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Binding Specificity Challenges in Biosensor-Based Platforms: Non-specific Binding and Potential Mitigation Factors

Introduction

Biosensor Based Affinity Characterization

Affinity characterization is a critical process in the development of biotherapeutic drugs and describes the measurement of the strength of binding between a drug (analyte) and its potential receptor or binding partner. This measurement can be performed either through end-point analysis using, for example, enzyme-linked immunosorbent assays (ELISAs), or through kinetics-based assays, such as those using biosensors. In biosensor-based kinetic assays, one molecule is immobilized on the sensor surface (ligand) and the other binding partner is kept in solution (analyte). Kinetics based assays are typically information rich affinity characterization processes as they provide the researcher not just with the strength of binding (affinity; K_D) but also how fast the complex forms (association rate constant; on-rate; k_a) and how fast it decays (dissociation rate constant; off-rate; k_d). The accuracy of these key parameters depends on the specificity of binding between the partners.

Binding Specificity

Binding specificity is comprised of two factors; the specific, functional interactions that occur between a molecule (e.g., a protein) and its target, and the absence of binding interactions with any other targets.[1] Specific interactions are essential to many biological functions, for example, cell signaling, drug activity and antigen recognition by the immune system. The strength of these interactions can be described using binding affinity and are generally reported using the equilibrium constant (K_D). Proteins can also bind to molecules or surfaces other than their target, in a non-functional, non-specific binding (NSB) event. A single molecule may bind specifically and non-specifically to multiple different partners, both in the body and *in vitro* applications.

NSB can create a problem when using biosensor-based techniques, such as biolayer interferometry (BLI), to measure binding affinity. These interactions can mask the true, specific binding events being measured and lead to inaccurate calculation of kinetic parameters, such as k_a, k_d and K_D. NSB can occur when the analyte of interest binds non-specifically to something other than the target molecule (such as the biosensor surface or coating), or it can occur when another molecule in the sample binds non-specifically to the target protein or sensor, as is more common in unpurified or crude samples.

Potential Sources of NSB in BLI

BLI biosensors are developed for a variety of applications and therefore have a diverse range of chemistries (i.e., are coated with different ligands). These differences in chemistry mean that the level of NSB that can occur between each sensor type and the analyte of interest can vary greatly. This variation could be a result of direct interactions between the molecule coating the sensor and the analyte. It could also occur because the coating ligands are immobilized at different densities or have different sizes /structures that may influence how much access there is to the biosensor surface.

Of critical importance to the occurrence of NSB are the biophysical properties of the analyte of interest in relation to either the biosensor coating chemistry or the ligand to be immobilized. For example, if a protein has a low isoelectric point (pI), it will be negatively charged at the neutral (or close to neutral) pHs generally used for BLI assay buffers. This means the protein may be more likely to bind non-specifically to immobilized proteins containing positively charged groups. The reverse is true for proteins with a high pI. Moreover, since the biosensor surface itself is negatively charged then proteins with a high pI may exhibit increased NSB to the sensor itself. A similar effect may be seen if there are opposing hydrophobic and hydrophilic interactions between the proteins pre-immobilized onto the biosensor and the analyte of interest.

There are also instances where the amino acid sequence of the analyte protein includes groups that directly recognize groups on the biosensor coating protein, despite these not being a specific binding partner for these molecules. An example of this can be seen with the fibronectin protein which contains an RGD sequence (Arg-Gly-Asp). This sequence acts as a streptavidin recognition site and therefore induces high levels of NSB of the fibronectin protein to streptavidin loaded biosensors.[2]

When NSB is encountered, it is advisable that the user first evaluates the use of a different biosensor surface as well as changing the assay orientation such that the suspect "sticky" molecule is immobilized / captured on the biosensor while the "good" molecule is kept in solution. Generally, however, the process to eliminate NSB starts with the understanding of the analyte properties and the subsequent incorporation into the assay of mitigators that may counter these properties.

Mitigating NSB in BLI

There are a vast number of different conditions and NSB mitigators that can be used to reduce NSB in BLI (outlined in Table 1). One example is to use protein-based blockers such as bovine serum albumin (BSA), caseins, dry milk or fish gelatin. The presence of these blockers in the BLI assay buffer can inhibit hydrophobic, ionic, or electrostatic interactions between proteins and thus can greatly reduce their NSB.[3] Another common way to reduce NSB is to add detergent into the assay buffer, with these, again, acting to disrupt protein-protein interactions, such as hydrophobic contacts. Detergents can have different properties which may also affect how they disrupt protein-protein interactions, for example they may be non-ionic (uncharged), such as TWEEN® 20 or Triton® X-100, or zwitterionic (both positively and negatively charged but with net charge of zero), such as CHAPS.[4] Increasing the concentration of salt (e.g., NaCl) in the buffer is another method used to reduce NSB in a system where charge-based interactions are an issue.

In addition to the described general tactics for NSB reduction (salts, blockers, pH, etc.) it is also possible to make informed changes based on the specific assay chemistry in order to reduce NSB. For example, since HIS repeats can occur naturally in proteins, NSB with the Octet® Ni-NTA Biosensors is common. In this scenario, switching from the Ni-NTA to another biosensor type and changing the immobilization method may eliminate the NSB from the system. Another approach, which could be used if an initial test shows there is high NSB due to a 'sticky' analyte, could be to swap which molecule is used as the loaded ligand and which is the analyte. In some systems, it is possible to reduce NSB by physically blocking the biosensor surface or by quenching unused pre-immobilized groups. An example of this is to block streptavidin coated biosensors using molecules such as biotin, D-Desthiobiotin or biocytin.

In this case, the ligand would be immobilized to the streptavidin biosensor, then the sensor would be dipped into the biotin or biocytin, which would bind to and block streptavidin-biotin binding sites. When the sensor is then dipped into the analyte, NSB due to interactions with the biotin binding site should be reduced. If the NSB is linked to another region on the streptavidin molecule or with the biosensor itself, the biotin/biocytin could be replaced with a larger biotinylated molecule that presents an even greater physical block of both the streptavidin sensor surface, such as polyethylene glycols (PEGs).

The 'standard' method for reducing NSB in Octet® BLI is to use the Octet® Kinetics Buffer (KB), at either a 1X or 10X concentration, as the assay buffer. The 1X buffer contains 0.01% BSA and 0.002% TWEEN® 20, both of which are very commonly used mitigators of NSB in several applications. The 10X buffer contains 1X PBS with 10X BSA and TWEEN compared to the 1X buffer. However, there are certain model systems where this buffer has not provided sufficient NSB reduction for accurate kinetics to be measured. Therefore, we aimed to design a method for determining other potential 'optimal' conditions that reduce NSB in less straightforward scenarios.

Table 1: *Common NSB mitigators and their properties*

Mitigator	Type	Properties
BSA	Protein	Universal blocker from bovine serum
Casein	Protein	Universal blocker from bovine milk
Dry milk	Protein	Universal blocker from bovine milk
Fish gelatin	Protein	Universal blocker from fish skins
Biotin	Small molecule	Forms strong non-covalent bonds with streptavidin
Biocytin	Small molecule	Biotin molecule, with an additional lysine group, forms strong non-covalent bonds with streptavidin
D-Desthiobiotin	Small molecule	Modified form of biotin molecule, forms strong non-covalent bonds with streptavidin
NaCl	Salt	Common salt reduces charge based NSB interactions
MgCl	Salt	Common salt reduces charge based NSB interactions
Sucrose	Sugar	Common sugar may reduce NSB interactions when combined with other mitigators
Glucose	Sugar	Common sugar may reduce NSB interactions when combined with other mitigators
Trehalose	Sugar	Common sugar may reduce NSB interactions when combined with other mitigators
TWEEN® 20	Detergent	Non-ionic, uncharged
TWEEN® 40	Detergent	Non-ionic, uncharged
TWEEN® 80	Detergent	Non-ionic, uncharged
Triton® X-100	Detergent	Non-ionic, uncharged
n-dodecyl-β-D-maltoside (DDM)	Detergent	Non-ionic, uncharged
Pfluronic-F68	Detergent	Non-ionic, uncharged
Cetyl Trimethyl Ammonium Bromide (CTAB)	Detergent	Cationic, positively charged
Tetradecyltrimethylammonium bromide (TTAB/MTAB)	Detergent	Cationic, positively charged

Design of Experiments (DOE) Approach

Using MODDE® to Screen NSB Mitigators

To quickly screen multiple mitigation conditions for their ability to reduce NSB, a design of experiment (DOE) approach was conducted using the Sartorius MODDE® software. MODDE® combines a guided method for planning DOEs with integrated, advanced data analysis tools that can be used to deduce relationships and trends in complex data sets. This technique utilizes experimental designs that negate the need to test every single combination of factors, which saves both time and resources. Links to user guides, tutorials and videos on how to use the MODDE® platform can be found on the Sartorius website **(MODDE® - Design of Experiments Software | Sartorius).** The inputs into MODDE® are the responses to be measured, for example the magnitude of NSB and specific binding, as well as factors to be varied, such as BSA and TWEEN® 20 concentration. A design is then selected which will determine how many different factors and concentrations will be assessed.

Methods & Results

This outputs a numbered list of 'experiments', with each corresponding to a different condition to be tested. For the Octet® BLI application, each experiment was taken to correspond to an individual biosensor within the Octet® BLI run. Following completion of the experimental phase of the DOE, the results (in this case the nm shifts for NSB, specific binding and loading) are copied into MODDE® and analyzed to reveal trends in the data. In this case an assessment of how each mitigator effects NSB was performed, examples of which are shown in Figures 2 to 6.

Model System

It was important for these experiments that a relevant model system was chosen to test the DOE method. This required the selection of biosensors and a protein model pair. Streptavidin biosensors are by far the most widely used for kinetics studies on Octet® BLI and so these were chosen as the model system for the DOE. In general, most NSB mitigating conditions on streptavidin surfaces are expected to be applicable across the multiple streptavidin-based biosensors (SA, SAX or SAX2). In these studies, we selected a variety of protein molecules with diverse biophysical properties that we expected may bind non-specifically to streptavidin-based biosensors. These proteins vary in characteristics such as their molecular weight (MW) and pI. Examples of the biological functions of these proteins are described in Table 2. Common themes within the functions of these proteins are to act as adhesion proteins or to require binding to multiple different binding partners. This provides some explanation as to why these proteins may have proven to be 'sticky' in Octet® BLI experiments, with their functions and characteristics leaving them more prone to NSB.

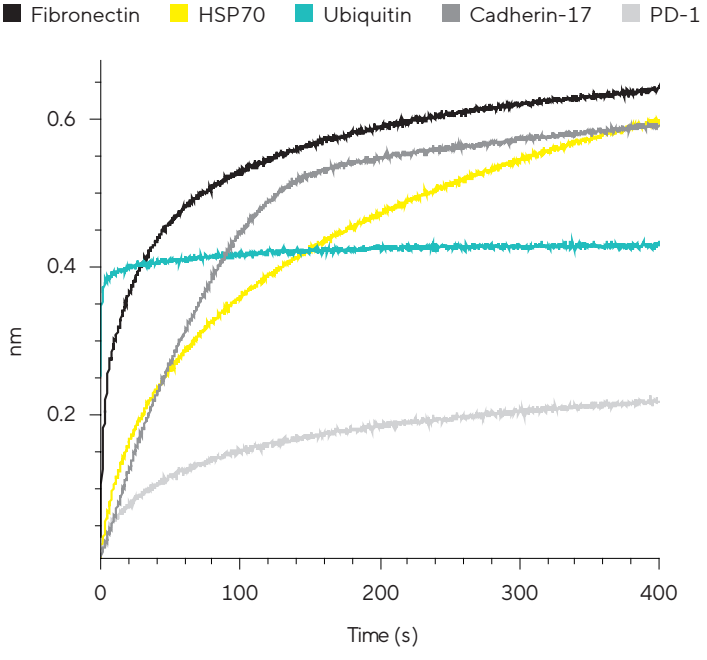
Table 2: *Example 'sticky' proteins and characteristics*

pI	Function	pI	MW (kDa)
Fibronectin	Glycoprotein with roles in cell-cell adhesion, wound healing and migration[5]	5.2	220
HSP70 (heat shock protein 70)	Molecular chaperone that binds protein substrates and stabilizes them against denaturation during stress[6]	5.2	70
Ubiquitin	Regulates many processes, including immune response, angiogenesis, cell proliferation, apoptosis, and DNA repair[7]	6.7	8.5
Cadherin-17	Calcium-dependent cell adhesion proteins[8]	4.9	89
PD-1	Cell surface receptor on T cells and B cells with a role in immune response down-regulation[9]	8.25	32

NSB of Proteins With SA Sensors

As a first assessment of NSB exhibited by each protein, commercially available samples were diluted in PBS buffer and binding to SA biosensors measured using an Octet® RH16 system (Figure 1). Analyte concentrations were determined based on empirical knowledge or from literature, with the aim of using a concentration around 20 times larger than the KD. Since none of these proteins contained a biotin-tag, any binding that was observed to the streptavidin coated sensors could be considered non-specific (although fibronectin has the unique SA binding group as discussed above). All the proteins showed considerable levels of NSB to the sensors, suggesting that they can either bind to streptavidin, or to the base sensor itself. Due to the high level of NSB, Cadherin-17 protein with biotinylated anti-Cadherin-17 antibody was selected for further exploration in the DOE experiments. As shown in Table 2, Cadherin-17 functions as a cell adhesion protein, which likely requires it to bind to multiple partners and may explain why it has a propensity to exhibit NSB.

Figure 1: *Non-specific binding of proteins to SA sensors*



Note. Octet® BLI measurement of the association of a range of proteins to streptavidin coated (SA) biosensors over 400 seconds. Fibronectin, HSP70, Cadherin-17 and PD-1 proteins were diluted to 100 nM in PBS. Ubiquitin was diluted to 11700 nM. A PBS only reference sample has been subtracted from each trace.

DOE 1: BSA, TWEEN® 20, Salt and Buffer / pH, Protein and Sensor Only

The first DOE experiments measured the effects of various mitigators and buffer choice on the level of NSB of Cadherin-17 (150 nM) protein to SA sensors. The presence or absence of 1% BSA and 0.02% TWEEN® 20 in three different buffer types (each at a different pH) were evaluated. The three buffers used were pH 6.2 citrate buffer, pH 7.4 PBS and pH 8.2 HEPES. The addition of up to 300 mM NaCl was also tested.

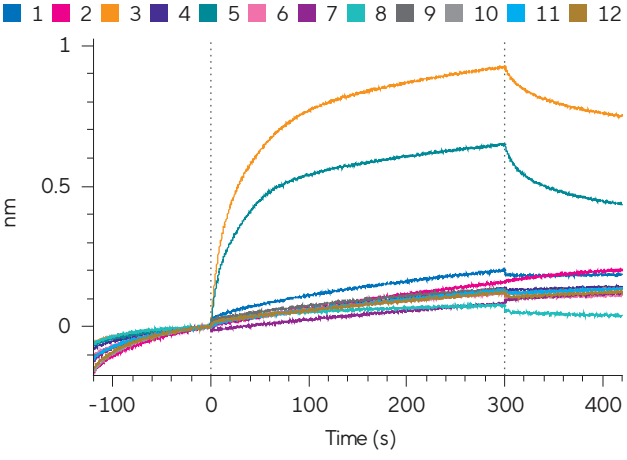
It should be noted that the NaCl concentrations only describe the NaCl added on top of the NaCl already present in the base buffers (for example PBS contains 137 mM NaCl and up to 300 mM NaCl was added to achieve a final NaCl concentration of 437 mM NaCl). Inputting these variable factors into MODDE® and using a D-optimal design with 3 center points output a total of 12 experiments (i.e., 12 biosensors in different assay buffers) to be tested (Figure 2A). This is much lower than the 27 experiments that would be needed to test all combinations of buffer and mitigators at a single concentration. The buffers in experiments 10, 11 and 12 are identical with mid-range levels of each quantitative variable and aim to investigate the intra-experiment variability.

Figure 2: *Combining Octet® BLI with MODDE® DOE software to screen common mitigators for NSB reduction*

A

Exp No	Exp Name	Run Order	Incl/Excl	BSA	TWEEN-20	NaCl	Buffer/pH	NSB
1	N1	10	Incl	1	0	0	pH 6.2 citrate	0.1931
2	N2	5	Incl	0	0.02	0	pH 6.2 citrate	0.1559
3	N3	2	Incl	0	0	300	pH 6.2 citrate	0.9173
4	N4	4	Incl	1	0.02	300	pH 6.2 citrate	0.1348
5	N5	6	Incl	0	0	0	pH 7.4 PBS	0.6434
6	N6	9	Incl	1	0.02	0	pH 7.4 PBS	0.1159
7	N7	11	Incl	0	0.02	300	pH 7.4 PBS	0.0845
8	N8	1	Incl	0	0.02	0	pH 8.2 HEPES	0.0682
9	N9	12	Incl	1	0	300	pH 8.2 HEPES	0.1283
10	N10	8	Incl	0.01	0.002	150	pH 8.2 HEPES	0.1229
11	N11	7	Incl	0.01	0.002	150	pH 8.2 HEPES	0.1276
12	N12	3	Incl	0.01	0.002	150	pH 8.2 HEPES	0.1163

B Cadherin-17 NSB



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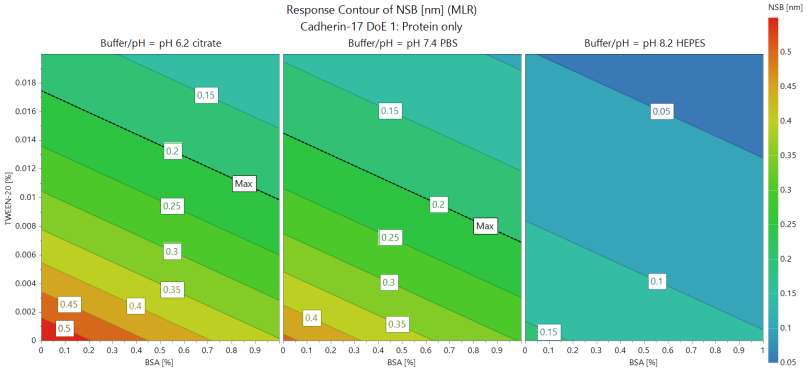

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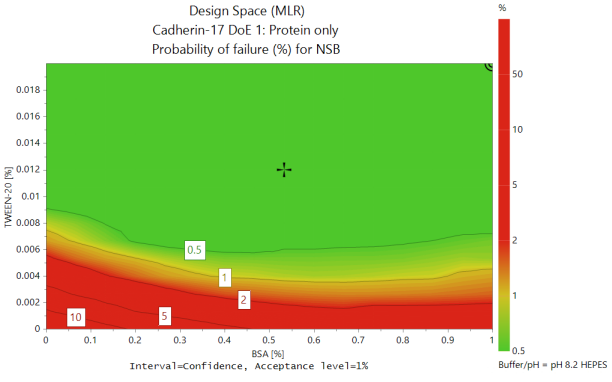


Note. (A) Specification table from MODDE® defines 12 experiments from a D-optimal design with 3 center points. Three base buffers were tested, each with a different pH (citrate buffer, pH 6.2, PBS, pH 7.4 and HEPES pH 8.2). Concentrations of BSA (0-1%), TWEEN® 20 (0-0.02%) and additional NaCl (0-300 mM) were varied. The NSB response was copied from the Octet® Analysis Software and describes the nm shift in the association phase. (B) Octet® traces show association of 150 nM Cadherin-17 protein to SA biosensors in each condition. (C) 4D contour plot from MODDE® profiles NSB. The salt metric was removed due to insignificance. A max NSB threshold of 0.2 nm was defined. (D) Design space plots the region with probability of failure >1% for NSB being below threshold. The robust and optimal setpoints mark the center of the design space and the lowest NSB conditions, respectively.

Figure 2B shows the association of Cadherin-17 to SA biosensors over a time period of 300 seconds, as measured using the Octet® RH16. Experiment 5 (dark teal) describes protein binding when PBS alone is used as the assay buffer, which is in line with Figure 1. Of the other conditions tested only experiment 3 (orange, pH 6.2 citrate buffer with 300 mM NaCl) did not reduce NSB compared to PBS. For the other 10 experiments, the level of NSB was very low, suggesting the mitigators were acting to reduce non-specific interactions with the biosensor. The nm shifts for association were exported from the Octet® Analysis Studio and pasted into the MODDE® platform (final column in Figure 2A). The three midpoint experiments (10, 11 and 12) showed minimal variation which suggested good intra-experiment reproducibility.

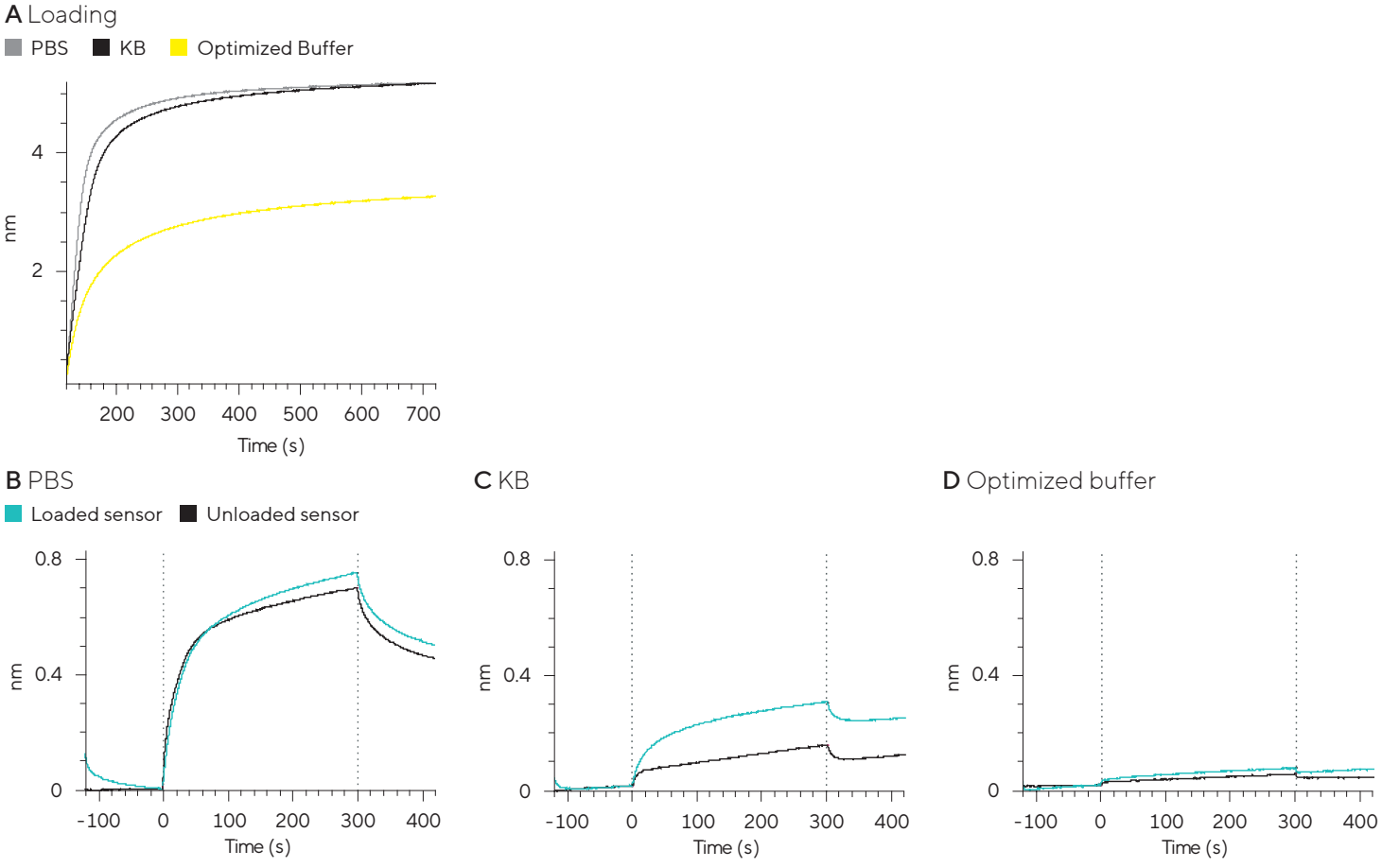
The analysis wizard in MODDE® was used to fit the generated data to the model. During these steps, parameters such as the skew of the data and the quality of the model are assessed, with the option for the user to change aspects of the model fit to improve its quality. Part of this includes the use of coefficient plots to assess the significance of each variable in terms of its impact on the NSB response. This led to the exclusion of the salt factor from the model as it was deemed an insignificant term. MODDE® was then used to generate 4D contour plots which enabled clear visualization of the impact of each variable on the NSB response (Figure 2C). A maximum NSB threshold of 0.2 nm was applied. This plot showed that NSB in the HEPES 8.2 buffer was lowest, with all conditions tested within this buffer below the 0.2 nm threshold.

The robust setpoint marks the center of the design space region with very low probability of failure, whilst the optimal setpoint predicts the conditions with the lowest NSB. In this case, the optimal setpoint (shown in the design space plot in Figure 2D), suggested that the optimal conditions for low NSB are pH 8.2 HEPES buffer with high levels of BSA and TWEEN® 20.

A subsequent experiment was performed to compare the ligand loading, NSB and specific binding in the Cadherin-17/anti-Cadherin-17 model system in the new optimized buffer (pH 8.2 HEPES + 1% BSA + 0.02% TWEEN® 20) with standard base buffers, PBS and Octet® BLI Kinetics Buffer (KB). The loading data showed that the biotinylated antibody loaded onto the SA sensors in all 3 conditions, but the magnitude of loading was 40% lower in the new 'optimized' buffer (Figure 3A). In addition, the association of Cadherin-17 to these loaded sensors compared to unloaded sensors showed that whilst NSB had successfully been reduced in the optimized buffer (Figure 3D) the levels of specific binding were also so low that they couldn't be distinguished from the reference sensor. The overall profile of specific binding compared to NSB in the KB were much preferable, with a clear increase in protein binding to the antibody-loaded sensor, suggesting specific binding is being measured (Figure 3C). We therefore conclude that while the optimized buffer may be optimal for preventing non-specific binding to the biosensor surface, it may not be optimal for the reaction between the ligand (anti-Cadherin 17) and the analyte (Cadherin-17). It is therefore essential to measure both specific binding and NSB during the DOE process to ensure the optimized result balances both high specific binding and low NSB.

It should be noted that, ideally, the reference sensor should be a sensor that is loaded with a similar, but non-active ligand of interest, rather than a blank sensor. This is because the access to the base biosensor itself will differ depending on whether it is loaded or not, and this in turn could impact the level of NSB. However, as these experiments aimed to screen mitigators for their ability to reduce non-specific interactions between the protein and biosensor, a blank reference sensor was used.

Figure 3: *Comparing the optimized Buffer from DOE 1 with PBS and KB*



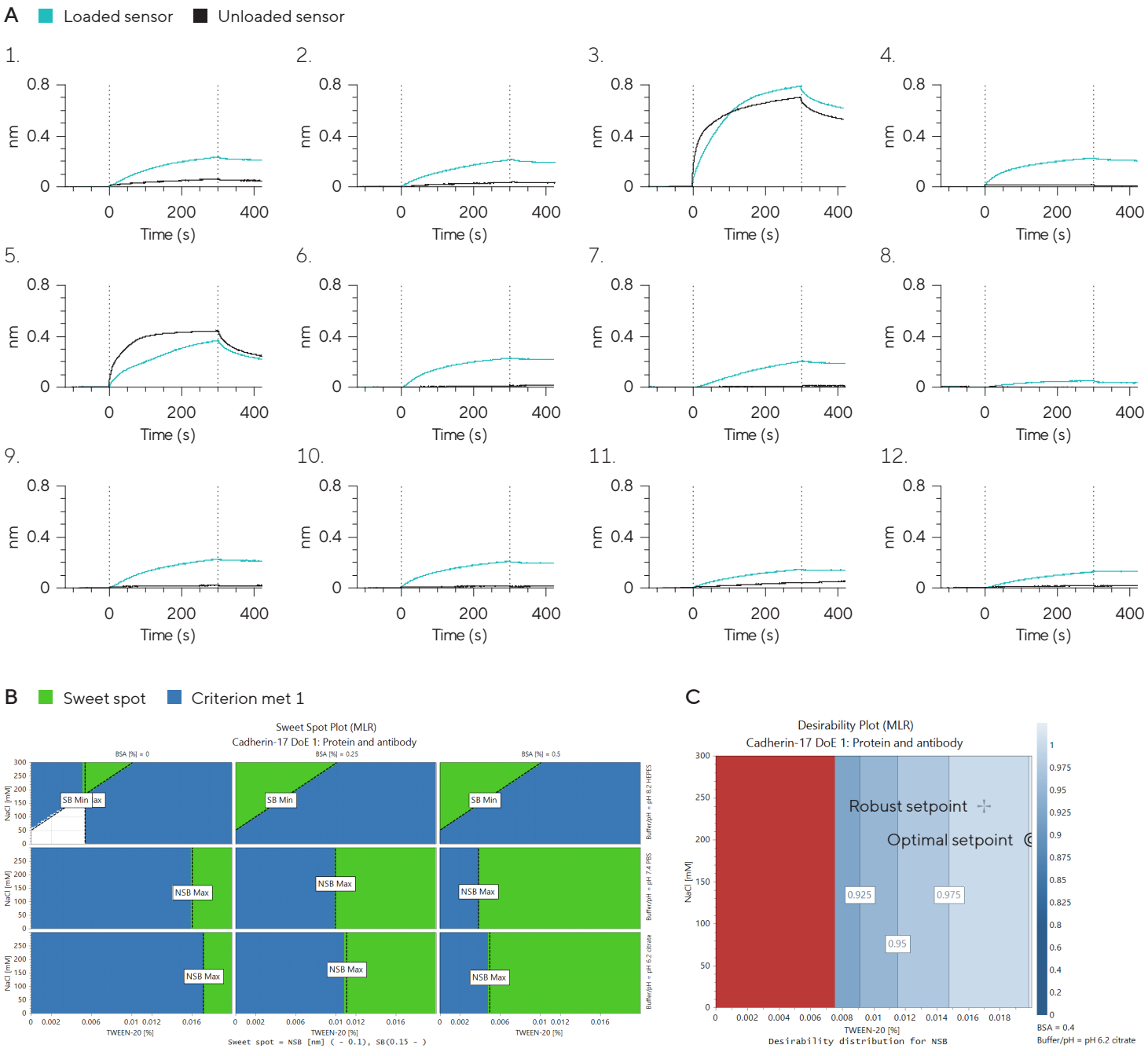
Note. Octet® BLI measurement of the association of a range of proteins to streptavidin coated (SA) biosensors over 400 seconds. Fibronectin, HSP70, Cadherin-17 and PD-1 proteins were diluted to 100 nM in PBS. Ubiquitin was diluted to 11700 nM. A PBS only reference sample has been subtracted from each trace.

DOE 1 Repeat With Antibody Loading and Protein Association

To test the conclusion that protein association to the loaded ligand must be assessed during the DOE if meaningful results are to be produced, DOE 1 was repeated, this time with the inclusion of a loading step. The sensors were either loaded with 1 µg/mL biotinylated anti-Cadherin-17 antibody or left unloaded (i.e., no antibody added) (Figure 4).

SAX sensors were used for the remaining experiments in this note. Using 12 biosensors, all steps, from antibody loading to Cadherin-17 protein association were tested in one of each of the 12 conditions outlined in Figure 2A.

Figure 4: *Repeat of DOE 1 conditions with comparison between antibody loaded and unloaded sensors*



Note. (A) SAX sensors were either loaded with 1 µg/mL biotinylated anti-Cadherin-17 antibody or no antibody. The 12 different buffer conditions defined in Figure 2 were tested. Pink traces show binding of Cadherin-17 protein to the antibody loaded sensor; black traces show protein binding to an unloaded SAX sensor. (B) MODDE® sweet spot plot shows conditions with specific binding (SB) above 0.15 nm and NSB below 0.1 nm. (C) The desirability plot maps the probability of fulfilling the set criteria. BSA concentration is set to 0.4% and the buffer is set to pH 6.2 citrate. Robust and optimal setpoints have been plotted.

Figure 4A compares Cadherin-17 binding to antibody loaded sensors (pink traces) to unloaded sensors (black traces) in each of the 12 buffers. As shown in Figure 2, all conditions except for condition 3 (pH 6.2 citrate buffer with additional 300 mM NaCl) induced a considerable reduction in the level of NSB compared to the PBS base case (condition 5). The association data was transferred into MODDE®, with the nm shift for protein binding to the loaded sensor and the unloaded sensors defining the specific binding and NSB responses, respectively.

To determine conditions with specific binding above the defined threshold (0.15 nm) and NSB below the defined threshold 0.1 nm, MODDE® was used to generate a 4D sweet spot plot (Figure 4B). This suggested that the two criteria would be met most efficiently in either pH 7.4 PBS or pH 6.2 citrate buffer with high levels of BSA and TWEEN® 20. As we were aiming to use the platform to find an alternative to KB (i.e., PBS with BSA and TWEEN® 20), the citrate buffer was focused on in the desirability plot in Figure 4C. In this plot, the BSA concentration had been fixed to a concentration deemed optimal by the software (0.4%). The optimal and robust setpoints confirmed that high TWEEN® 20 is optimal but also showed that a high level of NaCl should be added to the buffer (>200 mM). Analysis of the coefficient plots during variable significance assessments revealed that this is due to a small increase in specific binding in the presence of high NaCl concentrations (data not shown). This explains why NaCl was deemed insignificant in the first attempt at DOE1, when only NSB was being assessed.

The sweet spot plot (Figure 4B) also confirmed what was revealed from the testing of the optimized Buffer in Figure 3D, which suggested that in pH 8.2 HEPES buffer with high TWEEN® 20 and high BSA, specific binding is very low and does not meet the minimum threshold. This reiterates the advantage of measuring both specific binding and NSB during the DOE and finding conditions which meet both criteria.

DOE 2: Recombumin®, TWEEN® 20 and Buffer / pH

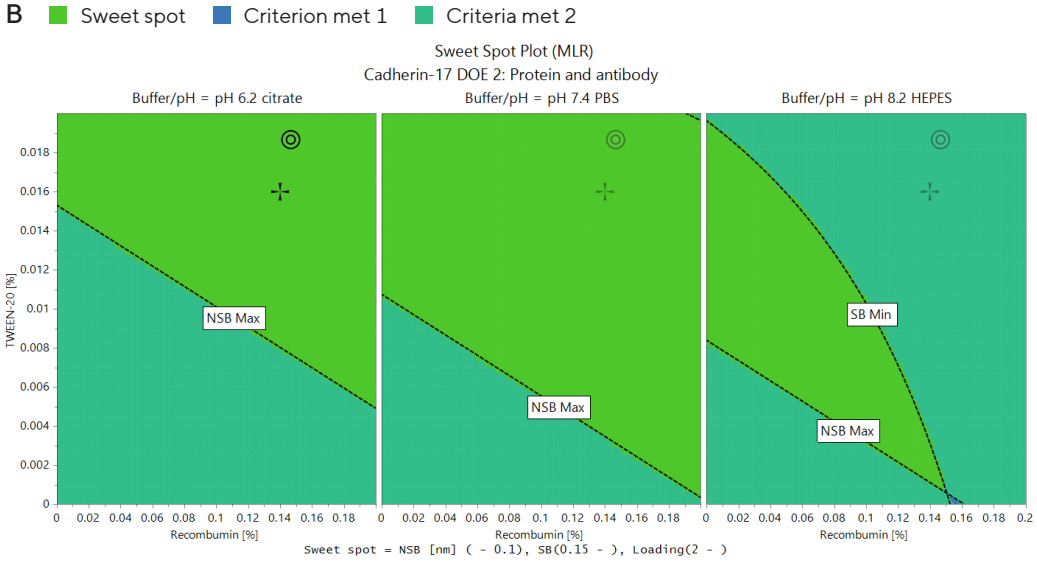
A second DOE was designed to assess the capability for Recombumin® Elite (Sartorius, Recombinant Human Albumin, 205-005) to reduce NSB as an alternative to BSA. Concentrations of both Recombumin® and TWEEN® 20 were varied in the same three base buffers as tested previously (Figure 5A). In these experiments, due to the reduced number of variable factors, a full factorial experimental with three center points was used. This tests all the possible combinations of factors at a single concentration (with the center points at a mid-concentration). As this only utilized 15 sensors, this could fit into a single read on the Octet® RH16 system. The benefit of this design is that each interaction is assessed separately with no confounding variables. Sensors were loaded with 2.5 µg/mL biotinylated Cadherin-17 or left unloaded. The measured responses are the nm shift of association of the Cadherin-17 protein to the unloaded reference sensor (NSB), antibody-loaded specific binding (SB) sensor and the antibody loading. Thresholds for these three metrics were set (NSB < 0.1 nm, SB > 0.15 nm, Loading > 2 nm) to enable production of a 4D sweet spot plot (Figure 5B). Like in the previous DOE, the robust and optimal set points were defined in the pH 6.2 citrate buffer. These determined that mid to high levels of Recombumin® and high levels of TWEEN® 20 would lead to low levels of NSB balanced with relatively high levels of specific binding.

Figure 5: *Varying Recombumin®, TWEEN® 20 and buffer/pH in DOE 2*

A

Exp No	Exp Name	Run Order	Incl/Excl	Recombumin	TWEEN-20	Buffer/pH	NSB	SB	Loading
1	N1	2	Incl	0	0	pH 6.2 citrate	0.9044	0.9645	3.61
2	N2	10	Incl	0.2	0	pH 6.2 citrate	0.1561	0.2999	4.43
3	N3	1	Incl	0	0.02	pH 6.2 citrate	0.0323	0.2588	4.32
4	N4	15	Incl	0.2	0.02	pH 6.2 citrate	0.0235	0.2023	4.34
5	N5	14	Incl	0	0	pH 7.4 PBS	0.6867	0.8127	3.46
6	N6	9	Incl	0.2	0	pH 7.4 PBS	0.1245	0.2182	4.32
7	N7	5	Incl	0	0.02	pH 7.4 PBS	0.0296	0.1871	4.02
8	N8	8	Incl	0.2	0.02	pH 7.4 PBS	0.0018	0.1905	3.99
9	N9	13	Incl	0	0	pH 8.2 HEPES	0.3083	0.5884	2.31
10	N10	6	Incl	0.2	0	pH 8.2 HEPES	0.0667	0.1176	3.39
11	N11	3	Excl	0	0.02	pH 8.2 HEPES	0.1219	0.12	-0.06
12	N12	12	Incl	0.2	0.02	pH 8.2 HEPES	0.0046	0.0527	3.26
13	N13	7	Incl	0.1	0.01	pH 7.4 PBS	0.0643	0.2244	4.13
14	N14	11	Incl	0.1	0.01	pH 7.4 PBS	0.0504	0.2133	4.14
15	N15	4	Incl	0.1	0.01	pH 7.4 PBS	0.061	0.2444	4.23

B



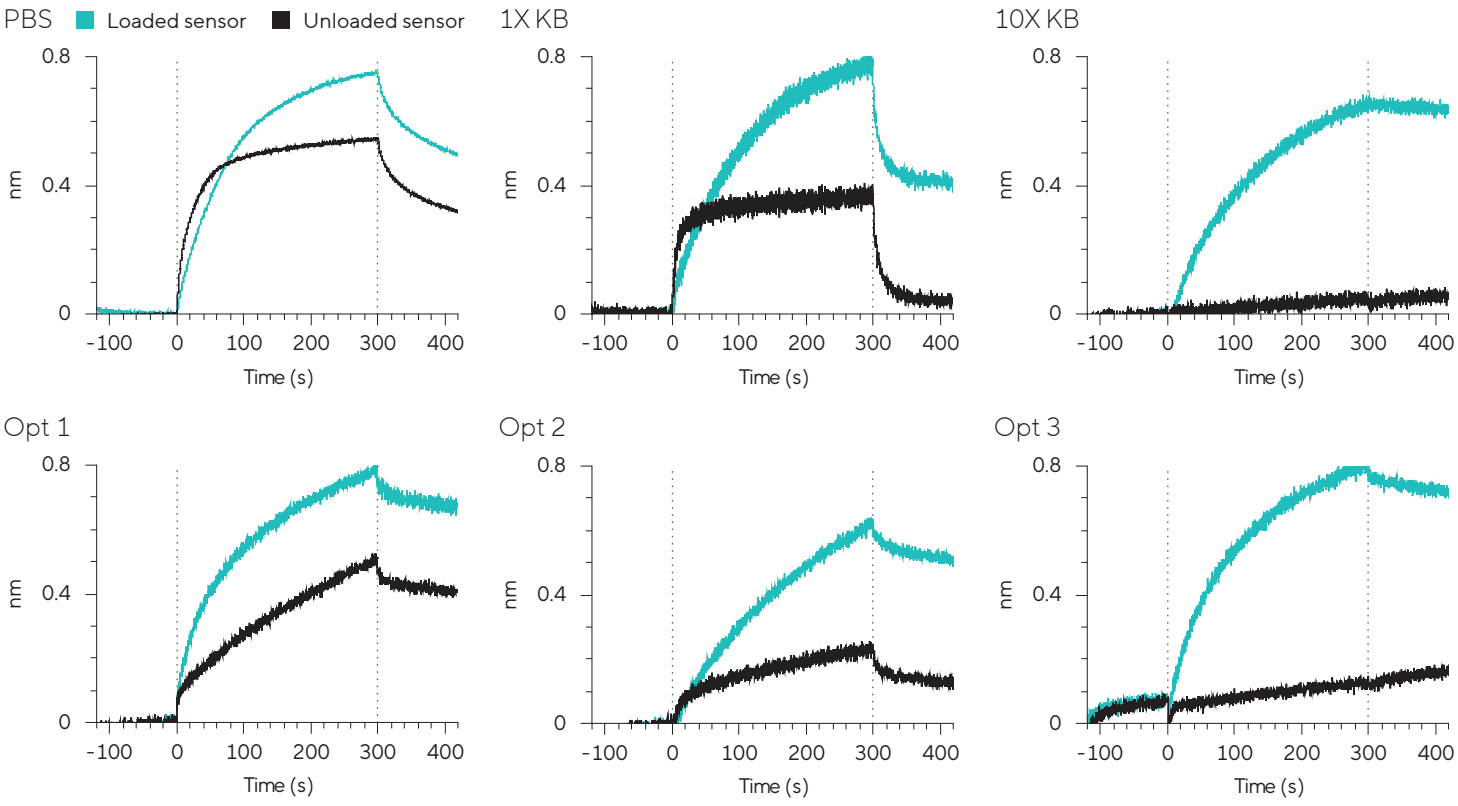
Note. (A) Specification table from MODDE® defining 15 experiments from a full factorial design with 3 center points. Three base buffers were tested (citrate buffer, pH 6.2, PBS, pH 7.4 and HEPES pH 8.2), with varying levels of Recombumin® (0-0.2%, Recombinant Human Albumin, Recombumin® Elite Vial, Sartorius, 205-005) and TWEEN® 20 (0-0.02%). Sensors were loaded with 2.5 µg/mL biotinylated Cadherin-17 or unloaded. (B) 4D sweet spot plot defines conditions response thresholds are met (NSB < 0.1 nm, SB > 0.15 nm, Loading > 2 nm). Optimal and robust set points were calculated in pH 6.2 citrate buffer data.

Comparing the 'Best' and 'Worst' NSB From the DOE Process

Final experiments compared the specific binding and NSB of Cadherin-17 protein in the initial 'worst' case conditions (PBS) with the current standard conditions (KB at either a 1X or 10X concentration) and three optimized buffer conditions as ascertained from the two DOE experiments (Figure 6). As the best case from DOE 1, the 'Opt 1' buffer, (pH 6.2 citrate with BSA (0.5%), TWEEN® 20 (0.02%) and additional NaCl (300 mM)) was chosen.

'Opt 2' from DOE 2 (pH 6.2 citrate with Recombumin® (1 mg/mL) and TWEEN® 20 (0.02%)). Since the effect of NaCl concentration had not been explored in DOE 2 but it had a positive effect in DOE 1, the 'Opt 2' buffer was also tested with the addition of NaCl (300 mM), and this formed the 'Opt 3' buffer.

Figure 6: *'Best' and 'worst' condition comparisons from DOEs 1 and 2 with standard buffers*



Note. SAX sensors were either loaded with 1 µg/mL biotinylated anti-Cadherin-17 antibody or no antibody. Pink traces show binding of Cadherin-17 protein to the antibody loaded sensor; black traces show protein binding to the unloaded SAX sensor. Six different buffer conditions were used across the loading, association and dissociation steps. These were PBS, 1X KB, 10X KB, Opt 1 (Optimized media defined from DOE 1, citrate pH 6.2 + BSA (0.5%) + TWEEN® 20 (0.02%) + NaCl (300 mM)), Opt 2 (Optimized media defined in DOE 2, pH 6.2 citrate + Recombumin® (1 mg/mL) + TWEEN® 20 (0.02%) and Opt 3 (Opt 2 + 300 mM salt).

Conclusions & Recommendations

This article has explored some of the challenges with NSB that can occur in Octet® BLI and highlighted potential solutions to mitigate this issue. Specifically, we have shown the power of the Sartorius MODDE® software for conducting DOE type experiments that reduce both the time and resources needed to screen NSB mitigation factors. The integrated data analysis tools provide a clear, informative, and visual representation of your data which simplifies the process of drawing conclusions from large data sets. To summarize, recommendations based on the findings from this study include:

- If using 1X or 10X KB does not effectively reduce the level of NSB in your model system, a DOE-based approach can facilitate screening of multiple other NSB mitigators.
- The Sartorius MODDE® platform provides an all-in-one design and analysis package for DOE experiments and can reveal trends and relationships between variables in complex data sets.
- Assessing multiple criteria simultaneously, such as NSB, specific binding and ligand loading, can lead to more confidence in results compared to when each metric is evaluated individually.
- Some prior knowledge of the proteins being tested, for example their hydrophobicity, structure, and pI, can help to inform why they are binding non-specifically and could indicate which mitigators are most likely to negate that type of NSB.
- If issues with reproducibility are seen, it may be beneficial to repeat the DOE at least once to confirm that results are stable before proceeding with kinetic quantification.

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