial-value problem with coupled ordinary differential equations, assuming a quasi-steady state condition for the analyte surface concentration.
We avoided the need to make this assumption by solving the transport equations numerically using the “ode15s” solver in Matlab 6.5 (The MathWorks, Natick, MA). $k_0$, values of $3.3 \times 10^4$ and $7.1 \times 10^6$ RU M$^{-1}$ s$^{-1}$ were used for flow rates of 5 and 50 µl min$^{-1}$, respectively.

**Competitive Binding Analysis**

The effects of aptamer sequence modifications on VEGF recognition were studied by performing competitive binding experiments with modified DNA aptamers. The internal sequence substitutions were chosen based on the aptamer secondary structure predicted by mfold$^{40,41}$ at 25°C in 0.1M NaCl (Figure 6a). Mfold also predicts a similar structure of nearly identical low stability ($\Delta G = -2.04$ kcal mol$^{-1}$ vs. $-1.80$ kcal mol$^{-1}$ for that shown) in which the base pair between nucleotides 6 and 10 is absent, but no other predictions were of similar stability. Single-nucleotide substitutions were made such that purines were replaced with pyrimidines and vice versa. For example, substitution of the third base from the 5’-end, G, with an A was denoted by G3A. All possible single-nucleotide substitutions of the bases 1, 7, and 10 were also made, and all possible single-nucleotide extensions at the 5’- and 3’-ends were also independently tested. VEGF (66 nM) was incubated with 660 nM of each modified aptamer for 30 min, and the complex was then passed over a CM5 sensor chip loaded with 108 RU of biotin-modified anti-VEGF DNA aptamer; a higher concentration of the DNA aptamer was immobilized on the chip so that lower concentrations of VEGF could be used. The binding of aptamer to VEGF may be represented by:

$$L + P \rightleftharpoons LP$$

(4)

where $L$ is the ligand (aptamer), $P$ is the protein (VEGF-homodimer), $LP$ is the complex, and $k_{+1}$ and $k_{-1}$ are the association and dissociation rate constants, respectively. The rate of formation of the complex $LP$ may be written as:

$$\frac{d[LP]}{dt} = k_{+1}[L][P] - k_{-1}[LP]$$

(5)

Assuming VEGF concentration ([P]) to be constant and aptamer concentration to be $[L] = [L_0] - [LP]$, the solution to the above differential equation is:

$$[LP] = \frac{k_{+1}[L_0][P]}{k_{+1}[P] + k_{-1}} \left(1 - e^{-\left(k_{+1}[P] + k_{-1}\right)t}\right)$$

(6)

where $[L_0]$ is the aptamer concentration immobilized on the surface of the sensor chip. Equation (6) may also be written in terms of RU as:

$$[LP]_{RU} = \frac{k_{+1}R_{max}[P]}{k_{+1}[P] + k_{-1}} \left(1 - e^{-\left(k_{+1}[P] + k_{-1}\right)t}\right)$$

(7)

The association data from the competitive binding experiments were fit to Eq. (7) using Igor Pro (WaveMetrics, Lake Oswego, OR) to predict the parameter $[P]$ (VEGF concentration available for binding), whereas the parameters, $k_{+1}$, $k_{-1}$, and $R_{max}$ were held constant at the values obtained from the competitor-free experiments. The fraction of VEGF bound to the competitor (modified aptamer) was found by subtracting the ratio of VEGF concentration (available for binding) with and without competitor from unity.

**Two-Conformer Kinetics**

The complex kinetics of Figs. 6 and 7 were analyzed in terms of a hypothesized existence of two conformers of the aptamer with different kinetic properties. Assuming that the aptamer ligand exists as two conformers, $L_1$ and $L_2$, the “two-form” binding process may be represented by:

$$L_1 + P \rightleftharpoons L_1P \quad \text{and} \quad L_2 + P \rightleftharpoons L_2P$$

(8)

For the dissociation phase, assuming the forms $L_1$ and $L_2$ are not altered on VEGF association, the expression for the fraction of dissociating complexes (assuming no re-binding) is:

$$\frac{[L_1P] + [L_2P]}{[L_1P]_0 + [L_2P]_0} = be^{-k_1t} + (1 - b)e^{-k_2t}$$

(9)

where $[L_1P]_0 + [L_2P]_0$ is the concentration of the complex (in RU) before dissociation and “b” is the fraction of aptamer/VEGF complex that dissociates with a dissociation rate constant of $k_1$. For the association phase, the solution [refer to Eq. (7)] of Eqs. (8a) and (8b) in terms of RU is:

$$\frac{[L_1P] + [L_2P]}{[L_1P]_0 + [L_2P]_0} = a\frac{k_{+1}R_{max}[P]}{k_{+1}[P] + k_{-1}} \left(1 - e^{-\left(k_{+1}[P] + k_{-1}\right)t}\right)$$

$$+ (1 - a)\frac{k_{+2}R_{max}[P]}{k_{+2}[P] + k_{-2}} \left(1 - e^{-\left(k_{+2}[P] + k_{-2}\right)t}\right)$$

(10)

where “a” is the fraction of aptamer which exists as form $L_1$.

The dissociation data for aptamer/VEGF interactions (in RU vs. time) was converted to fraction of aptamer/VEGF complex remaining bound vs. time by dividing the dissociation data by the maximum response (RU) of VEGF bound. The resulting data were globally fit to Eq. (9) using Igor Pro with $k_1$ and $k_2$ as global parameters and “b” as a local parameter. The association rate constants for aptamer/VEGF interactions were obtained by globally fitting the association phase data to Eq. (10) using Igor Pro with $k_{+1}$, $k_{-1}$, and “a” as global parameters, and $R_{max}$ as a local parameter. Additional constraints were imposed by holding the parameters, $k_{+1}$ and $k_{-1}$ constant at the values obtained from the dissociation data fits.

**Synthesis of RNA Control Aptamer**

To create a double-stranded DNA template for in vitro transcription of the 35-nucleotide anti-VEGF RNA control aptamer, “84t$^{42}$”, the oligos 5’-GCTCTACGGCGAGGACAGGCCTCCAACTATTGAGC GCTCTACGGCGAGGACAGGCCTCCAACTATTGAGC TAIAGTGAGTGATGATTTA-3’ (italicized and underlined nucleotides correspond to DNA analog of the complement of “84t”) and 5’-TAACTGACTCTAGTTAGG-3’ were annealed in a GeneAmp 2400 thermal cycler (Perkin Elmer, Waltham, MA). To the annealed oligos, Epicentre Failsafe Enzyme Mix and Failsafe buffer “E” containing dNTPs were added, and allowed to extend to create the full length double-stranded template which was amplified using polymerase chain reaction (PCR). The RNA aptamer “84t” was then generated by in vitro transcription of the PCR-amplified dsDNA.

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RESULTS

Fluorescence Anisotropy
Equilibrium fluorescence anisotropy titration curves (see Figure 1) fit well to a 1:1 (aptamer:VEGF homodimer) binding model and not to a 2:1 binding model, suggesting that the aptamer binds to the dimer-forming interface of VEGF. As shown in Table I, the equilibrium dissociation constant ($K_d$) increases with salt concentration, suggesting counter-ion liberation on binding. Titration of the 5'-fluorescein-labeled DNA aptamer with VEGF in PBS buffer gave a $K_d$ of 404 ± 67 nM. Addition of 5 mM MgCl$_2$ to the buffer reduces affinity, increasing $K_d$ 2.6-fold to 1070 ± 198 nM (Table I). To identify the number of ion pairs formed during protein/nucleic acid interactions, we used the approach developed by Record et al.$^{45}$ The log–log plot of equilibrium dissociation constant vs. salt concentration was fit to a straight line with a slope of 1.5 ± 0.2. The slope of this plot gives the number of ions released on protein–nucleic acid interaction, and the ratio of the number of ions released to the fraction of counter-ions thermodynamically bound per phosphate gives the number of ion pairs formed between protein and nucleic acid.$^{45}$ Taking the fraction of counter-ions thermodynamically bound per phosphate as 0.71$^{46}$ yielded a value of 2.1 ± 0.3 for the number of ion pairs formed on aptamer-VEGF binding.

Isothermal Titration Calorimetry (ITC)
Calorimetric titration of the aptamer with VEGF at 20°C in PBS buffer gives a well-defined sigmoidal curve (see Figure 2). The fit gives an equilibrium dissociation constant of 40.5 ± 4.1 nM and aptamer/VEGF-homodimer stoichiometry of

![FIGURE 1](https://example.com/fig1.png)

**FIGURE 1** Equilibrium fluorescence anisotropy binding isotherms obtained from titration of 5'-fluorescein-labeled DNA aptamer (100 nM) with VEGF in 20 mM Tris, pH 7.4 + different salt concentrations: 0 mM (■), 50 mM (●), 100 mM (▲), 150 mM (▲▲), and 200 mM (×) NaCl at 25°C. The binding of the aptamer in PBS (△; dashed line) and PBS +5 mM MgCl$_2$ (●; dotted line) are also shown. The nonlinear curves are fits of the 1/aptamer:1 (VEGF dimer) binding model.

![FIGURE 2](https://example.com/fig2.png)

**FIGURE 2** Calorimetric titration of 2 μM VEGF with 20 μM anti-VEGF DNA aptamer in PBS buffer at 20°C. The integrated heat change per injection (■) along with the single-site binding model fit are shown.

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**Table I** Aptamer/VEGF Complex Equilibrium Dissociation Constants in Different Buffers and Salt Concentrations at 25°C Obtained by Fitting the Binding Data to a 1(Aptamer):1(VEGF Homodimer) Binding Model.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>$K_d$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM Tris, pH 7.4 + NaCl</td>
<td>0 mM 1.9 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>50 mM 67.0 ± 8.6</td>
</tr>
<tr>
<td></td>
<td>100 mM 146.1 ± 35.1</td>
</tr>
<tr>
<td></td>
<td>150 mM 222.6 ± 54.6</td>
</tr>
<tr>
<td></td>
<td>200 mM 855.7 ± 284.0</td>
</tr>
<tr>
<td>PBS</td>
<td>403.6 ± 66.6</td>
</tr>
<tr>
<td>PBS + 5 mM MgCl$_2$</td>
<td>1066.4 ± 198.0</td>
</tr>
</tbody>
</table>

Mean ± 1 standard deviation.
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Dissociation kinetic profiles for the binding of VEGF to aptamer are shown in Figure 3 along with the global fits of the two global parameters: association constant ($k_{on}$) and dissociation constant ($k_{off}$). Global fitting with and without correction for mass-transfer limitations resulted in association constants of $4.79 \pm 0.03 \times 10^4 \, M^{-1} \, s^{-1}$ and $5.14 \pm 0.03 \times 10^4 \, M^{-1} \, s^{-1}$, respectively, and dissociation constants of $5.21 \pm 0.02 \times 10^{-4} \, s^{-1}$ and $4.64 \pm 0.03 \times 10^{-4} \, s^{-1}$, respectively. The ratio of $k_{off}$ to $k_{on}$ gave a $K_d$ of 10.9 ± 0.1 nM, when corrected for mass-transfer effects.

**Effects of Aptamer Modifications in the Stem-Loop Region**

The fraction of VEGF bound to initial DNA aptamer in the presence of each competitor is shown in Figure 5. The RNA control aptamer used in the competitive binding studies exists as two different conformers which bind to VEGF with equilibrium dissociation constants of 1.8 and 31 nM. The RNA control aptamer binds to VEGF with higher affinity than the initial DNA aptamer suggesting that the RNA aptamer sequesters VEGF completely and prevents VEGF from binding to the immobilized aptamer. The DNA aptamer modifications tested here were chosen based on the mfold-predicted secondary structure of the aptamer. Mfold predicts nucleotides 3–13 to be involved in a stem-loop structure and substitutions were primarily introduced in this region. Substitutions in the mfold-predicted loop region T7A, T7C, T7G, C8T, and C9T show similar or slightly higher affinity compared with the initial aptamer sequence. In contrast, the substitutions in the mfold-predicted stem T4C, C5T, A10G, G11A, A12G, and C13T result in considerable loss of aptamer affinity. The loss of affinity for the substitu-

Matlab Simulations

Modeling of mass transfer effects as a function of flow rate is shown in Figure 3. Operating the Biacore instrument with high flow rate and low immobilized aptamer density reduced mass transfer effects to keep the VEGF concentration near the surface ($A_{surface}$) closer to the bulk concentration ($A_{bulk}$). With an $R_{max}$ of 1000 RU and $A_{bulk} = 200 \, nM$, $A_{surface}$ at 2.0 s reaches 150 nM and 192 nM with flow rates of 5 and 50 $\mu l\, min^{-1}$, respectively. However, with a lower $R_{max}$ of 100 RU and a flow rate of 50 $\mu l\, min^{-1}$, $A_{surface}$ reaches 199 nM (within 1% of the bulk concentration) in 2.0 s. The kinetic parameters obtained when operating under high flow rate and low ligand (aptamer) density would closely approximate true kinetics with minimal effects from mass-transport limitations. The experimental rate constants obtained with and without the mass transfer correction fitting term matched closely, suggesting that the experimental conditions chosen were suitable for kinetic measurements.

Kinetics of Aptamer-VEGF Interactions

Association and dissociation kinetic profiles for the binding of varied concentrations of VEGF in buffer A to aptamer are shown in Figure 4 along with the global fits of the two global parameters: association constant ($k_{on}$) and dissociation constant ($k_{off}$). Global fitting with and without correction for mass-transfer limitations resulted in association constants of $4.79 \pm 0.03 \times 10^4 \, M^{-1} \, s^{-1}$ and $5.14 \pm 0.03 \times 10^4 \, M^{-1} \, s^{-1}$, respectively, and dissociation constants of $5.21 \pm 0.02 \times 10^{-4} \, s^{-1}$ and $4.64 \pm 0.03 \times 10^{-4} \, s^{-1}$, respectively. The ratio of $k_{off}$ to $k_{on}$ gave a $K_d$ of 10.9 ± 0.1 nM, when corrected for mass-transfer effects.

**FIGURE 3** Matlab simulations demonstrating the influence of flow rate and $R_{max}$ on mass transport limitations. The calculated surface concentration of VEGF is plotted as a function of time for varied $R_{max}$ and flow rates. The parameters $k_{on} = 3.0 \times 10^4 \, M^{-1} \, s^{-1}$, $k_{off} = 5.0 \times 10^{-4} \, s^{-1}$, and $A_{bulk} = 200 \, nM$ were held constant whereas $R_{max}$ and flow rate were varied.

**FIGURE 4** Sensorgrams for the binding of different concentrations of VEGF (6.25, 12.5, 25, 50, and 100 nM) to 3'-biotin-modified anti-VEGF aptamer immobilized on streptavidin-functionalized CM5 chips. Association and dissociation were monitored for 5 and 10 min, respectively; duplicates are shown along with global fits obtained using Scrubber 2.0 with $k_{on}$, $k_{off}$, and $k_{on}$ ($k_{on}$ held constant at $7.1 \times 10^9 \, RU \, M^{-1} \, s^{-1}$) as global parameters and $R_{max}$ as local parameter. A flow rate of 50 $\mu l\, min$ was used to minimize mass transport limitations.

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tions T4C and A12G (85 vs. 84%), and C5T and G11A (89 vs. 84%) is remarkably similar, suggesting that these bases form base-pairs in the context of Stem 1 (see Figure 6a). The other internal modification studied (G18A) shows that a large effect on the fraction of VEGF bound is potentially due to disrupting the nucleotide/VEGF interface contacts.

Effects of Aptamer Extensions/Truncations
Truncation by 1 nucleotide (1tr) or 3 nucleotides (3tr) at the 5’ end results in complete loss of affinity for VEGF, but a substitution of the same nucleotide, C1A, has very limited effect while the substitution C1T results in 62% loss of affinity (see Figure 5). One-nucleotide extensions at the 3’-end A26, G26, C26, and T26 result in 31, 36, 33, and 11% loss of affinity, respectively. On the other hand, 1-nucleotide 5’-end extensions 0A, 0G, and 0T show slight improvements in affinity, whereas 0C (a truncated aptamer selected by Gold and Janjic10) shows considerable enhancement in affinity (see Figure 5). Based on the above results and the mfold-predicted secondary structure, we hypothesize that addition of a single cytosine to the initial aptamer may produce additional structural stabilization from pairing of 5’-CCC and 3’-GGG (Figure 6a).

To gain better insight into 0C aptamer/VEGF interactions, a separate kinetics study was performed by immobilizing 3’-biotin modified 0C aptamer on streptavidin-functionalized CM5 sensor chips (as described in the Methods section). Association and dissociation profiles for 0C aptamer/VEGF interactions were obtained by passing 50 and 100 nM VEGF through a 0C aptamer-immobilized flow cell. As compared to the initial aptamer, 0C/VEGF interaction (assuming 1:1 binding and with correction for mass-transfer limitations) is marked by a 11-fold higher association rate ($k_{on} = 5.25 \pm 0.03 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$) and similar dissociation rate ($k_{off} = 4.83 \pm 0.03 \times 10^{-4} \text{s}^{-1}$) resulting in a 12-fold lower $K_d, 0.92 \pm 0.01 \text{nM}$, which agrees closely with the value of 0.7 nM obtained by Gold and Janjic.10 In contrast to the statistically reliable fits in Figure 4, the 1:1 binding model fits in Figure 7a significantly deviate from the experimental data (as observed from visual inspection of the fits and residuals). Therefore, an alternate binding mechanism involving two different conformers of the aptamer (two-conformer model) was explored to fit the kinetics data and gain better understanding of the binding mechanism.

Fitting the dissociation phase data to Eq. (9) resulted in two-conformer binding model parameters: $k_{-1} = 2.60 \pm 0.03 \times 10^{-4} \text{s}^{-1}$, $k_{-2} = 1.47 \pm 0.03 \times 10^{-2} \text{s}^{-1}$, $b_{100 \text{nM}}$ =
0.87, and $b_{50\text{ nM}} = 0.90$ (Figure 7b). Using the above-estimated parameters and fitting the association phase data to Eq. (10) resulted in $k_{11} = 2.65 \pm 0.10 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, $k_{12} = 6.75 \pm 0.41 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, $R_{\text{max(100 nM)}} = 182.6 \pm 2.2$, $R_{\text{max(50 nM)}} = 135.2 \pm 2.8$, and $a = 0.45 \pm 0.03$ (Figure 7b), suggesting that the kinetic data could be explained by assuming a 45% initial population of the higher affinity aptamer species which forms 87–90% of the final complex. The two-conformer binding analysis shows that the two conformers bind with equilibrium dissociation constants of $k_{-1}/k_{11} = 1.0 \text{ nM}$ and $k_{-2}/k_{12} = 21.7 \text{ nM}$. Compared with the initial aptamer, ~90% of 0C extended aptamer/VEGF complex binds tightly with an 11-fold higher association rate and similar dissociation rate, and the remaining 10% exhibits weak affinity due to a 28-fold higher dissociation rate but a similarly accelerated association rate. The increase in the association rate may be attributed to an enhancement in the stability and/or population of a DNA secondary structure that preorders the unpaired nucleotides in the aptamer to a preferred orientation for interacting with VEGF.

**Proposed Aptamer/VEGF Binding Mechanism**

Based on the above observations, we propose that aptamer/VEGF binding follows a mechanism in which conformational
rearrangements of the aptamer affect the association process. We believe that the stem regions provide stability to the aptamer, enhancing binding competence, whereas the nucleotides in the loop regions are involved in close-range, relatively sequence-independent interactions offering stability to the aptamer/VEGF complex. Aptamer/VEGF binding has an unfavorable entropy change (ITC data) consistent with a loss in the conformational flexibility of the aptamer on binding. Therefore, an attempt to lower this unfavorable entropy contribution and further improve aptamer/VEGF binding was made by enhancing aptamer stability. To achieve this, the 0C aptamer was extended with 5'-G and 3'-C; this modification was performed specifically to extend “Stem 2” formed between the termini by an additional GC pair (Figure 6a). A two-conformer binding analysis of the kinetic profiles with 50 and 100 nM VEGF resulted in the parameters: \( k_{+1} = 1.87 \pm 0.13 \times 10^6 \text{ M}^{-1} \text{s}^{-1}, k_{-1} = 7.00 \pm 0.07 \times 10^{-4} \text{ s}^{-1}, \) and \( a = 0.19 \pm 0.03 \) for the first species, and \( k_{+2} = 4.33 \pm 1.44 \times 10^4 \text{ M}^{-1} \text{s}^{-1}, k_{-2} = 1.75 \pm 0.05 \times 10^{-2} \text{ s}^{-1} \) for the second species (see Figure 8). The two conformers bind with equilibrium dissociation constants of 0.37 nM (30-fold higher affinity than the initial sequence) and 404 nM, with the initial population containing 19% of the higher-affinity species which forms a disproportionate share (83–90%) of the final complex due to its more rapid association and slower dissociation.

**DISCUSSION**

As shown in Figure 1 and Table I, equilibrium binding studies using fluorescence anisotropy demonstrate the role of electrostatics on aptamer-VEGF binding, as expected from the overall positive charge of VEGF at pH 7.6 and the polyanionic nature of the aptamer. The 2.6-fold increase of \( K_d \) in the presence of Mg\(^{2+}\) ions may be attributed partly to a 15 mM increase in the ionic strength. Another major contribution could be magnesium’s conformational stabilization of nucleic acids which may reduce the conformational flexibility of the aptamer and/or result in a conformation that is not capable of recognizing VEGF.47–49

From a thermodynamic standpoint, aptamer/VEGF interaction is enthalpically driven with an unfavorable entropic
The kinetics of aptamer-VEGF interaction is characterized by moderate association and slow dissociation rates (see Figure 4). The diffusion coefficient for VEGF was calculated to be 9.8 \times 10^{-11} \text{ m}^2 \text{ s}^{-1} as discussed earlier. For single-stranded DNA, the diffusion coefficient (obtained based on fluorescence recovery after photobleaching measurements on ssDNA ranging from 280 to 5386 nucleotides) scales as $N^{-0.49}$, where $N$ is the number of nucleotides. Using this relation and taking the diffusion coefficient for a 280-nucleotide ssDNA to be $1.9 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$, the diffusion coefficient for a 25-nucleotide ssDNA molecule was estimated to be $6.2 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$. From the diffusion coefficients, the hydrodynamic radii of the molecules were estimated using the Stokes-Einstein relation and the diffusion-limited association rate constant was estimated using the modified Smoluchowski equation to be $7.4 \times 10^{-10} \text{ M}^{-1} \text{ s}^{-1}$ (assuming the unitless interaction parameter to be 0.01 based on a rough estimate of 10% active binding surface for each molecule), which is several orders of magnitude higher than the experimentally determined values of $4.8-53.0 \times 10^{4} \text{ M}^{-1} \text{ s}^{-1}$. This estimate, however, does not accurately account for long-range electrostatic interactions and the finite probability of encounter pairs forming stable complexes depending on their position and orientation, but some of the disparity may arise from the presence of only a minority of aptamer molecules preorganized for VEGF association or from rate-limiting conformational rearrangements.

To study the effect of aptamer sequence modification on VEGF recognition, we used a competitive binding analysis on an SPR chip. A competitive-binding strategy was used such that the most important interactions occurred in solution. Further, we address the key issue of mass transport limitations as outlined in the Matlab Simulations (see Figure 3) to minimize any effect on the results. Our results clearly indicate that substitutions in the predicted loop region of the DNA aptamer have very limited effect on binding, suggesting that target recognition does not depend on base-pairing or base-specific interactions of these nucleotides. However, substitutions in the predicted stem region cause almost complete loss of affinity suggesting that the stem plays an important, although potentially indirect, role in VEGF recognition (see Figure 5). The substitutions at nucleotides T6 and A10 reduce affinity by 20% and 71%, respectively. Although the most stable mfold-predicted secondary structure of the aptamer does not involve a T6-A10 base pair, the decrease in affinity on replacement of T6 or A10 suggests that these are base-paired at least part of the time. Pairing of the 5’- and 3’-ends is key to stabilization of the free aptamer and VEGF recognition, as a 1-nucleotide deletion at the 5’-end causes complete loss of affinity and the substitutions C1A, C1G, and CIT reduce binding by 12, 46, and 62%, respectively (see Figure 4). All four 1-nucleotide extensions at the 3’-end result in loss of affinity which could be attributed to the destabilizing effect of 3’-end overhangs (Figure 6a). In contrast, all four 1-nucleotide 5’-end extensions result in enhanced affinities with 0C increasing affinity to $K_d = 0.9 \text{nM}$ primarily due to an increased association rate constant. Hence a role of the secondary structure identified within the free aptamer, stem loop, and 5’- and 3’-pairing, may lie in preorganization of the extensive unpaired bases (A14-A22) in an orientation that allows for high affinity binding of the VEGF homodimer (see Figure 7). These observations are consistent with the recent data of Katilius et al. on an anti-IgE DNA aptamer, who identified the unpaired residues in a loop as essential for binding and that the paired 5’ and 3’ ends organized the overall topology of the molecule. Further, single molecule studies indicate that divalent cations compact this DNA aptamer and that a more open structure is formed on formation of a complex with VEGF. Foote and Milstein have observed complex biphasic and triphasic kinetics using stopped-flow fluorophores in antibody–hapten binding. They proposed binding mechanisms involving conformational isomers along with multiple association steps. As shown in Figure 7b, a minority unordered population of the aptamer might contribute to the slightly multiphasic character of this aptamer’s interaction with VEGF. The kinetics indicate that approximately 90% of the aptamer/VEGF complex binds tightly with a $K_d$ of 1.0 nM, whereas 10% binds with a $K_d$ of 21.7 nM. Addition of the 5’-cytosine induces an 11-fold increase in the association rate constant; however, no such increase in affinity was observed with other base extensions (see Figure 5) suggesting that this increase is not purely due to electrostatic interactions but from enhanced conformational organization of the aptamer before VEGF binding (also note that the enhanced association rate is still two orders of magnitude lower than the Smoluchowski diffusion-limited association rate). Interestingly, the 5’-cytosine extension does not significantly improve the stability of the aptamer/VEGF complex as the change in dissociation rates is negligible, suggesting that short-range interactions are not greatly affected by the extension. The biphasic nature of these
interactions could arise from a heterogeneous distribution of the aptamer as explained by the two-form model (Figures 6 and 7). The model predicts ~10% population of the 0C extended aptamer/VEGF complex to bind weakly with a dissociation rate constant of $1.47 \times 10^{-2}$ s$^{-1}$, which is 28-fold faster than the initial aptamer $(5.21 \times 10^{-4}$ s$^{-1}$), and the remaining 90% to bind tightly with a rate constant of $2.6 \pm 0.03 \times 10^{-4}$ s$^{-1}$, half the rate of the initial aptamer. Kinetic analysis of the 5'-GC-, 3'-C-extended aptamer indicates the presence of two conformers: a high affinity conformer ($K_d = 0.37$ nM) and a lower affinity conformer ($K_d = 404$ nM). The high affinity conformer has a 39-fold higher association rate than the initial aptamer sequence and a 7-fold higher association rate than the 0C extended aptamer which could be attributed primarily to a more stable aptamer conformation. However, the increase in stability may result in reduced conformational flexibility of the aptamer as observed from an increase in the dissociation rate from $5.21 \pm 0.02 \times 10^{-4}$ s$^{-1}$ (for the initial sequence) to $7.00 \pm 0.07 \times 10^{-4}$ s$^{-1}$.

In conclusion, our data clearly indicate that aptamer stabilization is a key component of target recognition. This observation strongly agrees with our recent single-molecule fluorescence resonance energy transfer studies in which addition of VEGF shifts the aptamer’s conformational equilibrium and the interaction is mediated by a stable but reversible conformation. Improving aptamer stability results in higher association rates, but the stability of complex is reduced due to lower aptamer conformational flexibility. Therefore, adding additional stability to the aptamer has to be undertaken cautiously such that conformational flexibility results in higher association rates, but the stability of complex is reduced due to lower aptamer conformational flexibility. Improving aptamer stabilization is a key component of target recognition. This observation strongly agrees with our recent single-molecule fluorescence resonance energy transfer studies in which addition of VEGF shifts the aptamer’s conformational equilibrium and the interaction is mediated by a stable but reversible conformation. Improving aptamer stability results in higher association rates, but the stability of complex is reduced due to lower aptamer conformational flexibility. Therefore, adding additional stability to the aptamer has to be undertaken cautiously such that conformational flexibility is not substantially compromised. In this view, we believe internal substitutions based on the predicted secondary structure of the library-selected aptamer along with 5’- and 3’-end extensions are excellent candidates to be tested combinatorially. Clearly a selective mutagenesis approach to map aptamer secondary structure and binding mechanism could be coupled with selective covalent modifications to further improve aptamer binding characteristics.

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