APPLICATION NOTE

Binding Kinetics of the GLP-1R Agonist Semaglutide: Practical Methods for Studying Peptide Therapeutics with SPR

Overview

Glucagon-like peptide-1 receptor (GLP-1R) agonists are a rapidly expanding class of therapeutics for the treatment of various metabolic diseases. Characterization of their binding kinetics is essential for understanding mechanism of action, optimizing receptor engagement, and differentiating candidate molecules early in development.

When analyzing peptides using label-free biosensors, the complexity involved in assay development requires a high level of expertise and places hurdles in accessing high-quality data. Optimal assay methods may be challenging and time-consuming to develop. Key challenges include non-specific binding, maintaining the peptide's stability and activity, the difficulty in detecting small molecules/peptides, and challenges with site-specific immobilization.

In this application note, we demonstrate how Nicoya's Digital Surface Plasmon Resonance (SPR) instruments offer a powerful platform for measuring real-time binding interactions of peptides. This application note outlines impactful experimental strategies for measuring kinetics and binding affinity of the GLP-1R agonist semaglutide with Digital SPR. Various assay formats and capture surfaces will be discussed, including best practices for assay optimization and data analysis.

Introduction

GLP-1R agonists

GLP-1 receptor (GLP-1R) agonists are widely used to treat metabolic disorders such as type 2 diabetes and obesity. These therapeutics are typically peptide analogs of human glucagon-like peptide-1 (GLP-1) that activate GLP-1R, stimulating insulin secretion, delaying gastric emptying, suppressing glucagon, and reducing appetite via hypothalamic signaling (Figure 1).[1] These effects have driven their recent popularity as weight-loss drugs.[2]

Beyond metabolic disease, GLP-1R agonists have shown potential in treating musculoskeletal inflammation,[3,4] cardiovascular disease,[5] non-alcoholic fatty liver disease,[6] neurodegenerative disorders,[7] and certain cancers.[8-11]

Although GLP-1R agonists target the same receptor, they differ in kinetics, signaling bias, tissue distribution, and half-life, resulting in variable efficacy, glycemic control, and dosing frequency.[12] Semaglutide, marketed as Ozempic, Rybelsus, and Wegovy, is the most well-known example. Despite its small size (~4 kDa), semaglutide exhibits complex binding behavior, making kinetic characterization essential for understanding its potency, stability, clearance, and mechanism of action. Its extended half-life is achieved through fatty-acid acylation, which promotes binding to human serum albumin (HSA) and resistance to enzymatic degradation.

While GLP-1R binding is critical for potency, semaglutide's interaction with HSA also influences bioavailability and *in vivo* residence time. These interactions can be characterized using SPR to identify candidates with optimal properties. Accordingly, this application note examines the kinetics of semaglutide binding to both GLP-1R and HSA.

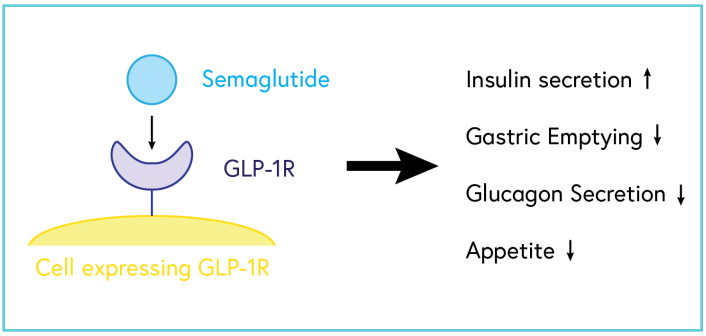

Figure 1. *Schematic representation of the mode of action and metabolic effects of GLP-1R agonists*

Characterizing binding of a GLP-1 receptor agonist to the GLP-1 receptor

Characterizing the binding kinetics of semaglutide to GLP-1R is essential for understanding its therapeutic function. Peptide therapeutics often exhibit complex conformational dynamics and can interact with their target receptor through diverse binding mechanisms. SPR provides an ideal platform for dissecting these kinetic behaviors, allowing researchers to measure the association rate constant (k_a) and dissociation rate constant (k_d) in addition to binding affinity. The parameters reveal how quickly a peptide binds to or dissociates from its target, which is critical in modulating the efficacy of a therapeutic.

Typical SPR assay design and development must consider some assay complexities. Potential interference from non-specific binding to the sensor surface, steric hindrance of peptides upon immobilization, low binding response of peptides in solution, difficulty in distinguishing subtle binding affinity differences, and mass transport limitations can all impact the accuracy and reliability of the binding data. Effective assay development and optimization can be time, labor, and reagent consuming. An assay platform that helps rapidly optimize assay conditions and obtain high data quality can significantly accelerate time to results.

Nicoya's Digital SPR products are the only instruments on the market integrating digital microfluidics (DMF) and nano-biosensors into an instrument that combines the industry-leading data quality of traditional SPR with the ease of use of BLI platforms. Digital SPR instruments offer the data quality standards needed to study the low signals generated by peptide analytes while requiring up to 200X less sample than traditional systems. Their discrete fluidic handling also make Digital SPR instruments uniquely suited to measuring the fast kinetics often seen with early peptide candidates by eliminating sample diffusion. Finally, with various throughput options, Digital SPR instruments offer users the flexibility they need to multiplex conditions, ligands, and analytes to rapidly run complete characterization of candidates.

Immobilization strategies for peptide kinetics

An important consideration for SPR experiments is the assay orientation, i.e., which of the semaglutide or GLP-1R will be immobilized. The optimal immobilization method depends on the size difference between the molecules, availability of primary amines on the peptide, and presence of affinity tags such as biotin on the peptide and/or receptor. This is especially important when working with peptides, which may have large size differences with their targets and are less likely than proteins to have residues or tags available for immobilization.

Direct kinetics with streptavidin is an assay format that can be used when analyzing biotinylated molecules. This format utilizes either the Digital SPR Streptavidin Kit (Figure 2a) or the Streptavidin Cartridge (Figure 2b). Streptavidin surfaces are extremely robust and have reliable, consistent, and directional capture of biotin-tagged molecules.

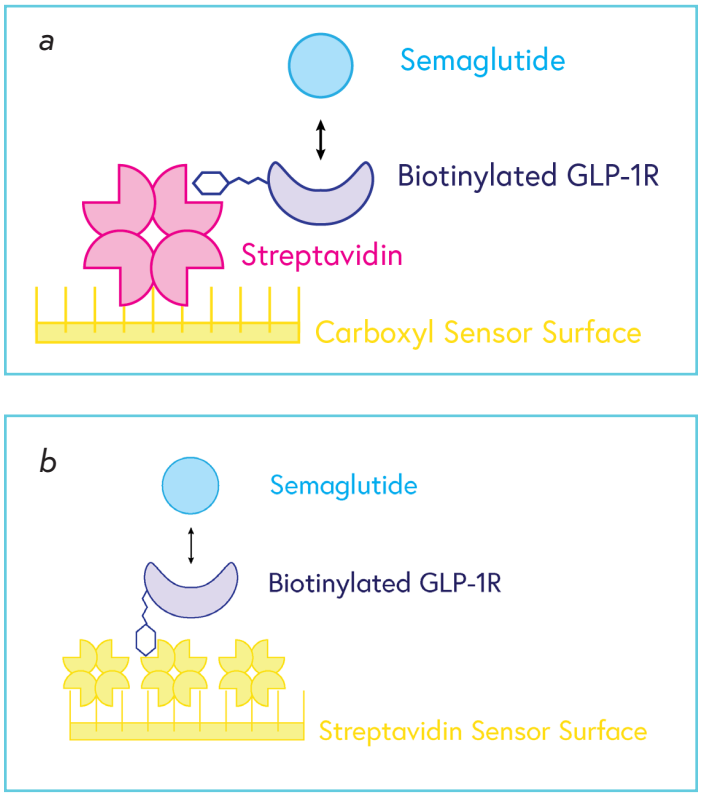

Figure 2. *Schematic representation of binding assay with biotinylated GLP-1R immobilized on streptavidin using (a) Streptavidin Kit and (b) Streptavidin Cartridge.*

Immobilizing biotinylated peptides is one of the best ways to analyze peptide-receptor interactions. It is advantageous to use peptides as the ligand where possible due to the larger SPR response that will be measured. Functionalizing a peptide with a small tag such as biotin also minimizes the likelihood of steric interference upon immobilization compared to larger affinity tags. The semaglutide used in this application note was not biotinylated so this strategy was not used. Besides biotin, Digital SPR is also compatible with a wide range of other affinity tags, including but not limited to: Fc, HIS, and Twin-Strep-tag.

Direct kinetics by amine coupling is a general assay format that can be used to analyze most binding pairs, provided there is at least one free primary amine group in the molecule being immobilized.

Direct kinetics is the go-to protocol for immobilizing ligands without affinity tags, although it can still be used when affinity tags are present. When affinity tags are present, it is generally preferred to use a capture protocol so that the molecule is oriented directionally.

In this study, HSA did not have any affinity tags, so it was immobilized directly to measure semaglutide binding (Figure 3). The SPR response is proportional to the size difference between the ligand and analyte, therefore it can be advantageous to immobilize peptides when possible. However, if there are no amine groups present or if the amine groups present are involved in receptor binding, it may not be possible to use this immobilization strategy. The HSA-semaglutide interaction measured here demonstrates that Digital SPR is easily able to measure kinetics for an analyte that is approximately 16x smaller than the ligand.

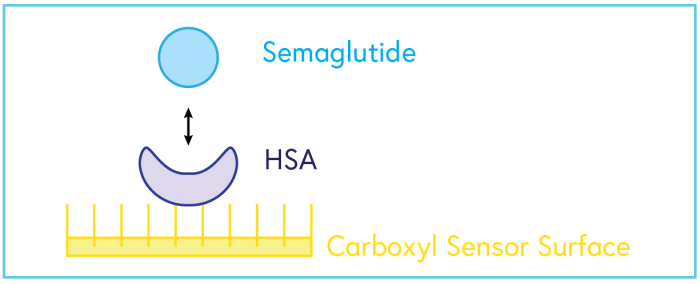

Figure 3. *Schematic representation of a binding assay with HSA amine coupled to a carboxyl sensor to measure semaglutide binding.*

Results & Discussion

In this study, Digital SPR was used to compare the binding kinetics of semaglutide against GLP-1R and HSA using direct amine coupling and a biotin-streptavidin capture method. The immobilization of ligands on the carboxyl and streptavidin surfaces was optimized to obtain a low ligand density while allowing for the detection of all analyte concentrations sampled. Full regeneration was achieved using Gly-HCl, pH 1.5.

GLP-1R kinetic values were calculated using a 1:1 Langmuir fit (Figures 4a-d) since semaglutide binding to GLP-1R has been shown to be a 1:1 interaction in previous studies.[13] HSA kinetic values were also calculated using a 1:1 Langmuir fit (Figure 4e). Previous studies indicate that the most favorable binding site on HSA is the FA3-FA4 region (referred to as FA3), with a 1:1 binding ratio for the high-affinity interaction.[14]

The kinetic parameters calculated for all assays are summarized in Table 1. The kinetic parameters measured are nearly identical across all assay methods as expected, with all K_D values falling in the range of 10.0-15.5 nM. This data confirms that the same kinetics should be measured whether using the Streptavidin Kit or Streptavidin Cartridge for biotinylated ligands. The kinetics measured using the Streptavidin Kit also demonstrate that SCK and MCK assay formats will generate equivalent kinetics.

The kinetic parameters calculated for semaglutide binding to HSA using direct immobilization are also summarized in Table 1, and indicate a K_D of 2.37 µM. This is over 100x lower affinity compared to the affinity of GLP-1R for semaglutide.

The affinities of these molecules differ due to the fundamentally different purposes of the binding. Semaglutide was specifically designed with a hydrophobic tail to have a modest but reversible affinity for HSA. Binding to HSA extends the half-life of semaglutide in the bloodstream, while leaving an unbound population available for GLP-1R binding.[14] Semaglutide is a GLP-1 receptor agonist, meaning it mimics the natural human GLP-1 hormone and directly binds to GLP-1R with high affinity, activating it.[1] Together, these two binding mechanisms allow for less frequent dosing and maximum drug efficacy. These results demonstrate that Digital SPR is a powerful tool for understanding binding mechanisms and enabling scientists to select and design drug candidates with optimal receptor binding properties.

Conclusions

SPR is a critical tool in the discovery and development of peptide therapeutics such as GLP-1R agonists. Digital SPR eliminates the common challenges of using SPR to provide a more cost-effective and efficient solution for biologics discovery and development. Here, we have demonstrated approaches to measure high-quality kinetics of semaglutide interactions with GLP-1R and HSA using popular assay methods on Digital SPR. The DMF platform offers great flexibility in assay design and requires only 2 µL of sample for full kinetics. Effective assay design, optimization of the immobilization method, ligand density, analyte concentration and regeneration solutions are critical to running successful assays that give the most reliable results possible. With a wide range of available assays, and consistent, reproducible data, Digital SPR is an essential platform to accelerate the development of peptide therapeutics.

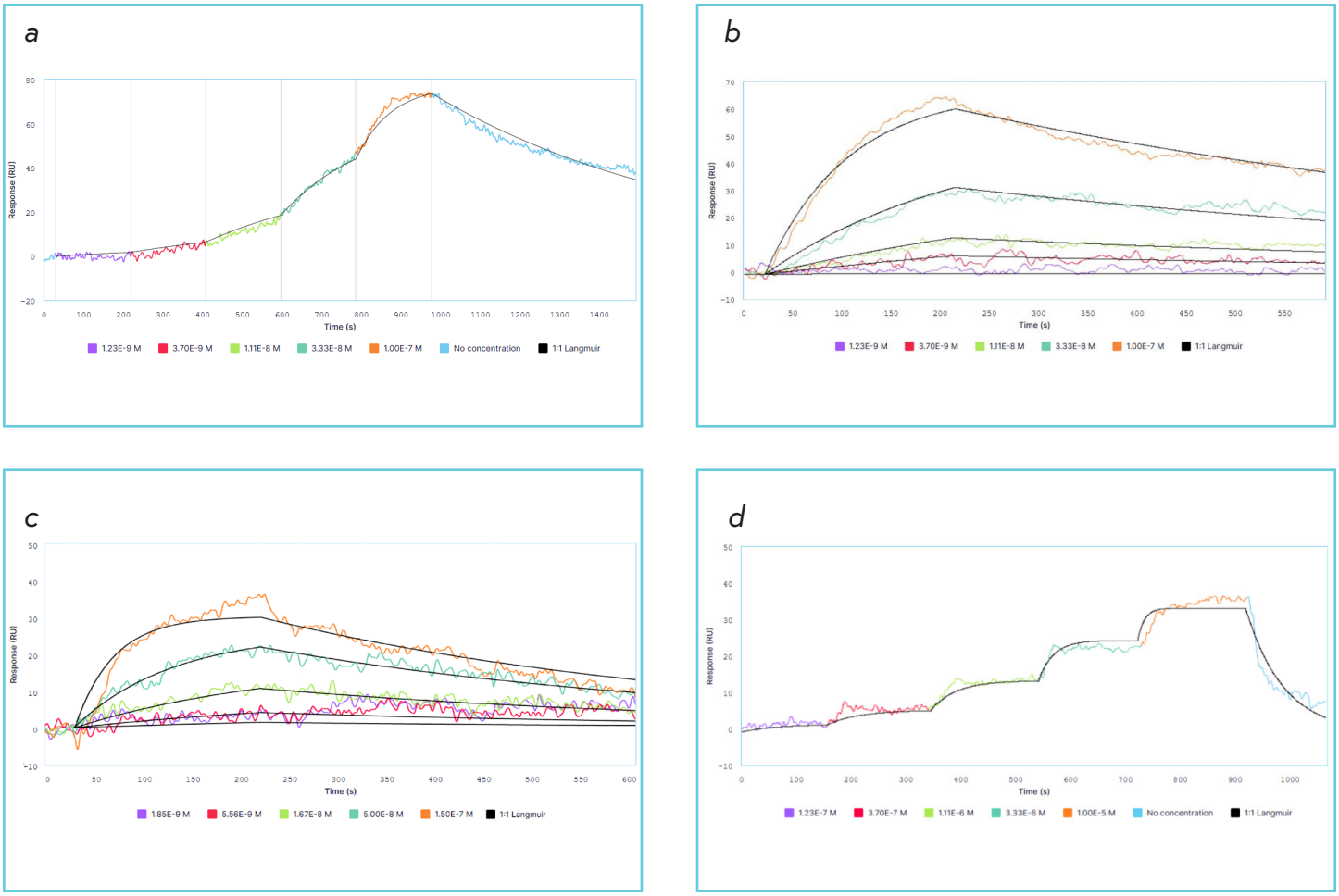

Figure 4. *Representative kinetic fits for (a) Semaglutide binding to GLP-1R immobilized using the Streptavidin Kit in SCK format, (b) semaglutide binding to GLP-1R immobilized using the Streptavidin Kit in MCK format, (c) semaglutide binding to GLP-1R immobilized on a Streptavdin Cartridge and (d) semaglutide binding to HSA immobilized on a carboxyl sensor. (a) & (d) were single-cycle kinetics (SCK) assays, and (b) & (c) were multi-cycle kinetics (MCK) assays. Analyte concentrations are shown in the figure legends. The black curve represents the Langmuir 1:1 binding fit.*

Surface	Assay	Ligand	k_a (M^{-1} s^{-1})	k_d (s^{-1})	K_D
Streptavidin Kit	SCK	GLP-1R	$1.71 \times 10^5 \pm 5.37 \times 10^4$	$1.89 \times 10^{-3} \pm 7.16 \times 10^{-4}$	11.6 ± 4.3 nM
Streptavidin Kit	MCK	GLP-1R	$1.17 \times 10^5 \pm 3.15 \times 10^4$	$1.89 \times 10^{-3} \pm 8.58 \times 10^{-4}$	15.5 ± 4.5 nM
Streptavidin Cartridge	MCK	GLP-1R	$1.52 \times 10^5 \pm 3.82 \times 10^4$	$1.58 \times 10^{-3} \pm 9.31 \times 10^{-4}$	10.0 ± 3.6 nM
Carboxyl	SCK	HSA	$6.41 \times 10^3 \pm 9.83 \times 10^2$	$1.50 \times 10^{-2} \pm 2.83 \times 10^{-4}$	2.37 ± 0.30 µM

Table 1. *Binding kinetics of semaglutide with GLP-1R and HSA using several immobilization strategies.*

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Appendix

Materials & Equipment

- Digital SPR 16-Channel Instrument with Nicosystem Pro Software (DSPR16-PRO)
- 16-Channel Carboxyl Cartridge (KC-CBX-CMD-16)
- 16-Channel Streptavidin Cartridge (KC-STV-CMD-16)
- Running Buffer: PBS-T (0.1% Tween 20), pH 7.4 (DSPR-PBST)
- Carboxyl Surfacing Kit: cleaning, normalization, activation (DSPR-CBX-SURF)
- Streptavidin Kit: (DSPR-STV-KIT)
- Immobilization Buffer: 10 mM Sodium Acetate Buffer, pH 4.0 (DSPR-IMB-4.0)
- Regeneration Solution: 10 mM Glycine-HCl, pH 1.5 (DSPR-GLYHCl-1.5)
- Semaglutide (Targetmol, Cat # T19850)
- GLP-1R (Sino, Cat # 13944-H49H-B)
- Human Serum Albumin (Sigma, Cat # A3782)

Experiment design

The experimental setup was completed remotely on the Digital SPR Nicosystem™ User Portal, followed by run initiation on the instrument:

1. From a laptop, the experiment was designed and saved in the Nicosystem.
2. On the instrument, the designed method was selected to launch the Digital SPR on-screen setup guide.
3. A Digital SPR 16-Channel Carboxyl Cartridge was placed in the instrument, and samples were loaded into the cartridge following the experiment setup guide.
4. The experiment was initiated on the Digital SPR by selecting "Run Method".

Assay protocols

Nicoya's eBook: Mastering kinetic binding assays walks through the assay development steps for determining the optimal experimental conditions for analyzing kinetics on Digital SPR platforms. This includes guidance on ligand loading density, analyte concentration, buffer conditions, and regeneration for obtaining accurate and reliable kinetics and affinities.

Direct Kinetics

1. Carboxyl sensors were normalized with normalization solutions.
2. Carboxyl sensors were primed with 10 mM HCl for 60 s.
3. Carboxyl sensors were activated with 200 mM EDC/NHS for 600 s.
4. 10 ug/mL Human serum albumin diluted in 10 mM Sodium Acetate, pH 4.0, was immobilized onto even sensors for 600 s.
5. All sensors were blocked with 1 M ethanolamine for 300 s to quench any remaining active carboxyl groups.
6. Functionalized carboxyl sensors were conditioned with 10 mM Gly-HCl, pH 1.5 for 60 s.
7. Digital SPR automatically executed three-fold dilutions from stock concentrations loaded. A semaglutide sample diluted from a 30 uM stock produced concentrations of 0.12 uM, 0.37 uM, 1.1 uM, 3.3 uM and 10 uM.
8. In SCK assays, the five concentrations were exposed to each sensor from low to high for 180 s, followed by a single dissociation in the running buffer for 600 s, and a single 60 s regeneration step with 10 mM glycine-HCl, pH 1.5.
9. In MCK assays, the lowest analyte concentration was exposed to each sensor for 180 s, followed by dissociation in the running buffer for 600 s, and a 60 s regeneration step with 10 mM glycine-HCl, pH 1.5.
10. Step 9 was repeated for the remaining four GLP-1R or HSA analyte concentrations, which constitutes a full multi-cycle kinetics (MCK) round.

Direct kinetics of semaglutide to GLP-1R with Streptavidin Kit

The following steps were completed automatically by Digital SPR with no operator supervision.

1. Carboxyl sensors were normalized with normalization solutions.
2. Carboxyl sensors were primed with 10 mM HCl for 60 s.
3. Carboxyl sensors were activated with 200mM EDC/NHS for 600 s.
4. The streptavidin from the Streptavidin Kit diluted in 10 mM Sodium Acetate, pH 5.0 was immobilized onto all sensors for 600 s.
5. All sensors were blocked with the 1 M ethanolamine for 300 s to quench any remaining active carboxyl groups.
6. Sensors were conditioned with 10 mM Gly-HCl, pH 1.5 for 60 s.
7. 10 µg/mL of biotinylated GLP-1R ligand in the running buffer was introduced to each even-numbered active sensor for 300 s.
8. Digital SPR executed five automated Semaglutide serial dilutions on the cartridge. A semaglutide sample diluted from a 300 nM stock produced 1.23 nM, 3.7 nM, 11.1 nM, 33.3 nM, and 100 nM solutions in the running buffer.
9. In SCK assays, the five concentrations were exposed to each sensor from low to high for 180 s, followed by a single dissociation in the running buffer for 600 s, and a single 60 s regeneration step with 10 mM glycine-HCl, pH 1.5.
10. In MCK assays, the lowest analyte concentration was exposed to each sensor for 180 s, followed by dissociation in the running buffer for 600 s, and a 60 s regeneration step with 10 mM glycine-HCl, pH 1.5.
11. Step 10 was repeated for the remaining four GLP-1R or HSA analyte concentrations, which constitutes a full multi-cycle kinetics (MCK) round.

Direct kinetics of semaglutide to GLP-1R with Streptavidin Cartridge

The test with a streptavidin cartridge followed steps 1, 6-8 & 10-11 from 'Direct kinetics of semaglutide to GLP-1R with Streptavidin Kit.'