Encoded particle microfluidic platform for rapid multiplexed screening and characterization of aptamers against influenza A nucleoprotein

Pelin Leblebici, Karen Leirs, Dragana Spasic, and Jeroen Lammertyn *

Department of Biosystems, Biosensors group, KU Leuven, Willem de Croylaan 42, 3001 Leuven, Belgium

Corresponding Author: jeroen.lammertyn@kuleuven.be

ABSTRACT

Aptamers represent interesting bioreceptor alternatives to antibodies when developing a bioassay and are selected by the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) process. After selection, an extensive characterization process is essential to verify the binding affinity and specificity of aptamer candidates, which is the most time-consuming and costly step. In this study, we assessed a new microfluidic platform, namely Evalution™, as a rapid and high throughput aptamer characterization platform. To do this, we first selected aptamers against influenza A nucleoprotein (infA NP) by performing magnetic bead-based SELEX. The selected aptamer candidates were subsequently screened using Evalution™ for their binding kinetics and specificity towards infA NP. All aptamers showed dissociation constants (K_D) in the low nanomolar range (from 13 to 41 nM), and differential binding behavior towards control proteins, such as BSA and influenza B nucleoprotein (infB NP). Among 5 selected candidates, one aptamer (NP5) exhibited a significant discrimination between infA NP and infB NP and was further used to benchmark the kinetic analysis of Evalution™

($K_D = 41$ nM) with an SPR platform ($K_D = 17$ nM). These results suggested that NP5 has the potential to be used for developing sensitive and infA NP specific aptamer-based assay. Moreover, the presented platform proved to be an efficient aptamer characterization tool for performing typical aptamer characterization experiments like binding kinetics (due to the real-time monitoring feature) and specificity assessment in a high-throughput manner due to the multiplexing capacity.

KEYWORDS: aptamer, influenza A, nucleoprotein, high throughput, multiplexing, microfluidic

1. INTRODUCTION

Aptamers are short single stranded DNA (ssDNA) or RNA molecules that can bind to their target with high selectivity and specificity by folding into three dimensional structures. Once selected, aptamers offer numerous advantages compared to antibodies, including the ease and low cost of production, the simplicity of chemical modification and their resistance to denaturation and degradation [1]. Therefore, despite antibodies still being frequently used as receptors in immuno-based bioassays, aptamers constitute promising alternatives. However, the lengthy and tedious procedure of their selection and characterization hampers this potential.

Aptamers are selected in vitro from combinatorial libraries using Systematic Evolution of Ligands by Exponential Enrichment (SELEX) technology against targets ranging from small organic molecules [2] to proteins [3–5] and even whole cells [6]. SELEX is a multistep process starting with a randomized DNA oligonucleotide library (10^{15} sequences) and consists of four main steps: binding to the target, separation of bound strands, amplification and generation of a new ssDNA library. Usually, additional steps like negative/counter SELEX are introduced to minimize the enrichment of unspecific oligonucleotides. After performing several iterative cycles of SELEX with the aforementioned steps (typically 6-20 cycles), the initial library is enriched with aptamer candidates having high affinity towards their target. At this stage, typically 50 aptamer clones are sequenced to identify those with identical sequences/sequence motifs, which are further explored in affinity studies [7]. This

characterization is generally done by performing affinity studies with the target molecule to determine the dissociation or affinity constant (K_D or K_A). Considering the large number of sequences found after selection, characterization of each individual aptamer for its binding properties and specificity is lengthy and costly, yet very necessary to identify the best performing candidate. It is also known that the affinity and specificity towards the target may change depending on the operating conditions [8]. Therefore, it is equally important to monitor the aptamers under different assay conditions during the characterization step. For that reason, a platform that would enable high throughput analysis of a sufficiently large number of aptamer candidates simultaneously, would offer a huge advantage compared to the conventional practices. Currently, Surface Plasmon Resonance (SPR) platforms are commonly used for K_D determination, with Biacore being a front-runner in this domain. Despite its main advantage of label free detection, limited multiplexing capacity makes screening of numerous aptamer candidates a lengthy and costly process.

Recently, a new platform has been developed, namely Evalution™ (MyCartis NV, Belgium), for multiplex biomarker analysis (Figure 1) [9]. This platform is based on dynamic multi-analyte technology (DMAT) and has 3 distinctive features: a novel type of digitally encoded microparticles (μ Ps), an assay plate composed of 16 individually actuated microfluidic channels and an instrument that integrates incubation, washing, and optical real-time readout. While the platform allows high throughput multiplexing (16 channels can be used in parallel with up to 150-plex assays per channel thanks to the different codes of μ Ps), it also uses the advantage of microfluidic-based systems that enables fast assay time and less sample consumption. Furthermore, binding reaction occurs in the reaction limited regime due to the continuous supply of analytes to the capture molecules with flow, which provides monitoring of kinetic profiles independent of diffusion effect. These unique features of the system make it a promising tool to render the task of multiple aptamer screening.

In this work, we assessed for the first time, the potential of Evalution™ to be used in a time-efficient and high throughput characterization process of the selected aptamer candidates. To do so, we first

selected aptamers against recombinant influenza A nucleoprotein (infA NP) using a magnetic bead-based SELEX [10]. InfA NP was selected as a target molecule rather than the whole virus. It is known that glycoproteins on the surface of the virus has high mutation rates while internal proteins like NP are more conserved within the influenza strains. Thus, targeting infA NP, would allow a universal detection without the risk of missing an infection due to mutations of the virus [11]. Although all amplification-free detection techniques for influenza viruses use antibodies as receptors, due to their high sensitivity, there has been a significant research ongoing in the recent years for utilizing aptamers that either target the whole virus [12–14] or surface protein hemagglutinin [15–17]. Therefore, an aptamer that can detect infA NP with high affinity would be of great significance for the development of these detection platforms. However, to our knowledge, such aptamer is not available for open access (except for the commercially available AptaRes polyvalent anti-influenza aptamer (AptaRes AG, Mittenwalde, Germany) [18]. Selected aptamer candidates were further screened for their binding kinetics to infA NP using Evaluation™. Finally, their specificity towards infA NP was challenged with different proteins, such as bovine serum albumin (BSA) and influenza B nucleoprotein (infB NP). The most promising candidate was used to compare the viability of the new platform against Biacore 3000.

2. MATERIALS AND METHODS

2.1. Reagents

All chemicals for buffer preparation were of analytical grade and purchased from Sigma-Aldrich except for Tris(hydroxymethyl) aminomethane (Bio-RAD Laboratories), NaCl and KCl (Merck). Overview of buffers and solutions prepared for the experimental study is given in Table 1.

2.2. Proteins and antibodies

The custom made recombinant infA NP was obtained from Imgenex, (United States). Recombinant infB NP was purchased from Orla Protein Technologies (UK) and BSA from Rockland Immunochemicals (US). Monoclonal (ab128193) and polyclonal (11675-RP01) anti-Influenza A Virus Nucleoprotein antibody (anti-InfA NP ab) were purchased from Abcam plc (US) and Sinobiological Inc (China), respectively. HRP-labelled goat-anti-mouse antibody (HRP-GAM) highly cross adsorbed and biotin labelled goat-anti rabbit antibody (biotin-GAR) highly cross adsorbed were purchased from Novex Life Technologies (Belgium). Biotinylated anti-his tag antibody (6-Histidine Epitope Tag Antibody (33D10.D2)) was purchased from Bio-technie Ltd. (UK).

2.3. Random oligonucleotide library, primers and aptamers

The random oligonucleotide library, primers and selected aptamer sequences were purchased from Integrated DNA Technologies (Haasrode, Belgium). The 80-nucleotide ssDNA library contained a central random sequence of 40 nucleotides (N40) flanked by 20-nucleotide primer hybridization sites (5'-/56-FAM/TCG CAC ATT CCG CTT CTA CC-(N40)-CGT AAG TCC GTG TGT GCG AA-3'). The following forward and reverse primers were used in PCR for amplification of the library, respectively: 5'-/56-FAM/TCG CAC ATT CCG CTT CTA CC-3' and 5'-TTC GCA CAC ACG GAC TTA CG-3'. The forward primer was labelled with fluorescein (6-FAM), in order to identify relevant DNA sequence on urea denaturing polyacrylamide gels. The 5 selected aptamer sequences were labelled with ATTO550 label at 5'-end and used in characterization experiments (Table 2).

2.4. Instruments

Aptamer characterization experiments (kinetic analysis and specificity assessment) were performed using the Evalution™ platform (Figure 1), which consists of three major components: μ Ps as solid support to tether molecules, the cartridge (assay plate) composed of 16 individually actuated

microfluidic channels and the instrument with pump, camera and software that provides assay control and data processing [19]. The disk-shaped silicon μ Ps (diameter 40 μ m and thickness of 10 μ m) (Figure 1A) are the central component of the platform and act as solid support to tether capture molecules, such as antibodies and nucleic acid (NA) probes. μ Ps have at their outer ring holes or no holes at defined places, creating a 10-bit binary code that can be identified and read by the software at all times. The central area of the μ Ps is dedicated to measure fluorescence intensity. By coupling different capture molecules to μ Ps with different codes and loading these together in a microfluidic channel, a multiplexing environment is created, allowing the monitoring of molecular interactions between different biomolecules in a single run [9,20,21].

The main steps of a typical assay on the platform can be summarized as follows: (1) mixing different populations of μ Ps, each coupled with a different capture molecule (multiple particles with identical codes form a population); (2) loading of μ P mix into the microfluidic channels of the cartridge using a loading device (a pumping system); with the aid of the filter structure, loaded μ Ps are tiled in a monolayer in the detection zone and stay static during the sample flow; (3) insertion of the loaded cartridge into the instrument, and the execution of a defined protocol; in any type of protocol (kinetic or end-point), the first step is to scan the μ Ps before the assay starts for determining background fluorescence; (4) loading of samples containing fluorescently labelled detection molecules (antibody or DNA); this solution is then flowed into the channels by the application of a differential pressure (0–2000 mbar) between inlet and outlet wells; (5) recording of the fluorescent signal on μ Ps either at repeated time intervals during a real-time measurement or at the end of the run for end-point measurements [9].

During the scanning of the channel, μ Ps are imaged from the bottom of the cartridge (Figure 1B). μ P decoding in bright field and assay quantitation in fluorescent mode are done consecutively so that the relative position of each μ P is the same in both images. The imaging system of the instrument is a high-sensitivity CMOS camera that is mounted on an automated x-y-z stage for scanning the channels

in real-time (i.e. while the reaction is occurring) or at endpoint. Each μP generates approximately 1800 results, one per pixel. The instrument is equipped with a 532 nm laser. The output of each run is the population fluorescence, which is computed by the arithmetic mean of fluorescence obtained from all μPs of the given population.

2.5. Aptamer selection

2.5.1. Magnetic bead-based SELEX

Aptamers binding to infA NP were isolated from a random ssDNA library using magnetic bead-based SELEX method (Figure 2). InfA NP was immobilized on M-280 Tosyl-activated Dynabeads (Life Technologies, Belgium) according to the manufacturer's instructions. The protocol for preparation of infA NP functionalized beads (infA NP- beads) is given in supporting info (SI), Section 1 (Figure S1).

Prior to each SELEX round, a counter-SELEX was performed in order to remove any unspecific DNA sequences that would bind to BSA and/or the solid support. An aliquot of 9.6×10^6 BSA-beads (0.144 mg, prepared as described in SI, Section 1) was washed twice with SELEX buffer (Table 1) [10]. The ssDNA library was heated at 80 °C for 15 min on a thermomixer (Eppendorf, Belgium), rapidly cooled down on ice for 5 min and brought to room temperature (RT) before use.

In the initial round, 1.5 μmol of ssDNA library was suspended in 185 μL SELEX buffer and added to BSA-beads, followed by rotation for 30 min at RT. Cleaned library was pooled by removing BSA-beads with magnetic capture and incubated immediately with an aliquot of 9.6×10^6 (0.144 mg) infA NP-beads. After rotation for 30 min at RT, aptamer bound infA NP-beads were recovered and washed four times with SELEX buffer. Upon re-suspension in molecular biology grade water (5Prime GmbH, Belgium), a first PCR amplification was performed on the beads with 20 cycles. Next, the magnetic beads were removed and the product from the first PCR was further amplified in seven parallel PCR reactions. Each PCR reaction contained 1 μM of each primer, 0.5 μM of dNTP mixture (Eurogentec, Belgium), 0.5 μM Phusion DNA Polymerase (Thermoscientific, Belgium) and 10 μL Phusion High

Fidelity Buffer (Thermoscientific, Belgium) in a total volume of 50 μ L. The amplification conditions for both the first and second PCR were: 5 min at 98 °C, 20 cycles of 5 s at 98 °C, 15 s at 68 °C and 5 s at 72 °C.

To generate new ssDNA library, relevant DNA strands labelled with FAM were separated from double stranded PCR products by denaturing polyacrylamide gel electrophoresis (PAGE). All PCR products from the second PCR round were pooled and diluted in 1:1 ratio using Novex TBE urea sample buffer (Invitrogen, Belgium). The sample was run on precast 6 % Novex TBE urea gels with 1X Novex TBE running buffer. The relevant FAM labelled DNA strands were identified on the urea gels using a UV transilluminator (Safe imager, Invitrogen) and cut out. Extraction of DNA from gel was done by a crush and soak method. Briefly, the cut gel piece was frozen at -20 °C, crushed into small pieces and incubated in 1 mL of crush and soak buffer overnight at 37 °C with gentle shaking at 300 rpm on a thermomixer. Subsequently, the supernatant was pooled and DNA was extracted by ethanol precipitation. Purified ssDNA was re-suspended in SELEX buffer and used as the new ssDNA library in the next SELEX round.

After the completion of the first round, the entire ssDNA library generated was used in the next round, starting with the counter-SELEX. The sequence of counter SELEX proceeded with SELEX round was applied in all subsequent rounds. In total, eight rounds of SELEX were performed and in the last round the amount of target was decreased by 25 % in order to enrich the library with aptamer candidates that have high affinity. The progress of the SELEX procedure was monitored using reMelting Curve Analysis method (rMCA) [22] as explained in SI, Section 2 (Figure S2).

2.5.2. Cloning, sequencing and structure analysis of selected aptamers

After the completion of round 8, the pool of ssDNA molecules obtained was cloned and sequenced. First, a final PCR amplification was performed with the same primers used in the SELEX rounds, except that the forward primer was without FAM-label. The PCR products were further purified using a GeneJet PCR purification kit (Thermoscientific, Belgium) following manufacturer's instructions and

subsequently cloned into the pGEM-T easy plasmid DNA vectors (Promega, Madison, WI, USA). DNA sequences with A-overhang were obtained by performing an A-tailing procedure at 70 °C for 15 min using Taq polymerase (Life technologies, Belgium) and were then ligated with a 1:1 ratio into the vector by incubating for 1 h at RT. Subsequently, transformation was performed using Subcloning efficiency™ DH5α™ competent cells (E. coli) (Invitrogen, Belgium). Briefly, 2 µL of the ligation mixture from previous step was added to 50 µL of cells and kept on ice for 30 min. The solution was incubated for 45 s at 42 °C and for 2 min on ice. 250 µL of SOC medium (Invitrogen, Belgium) was added and incubated for 1 h at 37 °C. The cells were plated on LB agar plates containing 200 µg mL⁻¹ ampicillin and incubated overnight at 37 °C. Then, the colonies were picked and incubated overnight at 37 °C in 2 mL LB medium (Becto Dickinson and Company, Belgium) containing 40 µg mL⁻¹ ampicillin. The plasmid DNAs of 50 clones were purified using GeneJet miniprep kit (Thermoscientific, Belgium) and sequenced at Genetic service facility, University of Antwerp, Belgium. Shared sequence motifs were examined using Clustel X program [23] and the secondary structure analysis of the selected aptamers were performed using the Mfold program [24].

2.6. Aptamer characterization on Evalution™

For the characterization experiments, µPs were prepared by coupling target protein (infA NP) and negative controls (infB NP and BSA) to the µPs (will be referred to as infA NP µPs, infB NP µPs and BSA µPs, respectively). To obtain the optimal coupling condition, a screening process was first performed using only infA NP as described below in Section 2.6.1. Following, using the optimal coupling condition, a large-scale coupling was done for each protein as described in SI, Section 3.1. Finally, binding kinetics and specificity tests were performed using these µPs as explained in Section 2.6.2 and 2.6.4, respectively.

2.6.1. In-channel optimization of infA NP coupling to μ Ps

In this screening, an infA NP coupling protocol was executed directly in the microfluidic channels of the cartridge (MyCartis NV, Belgium) using different coupling conditions, i.e. 8 different buffers: MEST (pH 2.5, 3.5, 4.5, 5.4 and 6.6), PBST (pH 7.4 and 8.3) as well as carbonate-bicarbonate buffer solution (pH 9.1). All conditions were tested simultaneously in parallel channels during the same run on the Evalution™ platform. Two steps were followed for in-channel coupling: activation of μ Ps (MyCartis NV, Belgium) and coupling of infA NP to μ Ps. In the activation step, μ Ps with carboxyl (COOH) groups grafted on their surface (COOH μ Ps) were re-suspended in 500 μ L of activation solution (Table 1) and loaded into the microfluidic channels of the cartridge (for the loading procedure, the reader is referred to SI, Section 3.2). Then, COOH groups were activated by flowing 50 μ L of EDC/NHS mixture (1:5 volumetric ratio) using 50 mg mL⁻¹ EDC.HCl and 10 mg mL⁻¹ sulfo-NHS for 10 min. Following, the channels were washed with activation solution for 60 s. Subsequently, coupling of infA NP was done by flowing 100 μ L of a 10 μ g mL⁻¹ infA NP solution, each with a different pH, in 8 separate channels for 20 min. After coupling, channels were flushed with PBST (pH 7.4), which also served as washing buffer.

Finally, the quality of coupling was checked by performing an antibody-based assay and visualizing μ Ps. To do that, coupled infA NP was detected using a polyclonal anti-infA NP ab, that is selected based on a previous study [25]. In the first step, μ Ps were incubated with 100 μ L of 3 μ g mL⁻¹ polyclonal anti-infA NP ab in LC buffer (Table 1) for 20 min. After a short (60 s) wash with PBST, 100 μ L of 5 μ g mL⁻¹ detection antibody (biotin-GAR) in LC buffer was flowed for 20 min to form the immuno-complex on the μ Ps. This step was followed by a short wash (60 s) with PBST. Subsequently, 40 μ L of 3 μ g mL⁻¹ streptavidin-labelled phycoerythrin (SAPE) in PECD-100 (Moss Inc., USA) was added and flowed for 5 min. After the final washing step, the channels were scanned, and fluorescence read-out was recorded. During all assay steps, the differential pressure applied between inlet and outlet was 300 mBar, which corresponds to a flow rate of approximately 30 nL s⁻¹.

2.6.2. Determination of binding kinetics on Evalution™

The selected aptamer candidates were screened for their affinities by conducting kinetic measurements using SELEX conditions: RT (25 °C) and SELEX buffer as the running buffer. All aptamer candidates were purchased with ATTO-550 modification (post-synthesis conjugation of ATTO NHS ester) at their 5' end. For each kinetic measurement, a μP mix of 5000 $\mu\text{P mL}^{-1}$ was prepared in PBST (pH 7.4), using infA NP μPs and non-functional COOH μPs with a population ratio of 1:2, respectively. Each channel was loaded up to 1 mm field of view (FOV) which accommodates approximately 250 μPs . To determine the association and dissociation rate constants, aptamer solutions prepared in SELEX buffer (37.5, 75, 150, 300 and 600 nM) were flowed through the channels at 300 mBar (~ 30 nL s^{-1}). After the association step, aptamer solutions were replaced with SELEX buffer and flowed under the same conditions to allow dissociation. The fluorescent signal was recorded with 2 min time intervals using the green laser (excitation at 532 nm, laser power 10 mW, exposure time of 1000 ms) and CMOS camera (10x objective) present on-board of the instrument. Five neighbouring channels were used in a single run, each channel accommodating a different aptamer concentration. Before the start of each run, a background read was performed with the same laser settings mentioned above. After subtraction of the COOH μP signal from infA NP μP signal, association and dissociation curves were fitted globally using an 'association then dissociation' equation (GraphPad Prism version 7.02, USA) based on the Langmuir model with the assumption of 1:1 binding. For each aptamer, 3 independent experiments were performed using 5 concentrations of the aptamer. The binding curves obtained in each experiment were fitted globally and k_{on} , k_{off} and K_{D} values were estimated. The reported k_{on} , k_{off} and K_{D} values (Table 3) were calculated as the average of the values obtained from global fittings of 3 independent repetitions.

2.6.3. Determination of binding kinetics on Biacore

In order to demonstrate the potential of the Evalution™ platform as an aptamer characterization tool, a comparison of kinetic analysis for NP5 was done using Biacore 3000 as a benchmark. The experimental protocol is given in SI, Section 4.

2.6.4. Specificity tests for selected aptamers

Selected aptamers were further tested for their specificity against infA NP. For each experiment, a μP mix of $10000 \mu\text{P mL}^{-1}$ was prepared in storage buffer (undisclosed composition of MyCartis NV) by mixing three μP populations (infA NP μPs , infB NP μPs and BSA μPs) in 1:1:1 ratio (details of the coupling procedure for all particles is given in SI, Section 3.1). Then, the μP mix was loaded on the cartridge. The assay protocol consisted of two parts: binding of aptamers to functionalized μPs in SELEX buffer (30 min) and washing step (11 min). Three different washing buffers (Table 1) were tested here, the original SELEX buffer and the SELEX buffer containing increased NaCl concentration (i.e. 150 mM and 300 mM). In the first part, aptamer solutions (150 nM prepared in SELEX buffer) were flowed through the channels and the fluorescent signal was recorded (F1). In the second part, aptamer solutions were replaced with one of the washing buffers. Therefore, for the same aptamer, three different washing conditions were monitored in separate channels. At the end of the washing step, fluorescent signal was recorded (F2) and signal drop was calculated as $(F1-F2) / (F1)$. Two replicate runs were performed and before the start of each run, a background read was done with the same laser settings mentioned in Section 2.6.2. During all assay steps, the differential pressure applied between inlet and outlet was 300 mBar ($\sim 30 \text{ nL s}^{-1}$).

3. RESULTS AND DISCUSSION

3.1. Selection of aptamers against infA NP

Eight SELEX rounds were performed in total in the process of selecting an aptamer against infA NP, as explained in Section 2.5.1. The progress of the SELEX procedure was monitored using rMCA for the ssDNA libraries from round 6 to 8 with the described method in SI, Section 2 [22]. The 2-consecutive high-resolution melting (HRM) analyses (HRM 1 and HRM 2) with a short reannealing step (1 min at 70 °C) in between, only allowed the hybridization of the sequences that are abundantly present. Thus, the melting profile of the 2nd HRM analysis indicated the potential presence of aptamer candidates. Although the differences between the rounds were not very distinctive, round 8 showed a slight difference in melting behavior around 80 °C (Figure S2), which is a typical melting temperature of aptamers. Considering this information, the aptamer pool collected after round 8 was cloned and sequenced for characterization, as detailed in Section 2.5.2. Out of 50 sequenced clones, 5 sequences (NP5, NP15, NP26, NP34, NP40) showing similar motifs and having the lowest ΔG values were selected for further characterization as listed in Table 2.

Secondary structures of the selected aptamers were predicted using web-built MFold software [24], which is based on a free energy minimization algorithm. A stem-loop structure appeared as common among the candidates, mainly with one dominant loop and 1 to 3 protruding hairpins (Figure 3).

3.2. Aptamer characterization on Evaluation™

The main features of Evaluation™ such as, multiplexing, real-time reaction monitoring and parallel channel configuration of the cartridge were explored in the context of the demanding aptamer characterization process, which often requires screening of multiple aptamer sequences under different conditions. To do this, here we used 5 aptamer candidates selected against infA NP. The aptamer characterization process in this study consisted of 3 crucial steps (schematically depicted in

Figure 4): (1) providing functional μ P surface with immobilized infA NP (for all downstream analysis); this was achieved through an extensive optimization of the coupling reaction conditions (8 conditions) by exploiting multiple parallel channels on a single cartridge (Section 3.2.1); (2) determining the binding kinetics of the selected aptamer candidates by exploring the real-time reaction monitoring feature of the device (Section 3.2.2) and (3) evaluating the specificity of the selected aptamers towards infA NP versus other proteins, such as infB NP and BSA, enabled by the multiplex capacity of Evalution™ (Section 3.2.3).

3.2.1. Optimization of NP coupling to μ Ps

One of the unique features of the system is the easy screening of numerous reaction conditions simultaneously in parallel channels within a single and short assay [26]. Exploiting this feature, the optimal coupling condition of infA NP to the μ P surface was determined by testing different buffers, namely MEST pH 2.5, 3.5, 4.5, 5.4, 6.6, PBST pH 7.4 and 8.3, carbonate-bicarbonate buffer pH 9.1. The in-channel coupling procedure was conducted as described in Section 2.6.1 and visualized following the assay configuration given in Figure 5A.

Here the optimal coupling condition was defined as the one displaying minimal aggregation of infA NP to be able to accurately monitor the binding kinetics between aptamer and target. Ideally, the images should show homogenous fluorescence signal on the μ P surface with minimum localized bright points and aggregates between the μ Ps. The fluorescence images showed significant aggregation when NP was coupled to the μ Ps in MEST buffers (pH 2.5 to 6.6). Higher pH values (pH > 7.0) showed less aggregation on top and in between the μ Ps and resulted in a more homogenous distribution of the protein on the μ P surface (Figure 5B). This may be explained by the fact that infA NP has an isoelectric point (pI) of 9.39 [11].

Considering the run time spent for the screening (~ 2h), the parallel channel configuration of the cartridge allowed us to eliminate non-ideal coupling conditions in a time-efficient way and with minimum material consumption. According to the screening results, 2 of the high pH values (i.e. pH

7.4 and 9.1) were further tested in a large-scale (in tube) coupling (SI, Section 3.1) in order to choose the best performing coupling buffer. The bicarbonate-carbonate buffer solution showed higher signal intensity (158 a.u) compared to the PBST (107 a.u) with less aggregation. Therefore, we used bicarbonate buffer pH 9.1 for coupling new infA NP μ Ps for all subsequent experiments and proper coupling was confirmed with the same protocol described in SI, Section 3.2 (Figure S3, S4).

3.2.2. Determining binding kinetics of aptamers

Using the real-time monitoring feature of the instrument, we first determined association and dissociation constants of the selected aptamer candidates. To do that, we used the ATTO-550 labelled aptamers for the fluorescent read-out and monitored binding to the InfA coupled to μ P surface (Figure 4). In addition, by utilizing the parallel channel configuration of the cartridge, we could test different concentrations of the same aptamer (ranging from 37.5 to 600 nM) simultaneously during the same run. Each aptamer was flowed in separate channels that was loaded with the infA NP μ Ps and COOH μ Ps and fluorescent intensities were recorded with time intervals of 2 min. The obtained association and dissociation curves with corresponding fittings for all 5 tested aptamers are shown in Figure 6. All aptamers showed slow dissociation, which is a preferable property of a bioreceptor in assay design. This property corresponds to a previous finding where an SPR experiment indicated a slow dissociation between monomeric NP and an RNA strand [27]. Moreover, dissociation constants (K_D) of all aptamers were in the low nanomolar range (Table 3), suggesting strong binding to the infA NP, with NP26 showing the lowest K_D of 13 nM [28]. Although NP26 shared identical and long sequence motifs with NP5 (Figure 3), their binding profiles were rather different. NP26 showed a more than three times higher association rate (k_{on}) and a slightly lower K_D than NP5. On the contrary, NP34 gave the closest K_D value to NP26, despite having very few bases in common. The differences in K_D can hypothetically be attributed to the hairpin structures protruding from the main loop, which is smaller in NP26 and NP34 compared to NP5. Based on Figure 3 and Table 3, we can say that aptamers

with more protruding hairpin structures (NP26, NP34 and NP40) showed higher affinity towards infA NP.

3.2.3. Evaluating aptamer specificity

Following kinetic analysis, the multiplexing capacity of the platform was utilized to assess the specificity of the aptamers towards infA NP. To do this, the interaction of the aptamers was monitored with 3 different proteins (infA NP, infB NP and BSA) within the same channel, in a short binding assay (~40 min total assay time). Moreover, due to the parallelization capability of the microfluidic cartridge, all 5 aptamers were tested simultaneously in parallel channels during the same run. Aptamer solutions, prepared in SELEX buffer at 150 nM concentration, were flowed over the μ P mix (infA NP, infB NP and BSA μ Ps) for 30 min and then the channels were washed for 11 min to remove unspecific interactions. The signal recorded after binding (F1) and washing (F2) was used to evaluate the specific and unspecific interaction and reported as the signal drop (%) on each μ P population in the same channel. The results, as summarized in Figure 7A, indicated a good discrimination for BSA by each aptamer with more than 50 % drop in fluorescence signal after washing, suggesting that the generated signal was mostly unspecific interaction of aptamers with BSA. This result was expected since a counter SELEX using BSA-beads was performed in the beginning of each SELEX round to eliminate DNA sequences that bind unspecifically to BSA. Although not as high as BSA, a moderate discrimination towards infB NP was also observed for all aptamers, with NP5 showing the highest signal drop upon washing. Only 13 % signal drop was seen for infA NP while 35 % was observed for infB NP μ Ps, which indicated a stronger interaction with infA NP. The other aptamers also followed the same pattern of lower signal drop for infA NP compared to infB NP, indicating the overall higher affinity towards infA NP.

Furthermore, we evaluated the effect that increased NaCl concentrations in the washing buffer might have on the discrimination between different proteins. Here, benefitting from the high-throughput analysis capacity of the cartridge design, it was possible to monitor 3 different washing buffers for 5

aptamers (45 data points) simultaneously in single run. The salt concentration was changed by increasing it from 100 mM in the SELEX buffer to 150 mM and 300 mM in the washing buffer. Only the results of aptamer NP5 are given in Figure 7B for 100 mM and 300 mM NaCl (the results of the other aptamers are given in SI, Section 3.3, Figure S5). A higher signal drop was observed when increasing the NaCl concentration (300 mM) compared to the initial condition (100 mM) on both infA and B NP μ Ps, while there was no significant change for BSA μ Ps. On the other hand, the signal drop difference observed between infA NP and infB NP μ Ps remained similar in both high and low salt concentration conditions. This result indicated the limited effect of buffer stringency on the discrimination. Nevertheless, under the higher stringency condition, NP5 still conserved the higher discrimination ability compared to the other aptamers.

The low discrimination between inf NP types can be explained with structural and functional similarities of NP from infA and infB viruses. It is known that infA and infB NPs show 37.7% identity and 76.2% similarity over a 496-amino acid overlap [29,30]. Furthermore, it was reported that NP binds to viral RNA in the virus [31] and both infA NP and infB NP have identical sites in the RNA binding groove [32]. Deletion or mutations in the residues in the binding groove showed decrease in RNA binding affinity [33]. Considering the similarities in structure and NP binding function, discrimination between inf NP types may be intrinsically limited when using aptamers. However, performing a counter SELEX against inf B NP might improve the discrimination ability of selected aptamers.

Although the binding mechanism of NP to RNA is mostly governed by the interaction of the negative phosphate backbone of the RNA with positive residues of the protein [34], a significant preference in aptamer binding to infA NP compared to infB NP was observed in our results, which cannot be solely explained by electrostatic attraction. The difference in the binding can be attributed to the specific secondary structures of the aptamers that were selected under the same assay condition. It might be that some sequence motifs found on the random region contribute to the binding of aptamers to infA

NP more than infB NP. Moreover, it was found that NP has a preference for single stranded polynucleotide structures rather than for double stranded ones [34]. This might explain our results that the aptamer candidate with the longest single stranded structure (NP5) also showed the best performance in specificity. According to present results (Table 3 and Figure 7A), NP5 is the most promising candidate as it showed both high affinity (comparable to the other candidates) and the best specificity towards infA NP.

3.3. Benchmarking kinetic results against Biacore

Finally, in order to benchmark the present platform as a kinetic characterization tool for aptamers, the binding experiment of NP5 was also performed on a Biacore 3000 platform (experimental details are given in SI, Section 4). NP5 was especially selected for this experiment since it showed high affinity and the best specificity towards infA NP. Furthermore, its sequence was found more than once during the selection process. The aptamer was immobilized on the sensor chip surface using the biotin-streptavidin method and NP was injected at different concentrations ranging from 0 to 200 nM. Both association and dissociation rates were determined by fitting the response curves globally using a 1:1 Langmuir model (Figure 8).

K_D of the aptamer was calculated as 17.7 ± 3.5 nM. Although the K_D differed slightly from the results of the Evaluation™ platform (41 ± 5 nM), the values were still in the same order of magnitude being the low nanomolar range. These subtle differences might be attributed to the opposite ligand-analyte orientation on the two platforms: NP in solution on Biacore, while being immobilized on the μP surface on Evaluation™. Immobilization of NP might have affected the availability of residues in the binding groove to some extent, resulting in slightly different dissociation constants [35]. Due to the unavailability of fluorescently labelled NP, using the same assay format was not possible on both platforms. On the other hand, the assay format used on the Evaluation™ resembles better the SELEX procedure where NP was immobilized to the solid surface. Moreover, using the presented assay configuration, the multiplexing capacity of the platform was easily applied in testing the specificity of

the aptamers against different targets in a single channel. In addition, during the same run multiple assay conditions (buffer type, salt concentration, etc.) were monitored simultaneously by using different channels in parallel, thus offering the possibility to acquire substantial amount of data in limited time.

4. CONCLUSIONS

In this study, the potential of Evalution™ as an aptamer characterization platform was assessed using 5 aptamers selected against infA NP. The aptamers were selected in 8 rounds of a magnetic bead-based SELEX method. Based on the secondary structure predictions, stem-loop appeared as a common structure among the candidates, mainly with 1 dominant loop and 1 – 3 protruding hairpins. The selected candidates shared 5 sequence motifs in common.

The platform features, such as real-time monitoring and multiplexing, were utilized to characterize the selected aptamers. Binding studies were performed by monitoring the interaction between aptamers and infA NP in real-time and revealed K_D values in the low nanomolar range for all 5 aptamers. Furthermore, with the aid of multiplexing feature, a rapid specificity (2 runs of 40 min) assessment was also performed. This experiment revealed that all aptamers were capable of distinguishing between infA NP and BSA while also showing some binding to infB NP, probably because of high sequence similarity between the two NPs. Among the 5 candidates, NP5 was recognized as the most promising aptamer because it showed as good affinity towards infA NP as other aptamers but also the highest specificity. Using NP5 as the most prominent aptamer candidate, the affinity studies performed on the Evalution™ platform were further compared to K_D value obtained with SPR analysis, a technology commonly used in the kinetic assessment of binding reactions. Similar K_D value in low nanomolar range was obtained by the SPR platform, demonstrating thus the Evalution™ potential for reliable kinetic characterization studies with a great advantage of high multiplexing capacity in a single channel, which is limited in current SPR platforms.

The present work revealed the potential of Evalution™ platform as an alternative aptamer characterization platform. The platform has been evaluated in this study using only 5 aptamers, however, it can easily monitor 16 aptamer sequences in one cartridge using the same assay. Considering the preparation time for the target coupling to the μ Ps and cartridge loading (3-4 h), a quick overview of the binding profile for a short association time (i.e. within 30 min) can be already obtained in a single run, which can help to select only the most prominent aptamer candidates. Furthermore, using the presented assay configuration (Figure 4), it is also possible to monitor both kinetics and specificity at the same time under multiple assay conditions (buffer type, salt concentration, etc.). Knowing that aptamers have been considered as promising bioreceptors in bioassay development, this work may serve as a template for future aptamer-based studies in simplifying and speeding up the aptamer characterization and assay development process.

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AUTHOR CONTRIBUTIONS

All authors have given approval to the final version of the manuscript.

Notes: The authors declare no competing financial interest.

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Figure 1. Schematics of the components of Evaluation™ (Reproduced from [9])

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related to the material excerpted should be directed to the ACS): (A) the digitally encoded μ P and (B)

the microfluidic cartridge and signal read-out.

Figure 2. Schematic representation of the magnetic bead-based SELEX.

Figure 3. Predicted secondary structural folding of selected ssDNA aptamers for InfA NP. Common motifs identified in boxes.

Figure 4. Representative scheme of assay configuration in the channel (left) and features of the system utilized for characterization experiments (right).

Figure 5. Assay configuration and representative fluorescent images of the in-channel screening test for coupling conditions: (A) Assay configuration for visualization. Anti-infA NP antibody and subsequently biotinylated detection antibody were flowed over coupled particles for 20 min. The signal was generated by incubation of SAPE for 5 min, (B) Fluorescence images of the channels for each tested buffer.

Figure 6. Association and dissociation curves and their corresponding fitting for NP26, NP34, NP40, NP15 and NP5. One replicate run for each aptamer is shown as representative curves. Curve fittings were performed using 1:1 Langmuir model with global fitting (GraphPad Prism version 7.02).

Figure 7. Discrimination capacity of the aptamers against different target proteins in the same channel: (A) comparison of all aptamers with signal drop (%) after the washing step, (B) Effect of increased NaCl concentration on the signal drop (%) after washing using aptamer NP5. The NaCl concentration was increased from 100 mM (gray) to 300 mM buffer (black) for improved discrimination. Errors are standard deviations based on 2 repetitive runs.

Figure 8. Association and dissociation curves and their corresponding fitting for NP5. Errors are standard deviations based on 3 repetitive runs. Curve fittings were performed using 1:1 Langmuir model with global fitting (GraphPad Prism version 7.02).

LIST AND COMPOSITION OF BUFFERS USED IN THE PAPER

1	100 mM sodium phosphate buffer
2	300 mM ammonium sulfate in 100 mM sodium phosphate buffer
3	PBS with 0.2 % BSA and PBS with 0.1 % BSA
4	SELEX buffer: 20 mM Tris-HCl, 100 mM NaCl, 5 mM KCl, 2 mM MgCl ₂ , 1 mM CaCl ₂ , 0.02 % Tween 20, pH 7.6
5	Crush and soak buffer: 500 mM NH ₄ OAc, 0.1 mM EDTA and 0.1 % SDS
6	MES buffer with Tween 20 (MEST): 100 mM MES, 0.3 % Tween 20, at pH 2.5, 3.5, 4.5, 5.4 and 6.6
7	PBS with Tween 20 (PBST): 100 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, 0.3 % Tween 20, pH 7.4 and 8.3
8	Coupling buffer: 100 mM carbonate-bicarbonate buffer solution, pH 9.1
9	Activation solution: MEST, pH 3.5
10	Washing buffer for Evalution™ runs: PBST, pH 7.4
11	Washing buffer for specificity tests: SELEX buffer with 150 mM or 300 mM NaCl
12	LowCross-Buffer® (LC buffer): Candor Bioscience, Germany
13	Storage buffer: undisclosed formulation of MyCartis NV
14	PECD-100: Stabilizing diluent for phycoerythrin (PE) conjugates (Moss Inc., USA)

Table 1 List of buffers used in the manuscript and their composition.

SEQUENCE NAME	RANDOM REGION	ΔG (KCAL/MOL)
NP26	TGCATCTTCCTGTTTCATCATTCTCCTATTACGTTCTTA	-5.84
NP5	TATTGTCTATTGATTCTGTTTCGGTCTATCTCTTTTT	-4.89
NP40	TGGTGTATGATGAAATTGTTGAATATTTCCCTCTCTATA	-6.37
NP34	TCCACCATGTGTCCTTTTAATAATCCTTCTATTT7CACCA	-6.51
NP15	CTCCCCATTTTCGCACATATTTCTCCTTTCTATCTCGCAT	-5.26

Table 2 Selected aptamer sequences and ΔG values. Shared sequence motifs are grouped as: Motif1 (red) and motif2 (green), shared between NP5 and 26; Motif3 (orange), shared between NP15, 34 and 40; Motif4 (underlined in blue), shared between NP5, 15 and 34; Motif5 (gray), shared between NP5 and NP15. Motif4 was located in the loop of both NP34 and NP15 and with only one base difference (italic, C→T) in the loop of NP5.

	NP26	NP34	NP40	NP15	NP5
K_{ON} (M × MIN)⁻¹	$(16.8 \pm 0.7) \times 10^4$	$(7.0 \pm 0.6) \times 10^4$	$(7.7 \pm 0.7) \times 10^4$	$(10.4 \pm 1.2) \times 10^4$	$(5.3 \pm 0.3) \times 10^4$
K_{OFF} (MIN⁻¹)	$(2.2 \pm 0.4) \times 10^{-3}$	$(1.4 \pm 0.1) \times 10^{-3}$	$(2.3 \pm 0.4) \times 10^{-3}$	$(3.4 \pm 0.6) \times 10^{-3}$	$(2.2 \pm 0.2) \times 10^{-3}$
K_D (M)	$(13 \pm 3) \times 10^{-9}$	$(20 \pm 1) \times 10^{-9}$	$(30 \pm 7) \times 10^{-9}$	$(34 \pm 9) \times 10^{-9}$	$(41 \pm 5) \times 10^{-9}$

Table 3 Affinity constants of aptamer candidates (1:1 Langmuir fitting). Errors are standard deviations based on 3 repetitions for each aptamer.