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Comprehensive Analytical Comparison of Strategies Used for Small Molecule Aptamer Evaluation

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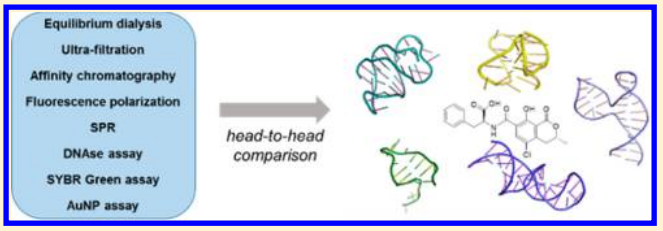
Supporting Information

ABSTRACT: Nucleic acid aptamers are versatile molecular recognition agents that bind to their targets with high selectivity and affinity. The past few years have seen a dramatic increase in aptamer development and interest for diagnostic and therapeutic applications. As the applications for aptamers expand, the need for a more standardized, stringent, and informative characterization and validation methodology increases. Here we performed a comprehensive analysis of a panel of conventional affinity binding assays using a suite of aptamers for the small molecule target ochratoxin A (OTA). Our results highlight inconsistency between conventional affinity assays and the need for multiple characterization strategies. To mitigate some of the challenges revealed in our head-to-head comparison of aptamer binding assays, we further developed and evaluated a set of novel strategies that facilitate efficient screening and characterization of aptamers in solution. Finally, we provide a workflow that permits rapid and robust screening, characterization, and functional verification of aptamers thus improving their development and integration into novel applications.



Nucleic acid aptamers are versatile molecular recognition agents that bind to their targets with high selectivity and affinity.[1] The past few years have seen a dramatic increase in aptamer development and interest for diagnostic and therapeutic applications. For example, aptamer technology finds uses in analytical detection,[2,3] therapeutics,[4−6] and for cell-based engineering.[7] Aptamers are selected by an *in vitro* procedure called systematic evolution of ligands by exponential enrichment (SELEX).[8−10] Compared to antibody generation, SELEX allows greater control over binding conditions and allows selection under nonphysiological conditions. Therefore, high-affinity, chemically stable aptamer probes can be generated for targets that are highly toxic or for targets that do not elicit an immune response *in vivo*. Furthermore, compared to other molecular recognition elements, aptamers can be easily labeled or modified with a variety of reporter molecules at precise locations with little-to-no effect on their function or activity, thus enabling their implementation into the wide variety of applications described above.[11]

Major technological advances for *in vitro* selection have improved our ability to generate thousands of potential aptamer candidates; however, our ability to characterize and validate individual aptamers remains low-throughput and laborious, creating a major bottleneck in the aptamer discovery pipeline.[12] Typically, a few aptamer candidates identified by SELEX (<10) are synthesized and assayed using precise analytical instrumentation. Such analytical methods, including surface plasmon resonance (SPR),[12] isothermal calorimetry (ITC),[13] and capillary electrophoresis (CE)[14] have been useful for elucidating precise aptamer metrics; however, these technologies are costly and not always available in standard molecular biology laboratories where SELEX is typically performed. These challenges are particularly problematic in the context of small molecule-binding aptamers because most affinity binding assays are not sufficiently sensitive to measure the interaction of low molecular weight targets (<1 000 amu) compared to their larger aptamer binding partners (>10 000 amu).[15] As a consequence, many researchers use column binding assays to quickly screen panels of aptamers. However, these assays suffer from high nonspecific binding that can make it difficult to compare different aptamer candidates.

Additional experiments are required to obtain more quantitative metrics, such as equilibrium binding affinity constants (K_D), selectivity, and specificity.[16] These binding affinity metrics sometimes go unreported and do not eliminate the variability of aptamer functionality under various conditions. These challenges are not isolated to aptamer-based molecular recognition probes. For example, it is well-known

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Table 1. Characterization of Various OTA Aptamers Using Quantitative and Qualitative Binding Assays[a]

OTA aptamer	K_D values (nM) by using different analytical approaches							qual. binding	Reported K_D (nM)[ref]
	equil. dialysis	ultrafiltration	affinity chrom. (mag beads)	FP	SPR	DNase assay[b]	SYBR Green	AuNP assay	
1.12.2	287 ± 56	255 ± 49	374 ± 255	125 ± 23	163 ± 15	NB[c]	146 ± 43	yes	200[18]
T22-O36-T3	160 ± 21	250 ± 49	n/a	77 ± 9	63 ± 12	n/a	31 ± 9	yes	88 ± 14[19]
A08	NB	NB	286 ± 149	NB	NB	200 ± 157	108 ± 61	yes	290 ± 150[20]
A08 min	n/a	n/a	406 ± 166	n/a	NB	NB	169 ± 52	yes	n/a
B08	n/a	n/a	125 ± 44	n/a	NB	670 ± 331	17 ± 5	no	110 ± 50[20]
H8	NB	NB	14 ± 7	NB	NB	54 ± 23	NB	no	130[21]
H12	NB	NB	40 ± 14	NB	NB	270 ± 201	NB	no	96[21]

[a]All reported K_Ds are measured in the working buffers (Table S1 in the Supporting Information) unless indicated otherwise. Reported K_Ds are the mean and standard deviation of at least two independent experiments. 95% confidence intervals are reported in Table S2 in the Supporting Information. n/a, Not tested; NB, no binding detected. [b]This assay was performed in standard DNaseI buffer conditions. [c]Only the full length 1.12 resulted in DNA cleavage (see discussion and Figure S7 in the Supporting Information).

that the performance of an antibody, which remains the gold standard of molecular recognition, in one application cannot predict its performance in another application. Thus, researchers demand more strict validation guidelines of antibodies from suppliers that include stringent, application-specific verification and functional characterization using multiple techniques, enabling them to focus on obtaining results more quickly and cost-effectively.[17]

Despite increasing demands for high-quality aptamers, there is no universally accepted quality guidelines or standards for the characterization and reporting of aptamers, thus limiting their widespread use in research and medicine. Similar to antibodies, biological conclusions drawn from aptamer-target binding experiments are more likely to be replicated when the hypothesis has been tested using multiple techniques and applications and are desperately needed to advance aptamer applications.

We performed, for the first time, an analytical comparison of a large spectrum of affinity assays typically used to characterize aptamers to small molecules. We evaluated the methods using a suite of previously characterized aptamers that bind to the mycotoxin ochratoxin A (OTA). We assessed the utility of these techniques based on several metrics including throughput, general applicability, cost, labor, and reliability (CV, %). We further evaluated three assays that permit rapid screening and characterization of aptamers. On the basis of our analysis, we provide a systematic strategy to rapidly screen, characterize, and functionally verify aptamers. Our recommended workflow will improve the aptamer development pipeline and enable rapid integration of aptamers into important biosensing applications.

Each aptamer binding assay was performed using a dilution series of either aptamer or OTA, spanning at least a 100-fold concentration range surrounding the previously reported K_Ds. Each assay was performed in duplicate for each aptamer candidate using the corresponding working buffer, matching that of the original selection (where appropriate, details in the Supporting Information). The K_D values were determined by obtaining saturation binding isotherms based on the titration of a constant concentration of OTA with an increasing concentration of the aptamer or vice versa. The stoichiometry between OTA and aptamer was assumed to be 1:1 and the K_D was determined by applying the Langmuir model through nonlinear regression analysis by fitting the experimental data with the one site specific binding equation using GraphPad Prism 6 software (version 6.04):

$$Y = B_{max}\frac{X}{K_D + X} \quad (1)$$

where X is the concentration of the variable molecule (either OTA or aptamer), Y is the signal associated with specific binding and B_{max} is the maximum signal associated with specific binding (in the same units as Y).

Characterization of aptamer binding is critical for studying aptamer molecular recognition and integrating them into diverse applications.[16] To elucidate the benefits and limitations of current affinity methods, we performed the first parallel comparison of a panel of aptamers for the same target. A number of aptamer binding assays are available, and conceptual challenges associated with each assay have been reported;[15] however, very little is known about the analytical capabilities of each assay and how they may ultimately inform or confound the integration of aptamers into novel application platforms.[15] Currently, there is no one technique that can be considered generally applicable to all aptamer cases, which poses challenges when trying to characterize, functionally test, or compare aptamer candidates for a new application. Importantly, aptamer performance in solution or immobilized may vary; therefore, affinity must be measured to match the application conditions.

We chose to perform this work using a suite of aptamers that bind to the important small molecule OTA for three reasons. First, OTA is a mycotoxin produced by several *Penicillium* and *Aspergillus* species that frequently contaminates a wide range of foods and beverages. OTA has been implicated in several animal diseases and has been classified as a possible human carcinogen.[22−24] Because of its toxic effect, the European Commission has set maximum permitted levels for OTA in several food matrixes.[25] Second, OTA is the only small molecule target for which three independent groups have isolated distinct aptamer sequences through separate SELEX techniques.[18,20,21] These aptamers display vastly different sequence and structure, however, have similarly reported binding affinity to OTA. We hypothesized that having several previously tested aptamer candidates would serve as biological replicates for our method comparison and would further facilitate elucidation of key challenges within individual assay methods that might be aptamer-specific. Finally, over the past few years, these aptamer candidates have been integrated into diverse applications for OTA analysis[26] providing precedent of the robust binding affinity of these aptamer candidates and further highlighting the importance of this molecule.

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Table 2. Workflow Diagram for Aptamer Screening, Characterization, and Functional Verification for Optimal Integration into Aptamer-Based Applications[a]

	1. Candidate screening	2. Truncation & optimization	3. Characterization	4. Functional validation
Method milestones	Reduce the number of putative aptamer sequences from SELEX from hundreds to ~3 candidates	Determine minimal binding sequence	Determine the K_D, selectivity and other parameters	Assess the robustness of the aptamer for use in different application platforms
Important assay considerations	1. High-throughput 2. Cost-effective	1. High-throughput 2. Cost-effective 3. Provides insight about structure or important binding residues	1. Quantitative 2. Precise 3. Measurement of multiple parameters in parallel	1. Validate function with at least two separate methods 2. Determine functionality in solution and immobilized 3. Does not need to be quantitative
Assay options (ascending order)	1. Fluorescence Polarization (FP) 2. SYBR Green (SG) 3. AuNP Assay 4. Affinity Chrom. (mag. Beads) 5. SPR	1. DNase Assay 2. FP 3. SG 4. Affinity chrom. (mag. Beads) 5. SPR	1. SPR 2. FP 3. Equil. dialysis 4. SG 5. Affinity Chrom. (mg. beads)	*Choose at least 1 assay from group not used in step 3* **In solution** FP, Equil. dialysis, SG, Affinity Chrom. (either), Ultrafiltration, DNase **DNA immobilized/constrained** SPR, AuNPs

[a]The assay considerations and careful measurement choices assist in achieving method milestones and ensure that produced aptamers display the appropriate functionality for incorporation into novel applications.

We first evaluated the set of aptamers using standard techniques, in this case, equilibrium dialysis, ultrafiltration, and affinity chromatography with an agarose resin or magnetic beads. Each assay was evaluated by determining K_Ds (see Table 1 and Figures S1, S2, S4 in the Supporting Information). In addition to known technical challenges associated with these assays (e.g., intrinsic fluorescence/absorbance needed for target quantification for equilibrium dialysis and ultrafiltration or the need for immobilization of target for affinity chromatography),[15] these separation-based assays were more variable resulting in larger standard deviations in measured K_D. Additionally, K_D could not be determined with affinity chromatography with agarose due to the large nonspecific binding to the matrix itself (see Figure S5 in the Supporting Information).

We also tested two frequently applied methods, SPR and fluorescence polarization (FP). Consistent with previous reports, these methods displayed improved precision and improved accuracy compared to previously reported values.[12,16] However, FP requires intrinsic fluorescence of the target or target labeling and thus is not scalable to all small molecule targets. In some cases the aptamer can be labeled, but this may not always result in an observable change in rotation upon target binding. SPR requires immobilization of either the target or the aptamer. Given the known limitations of target immobilization (limitations of functional groups, altered binding), it is preferential to immobilize the aptamer to the SPR surface. However, our analysis indicates that immobilization at one end of the aptamer may abolish binding (Table 1). Further work will investigate the reasons for this, but it is likely that this is due to steric hindrance or alteration of aptamer structural conformation. Thus, while SPR and FP are rapid, accurate, and precise methods, they are not generally applicable. Given that our goal was to find rapid, inexpensive, parallelizable methods that would help elucidate information about aptamer binding for diverse applications, we further evaluated three new approaches for measuring aptamer K_D.

The first method, the DNase I digestion assay[27] is a new application of classic molecular biology technique typically used for locating specific binding sites of proteins on DNA (footprinting). Using this assay, we were able to rapidly determine the K_D values of the OTA aptamers in solution (Table 1 and Figure S6 in the Supporting Information), without the need for labeling or intrinsic fluorescence of the target molecule. Furthermore, the digestion pattern from this assay can offer insight into important aptamer regions required for binding, similar to inline probing assays with RNA. It is critical to note that one drawback of this assay is that it is not compatible with previously truncated (minimized) aptamers, as observed in our experiments (Table 1). For example, our results show that very little cleavage occurs for the minimized aptamers 1.12.2 and A08 min and it was not possible to use the change in the cleavage as a readout for aptamer affinity (see Figure S7 in the Supporting Information). In the case of fully minimized aptamers, typically the entire length is required for target binding. Given that DNase I digestion is classically used for locating binding sites, it is not surprising that fully minimized candidates are protected from DNase I digestion.

The next two methods leveraged a displacement-based mechanism that is frequently applied to analytical aptamer applications. While these assays are performed in solution, one complication is that steric hindrance and/or nonspecific adsorption complications may be introduced that could interfere with the nature of the interaction between the aptamer and target. Regardless, we determined that the SYBR Green I assay (SG) was facile and rapid[20] and allowed accurate determination of aptamer K_Ds. Furthermore, the precision of this assay was comparable to more commonly applied method SPR and FP (see Table 1 and Table S3 in the Supporting Information). In the gold nanoparticle (AuNP) assay, binding of the aptamer to the target leaves AuNPs unprotected and susceptible to salt-induced aggregation which can be detected using transmission electron microscopy (TEM) or by measuring the color change associated with the aggregation using UV−visible spectroscopy[28] (see Figure S11 in the Supporting Information). While we found this assay to be extremely rapid and useful, the K_D values obtained in this way were significantly improved compared to the reported values (appropriately 3 orders of magnitude, see Figure S10 in the Supporting Information). This effect is likely due to the signal

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enhancing property of the surface plasmon phenomenon of the AuNPs. From our analytical comparison of these assays, we have compiled the capabilities and limitations for each assay. Table S3 in the Supporting Information summarizes these capabilities. However, one important finding is that aptamer binding varies when either the target or aptamer is used in solution vs immobilized, and the sensitivity of each technique affects the apparent affinity. Second, our results highlight that each assay has at least one substantial limitation in the aptamer development pipeline. One strategy to effectively confirm aptamer binding would be to determine aptamer binding using more than one method. However, from our results, no two methods can be considered generally applicable; therefore, multiple combinations of methods must be included that take into account the limitations associated with each individual assay method and it is critical to employ methods that reflect the final desired aptamer application.

Given that *de novo* aptamer development is time-consuming and requires screening of multiple potential aptamer candidates to discover the best aptamer for a particular application, it is not practical to thoroughly test hundreds of potential candidates using multiple binding assay methods. Therefore, to address this complex challenge, we assembled an inclusive workflow that supports efficient and effective functional screening and verification of aptamer binding that can be applied from the initial putative sequences isolated from SELEX. For each stage of aptamer development, we highlighted the important assay characteristics and considerations. By comparing our results (Table S3 in the Supporting Information) with the assay considerations, an ascending order of possible aptamer binding assays is recommended. For example, for screening aptamers, it is most important to implement high-throughput methods so that more candidates can be screened. However, one limitation is the cost of the high-throughput screen. Thus, to rapidly and reliably screen aptamer candidates, we propose that FP, SG, AuNP, affinity chromatography (mag. beads) or SPR are used, in that order, to strike a balance between reliable high-throughput measurement and cost effectiveness. Our proposed workflow is described in Table 2. Note that alternative methods with similar capabilities not reviewed here can be substituted into the workflow (e.g, isothermal calorimetry, microscale thermophoresis). The workflow provides a cost-effective, efficient, and rapid strategy for screening, characterizing, and functionally verifying aptamers. It is a flexible methodology that can be easily implemented in diverse laboratories spanning molecular biology, analytical chemistry, and bioengineering, thus, making our workflow scalable to all potential aptamer researchers. As it was developed and evaluated in the context of OTA-binding aptamers, we expect that the proposed workflow can be used to reliably functionally verify small-molecule binding aptamers, ultimately permitting their direct, successful implementation into a variety of applications.

In conclusion, we performed an analysis of a diverse set of aptamer affinity assays by leveraging a panel of previously reported aptamers that bind to OTA. By quantifying the precision and accuracy of the measured K_D of each aptamer within the context of each assay, we were able to critically compare assay performance. After evaluating conventional aptamer affinity assays, as well as three new strategies to characterize and functionally verify aptamer affinity, we elucidated a streamlined workflow that supports (i) high-throughput screening of aptamer candidates, (ii) quantitative characterization of aptamer candidates, and (iii) reliable functional confirmation of aptamers within more than one assay platform. The workflow proposed here provides specific recommendations for each step that will ensure that new aptamers are thoroughly and reliably reported. However, we have included flexibility within each step to ensure that the recommendations are scalable and translatable to laboratories working with aptamers regardless of the field. Incorporation of this workflow as a standard practice in aptamer development allows for a more streamlined discovery process as well as accelerating the associated design cycle for incorporating aptamers into novel applications. We anticipate that proper employment of the workflow recommendations will also mitigate many of the current challenges involved in integrating aptamers into new application platforms and thus will extend their application beyond the small subset of aptamers used in proof-of-concept studies.

■ ASSOCIATED CONTENT

Supporting Information

Materials and methods, 11 additional figures (Figures S1−S11), and 3 tables (Tables S1−S3). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b02102.

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Author Contributions

The manuscript was written through contributions of all authors. M.M., A.D., S.V., M.P. and M.C.D. designed the research; M.M. and A.D. analyzed the data; M.M., A.D., S.V., A.R., R.V., N.R.F., K.H., M.S., and E.M.M. performed the experiments; M.M., A.D., and M.C.D. wrote the article.

Notes

The authors declare no competing financial interest.

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