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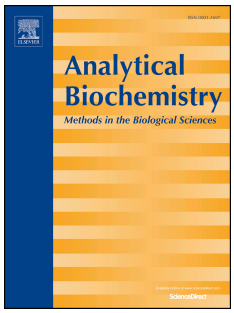

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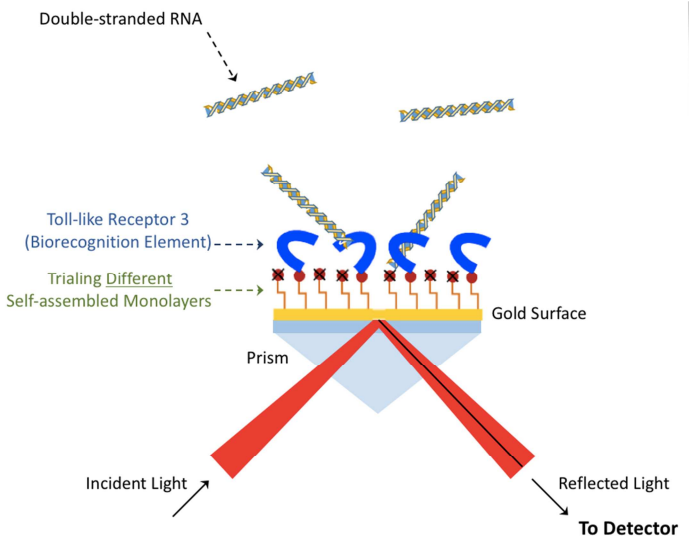

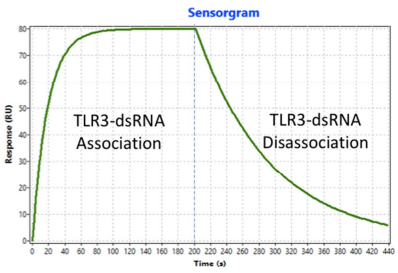

INVESTIGATION OF BINDING CHARACTERISTICS OF IMMOBILIZED TOLL-LIKE RECEPTOR 3 WITH POLY(I:C) FOR POTENTIAL BIOSENSOR APPLICATION

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Abstract

Toll-like receptor 3 (TLR3), a pathogen recognition receptor of the innate immune response, recognizes and is activated by double-stranded RNA (dsRNA), which is indicative of viral exposure. A sensor design exercise was conducted, using surface plasmon resonance detection, through the examination of several immobilization approaches for TLR3 as a biorecognition element (BRE) onto a modified gold surface. To examine the TLR3-dsRNA interaction a synthetic analogue mimic, poly(I:C), was used. The interaction binding characteristics were determined and compared to literature data to establish the optimal immobilization method for the TLR3 BRE. A preliminary evaluation of the efficacy of the selected TLR3 surface as a broad-spectrum viral biosensor was also performed. Amine-coupling was found to be the most reliable method for manufacturing repeatable and consistent TLR3 BRE sensor surfaces, although this immobilization schema is not tailored to place the receptor in a spatially-specific orientation. The equilibrium dissociation constant (K_D) measured for this immobilized TLR3-poly(I:C) interaction was 117 ± 3.30 pM. This evaluation included a cross-reactivity study using a selection of purified *E.coli* and synthetic double- and single-stranded nucleic acids. The results of this design exercise and ligand binding study will inform future work towards the development of a broad-spectrum viral sensor device.

Key Words: toll-like receptor, biorecognition element, pathogen-associated molecular pattern, surface plasmon resonance, sensor design

1. Introduction

The innate and adaptive immune response of higher order vertebrate species provide an integrated protection and defence system against invasion by foreign pathogens. The innate immune response acts as an “early warning system” by recognising and using a signalling mechanism to announce the presence of pathogenic antigens based on the innate immune systems response to conserved pathogen structures. This broadcast, via established signalling pathways, is coordinated through the use of several classes of pattern recognition receptors (PRRs), which are expressed on, within, or are secreted by many cell types [1]. The activation of the innate immune response does not translate into long-term protection of the host against repeat

exposures to the same or similar pathogens. The adaptive immune response is more specific, employing highly specialised processes that are initiated by the innate immune system thus increasing a host's ability to respond to future pathogen challenges.

The present work focuses on one suite of the PRRs that are vital components of the innate immune response, toll-like receptors (TLRs). TLRs are type I transmembrane glycoproteins that are expressed by a variety of immune and non-immune cells. There are suites of ten and twelve TLRs that have been identified in humans and mice, respectively, with TLR1 – TLR9 present in both species [2,3]. TLRs are activated by a collection of evolutionarily conserved pathogen-associated molecular patterns (PAMPs), which are unique and recurring for each generic microbial classification, Gram-negative or Gram-positive bacteria, viruses and fungi, regardless of species, strain or pathogenicity [4]. TLRs 1, 2, 4, 5 and 6 are expressed extracellularly and primarily recognise microbial surface components such as proteins, lipids and lipoproteins [5]. Several of the extracellular TLRs have the ability to bind to an assortment of PAMPs of varying pathogenic origin as long as they contain the structural moiety that is required for recognition [6]. TLRs 3, 7, 8 and 9 are found in intracellular compartments such as endosomes, lysosomes and endoplasmic reticulum and their ligands are nucleic acids [5]. There are significant structural similarities between each of these TLRs including a horseshoe-shaped extracellular domain (ECD) consisting of 18 – 25 leucine-rich repeats (LRRs) that are directly involved in ligand recognition. A similar cytoplasmic signalling domain, referred to as the Toll IL-1 receptor (TIR) domain also occurs, which, upon PAMP recognition, recruits adaptor proteins for the proliferation of a signalling cascade [7,8].

Of interest to the present work is toll-like receptor 3 (TLR3) which specifically recognises, and is activated by, double-stranded RNA (dsRNA). Double-stranded RNA is indicative of the presence of viruses or virus-infected cells. Double-stranded RNA is not a PAMP signature solely attributed to the genome structure of dsRNA viruses but rather can be found in all members of the viral pathogen suite. In DNA viruses, dsRNA is a by-product of symmetric transcription and in single-stranded RNA viruses it is a replication intermediate [9].

In this study, the TLR3-dsRNA interaction is examined using the synthetic analogue mimic, poly(I:C). Utilising surface plasmon resonance (SPR), a sensor design exercise has been conducted through the examination of several novel immobilization approaches for the placement of TLR3 as a biorecognition element on a modified gold sensor surface. Previous

work using SPR to examine the TLR3-dsRNA interaction exploited the nucleic acid as the immobilized receptor [10]. The interaction binding characteristics were determined and compared to existing data from the literature, including both *in vivo* and *in vitro* studies, to establish the optimal immobilization method for the TLR3 biorecognition element. In addition, a preliminary evaluation of the efficacy of the selected TLR3 surface as a broad-spectrum viral biosensor was performed. This evaluation included a cross-reactivity study using a selection of purified *E.coli* and synthetic double- and single-stranded nucleic acids.

2. Materials and Methods

All SPR analyses were performed on a BIAcore 3000 instrument. Carrier-free, recombinant human TLR3, with a C-terminal 10-Histidine tag was purchased for use in these experiments. The predicted molecular mass (MM) for this biorecognition element is 116 kDa [11]. All solutions used are aqueous. The experimental reagent, biorecognition element, ligand and sensor chip acquisition information are described in the supplementary information.

2.1 TLR3 Immobilization Method #1: Ni(II) Chelation + Amine-coupling

This immobilization method employed the BIAcore nitrilotriacetic acid (NTA) sensor chip. The procedure was run at 25^{o}C using a running buffer of HEPES-buffered saline (HBS-EP, pH = 7.4), consisting of 10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 150 mM sodium chloride (NaCl), 50 µM ethylenediaminetetraacetic acid (EDTA) and 0.05 % Surfactant Polysorbate 20 (P20), with a flow rate of 5 µL/min. All buffer solutions are degassed prior to use. The analysis and reference channels of the chip were preconditioned using a two-step application cycle: 1 min, 350 mM EDTA; and 2 min, HBS-EP. The analysis channel was then activated using 0.5 mM nickel (II) chloride ($NiCl_2$) for 1 min. The unmodified carboxymethyl groups on both the analysis and reference channels were activated using 0.1 M *N*-hydroxysuccinimide (NHS)/0.5 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) for 9 min total. TLR3 recombinant protein, with a 10-Histidine tag, at a concentration of 40 nM in HBS-EP, was applied only to the activated analysis channel by manual injection until a mass of approximately 530 resonance units (RU) was deposited. This corresponds to an immobilized protein concentration of 530 pg/mm^2 [12]. Both channels were then rinsed with 350 mM EDTA (3 min); HBS-EP (4 min); and, finally, 1 M ethanolamine (14 min). Ethanolamine is used to

deactivate the unreacted NHS-esters on the sensor surface. The final surface density of the immobilized TLR3 protein was approximately 500 RU or 500 pg/mm^2.

2.2 TLR3 Immobilization Method #2: Thiol-coupling using Maleimide

A BIAcore CM5 chip (carboxymethylated dextran matrix) is used for this immobilization method. The same HBS-EP buffer (pH = 7.4) was used as the running buffer and the SPR system was run at 25^{o}C with a flow rate of 5 µL/min. The analysis and reference channels of the chip were preconditioned using a three-cycle HBS-EP running buffer rinse of 4 min each. It is a three-stage process to activate the surface prior to TLR3 immobilization: 0.1 M NHS/0.5 M EDC (7 min); 1M ethylenediamine in 0.1M sodium borate (pH = 8.5, 7 min); and 50 mM 4-maleimidobutyric acid sulfo-*N*-succinimidyl ester sodium salt (sulfo-GMBS) in 0.1M sodium borate (pH = 8.5, 5 min). Two different maleimide thiol-coupling experiments were carried out, on separate chips, by using immobilization buffers with different pHs for the TLR3 application step across the analysis channel:

Trial #1: TLR3 was applied at a concentration of 40 nM, in 10 mM acetate buffer (pH = 5.5). Manual injection was used for this application until a TLR3 mass of approximately 185 RU or 185 pg/mm^2 was obtained.

Trial #2: TLR3 was applied at a concentration of 40 nM, in a 10 mM HEPES buffer (pH = 7.4). As for trial #1, manual injection was employed and approximately 100 RU or 100 pg/mm^2 was collected at the surface.

In both cases, excess maleimide groups on both the analysis and reference channels were capped using a 50 mM cysteine and 1M NaCl solution in 0.1M sodium acetate (pH = 4.0, 10 min). The final surface density of the immobilized TLR3 protein was approximately 140 RU and 55 RU, respectively for each trial.

2.3 TLR3 Immobilization Method #3: Amine-Coupling

This immobilization method also uses a BIAcore CM5 chip; the running buffer was HBS-P (pH = 7.4, 10 mM HEPES, 150 mM NaCl and 0.05% Surfactant P20). The SPR system was run at 25^{o}C with a flow rate of 5 µL/min. The analysis and reference channels of the chip were preconditioned using a three-cycle HBS-P running buffer rinse of 4 min each. To activate the

surfaces of both channels 0.1 M NHS/0.5 M EDC is applied for 7 min. TLR3 protein at a concentration of 40 nM, in a 10 mM acetate buffer (pH = 5.5) is applied only to the analysis channel by manual injection until a mass of between 500 – 700 RU was deposited. 1 M ethanolamine is used to deactivate the unreacted NHS-esters on the analysis and reference channel surfaces. This immobilization method was found to produce a stable surface and therefore was progressively refined; various protein immobilization levels were trialed. At the end of each immobilization trial the final surface density was recorded.

2.4 TLR3 Ligand Binding Experiments

The running buffer for all ligand binding experiments across the immobilized TLR3 sensor surface is 10 mM acetate buffer (pH = 5.5) with 150 mM NaCl and 0.05% Surfactant P20. The SPR instrument operated at 25^{o}C with a flow rate of 40 µL/min. Prior to commencing binding experiments the analysis and reference channels are flushed with two acetate buffer injections (2 min each). In all binding experiments, shifts in bulk refractive index were accounted for by running blank sample injections in addition to the samples, which would then be used for correction during the data analysis stage.

Poly(I:C)-binding Experiments: A low molecular mass variant, average 200 kDa, 200-1000 base pairs,[13] of the synthetic TLR3 ligand, polyinosinic-polycytidylic acid (poly(I:C) was used for these experiments. To assess binding constants, a calibration series ranging from 0.25 nM to 3.0 nM was injected over both the analysis and reference channels using the BIAcore kinject injection profile (3 min, 120 µL) followed by a 4 min dissociation period. The channel surfaces were regenerated down to their baseline TLR3 immobilization levels, removing any remaining poly(I:C) ligand, by applying a single 30 second pulse of 10 mM borate buffer (pH = 8.5, 150 mM NaCl) followed by 3 – 4 injections (2 min) of acetate buffer to flush the remaining borate regeneration buffer from the surfaces.

Sensor Cross-reactivity Study: Endotoxin-free DNA from *E. coli* K12 was purchased from Invivogen. Gel electrophoresis was used to determine the number of base pairs and, subsequently approximate molecular mass of this nucleic acid sample (820 base pairs, ~ 500 kDa). The calibration series used for this bacterial DNA cross-reactivity trial ranged from 10 nM to 300 nM. The kinject injection profile was applied across both the analysis and

reference channels (5 min, 200 μL) followed by a 6 min dissociation period. The regeneration injection sequence was identical to that for the poly(I:C) ligand. Other nucleic acids that were studied include: poly(U), a single-stranded RNA polymer; ORN 02, an AU-rich oligonucleotide; and single-stranded, sheared *E. coli* K12 DNA, were purchased from Invivogen.

2.5 Data Analyses

Data sets were analysed using BIAcore 4.1 evaluation software to calculate the equilibrium dissociation constants (K_D). The maximum response unit, or binding response (Rmax) is also evaluated in order to determine the protein-ligand binding efficiency based on the amount of immobilized TLR3 protein.

3. Results and Discussion

As previously stated, TLR3 immobilization was originally evaluated using a synthetic analogue of dsRNA, polyinosinic-polycytidylic acid (poly(I:C)) [10]. To understand the immobilization approaches chosen and pre-immobilization considerations, such as the choice of buffer solutions, etc., it is first important to deliberate on the overall structure of the TLR3 protein, its behaviour in solution and within a ligand binding environment. The elucidation of the crystal structures of, first, the TLR3 monomer [14] and then the TLR3 homodimer bound to poly(I:C) [15] indicates that TLR3 exists as a monomer in solution and that dimerization only occurs upon ligand binding. The dimerization interface is localized and small thus, a significant attractive force would be required to bring the TLR3 monomers close enough and in the correct orientation for proper *in situ* binding to occur.

Optical detection methods have examined the TLR3 interaction with its dsRNA PAMP [10]. Surface plasmon resonance (SPR) concluded that dsRNA binds to a defined site or sites on the TLR3 ectodomain. Leonard *et. al.* also demonstrated that the TLR3 ligand binding affinity increases as the buffer acidity is modified away from neutral and as the number of base pairs present in the ligand is increased. This finding is consistent with TLR3 being located primarily internal to the cellular membrane in the acidic environment of the endosomal vesicles [16]. Several conserved histidine residues within the N- and C-terminal of the TLR3 ectodomain have been demonstrated to interact electrostatically with the phosphate groups of the dsRNA backbone once they have become protonated in an acidic environment [17]. Finally, it was established that

TLR3 ligand-induced homodimerization is highly cooperative, confirming the conclusion of Liu *et.al.*, and that multiple TLR3 dimers can form along an extended length of dsRNA ligand strand, as long as each dimer has the requisite 40-50 base pair space required to form a stable TLR3-ligand-TLR3 complex. Notably, dsRNA was immobilized on the surface in an end-to-end oriented fashion in these studies. TLR3 was then passed over the dsRNA-modified surface in order to ensure that the receptor's orientation was not hindered by the surface immobilization process. This surface assembly model, while wholly suited for the bioanalytics described above, does not address the suitability of immobilized TLR3 as a potential sensor biorecognition element. Hence, the immobilization approaches trialed within this body of work, where TLR3 becomes the biorecognition element.

3.1 TLR3 as a Biorecognition Element

Based on the literature, TLR3 exists as a monomer [15]. Moreover, its orientation on the surface is key to ensure access to the ligand binding sites, which are located in isolated regions on the lateral side of the convex surface of both the C- and N- terminal sites. The ligand binding regions of TLR3 only comprise 4.4% of the total ectodomain surface area [17]. The majority of the TLR3-ligand interactions are electrostatic or hydrogen bonds between the positively-charged residues of TLR3 and the negatively-charged sugar-phosphate dsRNA backbone. TLR3 does not distinguish between the base sequences of dsRNA or the order in which they present, unlike TLRs 7 and 8 which identify specific amino acids [18].

The ability of TLR3-ligand complex to homodimerize will likely be significantly limited by the monomer complexes' inability to access an appropriately oriented TLR3 protein (either due to surface-bound orientation or spatial restrictions). Homodimerization is a requirement for the unique specificity of TLR3 for the dsRNA molecule [15,19]. It is the dsRNA α-helix backbone structure, based on the molecular placement of the grooves within the ribose phosphate backbone and the unique length of the resulting rotation, which allows for the specificity of binding to TLR3 *in vivo* [15]. While the β-helical structure of double-stranded DNA would structurally preclude compatibility with the two binding sites and the TIR signalling domain of the TLR3 structure, it would not prevent electrostatic bonding of its sugar-phosphate backbone to a single binding site on the biorecognition element. Hydrogen-bonding of the DNA backbone to TLR3 would be less likely as DNA lacks the 2'-hydroxyl groups of dsRNA. When examining potential

TLR3 sensor cross-reactivity, the same consideration must be given to a non-conformational, complex 3-D structure of a single-stranded nucleic acid ligand.

The following diagram outlines the four immobilization approaches that were trialed based on the hypothetical orientation of TLR3 on the surface and access to the ligand binding sites.

Figure 1: Immobilization schema trialed, from top to bottom: thiol-coupling using a maleimide reagent, the TLR3 immobilization step is examined in two different buffer pHs (A, B); amine-coupling (C); and nickel(II) chelation + amine-coupling (D).

3.2 TLR3 Immobilization Method #1: Ni(II) Chelation + Amine-coupling

The first method assessed was through the immobilization of TLR3 by nickel (II) chelation of the histidine tag (10-His) that is located on the C-terminal. A polyhistidine tag is an amino acid sequence that is added to the terminus end of a protein of interest in order to aid in a variety of applications including protein purification, fluorescent tagging and binding assays. Surface immobilization via His-tag is advantageous in that it would allow for the specific orientation of the TLR3 biorecognition element in a standardised manner. It should be noted that there are also histidine residues within the TLR3 N- and C- terminal binding domains that are essential binding residues, including a histidine cluster formation (His108, His39 and His60) that Ni(II) is suspected to interact with in a non-specific manner [15].

The results for the Ni(II) chelation immobilization application schema as well as the resulting SPR spectra can be found as Figure 1 in the corresponding Data in Brief article [20]. TLR3 was immobilized in a mobile phase consisting of HBS-EP (pH = 7.4). The final surface density of the sensor after immobilization was measured at 500 RU or approximately 500 pg/mm^2 of the TLR3 biorecognition element. Whilst immobilization occurs, the poly(I:C) ligand binding was not successful. The SPR spectra for the poly(I:C) applications onto the TLR3 sensor surface coupled by Ni(II) chelation are within Figure 4 of Ref [20]. From these results, it is apparent that there is no interaction indicated during the poly(I:C) application phase of the SPR run sequence. The acetate binding buffer (pH = 5.5) produced the same effect on the TLR3 sensor as the samples containing the poly(I:C) ligand.

While Ni(II) chelation is the most popular and ubiquitous metal chelation method for the immobilization of his-tagged moieties, it does have some significant drawbacks [21]. Other amino acid residues, such as the clusters of cysteine at the C- and N-terminals and the pervasive lysine residues threaded throughout the TLR3 protein [14], may participate in binding to Ni(II), although with significantly lower affinity. Thus, it is unclear whether the lower affinity of nickel(II) binding is balanced by the prevalence of these groups. Prolonged exposure to the required poly(I:C) binding buffer pH of 5.5 may also have had a progressive negative impact on the sensor surface, inducing the observed deleterious effect during the dissociation phase.

3.3 TLR3 Immobilization Method #2: Thiol-coupling using Maleimide

Thiol-coupling was the second method trialed to immobilize the TLR3 protein in a specific orientation, in an attempt to ensure ligand access to the binding sites. This coupling method exploits cysteine residues within the protein as attachment sites to the sensor dextran surface. Within the TLR3 biorecognition element, cysteine residues are scarce and are isolated to areas away from the ligand binding sites, either at the very ends of the C- and N-terminal or on the medial, glycosylated side of the protein, making thiol-coupling an attractive potential option for immobilization [15].

There are two common methods for thiol-coupling, ligand immobilization by thiol-disulphide exchange or maleimide coupling. The former method is not suitable for experiments where the buffer pH will be in cyclic variance (ex. binding pH = 5.5 to the regeneration pH = 8.5) as the coupling bond is unstable in these conditions. In this alternative approach, maleimide reagents are used to attach the thiol groups of the cysteine residues to the dextran matrix resulting in a thioether bond that is much more stable under the rigors to be applied during this study. BIAcore have published a protocol for the use of sulfo-GMBS as the coupling reagent [22]. Within this protocol detail was provided regarding pH for the architecture pre-immobilization stages and the capping stage. No detail was provided for the pH of the protein immobilization buffer.

During immobilization the intent is to pre-concentrate the TLR3 protein to the surface in order to maximize the immobilization potential. At pH values above 3.5 the carboxymethylated dextran on the BIAcore sensor chip is negatively-charged, this allows for an electrostatic attraction to occur between positively-charged proteins. The requirement for electrostatic pre-concentration at the surface is that the pH of the solution containing the substrate to be immobilized be between 3.5 and the isoelectric point of the substrate. BIAcore specifically

recommends that, for protein immobilization the buffer pH be at least one unit lower than the isoelectric point of the protein [11]. The isoelectric point for TLR3 is 6.73, meaning the ideal buffer for immobilization has a pH no higher than 5.73 [23].

It was determined that maleimide groups specifically react with sulfhydryl groups when the pH of the reaction mixture is between 6.5 and 7.5 to form the thioether bond [24]. Thus, the combined pre-concentration and thioether bond formation steps were compared in separate experiments at pH values favourable to the former (pH = 5.5) and the latter (pH = 7.4).

Trial #1: The immobilization schema and the sensorgram for the thiol-coupling of TLR3 in acetate buffer pH = 5.5, in addition to the resulting spectra for the poly(I:C) applications on this sensor surface, are in the correlated Data in Brief article [20] as Figures 2 and 5, respectively. TLR3 was applied over the prepared surface to a level of 185 RU. Due to the promotion of electrostatic attraction between the protein and the carboxymethylated dextran sensor surface through the use of a low pH immobilization buffer, the TLR3 protein was visibly pre-concentrating to the surface (see Figure 2 in Ref. [20]). However, formation of stable thioether bonds was limited, as only 140 RU TLR3 remained on the surface following capping.

A very high concentration of poly(I:C), 2000 nM, was required to differentiate the ligand binding signal from that produced by a blank acetate injection (Figure 5, Ref. [20]). This reflects the small quantity of TLR3 bound to the surface and the potential for TLR3 to be bound in a non-ideal orientation with the cysteine sites facing toward the gold surface. Rapid dissociation of the poorly bound poly(I:C) prevents the determination of a meaningful binding constant.

Trial #2: The immobilization schema and the sensorgram for the thiol-coupling of TLR3 in acetate buffer pH = 7.4, in addition to the resulting spectra for the poly(I:C) applications on this sensor surface, are in the correlated Data in Brief article [20] as Figures 3 and 6, respectively. Visible preconcentration during the TLR3 immobilization step is not evident during this trial (see Figure 3, Ref. [20]). The maximum surface density of TLR3 achievable at this pH was 100 RU, following cysteine capping the surface density was depleted to 50 RU. This was an early indication of the importance of the pre-concentration of the protein to the sensor surface for the efficacy of TLR3 immobilization.

The results from the poly(I:C) applications onto this thiol-coupled surface also indicate that the pre-concentration factor was significantly reduced and it appears that no TLR3 was

immobilized as there is no difference between the signal achieved by the blank sample and those containing poly(I:C) ligand (Figure 6, Ref. [20]). Based on the brief results of the poly(I:C) binding studies, it is clear that pre-concentration on the surface is an important factor in drawing the PAMP closer to the surface for the immobilization stage. However, pre-concentration of the PAMP does not directly correlate to increased immobilization levels. Thiol-coupling is not ideal due to the mismatch in pH requirement for the formation of the thioether bond and it is not evident that further adjustment of the buffer pH will optimize the results.

3.4 TLR3 Immobilization Method #3: Amine-Coupling

Amine-coupling at the ε-lysine residues or the terminal NH_2 groups in the protein structure is problematic within the TLR3 ectodomain. Based on the elucidated structure of TLR3 [14,15], there are several of these chemical groups at the N- and C-terminals however, most are scattered indiscriminately, to the present purpose, throughout the protein's structure. Only one residue, K619, at the C-terminal has been identified as being involved in ligand binding; several lysine residues and terminal NH_2 groups surround the C- and N-terminal binding sites on the glycan-free lateral side of the TLR3 ectodomain. This increases the probability that TLR3 will immobilize with the binding interface facing away from the ligand in the mobile phase and toward the surface, thus blocking a potential ligand interaction. However, it does increase the likelihood that the TLR will immobilize to the surface in a repeatable manner even though there will be a smaller number of correctly-oriented TLR3 molecules dispersed across the surface. If TLR3 binding sites are available, and a nucleic acid with an optimal negatively-charged sugar-phosphate backbone is accessible, receptor-ligand interaction is possible, which is enough for sensor design purposes.

Figure 2 depicts the amine-coupling immobilization schema as well as the resulting SPR spectra. The final surface density of the sensor after immobilization was measured at 500 RU or approximately 500 pg/mm^2 of the TLR3 biorecognition element.

Figure 2: Amine-coupling immobilization schema aligned to the resulting SPR spectra for the step-wise application. TLR3 was immobilized in a mobile phase consisting of acetate buffer (pH = 5.5). The numbered labeling of the schema application steps correspond to the numbered labeling on the SPR spectra results.

3.5 Poly(I:C)-binding Experiments on the Amine-coupled TLR3 Surface

Despite having immobilized approximately 500 RU of TLR3 on the surface of the CM5 chip, the maximum poly(I:C) binding level achieved was ~ 10-12 RU. Assuming a 1:1, straightforward binding model between TLR3 and poly(I:C) and using Equation (1) below, this equates to a binding efficiency of approximately 1.4%.

Equation (1)

$$\text{Analyte Binding Capacity} = \left(\frac{\text{Analyte MM}}{\text{TLR3 MM}} \times \text{Immobilized TLR3 Level (RU)}\right) \times 100\%$$

Using the amine-coupled TLR3 sensor profile an equilibrium dissociation constant (K_D) value was estimated by applying a simple, single-step Langmuir binding isotherm for a receptor-ligand equilibrium to model the interaction. Figure 3 shows the poly(I:C) sensorgrams and calibration curve fit on an immobilized TLR3 BIAcore CM5 chip; all ligand injections were performed in triplicate for statistical relevance.

Figure 3: Poly(I:C) sensorgrams and calibration curve fit on an immobilized TLR3 BIAcore CM5 chip. Poly(I:C) concentrations in acetate buffer (pH 5.5, 150mM NaCl). Ligand injections were performed in triplicate for statistical relevance.

The K_D for this interaction (pH = 5.5) was estimated at 117 ± 3.30 pM. The K_D value for this TLR3 sensor model is showing a higher binding affinity than the established norm obtained in previous studies, where dsRNA was immobilized to the surface. Leonard, *et. al.* verified a K_D apparent for pH = 5.5 ranging from 4.64 ± 0.11 nM to 72.6 ± 1.7 nM for dsRNA ranging in size from 540 to 39 base pairs respectively using a 1:1 binding model. As previously stated, this study immobilized dsRNA to the surface, rather than the protein, enabling the researchers to prove that binding between TLR3 and dsRNA is positively cooperative [10]. Fukuda, *et. al.*, using a quartz crystal microbalance method that exploited a dsRNA-coated (48 base pairs) sensor tip to evaluate the TLR3 interaction (pH = 5.0), found a K_D of 19 ± 0.9 nM [25].

Attempts were made to fit the data obtained in this study using both a single-step binding model and a cooperative-binding model as suggested by the work done by Leonard, *et. al.*; only the 1:1 model was successful. The dispersed nature of the TLR3 across our surface likely

limited any potential cooperative dimerization or multi-protein binding to the same ligand length (i.e. poly(I:C) chains longer than 50 base pairs). This TLR3 self-assembled mono-layer also proved to be the simplest to manufacture and it could be repeated in a consistent manner. Comparing replicate TLR3 amine-coupled sensor surfaces, manufactured and tested using the same experimental parameters outlined above and accounting for only slight variations due to human error (different batches of buffer solutions, etc.), the estimated K_D values based on replicate poly(I:C) calibration injections were 22.1 ± 0.637 pM and 50.9 ± 1.29 pM.

3.6 Sensor Cross-reactivity Study

The last stage of these experiments was to conduct preliminary challenges of this amine-coupled TLR3 sensor surface against non-PAMP ligands, such as DNA and single-stranded nucleic acids. The first non-PAMP challenge was endotoxin-free DNA from *E. coli* K12. Although TLR3 does not discriminate by base pair, the double-stranded β-helical backbone of DNA is much different from that of the α-helical structure of dsRNA. *In vivo*, the TLR3-DNA interaction would not induce the pro-inflammatory signalling cascade that invokes the upregulation of the immune response because the TIR domain at the end of the TLR3 C-terminals would not reach their required orientation due rotational length of the DNA structure. *In vitro*, while this reaction is not ideal, it is still possible through electrostatic bonding of the DNA sugar-phosphate backbone to a single binding site on the TLR biorecognition element. TLR3 and dsRNA more traditionally interact by hydrogen-bonding but this is unlikely as DNA lacks the 2'-hydroxyl groups of dsRNA.

The TLR3 amine-coupling immobilization protocol produced binding of approximately 700 RU onto the surface of the CM5 chip and a maximum binding level of the non-viral nucleic acid PAMP of ~ 17 RU was achieved during the calibration interaction trial. Assuming a simple, 1:1 binding model for TLR3 and the *E.coli* DNA and using Equation (1), this achieved a binding efficiency of approximately 0.56%. Figure 4 shows the DNA sensorgrams and calibration curve fit on an immobilized TLR3 BIAcore CM5 chip.

Figure 4: DNA sensorgrams and calibration curve fit on an immobilized TLR3 BIAcore CM5 chip. DNA concentrations in acetate buffer (pH 5.5, 150mM NaCl). Ligand injections were performed in triplicate for statistical relevance.

The equilibrium dissociation constant for this TLR3-DNA interaction was estimated at 1360 ± 184 pM. This binding affinity is approximately ten times weaker than that measured for the TLR3-poly(I:C) interaction. While the importance of dimerization for TLR3 specificity *in vivo* is not in question, the fact that this evaluated equilibrium constant is distinctly different from that for the traditional PAMP is a starting point for understanding how a detection library can be built for TLR3 as a sensor surface. As TLR3 generically scouts for the dsRNA phosphate backbone to bind with, in its monomeric form, that opens it up to a host of other potential nucleic acid PAMP binding opportunities without broad-spectrum differentiation or preference.

Finally, several single-stranded nucleic acid moieties were also used to interrogate the TLR3 surface to test for potential cross-reactivity. The first two evaluated were single-stranded RNA structures: poly(U), a single-stranded RNA polymer; and ORN 02, an AU-rich oligonucleotide.

Figure 5: SPR spectra for the **A: poly(U)** and **B: ORN 02** applications of known concentrations: 10 nM and 100 nM, respectively onto the TLR3 sensor surface using the established amine-coupling method.

Figure 5A shows the preliminary application results for poly(U) and Figure 5B for ORN 02. The ORN 02 sample was complexed with LyoVec™ a cationic lipid-based transfection reagent [26] which likely contributed significantly to the non-specific binding observed. Poly(U) was synthetic and not complexed with any other transfection agent. Both non-PAMP samples showed clear non-specific binding and little differentiation between the variation in injected concentrations. Further examination of a wider range of single stranded nucleic acid sample types and concentrations is required before a definitive conclusion can be reached.

The last single-stranded nucleic acid that was examined was single-stranded, sheared *E. coli* K12 DNA. This ligand was also complexed with LyoVec™ which contributed to non-specific binding. The results shown in Figure 6 for the ssDNA application are very interesting. The consequence of the non-specific binding effect rendered the spectra impossible to model, however the initial profile is characteristic of a Langmuir binding isotherm. It appears that ssDNA may bind to TLR3 binding sites, as was acknowledged by Sköld, *et. al.*, in 2012 after *in vivo* study [27]. Further examination without cross-contamination with a cationic transfection agent is required to establish an equilibrium dissociation constant.

Figure 6: SPR spectra for ssDNA applications of known concentrations: blank acetate buffer (pH = 5.5), 10 nM and 100 nM, respectively onto the TLR3 sensor surface using the established amine-coupling method.

4. Conclusions

This study represents the first sensor design exercise, whereby, TLR3 is sequestered as a biorecognition element by several novel immobilization approaches onto a gold-modified dextran surface matrix. Surface plasmon resonance was the used to examine the surface throughout the immobilization and ligand challenge phases. The TLR3-modified surface was tested with its dsRNA synthetic analogue, poly(I:C), in addition to several non-PAMP single- and double-stranded nucleic acids. Amine-coupling, while not ideal for placing the TLR3 in a spatially-specific orientation, was found to be the most reliable with respect to repeatable surface manufacturing from one sensor to the next and the subsequent resulting equilibrium dissociation constants were within satisfactory range. The K_D measured for the immobilized TLR3-poly(I:C) interaction was 117 ± 3.30 pM. This binding affinity is higher than that measured in previous studies [10,24], where the TLR3-dsRNA interaction was examined using the dsRNA as the immobilized receptor. It appears that although the binding efficiency was low, at 1.4%, the poly(I:C) that was bound to the immobilized TLR3 was tightly bound to the binding regions on the ectodomain that were exposed to the mobile phase.

This evaluation included a brief cross-reactivity study using a selection of purified *E.coli* and synthetic double- and single-stranded nucleic acids. Most notably, DNA produced a measurable K_D using the 1:1 Langmuir binding isotherm of 1360 ± 184 pM. This difference is an important factor in terms of sensor design as it establishes the requirement for a thorough cross-reactivity study on the interaction of immobilized TLR3 with other non-PAMP ligands to establish a detection response library. This binding affinity is a full order of magnitude weaker than that measured for the TLR3-poly(I:C) interaction. Single-stranded DNA also displayed results that seem to imply the impedance of immobilized TLR3, although further study is required before a definitive conclusion can be drawn.

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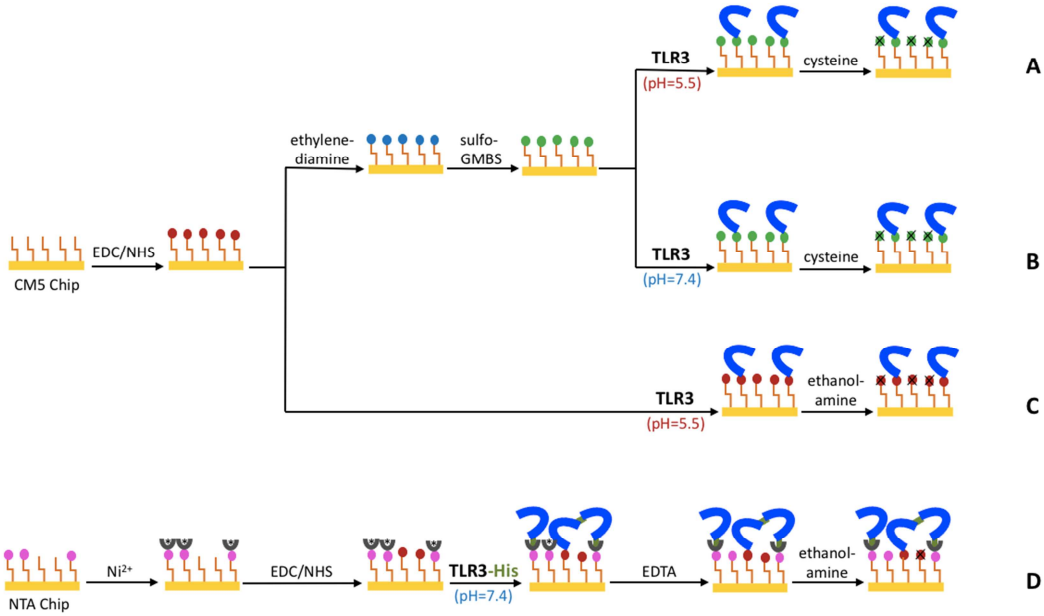

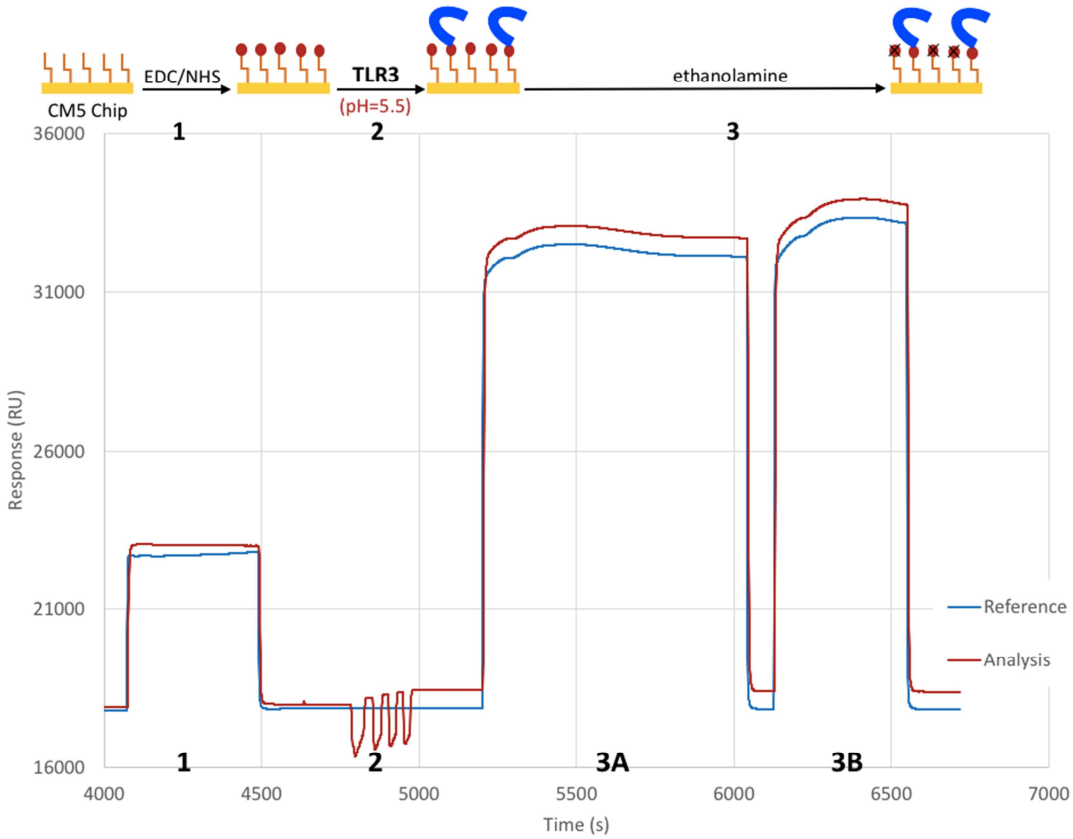

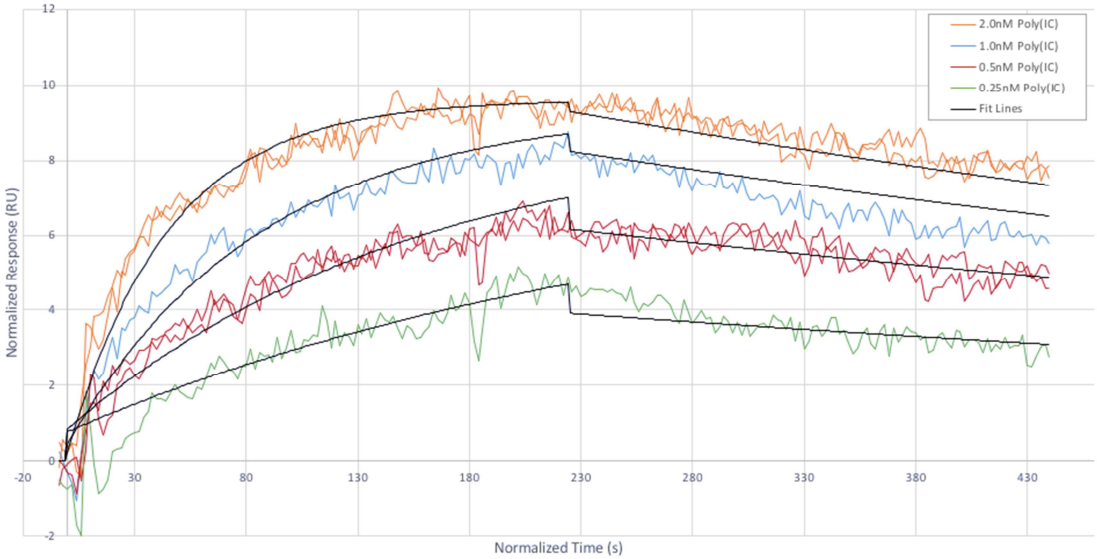

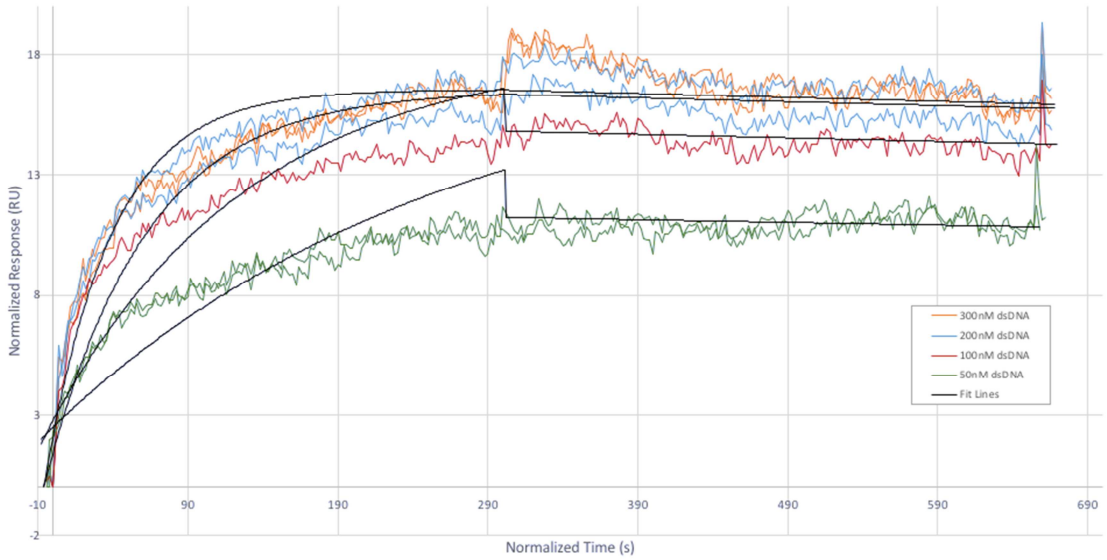

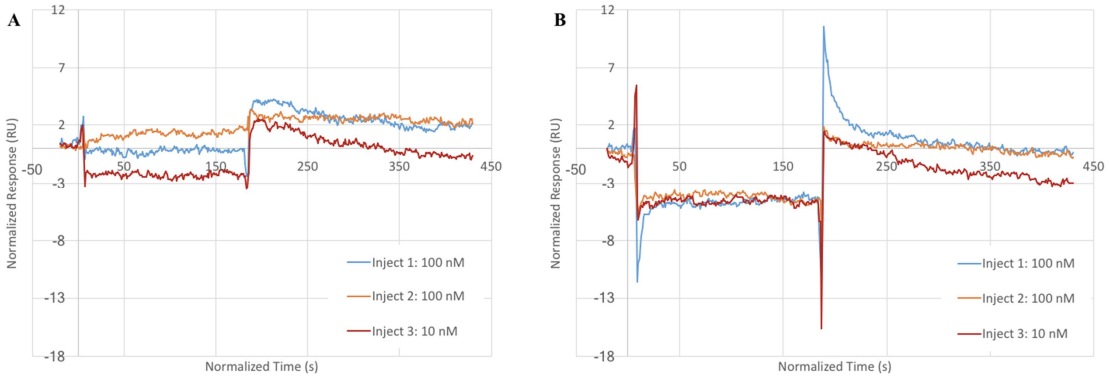

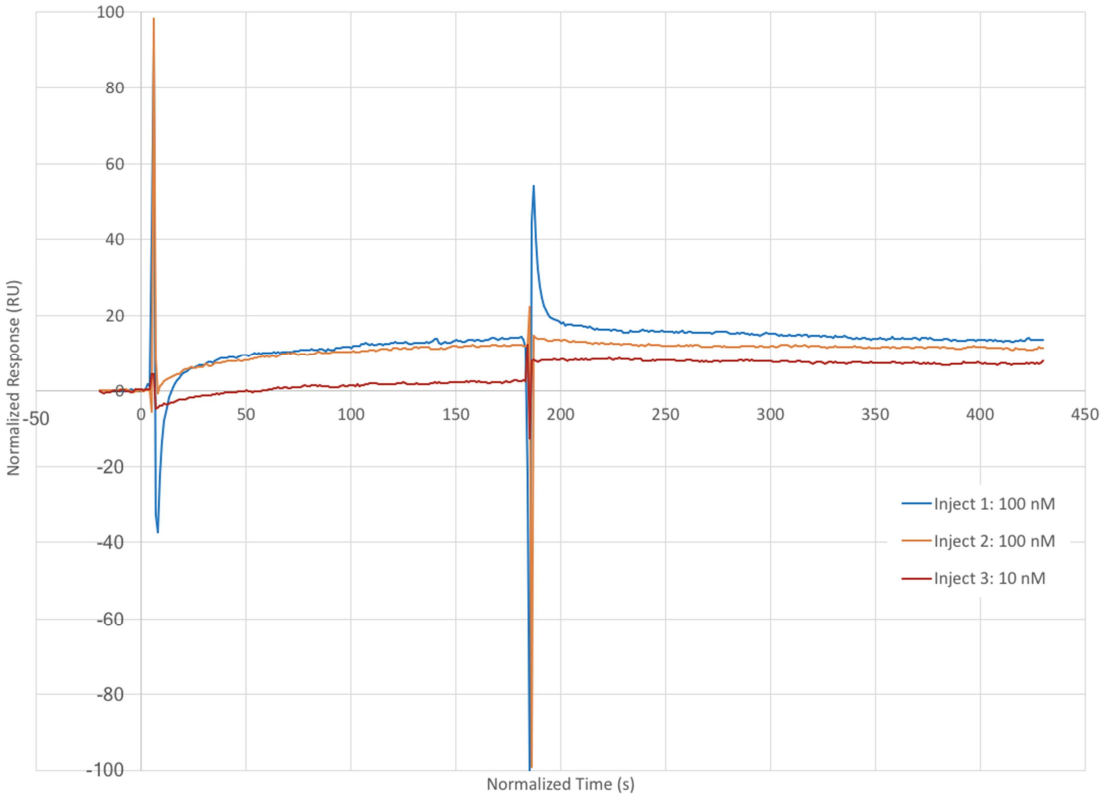

Highlights

INVESTIGATION OF THE BINDING CHARACTERISTICS OF IMMOBILISED TOLL-LIKE RECEPTOR 3 WITH POLY(I:C) FOR POTENTIAL BIOSENSOR APPLICATION

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- Sensor design exercise for the immobilization of TLR3 as a biorecognition element
- TLR3-dsRNA interaction measured using poly(I:C) for design exercise
- Amine-coupling was most reliable and repeatable method; K_D = 117 ± 3.30 pM
- Cross-reactivity study using purified *E.coli* and synthetic nucleic acids also performed