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APPLICATION NOTE – JAGIELLONIAN CENTER OF INNOVATION

BINDING AFFINITY OF ANTI-THROMBIN APTAMERS MEASURED USING SPR AND MST TECHNIQUES

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ABSTRACT

DNA aptamers are small DNA molecules with secondary and tertiary structures that specifically bind proteins or other molecular targets. In this respect, DNA aptamers serve as a chemical equivalent of antibodies and can be used for both research and clinical applications. Here, we characterized affinity of two thrombin-binding aptamers: HD1 (Bock et al., 1992) and HD22 (Tasset et al., 1997). Our results confirm that both aptamers bind thrombin with high affinity since the obtained K_D values were within the nanomolar range (K_D=7.94 nM for HD1 and K_D=0.48 nM for HD22 as determined by Surface Plasmon Resonance, and K_D=5.91 nM for HD1 and K_D=0.53 nM for HD22 as determined by Micro Scale Thermophoresis). Importantly, the obtained results are in agreement with previously published data (Trapaidze et al., 2016).

INTRODUCTION

Thrombin is a serine protease that converts soluble fibrinogen to insoluble fibrin. Due to its relevance in the clotting process, thrombin is an important therapeutic target and a diagnostic marker. HD1 and HD22 were developed as oligonucleotide inhibitors of human thrombin (Bock et al., 1992; Tasset et al., 1997). Here, we analyzed the affinity of these aptamers for thrombin using two different approaches: SPR (Surface Plasmon Resonance) technology (Biacore 100X, GE Healthcare) and MST (MicroScale Thermophoresis) technology (MonolithNT.115, NanoTemper). The SPR experiments, including multi- and single-cycle kinetics, allowed us to obtain the association rate constant, dissociation rate constant and dissociation constant values (k_a, k_d, and K_D, respectively), while the MST experiments provided us with K_D values without the kinetic

RESULTS AND CONCLUSIONS

Prior to SPR analysis, we diluted biotinylated HD1 and HD22 in a HEPES-based buffer containing 5 mM Mg^{2+} since magnesium ions are necessary for ensuring proper conformation of DNA molecules. Diluted aptamers were heated at 95°C and then cooled on ice which, together with magnesium ions, ensures proper folding of these molecules. The presence of biotin allowed us to immobilize HD1 and HD22 (ligands) on the surface of Sensor chip SA with pre-immobilized Streptavidin (GE Healthcare) for the SPR analysis.
Following immobilization, we performed two kinds of experiments using SPR technology: multi-cycle kinetics and single-cycle kinetics. Multi-cycle kinetics employed several injections of thrombin (analyte) in separate cycles. Thrombin was removed by two injections of 2 M KCl at the end of each cycle. In single-cycle kinetics, increasing concentrations of thrombin were run in one cycle without the regeneration step between them.

The affinity of HD22 to thrombin was assayed in multi- and single-cycle kinetics experiments providing us with k_a of 1.45 x 10^7 $M^{-1}s^{-1}$, k_d of 6.50 x 10^{-3} s^{-1} giving the K_D of 4.48 x 10^{-10} M (448 pM) and k_a of 1.38 x 10^7 $M^{-1}s^{-1}$, k_d of 3.50 x $10^{-3}s^{-1}$ and K_D of 2.54 x 10^{-10} M (254 pM), respectively (Figure 1 A and B).

The K_D for HD1 was calculated as 5.91 nM and for HD22 as 0.53 nM with response amplitudes 15.5 for HD1 and 19.3 for HD22 (Figure 3 and 4).

Importantly, the results provided by SPR and MST are comparable. Therefore, both the techniques can be successfully applied for affinity measurement of these

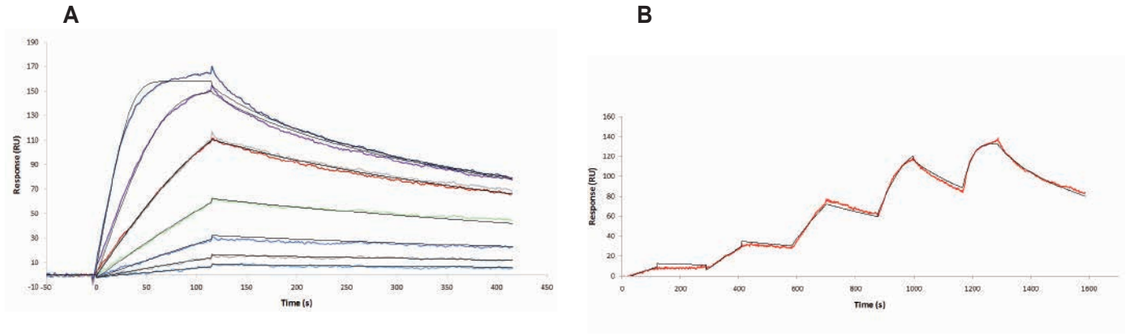

Figure 1. Binding of HD22 to thrombin determined by SPR in multi- (A) and single-cycle kinetics (B). *For the multi-cycle kinetics, thrombin concentrations ranging from 0,24 to 125 nM were used, whereas for the single-cycle kinetics the following concentrations of thrombin were run over the Sensor chip SA: 5, 2.5, 1.25, 0.625 and 0.313 nM. Analysis of these experimental data was performed using the Langmuir 1:1 kinetic model.*

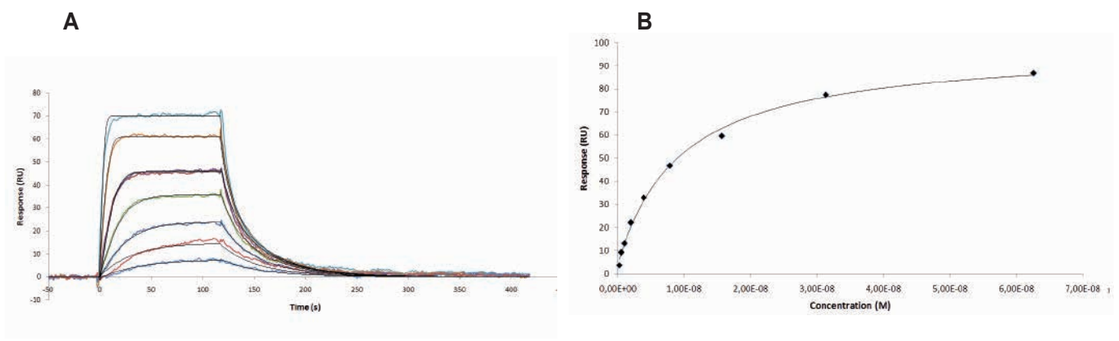

Figure 2. Binding of HD1 to thrombin determined by SPR in multi- and single-cycle kinetics analyzed using the Langmuir 1:1 kinetic model (A) and a steady state model (B). *Thrombin concentrations ranging from 1.95 nM to 31.25 nM (serial 2-fold dilutions) were used.*

For the binding of HD1 to human thrombin, the following parameters were obtained in multi-cycle kinetics: k_a of 9.03 10^{11} $M^{-1}s^{-1}$, k_d of 4.3 10^3 s^{-1} resulting in the K_D of 4.80x10^{-9} M (4.8 nM) (Figure 2 A). However, since dissociation of thrombin was very rapid resulting in the k_d that was beyond the detection limit of Biacore X100 (0.1 s^{-1}), we decided to analyze the binding of HD1 to thrombin using a steady state model to obtain an accurate K_D, which was estimated for 7.94 x 10^{-9} M (7.94 nM) (Figure 2 B).

KD for HD1 was calculated as 5.91 nM and for HD22 as 0.53 nM, with response amplitudes of 15.5 for HD1 and 19.3 for HD22 (Figure 3 and 4).

Importantly, the results provided by SPR and MST are comparable. Therefore, both the techniques can be successfully applied for affinity measurement of these DNA aptamers

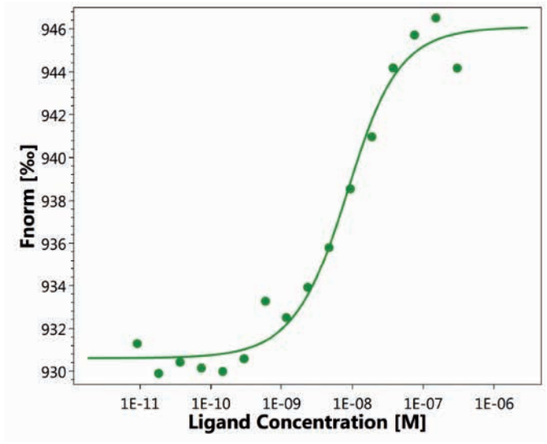

Figure 3. Binding of thrombin to HD1-Cy5 on MST. *The fluorescently labeled aptamer HD1 was used at the constant concentration with varying concentrations of thrombin.*

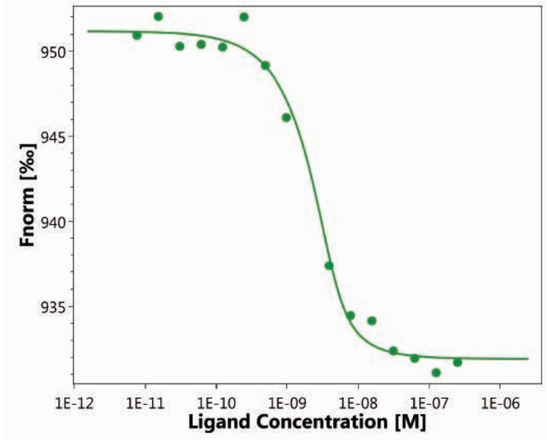

Figure 4. Binding of thrombin to HD22-Cy5 on MST. *The fluorescently labeled aptamer HD22 was used at the constant concentration with varying concentrations of thrombin.*

MATERIAL AND METHODS

SPR ANALYSIS

ASSAY CONDITIONS

Prior to the SPR experiments, biotinylated aptamers were diluted to a concentration of 1 nM in HBS-P buffer pH 7.4 (GE Healthcare) containing 5 mM Mg^{2+}, boiled for 10 min at 95°C and cooled on ice for 10 min. 300 resonance units (RU) of biotinylated HD1 and HD22 were immobilized on the surface of SA chip in flow cell 2 (GE Healthcare) as per the manufacturer's protocol. The negative biotinylated aptamer was immobilized on the same chip in flow cell 1 using the same conditions as for the positive aptamers. Binding kinetics assay was performed in HBS-P+ buffer pH 7.4 (GE Healthcare) containing 5 mM Mg^{2+}. Two pulses of 2 M KCl were used for chip regeneration in multi-cycle kinetic experiments. The binding data were fitted to the Langmuir 1:1 binding model using Biacore X100 Evaluation software.

INSTRUMENTATION

The SPR experiments were performed using Biacore X100.

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MST ANALYSIS

ASSAY CONDITIONS

Assays were performed using MST buffer. HD1-Cy5 was used at the concentration of 5 nM and HD22-Cy5 was used at the concentration of 4 nM with varying concentrations of thrombin. After 20 minutes of incubation, samples were loaded into Premium Coated MST capillaries and capillary scan was performed, followed by MST analysis at medium MST power and autodetect (60% for HD1 and 100% for HD22) LED power.

INSTRUMENTATION

The MST experiments were performed at Nanotemper Monolith NT.115 instrument with MO.Control v.1.5.2. software

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