APPLICATION NOTE

Regeneration of His-tagged Streptavidin on OpenSPR® NTA Sensors

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Summary

Capture coupling techniques are advantageous to investigate biomolecular interactions as they provide specificity for immobilization on the OpenSPR real-time, label-free biosensor platform. Streptavidin sensors offer a streptavidin coated sensor surface which can capture biotin-tagged ligands with high affinity. However, the biotin-streptavidin interaction is irreversible, limiting researchers to one biotinylated ligand per sensor. In this application note, Eliza Hanson, a member of Rebecca Whelan's research team at the University of Kansas explored the use of His-streptavidin on OpenSPR NTA sensors to create a regeneratable surface and measure binding interactions for multiple biotinylated aptamer ligands on a single sensor.

Introduction

Thrombin

Thrombin is a serine protease integral to the clotting process in humans. Soluble fibrinogen is converted into insoluble fibrin during the clotting process as coordinated by thrombin. As such, it is an important target for therapeutics related to clotting disorders. One such tactic uses anti-thrombin aptamers that can inhibit thrombin from interacting with its binding partners and thus act as anticoagulants and reduce platelet aggregation.[1]

Aptamers

Aptamers are molecules composed of short sequences of DNA engineered to bind a specific target. Aptamers are isolated and amplified through a process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX).[2] As increasing selective pressure is applied, high affinity oligonucleotides are isolated from a pool of ~10^{15} randomized oligonucleotides. Two single-stranded DNA (ssDNA) aptamers have been found to bind thrombin, a 15mer (5'-GGT TGG TGT GGT TGG-3') and 29mer (5' -AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3'). These aptamers and thrombin are one of the most widely used model aptamer-protein binding systems given their high affinity and specificity.[3] These aptamers target separate positively charged exosites on thrombin. The 15mer binds the fibrinogen-binding sites through electrostatic interactions. In contrast, the 29mer targets the heparin-binding site of thrombin through hydrophobic interactions. Previous studies have suggested the 29mer has slightly higher affinity for thrombin than the 15mer.[4]

Biotinylated ligand capture for SPR

Biotinylated ligands captured via immobilized streptavidin on biosensors provide a robust surface for measuring interactions on OpenSPR biotin-streptavidin sensors. This system provides some advantages, notably:

1. The ability to immobilize ligands, which amine couple poorly due to strong negative charges (ex. DNA and RNA).
2. Site-directed biotin-tagged ligands will result in specific orientation of the protein being achieved on the surface.

The biotin-streptavidin bond is extremely strong, with an affinity on the order of 10^{-14} M.[5] While this makes a highly stable surface on which to test biomolecular interactions, it also makes this system extremely difficult to regenerate. This limits a researcher's ability to screen multiple biotinylated-ligands on the same sensor.

As an alternative, OpenSPR NTA sensors provide a convenient capture coupling surface on which the ligand of interest can be removed and recaptured. OpenSPR NTA sensors capture ligands via a His-tag, which is typically

used for protein purification using Ni^{2+}-NTA columns. $NiCl_2$ coordinates with nitriloacetic acid (NTA) groups on the sensor surface, allowing one to capture the His-tag of the protein of interest. We have previously demonstrated His-streptavidin can be successfully recaptured with 2.9% variability with over 27 cycles on the same sensor. The use of His-streptavidin as a capture surface on a NTA sensor allows screening of multiple biotinylated ligands on a single sensor.

This study investigates the creation of a regeneratable His-streptavidin capture coupling surface for the study of biotinylated ligands on an OpenSPR NTA sensor. A model aptamer-protein system (29mer and thrombin) was employed to evaluate the viability and reproducibility of this capture method.

Materials & Equipment

- Nicoya OpenSPR-XT benchtop instrument
- NTA Sensors (Nicoya, SEN-AU-100-10-NTA)
- NTA Reagent Kit (Nicoya, NTA-RK)
- Running Buffer 1X PBS-T pH 7.4 (Nicoya, PBS-T)
- 2 M NaCl
- Ligands, analytes and capture molecules:
  - His-tagged streptavidin (MyBioSource, Cat: MBS203163) and
  - Biotinylated Anti-Thrombin 29-mer (Integrated DNA Technologies),
  - Thrombin (Millipore Sigma, Cat: T6884-1KU)
- 96-well plates (Nicoya, WP-10)
- Well plate covers (Nicoya, WPF-10)
- Various pipettes and associated tips

Method

Instrumentation

OpenSPR is a powerful instrument providing in-depth label-free binding kinetics for a variety of different molecular interactions. The determination of kinetic binding constants for antibody-antigen interactions is critical in many research and development applications. These are often high-affinity interactions which can make them challenging for analysis.

Method

A preliminary binding assay was completed using one standard NTA sensor on an OpenSPR-XT instrument. A second test was completed using a new standard NTA Sensor on an OpenSPR-XT instrument to evaluate the reproducibility of the capture surface. This test was performed over the course of two days for a total of three replicates per day. For each test, 200 µL of reagents and samples were loaded into 96-well plates and the test procedure was set up in the OpenSPR-XT software, after which all injections were automated. NTA Sensors were prepared and run according to Nicoya's NTA Sensor Tech Guide. Below outlines the procedures followed for all tests:

1. Sensor conditioning: NTA Sensor surfaces were conditioned with two injections of 10 mM HCl (150 µL/min), followed by one injection of 350 mM EDTA (100 µL/min).
2. Surface activation: An injection of $NiCl_2$ (40 µL/min) was performed to activate the NTA surface.
3. Capture Surface Preparation:
   a. His-streptavidin (ligand) was diluted into PBS-T at a concentration of 1 µM and injected at 20 µL/min in both channels.
   b. Biotinylated 29-mer was diluted to 1 µM in PBS-T and injected into channel 2 only at 20 µL/min.
4. Analyte Injections:
   a. Thrombin at concentrations of 5, 10, 25, 50, 75, 100, 200, and 300 nM was injected into the instrument at 20 µL/min in both channels. Each concentration was repeated in triplicate.
   b. 2 M NaCl was used to regenerate the 29-mer surface without stripping the His-Streptavidin or captured 29-mer from the NTA sensor surface between each Thrombin injection (150 µL/min; both channels).
5. Surface conditioning and surface activation (Steps 1-2) were repeated prior to leaving the sensor in stand-by with running buffer overnight to remove bound His-streptavidin.
6. For the reproducibility assessment, the instrument was put into Standby and steps 1-5 were repeated on day two.

Results & Discussion

This study evaluated the application and reproducibility of His-streptavidin on OpenSPR NTA sensors to enable regeneratable capture of biotinylated ligands. His-streptavidin captured on an OpenSPR NTA sensor was used to successfully bind an anti-Thrombin aptamer (29-mer) (Figure 1). 322 RU of 29-mer was successfully immobilized on the sensor.

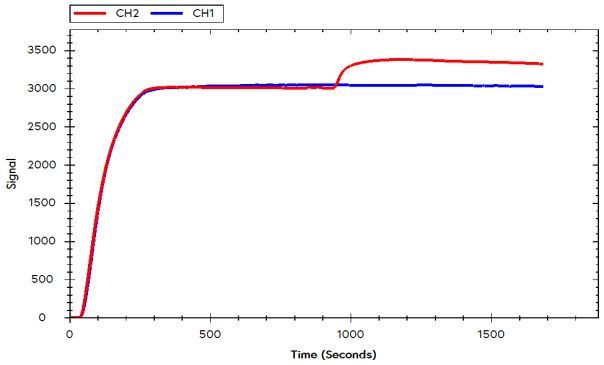

Figure 1: *Immobilization of Biotinylated 29-mer on a His-streptavidin coated NTA sensor. 3037 and 3011 RU of His-streptavidin were immobilized on Channels 1 (blue) and 2 (red) respectively. 322 RU of 29-mer was immobilized on Channel 2.*

The analyte response was measured against the 29-mer ligand using Thrombin. Specific binding could be observed for all concentrations tested and there was no significant contribution from non-specific binding to the reference channel (Figure 3). Since the interaction between 29mer and thrombin is hydrophobic in nature, 2 M NaCl removedthe bound thrombin without affecting the His-streptavidin or 29mer bound to the sensor. In cases where more acidic conditions are needed for regeneration, the capture surface and the ligand can be bound to the sensor again prior to the next analyte injection.

Binding assays were completed on the same NTA sensor over the course of two days to evaluate the reproducibility of the regenerated His-streptavidin surface. Stripping the His-streptavidin from the surface of the NTA sensor did not affect the amount of ligand captured after re-functionalization of the sensor with His-streptavidin. Captured ligand was 260 RU on Day 1 and 244 RU on Day 2 (Figure 4). This demonstrates that the regenerated His-streptavidin surface can produce similar immobilization over the course of a long experiment. Moreover, analyte injections at the same concentrations were similar to each other on day 1 and day 2 (Figure 5, Table 1).

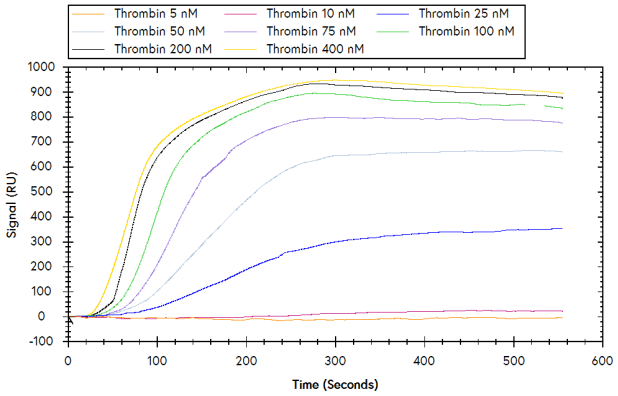

Figure 2: *Thrombin (analyte) binding to immobilized 29-mer (ligand) on OpenSPR. Analyte was injected at concentrations of 5, 10, 25, 50, 75, 100, 200, and 300 nM. The surface was regenerated with 2M NaCl between each thrombin injection. Curves were double reference subtracted to obtain the resultant corrected curves.*

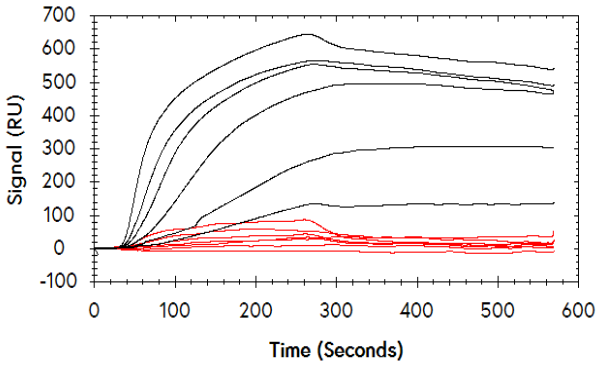

Figure 3: *Comparison of Thrombin binding on channel 1 (red) and channel 2 (black). Thrombin (analyte) binding to immobilized 29-mer (ligand) on OpenSPR. Analyte was injected at concentrations of 5, 10, 25, 50, 75, 100, 200, and 300 nM. The surface was regenerated with 2M NaCl between each thrombin injection. Specific binding in the response channel is shown in black and non-specific binding in the reference channel is shown in red.*

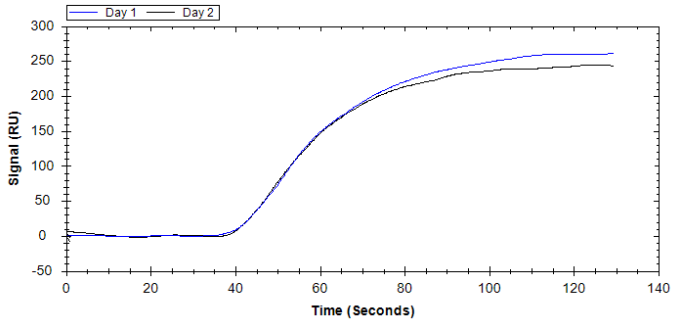

Figure 4: *Capture of Biotinylated 29-mer on His-streptavidin coated NTA sensor which was stripped after day 1 and subsequently re-functionalized with His-streptavidin prior to immobilization on day 2. Captured ligand was 260 RU on Day 1 and 244 RU on Day 2.*

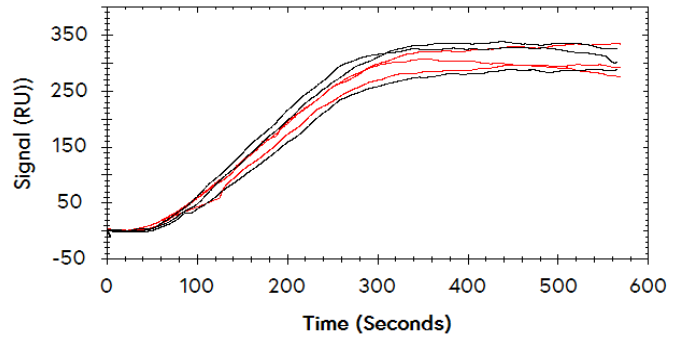

Figure 5: *Comparison of the binding response of 10 nM thrombin to immobilized 29-mer on Day 1 (black) and Day 2 (red).*

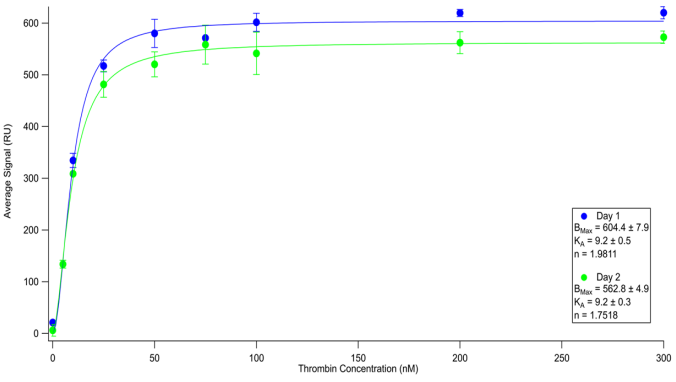

Figure 6: *Average response per day at the end of the analyte dissociation step plotted with respect to the thrombin concentration. Data were fit according to the Hill Equation in IGOR.*

The kinetic measurements were comparable for both days. The average signal at the end of the analyte dissociation was plotted with respect to the thrombin concentration and fit according to the Hill Equation in IGOR (Figure 6). The aptamer-thrombin binding interaction is most precisely fit according to a 1:2 protein:aptamer model which is best modeled by the Hill Equation.6 The positive cooperative binding interaction can be seen in the sigmoid shape of the curve and the value of $n > 1$ per the Hill Equation. The resultant kinetic coefficients were similar for day 1 and day 2 with KA's of 9.2 ± 0.5 nM and 9.2 ± 0.3 nM, respectively. The value of the n coefficient was 1.98 for day 1 and 1.75 for day 2, both of which indicate positive binding cooperativity.

Table 1: *Thrombin (analyte) binding to immobilized 29-mer (ligand) on OpenSPR. Analyte response was measured in triplicate per day.*

Day	Thrombin Concentration (nM)	Average Response (RU)	Standard Deviation (RU)
1	10	314.72	24.72
	25	500.74	19.10
	100	583.23	26.61
	300	601.82	20.39
2	10	305.11	23.47
	25	477.79	42.00
	100	539.48	65.96
	300	570.50	36.60

Conclusion

This study demonstrated that His-streptavidin on NTA sensors can be used to produce a regeneratable surface on which to study biotinylated-ligands. Consistent ligand re-capture was observed on the same sensor over the course of a two day experiment. Analyte responses were similar and resulted in robust kinetic data on OpenSPR.

References

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