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Detection Methods for Small Analytes: Improvement of Novel SPR Technology Detection of Toxins and Toxicants

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**Detection Methods for Small Analytes:
Improvement of Novel SPR Technology
Detection of Toxins and Toxicants**

William Ollayos

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1.0 Abstract

Humans are exposed to a variety of toxic substances in their day-to-day activities that may have adverse health effects if not properly identified. An integrated dual-mode instrument that combines grating-coupled surface plasmon resonance (GCSPR) and grating-coupled surface plasmon coupled emissions (GCSPCE) should allow for the efficient detection of toxic analytes in a high-content system using gold-coated superparamagnetic nanobeads. The goal of this study is to validate the binding characteristics of the reagents intrinsic to this innovative assay methodology. Competition enzyme-linked immunosorbent assays (ELISA) and surface plasmon resonance (SPR) assays were conducted on NNK toxicants and SEB toxins, which were the prototype toxicant and toxin being analyzed.

2.0 Introduction

The prevalence of foreign antigens ingested by the human body has become an issue of great concern for the Food and Drug Administration [1]. Many of the products at local supermarkets come with warning labels, cleanly printed tags that list exactly which chemicals are included in the item's biochemical makeup. However, humans come into contact with a variety of toxic substances in their day-to-day activities that cannot be so easily identified. Such materials may have adverse influences on the human physiology that can only be treated if properly identified [2]. Ciencia, Inc and the laboratory group of Dr. Michael Lynes have developed an integrated dual-mode instrument that combines grating-coupled surface plasmon resonance (GCSPR) and grating-coupled surface plasmon coupled emissions (GCSPCE) in a manner that should allow for more efficient detection of toxic analytes in a high-content system. The instrument system enables the simultaneous measurement of thousands of different analytes in a single sample. Diagnostic processes are expedited by efficient detection mechanisms that can identify toxic agents. The novel technology will allow for simultaneous, rapid, efficient and cost-effective detection of a broad range of toxic analytes that humans might encounter in their everyday environment. The intention of this study is to validate the binding relationship of the reagents intrinsic to this innovative assay methodology.

The hybridization of GCSPR and GCSPCE technology will improve detection methods of small analytes as compared to current SPR technologies. Label-based methods, for example, can compromise protein function and therefore result in erroneous data [6]. Specific protein-binding molecules can be immobilized upon GCSPR sensor chips. Illumination of the chip by light of the proper wavelength, angle of incidence and polarization will cause energy from the incident light to couple with electrons of the gold metal coating [6]. The coupling creates what is

known as a surface plasmon at the metal-dielectric interface. Binding events that occur at the surface of the gold-coated chip will cause a shift in the SPR angle, which can be measured by a CCD camera as an indication of binding. SPR measures binding of unlabeled proteins to the immobilized, specific protein-binding molecules; GCSPR can therefore detect protein binding at the sensor surface [6].

GCSPCE is a fluorescent protein microarray technology that has higher sensitivity than similar assays. The surface plasmons generated at the metal-dielectric interface provide directional excitation to fluorophores positioned within 200 nm of the chip surface [7]. The strength of the excitation field results in an increase in fluorescent signal when compared to standard fluorescent immunoassays. GCSPCE can therefore effectively detect binding within regions of interest printed upon a gold-coated chip [7].

The novel GCSPR/GCSPCE technology employs spatially encoded capture molecules specific to a single analyte type. Therefore, this technology can be used to determine if a certain analyte is present in a sample. The novel GCSPR/GCSPCE technology will allow for the simultaneous detection of multiple toxins and toxicants in a cheaper and less labor-intensive manner than current label-based immunoassays. The integrated GCSPR/GCSPCE dual-mode instrument employs superparamagnetic nanobeads that boost the signal typically acquired through SPR assays, enabling the simultaneous measurement of thousands of different analytes in a single sample.

Differing configurations of the analytes and antibodies on the sensor chip surface versus the nanobead surface enhance the signal retrieved during SPR assays, indicating the binding between reagents (Figure 4). The dual mode nature of this technology should allow for a large dynamic range with high sensitivity and a low limit of detection [7].

The toxic analytes studied are categorized as substances that can be easily spread among individuals and into the human environment. Toxins are poisonous substances generated by living cells. Toxicants, however, are forms of poison that are created by humans and introduced by their activity into the surrounding environment. Cigarette smoke is a complex mixture of toxicants and it is known to contain a variety of dangerous carcinogens that can be exhaled into the environment [2]. Humans can come into contact with these carcinogens, specifically benzo(a)pyrene (B(a)P) and nicotine-derived nitrosamine ketone (NNK), either by smoking the cigarettes themselves or through second-hand inhalation of the toxic smoke [2]. NNK is a metabolite of nicotine and B(a)P is a product of combustion. SEB is produced by the gram-positive bacteria *Staphylococcus aureus* and is an enterotoxin with pathological effects on the human body. The toxicants (B(a)P and NNK) and toxins (SEB) studied were selected because of their relevance to human health.

Mechanisms of toxic action can follow either non-specific or specific mechanisms [10]. Non-specific modes of toxic action cause narcosis, the generalized and broad-sweeping depression of biological activity that results from toxic exposure. A mechanism of toxic action is considered specific when the toxins or toxicants modify physiological processes by binding to a specific site at low concentrations. Specific modes of toxic actions include uncouplers of oxidative phosphorylation (which prevent ATP production), respiratory blockers and irritants that inflame tissue at the site of contact [10].

Small molecules cannot be directly detected because SPR technology measures mass, and the small molecules contribute little on a molar basis. The competition approach to these GCSPR/GCSPCE assays may therefore improve the current limits of detection. This study developed measurements for NNK and SEB in order to validate the essential binding relationship

between these antigens and their antibodies. In order to do so, a standard curve was generated of the detection of these two substances using a reference “gold standard” technology acting as a basis for comparison. This comparison technology was an ELISA-based detection. While an ELISA-based detection determines the presence of binding between one specific antibody/antigen pair through color-changing enzymes read in microtiter plates (Figure 11), an SPR approach allows for the detection of multiple antibody/antigen pairings through label-free platforms [6].

2.1 Small Analytes

2.1.1 NNK

4-(N-Methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone (Figure 1) is a tobacco-specific nitrosamine (TSNA) known as NNK [3]. NNK can be metabolized to form the derivative *N*-NAL, which can be detected in smokers’ urine and plasma samples [2]. Animal studies have proven that NNK is one of the most potently carcinogenic substances of the TSNA family. P-450 mediated α -hydroxylation reactions involving NNK create electrophiles able to interact with DNA and other crucial macromolecules, leading to carcinogenic products [3].

Covalent modification of DNA induces irreparable mutations in the genome that can cause malfunctioning repair processes and uncontrolled DNA replication during the cell cycle’s S-phase [3]. Exposure to NNK can therefore predispose contaminated cells to tumorigenesis.

2.1.2 SEB

Staphylococcal enterotoxin B (Figure 2) is an enterotoxin produced by *Staphylococcus*

aureus, a gram-positive bacteria. Exposure to SEB is known to promote the initiation of immune disorders in human intestines [9]. SEB can be found in food that, upon ingestion by humans, leads to classic food poisoning symptoms such as severe diarrhea, nausea and cramping. The immune system will release a massive amount of cytokines in response to SEB exposure, resulting in severe inflammation of the human body. SEB can also result in a potentially fatal illness known as toxic shock syndrome.

2.1.3 B(a)P

Benzo(a)pyrene (Figure 3) is a polycyclic aromatic hydrocarbon that is a product of combustion and is most often associated with human exposure as a consequence of smoking tobacco [2,4]. Exposure to B(a)P has been shown to cause DNA damage similar to that of NNK. B(a)P's mutagenic effects have been proven on somatic cells and several environmental protection agencies have registered B(a)P as a reproductive toxicant. Benzo(a)pyrene diolepoxide (BPDE-DNA), a diol epoxide adduct, levels increase with B(a)P metabolism, which cause DNA breaks during attempts to excise and repair the areas of mutation in exposed cells. Direct genotoxicity can also increase from the production of reactive oxygen species that is attributed to B(a)P exposure [5].

2.2 Detection Methods

2.2.1 ELISA

An enzyme-linked immunosorbent assay (ELISA) is a high sensitivity detection method [7]. In a sandwich ELISA, specific antibodies will coat the surface of a polystyrene well and can

subsequently capture the cognate analyte. A second antibody is then added to the wells so that the analyte is bound on either side, forming a “sandwich.” This secondary antibody will be coupled to an enzyme marker that causes a substrate to change color in proportion to the amount of analyte in the well [8]. Antibodies with the proper binding sites will enable specific capture of the analyte in the solution.

In order to evaluate the proper antibody sets for B(a)P and NNK, reagent validation was performed using ELISA and standard curves were generated. The ELISA method employs immobilizing antibodies that will bind their cognate analytes when mixed in separate wells; if the antibodies selected work with ELISA, then it is reasonable to assume that the same antibodies will work in SPR. A standard curve produced by the ELISA assay will verify that the proper binding occurred between antibodies and their cognate antigens. Once the limits of detection (LOD) and the sensitivity have been determined by ELISA, implementation of the SPR technology can begin. The antibodies will only be able to detect certain concentrations of analyte [8]. These values can then be used on the surface-bound receptors of the SPR to investigate what concentrations of the analyte must be present in the sample for the technology to detect it.

2.2.2 SPR

The GCSPR/GCSPCE integrated technology functions more effectively than current SPR measuring devices. SPR, or surface plasmon resonance, is the oscillation that occurs when valence electrons are stimulated by light. A grating-coupled surface plasmon resonance (GCSPR) sensor chip employs a layer of gold on a diffraction grating and a variety of immobilized specific capture molecules arranged in regions of interest (ROIs) on the gold surface [6]. When this chip is exposed to light of the appropriate wavelength, phase velocity, and

angle, the electrons of the metal will become excited as “surface plasmons.” As this coupling of light to the electrons occurs at the surface of the chip, the amount of reflected light decreases in proportion to the energy transferred to the plasmons. Proteins that may bind to the surface of the chip affect the local refractive index and thus the angle at which this coupling occurs. SPR can therefore be used to measure analyte-binding to surface-bound capture molecules, which can be monitored with an imaging detector known as a charge-coupled device (CCD) camera [6].

The introduction of gold-coated superparamagnetic nanobeads should increase the signal typically acquired through SPR assays by boosting the mass change that occurs after binding between the antibodies and their cognate antigens. The antibodies may be conjugated to the chip and the analytes conjugated to nanobeads or vice versa (Figure 4). These configurations should ultimately yield a more effective diagnostic tool for use in instances of toxic exposure.

3.0 Materials and Methods

3.1 BCA Protein Assay

NNK, anti-NNK, NNK conjugated to BSA and NNK conjugated to ovalbumin were generously provided by Dr. Lawrence Silbart and Dr. Amy Howell. The BCA protein assay (using PierceTM BCA Protein Assay Kit) was run to determine the concentrations of NNK-OVA and NNK-BSA. The BCA assay relies on the reduction of copper ions in an amount proportional to the quantity of protein found in solution. Comparing the absorption spectra of the unknown samples to known standards indicated the amount of protein present in solution. A dilution series of known albumin standards was added to separate wells in the microtiter plate, and compared to 10 ul of NNK-ova, 10 ul of 1:10 dilution of NNK-ova, 10 ul NNK-BSA and 10 ul of 1:10 dilution of NNK-BSA. BCA working reagents were then made in a ratio of 1:50 and added to each well. The absorption spectrum was measured at 560 nm. This assay was also used to determine the concentration of SEB aliquots using 10 ul of SEB and 10 ul of 1:10 dilution of SEB.

3.2 Titration ELISA

Titration ELISAs were done to determine the proper concentration of NNK reagents to use in subsequent SPR assays. The titration began by coating Immulon 2 HB plate with 10 ug/ml of NNK, NNK-OVA or NNK-BSA and incubating for 1 hour at 37°C. The plates were washed and then blocked with 2% BSA. The plate was then incubated for 1.5 hours at 37°C and then washed again. The primary antibody dilution series began by mixing 20.8 ul of NNK antibody in 629.2 ul of binding buffer (0.25 g BSA in 100 ml PBST). 100 ul of each dilution was then added

across the columns of wells. The plate was incubated for 1 hour at 37°C and then washed. The secondary antibody (Goat anti-mouse Ig-AP 10.8) was mixed with binding buffer, incubated on plate for 1 hour at 37°C and then washed. P-nitrophenyl phosphate (PNPP) tablets were dissolved in substrate buffer and 100 μ l were added to each well. The absorption spectrum was then read kinetically at 450 nm across 10 minutes. The experiment was repeated with SEB and anti-SEB reagents in order to determine the proper concentration of SEB for use in subsequent SPR assays.

3.3 SPR

Reagents included 95% ethanol and ultrapure H₂O (18 m Ω). Spotting was done with the SpotBot II Automated Spotting System (ArrayIt Microarray Technology) on gold GCSPRI/SPCE dual mode sensor chips provided by Ciencia, Inc. 384 well low-protein binding V bottom Microplates were purchased from ThermoScientific, Inc. Capture antibody to be immobilized on sensor chips was dissolved in PBS.

3.3.1 Preprinting of Sensor Chip

Aliquots of capture and control antibodies were added to wells of a 384 well microplate. The plate was then covered with adhesive foil and spun at 500 rpm for 4 minutes at 4°C to ensure all liquid had reached the bottom of the wells. Gold sensor chips were rinsed with 95% ethanol and H₂O (18m Ω) for 10 seconds each. Chips were then dried using pressurized filtered air and placed in a plastic petri dish, leaving the coated gold surface facing up. Nonspecific absorption occurred between reagents and the chip surface.

3.3.2 Preparing the Robotic Spotter

The wash buffer, vacuum and humidity of spotting apparatus were activated. A new spotting procedure was built that designated for printing the number of required replicates onto the sensor chip. The pin head was removed from the machine using gloves and washed with 95% ethanol and then sonicated in H₂O (18 mΩ) and dried with pressurized filtered air. The pin was studied under 10x magnification of microscope to ensure that it was clean. The plate was placed into the spotting machine.

3.3.3 Sensor Chip Printing

The chips were washed with H₂O (18 mΩ) for 10 seconds and pressurized filtered air was used to dry them. The chips were inserted into the spotting apparatus. Once the spotting had finished, the plate and the spotting pin were removed. The wash, vacuum, and sonication components of the spotting machine were deactivated. The chips were incubated in a humid environment for approximately 1 hour and then stored at 4°C in the dark for up to two weeks.

3.3.4 Competition Assay

Each chip was blocked with 2% bovine serum albumin (BSA) for thirty minutes. In separate experiments, a dilution series starting with a 1:32.5 dilution of SEB antibody and NNK antibody were incubated with 10 ug/ml of free SEB or free NNK, respectively, for thirty minutes for use in the competition chambers. Uncompeted SEB or NNK was added to the other chamber on the dual chamber SPR chip. Both chambers were washed with PBST three times and then incubated with goat anti-mouse IgG-Alexa 647 at 2 ug/ml. The chips were then washed three times with PBST and read fluorescently with an exposure of five seconds.

4.0 Results

4.1 BCA Results

NNK-BSA was found to be at a concentration of 1.1075 mg/ml. NNK-OVA was found to be at a concentration of 0.605 mg/ml. SEB was found to be at a concentration of 0.327 mg/ml (Figure 5).

4.2 Titration ELISA Results

The results of the BCA identified the concentration of the NNK-ova and NNK-BSA solutions, but the concentration of the NNK antibody was still unknown. Titrations produced a curve that ranged from 1:62.5 to 1:6400 doubling dilutions. The titrations allowed for the determination of the dilution factor that will be most effective in subsequent SPR assays (Figure 6).

The laboratory of Dr. Michael Lynes had access to four separate sources of secondary antibody (Goat anti-mouse Ig-AP 10.8). A titration was performed to determine which vial gave the highest signal on the plate reader and should therefore be used in all titrations of NNK and SEB. Tube 3 was found to give the highest reading and was therefore used in subsequent titrations (Figure 7).

Titrations were performed using purchased SEB and SEB antibody (Figure 8). Neither titration formed a typical titration curve, but instead dropped off where it would typically plateau. When graphed on log scale, both graphs showed a distinct peak at 1:1000 dilution of SEB antibody.

4.3 SPR Results

A two chamber gasket was used on the chip in order to test the competition between NNK-OVA/NNK-BSA conjugated to the chip versus free NNK flowed over the chip. Each ROI showed significant competition with free NNK (Figure 9). The fluorescent units per second exposure sharply decreased when competing with free NNK.

A similar assay was conducted that competed free SEB with SEB preincubated with SEB antibody. Each ROI also showed significant competition with free SEB (Figure 10). The fluorescent units per second exposure sharply decreased when competing with free SEB.

5.0 Discussion

The results of the BCA identified the concentration of the NNK-OVA and NNK-BSA solutions, but the concentration of the NNK antibody was still unknown. The solution of NNK-antibody provided by Dr. Silbart was a mixture of antibody and tissue culture media supplemented with fetal bovine serum proteins; the presence of irrelevant proteins in the NNK-antibody sample required that it be regarded in terms of relative dilution factor, and not absolute concentration. The titrations would allow for the determination of which dilution factor will be most effective in subsequent SPR assays. Furthermore, the titration allowed for the construction of a curve to examine the activity at a range of concentrations. The shape of these curves is indicative of effective binding between antibodies and their antigens. The titrations therefore demonstrated that the NNK-antibody was effectively binding the NNK analytes when tested in polystyrene wells and would likely do the same on the SPR chip (Figure 6).

The NNK-OVA and NNK-BSA yielded curves when titrated. However, the NNK (which was not conjugated to a large protein) yielded relatively flat lines that did not form the expected curves. The unconjugated NNK failed to bind effectively to the plate, though the reason for this failure is unclear. It is understood that the large ovalbumin and bovine serum albumin proteins are able to bind effectively to the polystyrene well (Figure 11). The larger proteins will bind to the well and present the NNK analyte to the primary antibody.

The SPR data revealed that competition assays can be successfully performed on an SPR chip using the NNK-OVA and NNK-BSA reagents. The presence of free NNK significantly reduced the resulting signal, indicating that the proper binding between antibody and antigen was occurring.

The SEB titrations did not form typical curves when titrated. The curves would begin

sloping upwards and then drop steadily down where it would normally plateau. The drop in the curve was likely caused by nonspecific-binding; the primary antibodies may have bound to the well instead of the SEB antigens. Furthermore, inefficient hand washing with PBST may have left some unbound primary antibody in the wells, allowing the secondary antibodies to bind nonspecifically. Nevertheless, a distinct peak was visible at the 1:1000 dilution factor, indicating the optimal dilution for use in subsequent SPR assays.

The SPR data performed with the SEB reagents gave results similar to the SPR assays performed using NNK reagents; competition assays can be successfully performed on an SPR chip using the SEB reagents. Binding between the SEB antibody and the SEB antigen did occur because the presence of free SEB significantly reduced the resulting signal. The SPR data of both the SEB and NNK reagents showed high signals even at the lowest dilutions, indicating that far lower concentrations of the small molecules could be detected using this integrated technology.

In the future, configuring NNK reagents using the superparamagnetic nanobeads should efficiently yield even more distinct readings because of the increase in mass. The steep increase will affect the dielectric interface of the SPR assay in such a way as to give even more prominent readings.

B(a)P reagents still need to be validated through titrations and SPR assays. B(a)P, similarly to the titrations of unconjugated NNK, failed to bind effectively to the plate during initial titrations. It is theorized that the small size of B(a)P and NNK render it unable to grip the polystyrene well, though other possibilities still exist. B(a)P reagents that are conjugated to larger proteins (in such a way as the NNK reagents are conjugated to ovalbumin and bovine serum albumin) are required before B(a)P titrations can proceed.

The heightened sensitivity of the integrated GCSPR/GCSPCE technology surpasses that

of typical SPR and ELISA based detections, holding potential for improved utility in the medical field. A more effective understanding of the binding relationships between antibodies and their antigens allows for expedited and more thorough diagnostic methodologies. Point-of-care testing (POCT), for example, can be enhanced through the implementation of this novel integrated technology. POCT is simple blood tests performed at a patient's bedside and often involves transportable or handheld instruments. Widespread use of the integrated GCSPR/GCSPCE technology could allow for more effective diagnoses of patients afflicted with toxic exposures. A patient would be able to provide a blood sample to a medical practitioner, who could then introduce the sample into the integrated SPR technology and verify which toxin or toxicant was affecting the patient. The integrated GCSPR/GCSPCE technology has immense potential to revolutionize the medical field of toxic detection methodologies.

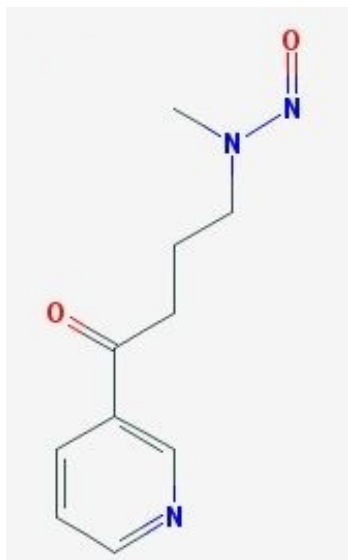
Figures

FIGURE 1. NNK Structure. Nicotine-derived nitrosamine ketone (NNK) is present in tobacco products and acts as an exceptionally powerful carcinogen. It is an aromatic compound and a yellow, crystalline solid with a molecular weight of 207.23 g/mol.



Figure 2. SEB Structure. Staphylococcal enterotoxin B (SEB) has a molecular weight of 28 kD and contains 239 amino acids residues [11]. SEB has two domains with complex tertiary structures that allows SEB to become resistance to proteases such as trypsin and papain.

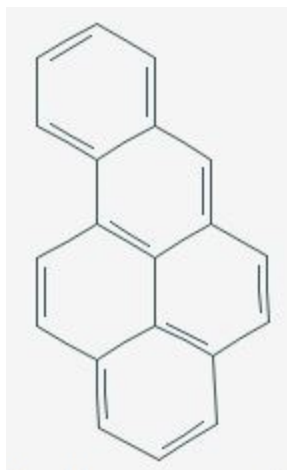


FIGURE 3. B(a)P Structure. Benzo(a)pyrene is an aromatic hydrocarbon with a molecular weight of 252.31 g/mol. Its bulky shape can inflict serious damage to exposed DNA.

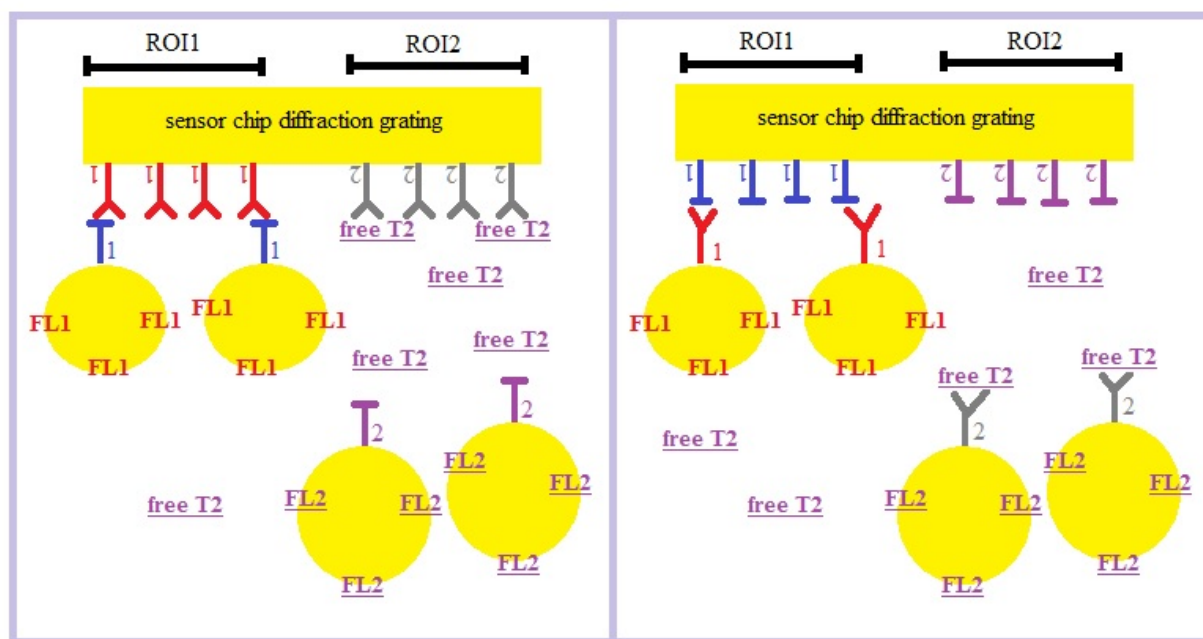


FIGURE 4. Improvement of analyte detection. The left panel displays ROIs populated by antibodies (Y1 and Y2) with each analyte (T1 and T2) coupled to the gold nanobead surface. The right panel shows the antibodies coupled to the nanobead surface while the analytes have been immobilized upon the sensor chip surface.

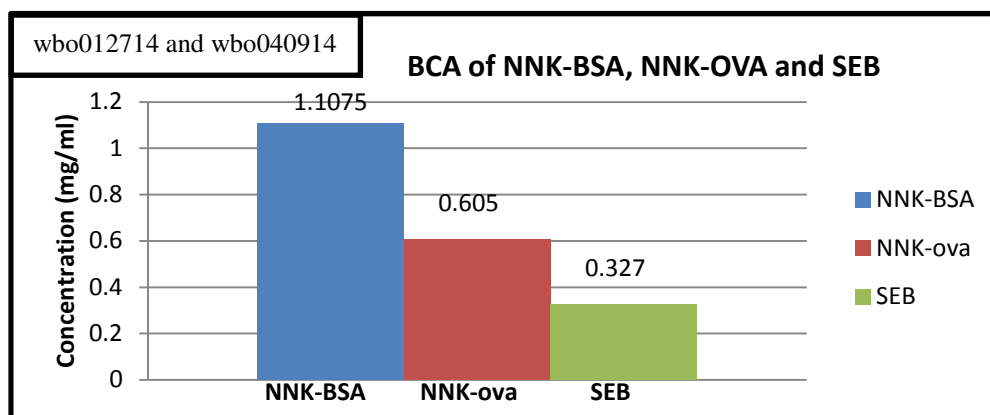


FIGURE 5 A. BCA of NNK-OVA, NNK-BSA and SEB. NNK-BSA was found to be at a concentration of 1.1075 mg/ml and NNK-OVA was found to be at a concentration of 0.605 mg/ml through BCA. SEB was found to be at a concentration of 0.327 mg/ml.

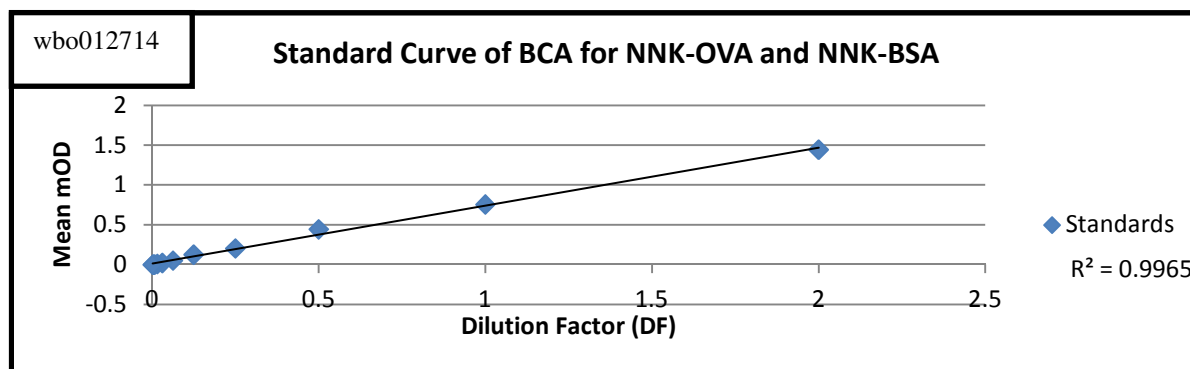


FIGURE 5 B. Standard curve of BCA for NNK-OVA and NNK-BSA. The above curve was generated from the albumin standards used in the BCA of the SEB aliquots.

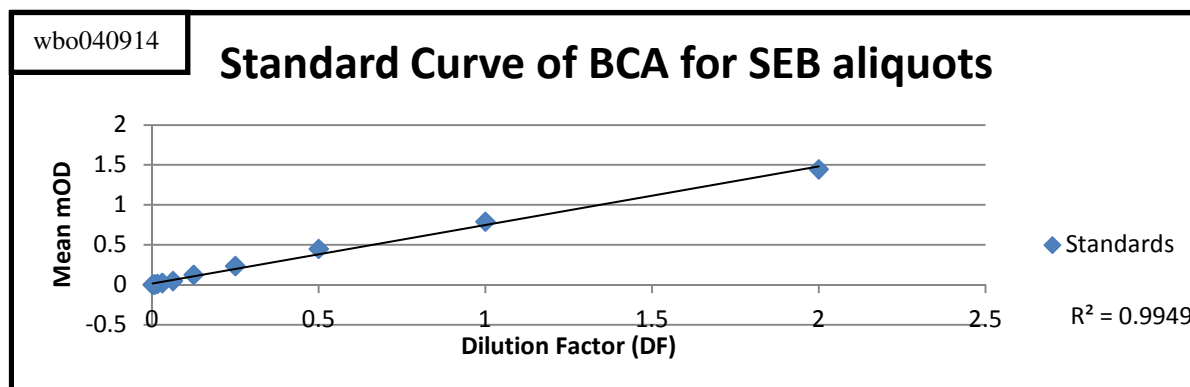


FIGURE 5 C. Standard curve of BCA for SEB aliquots. The above curve was generated from the albumin standards used in the BCA of the SEB aliquots.

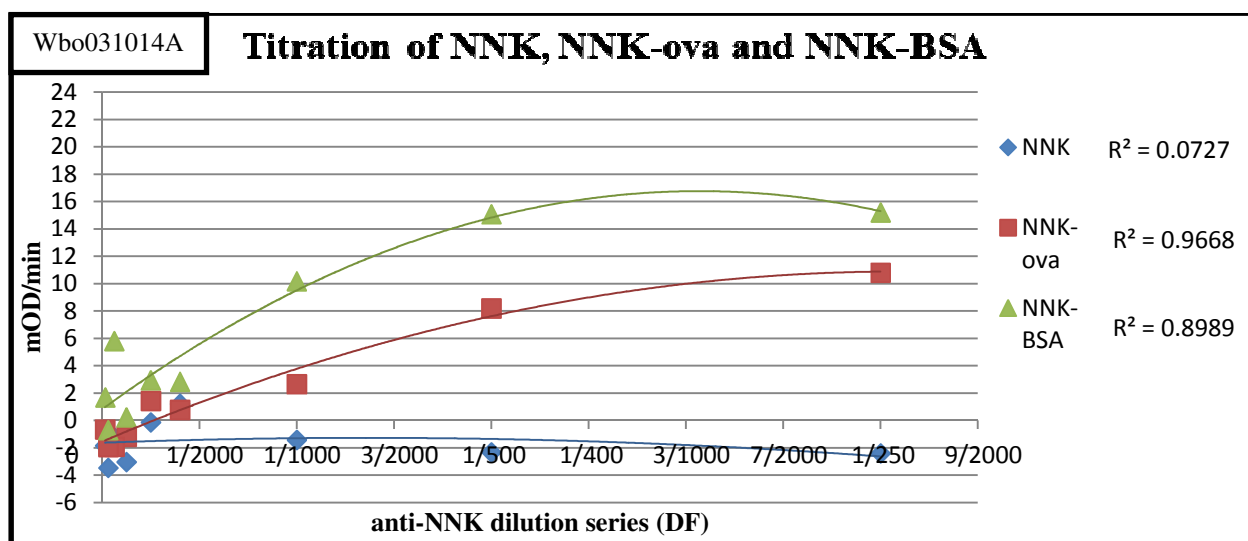
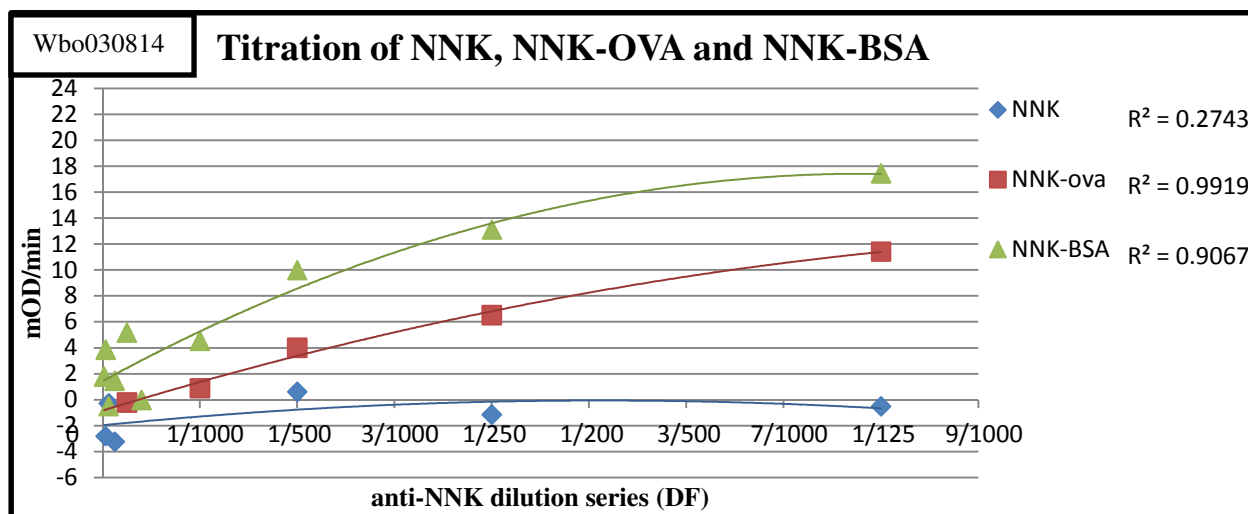


FIGURE 6. Titration of NNK, NNK-OVA and NNK-BSA. A plate was coated with NNK, NNK-OVA and NNK-BSA, each at 10 ug/ml. The plate was blocked with 200 ul/ well of 2% BSA. A dilution series of primary NNK antibody was added across all rows. Secondary antibody (Goat anti-mouse Ig-AP 10.8) was added across all rows. 1 mg/ml PNPP in AP Substrate Buffer was added across all rows. The plate was read kinetically at 405 nm for 10 minutes.

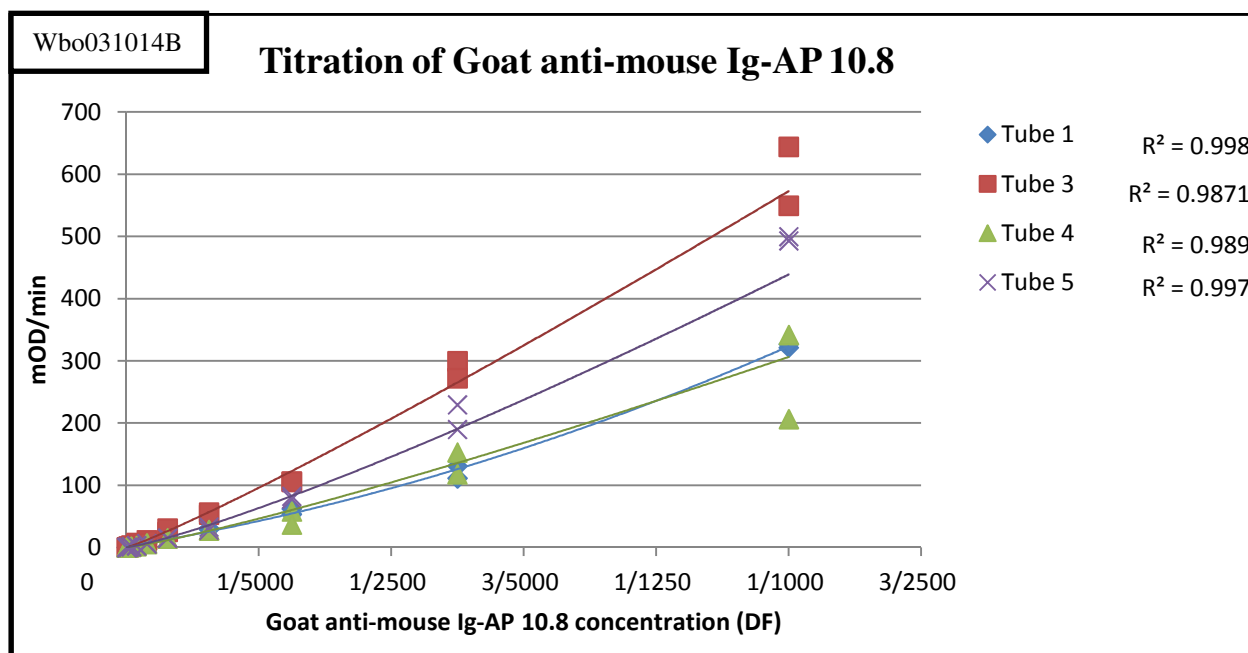


FIGURE 7. Titration of Goat anti-mouse Ig-AP 10.8. All 10.8 antibody tubes were collected; they were numbered 1, 3, 4 and 5. 1000 ul of PBS was mixed with 1 ul of each of the four antibodies (one from each set) and then made into four series of doubling dilutions. 100 ul of each tube in each series was put into a well and incubated in Immulon 2 HB plate for 1 hour at 37°C. The plate was washed. Two PNPP tablets were dissolved in 10 ml of substrate buffer and 100 ul was added to each well. The plate was read at 405 nm for 10 minutes with a 37 second interval.

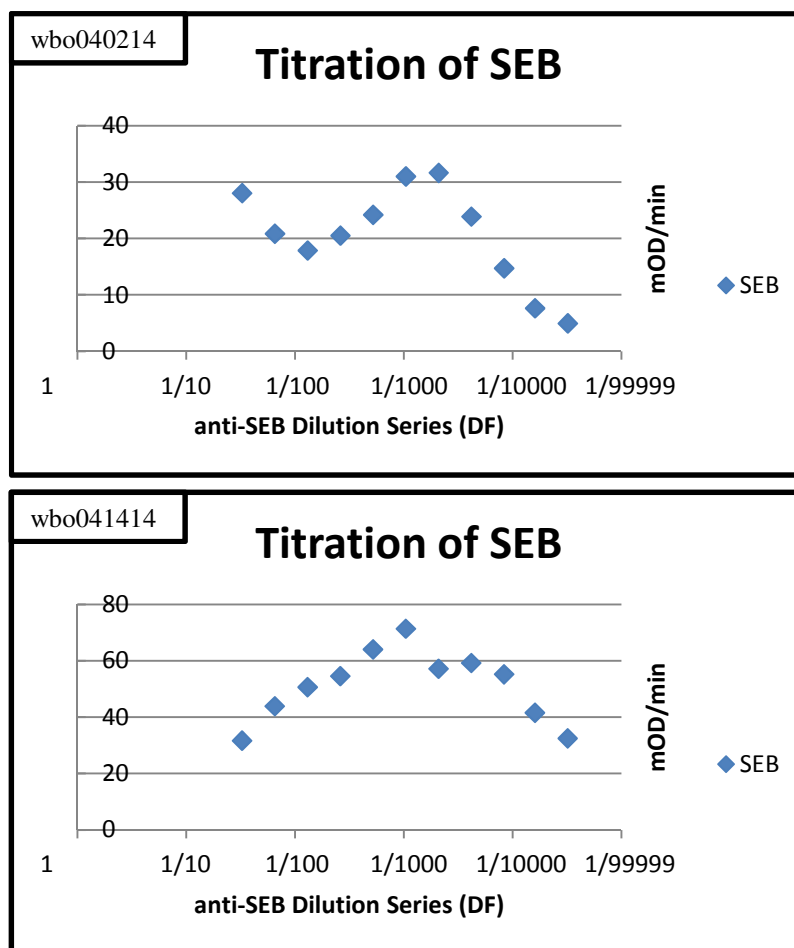


FIGURE 8. 123 μ l of SEB aliquots (each at a concentration of 0.327 mg/ml) was mixed with 3877 μ l of coating buffer to yield a solution of 10 μ g/ml. Three rows of Immulon 2 HB plate were coated with 100 μ l of solution and then incubated 1 hour at 37°C. The plate was washed and blocked with 200 μ l/well of 2% BSA. Dilution series of SEB antibody, starting with a 1:32.5 dilution and going down by half, was made in binding buffer and 100 μ l from each tube was added down the three rows. The wells were incubated for 1 hour at 37°C. 100 μ l of secondary antibody (Goat anti-mouse Ig-AP 10.8) was added to each well and incubated for 1 hour at 37°C. PNPP tablet was dissolved in 5 ml of substrate buffer and 100 μ l was added to each well. The plate was read at 405 nm for 10 minutes with a 37 second interval.

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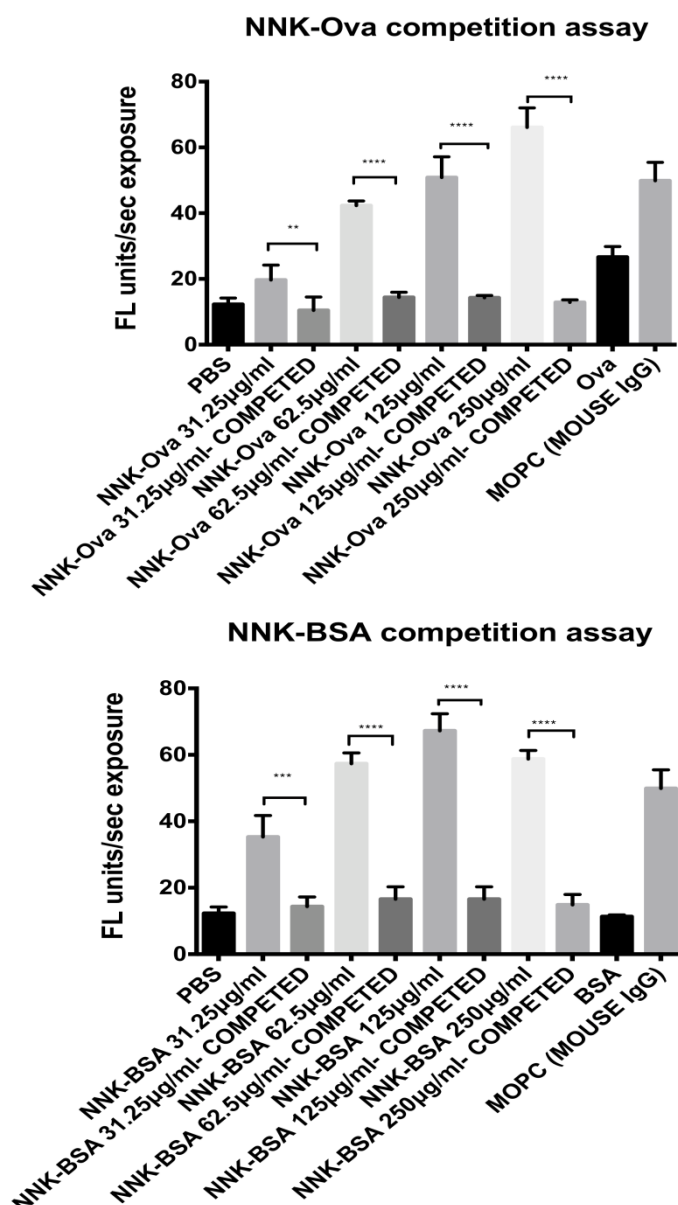


FIGURE 9. Significant competition with free NNK on an SPR chip. There was significant competition between NNK-OVA and NNK-BSA with free NNK on chip. Free NNK was able to compete the binding between NNK antibody and NNK conjugated to the chip. For competition, free NNK was preincubated with 1:160 dilution of monoclonal NNK antibody for 1 hour. The competed and uncompleted NNK antibodies were added to the chip. The chip was incubated with goat anti-mouse IgG-Alexa 647 at 2 ug/ml. The chip was washed with PBST and read under FL mode for 5 seconds exposure. **P = < 0.01; ***P = < 0.0005; ****P = < 0.0001

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Staphylococcus Enterotoxin B (SEB) competition assay

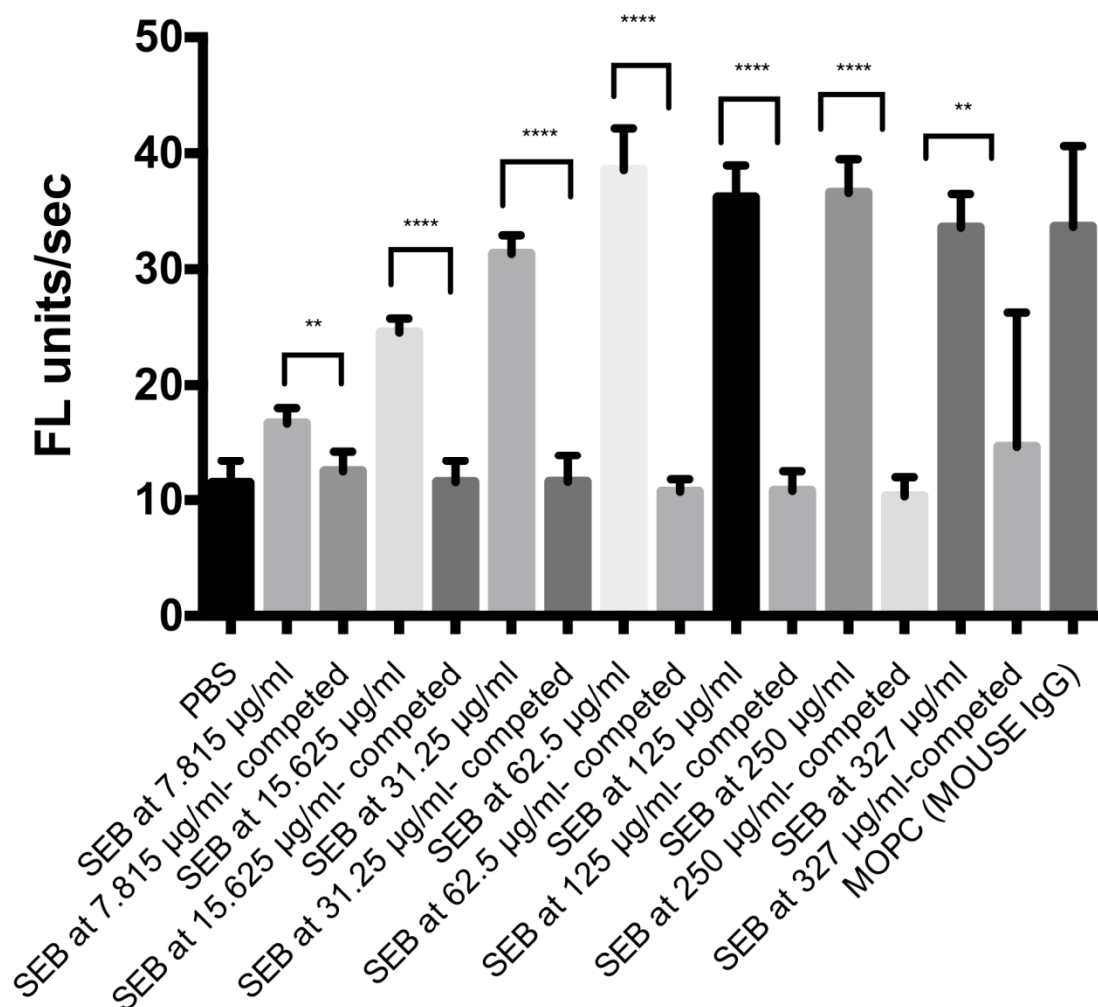


FIGURE 10. Significant competition with free SEB on an SPR chip. There was significant competition between SEB and SEB antibody at all concentrations. Free SEB was able to compete the binding between SEB antibody and SEB conjugated to the chip. The chip was blocked with 2% BSA for 30 minutes. For competition, free SEB was preincubated with 1:500 dilution of monoclonal SEB antibody for 1 hour. The competed and uncompleted SEB antibodies were added to the chip. The chip was incubated with goat anti-mouse IgG-Alexa 647 at 2 ug/ml. The chip was washed with PBST and read under FL mode for 5 seconds exposure. **P = < 0.01; **** P < 0.0001.

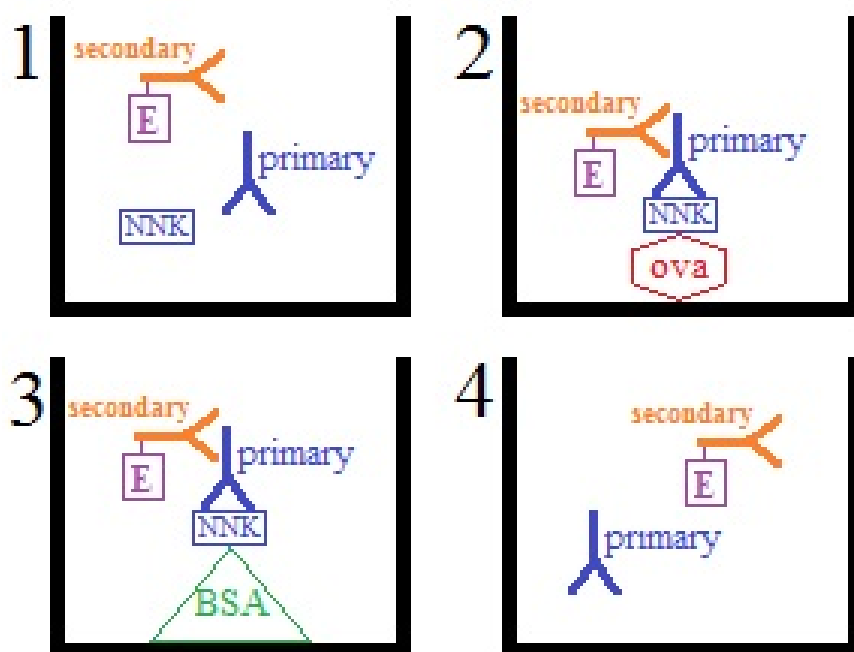


FIGURE 11. Configuration of titration ELISAs. In Well 1, the NNK analyte binds to the plate at an undetectable level. The large ovalbumin in Well 2 and bovine serum albumin in Well 3 bind to the plate and present the conjugated NNK analyte to the primary NNK antibody. The anti-NNK is followed by a secondary antibody (Goat anti-mouse Ig-AP 10.8) that is conjugated to an enzyme. Well 4 served as a blank and did not receive any NNK analytes.

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