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PROTOCOL

Quantification of aptamer-protein binding with fluorescence anisotropy

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ABSTRACT

At the turn of the 21st century, aptamers selected *in vitro* have secured their place in the mainstream study of nucleic acids and are commonly evolved via a process termed SELEX. While new sequences can be isolated from SELEX in as little as a 14-day time period, characterizing putative aptamer sequences poses a greater challenge and often goes unreported in the literature. Rigorous characterization and validation of individual sequences are required for reliable reporting of aptamer sequences with high affinity and specificity to their targets. Here, we present a protocol for the use of fluorescence anisotropy to characterize aptamers towards protein targets. This protocol also serves as an introduction for new aptamer researchers, in particular students, who wish to understand and perform aptamer binding experiments.

KEYWORDS: Aptamer, fluorescence anisotropy, dissociation constant, protein targets

INTRODUCTION

Aptamers are short nucleic acids that are evolved *in vitro* in a process known as Systematic Evolution of Ligands by Exponential enrichment (SELEX) (Ellington and Szostak, 1990; Tuerk and Gold, 1990). SELEX contributed greatly to the arsenal of available nucleic acid ligands that bind to specific small molecules, peptides, proteins, and cells (McGown et al, 1995; McKeague and DeRosa, 2012; Ababneh et al, 2013; Alshaer et al, 2015; Honget al, 2015; Hong and Sooter, 2015; McKeague et al, 2015;). Although the timeline of isolating new sequences is very short, characterizing possible candidate aptamer sequences often goes missing in the scientific literature. However, thorough validation of individual sequences and characterization of their metrics are essential for implementing functional aptamers into diverse applications (McKeague et al, 2015).

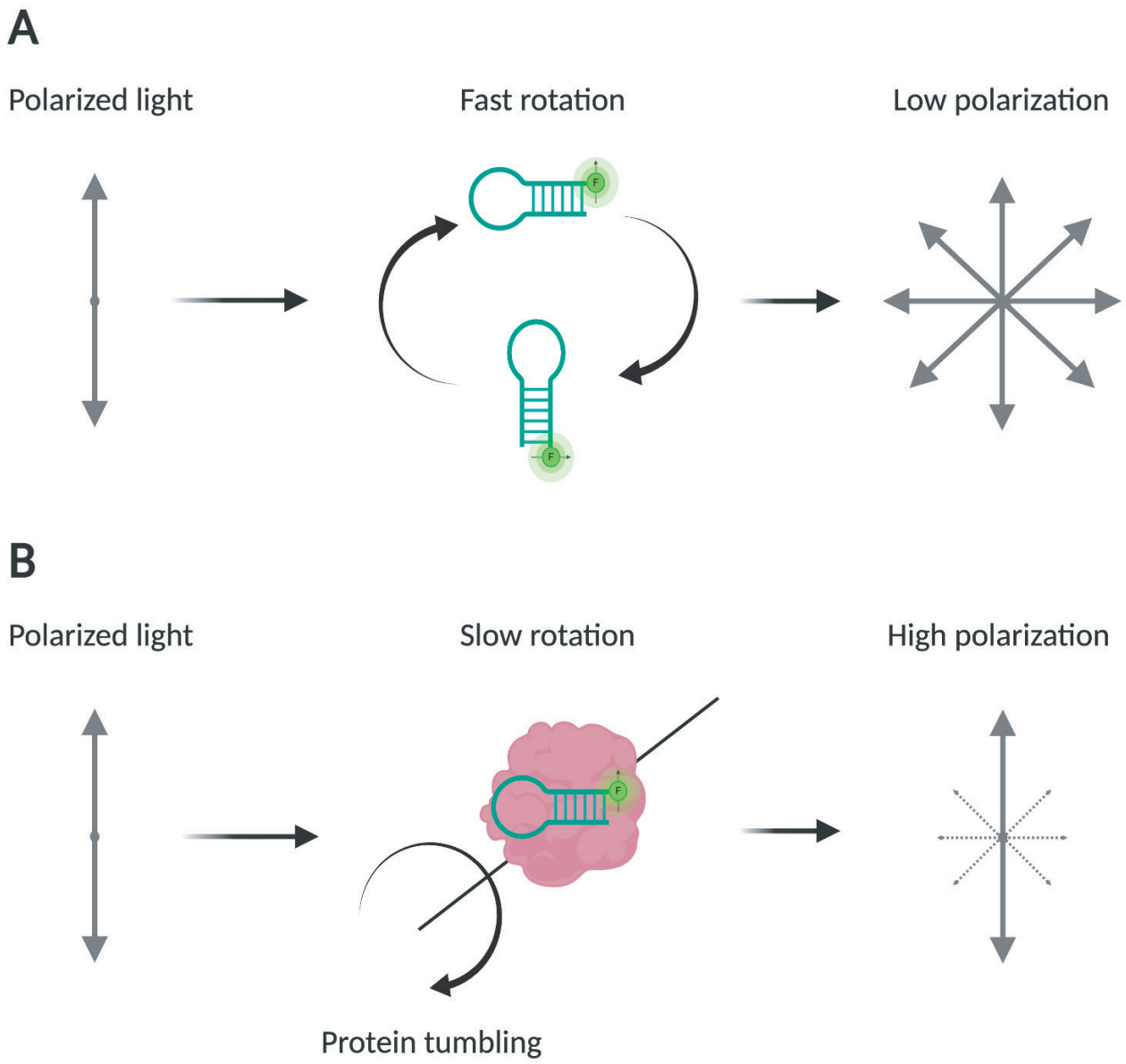
Several methods exist for the assessment and characterization of novel aptamers. As one example, the Johnson group published a protocol detailing the use isothermal titration calorimetry (ITC) that is particular effective for small molecules, and if binding entails a significant change in conformation. ITC relies on the heat released by aptamer–target complex formation and can provide valuable information on stoichiometry as well as thermodynamic properties (McKeague and DeRosa, 2012). One drawback is that ITC often requires large quantities of the sample and is very sensitive to various impurities that interfere with the measurement (Slavkovic and Johnson, 2018). As another useful method for measuring the affinity of aptamers includes surface plasmon resonance (SPR), which provides a real-time signal of interactions at the surface. The precision is affected to some degree when the surface is regenerated, and the immobilized agent does not always reflect the in-solution activity (Chang et al, 2014). Indeed, ITC and SPR are robust and important methods, however, they rely on specialized equipment not always available to all aptamer research groups. More essentially, it is important to use multiple methods are used to fully validate the binding affinity of aptamers (McKeague et al, 2015). In particular, a dissociation constant K_D, which in some instances goes unreported,

is considered the most critical metric of aptamer binding. This mathematical representation describes the extent to which the complex aptamer-target dissociates to its free constituents and is the inverted association constant (Thevendran et al, 2020) (see Eq. (1) for 1:1 stoichiometry).

$$Apt + Tar \rightleftharpoons Apt \cdot Tar$$

$$K_D = K_A^{-1} = \frac{[Apt][Tar]}{[Apt \cdot Tar]} (1)$$

Here, we present a protocol for the use of fluorescence anisotropy to characterize aptamers towards protein targets. This protocol also serves as an introduction for new aptamer researchers, in particular students, to understand aptamer binding experiments. This particular assay is a homogeneous high-throughput method that allows rapid determination of binding events and their associated affinities (Ruta et al, 2009). The phenomenon of fluorescence anisotropy, or less commonly fluorescence polarization, is observed when a fluorophore molecule is excited by the polarized light and emits light either back in the polarized plane i.e., retains high polarization, or in another plane i.e., becomes depolarized. When covalently attached to a small entity such as aptamer, it rotates and tumbles uninhibitedly; the light is depolarized. When the same species is associated with a large protein molecule (>10,000 Da), or found in a viscous environment, this limits the fluorophore's motion, which in turn does not produce major changes in the polarization of the light (Moerke, 2009). In FA measurement, the fluorescently tagged molecule is incubated with



Scheme 1. Principle of the fluorescence anisotropy assay. **A.** A free ligand molecule with the fluorophore (green) undergoes rotational movements upon excitation, leading to depolarization of light during the emission. Note that the molecules whose dipoles are aligned with the polarization of the light are preferentially promoted to the excited state. Direction of electric dipole moment is represented by an arrow. **B.** When the molecule is bound to a bigger entity such as protein (pink), its mobility is restricted, and the light remains largely polarized.

an array of concentrations of the target, and the output is analyzed in both parallel and perpendicular planes to the polarization plane of the incident light. This method has been successfully applied in the literature to determine the K_D of an aptamer-target interaction (Chang et al, 2014; Rangel et al, 2018).

MATERIALS AND METHODS

Fluorescence anisotropy equipment

Any fluorescence polarization-capable plate reader is suitable for this experiment. Examples include the Spark (Tecan), Cytation (BioTek), SpectraMax (Molecular Devices), FluoroMax (Horiba). Fluorimeters without a plate-reader capability can also be used for this assay, although more labour-intense and time-consuming. Here, we used the BioTek Cytation 5 cell imaging multi-mode reader, with fluorescence polarization filter cube with 96-well black plate with black bottom.

Chemicals

Chemicals necessary to prepare the suitable buffer for your aptamer/target. When preparing to perform the FA measurement, it is best if the buffer solution is the same buffer used in the aptamer selection. For example, we measured thrombin binding aptamer (TBA) *6FAM-GGTTGGTGTGGTTGG* in 20mM Tris, pH = 7.4, 140mM NaCl, 20mM KCl, 1mM $CaCl_2$, 1mM $MgCl_2$, 0.01% (v/v) Triton X-100. This recreates the selection buffer, except the higher K^+ concentration, which is known to help G-quadruplexes stabilize their structure and in doing so retain binding (Nagatoishi et al, 2007) as well as non-ionic surfactant to prevent non-specific interactions (Moerke, 2009). All chemicals were purchased from BioBasic (Markham, ON).

Aptamers

DNA or RNA aptamers against a specific target can be purchased commercially from a variety of sources (e.g., IDT, ChemGenes, TriLink, Sigma Aldrich) or synthesized using standard phosphoramidite chemistry (Beaucage and Caruthers, 1981; Nielsen et al, 1986). If the protein is unlabelled, the aptamer must be tagged with a fluorescent label at either the 5' or 3' end. As an example, the TBA in our experiment was tagged with a 5' fluorescein modification, 6-Fluorescein Phosphoramidite from Glen Research (Sterling, VA) (Bock et al, 1992). The concentration of the aptamer, another point of consideration, should be held constant and is usually around 20 nM with binders in the nanomolar range (Gokulrangan et al, 2005). If significant variability is observed in the data, adjusting the concentration (usually lowering it) should remedy this.

Target

The protein target of interest. As example, we used lyophilized powder human thrombin from plasma purchased from Sigma-Aldrich, cat. T6884.

Plasticware

Black plate (96-well or 384-well) – uncoated, black or clear bottom. Tips and tubes as needed. For example, we have used Falcon 96-well black plates with clear flat bottom and lid (tissue culture treated, sterile), cat. 353219 and Thermo Fisher 96-well black plate with non-treated surface, no lid (non-sterile), cat. 237108.

Preparation

All the working surfaces should be cleaned prior to start of the assay. If working with RNA aptamers, follow appropriate technique to avoid contamination with RNase – gloves, RNase-free water and appropriate decontaminating solution.

If the goal is to determine an in-solution dissociation constant of the aptamer-protein complex, serial dilutions of the protein or aptamer are necessary. Usually the aptamer is fluorescently tagged, and therefore the dilutions of the target protein are prepared. In particular, if this is the first binding experiment, it is advised that the target concentration span over from low picomolar to high micromolar to determine the appropriate range. If an approximate K_D value is already available, using concentrations that span approximately an order of magnitude below and above the K_D is ideal.

Our approach is to prepare the aptamer in buffer at 2x the final concentration. This can be stored at −20°C for several months. The protein dilutions in buffer, on the other hand, are preferably made fresh (also at 2x the final concentration), although the nature of the protein may allow its storage at −20°C. Before the assay is performed, thawed aliquots of aptamer should undergo a denaturation-renaturation cycle to ensure that the favourable aptamer structures are formed. Typically, this involves a short high-temperature heating step, followed by cooling on ice. For example, the TBA sample was heated to 95°C for 3min, cooled down at room temperature for 2min and kept on ice for 15min.

Note: the nature of the protein dictates the handling procedures; however, it is generally recommended to store on ice and to avoid excessive vortexing and pipetting.

Binding assay

Once both the aptamer and the protein dilutions are prepared, they should be transferred to an appropriate multi-well plate, as described above. The aptamer and protein solution is added to each well in a 1:1 ratio, *e.g.*, 50µl and 50µl. A suggested order of addition is aptamer first (or whichever biomolecule is kept at a constant concentration). The plate should be incubated on a slow shaker prior to measurement. The length of incubation can be adjusted to match the conditions of the SELEX experiment; however, 30min is a typical incubation time. Some plate readers have the shaking capacity built in; a separate benchtop shaker can be used as well. The well plate, as well as all the solutions of fluorescently tagged compounds, should be kept in the dark at all times to avoid photobleaching, *e.g.,* wrapped in a piece of aluminum foil.

Measurement

Set the excitation and emission wavelengths for the fluorophore or choice. For this experiment, 6-fluorescein was used, thus the excitation wavelength should be set to 495nm and emission collected at 520nm. Sensitivity of the machine is set to 100%. The experiment is run in triplicate, to rule out the error. The FA measurement itself (after

shaking) takes 2-3min. The shaking step is essential for aptamer binding, as it allows for the protein, aptamer and protein-aptamer associate to equilibrate, ensuring the correct determination of K_D. Premature FA measurement may skew the measurement results.

Data analysis

The raw data include parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) fluorescence intensity, from which it is suggested to subtract the corresponding values of a blank buffer solution in order to minimize the error. Fluorescence anisotropy (r) can be then calculated following the Eq. (2). While the anisotropy can be used for further calculations, fluorescence polarization Eq. (3), is still commonly used in the scientific literature. The two values can be transformed into each other using Eq. (4). It is worth mentioning that fluorescence anisotropy is the preferred measure, as it accounts for the total intensity, I_T, Eq. (5). The following equation also explains the coefficient 2 before $I_{\perp}$. The output of the measurement is anisotropy (r), with theoretical values spanning from −0.2 to 0.4 or −0.333 to 0.5 for polarization (P). In practice, the extreme values do not appear in usual assays and instead, a more in-reach bioanalytical range of 0.05 to 0.222 for anisotropy and 0.01 to 0.3 for polarization can be expected (Walla, 2014), with temperature, viscosity having an effect on the final anisotropy/polarization value. After obtaining the anisotropy value, change in anisotropy Δr or polarization ΔP is calculated for each concentration point by subtracting the value r_0 or P_0, the value of anisotropy/polarization of aptamer in the absence of the target, Eq. (6). These values are then plotted to extract the K_D.

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (2)$$

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}} \quad (3)$$

$$P = \frac{3r}{r+2} \quad (4)$$

$$I_T = I_x + I_y + I_z \quad (5)$$

$$I_x = I_y = I_{\perp}, \quad I_z = I_{\parallel}$$

$$\Delta r = r - r_0 \quad (6)$$

Binding data

The FA or FP data can be used to compare the relative binding of many aptamer sequences across a fixed concentration of target protein. Furthermore, specificity of the aptamer can be tested at fixed concentrations of many different proteins. Here, we describe the details for obtaining the in-solution dissociation constant of the aptamer with the target. Typically, the stoichiometry between the aptamer and the protein is 1:1. Therefore, for calculating K_D, the Langmuir model applies via non-linear regression analysis by fitting the experimental data with the one-site specific binding Eq. (7) utilizing GraphPad Prism software or similar.

A common approach to quantifying the K_D values of aptamers is the one that involves the titration of a fixed concentration of an aptamer with varying concentrations of the aptamer's specific target. The fraction of aptamer bound (f_a) depends on both K_D and target concentration [Tar] Eq.(7).

$$f_a = \frac{[Apt \cdot Tar]}{[Apt]_T} = \frac{[Tar]}{K_D + [Tar]} B_{max} \quad (7)$$

This equation is obtained via an introduction of two new variables, maximum binding site, B_{max} and total concentration of aptamer, $[Apt]_T$. Since the concentration of aptamer in equilibrium can be expressed as $[Apt] = [Apt]_T - [Apt \cdot Tar]$, put into the Eq. (1) and rearranged to give the bound aptamers fraction, thus the Eq. (7) yields a hyperbola that approaches the binding saturation point (B_{max}), Figure 1 (Thevendran et al, 2020). The K_D calculated from this data (K_D = 18 ± 4 nM) is well within the region of reported K_Ds for the thrombin-binding aptamer (Li et al, 2002), thus validating the reliability of the method.

From the fitted curve, the estimation is done at the $f_a = 0.5$, because at this value $K_D = [Tar]$ and thus defined as a point at which 50% of the aptamer is bound to the target in equilibrium. This, however, applies only to the 1:1 stoichiometry, Eq. (8).

$$Y = B_{max} \frac{X}{K_D + X} \quad (8)$$

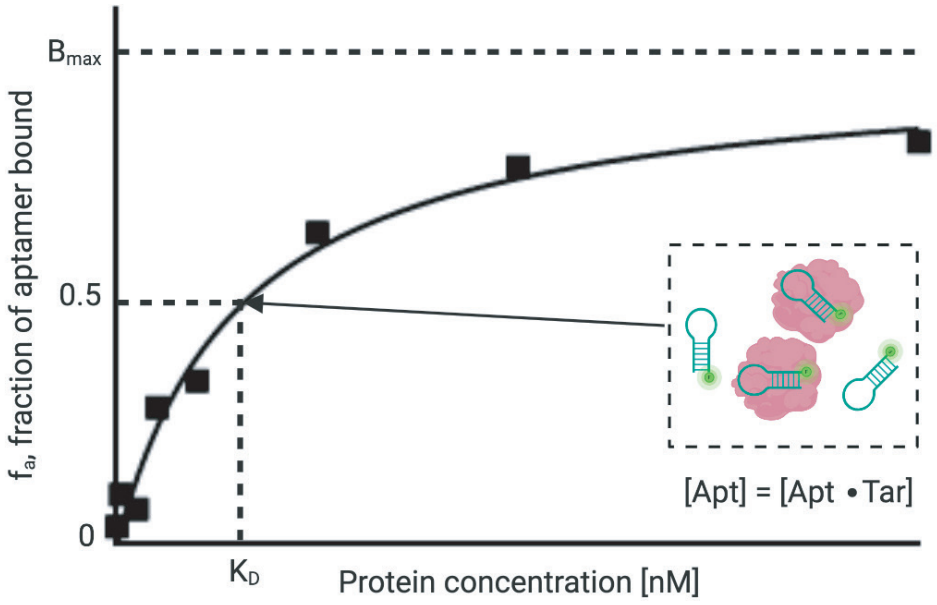
Y is associated with specific binding, B_{max} is the maximum signal of the specific binding, and X is the concentration of analyte. Similarly, the point at which Y(X) = ½B_{max} has X = K_D, thus allowing us to estimate the K_D with the help of a standard curve fitting run on an appropriate software. Finally, with the K_D in hand, it is more straightforward to compare the aptamer with other reporter aptamers, other putative aptamers from the selection, to compare the aptamer binding to other targets, and to determine the suitability of the aptamer for novel applications (Tan et al, 2020).

TROUBLESHOOTING AND CONSIDERATIONS

The fluorescent anisotropy assay is a precise and sensitive technique that allows high-throughput analysis of multiple samples and is an excellent tool in the study of aptamers. The output generated by the assay should fall into the usual range for FA produce a hyperbola (see above). Some reasons may include:

1. Alignment of the light source with the plate or distortion;
2. Impurities (e.g., glycerol, affects viscosity);
3. Denaturation of the substrate while handling;
4. Insufficient incubation time.

To address these issues, the light source has to be properly aligned according to the manufacturer's manual with the plate in use and/or using the FP One-Step Reference Kit (Thermo Fisher Scientific). Distortion in the centre of the



[Tar], nM	Δr × 1000		Δr × 1000, average	ΔP × 1000		ΔP × 1000, average	KD, nM	Bmax
0.032	-2	0	-1	-3	0	-1	18±4	31±2
0.16	0	-2	-1	0	-3	-1		
0.8	3	2	3	5	3	4		
4	1	9	5	2	14	8		
20	17	16	17	25	24	25		
100	27	26	27	40	39	40		
500	28	26	27	42	39	41		
1000	32	32	32	47	47	47		

Figure 1. **(top)** Fraction of bound aptamer (f_a) plotted against the target concentration with a curve fitted to estimate the dissociation constant K_D. The point where concentration of bound protein equals to that of free aptamer corresponds to K_D on X-axis; **(bottom)** Example data: the respective target concentration, fluorescence polarization (P) and fluorescence anisotropy (r) values, calculated K_D and B_{max}.

plate may occur, especially in the low-volume plates (Zuck et al, 2005). This measurement artifact can be offset by using greater volume and in general is less of a challenge in FA. Impurities that may affect tumbling of the aptamer have to be removed from the analyte by standard procedures of purification (*e.g.,* dialysis, chromatographic methods, precipitation). Some substrates can be protected from denaturation by applying vortexing sparingly, as well as limiting pipetting. Incubation time has to be long enough to allow substrates to equilibrate. When dealing with proteins, it is worth remembering that many commercially available proteins come in glycerol, which affects the viscosity of the solution, and in turn affects the polarization. This contribution consists of a slight rise in baseline polarization, which is negligeable if the following recommendation is heeded. As the serial dilution is applied, every next dilution will have a smaller amount of glycerol than the previous one, and this would lead to different contribution of viscosity to FA. Ideally, lyophilized proteins should be purchased. If not, glycerol should be added accordingly to each dilution, ensuring that all dilutions have the same concentration of glycerol, i.e., contribute equally to the polarization. This contribution is offset when the change in anisotropy is calculated, Eq. (6).

An important consideration is the choice of fluorophore and its position. The standard option for labelling is fluorescein, thanks to its wide availability both as a synthetic precursor and as tag on commercially synthesized oligonucleotides. Despite that, fluorescein suffers from low photostability, pH sensitivity and a relatively broad spectrum of emission. In that regard, alternative dyes such as Alexa 488 and Cy5 have an advantage over fluorescein in that they are more photostable. Labelling of the aptamer is typically done at its 5′ or 3′ end, preferably with minimal linker length to minimize the mobility of the fluorophore in a bound state. In some circumstances, instead of tagging the aptamer, incorporation of fluorescent base analogues may prove useful (Lawson et al, 2018). The challenge with aptamers is choosing an appropriate position: specifically, a position must be selected where binding is not reduced/impacted by the inclusion of the analogue. Overall, in cases where there is a limited information available about an

aptamer, it is useful to try multiple labelling sites to find the best option.

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LIST OF ABBREVIATIONS

FA: **F**luorescence **A**nisotropy
FP: **F**luorescence **P**olarization
ITC: **I**sothermal **T**itration **C**alorimetry
K_A: Association equilibrium constant
K_D: Dissociation equilibrium constant
SELEX: **S**ystematic **E**volution of **L**igands by **Ex**ponential Enrichment
SPR: **S**urface **P**lasmon **R**esonance
TBA: **T**hrombin **B**inding **A**ptamer, *5'-GGTTGGTGTGGTTGG-3'*

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