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Publication Date

2014-09-01

DOI

10.1016/j.chroma.2014.07.002

Peer reviewed

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1 **Probing and Quantifying DNA-protein Interactions with Asymmetrical Flow**

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Field-Flow Fractionation

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21Abstract

22 Tools capable of measuring binding affinities as well as amenable to downstream
23sequencing analysis are needed for study of DNA-protein interaction, particularly in discovery of
24new DNA sequences with affinity to diverse targets. Asymmetrical flow field-flow fractionation
25(AF4) is an open-channel separation technique that eliminates interference from column packing
26to the non-covalently bound complex and could potentially be applied for study of
27macromolecular interaction. The recovery and elution behaviors of the poly(dA)_n strand and
28aptamers in AF4 were investigated. Good recovery of ssDNAs was achieved by judicious
29selection of the channel membrane with consideration of the membrane pore diameter and the
30radius of gyration (R_g) of the ssDNA, which was obtained with the aid of a Molecular Dynamics
31tool. The R_g values were also used to assess the folding situation of aptamers based on their
32migration times in AF4. The interactions between two ssDNA aptamers and their respective
33protein components were investigated. Using AF4, near-baseline resolution between the free and
34protein-bound aptamer fractions could be obtained. With this information, dissociation constants
35of ~16nM and ~57 nM were obtained for an IgE aptamer and a streptavidin aptamer,
36respectively. In addition, free and protein-bound IgE aptamer was extracted from the AF4 eluate
37and amplified, illustrating the potential of AF4 in screening ssDNAs with high affinity to targets.
38Our results demonstrate that AF4 is an effective tool holding several advantages over the existing
39techniques and should be useful for study of diverse macromolecular interaction systems.

40

41**Key words:** field-flow fractionation, DNA-protein interaction, aptamer, DNA folding, affinity
42measurement

43 1. Introduction

44 DNA-protein interactions control diverse cellular functions, such as transcription regulation,
45DNA replication and repair, chromosome maintenance, etc. Interruption of such interactions
46could lead to numerous diseases, including cancers.[1,2] To achieve a better understanding of
47the nature of DNA-protein interaction, numerous research efforts have been devoted to
48measurement of binding kinetics and affinity, and identification of DNA sequences with affinity
49to transcription factors, polymerases, histones, etc.[3-6] In addition to endogenous interactions,
50synthetic DNA probes - DNA aptamers - that can interact with proteins and other biological
51targets strongly and specifically, have been discovered [7,8] with high utility in molecular
52detection and great potential as therapeutic agents.[9-12] Discovery of additional aptamers with
53high functionality and obtaining more knowledge on aptamer-target interaction are thus in great
54demand.

55 Many techniques have been developed for mapping of DNA-protein interaction.[3] Although
56high-throughput next generation sequencing techniques have greatly enhanced the discovery rate
57and reliability of DNA-protein interactions,[5,13] the first step of isolating the DNA-protein
58complex is limited to immunocapture on a solid support. Similar immobilization is also required
59for interaction studies using surface plasmon resonance [14] and protein arrays.[6,15]
60Immobilization could reduce protein stability, change binding epitopes, and introduce
61interference to the binding from the support surface.[16]

62 On the other hand, open channel separation methods like capillary electrophoresis (CE) can
63carry out affinity and kinetic measurements as well as binding screening without protein
64immobilization, in which the absence of channel packing imposes minimal disturbance to the
65interacting molecules. CE also provides the advantages of rapid separation and high resolution

66for study of various interaction systems, including DNA-protein, protein-nanoparticles, ligand-
67receptor, etc.[17-20] It may also be used for the selection of aptamers.[21] However, severe
68Joule heating that destroys separation resolution imposes big problems to CE, since buffers with
69high salt contents are needed to maintain good interaction,[22] appropriate DNA folding, and
70intact protein structure.[23-25] CE's low sample capability also makes it difficult to be coupled
71with downstream discovery of new sequencing with high affinity to targets.

72 Another open channel separation technique, asymmetric flow field-flow fractionation (AF4),
73should be useful for study of biological complexes as well.[26,27] Capable of separating analytes
74based on hydrodynamic size, AF4 employs an asymmetrical trapezoidal channel, the breadth of
75which decreases from inlet toward outlet. (Supporting Information Figure S1).[26] The carrier
76solution entering the channel from the inlet continuously escapes through the accumulation wall
77made of an ultrafiltration membrane, splitting into a cross flow and a decreasing axial flow. All
78analytes are pressed against the accumulation wall by the cross flow, and simultaneously diffuse
79away due to Brownian motion. Analytes with larger sizes diffuse slower and thus are located at a
80position closer to the wall, being eluted by the axial flow at later times than those with smaller
81sizes.

82 Compared to CE, AF4 can use carrier solutions with divalent cations and high ionic strength
83that is beneficial for maintaining complex integrity.[28] Additionally, its high sample capacity
84allows convenient after-column collection for down-stream analysis.[29] These advantages have
85been used in determining the affinity of protein-protein interactions[30] and for characterization
86of protein complexes,[31,32] and should also be useful in the discovery of ssDNAs with high
87affinity to targets. However, few reports on using AF4 to analyze DNA, in particular short
88ssDNAs containing motif for protein binding, have been found.[29,33-36] Giddings *et al.*

10

89demonstrated the initial ability of symmetrical F4 to fractionate ssDNA and dsDNA of similar
90length,[29] while Moon *et al.* demonstrated the ability of miniaturized AF4 to fractionate 50 and
91100nt ssDNA, as well as a complex between ssDNA and replication protein A.[33] In addition,
92Winnik *et al.* utilized AF4 to characterize DNA-chitosan complexes.[34] However, these
93previous applications did not specifically investigate the affinity of DNA towards specific
94targets. Herein, to assess AF4's capability in studying ssDNA-protein interaction, the present
95work explored the recovery and retention behaviors of a series of poly(dA)_n strands and several
96aptamers; evaluated whether aptamers could maintain certain degrees of folding during
97separation; measured the binding affinity of two aptamer-protein systems; and investigated the
98feasibility of using AF4 as an aptamer selection tool through collection and amplification of the
99eluted protein-bound aptamer.

100

101 2. Materials and Methods

1022.1 Chemicals

103 Phosphate buffered saline (1× PBS), used as the carrier solution in the recovery and elution
104studies, contained 137 mM NaCl, 10 mM phosphate sodium salts, 2.7 mM KCl, and 1.0 mM
105MgCl₂. Its pH was adjusted to 7.40 (± 0.03) with 6N HCl. This was also the buffer when
106preparing the IgE-aptamer binding mixture. Analysis of streptavidin-aptamer binding employed
107a buffer consisting of 20 mM Tris HCl (pH 7.4), 100 mM NaCl, 5 mM KCl, 2 mM MgCl₂, and 1
108mM CaCl₂. All carrier solutions for AF4 were filtered with a 0.2-μm nitrocellulose filter
109(Whatman, NJ, USA). Sources and preparation of biochemicals, including proteins and DNAs,
110were listed in Supporting Information.

111

1122.2 AF4 conditions

113 An AF2000 system manufactured by Postnova Analytics (UT, USA) was used in this
114study. The trapezoidal separation channel was 0.350 mm thick (thickness of the spacer), and its
115tip-to-tip length was 275 mm, with an inlet triangle width of 20 mm and an outlet width of 5 mm.
116The injection loop volume was 20 μ L. The surface area of the accumulation wall was 3160 mm²,
117which was made out of the regenerated cellulose ultrafiltration membrane (Postnova Analytics)
118with the molecular weight cutoff (MWCO) value of 5- or 10-kDa, definition useful for both
119proteins and nucleic acids. A longer tubing connecting the channel outlet to the detector was
120used with the 5-kDa membrane to correct for the backpressure change induced by the pore size
121difference, so that the same axial-outlet flow rates could be used for both membranes.

122 All separations were done with a channel-inlet flow of 3.30 mL/min, a crossflow of 3.00
123mL/min, and an axial-outlet flow of 0.30 mL/min. During the focusing/relaxation step, the
124channel-inlet flow was 0.30 mL/min and the focusing flow (a flow entering at a position further
125down from the inlet to focus the analyte into a narrow sample zone) was 3.00 mL/min. The
126focusing/relaxation step lasted for 8 minutes. A SPD-20A Prominence UV/Vis detector
127(Shimadzu, MD, USA) at 280 nm was used to monitor the eluent for unlabeled DNAs, and a 474
128scanning fluorescence detector with an excitation wavelength of 495 nm and an emission
129wavelength of 525 nm (Waters, MA, USA) was employed to monitor the fluorescently labeled
130DNAs. Fluorescence detection was used in the analysis of binding affinity between fluorescently
131labeled aptamers and their corresponding proteins, while absorbance detection was used in all
132other measurements.

133

1342.3 Recovery Calculation

135 Recovery of ssDNAs in AF4 was calculated using the peak areas obtained with or
 136 without a global crossflow, i.e. the apparent crossflow rate was zero. We defined the peak area of
 137 ssDNA obtained using no global crossflow but both the channel-inlet and axial-outlet flows at
 138 0.30 mL/min as A_{NCF} , which should be proportional to the concentration of ssDNA in the injected
 139 sample. The axial-outlet flow passes through the detector. The peak area obtained with the same
 140 axial-outlet flow rate at 0.30 mL/min but a channel flow at 3.30 mL/min and a crossflow at 3.00
 141 mL/min, was defined as A. Any reduction from A_{NCF} to A would indicate sample loss inside the
 142 AF4 channel. Thus, the ratio of A and A_{NCF} can be used to calculate sample recovery:

$$143 \quad \% \text{ Recovery} = (A/A_{NCF}) \times 100\% \quad (1)$$

144

145 2.4 Calculation of Dissociation Constant

146 For affinity measurement, a Alexa Fluor 488-labeled aptamer was first denatured in
 147 boiling water for 5 minutes and kept on ice until mixed with the target protein. Increasing
 148 amounts of protein were added to 2 nM of the Weigand aptamer (IgE) or 10 nM of the
 149 streptavidin aptamer. The mixtures were injected into the AF4 and fluorescence fractograms
 150 obtained. From the fractograms, the amount of free aptamer and protein-bound aptamer (peak
 151 area) was obtained. The bound ratio was determined through division of the area of the protein-
 152 bound aptamer peak by the sum of both free and bound aptamer peaks, which was then used to
 153 calculate the dissociation constant (K_d) via the Hill equation[34, 35]:

$$154 \quad \text{Bound ratio} = \text{Bound}_{\max} \times [P]^n / (K_d^n + [P]^n) \quad (2)$$

155 The Hill equation is commonly used to describe ligand-receptor binding. $\text{Bound}_{\max}$ is the bound
 156 ratio when the binding curve reached plateau; [P] represents free protein concentration in the
 157 mixture; and n is the cooperativity of binding.

1592.5 Molecular Dynamics (MD) Simulation and Folded Percentage Calculation

160 The initial 3D structures of the poly (dA)_n strands and the unfolded aptamers were in the
161linear B-form; and those of the folded aptamers were in the forms built using w3DNA
162(<http://w3dna.rutgers.edu>)[36] as predicted by Mfold, oligonucleotide secondary structure
163prediction software (<http://www.bioinfo.rpi.edu/applications/mfold>),[37] except for the thrombin
164aptamer. The thrombin aptamer folds into a G-quadruplex not predictable by Mfold; and its
165initial structure adopted the reported crystal structure (PDB ID: 4DIH), to which three more
166deoxyribonucleotides (AGT) were added to the 5' terminal. MD simulations were performed on
167the Stampede supercomputer resource (Texas Advanced Computing Center) provided by XSEDE
168Science Gateways with the Amber 12 program package.[38, 39] An AMBER 99 force field[40]
169and TIP3P water model[41] were applied. The counter ions of Na⁺, Mg²⁺, K⁺ and Cl⁻ were used
170in modeling at the same concentrations as in the AF4 carrier solution to mimic the actual solution
171environment for the ssDNAs. After energy minimization of the starting molecule structure, 1000
172picoseconds (ps) equilibration without restraints was performed with constant pressure and the
173periodic boundaries maintained by the particle mesh Ewald method,[42] while the volume of the
174box was allowed to change. The simulated 3D structure allowed the measurement of d_{xyz} of each
175atom, which was the distance from the atom to the center of mass of all selected atoms. The d_{xyz}
176values of all atoms were then used for calculation of the radius of gyration (R_g) of the worm-like
177ssDNA, using the mathematic function ($\sqrt{\text{SUM}[d_{xyz}^2 \cdot \text{mass}] / \text{total mass}}$) available in Amber.
178 R_g measures the root mean square distance of the parts from its center of gravity, and is directly
179proportional to the hydrodynamic radius of the DNA coil. The calculations were performed for

180every coordinate frame obtained for every 2 ps. More details about the MD process can be found
181in Supporting Information.

182 Using the R_g and the measured retention time of the poly(dA)_n strands, we obtained the
183calibration plot for Log t vs. Log R_g , which in turn was used to calculate the theoretical elution
184time of the unfolded or folded form of each aptamer, depicted as t_{UF} and t_F , respectively, based on
185their corresponding R_g values. The measured elution time (t_M) of each aptamer is in fact the
186weighted sum of t_{UF} and t_F with the weights being the percentage of each form in the entire
187population. Therefore, the percentage of aptamer molecules staying in a folded structure during
188AF4 separation can be calculated by:

$$189 \quad \% \text{Folded} = (t_{UF} - t_M) / (t_{UF} - t_F) \times 100\% \quad (3)$$

190

191 3. Results

192 3.1 Properties of ssDNAs selected for the study.

193 For better understanding of the elution behaviors of ssDNAs, we investigated ten strands, the
194sequences and molecular weights of which are listed in Table 1. Five were poly(dA)_n that should
195have the lowest possibility of forming intra-molecular H-bonding than other poly(dN)_n. Much
196lower dissociation energy and longer distance were predicted for the H-bond formed from
197mismatching base-pairing between two A bases than that of T-T, C-C, and G-G, as reported by
198Otero-Navas *et al.*[37] No folding was predicted by Mfold for all poly(dA)_n used in our study.
199The other five were capable of forming unique secondary structures as predicted by Mfold
200(Supporting Information, Figure S2), and referred to as aptamers in the following text. Among
201them was the anti-IgE aptamer with a stem-loop structure firstly reported by Weigand, *et al.* in
2021996[38] and thus called the Weigand aptamer (WA) throughout the text. Its complementary
203strand, cWA, can form two small stem-loops separated by 4 nucleotides and was also included.

204 Another anti-IgE aptamer identified by the Liu group,[39] the I9-102 strand, was tested as well,
205 the secondary structure of which has two bulges on its long stem (>13 base-pairs) and a long
206 overhang at the 5'-end of the stem. The 60-mer aptamer against streptavidin (SA) was
207 discovered as Aptamer 31 by Stoltenburg *et al.* in 2005.[40] The free energy of folding
208 calculated by Mfold for these aptamers were listed in Table 1. The last aptamer was the anti-
209 thrombin aptamer (TA) with a G-quadruplex structure, but its folding energy could not be
210 calculated by Mfold.

211 To better explain their recovery and elution behaviors, we employed MD simulation for
212 calculation of the radius of gyration, R_g , of the ssDNA in 1 mM Mg^{2+} solution and after energy
213 minimization. MD modeling has been found to be a promising tool for studying the structural
214 and dynamic conformation of DNAs.[41-43] To evaluate the ssDNA structure stability during
215 the simulation, the root-mean-squared deviation (RMSD) of all heavy atoms of each ssDNA was
216 obtained. Although we used a shorter simulation time, 1000 ps, than the 50 ns in the cited
217 reports,[41-43] due to limitation of the total service units available to us from XSEDE, the
218 RMSD values in the last 200 ps of the simulation typically had < 5% relative standard deviation
219 (RSD), except for poly(dA)₁₀, poly(dA)₂₀, and the TA, the RSD of which varied between 8-10%
220 (Supporting Information, Figure S3a). Still, the volume or bulkiness of all strands changed very
221 little, shown by the fairly constant R_g during the simulation duration (Supporting Information,
222 Figure S3b). The R_g values of the ssDNA in the B-form (for both the poly(A)_n and aptamers) and
223 folded structure (for aptamers only) are also shown in Table 1.

224

225 3.2 Recovery of ssDNAs in AF4.

226 Recovery of ssDNAs in AF4 was investigated, because the ssDNAs may escape through the
227AF4 channel wall made by the porous membrane. The 5- and 10-kDa MWCO regenerated
228cellulose ultrafiltration membranes were studied. The recovery was calculated by Equation 1
229and plotted vs. Mw in Figure 1 for each poly(dA)_n (square) or aptamer (circle) strand. For
230ssDNAs in both groups, those with Mw close to or larger 10 kDa could yield a recovery > 90%
231on the 10-kDa membrane (the solid curves in Fig. 1), which agrees with the membrane's MWCO
232definition. However, on the 5 kDa membrane, only the poly(dA)_n with $n \geq 30$ ($M_w \geq 9.33$ kDa)
233and the aptamers with $M_w \geq 11$ kDa could be eluted out of the channel at > 95% (the dotted
234plots in Fig. 1) with negligible adsorption on the membrane. Moreover, the two aptamers, the
2355.66 kDa TA and the 8.99 kDa cWA, had much lower recovery than the poly(A)_n with
236comparable Mw.

237 The recovery results on the 5-kDa membrane could be explained by the relative size of the
238ssDNA and the membrane pore. The pore diameter for the 5-kDa regenerated cellulose
239ultrafiltration membranes has been estimated to be around 2 nm.[44,45] Indeed, both the
240poly(dA) strands showing low recovery on the 5-kDa membrane are with R_g smaller than 2 nm
241(Table 1). As for the two aptamers with very low recovery, the TA has the R_g values for both the
242unfolded B-form and the folded structure below 2 nm; and the cWA could fold into a secondary
243structure with a R_g around 1.6 nm, although its B-form has an R_g of 2.93 nm. If the folded form
244is the dominant species of cWA in AF4, its recovery on the 5-kDa membrane would be low.
245However, the R_g values of the 8.99 kDa cWA could not explain its high recovery on the 10-kDa
246membrane, the estimated pore size of which is 3 nm. Unlike the case of the 5-kDa membrane,
247using Mw seemed to be good enough to judge the retention of the ssDNA on the 10-kDa
248membrane. The discrepancy could be due to the higher membrane pore density of the 5-kDa

249membrane. It has been reported by Ramamurthy *et. al* that[46], the pore density for a 3.5 kDa
250MWCO membrane was about 3 fold higher than that of the 11 kDa membrane. Similar
251differences may exist between our 5- and 10-kDa membranes. Unlike ultracentrifugation, AF4
252lacks a strong centrifugal force that would push most of the ssDNA molecules to go into the
253membrane pores. While the crossflow presses the ssDNAs against the membrane, increasing
254their interaction with the pores, the channel flow generates a shear force to strip them away from
255the pores. Nevertheless, a higher pore density of the 5-kDa membrane could increase the
256probability of smaller ssDNA passing through the pores. As a result, both the relative size of the
257pore and its pore density can influence the recovery of ssDNA in AF4. For a larger MWCO
258membrane (10kDa), the lowered pore density still allows for a smaller degree of small ssDNA
259loss compared to the smaller yet denser 5kDa membrane.

260 It should be noted that, recovery difference between the 10-kDa and 5-kDa membrane could
261be attributed by the channel permeable elements as well. We measured the recovery under a zero
262global crossflow, which was achieved by balancing the crossflow forth (from channel through
263the membrane) and the crossflow back (from reservoir to the channel through the membrane).
264The characteristic length of the channel and the membrane permeability and thickness, could
265affect these flow rates[47,48] and lead to different levels of analyte adsorption on the membrane.
266Therefore, these parameters should be taken into account when explaining the recovery
267difference.

268 Overall, our results point out that, the R_g or hydrodynamic radius of a DNA and the pore
269diameter of the membrane are more appropriate than the values of Mw and MWCO, when
270determining which membrane to use in AF4 for DNA separation. The 10-kDa membrane in
271general resulted in higher recovery of ssDNA compared to the 5-kDa membrane. Besides, its

272larger pore size induces lower back pressure to the channel flow, and allows easy removal of
273contaminants with Mw much lower than the MWCO value, which may be needed for
274applications aiming to purify the target complexes. Thus, the 10-kDa membrane was chosen in
275our following study.

276

2773.3 Elution of ssDNAs in AF4.

278 Study DNA-protein binding using separation technologies requires sufficient resolution
279between the free components and the complex. Thus, the Mw difference necessary to obtain
280adequate resolution needs to be evaluated. AF4 separates analytes based on their diffusion
281coefficient D. Based on the Stokes-Einstein equation, D is proportional to the reciprocal value of
282the molecule's hydrodynamic radius, R_H . [49-51] For a free-draining polymer like DNA and
283protein, the relationship of R_H and M takes the form of $R_H \sim M^x$. For globular proteins, x
284equals to 1/3. For DNA, x ranges from 1 for a rigid rod to 1/2 for a random coil. Thus, ssDNAs
285would have larger hydrodynamic diameters than globular proteins with the same Mw and be
286eluted later in AF4.

287 We compared the elution times of ssDNAs and proteins under identical flow conditions. The
288fractograms were shown in Figure 2. Cytochrome c (12.4 kDa), streptavidin (60 kDa),
289transferrin (76.5 kDa), and IgG (150 kDa) were chosen to cover a wide Mw range. Indeed,
290ssDNAs were eluted at much later times than proteins with comparable Mw. For instance,
291poly(dA)₅₀ and SA with Mw around 15-20 kDa, came out much later than the 12.4-kDa
292cytochrome c, but at comparable times as the 80-kDa transferrin. The average peak widths at
293half-peak-height ($w_{0.5h}$) of all ssDNAs and proteins tested were calculated to be 1.46 min.
294Therefore, to achieve a resolution larger than 1 (Note: Resolution = time difference / $1.7 W_{0.5h}$ for

295a Gaussian peak), the time difference between the target protein and the ssDNA should be larger
296than 2.48 min under the current flow conditions. This corresponds to proteins larger than 120
297kDa if the ssDNA size is around 15-20 kDa, judged from the plots of retention time vs.
298molecular weight shown in the Supporting Information Figure S4.

299 Our result highlights that AF4 is more suitable to study ssDNA binding with larger targets,
300such as bulky proteins like IgG and biological complexes like microvesicles and viral particles,
301allowing for the ability to do aptamer selection towards these larger targets, such as what is seen
302in cell-SELEX. Reliable relationship between retention time and molecule hydrodynamic
303diameter in AF4 make it easy to predict whether sufficient resolution could be obtained for a
304given pair of ssDNA and protein, using calibration curves from protein and nucleic acid
305standards. Additionally, adjusting the inlet/crossflow ratio can help to improve resolution for the
306more challenging ssDNA-protein systems.

307

3083.4 Folding of ssDNA in AF4 and MD simulation

309 The aptamers need to be folded in order to bind to proteins; and in return, interaction with
310target proteins could stabilize the folding structures. As discussed above, the diffusion coefficient
311D of a molecule is proportional to $1/Mw^x$. The value of x could indicate the flexibility of the
312molecule.[49,51,52] For rod-like polymers, in which the monomers are tightly connected, x
313could be 1; and for polymers behaving like a self-avoiding random walk chain, x could be
314around 0.5. This means a folded ssDNA should have an x larger than an unfolded ssDNA
315because of its higher rigidity. Since in AF4, retention time t is proportional to D of the analyte
316molecule, we evaluated the folding situation of the aptamers in AF4 by plotting the Log
317(retention time t) against Log (Mw) in Fig. 3. Three linear plots were observed, meaning that

318they all followed the scaling law of $D \sim 1/Mw^x$. The poly(dA)_n had a $x = 0.56$, while the
319aptamers yielded a x of 0.73. The scaling exponent measured for poly(dA) and poly(dT) strands
320using Small Angle X-ray Scattering (SAXS) was 0.55 in NaCl solutions, to which our result has
321high agreement.[53] The higher exponent observed for the aptamer molecules supports that they
322have lower flexibility than the poly(dA)_n strands owing to the formation of secondary structures
323while being eluted by AF4.

324 Imagine that the aptamers rapidly change between the unfolded and folded forms.[54-57] The
325measured retention time (t_M) should then be the weighted sum of the retention times of the
326aptamer when it is in the unfolded (t_{UF}) and folded (t_F) structures. Since the poly(dA)_n molecules
327have no secondary structures, they can be used as the standards to obtain the linear relationship
328between R_g and retention time t (Supporting Information, Figure S5). Using this relationship, the
329theoretical retention times of the unfolded B-form and the folded structure, t_{UF} and t_F , can be
330calculated from their corresponding R_g values listed in Table 1. Then, with Equation 3, the
331percentage of the aptamer molecules in the folded form can be obtained (Table 2). In general,
332aptamers with larger free energy drop per base when it folds, such as the WA and cWA, exhibited
333a %Folding > 70%, and those for SA and I9-102 dropped below 40%. The folded structure
334should be beneficial for maintaining the binding between ssDNAs and their targets.

335

3363.5 Affinity Measurement.

337 After confirmation that there was negligible loss of WA on the 10-kDa membrane, we went
338on to measure its affinity to the target protein IgE using AF4. An incubated mixture of Alexa-488
339labeled WA and IgE was injected onto the AF4, and the bound and free aptamer fractions
340separated and fluorescently quantified. Using the bound ratio of WA, the affinity between protein

341and aptamer was determined, represented as the dissociation constant K_d . With 2 nM aptamer,
342steady increase of the protein-bound WA peak with protein concentration changing from 6.7 nM
343to 107 nM was observed (Figure 4a). Fitting the average peak area ratio and the protein
344concentration to Equation 2, K_d was found to be 16.3 ± 0.5 nM. This value was about 60% higher
345than the reported value of 10 nM.[38] The discrepancy between the K_d value obtained by AF4
346and the previously reported value can be attributed to the addition of a fluorophore to the short
347aptamer that may disrupt the binding interaction between aptamer and protein.

348 We also confirmed that there was no substantial dissociation of the WA-IgE complex during
349AF4, by measuring the peak area sum of the free WA and the complex peaks in four repeated
350injections. The representative fractograms for both samples were shown in Figure 5. With the
351peak area sum of the free and IgE-bound WA peaks being found to be 2565.2 ± 256.7 , the WA
352peak in the aptamer-only sample was 2417.3 ± 250.5 . No significant difference was revealed by
353the two-Sample *t*-test at the confidence interval of 0.95 ($n = 4$). In addition, the average
354symmetry of the bound peak was 0.987 ± 0.047 , a value very close to 1, indicating the peak was
355essentially Gaussian in nature. Overall, it was determined that the WA-IgE complex did not have
356any significant dissociation while traveling through the column, confirming the validity of using
357AF4 to obtain the K_d value of WA binding to IgE.

358 The WA used for obtaining results displayed in Fig. 5 carried the primer binding regions on
359its 3' and 5' ends. Thus, the free WA and the IgE-bound WA were collected (Supporting
360Information for the collection procedure) and amplified by PCR, the collecting regions being
361marked by the grey brackets in Fig. 5. The PCR products were analyzed by PAGE (insert in Fig.
3625). Successful amplification of the WA in collections from the WA-IgE incubation sample was
363observed in the collection regions of 1-5, which corresponded to the free WA peak, as well as in

364region 7 and 8, for the IgE-bound WA peak. No band was shown in region 6, the valley in
365between the free and IgE-bound WA peak, again proving no complex dissociation. Additionally,
366no DNA band was observed in the regions 7-8 in the WA only sample. Our experiment has
367proved the principle of collecting and amplifying of the protein-bound ssDNAs for downstream
368analysis by AF4.

369 The binding of SA to its target, streptavidin, was also tested. With the same buffer as used for
370discovery of the particular SA, the Tris-buffered saline containing 2 mM MgCl_2 and 1 mM
371 CaCl_2 , the K_d value was found to be 20.9 ± 1.8 nM (Figure 4b), ~63% lower than the reported
372value of ~ 56.7 nM.[40] The increased affinity between SA and streptavidin in AF4 could be
373attributed to the nature of the interaction – in the previously reported study, streptavidin was on a
374solid support. This immobilization could result in conformation difference in streptavidin,
375resulting in differences in affinity between the two. The low %Folding of SA did not prevent
376successful observation of the aptamer-protein complex in AF4, probably because the complex
377was formed before injection to AF4 and the binding stabilized the secondary structure of the SA,
378making it less unlikely to unfold. On the other hand, if a different solution, the 1×PBS with 1
379mM Mg^{2+} , was used as the carrier solution for testing the SA-streptavidin interaction, a much
380larger K_d was found (Supporting Information Figure S6) which was about 800% different than
381the reported value. As can be seen, the selection of buffer conditions can greatly affect the
382affinity between aptamer and target. Ideally, the buffer chosen for aptamer selection should be
383identical to that used in a subsequent assay. AF4, due to its tolerance to a variety of salts and
384reagents, is an ideal platform for this selection process.

385

3864 Conclusions

387 In this study, successful affinity measurement for ssDNA-protein binding was performed by
388AF4, after examining the recovery and elution behavior of ssDNAs. We also demonstrated that
389the sizes for the ssDNAs in unfolded and folded structures obtained by MD simulation can help
390to illustrate the folding situation based on the elution time in AF4. Both are new attempts not
391reported previously, based on our knowledge.

392 Additionally, our result demonstrates that AF4 has the freedom to use different carrier
393solutions with high salt concentrations and divalent cations like Mg^{2+} and Ca^{2+} . Such buffers
394could be necessary to produce the best binding environment for the interactive system. In
395particular, the divalent cations can stabilize the folding of ssDNAs and thus be beneficial for
396maintaining target binding. Although our AF4 measurement yielded K_d values for IgE and
397streptavidin with ~60% deviation from the reported values, the deviation can be attributed to the
398difference between the binding strengths towards free or immobilized subjects. Both reported
399values were from immobilized proteins, and AF4 measurement was performed over freely
400suspended proteins.

401 Moreover, our results highlight the additional advantage of AF4 over existing techniques in
402study of DNA-protein binding: its high sample capacity, open separation channel, and size-based
403separation mechanism of AF4 allow it to be employed for screening the target-binding ssDNAs
404from a complex mixture without immobilization of either the target. With its unique capability
405of analyzing objects with relatively large sizes, AF4 could be highly useful in study of binding
406systems not suitable for the existing techniques, for example, binding involves proteins larger
407than hundreds of kDa, pathogenic particles, nanoparticles larger than 50 nm, etc.

408

409**Acknowledgements**

410 This work was supported by the National Science Foundation CAREER Grant # CHE-
4111057113. J. Ashby was supported by the National Science Foundation Graduate Research
412Fellowship under Grant Number DGE-0813967. MD calculation used the Extreme Science and
413Engineering Discovery Environment (XSEDE), which is supported by National Science
414Foundation grant number OCI-1053575. All simulations were done on a TACC (Texas Advanced
415Computing Center) Dell PowerEdge C8220 Cluster with Intel Xeon Phi coprocessors
416(Stampede). The authors are grateful to Dr. Chia-en Chang for her guidance on the MD work.

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567**Tables**

568

569**Table 1.** Molecular weight, sequence, and unfolded/folded radius of gyration (R_g) of ssDNA
 570strands investigated. “A#” described the poly(dA)_n with # for the base number. Abbreviation for
 571each aptamer was explained in the text.

572

Name	MW	Sequence (5'-3')	R_g (nm) (B-form)	R_g (nm) (folded structure)	ΔG (kcal/J) of folding
Poly(dA) ₁₀	3.07 kDa	5'-(A) ₁₀ -3'	1.20	N/A	N/A
Poly(dA) ₂₀	6.20 kDa	5'-(A) ₂₀ -3'	1.94	N/A	N/A
Poly(dA) ₃₀	9.33 kDa	5'-(A) ₃₀ -3'	3.03	N/A	N/A
Poly(dA) ₄₀	12.47 kDa	5'-(A) ₄₀ -3'	3.69	N/A	N/A
Poly(dA) ₅₀	15.60 kDa	5'-(A) ₅₀ -3'	5.05	N/A	N/A
TA	5.66 kDa	5'-ATAGGTTGGTGTGGTTGG-3'	1.93	1.22	N/A
cWA	8.99 kDa	5'-CACGCCACTAGGAGGGACGGATAAACGTG-3'	2.93	1.64	-4.01
WA	11.29 kDa	5'-GGGGCACGTTTATCCGTCCCTCCTAGTGCGGTG CCCC-3'	3.64	2.21	-7.63
SA	18.31	5'-CTATACTCCACTTTGCTATTTCTCGGTTCTTCAC GCGCC GATCGCAGGCTGATGAATTG-3'	6.43	4.04	-2.49
I9-102	20.62	5'-CAGCTGACGTACGTGCATGGCAAACACACTTC ATCCGTACCTTCTAGTGGGTGTGTAGCAAGCGCG C-3'	5.57	3.61	-5.18

573

574

575**Table 2.** Determination of percent folding of aptamers. T_M , T_{UF} , and T_F were the measured
 576retention time and the predicted times for the fully extended and completely folded structures,
 577respectively.

578

Name	t_{UF} (min)	t_F (min)	t_M (min)	%Folded	ΔG (kcal/J) of folding per base
cWA	6.26	4.48	5.00	71%	-0.14
WA	7.10	5.32	5.61	84%	-0.21
SA	9.85	7.06	8.60	38%	-0.04
I9-102	9.08	7.54	8.79	31%	-0.08

579

Figure Captions

581

Figure 1. Percent recovery of poly(dA)_n and aptamers under AF4 conditions (3.3 mL/min inlet flow, 3 mL/min crossflow, 0.3 mL/min axial-outlet flow). Recovery is in comparison to crossflow free conditions (both the outlet and inlet flow rates at 0.3 mL/min), and based off of relative peak areas at 280 nm.

586

Figure 2. Stacked AF4 fractograms of poly(dA)_n strands (top), DNA aptamers (middle), and proteins (bottom). The last 1 or 2 peaks were labeled with the Mw of the corresponding ssDNA or protein to show that the ssDNAs would be eluted much later than proteins with comparable molecular weights. Poly(dA)₁₀ was not visible in figure 2a due to its low recovery. AF4 separations were conducted with 3.3/3,0/0.3 mL/min inlet/cross/outlet flow rates and absorbance detection at 280 nm. The theoretical system void time was calculated to be 0.86 min with the channel dimensions and the cross/output flow rates used in the study.[28]

594

Figure 3. Plotting of log(retention time) as a function of log(molecular weight) for the poly(dA)_n and aptamer strands, demonstrating the differences in rigidity between the unfolded and folded ssDNA.

598

Figure 4. Hill plots determining the affinity between IgE and the Weigand aptamer (a), or streptavidin and the streptavidin aptamer (b). Each Hill plot point is the average of three to five runs. Inset: Representative fractograms of the aptamer at a fixed concentration incubated with increasing concentrations of the target protein. The arrow indicated the change direction of the peak area along with increase in protein concentration: the area of the free aptamer peak decreased and that of the protein-bound aptamer peak increased. All fractograms were detected at 495 nm excitation and 520 nm emission wavelengths.

606

Figure 5. Successful collection of IgE-bound WA after AF4 separation. Collections were done on both the WA-only (dotted curve) and the WA-IgE incubated sample (solid curve). Fractions were collected every 2 minutes starting from the elution time of 6 min. Inset: PAGE images of AF4 eluates of WA only and the WA incubated with IgE. The gel band seen corresponds with WA isolated and amplified from each AF4 fraction. The gel was stained with SYBR Gold and imaged using a UV trans-illuminator.

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