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Quantifying Aptamer-Protein Binding via Thermofluorimetric Analysis

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Abstract

Effective aptamer-based protein assays require coupling to a quantitative reporter of aptamer-protein binding. Typically, this involves a direct optical or electrochemical readout of DNA hybridization or an amplification step coupled to the readout. However, method development is often hampered by the multiplicity of aptamer-target binding mechanisms, which can interfere with the hybridization step. As a simpler and more generalizable readout of aptamer-protein binding, we report that thermofluorimetric analysis (TFA) can be used to quantitatively assay protein levels. Sub-nanomolar detection (0.74 nM) of platelet-derived growth factor (PDGF) with its corresponding aptamer is shown as a test case. In the presence of various DNA intercalating dyes, protein-bound aptamers exhibit a change in fluorescence intensity compared to the intercalated, unbound aptamer. This allows thermal resolution of bound and unbound aptamers using fluorescence melting analysis ($-dF/dT$ curves). Remarkably, the homogeneous optical method allows subtraction of autofluorescence in human serum, giving PDGF detection limits of 1.8 and 10.7 nM in serum diluted 1:7 and 1:3, respectively. We have thus demonstrated that bound and unbound aptamers can be thermally resolved in a homogeneous format using a simple qPCR instrument—even in human serum. The simplicity of this approach provides an important step toward a robust, generalizable readout of aptamer-protein binding.

Introduction

Realizing the enormous analytical potential of aptamers for quantifying proteins, researchers have devised a variety of methods over the years for quantitative readout of aptamer-protein binding^{1–13}. Homogeneous quantification DNA ligation and amplification¹, real-time detection with quantum-dot aptamer beacons², direct micromagnetic⁴ or electrochemical sensing^{7, 9, 12}, and the use of chemiluminescence resonance energy transfer¹³ are but a few of many examples. Most of these methods include some exploitation of controlled DNA hybridization. DNA hybridization is sequence-specific, making it flexible and controllable, and downstream enzymatic amplification of the sequences^{1, 13} can provide exquisite assay sensitivities. However, method development can be hampered by the lack of generalizability

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Electronic Supplementary Information (ESI) available:

Confirmed aptamer quantification with SYBR Green (Figure S-1); confirmation of negligible protein-SYBR Green interactions (Figure S-2); data processing workflow for $-dF/dT$ difference plots (Figure S-3); evaluation of different probe concentrations for TFA (Figure S-4); and validation of thrombin TFA using thiazole orange (Figure S-5). See DOI: 10.1039/b000000x/

of each approach, often requiring customized method development for each aptamer-protein couple. Although dual probe proximity assays hold promise for improved generalizability^{1, 9, 11, 14–17}, this strategy requires aptamer pairs, of which only a few are available to date¹⁸.

Simplification of an assay's workflow is always an important goal in analysis, and this is particularly true in bioanalysis, where mere sampling of a biological system can be complex. As such, homogeneous protein assays^{1, 14, 19} have great potential, and aptamer-based homogeneous assays—reviewed recently by Sassolas et al.²⁰—could be argued as the most promising toward the bioanalytical ideal of arbitrary protein detection in real time⁷. In terms of readout, fluorescence-based optical detection provides high sensitivity and specificity, yet autofluorescence in biological samples results in high background and usually implores specialized instrumental workarounds such as time-resolved detection²¹ or chemiluminescence¹⁹.

We have recently introduced a novel approach for homogeneous protein quantification in biological matrices that exploits bivalent probes—either aptamers or antibody-oligonucleotide conjugates—and a homogeneous fluorescence readout enabled by thermal scanning²². This approach provides a method for robust mix-and-read fluorescence detection of proteins, and the thermal scanning allows subtraction of autofluorescence in human serum samples. However, dual proximity probes such as aptamer pairs¹⁸ are still required for this method. Inspired by the benefits of the bivalent approach²², here we introduce a more generalizable thermal scanning method that only requires a single aptamer for readout and does not require covalent labelling of the aptamers. By staining aptamers with a DNA intercalating dye, we demonstrate that the unbound aptamer and the protein-bound aptamer can be thermally resolved using a standard qPCR instrument that exploits thermofluorimetric analysis (TFA). Platelet-derived growth factor (PDGF) can be quantified in buffer at sub-nanomolar levels using TFA, and the methodology is functional with multiple intercalating dyes and other proteins. Importantly, the ability to negate autofluorescence in human serum²² is preserved with our single probe TFA, since background can be analytically separated from signal. Due to its simplicity and potential for generalizable protein quantification, this approach provides an important addition to the bioanalytical toolbox.

Results and Discussion

Protein-stabilized DNA melting transitions

It is known that the thermal stability of proteins can be increased in the ligand-bound state. Based on this principle, Pantoliano et al. developed a thermal shift assay capable of measuring protein-ligand binding constants, facilitating applications in screening and drug discovery²³. Their approach utilized protein staining dyes, which exhibit low quantum yields in aqueous solution but high quantum yields in low dielectric solvents, thus increasing fluorescence in the presence of denatured, or “melted,” proteins. The method was proven as generalizable to determine ligand binding constants with >100 protein targets, and the direct optical readout facilitated high-throughput analysis. A similar method was developed by Niesen et al., who showed that a standard qPCR instrument could be used to determine

protein-ligand binding constants due to its thermal scanning capability²⁴. However, these approaches were not used for quantitative measurement of a given protein in a biological matrix, presumably due to the inherent complexity and high signal from background proteins. Since others have shown DNA intercalating dyes capable of isothermal quantification of aptamer-protein interactions^{25, 26}, we reasoned that significant advantages could be gained by thermally resolving these complexes. Specifically, differential melting analysis of protein-stabilized DNA melt peaks should permit removal of fluorescence interference such as background autofluorescence²².

Herein, we demonstrate that thermofluorimetric analysis (TFA), when coupled with DNA-intercalating dyes, can be used to quantitatively report aptamer-protein interactions in biological fluids. Unlike the methods using differential protein melting^{23,24}, TFA monitors aptamer melting, leveraging the increased thermodynamic stability afforded by protein binding. Differential melting of dye-intercalated aptamers allows quantitative delineation of protein-bound aptamers from unbound aptamers, even in the presence of significant autofluorescence in human serum. In contrast to our previous work²², which utilized thermal scanning to measure binding of fluorescently labelled aptamers or antibody-oligonucleotide pairs and a connecting strand, this approach is simplified further through direct interrogation of the protein-aptamer stability. As shown by the schematic in Figure 1, a DNA-intercalating dye such as SYBR Green is used to quantify folded aptamers. In control experiments, this signal was confirmed to be proportional to aptamer quantity (Figure S-1), and SYBR Green-to-protein binding was shown to be negligible (Figure S-2). Upon melting of the aptamer, fluorescence decreases to baseline levels (equilibrium shifted left in figure). Since protein-bound aptamers are inherently more thermodynamically stable, their melting temperature ($T_{m,bound}$) should be measurably higher than that of the unbound aptamer ($T_{m,unbound}$). This should allow quantitative discrimination between bound and unbound aptamers, yet without physical separation. If so, background fluorescence could be easily subtracted, since such a well-defined thermal transition should not be present in this interfering fluorescence background²².

Thermofluorimetric analysis (TFA) of PDGF-aptamer interactions

As a proof-of-concept, TFA was applied as an assay of interactions of a single aptamer with its protein target. Platelet-derived growth factor B-chain (PDGF) is a homodimer that plays an important role in embryogenesis, connective tissue formation, and in cancer proliferation²⁷. A high-affinity aptamer for PDGF exists²⁸, with the K_d value reported to be ~ 100 pM¹, making this an excellent target for preliminary studies.

First, to verify the functionality of our slightly modified PDGF aptamer sequence, we carried out a microchip electrophoresis mobility shift assay²⁹. As shown in Figure 2A, the free aptamer migrated past the detector at around 47 s, while the aptamer-protein complex migrated significantly slower and passed the detector around 65 s. The decrease in free aptamer peak area at 47 s was observed to be PDGF concentration dependent, consistent with earlier work²⁹. The two peaks observed around 65 s could be attributed to the 1:1 and 2:1 aptamer:protein complexes, although this was not explicitly proven. These peak areas

were not significantly different at 150 and 300 nM PDGF, but saturation at 150 nM was expected with 300 nM aptamer and 2:1 complex formation.

Next, TFA was carried out using a standard qPCR instrument in the presence and absence of protein. It is clear from the derivative data ($-dF/dT$) in Figure 2B that a more thermally stable species was present in solution, having a melting temperature of approximately 63 °C compared to the free aptamer peak at 57 °C. Even at a PDGF concentration of 10 nM, the new peak was easily distinguished from the free aptamer peak. Furthermore, the methodology is functional with other DNA intercalating dyes, as shown in Figure 2C with SYTOX Orange and in Figure S-5 using Thiazole Orange. Figure S-5 also shows that other proteins can be assayed using TFA. Together, these data suggested that protein concentration could be measured without physical separation using TFA.

TFA for homogeneous protein quantification

By varying the PDGF concentration and holding aptamer levels at 100 nM, we demonstrate that TFA can be used as a quantitative readout of protein levels. In Figure 3A, the PDGF-bound aptamer melt peak is highlighted further by using $-dF/dT$ difference plots, where the $-dF/dT$ trace from the blank measurement ($[PDGF] = 0$) is subtracted from each protein-dependent $-dF/dT$ trace. Data processing details are included in Supporting Information, Figure S-3. The figure depicts a typical set of runs with varying $[PDGF]$. The inset image shows the same $-dF/dT$ difference plots presented as images, where grayscale colour density is used to depict the concentration dependence of the bound aptamer melt peak (darker pixels = more intense $-dF/dT$ peak). As shown in Figure 3B, triplicate TFA gave a linear response through at least 25 nM PDGF and maximal response at 50 nM. Plotted in the figure are peak heights of the protein-bound aptamer melt peaks in the $-dF/dT$ difference plots. Assay saturation at 50 nM is expected with 100 nM aptamer probes, since there are two aptamer binding sites on each PDGF molecule. With homogeneous fluorescence readout, this system afforded a sub-nanomolar limit of detection (LOD), at 0.74 nM PDGF.

TFA permits subtraction of serum autofluorescence

Finally, a major advantage of TFA is the ability to subtract background fluorescence that does not exhibit clearly defined melting transitions. We demonstrate this advantage using homogeneous, optical protein quantification in human serum. Figure 4A depicts the $-dF/dT$ difference plot images (grayscale colour density) of PDGF spiked into minimally diluted (1:7 and 1:3) human serum samples. Shown in Figure 4B are the peak heights as a function of protein concentration. As expected, general matrix effects from serum partially degrade assay performance, with sensitivity decreasing with increasing serum fraction. However, the assays in serum retained 61% and 42% of the sensitivity in buffer (for 1:7 and 1:3 dilutions, respectively), while isothermal versions of the assay (25 °C) were essentially nonresponsive to protein (Figure 4C). These results confirm that autofluorescence can be largely subtracted using TFA, allowing robust quantitative analysis of protein levels using a simple, mix-and-read, optical technique in human serum, even at a mere 1:3 dilution. LODs were 1.8 nM and 10.7 nM in the final 20 μ L serum samples diluted 1:7 and 1:3, respectively. If correcting to the whole serum LOD, the 1:7 dilution (12.5% serum) provides a more favourable result,

allowing direct quantification of as low as 14 nM PDGF in a serum sample. The whole serum LOD was not optimized in this work, since we analysed only spiked samples.

Experimental

Reagents and Materials

Reagents included deionized ultra-filtered water (Fisher Scientific), bovine serum albumin (BSA), SYBR Green I (10,000× concentrate in DMSO; Lonza), and recombinant human PDGF-BB (R&D Systems), which is a homodimer of the beta polypeptide of PDGF (referred to simply as “PDGF” herein). All oligonucleotides were purchased from Integrated DNA Technologies (IDT) and purified by standard desalting or HPLC. Pathogen-screened, normal human serum samples were purchased from BioreclamationIVT.

Aptamer preparation

The parent sequence of the DNA aptamer with affinity to PDGF was obtained from Green et al.²⁸. For purposes not related to the current work, additional nucleotides were added to this aptamer, which minimally affected protein binding. The PDGF aptamer sequence (parent sequence underlined) was as follows (5' to 3'): GGC GCA CCC AAC TCA AAA AAA TAC TCA GGG CAC TGC AAG CAA TTG TGG TCC CAA TGG GCT GAG TAT. 10 μM of the aptamer in buffer (10 mM Tris-HCl, pH = 8.0, 50 mM KCl, 3.3 mM MgCl₂) was folded by heating for 10 min at 95 °C then cooling on ice prior to experiments.

Thermofluorimetric analysis (TFA)

20 μL assay mixtures were prepared in buffer with final concentrations of 100 nM aptamer (see Figure S-4), 1× SYBR Green or 500 nM SYTOX Orange, and varying concentrations of PDGF. Specifically, 5 μL of 400 nM aptamer in assay buffer (10 mM Tris-HCl, pH = 8.0, 50 mM KCl, 3.3 mM MgCl₂, 0.1% BSA) was mixed with 5 μL of 4× SYBR Green or 2 μM SYTOX Orange in the same buffer. 10 μL volumes of varying concentrations of PDGF were prepared in protein buffer (1× PBS buffer, 10.1 mM Na₂HPO₄, 137 mM NaCl, 2.7 mM KCl, 1.8 mM KH₂PO₄, 1.0 mM MgCl₂, and 1% BSA). The workflow was slightly different for human serum samples, as follows: 2.5 μL of 800 nM aptamer in assay buffer was mixed with 2.5 μL of 8× SYBR Green; 5 μL of varying concentrations of PDGF in protein buffer were added to 10 μL aliquots of diluted serum; all solutions were mixed to give final serum ratios of 1:3 (25%) or 1:7 (12.5%). All final 20 μL mixtures of aptamer and protein (with or without serum) were then incubated at 37 °C for 15 min, and the temperature was scanned from 4 °C to 90 °C at 0.5 °C min⁻¹. At each set temperature, fluorescence was measured after a 10 s delay with 470 ± 20 nm excitation and 520 ± 10 nm emission filters for SYBR Green staining and 575 ± 15 nm excitation and 630 ± 20 nm emission filters for SYTOX Orange staining (Bio-Rad CFX96™ qPCR system). Assays were carried out in triplicate, with standard deviations presented in figures. The TFA data processing workflow is illustrated in Supporting Information, Figure S-3.

Microchip electrophoresis

Using an Experion microchip electrophoresis system (Bio-Rad) along with DNA 1K Chip and Reagents Kits (Bio-Rad), mobility shift assays were carried out as described by Hu and

Easley²⁹. 5 μ L of 7.2 μ M aptamer was mixed with 5 μ L of 7.2 μ M DNA adaptor to facilitate increased intercalator binding. The adaptor sequence was as follows: /55-TAMK/GAG GAG AGG AAC ACC CGA TTA AAT GTT GAT, with the underlined portion complementary to the arm of the aptamer; the TAMRA label on this adaptor was not relevant to the current study. The mixture was incubated for 15 min at room temperature. 1.5 μ L of the mixture was then added into 1.5 μ L of either 3.6 μ M or 1.8 μ M PDGF or into 1.5 μ L buffer for a control experiment. The final mixture was incubated for 15 min at 37 °C, 1 μ L was added to 5 μ L loading buffer, and microchip electrophoresis with electrokinetic injection was carried out as described by the manufacturer.

Conclusions

We have demonstrated homogeneous, aptamer-based protein quantification using direct fluorescence and thermal scanning, i.e. by using thermofluorimetric analysis (TFA). Results show that TFA permits delineation of unbound and protein-bound aptamers without physical separation. As shown in our recent work with bivalent probes²², a unique capability of TFA is the effective subtraction of autofluorescence in biological samples such as serum, permitting optical protein quantification in serum with low nanomolar detection limits using a simple, mix-and-read workflow and a standard qPCR instrument.

Since many aptamer-protein binding interactions could feasibly be interrogated in this manner, it is expected that a major advantage of TFA should be the generality of the readout. Judging from our prior work using microchip electrophoresis mobility shift assays²⁹ and from Figure S-5, it is likely that TFA could be successfully applied to various other aptamer-protein complexes or even to aptamer-small molecule complexes. However, it is yet to be determined whether differences in aptamer structure could preclude the use of TFA for certain aptamer/target combinations, and our data suggests that various intercalating dyes and aptamer/target combinations should be explored. In the future, based on the simplicity of TFA and its unique capability to overcome fluorescence interferences, studies should be directed toward evaluating responses to various analyte classes in complex matrices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Fredriksson S, Gullberg M, Jarvius J, Olsson C, Pietras K, Gustafsdottir SM, Ostman A, Landegren U. Nature Biotechnol. 2002; 20:473–477. [PubMed: 11981560]
2. Levy M, Cater SF, Ellington AD. Chembiochem : a European journal of chemical biology. 2005; 6:2163–2166. [PubMed: 16254932]

3. Xiao Y, Uzawa T, White RJ, Demartini D, Plaxco KW. *Electroanalysis*. 2009; 21:1267–1271. [PubMed: 20436787]
4. Csordas A, Gerdon AE, Adams JD, Qian J, Oh SS, Xiao Y, Soh HT. *Angew Chem Int Ed*. 2010; 49:355–358.
5. Leslie DC, Sohrabi A, Ikonomi P, McKee ML, Landers JP. *Electrophoresis*. 2010; 31:1615–1622. [PubMed: 20419707]
6. Lundberg M, Eriksson A, Tran B, Assarsson E, Fredriksson S. *Nucleic acids research*. 2011; 39:e102. [PubMed: 21646338]
7. Plaxco KW, Soh HT. *Trends Biotechnol*. 2011; 29:1–5. [PubMed: 21106266]
8. Olmsted IR, Xiao Y, Cho M, Csordas AT, Sheehan JH, Meiler J, Soh HT, Bornhop DJ. *Analytical chemistry*. 2011; 83:8867–8870. [PubMed: 22032342]
9. Hu J, Wang T, Kim J, Shannon C, Easley CJ. *J Am Chem Soc*. 2012; 134:7066–7072. [PubMed: 22452720]
10. Li J, Jia Y, Zheng J, Zhong W, Shen G, Yang R, Tan W. *Chem Commun*. 2013; 49:6137–6139.
11. Hu J, Yu Y, Brooks JC, Godwin LA, Somasundaram S, Torabinejad F, Kim J, Shannon C, Easley CJ. *J Am Chem Soc*. 2014; 136:8467–8474. [PubMed: 24827871]
12. Ren K, Wu J, Yan F, Ju H. *Sci Rep*. 2014; 4:4360. [PubMed: 24618513]
13. Mun H, Jo EJ, Li T, Joung HA, Hong DG, Shim WB, Jung C, Kim MG. *Biosensors & bioelectronics*. 2014; 58:308–313. [PubMed: 24658027]
14. Heyduk E, Dummit B, Chang YH, Heyduk T. *Analytical chemistry*. 2008; 80:5152–5159. [PubMed: 18491925]
15. Kim J, Hu J, Sollie RS, Easley CJ. *Analytical chemistry*. 2010; 82:6976–6982. [PubMed: 20704387]
16. Zhang H, Li F, Dever B, Wang C, Li XF, Le XC. *Angew Chem Int Ed*. 2013; 52:10698–10705.
17. Di Giusto DA, Wlasoff WA, Gooding JJ, Messerle BA, King GC. *Nucleic acids research*. 2005; 33:e64. [PubMed: 15817563]
18. Cho M, Oh SS, Nie J, Stewart R, Radeke MJ, Eisenstein M, Coffey PJ, Thomson JA, Soh HT. *Analytical chemistry*. 2015; 87:821–828. [PubMed: 25495696]
19. Ullman EF, Kirakossian H, Singh S, Wu ZP, Irvin BR, Pease JS, Switchenko AC, Irvine JD, Dafforn A, Skold CN, et al. *Proc Natl Acad Sci U S A*. 1994; 91:5426–5430. [PubMed: 8202502]
20. Sassolas A, Blum LJ, Leca-Bouvier BD. *The Analyst*. 2011; 136:257–274. [PubMed: 20949139]
21. Mathis G. *Clin Chem*. 1993; 39:1953–1959. [PubMed: 8375081]
22. Kim J, Hu J, Bezerra AB, Holtan MD, Brooks JC, Easley CJ. *Angew Chem Int Ed*. 2015 submitted.
23. Pantoliano MW, Petrella EC, Kwasnoski JD, Lobanov VS, Myslik J, Graf E, Carver T, Asel E, Springer BA, Lane P, Salemme FR. *Journal of biomolecular screening*. 2001; 6:429–440. [PubMed: 11788061]
24. Niesen FH, Berglund H, Vedadi M. *Nature protocols*. 2007; 2:2212–2221. [PubMed: 17853878]
25. Zhou C, Jiang Y, Hou S, Ma B, Fang X, Li M. *Analytical and bioanalytical chemistry*. 2006; 384:1175–1180. [PubMed: 16447044]
26. Chi CW, Lao YH, Li YS, Chen LC. *Biosensors & bioelectronics*. 2011; 26:3346–3352. [PubMed: 21306887]
27. Heldin CH, Westermarck B. *Physiological reviews*. 1999; 79:1283–1316. [PubMed: 10508235]
28. Green LS, Jellinek D, Jenison R, Östman A, Heldin C-H, Janjic N. *Biochemistry*. 1996; 35:14413–14424. [PubMed: 8916928]
29. Hu J, Easley CJ. *The Analyst*. 2011; 136:3461–3468. [PubMed: 21293790]

Figure 1.

Schematic of thermofluorimetric analysis (TFA) of aptamer-protein binding. Intercalator fluorescence is low in the melted, free state (left) and high in the folded aptamer or protein-bound state (middle & right). Protein binding adds stability, increasing aptamer melting temperature, i.e. $T_{m,bound} > T_{m,unbound}$.

Figure 2.

Aptamer-protein binding studies. (A) Binding of aptamers to the PDGF target was confirmed using a microchip electrophoresis mobility shift assay. Size markers (15 and 1500 bp) are labelled with asterisks. Thermofluorimetric analyses (TFA) using (B) SYBR green or (C) SYTOX Orange intercalating dyes were then validated as a readout mechanism for aptamer-protein binding.

Figure 3.

Quantitative protein detection with TFA. (A) Blank-subtracted $-\text{dF}/\text{dT}$ difference plots highlight the protein-bound aptamer melt peaks at 63 $^{\circ}\text{C}$. Grayscale colour density plots of the same data are inset (peaks labelled with red arrow). (B) Peak heights from $-\text{dF}/\text{dT}$ difference plots showed a linear assay response through at least 25 nM PDGF, with maximal response at 50 nM as expected. The assay LOD was 0.74 nM PDGF in buffer.

Figure 4.

Homogeneous quantification of proteins in human serum via TFA. (A) Grayscale colour density images of $-dF/dT$ difference data in 1:7 and 1:3 diluted serum. Protein-bound aptamer peaks (red arrows) show assay sensitivity decreasing with increasing serum fraction. (B) Homogeneous protein assays in serum using direct optical readout were enabled by TFA, (C) while corresponding isothermal protein assays were non-functional due to high background in serum.