

Reducing Non-Specific Binding in Surface Plasmon Resonance Experiments



Summary

- Reducing non-specific binding (NSB) is essential to generating accurate data with SPR
- The effect of bovine serum albumin, Tween 20, salt, and pH on NSB are examined
- Increasing salt and pH were the most effective methods to reduce NSB in this system
- Knowing the molecular forces that cause non-specific binding can guide the methods used to control it

Overview

Non-specific binding is an important experimental parameter to control when using SPR systems. Non-specific binding is the binding of analyte to non-target molecules on the sensor surface, as illustrated in *Figure 1* (all figures and tables can be found on the last page). The effect of non-specific interactions is a false positive contribution to the signal in a sensorgram. It is important for users to recognize non-specific binding and to implement strategies to reduce or eliminate its effects to get accurate kinetic data.

Non-specific binding is caused by molecular forces (charge-charge interactions, hydrophobic interactions, etc.) between the analyte and the sensor surface. To reduce and prevent nonspecific binding there are a number of experimental conditions that can be used. The most common methods include the addition of bovine serum albumin (BSA) as a blocking protein, the addition of a surfactant such as Tween 20, careful adjustment of the buffer pH, and the addition of salt. In this application note a biological system demonstrating non-specific interactions is examined and various prevention methods are tested to evaluate their effectiveness. The system comprises rabbit IgG antibody as the model protein analyte, which interacts non specifically with a carboxylated gold sensor surface. The effects of different methods for reducing NSB of rabbit IgG are analyzed and explained. SPR users can use this applicative note to help determine which conditions to use to reduce NSB in their experiments.

Materials and Equipment

- OpenSPR Instrument [SPR-01]
- TraceDrawer Kinetic Analysis software [TDS]
- COOH Sensor Chip [SEN-AU-10-COOH]
- 10 mM 2-(N-morpholino)ethanesulfonic (MES) buffer pH 6.0
- 1X phosphate buffered saline (PBS) buffer pH 7.4
- 1X PBS buffer pH 6.0
- Rabbit IgG antibody
- BSA solution
- Tween 20
- NaCl
- Regeneration solution: 10 mM HCl, pH 2.0

Procedure

1. OpenSPR is turned on and a COOH Sensor Chip loaded into the instrument
2. Buffer was pumped at 150 μ L/min for 30 minutes to stabilize the baseline
3. The pump speed was reduced to 100 μ L/min
4. 200 μ L of HCl regeneration solution was injected three times to prime the sensor surface
5. 100 μ L of 1 μ g/mL rabbit IgG solution was injected
6. After the sample passed through the flow cell, 100 μ L of regeneration solution was injected to remove any rabbit IgG from the surface and to bring the signal back to the baseline
7. The rabbit IgG injections were repeated at 5 μ g/mL, 10 μ g/mL, 50 μ g/mL and 100 μ g/mL with regeneration injections used between each concentration
8. To test the effect of different buffer compositions on NSB, the pump was stopped, and the inlet line was transferred from the original buffer to the new buffer. The pump was restarted, and the signal was allowed to return to baseline. With each new test, the rabbit IgG samples were prepared in the same buffer as the running buffer

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All experiments were performed in series on the same sensor chip. Control experiments were performed first to determine the level of nonspecific binding without the use of additives. Any non-specifically bound analytes were removed following a regeneration solution (HCl, pH 2). After the control experiments, the buffer solution conditions were changed and the injections of rabbit IgG were repeated. This allowed for the direct comparison of all results. An example sensorgram is shown in *Figure 2*.

Results and Discussion

Effect of pH

The isoelectric point (pI) of IgG antibodies ranges between 6.8 and 8.5. The isoelectric point predicts where a protein has a net overall charge of zero. By adjusting the pH of the buffer above or below the pI the overall charge of the protein can be made negative or positive. Non-specific experiments were conducted using three commonly used buffers: MES buffer pH 6.0, 1X phosphate buffered saline (PBS) pH 6.0, and 1X PBS pH 7.4. Rabbit IgG samples were dissolved in each respective running buffer. The rabbit IgG samples were injected into the OpenSPR instrument and allowed to flow over the carboxylated surface. The data was then analyzed for NSB.

The experiments using 10 mM MES buffer pH 6.0 repeatedly and consistently showed non-specific interactions of the antibody with the surface while the 1X PBS pH 7.4 showed little to no non specific interactions (*Figure 5*). Comparing the 1X PBS at pH 7.4 to the 1X PBS at pH 6.0, a similar result is seen. There is significant NSB at pH 6.0 with minimal at pH 7.4 (*Figure 4*). The cause of the non-specific interactions are likely due to the overall charge of the IgG at pH 6.0. The isoelectric point or pI of a protein is the pH at which its charges are balanced and has no net charge. At pHs above the pI, the protein (analyte) has a positive charge and at a pH below the pI it has a negative charge (*Figure 5*). At pH 6, the net positive charge on the rabbit IgG causes it to interact with the carboxylated surface. This effect is reduced at pH 7.4 because the buffer was near or above the pI of the analyte and its negative charges created a repulsive effect from the sensor surface (*Figure 5*). These results show that buffer pH plays a critical role in the level of non-specific binding. At pH 6, there were significant non-specific interactions between the rabbit IgG and the carboxylated sensor surface. By increasing the pH to near or above the isoelectric point, the level of nonspecific binding was significantly reduced.

To evaluate the effectiveness of other methods to reduce non-specific binding, 10 mM MES buffer pH 6 was used as the running buffer to produce an environment in which there was significant non-specific binding present.

Protein Blocker (BSA)

Bovine Serum Albumin (BSA) is a commonly used protein blocking additive that helps prevent nonspecific protein-surface interactions. BSA is a globular protein with hydrophilic and hydrophobic subgroups. It serves as a carrier protein that escorts low solubility molecules through the blood stream. Serum albumins are the most abundant protein found in blood. In some ways, it is nature's method of preventing non-specific binding in the body and it is used ubiquitously for in vitro biological applications to prevent proteins from binding to glass, plastic and to each other.

At pH 6.0 BSA has a negative charge (pI=4.7), which causes BSA to surround the positively charged protein analyte such as IgG (pI = 6.8-8.5) as illustrated in *Figure 6*. Therefore, at a high enough concentration, BSA molecules can fully surround IgG analyte preventing them from interacting with the negatively charged carboxylated surface. To test the effectiveness of BSA in reducing NSB, 0.1% w/v and 1% w/v BSA in 10 mM MES buffer pH 6.0 were used as the running buffers and the NSB of IgG to the COOH surface was tested.

The binding of IgG to the COOH surface at IgG concentrations of 1, 5, 10, 50, and 100 $\mu\text{g}/\text{mL}$ in the buffer with 0.1% BSA and the control (0% BSA) are shown in *Figure 7*. There is a small reduction in NSB seen at the highest IgG concentration, but otherwise the use of 0.1% BSA was not effective at preventing NSB. The binding of IgG with 0.1% w/v BSA present was actually higher than the control tests for 1 $\mu\text{g}/\text{mL}$ to 50 $\mu\text{g}/\text{mL}$ IgG concentrations. This result suggests that the presence of 0.1% w/v BSA increased non-specific binding of IgG to the sensor surface rather than reduced it. This is likely due to BSA coating the walls of the fluidic tubing, preventing the loss of IgG and increasing the concentration in the flow cell. BSA is often used to prevent losses of protein to tubing and container surfaces. It coats the plastic (hydrophobic) walls preventing proteins from binding to them, which would reduce the protein concentration in solution. The use of BSA keeps analyte concentrations more stable in biological assays where significant losses to container walls can occur. Therefore, additives such as BSA are used not just for their ability to prevent nonspecific binding but also to prevent analyte loss to the tubing walls, which is especially-

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evident at low concentrations. The increase in non-specific binding observed in this experiment suggests that there was an increase in free IgG concentration due to the presence of BSA and the prevention of loss to the container walls. While the BSA added was at a high enough concentration to prevent IgG losses to the walls, it was not high enough to prevent non-specific interactions between the IgG and the sensor surface.

When BSA was added at 1% w/v to the 10 mM MES pH 6.0 running buffer, an 88% reduction in non-specific binding was observed at every IgG concentration when compared to control experiments without BSA (*Figure 8*). The prevention of non-specific binding at this concentration demonstrates that enough BSA was present in the solution to coat the tubing walls and fully surround the IgG analyte to an extent that mostly prevented interactions with the surface. This demonstrates that the use of BSA can be an effective method to reduce NSB at sufficiently high concentrations.

Surfactant (Tween 20)

Tween 20 is a non-ionic surfactant that may be used to disrupt hydrophobic interactions. It is commonly added to biological systems at concentrations ranging from 0.01% v/v to 0.05% v/v to prevent hydrophobic based nonspecific interactions. It is also added to prevent analyte losses due to binding to tubing and other containers, similar to the function of BSA.

0.05% v/v Tween 20 was added to 10 mM MES pH 6.0 running buffer and IgG injections were made over the carboxylated sensor surface. Results are shown versus the control (0% Tween 20) in *Figure 9*. Tween 20 proved to be ineffective at preventing NSB. Similar to the case with 0.1% BSA, increases in non-specific binding are observed at lower IgG concentrations. These increases are due to Tween 20 blocking IgG from binding to the tubing walls and storage containers. Tween 20 was ineffective in preventing non-specific binding because its blocking effect is primarily on hydrophobic nonspecific interactions, while the primary forces for non-specific interactions in this system are assumed to be charge-based. However, the Tween 20 did prevent losses of analyte to container walls and would be useful in the prevention of non-specific interactions in a system where hydrophobic forces are the primary cause.

BSA and Tween 20 Combined

The combined effect of 1% BSA and 0.05% Tween 20 in reducing non-specific binding was also studied. Results

are shown in *Figure 10* and are similar to 1% BSA only, suggesting that BSA plays the dominant role in reducing non-specific binding in this protein surface system and that the two additives did not interfere or enhance their prevention of non-specific binding.

High Salt Concentration (NaCl)

Salts such as NaCl are able to shield charges in solution. This shielding can prevent the charges on the protein from interacting with charges on the surface and vice-versa. 200 mM NaCl was added to 10 mM MES pH 6 to examine its effect on reducing non-specific binding. Results are shown in *Figure 12* and indicate a dramatic reduction at all IgG concentrations tested, with essentially no non-specific binding observed. Similar results were demonstrated in an experiment performed at 300 mM NaCl (data not shown).

Unlike the 1% BSA experiment, all non-specific binding was eliminated at all Rabbit IgG concentrations with the addition of 200mM NaCl. This suggests that the shielding effect of NaCl is more effective than the addition of BSA under these buffering conditions and reaffirms that the primary source of non-specific interactions in this system are charge-based.

A summary of the reduction in NSB at 100µg/mL IgG concentration compared to the control for each condition tested is summarized in *Table 1*.

Conclusions and Summary

The prevention of non-specific binding is critical when performing any SPR experiment. The methods demonstrated in this article are commonly used but often with little explanation as to why those methods were chosen and why they were effective. When approaching a new biological system for study with SPR, knowledge of the biological molecules (pI, pKa, size, sensor surface, etc.) can help the user identify the types of intermolecular forces to expect (charge-charge, hydrophobic, etc.) and can help tailor the strategy to prevent non-specific binding. In this model system, the non-specific interaction was primarily charge-based, thus conditions that blocked charged-based interactions were most effective - pH and NaCl addition. The addition of BSA was moderately effective due to its capacity to be charged within certain pH ranges, while Tween 20 had minimal effect. One could easily imagine a different system, such as the binding of fatty acids, where the addition of Tween and BSA would be vital and the addition of NaCl would have minimal impact on non-specific interactions. However, the addition of BSA and Tween 20-

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demonstrated an additional benefit, which is the prevention of analyte losses to fluidic surfaces. This effect helps keep the concentration of analyte consistent and accurate and should be considered for all studies.

Efforts should always be made to reduce nonspecific binding as much as practically possible within the constraints (pH, additive concentrations) of each experiment depending on the biological constituents of a system, some additives or buffering conditions may not be useful. For instance, non-specific binding may be completely eliminated at pH 10, however the protein of interest may deactivate or denature under those conditions. There may be instances when the complete elimination of non-specific binding may not be possible. In those cases, efforts to enhance the specific binding signal may be more effective than the complete reduction of non-specific binding. When the specific binding signal is much larger than the non-specific binding signal, the effects of nonspecific binding can be accounted for or disregarded.

Figures and Tables

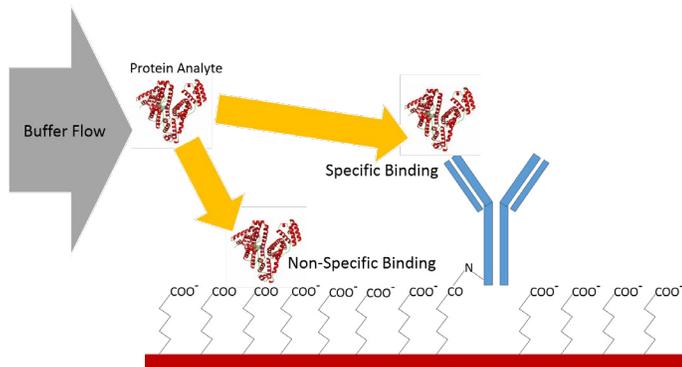


Figure 1
Non-specific binding vs specific binding of a protein analyte on a COOH coated SPR sensor chip with an immobilized ligand.

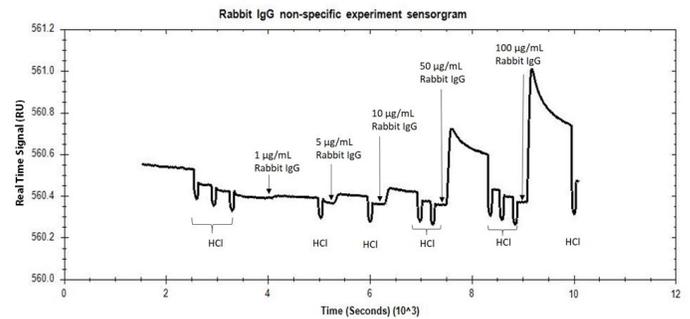


Figure 2
Example sensorgram of a non-specific binding experiment using different rabbit IgG concentrations with regeneration with HCl between each concentration.

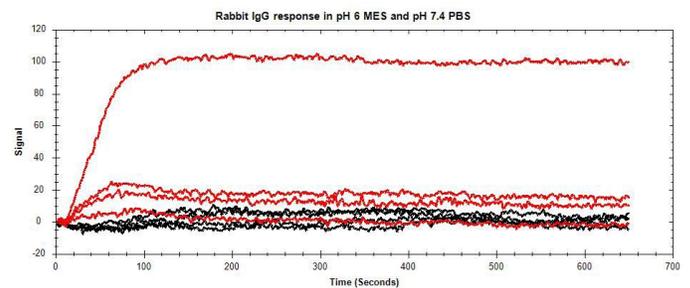


Figure 3
Rabbit IgG non-specific binding in MES buffer pH 6 (red) and 1X PBS buffer pH 7.4 (black). IgG concentrations injected were 1, 5, 10 and 50 µg/mL at both pHs.

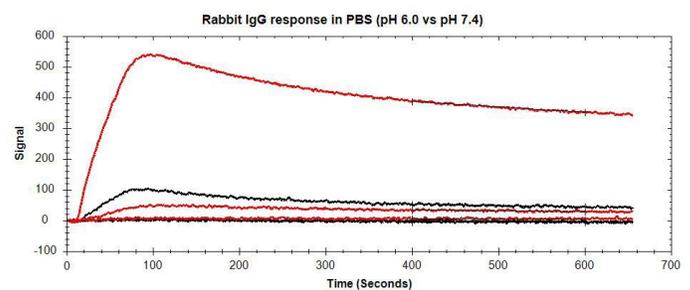


Figure 4
Rabbit IgG non-specific binding in MES buffer pH 6 (red) and 1x PBS buffer pH 7.4 (black). IgG concentrations injected were 1, 5, 10 and 50 µg/mL.

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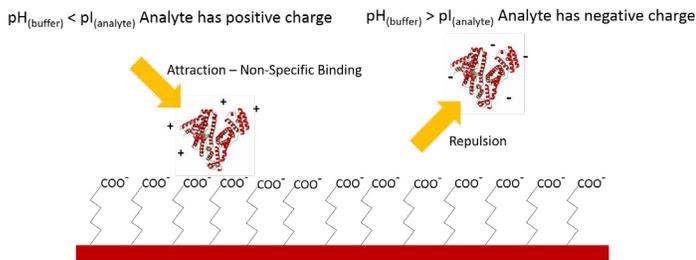


Figure 5

The effect of buffer pH on the overall charge of the protein and the resulting effect on non-specific binding to a charged surface.

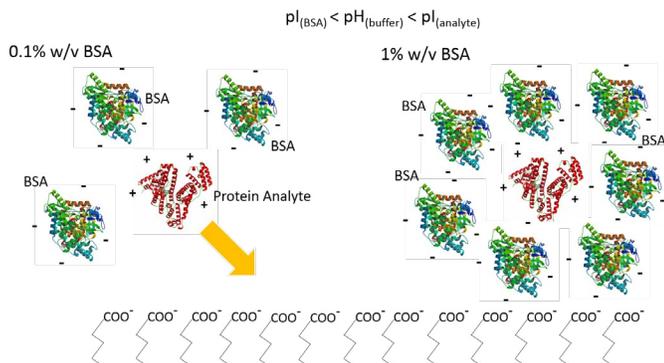


Figure 6

Concentration dependent BSA shielding a protein analyte and preventing non-specific binding with the carboxylated surface.

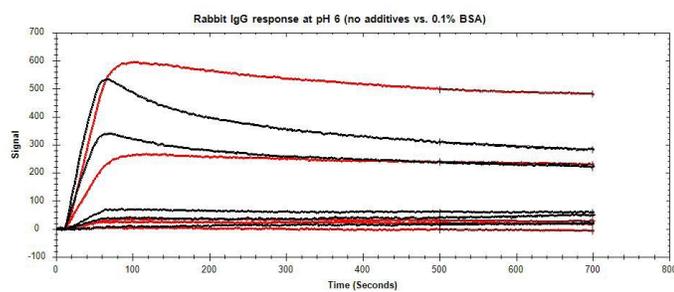


Figure 7

Rabbit IgG non-specific binding at pH 6 comparing no BSA (red) to 0.1% w/v BSA (black). Analyte concentrations used were 1, 5, 10, 50 and 100 $\mu\text{g}/\text{mL}$ of rabbit IgG.

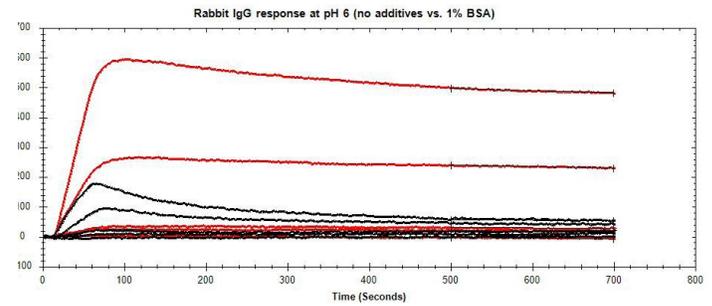


Figure 8

Rabbit IgG non-specific binding at pH 6 comparing no BSA (red) to 1% w/v BSA (black). Analyte concentrations used were 1, 5, 10, 50 and 100 $\mu\text{g}/\text{mL}$ rabbit IgG.

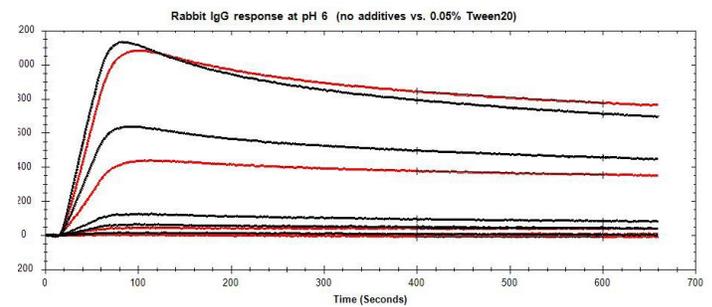


Figure 9

Non-specific binding with 0.05% v/v Tween 20 surfactant in the running buffer (black) and without Tween 20 (red). Rabbit IgG was injected at concentrations of 1, 5, 10, 50 and 100 $\mu\text{g}/\text{mL}$.

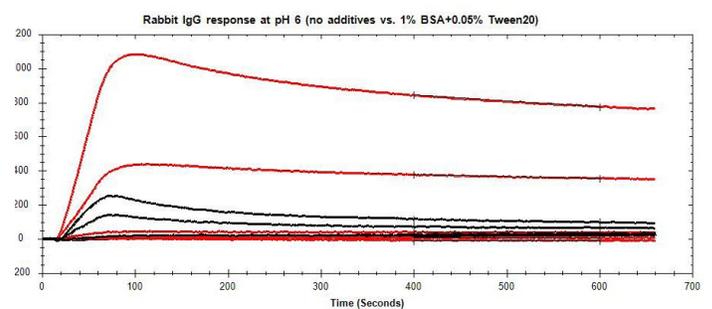


Figure 10

Rabbit IgG non-specific binding in MES buffer pH 6 with combined 1% w/v BSA and 0.05% v/v Tween 20 (black) versus the control with no additives (red). Rabbit IgG was injected at 1, 5, 10, 50, and 100 $\mu\text{g}/\text{mL}$ concentrations.

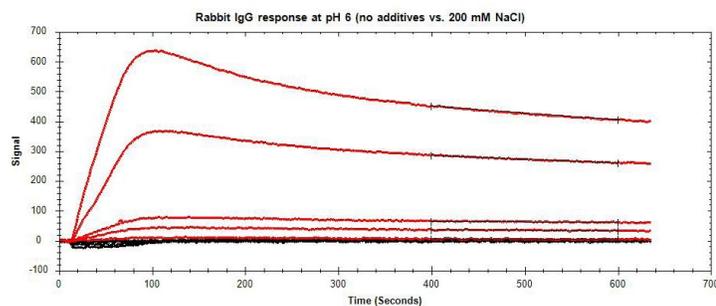


Figure 11

Rabbit IgG non-specific binding in MES buffer pH 6 with 200mM NaCl (black) versus the control with no NaCl (red). Rabbit IgG was injected at 1, 5, 10, 50, and 100 $\mu\text{g}/\text{mL}$ concentrations.

Additives/Conditions	Reduction in NSB for 100 $\mu\text{g}/\text{mL}$ IgG (%)
pH increase from 6.0 to 7.4	87%
0.1% w/v BSA	40%
1.0% w/v BSA	88%
0.05% v/v Tween 20	7%
0.05% v/v Tween 20 and 1.0% w/v BSA	87%
200 mM NaCl	100%
300 mM NaCl	100%

Table 1

Maximum reduction in NSB compared to control for each condition tested.