Protein–Small Molecule
# Biomolecular Interactions
– a Retrospective

Newsletter IV 2020

Surface Plasmon Resonance (SPR) is an invaluable technique that generates information-rich data for a variety of biomolecular interactions. Researchers use SPR to understand biological pathways and to develop and characterize a range of potential therapeutics to treat disease. These interactions include those occurring with and between the major classes of biological macromolecules.

This article is focused on using SPR to study biomolecular interactions involving small molecules binding to proteins. Many researchers rely on SPR to provide key information about these interactions; the technique is widely used for the determination of kinetic and thermodynamic parameters. For the purposes of this article, "small molecules" generally refers to molecules with molecular weight ≤1,000 Da. There are certain standard approaches that researchers follow to carry out these types of experiment. As a higher surface density is needed when there is a large molecular weight difference between the protein (the target/ligand) and the small molecule (the analyte) whose interaction is being studied, direct coupling is usually the preferred means of attaching a target to the sensor chip.

XanTec bioanalytics offer a broad range of sensor chips with high immobilization capacities for small molecule screening. Depending on the actual size of the ligand and analyte, there are different thicknesses, densities and even different polymer types to choose from. Thus, the results of screening campaigns or kinetic/thermodynamic analyses can be optimized. Several high capacity coatings are also available as derivatives, such as with NeutrAvidin/ streptavidin, or (poly)NTA for His-tagged ligands.

Since it is common for small molecules to show limited solubility in the aqueous running buffers used for SPR studies (eg. PBS, HEPES), analytes soluble in DMSO are typically diluted with running buffer to about 1-5% DMSO prior to SPR analysis. In some cases, detergents may also be used to help with solubility issues. Many of these experiments are carried out to aid in the development of small molecules as potential therapeutics for disease. We now highlight some recent articles that researchers have published using Reichert's SPR systems for investigating low molecular weight molecules interactions with proteins.
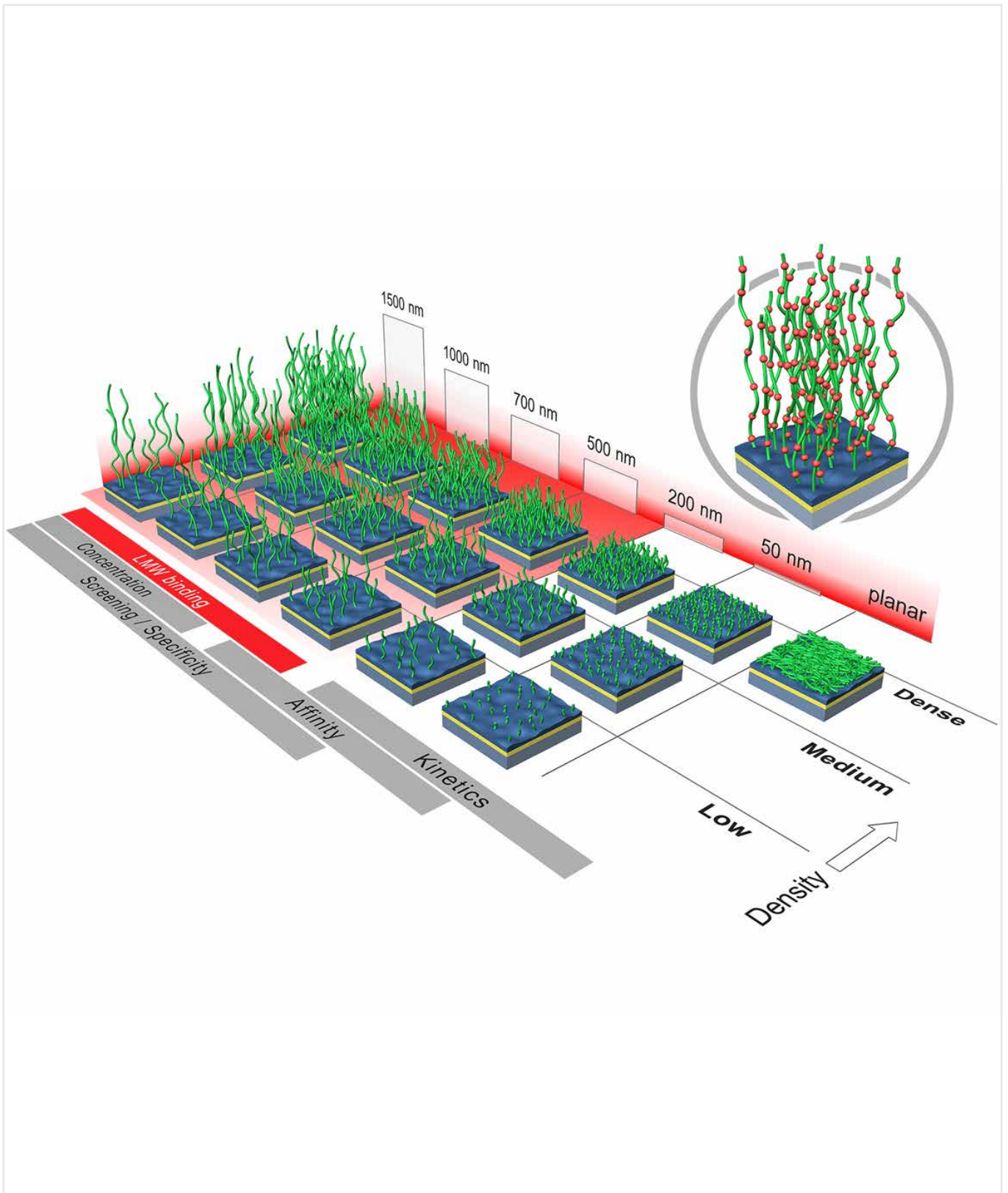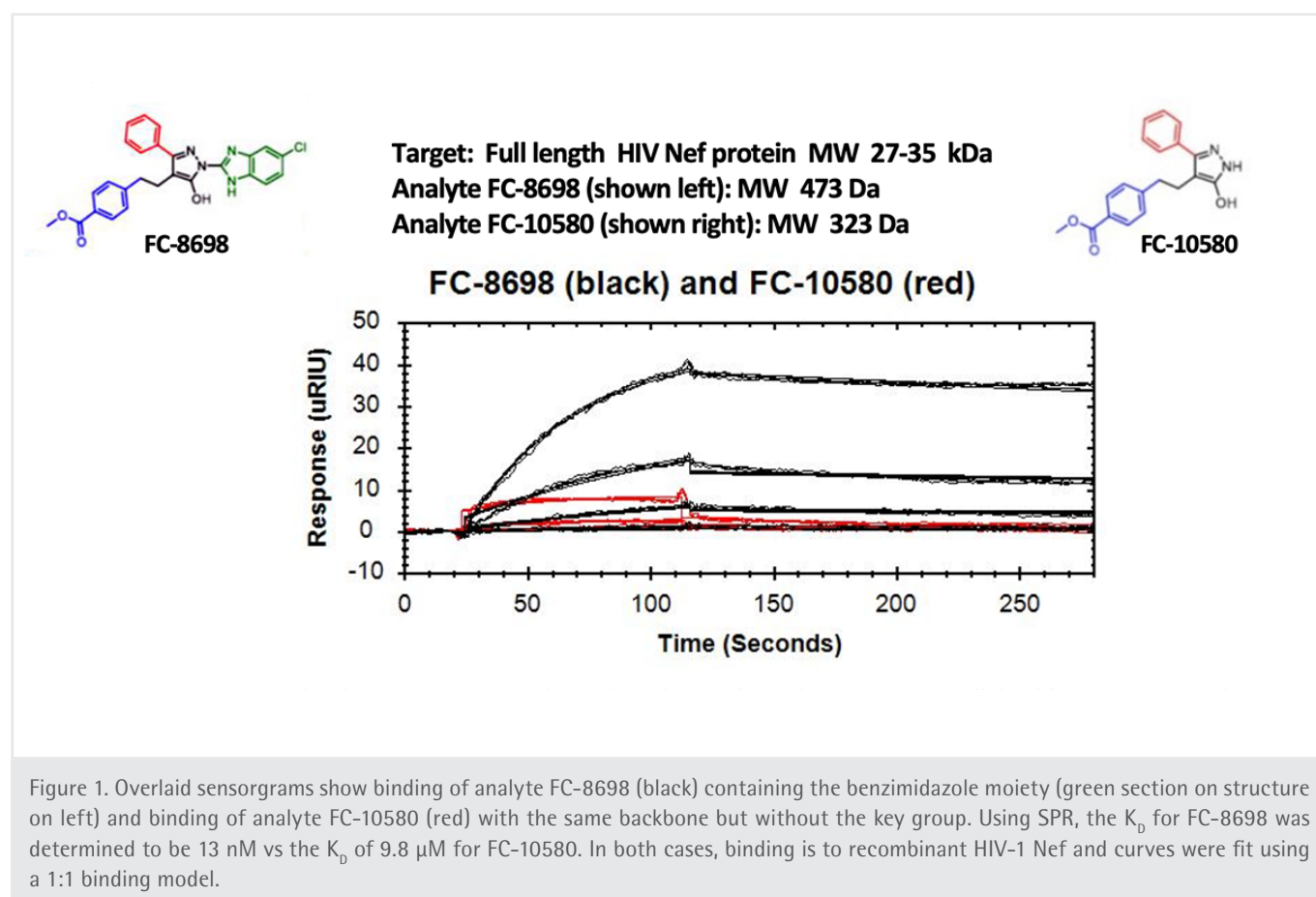
XanTec's sensor chip matrix: Different hydrogel thicknesses and densities allow a perfect fit to individual applications and to the molecular characteristics of the ligand and analyte.

Example 1: Target HIV – using a Reichert4SPR for screening

A 2019 article in ACS Infectious Diseases highlights how some of the unwanted functions of HIV Nef accessory protein, which plays a key role in HIV replication, can be blocked by small molecules[1].

For the results presented in this article, researchers developed analogs to an earlier small molecule that showed promise in testing. They had more than 200 analogs to analyze and ranked them based on binding results obtained using a Reichert4SPR system. Recombinant HIV-1 Nef was covalently bound to a dextran sensor chip as the target and binding interactions with the analogs were ranked in terms of affinity, residence time and binding response[1]. Of the 216 analogs screened, 45 exhibited no binding. The remainder of the responses were ranked based on the magnitude of the responses, their kinetics, including on- and off- rates, and activity from a separate assay[1].

SPR results for the two compounds shown below are representative of how the researchers determined that the benzimidazole moiety at position C is essential for Nef inhibitor action. As noted in the article, the percent inhibition of HIV-1 infectivity determined using the TZM-bl reporter cell assay goes down by nearly 90% if the benzimidazole moiety at position C (highlighted in green below) is not present on the scaffold. In addition, the affinity of the interaction decreases significantly from 13 nM to 9.8 µM[1] and the absolute magnitude of the responses decreases approximately fourfold (see overlays below). Based on these results, FC-8698 was ranked as one of the top overall binders of the compounds studied.[1]

Figure 1. Overlaid sensorgrams show binding of analyte FC-8698 (black) containing the benzimidazole moiety (green section on structure on left) and binding of analyte FC-10580 (red) with the same backbone but without the key group. Using SPR, the K_D for FC-8698 was determined to be 13 nM vs the K_D of 9.8 µM for FC-10580. In both cases, binding is to recombinant HIV-1 Nef and curves were fit using a 1:1 binding model.
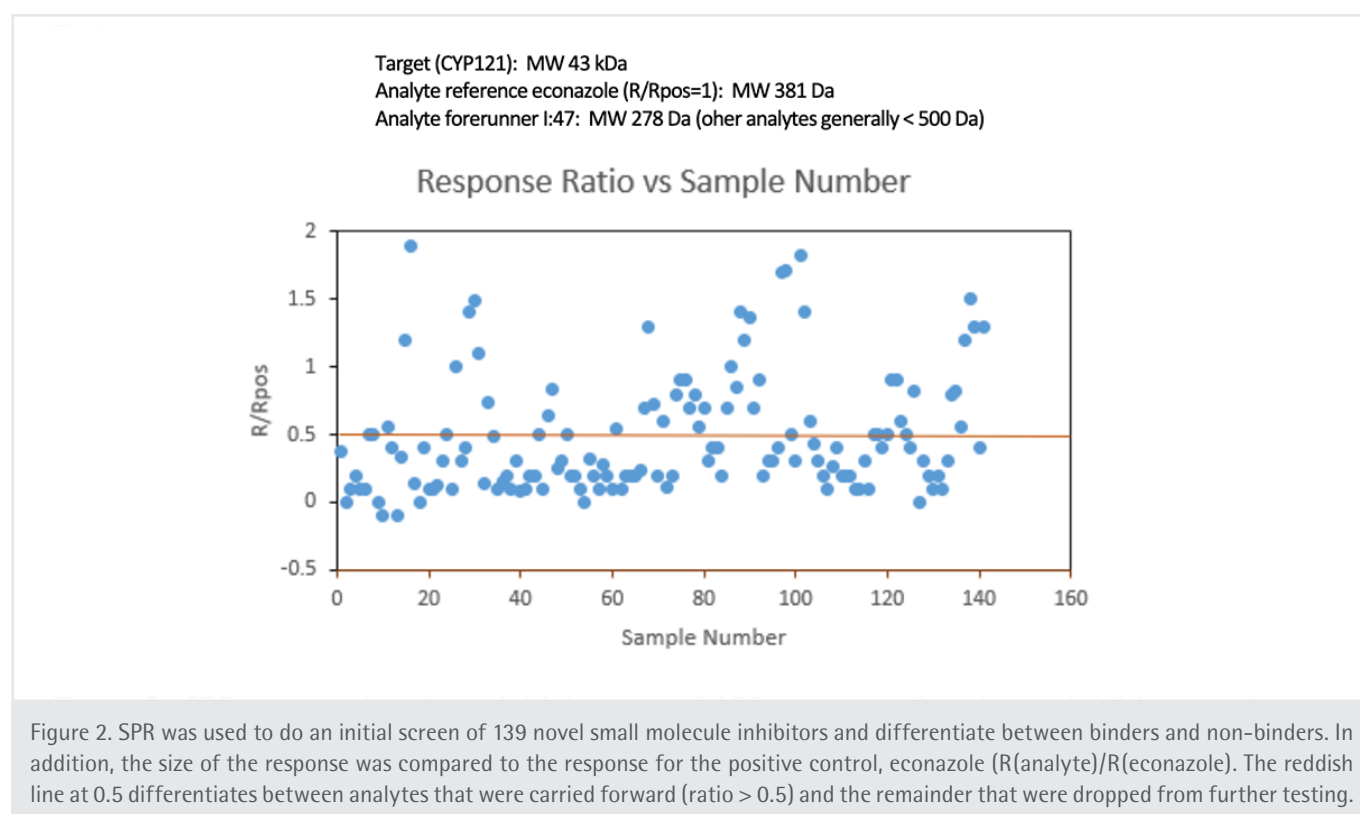
Example 2: Target Tuberculosis – using a two-channel Reichert SPR for initial screening

A 2017 article in *ChemMedChem* describes how an SR7500DC (2-channel Reichert SPR instrument) in combination with a high capacity streptavidin sensor chip from **XanTec bioanalytics** (SAD500M) was used for the screening of potential antimycobacterial agents against *Mycobacterium tuberculosis*[2].

In this article, researchers explain that there is an urgency to their work because multi-drug resistance and the need for long term treatment are making it difficult to treat this disease and, they note that there are millions of new cases of Mtb infections each year worldwide (as of the year 2017 when they wrote their article)[2]. Previous work showed that the enzyme CYP121 is essential for Mtb growth making it a potential target for Mtb treatment.[2]

Screening in this study was carried out based on biophysical and microbiological methods. The initial screen was carried out using Surface Plasmon Resonance (SPR) with a Reichert 2-channel instrument and involved capturing biotinylated CYP121 on a streptavidin chip as the target and then analyzing binding to the target of 139 novel small molecule antimycobacterial compounds across six classes. Researchers built up small molecule compounds on six different scaffolds to probe a wide range of structures. Of the compounds screened, 32% showed a response using SPR. Seventeen of the compounds had higher responses than econazole, the positive control.

When compared to the positive control econazole, forty-four of the compounds screened were found to be worthwhile for further testing (ie. to have responses R/R_{pos} of 0.5 or higher). Further testing with multiple techniques led the researchers to a promising target for further research, a compound they call I:47 which exhibited good antimycobacterial activity and bacterial selectivity with a favorable toxicity profile[2].

Target (CYP121): MW 43 kDa
Analyte reference econazole (R/Rpos=1): MW 381 Da
Analyte forerunner I:47: MW 278 Da (oher analytes generally < 500 Da)

Response Ratio vs Sample Number

Figure 2. SPR was used to do an initial screen of 139 novel small molecule inhibitors and differentiate between binders and non-binders. In addition, the size of the response was compared to the response for the positive control, econazole (R(analyte)/R(econazole)). The reddish line at 0.5 differentiates between analytes that were carried forward (ratio > 0.5) and the remainder that were dropped from further testing.

Example 3: Anti-Cancer Drug Candidates – comparison using a Reichert4SPR

A 2017 Article in PLOS 1 includes SPR data obtained using a Reichert4SPR for the binding of small molecule anti-cancer drugs to human serum albumin (HSA)[3]. In this study, researchers looked at two potential anti-cancer drug candidates, NSC48693 (which is hydrophilic) and NSC290956 (which is hydrophobic) and determined where they bind to HSA using fluorescence quenching and molecular modeling. For information regarding the kinetics and thermodynamics of the small molecule/HSA interactions, researchers used a Reichert4SPR.

HSA was amine coupled to a dextran sensor chip and then the two drug candidates were diluted and injected over the surface as analytes. Kinetic analysis resulted in the determination of the affinity for the HSA/NSC48693 interaction (K_D = 13.8 µM) to be almost 10 times higher than the affinity for the HSA/NSC290956 interaction (K_D = 116 µM).[3]

This information is expected to be particularly useful in later determination of the type of dosing that would be needed for clinical studies using each compound.
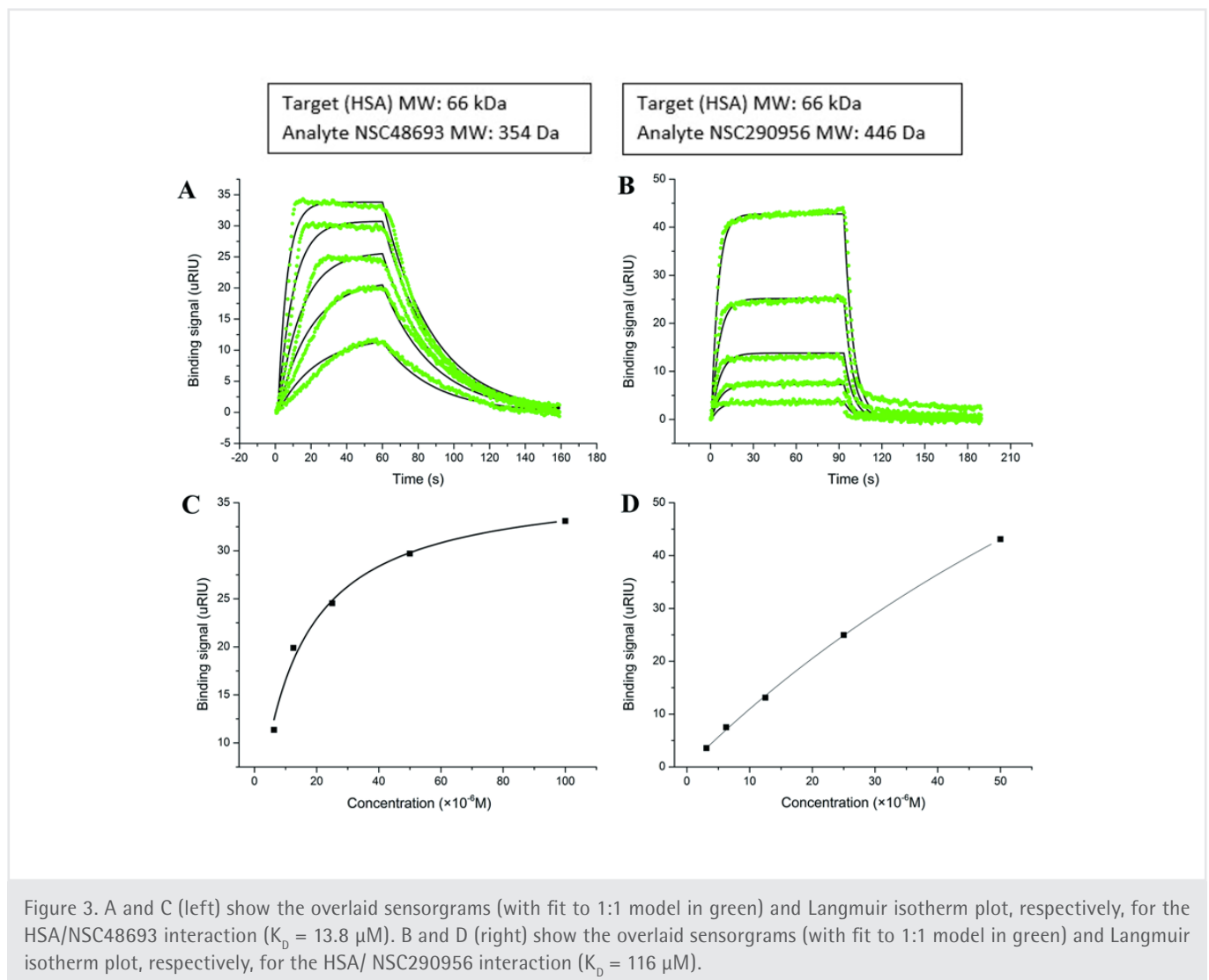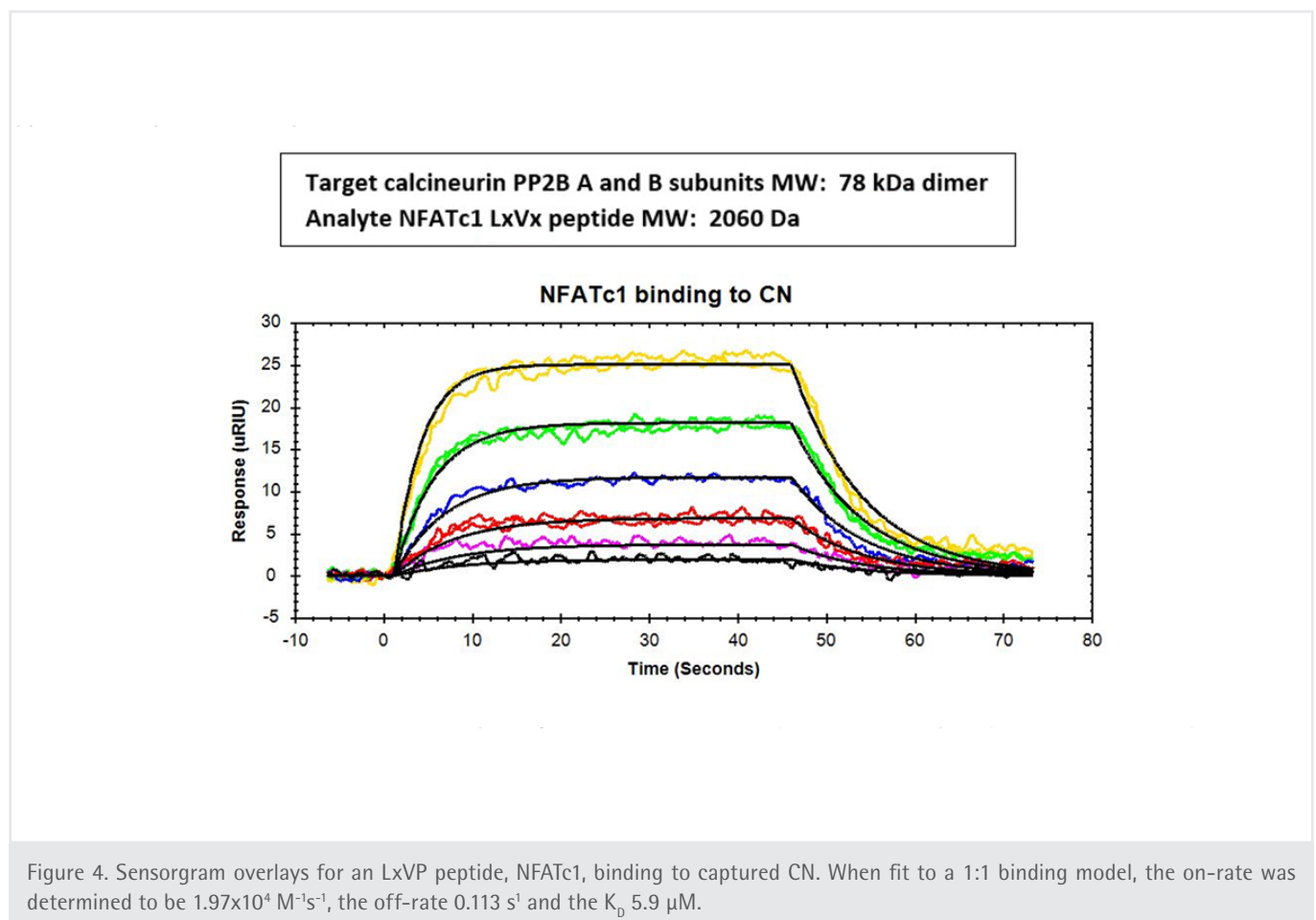

Figure 3. A and C (left) show the overlaid sensorgrams (with fit to 1:1 model in green) and Langmuir isotherm plot, respectively, for the HSA/NSC48693 interaction (K_D = 13.8 µM). B and D (right) show the overlaid sensorgrams (with fit to 1:1 model in green) and Langmuir isotherm plot, respectively, for the HSA/ NSC290956 interaction (K_D = 116 µM).

Example 4: The Binding of short linear motifs (SLiMs) to the Enzyme Calcineurin

In a 2019 paper in ACS Chemical Biology researchers present results from their research into two known short linear motifs (SLiMs), PxIxIT and LxVP, which bind to Calcineurin (CN), an enzyme involved in a number of biological functions including such things as development, heart function, and immune response[4]. Although most cellular signaling involves protein/protein interactions, some also occurs between proteins and short linear motifs which are about 3-10 amino acids in size and are typically located in intrinsically disordered regions. Using SPR to investigate these interactions is not trivial because the motifs are small and have low binding affinities.

For this project, researchers captured His-tagged CN on a NiNTA sensor chip and used a Reichert4SPR surface plasmon resonance (SPR) instrument to determine binding constants with peptides containing either PxIxIT or LxVP. Researchers employed Structure Based Shape Complementarity (SBSC) analysis which uses the shape of the preformed, rigid SLiM binding pocket to identify complementary amino acids for binding to expand the number of known SLiM sequences. Using this approach, they identified 119 CN regulators and substrates containing either PxIxIT or LxVP. Even though this type of interaction is usually weak, they were able to obtain kinetics. From SPR results, researchers concluded that LxVP SLiM binding to CN is on-rate-dependent[4].

Results for a typical compound they studied are shown here:

Figure 4. Sensorgram overlays for an LxVP peptide, NFATc1, binding to captured CN. When fit to a 1:1 binding model, the on-rate was determined to be 1.97x10^4 $M^{-1}s^{-1}$, the off-rate 0.113 s^1 and the K_D 5.9 μM.
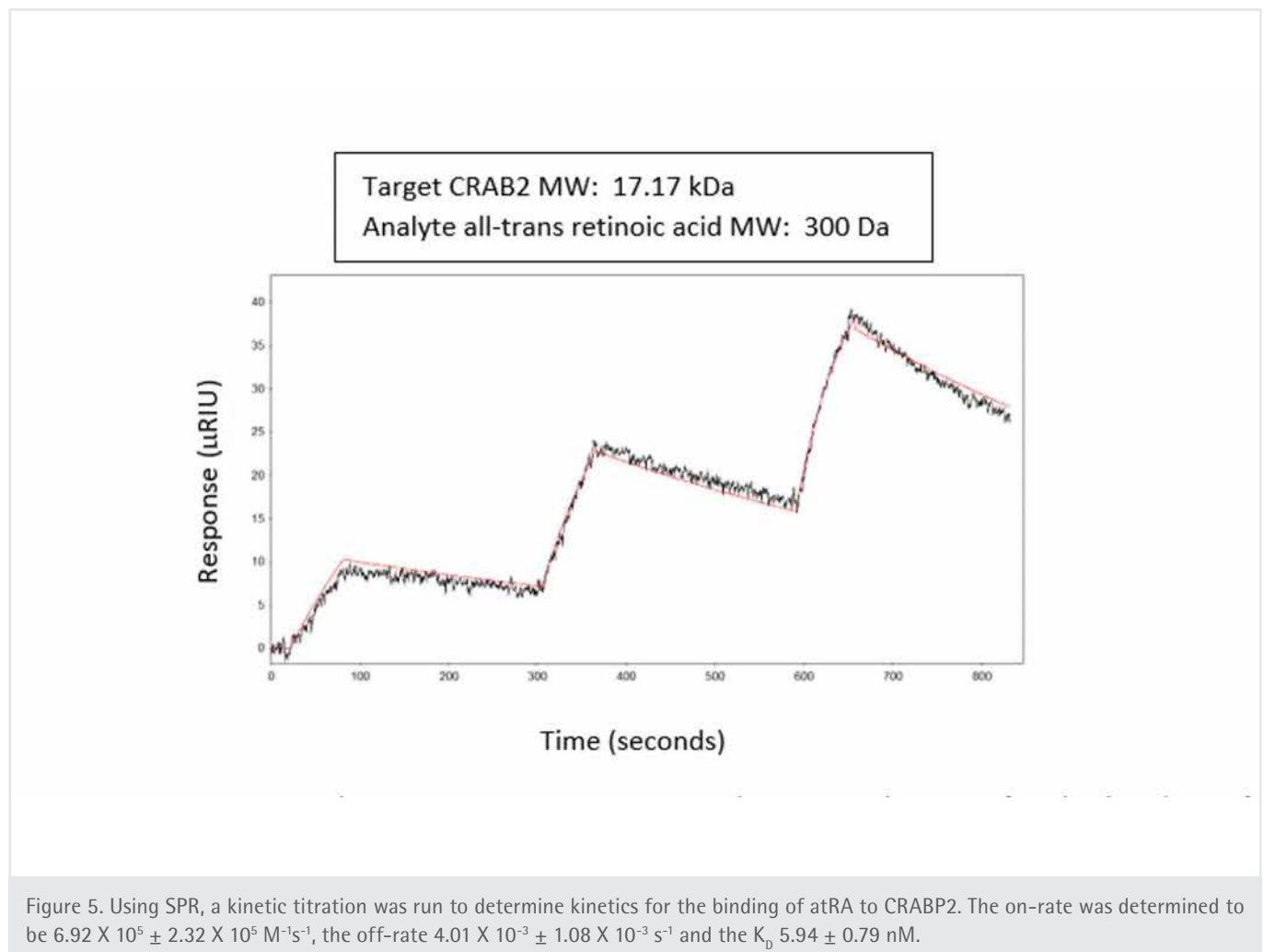
Example 5: Retinoic Acid – an important nutrient

In a 2019 article in Biochemistry, research on the binding of all trans Retinoic Acid (atRA), a metabolite of Vitamin A, to Cellular retinoic acid-binding protein 2 (CRABP2), an intracellular lipid- binding protein, is described[5]. CRABP2 delivers atRA to Retinoic Acid Receptors (RARs), which than leads to the activation of specific gene transcription. Using a Reichert4SPR, they captured His- tagged CRABP2 on a Ni-NTA sensor chip and injected the atRA over as analyte.[5] All-trans retinoic acid (atRA) is a hydrophobic molecule and CRABP2 controls its movement between the nucleus and cytoplasm.

They determined that the affinity for the CRAB2/atRNA interaction was in the nM range with a slow off rate. This information, combined with the hydrophobic nature of CRABP2, indicated to them that the atRA release from the CRABP2 binding pocket was probably mediated by external factors. They noted that the binding rate was determined by atRA's rate of diffusion out of detergent, which had to be added to the solution due to atRA's poor aqueous solubility[5].
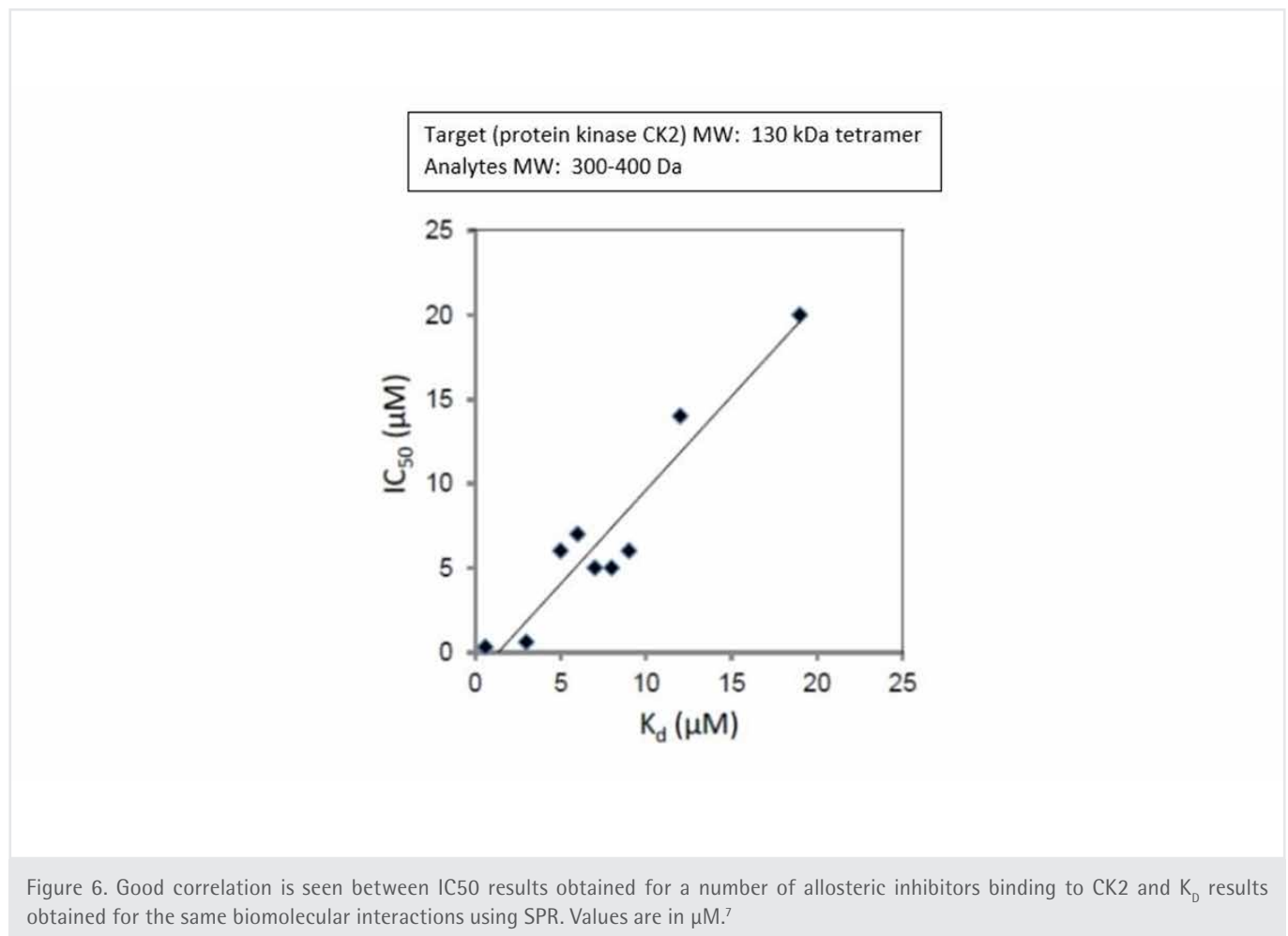
A typical result is shown here:

Target CRAB2 MW: 17.17 kDa
Analyte all-trans retinoic acid MW: 300 Da

Figure 5. Using SPR, a kinetic titration was run to determine kinetics for the binding of atRA to CRABP2. The on-rate was determined to be $6.92 \times 10^5 \pm 2.32 \times 10^5$ $M^{-1}s^{-1}$, the off-rate $4.01 \times 10^{-3} \pm 1.08 \times 10^{-3}$ s^{-1} and the K_D 5.94 ± 0.79 nM.

Example 6: Development of CK2 inhibitors for Cancer Treatment

In a 2019 article in the Journal of Medicinal Chemistry, researchers used the protein kinase CK2 as target and looked for potential inhibitors[6]. The protein kinase CK2 is known to play an important role in pro-oncogenic pathways that are critical to cells including proliferation, differentiation, and survival. Previous research involving development of inhibitors for the protein kinase CK2 were targeted toward the ATP binding pocket, but it was found that the compounds developed could also act on other targets in the body with ATP binding pockets which could then cause unwanted side effects. Researchers in this study previously determined there was a more specific, potentially druggable site on the protein CK2. In their current work they sought to prove it to be a viable option by developing 2-Aminothiazole Derivatives that they could test.
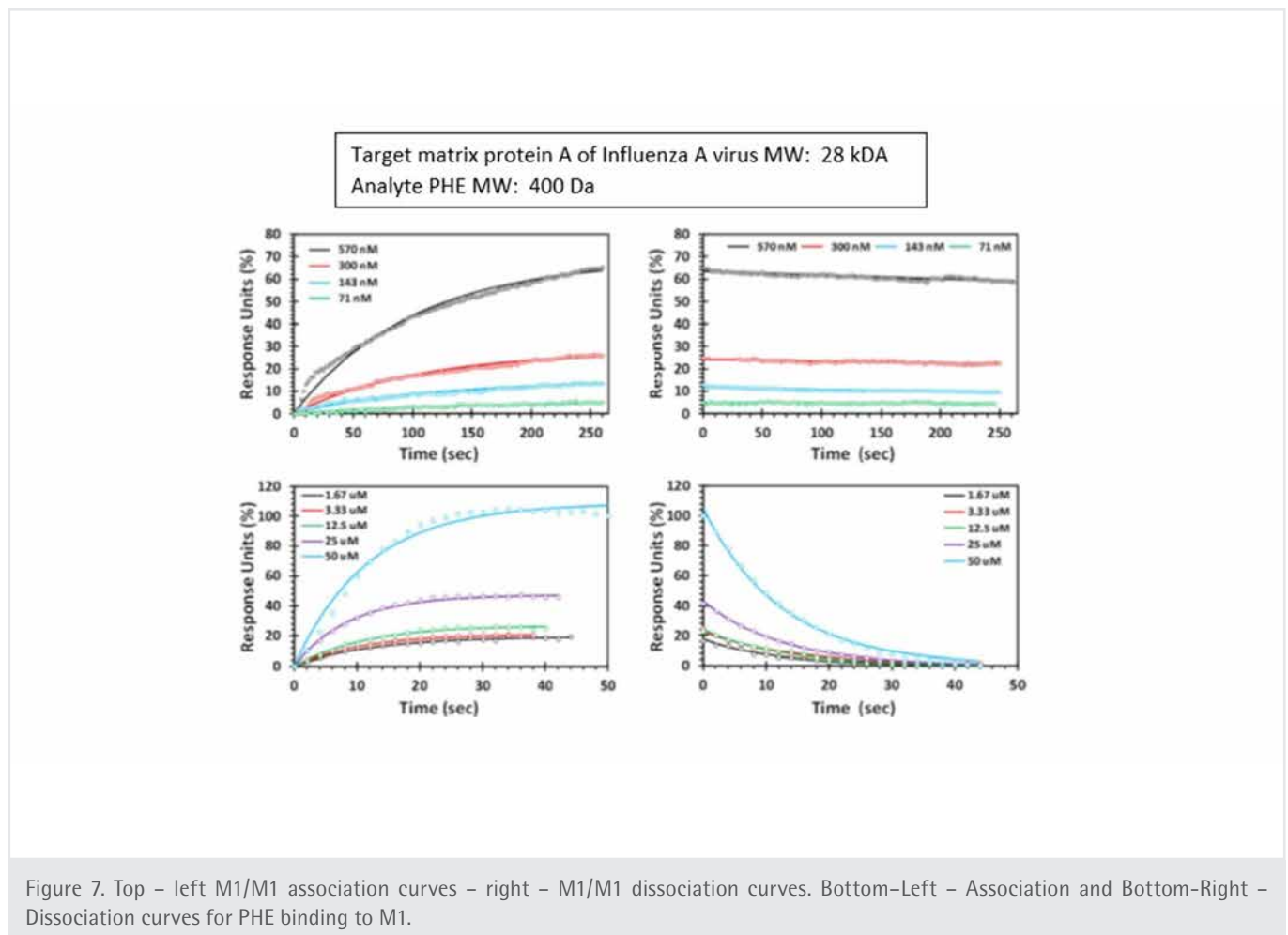
Researchers amine coupled Anti- GST to the surface of a dextran sensor chip and then captured GST-tagged CK2α over the surface as a target for the allosteric inhibitors they made[6]. Of the allosteric inhibitors they constructed and tested, they found one of the compounds, 2-hydroxy-4-((4-(naphthalen-2-yl)thiazol-2- yl)amino)benzoic acid to be the lead compound with a sub-micromolar IC50. Testing was carried out using a number of techniques including SPR[6]. Comparing IC50 (biochemical inhibition) results with SPR binding affinity constants obtained with a 2-channel Reichert SPR for a number of the inhibitors they tested, they found good agreement[7].

Target (protein kinase CK2) MW: 130 kDa tetramer
Analytes MW: 300-400 Da

Figure 6. Good correlation is seen between IC50 results obtained for a number of allosteric inhibitors binding to CK2 and K_D results obtained for the same biomolecular interactions using SPR. Values are in µM.[7]

Example 7: Potential Anti-Influenza Agent

In a 2016 paper in Scientific Reports, researchers describe how using a "wedge" could destabilize matrix protein 1 (M1) of influenza A virus (IAV) to and keep it from self-associating[8]. They started out testing about 70,000 potential compounds using virtual screening. Then, they took the top ten hits and did in vitro testing and found that six of the compounds showed antiviral activity. However, five of the six also exhibited substantial cytotoxicity. One small molecule which they call PHE only showed minimal cytotoxicity and became their top candidate to carry into further testing[8]. PHE is a hydrophobic compound with a molecular weight 400 Da and was found to be straightforward to synthesize in just a few steps so it was carried forward into further "proof of concept" testing. Researchers used a 2-channel Reichert SPR to determine kinetics for M1 to itself and M1 to PHE. Biotinylated M1 was captured over a neutravidin surface. M1 was then injected over at various concentrations and affinity was determined (Figure 7).

Next, they tested M1 binding to PHE and determined the affinity (Figure 7). The slow off rate for the M1/M1 interaction led researchers to conclude that self-association is an off-rate driven process[8]. The affinity decreases from about 50 pM for M1 to M1 binding to 870 nM for PHE binding to M1 which researchers stated is still fairly tight binding for a small molecule. Using molecular modeling, they found that PHE binds to multiple sites on M1 which they feel may give it a better chance of destabilizing M1. The method of inhibition is that the small molecule impedes M1 oligomerization.[8]

Figure 7. Top – left M1/M1 association curves – right – M1/M1 dissociation curves. Bottom–Left – Association and Bottom-Right – Dissociation curves for PHE binding to M1.

Example 8: Characterizing Inhibitors to Auxin Transport

Auxin is a plant growth regulator that has been widely studied. In a 2016 article in Plant Cell, researchers used a 2-channel Reichert SPR instrument to study binding interactions of several small molecule inhibitors with the protein Twisted Dwarf 1 (TWD1), which mediates the action of auxin transport inhibitors[9]. If a plant is grown on land, most of its development is dependenton the formation of auxin gradients called polar auxin transport or PAT. Synthetic auxin transport inhibitors, such as NPA (1-N-naphthylphthalamic acid), can inhibit the basal polar auxin flow required for plant development at low concentrations.

Using a 2-channel Reichert SPR, researchers immobilized TWD1 to the surface of a dextran chip using both amine and thiol coupling and flowed over small molecules at various concentrations. Using SPR, researchers determined that binding of small molecule inhibitors to the Twisted Dwarf protein was low affinity in vitro. This result was in agreement with NMR results[9].

SPR binding results for one of the small molecule inhibitors is shown here:
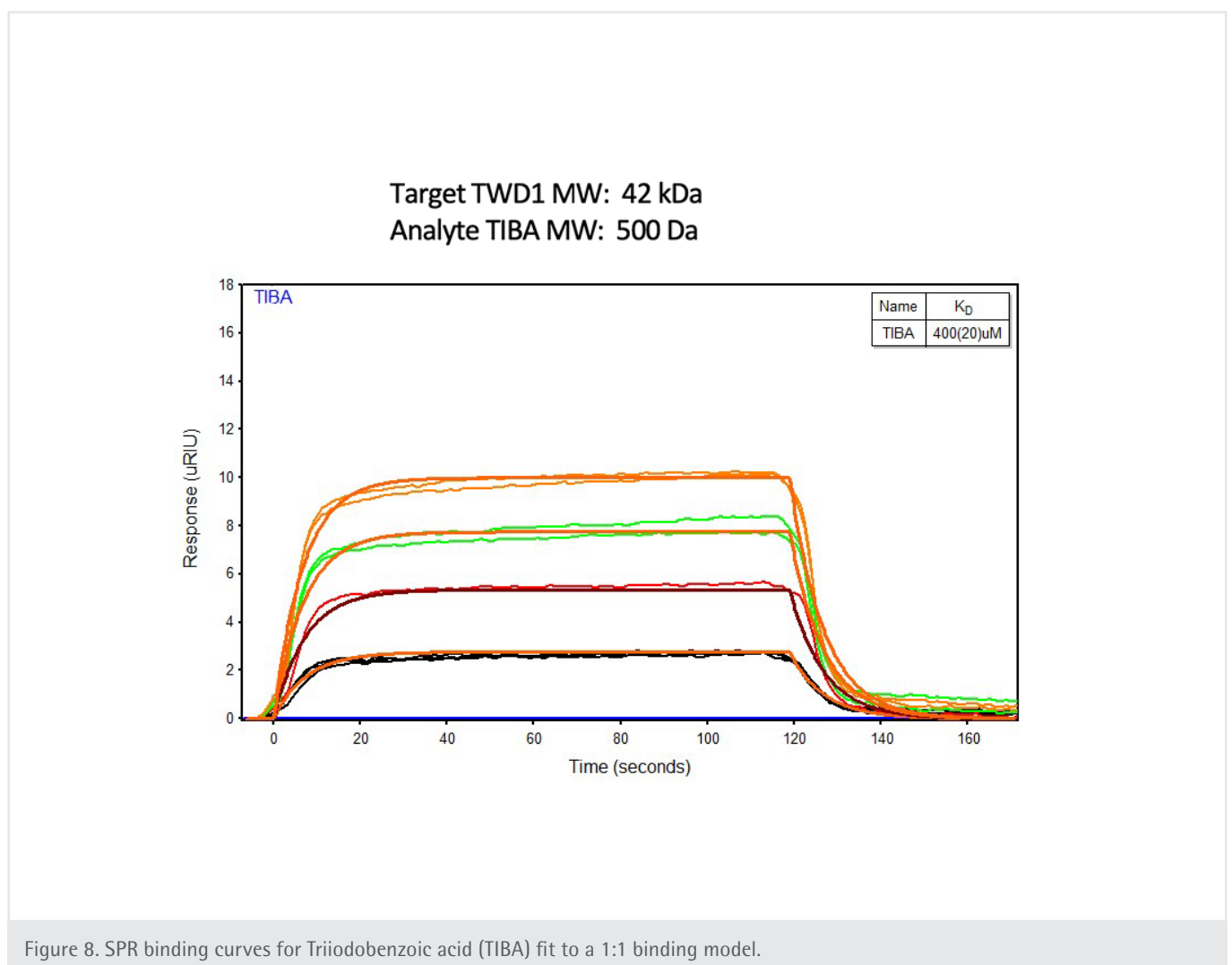

Figure 8. SPR binding curves for Triiodobenzoic acid (TIBA) fit to a 1:1 binding model.

Example 9: Honokiol Inhibition of the pathogen Staphylococcus aureus (S. aureus)

In a 2019 article in Emerging Microbes and Infections, researchers used SPR to study the binding of Honokiol, a natural plant polyphenol which they determined to be a potential inhibitor of a common pathogen Staphylococcus aureus (S. aureus).[10] Researchers used a Reichert4SPR to study this interaction. They immobilized α-Hemolysin (Hla) on a dextran chip and flowed over the Honokiol at various concentrations to determine the affinity.

Researchers in this study confirmed that α-Hemolysin (Hla), an extracellular toxin secreted by most pathogenic S. aureus strains, bound to Honokiol with a modest binding affinity. Additional research using other techniques showed that Honokiol, a low molecular weight natural product, can inhibit inflammasome activation without affecting oligomerization.[10]

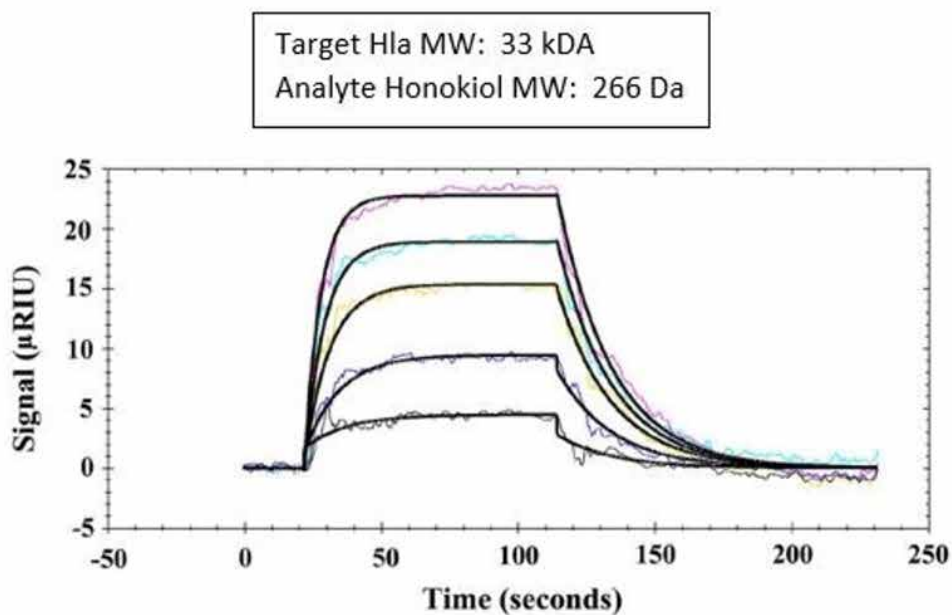The SPR binding curves are shown here:

Figure 9. SPR binding curves for Honokiol binding to Hemolysin. Data is fit to a 1:1 binding model. The association rate constant ka is 293 $M^{-1} s^{-1}$, the dissociation rate constant k_d is 5.03×10^{-2} s^{-1} and the equilibrium dissociation constant K_D is 172 μM.

Example 10: Small molecule zinc complexes binding to DNA

In a 2015 article in Dalton Transactions, researchers designed small molecule zinc complexes and determined how they fit into bulges and quadraplexes on DNA. Non-canonical nucleobases that are part of DNA bulges are common targets when designing small molecules for binding both organic and inorganic compounds. And Zn(II) complexes containing planar aromatic pendents with two fused rings bind to DNA Hairpin (T-bulge) more tightly than complexes with non-planarpendents.[11]

Both a better understanding of these types of interactions and the development of alternative ways to recognize unusual nucleic acid structures could lead to improved design of small molecules for future therapeutic applications. Researchers in this study used Reichert's 2-channel surface plasmon resonance (SPR) system to capture biotinylated DNA oligos on a planar streptavidin chip. They then flowed over the analytes at various concentrations to characterize the binding of Zn complexes to DNA T-bulge and T-loop structures.[11]
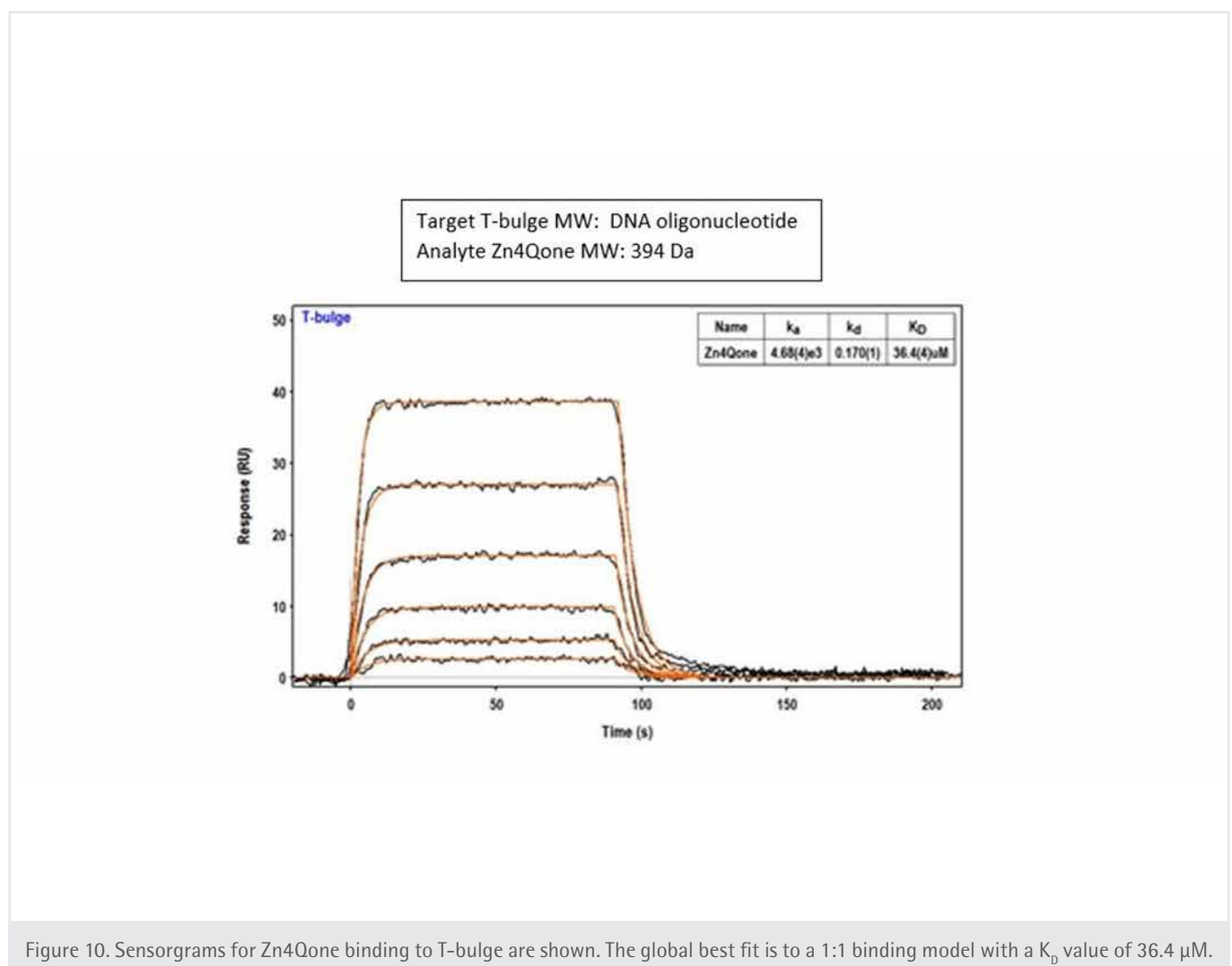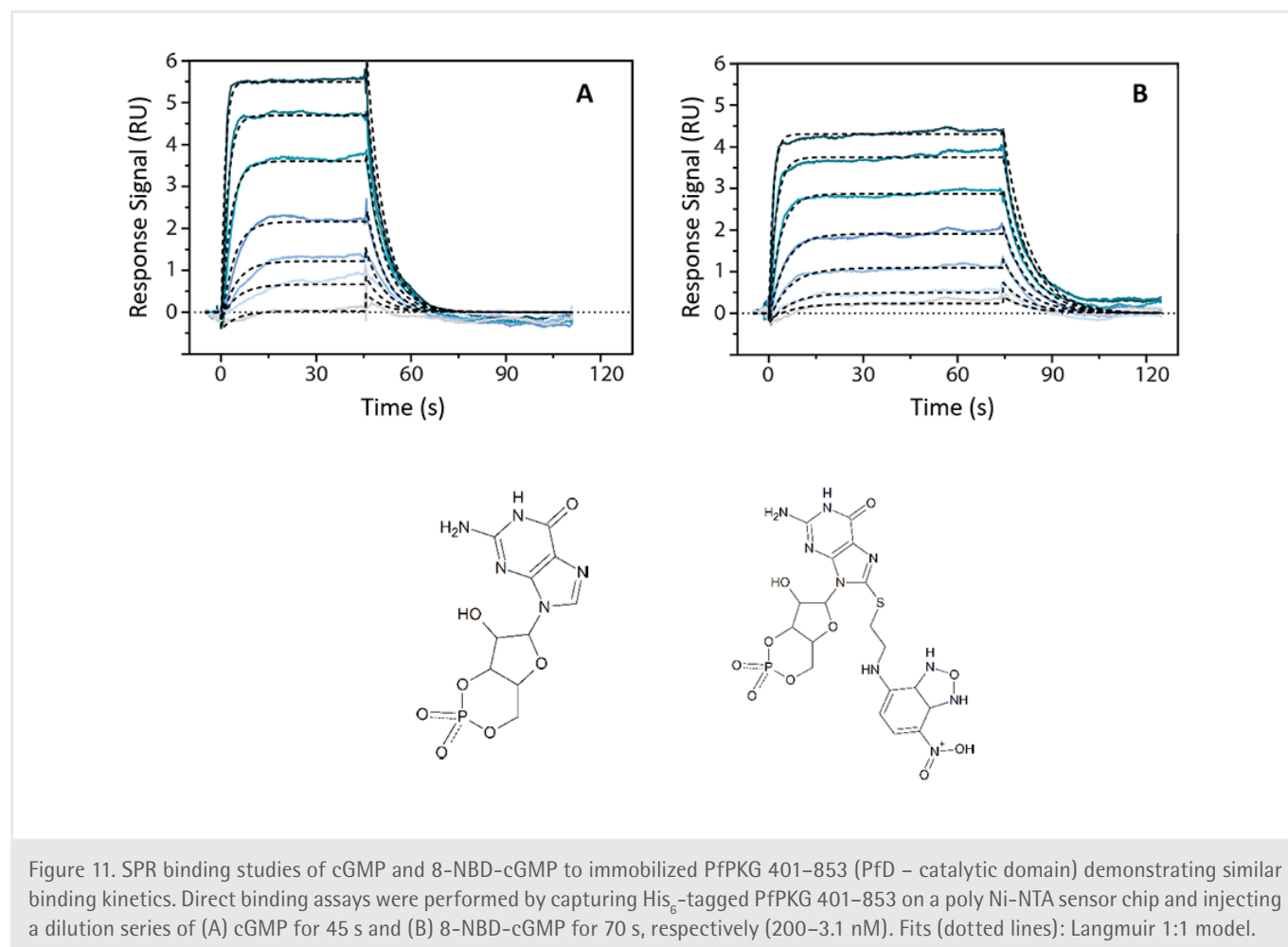
An example of a typical result is shown here:

Figure 10. Sensorgrams for Zn4Qone binding to T-bulge are shown. The global best fit is to a 1:1 binding model with a K_D value of 36.4 µM.

Example 11:

Most malaria deaths are caused by the protozoan parasite Plasmodium falciparum. Its life cycle is regulated by a cGMP-dependent protein kinase (PfPKG), inhibition of which is a promising antimalarial strategy. Allosteric kinase inhibitors, such as cGMP analogs, offer enhanced selectivity compared with competitive kinase inhibitors. However, the mechanisms underlying allosteric PfPKG inhibition are poorly understood. In this work, it was shown that 8-NBD-cGMP is an effective PfPKG antagonist[12].

The challenging part of the experiments was the site-directed immobilization of PfPKG via its His6-tag while maintaining full functionality. Typically, small molecule interactions require covalent immobilization strategies as the baseline drift that normally occurs with Ni-NTA sensor chips is too big for the analysis of small signals during the analyte interaction. This problem was solved by using a **XanTec** high affinity polydentate Ni-NTA sensor chip (NiHC1000M) which binds His6-tagged ligands with extremely high stability so that barely any baseline drift occurs. Thus, the small molecule inhibitors cGMP and 8-NDB-cGMP were accessible for kinetic analysis. It was found that both had similar affinities (K_d 59 nM for cGMP and 51 nM for 8-NDB-cGMP). This example demonstrates that **XanTec** sensor chips are compatible with other SPR instruments (in this case a Biacore© T200), where they are an excellent means to boost the instrument performance.

Figure 11. SPR binding studies of cGMP and 8-NBD-cGMP to immobilized PfPKG 401–853 (PfD – catalytic domain) demonstrating similar binding kinetics. Direct binding assays were performed by capturing His_6-tagged PfPKG 401–853 on a poly Ni-NTA sensor chip and injecting a dilution series of (A) cGMP for 45 s and (B) 8-NBD-cGMP for 70 s, respectively (200–3.1 nM). Fits (dotted lines): Langmuir 1:1 model.

Conclusion

SPR can play a pivotal role in development of therapeutics by the characterization of small molecule interactions with proteins. Reichert's SPR systems have been implemented in a variety of **research applications,** and have the sensitivity and performance characteristics to meet challenging experimental needs such as low molecular weight interaction analysis – especially, when combined with sensor chips from **XanTec bioanalytics.**
Reichert offers two main SPR platforms – the 2SPR and 4SPR systems – which are affordable, flexible and have outstanding performance. Please contact us to learn more about how Reichert SPR can help you with the design and development of small molecule therapeutics.

For further information regarding **XanTec's** range of SPR instruments, sensor chips and other consumables visit our homepage **www.xantec.com** or contact our team directly at **sales@xantec.com.**

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