Aptamers

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METHODS/PROTOCOLS

Surface plasmon resonance assay for screening diverse aptamer-target interactions

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ABSTRACT

Natural aptamers control biological processes in bacteria while synthetic nucleic acid aptamers have been applied to numerous biotechnological applications. Prior to developing aptamer technologies, sequence variants and specificity must be characterized. Here, we present a highly sensitive surface plasmon resonance (SPR) method adapted to rapidly screen aptamer variants against diverse molecules.

KEYWORDS: Aptamer, surface plasmon resonance, aptamer-target interaction, equilibrium constant, binding assay, kinetics

INTRODUCTION

Following identification of aptamer sequences from in vitro selection, many potential binding sequences must be validated for specificity and affinity (Mckeague et al, 2022). To characterize interactions between aptamers and their biomolecular targets, several biophysical techniques (DeRosa et al, 2023) are used including Isothermal Titration Calorimetry (ITC) (Slavkovic and Johnson, 2023), Flow Cytometry (Kelly et al, 2021), Microscale Thermophoresis (MST) (Breitsprecher et al, 2016), Bio-Layer Interferometry (BLI) (Lou et al, 2016) etc. Each techniques possess unique benefits and have been employed to quantify molecular interaction and binding kinetics or obtain thermodynamic and stoichiometric information (Yu et al, 2021). Here, we present the utility and adaptability of the Surface Plasmon Resonance (SPR) method to screen aptamer-target interactions. In particular, SPR stands out as a versatile biomolecular characterization technique for providing both kinetic and affinity data even for weak aptamer-target interactions (Chang et al, 2014; Chang et al, 2014).

SPR is a real time and sensitive optical sensing technique. The underlying principle of SPR relies on the change of refractive index near the chip sensor surface (Figure 1A) (Wang et al, 2022). Multiple formats can be used to measure aptamer-target interactions, whereby either the target or the aptamer can be immobilized to the surface with the other binding partner flowing through the flow cell. One highly versatile method involves capturing the aptamer onto the sensor surface through strong but reversible interactions such as hybridization or biotin-streptavidin interactions (Girolamo et al, 2018). Then, multiple different target solutions pass through the flow cell. When the target interacts with the immobilized aptamer, the refractive index increases in real time (Yu and Wu, 2019). The real time change in refractive index is plotted as a response (Resonance Unit, RU) over time, generating a sensorgram (Figure 1B) (Arney and Weeks, 2022). Sensorgrams can then be used to fit kinetic or equilibrium binding constants (Vo et al, 2019).

MATERIALS AND PROTOCOL METHOD

Surface plasmon resonance instrument

Any surface plasmon resonance instrument should work for this purpose: including Biacore T200 or Biacore 8K+ (Cytiva, USA), BI-4500 (Biosensing Instrument, USA).

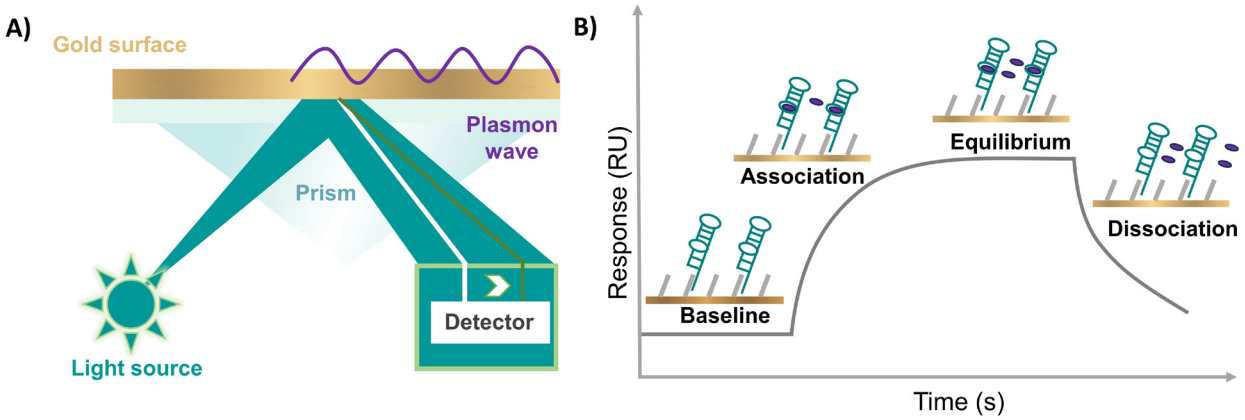

Figure 1. Surface plasmon resonance assay. **A)** Principle of SPR measurement **B)** Typical SPR sensorgram showing the baseline of immobilized aptamer; target association; equilibrium; and target dissociation.

Table 1. List of SPR chip immobilization conditions and possible applications.

Immobilization strategy	Application
Target	This is a suitable strategy when screening many different aptamer candidates or an aptamer library against a single target. Chips with a variety of functionalization handles are available including streptavidin, nickel-nitrilotriacetic acid, polyethylene glycol, and carboxymethylated dextran. Immobilizing the target can impact aptamer binding.
Aptamer immobilized through hybridization (Chang et al, 2014)	This is the most versatile strategy. Can be used for measuring different aptamer candidates as well as many targets. This method offers reversibility, enabling the regeneration of the sensor surfaces for measuring many different aptamer-target pairs. Note that immobilization on either end of the aptamer may impact aptamer folding and thus binding.
Aptamer immobilized via biotin/ streptavidin (Ostatná et al, 2008; Froehlich et al, 2023)	This is a suitable strategy to test a single aptamer against many targets. Due to the strong biotin-streptavidin interaction, surface regeneration is harsh. Due to the cost of RNA synthesis, modifying an aptamer with biotin is not recommended.

However, other systems might require different materials and reagents listed for compatibility. We used a Biacore X100 (Cytiva, USA) with Biacore X100 Evaluation Software version 2.0 (Cytiva, USA) for data processing and analysis.

Chemicals

Any running buffer suitable for the experiment and the equipment. For example, our protocol makes use of the running buffer 10mM HEPES pH 7.4; 150mM NaCl, 5mM $MgCl_2$ prepared with RNase-free purified milli-Q water (18.2MΩ·cm) followed by filtration through a 0.2µm filter. To dissolve some small molecules, dimethyl sulfoxide (DMSO, ThermoFisher Scientific, USA) can be used at low concentrations. For sensor surface immobilization purposes, we used an Amine Coupling Kit (Cat. BR100050; Cytiva, USA) containing 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), 1.0M ethanolamine-HCl pH 8.5. As surfactant we used hexadecyltrimethylammonium bromide (CTAB) (Cat. 52365; MilliporeSigma, USA).

Sensor surface immobilization

Different immobilization conditions are available (Table 1) depending on whether the aptamer or target is immobilized (Löfås and Mcwhirter, 2006).

To screen and characterize diverse small molecules for binding to the aptamer portion of a natural riboswitch, capturing the aptamer to the chip surface via hybridization is most suitable permitting many rounds of different small molecules to be tested for binding to the aptamer-immobilized chip surface. An immobilized "Complementary Small Oligo Sequence" (CSOS) for hybridization is ideal for this purpose such that we can easily capture an aptamer sequence extended with a sequence complementary to the CSOS. For example, a CSOS (/5AmMC6/-TTTTTTTTTTTTTTTTTTTTTTTT, IDT) with a 5' end amino modification can be covalently immobilized to a carboxymethylated dextran sensor chip (e.g., CM5 from Cytiva) via an amine coupling reaction, then any aptamer synthesized with a 24-mer polyA tail can be captured.

Before immobilization, a pre-concentration test should be performed to ensure sufficient interaction of the negatively charged CSOS to the negatively charged sensor surface decorated with carboxylic acid groups (carboxymethlated dextran). Notably, a surfactant carrier such as hexadecyltrimethylammonium bromide (CTAB, 1.2mM) prepared in 10mM HEPES buffer (pH 7.4) will improve CSOS interaction with the chip surface. Once the pre-concentration shows sufficient interaction of CSOS with the chip, an immobilization reaction is performed to covalently attach the CSOS onto both flow cells (FC1 as the reference cell and FC2 as the measurement cell) on the sensor chip. Specifically, a 1:1 volume ratio of EDC and NHS is injected to activate the reaction, followed by injection of CSOS carried by the CTAB micelles. Finally, 1M ethanolamine at pH 8.5 or tris buffer can be injected to block any remaining activated groups.

When preparing a chip in this way, we recommend aiming for approximately 3,000 RU of immobilized CSOS on each flow cell.

Aptamer constructs for immobilization via hybridization

Any putative DNA/RNA aptamer or aptamer construct can be used in the hybridization technique if there is a complementary domain for capture to the CSOS immobilized chip. For example, we tested the binding of the aptamer domain of the *Fusobacterium nucleatum* impX RFN element (FMN riboswitch). This RNA aptamer can be transcribed from the PCR product of a DNA aptamer domain template (**5'-TTCTAATACGACTCACTATAGGG**ATCTTCGGGGCAGGGTGAAATTCCCGACCGGTGGTATAGTCCACGAAAGTATTTGCTTTGATTTGGTGAAATTCCAAAACCGACAGTAGAGTCTGGATGAGAGAAGA*CCCCC***AAAAAAAAAAAAAAAAAAAAAAAA-3')** from Integrated DNA Technologies (IDT) (Coralville, USA) containing a T7 promoter for transcription at the 5' end and a polyA tail at the 3' end. Transcribed RNA should be purified (e.g., using Monarch RNA Cleanup Kit from New England Biolabs, USA) however, laborious purification of RNA via gel electrophoresis rarely improves binding measurements as shorter transcripts missing the polyA sequence will simply not be captured on the chip. Regardless of the immobilization strategy used, we recommend preparing a scrambled negative control sequence in a similar manner. This can be captured on the reference flow cell (FC1). For all aptamers (DNA and RNA), stock solutions are prepared to a final concentration of ~1-3 µM in the SPR running buffer.

Preparation of targets for binding to immobilized aptamers

Any protein or small molecule target can be tested. Small molecules with high solubility can be directly prepared in buffer. As an example, we prepared a flavin mononucleotide (FMN) (Cat. F6750; MilliporeSigma, USA) solution in running buffer. However, small molecules with low water solubility can be dissolved in 100% DMSO if the working concentrations are diluted to 0.1-0.5 % DMSO, ensuring the same concentration of DMSO in each dilution.

Aptamer-target binding assay

Prior to beginning an assay, the SPR should be primed with the running buffer followed by three to five startup cycles with aptamer to stabilize the sensorgram baseline (Chang et al, 2014). During each startup cycle, aptamers and the scrambled control sequence (~1-3µM) can be captured onto the active flow cell (FC2) and the reference flow cell (FC1) respectively for 40 seconds at a flow rate of 5µl/min. Longer times can be used but typically the low flow rate ensures efficient capture of the aptamer. A well-prepared chip should easily allow ~2000 RUs of aptamer captured onto the surface which is sufficient to detect binding to even small molecule targets as low as 75gm/mol in molecular weight.

Once aptamer capture is optimized, the target solutions and running buffer can be set up for injection over both flow cells (FC1 and FC2). High flow rates are used to measure association and dissociation, balancing consumption of the target. Therefore, typically 30µl/min is used for target association and 90µl/min of buffer is applied to monitor target dissociation. Finally, the surface can be regenerated using a variety of reagents: when working with aptamer-hybridized immobilization, 25mM NaOH for 30sec is highly effective; while high concentration of NaCl is recommended when proteins are immobilized. Adjustment of association and dissociation time may be necessary for certain interactions; whereas increasing the flow rate can help determine if there are mass transport limitations.

ANTICIPATED RESULTS

Applying SPR for aptamer-target screening

The SPR method can be used for screening variants of the parent aptamer sequence, including truncations and mutants. Rather than measuring binding of the target at many concentrations, a single concentration where binding is observed in a parent aptamer can be selected to screen the relative impact of different sequences on binding response. By comparing the binding responses, non-functional aptamer variants can be deduced, and promising ones can be selected for further evaluation to measure quantitative binding parameters (Figure 2) (Malmqvist, 1993).

On the other hand, the SPR method is valuable for rapidly determining specificity of an aptamer. In this case, the same aptamer is immobilized onto the surface, and the cognate binding target partner is used at a concentration greater than the K_D as a positive control. Next, one or two high concentrations of each counter molecule can be assayed to determine if there is any binding response. Due to the double-referencing method, non-specific interactions are accounted for. As such, small molecule concentrations of 500µM and micromolar protein concentrations can be applied. By comparing the binding responses, targets with no affinity for the aptamer can easily be discarded, while those with some interaction can be further evaluated using a greater concentration range to measure quantitative binding parameters.

Data analysis

To compare results from a large screening of aptamer variants or target molecules, the double reference subtraction including negative control and blank reference is essential to eliminate false positives (Arney and Weeks, 2022). Moreover, it is crucial to include a positive control to set a response range, enabling the comparison of the signal from the screened variants to the control (Figure 2). Note that different target molecules may exhibit distinct binding behaviors and thus result in sensorgrams with different shapes, described below.

Once the best target molecules or aptamer variants have been identified through the screening assay, the aptamer-target interaction can be characterized based via kinetic analysis or steady-state affinity analysis. The type of analysis performed depends on the sensorgram. When the sensorgram displays a curved pattern during both association and dissociation but fails to reach a stable state during association, kinetic analysis should be done (Figure 3A). However, if the association and dissociation occur too rapidly to exhibit a curved pattern suitable for kinetic assessment, affinity analysis is required (Figure 3B). In some cases, it is

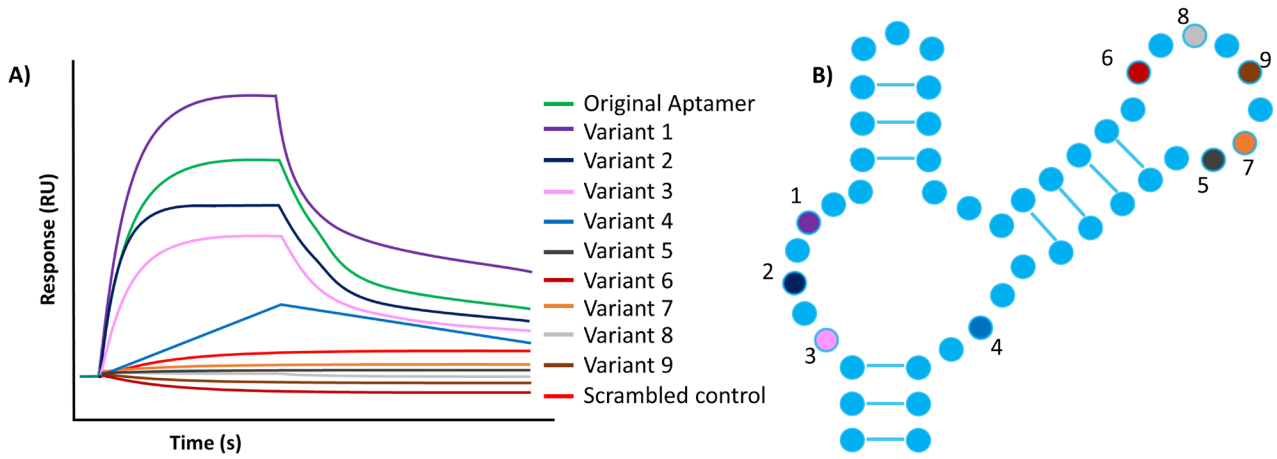

Figure 2. Example of an aptamer screening assay: comparing mutational variants of an aptamer and their impact on target binding. **A)** SPR results for each variant. **B)** Predicted aptamer structure and location of tested variants.

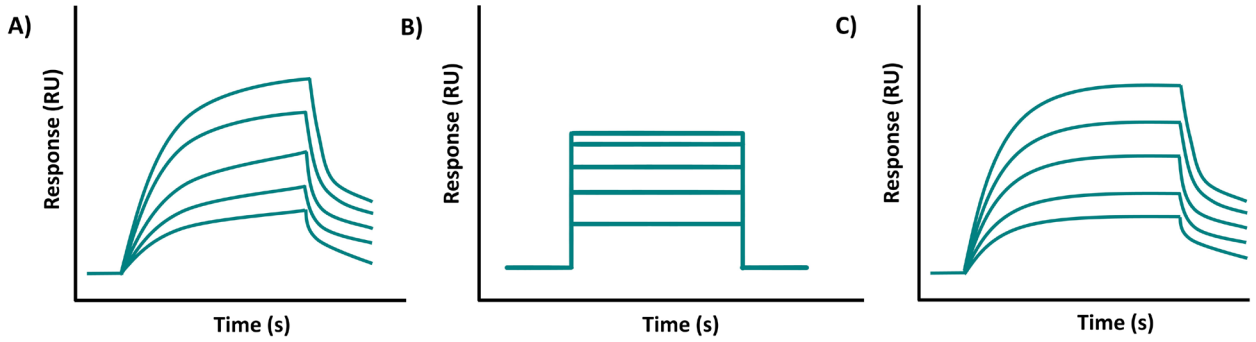

Figure 3. SPR Sensorgrams. **A)** Sensorgrams suitable for kinetic analysis **B)** Sensorgrams suitable for affinity analysis **C)** Sensorgrams suitable for both kinetic and affinity analysis.

possible to perform both kinetic and affinity analysis when the sensorgram reaches equilibrium along with sufficient association curvature (Figure 3C). All these curve types are possible with aptamers.

For kinetic analysis, the K_D can be calculated from Eq. 1, where R_t is the response with time, k_a represents association rate constant, k_d represents dissociation rate constant and [Target], represents target concentrations (Figure 4A). For equilibrium analysis, it is ideal to perform the assessment over a range of several concentrations of the target molecule and the equilibrium dissociation constant, K_D can be calculated using Eq 2 by plotting the steady state binding response (R_{eq}) against target concentrations (Fig. 4B). In both calculations, R_{max} is the maximum response regenerated by aptamer-target interaction.

$$R_t = \frac{R_{max}[Target]}{K_D + [Target]}\left[1 - e^{-(k_a[Target] + k_d)t}\right] \quad \text{Eq. 1}$$

$$R_{eq} = \frac{R_{max}[Target]}{K_D + [Target]} \quad \text{Eq. 2}$$

CRITICAL PARAMETERS AND TROUBLESHOOTING

Buffer mismatch and effects of DMSO

It is ideal to prepare fresh buffer. Buffer stored at room temperature can accumulate dissolved air creating air-spikes in the sensorgram (Nilvebrant, 2018). Another important consideration is buffer mismatch which can create negative binding response. This happens when organic solvents, such as DMSO, are used to prepare the small molecule solutions (Navratilova et al, 2007). Therefore, the same DMSO percentage in the running buffer should be used to avoid any buffer mismatch effects (Giannetti, 2011; Sparks et al, 2019).

Baseline drift

A commonly observed issue in SPR is baseline drift resulting from a non-equilibrated sensor surface particularly when docking a new sensor chip or changing the running buffer. To avoid baseline drift, it is recommended to equilibrate

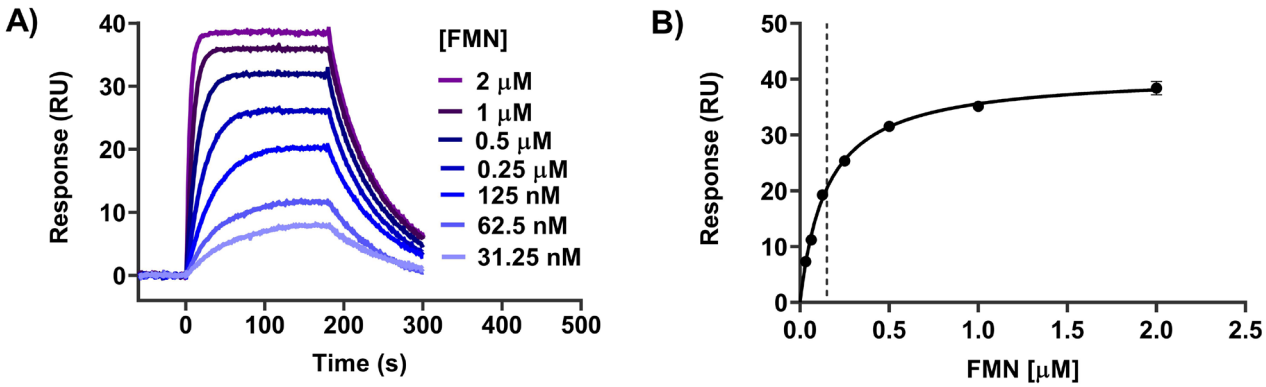

Figure 4. Representative SPR data of FMN binding to the aptamer portion of the FMN riboswitch. **A)** Sensorgram from a kinetic analysis with a good range of concentrations. **B)** Steady-state affinity fitting curve with a good range of target molecule concentrations. The dash line indicates the K_D. Error bars represent the standard deviation of technical duplicates.

the system by flowing running buffer over the sensor surface and performing priming before each assay (Drescher and Drescher, 2023). Three to five start up cycles are recommended to stabilize the baseline. This also provides an opportunity to stop or modify automated assays to optimize for sufficient aptamer capture.

CSOS Immobilization

HPLC purification is recommended for any functionalized CSOS.

The pre-concentration assay might show a weak interaction, indicating insufficient CTAB to balance negative charges of CSOS. To optimize this interaction, test a variety of CTAB to CSOS ratios during pre-concentration.

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STATEMENT OF COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

CSOS: Complementary Small Oligo Sequence
CTAB: hexadecyltrimethylammonium bromide
DMSO: dimethyl sulfoxide
FMN: flavin mononucleotide
K_D: dissociation equilibrium constant
RU: Response Unit
SPR: Surface Plasmon Resonance

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