Sensing of pathogenic bacteria based on their interaction with supported bilayer membranes studied by Impedance Spectroscopy and Surface Plasmon Resonance

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Abstract

Pathogenic bacteria secrete various virulence factors, including toxins, lipases and proteases that allow them to infect, breakdown and colonize host tissue. Among various modes of action that the pathogenic bacteria use to damage the host, pore formation (by pore forming toxins (PFTs)) and lipid hydrolysis (by phospholipases) modes are common in damaging the eukaryotic cell membrane. PFTs in their monomeric form are extracellular diffusible and able to form hydrophilic pores in cell membrane while phospholipases cleaves and hydrolyzes the ester bonds of most phospholipids in cell membrane. Both modes of action cause uncontrolled permeation of ions and molecules across cell membrane, leading to cell death by apoptosis or necrosis. In this work, the toxins secreted by two clinically important human pathogens, methicillin susceptible *S. aureus* (MSSA476) and *Pseudomonas aeruginosa* (PAO1) were studied via their interaction with a planar tethered bilayer lipid membrane (pTBLM) using surface plasmon resonance spectroscopy (SPR) and electrochemical impedance spectroscopy (EIS). Detection and discrimination is based on lipid-loss (lipid hydrolysis by phospholipases) or non lipid-loss (pore formation by PFTs) from pTBLM upon interaction with supernatant of pathogenic bacteria. Using EIS and SPR, it is demonstrated that major toxins of *S. auerus* are PFTs while most of toxin associated with *P. aeruginosa* are more lipid damaging lipolytic enzymes. Such a format might have future utility as a simple assay for measuring the presence membrane lytic virulence factors in clinical samples.

Keywords: Lipid bilayer membranes, pore-forming toxins, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, pathogenic bacteria

1. Introduction

Pathogenic bacteria have various virulence factors and pathogenic modes. There is clinical utility in being able to identify quickly and easily primary modes of bacterial virulence in an infection by an unknown organism, since this can both help to approximately identify the species and may affect the choice of drug / procedure to treat a patient. For example, two common species of bacteria: *S. aureus* and *P. aeruginosa* both have rather different primary virulence factors, which can affect the clinical outcome of a patient infected with such bacteria [Bukowski et al. 2010, Liu 1974]. This paper suggests a putative sensor construct that rather than identifying specific bacteria, instead detects the way in which secreted toxins and enzymes interact with a cell mimetic solid supported lipid membrane.

Human pathogenic bacteria infect host cells in a variety of ways. Many bacteria secrete toxins and enzymes which damage the cell membrane [Geny and Popoff 2006]. Normally, with the exception of transport through specific ion channels, the cell membrane acts as an ion-impermeable barrier. Bacteria such as *S. aureus* secrete various virulence factors including α-toxins, δ-toxins and leukocidins which can bind to cell membranes and form pores [Song 1996, Szmigielski et al 1999, Mellor et al. 1988]. Such pore forming toxins (PFTs) are highly lethal to targeted cells as they disrupt the controlled permeability of the cell membrane barrier which is vital for cell homeostasis [Parker and Feil 2005]. Many PFTs secreted by common pathogenic bacteria such as *Streptococcus, Listeria* and *Clostridium* take advantage of the presence of cholesterol in cell membranes and are specifically able to bind to cholesterol to form relatively large pores [Gonzalez et al. 2008]. These large pore formers are called cholesterol binding toxins (CBTs) or cholesterol dependent cytolysins (CDCs) and kill the host cells by uncontrolled permeation of ions and molecules, leading to lysis of the cell by necrosis or apoptosis [Gilbert 2002]. Many toxins produced by common pathogens are lipases which specifically hydrolyze the ester bonds of phospholipids and sphingomyelins making up cell membranes [Songer 1997, Titball 1993, Murakami and Kudo 2002, McDermott et al.

2004]. Other toxins activate intracellular targets of the host cell and require complex transport of enzymatic toxin fragments through binding of toxin-subunit onto particular receptors at extracellular sites of the cell membrane. These toxins include those that inhibit the release of neurotransmitters, e.g. botulinum toxin; over-activate the immune system e.g. super-antigens such as toxic shock toxin 1 (TSST-1); disrupt intracellular signaling and inhibit intracellular protein synthesis, e.g. cholera toxin [Schmitt 1999].

The aim of this work was to study membrane toxins from *S. aureus* and *P. aeruginosa* via their interaction with biomimetic planar lipid bilayer membranes on gold films; so-called planar tethered bilayer lipid membranes pTBLM. The change in electrochemical impedance of the pTBLM was measured when the hydrophobic seal created by the lipid bilayer was damaged or broken by PFTs and enzymatic lipases. Surface Plasmon Resonance (SPR) was used to gain information on the measured mass loss or mass gain at the bilayer interface which could be attributed to two primary modes of action: Pore formation by exotoxins such as α-hemolysin or direct loss of lipids via hydrolysis by enzymes such as phospholipase. The two modes of action are shown schematically in figure 1.

Figure 1

2. Materials and methods

The thiolipids used for the self-assembly of mono-molecular layers on gold surfaces were 2,3-di-O-phytanyl-glycerol-1-tetraethylene glycol-D,L-lipoic acid ester lipid (DPhyTL) and cholesterol-pentaethyleneglycol (CholPEG) [Schiller et al 2003]. A mixture of DPTL and CholPEG were used as the mixed-monolayer which served as the lower half of the surface-decoupled lipid bilayer. 1,2-di-O-phytanoyl-sn-glycero-2-phosphocholine (DPhyPC) and cholesterol are commercially available lipids, and were obtained from Avantis Polar Lipids. A mixture of DPhyPC and cholesterol were used to prepare vesicles which were fused onto the mixed-monolayer to form the upper half of lipid bilayer to complete the formation of pTBLM. For the purified toxins, α-hemolysin from *S. aureus*

in the form of lyophilized toxin powder (α-HL) and Phospholipase A2 from Bee Venom (PLA_2) were directly purchased from Sigma Aldrich UK, and used without further purification. Bacterial growth was carried out in Lauria Broth (LB), which was obtained from Invitrogen. SPR measurements were performed using gold coated BK7 glass disks (25 mm in diameter, 50nm thick gold layer) supplied by Windsor Scientific, UK. All the solvents used in preparation of lipids and the salts for preparing electrolytes were analytical and high purity grades respectively. High purity water (>18.2 MΩ) from Millipore was used to prepare the lipid solution, electrolyte and nutrient solutions for bacterial growth. 50 nm diameter polycarbonate porous membranes from Avestin Europe GmbH, Germany were used to prepare the small unilamellar vesicles (SUVs) by extrusion using an Avestin mini-extruder.

Preparation of pTBLM on gold surfaces has been previously described in previous communications [Thet and Jenkins 2010]. Briefly, 0.2 mg/ml of DPhyTL and CholPEG in 1:1 molar ratios was prepared in ethanol and the mixed monolayer was self-assembled onto gold surface of the BK7 glass disk for 24 hours. SUVs were prepared in MilliQ water with a lipid mixture of 67 mol% DPhyPC and 33 mol% cholesterol. The 2 mg/ml lipid and cholesterol mixture was first heated at 60°C for 30 minutes and then extruded through a 50 nm diameter nanoporous polycarbonate membrane 21 times. SUVs were directly fused onto the monolayer which was hydrated with a solution of 100 $mmol/dm^3$ NaCl + 10 $mmol/dm^3$ $CaCl_2$. Two hours later, the gold surface was rinsed with the same electrolyte and pTBLM formation was completed. pTBLM formation was characterized by measuring the electrical impedance of the bilayer with EIS and measuring the SPR resonance angle shift (not shown - figures S1 and S2 in supplementary section).

Human pathogenic bacteria studied in this work were clinically isolated strains of *S. aureus* (MSSA476) and *P. aeruginosa* (PAO1) [Holden et al 2004, Winsor 2009]. All bacterial species were cultured in autoclaved LB medium at 37 °C for 16 hours in a temperature controlled shaker incubator. The bacterial concentration was estimated by measuring the optical density (600 nm) of overnight cultured medium which was around 1.9 – 2A, absorbance in both MSSA476 and PAO1. Alternative plating and colony counting of overnight cultured bacterial growth LB medium gave approximately 10^9 colony forming unit per milliliter (CFU/mil). For SPR measurements with supernatant of

bacterial solution, the cultured bacterium was centrifuged at 12,000 rpm for 3 minutes. Then the supernatant was separated from bacterial pellet and filtered through a 0.22 µm diameter filter to eliminate cells from supernatant. All the LB media used for bacterial culture was pre-filtered and sterilized.

A micro-Autolab with inbuilt frequency response analyzer (FRA module, Ecochemie, Netherlands) was used to apply a sinusoidal 10 mV amplitude waveform in the frequency range of 5 kHz to 20 mHz, and the current response was measured to determine the impedance of the pTBLM. The gold surface on the BK7 glass substrate was used as the working electrode while a coiled-platinum wire and an Ag/AgCl were used as the counter and reference electrodes respectively. The impedance was measured for a gold surface area of 0.28 cm^2 which was defined by an O ring attached to a Teflon cell with an electrolyte capacity of 1 ml. The media used during the impedance measurement for purified toxins and bacterial supernatant were 100 $mmol/dm^3$ NaCl + 10 $mmol/dm^3$ $CaCl_2$ buffer and supernatant of overnight bacterial cultured LB medium respectively.

Surface Plasmon Resonance (SPR) spectroscopy was done on an Autolab ESPRIT (Metrohm) with two separate flow channels in parallel. Each channel has a sample volume of 150 µl and the exchange of electrolyte was operated by peristaltic pumps. The temperature of the electrolyte in the measuring channels was controlled and maintained using a thermal bath with a circulator (HAAKE, Thermo Electron Cooperation, UK). For optimum interaction of bacterial toxins and pTBLM, the electrolyte temperature was maintained at 37±0.1 °C during the SPR measurement. In control SPR experiment, 100 $mmol/dm^3$ NaCl + 10 $mmol/dm^3$ $CaCl_2$ buffer was used as a medium to add purified toxins (figure 4). During experiment with supernatant, saline buffer was first exchanged with sterilized LB medium which was kept at 37°C (data not shown – figure S7 in supplementary section) until SPR signal was stabilized. Then the medium was replaced with LB supernatant and SPR data was recorded at 37°C (figure 6a and 6b). All experimental trends were qualitatively reproducible over multiple experiments, but due to the difficulty in making pTBLMs with identical impedances typical, rather than average quantitative data are provided.

3. Results and discussion

3.1. Hemolytic action of test bacteria on blood agar

The bacteria, *S. aureus*, *P. aeruginosa* and a non-pathogenic species of *E. coli* DH5α, used in this investigation were initially tested for their direct hemolytic activity on a blood-agar plate. A pale halo around the streaked pathogenic species, the *P. aeruginosa* and *S. aureus*, was clearly observed, but not around the non-pathogenic *E. coli* (figure 2). This measurement confirms that the pathogenic species secrete considerable quantities of hemolytic lysing agents.

Figure 2

3.2. Impedance and SPR study of the effect of key bacterial toxins: phospholipase A_2 and α-hemolysin on pTBLM

The genome sequence of both *S. aureus* MSSA 476 and *P. aeruginosa* PAO1 has been published and genome mining for putative virulence factors reveals genes encoding for PFTs and membrane-lysing agents [Holden et al 2004, Winsor 2009]. The *S. aureus* MSSA 476 genome carries several genes that encode mostly the PFTs, hemolysin and leukocidin with a few lipases. The *P. aeruginosa* PAO1 genome carries genes for exotoxin A and hemolytic phospholipases. The only PFT is exotoxin A, which mainly acts as an inhibitor of intracellular protein synthesis. With this in mind, this study has looked at whether:

(a) The mode of interaction of the two primary virulence factors with lipids in a pTBLM could be distinguished when measured both by impedance spectroscopy and Surface Plasmon Resonance.

(b) Whether the bacterial supernatant of the two bacterial species growth media, containing impure toxins, could be seen to interact differently with lipids in the pTBLM.

The details of the impedance analysis, including spectra and fitting have been described in an earlier communication [Rose and Jenkins 2007]. In control experiment, the impedance of pTBLM was stable at room temperature with no significant change in impedance (data not shown – figure S3 in supplementary section). In a separate

impedance measurement, the sensitivity and minimum concentration of purified α-HL was tested at 37°C. Toxin concentration from 0.12 to 500 nM was serially added and change in pTBLM resistance was observed the toxin concentration as low as 3 nM (data not shown – figure S4 in supplementary section). Figure 3 summarizes the key findings with pTBLM resistance and capacitance being measured before and after addition of the PFT, α-hemolysin (α-HL), and the lytic enzyme, phospholipase A_2 (PLA_2). A large effect on the pTBLM resistance was observed, with both PLA_2 and α-HL giving an 80% reduction compared with the initial value. Both PLA_2 and α-HL adversely interfered with lipid bilayer by lipid hydrolysis and pore-formation respectively. This resulted rapid increase in ion-permeability of membrane which in turn lower the pTBLM resistance. It should be noted that some differences in bilayer re-constitution give variations in pTBLM resistance between membranes made at different times, but otherwise (as far as possible) identical conditions were used. An increase in capacitance is seen in both cases, but with a significantly greater change for PLA_2 than α-HL. This is probably attributable to significant PLA_2 catalyzed lipid hydrolysis and subsequent loss, while the α-HL forms pores in the membrane but has only a small effect on the bilayers dielectric properties. However, no information about the putative enzymatic binding is available from impedance measurements alone.

Figure 3

3.3. SPR studies of the stability of pTBLM at 37°C in LB and during toxin interaction

In order to test the effect of elevated temperature (37°C) of the medium, SPR was used to measure the stability of pTBLM by varying the temperature of 100 mmol/dm^3 NaCl + 10 mmol/dm^3 $CaCl_2$ buffer from 24°C to 37°C and vice versa. Effect of temperature was reversible and SPR data were stable over time at two different temperatures (data not shown - figures S5 and S6 in supplementary section). Additionally the stability of the pTBLM in LB media at 37 °C as a control was examined. SPR was used to follow the membrane stability for up to 5 hours (data not shown - figure S7 in supplementary

section) and the membrane was stable over this time. Binding of 0.5 µg/ml α-HL into the pTBLM and subsequent formation of heptameric α-HL pore was observed as positive shift about 852 m° in resonance angle (figure 4). This infers that the pTBLM stayed intact physically and maintained a bilayer structure which supported the insertion of α-HL toxin and formation of pores. Moreover general membrane degradation was not seen upon addition of α-HL.

Figure 4

The binding of 0.5 µg/ml PLA_2 was also measured and the data are presented in figure 4. These results show a very different change in SPR resonance angle, implying a very different mode of action. Initial binding of toxin appears to take place, as evidenced by an increase of around 200 m° in resonance angle in a very short time (20 seconds) followed by an almost instantaneous decrease in SPR resonance over the subsequent 900 seconds, giving a net decrease of 630 m°. A decrease in resonance angle is suggestive of a decrease in mass density at the surface. A reasonable interpretation of the data in figure 4 is that the PLA_2, after initially binding to the membrane surface, rapidly catalyses hydrolysis of the lipid head groups resulting in a large scale loss of material (lipids) from the gold surface.

The initial binding of PLA_2 to lipid membranes and delay before lysis is observed has been seen in previous studies which looked at the interaction of PLA_2 with lipid vesicles [Sanchez 2002]. In a study by Williams et al 2006, the permeation of the bilayer was observed via the leakage of encapsulated fluorescent dye from solid supported lipid vesicles. Both studies showed a delay between PLA_2 binding to a lipid membrane and lysis of around 8-10 minutes. This was longer than observed in this study, possibly due to this study deploying a planar bilayer rather than vesicles for detection, decreasing the mass-transport time required for a PLA_2 enzyme to ‘locate’ the membrane and lyses it, or greater strain in planar bilayer systems, perhaps leading to a catalytic ‘unzipping’ of the bilayer by PLA_2.

The binding of α-HL (figure 4) shows an increase in SPR resonance from the moment of applying the toxin. The binding curve resembles a standard Langmuir type binding isotherm, suggesting insertion of protein into or on the pTBLM but no lipid loss, in agreement with previous studies of the interaction of α-HL with planar bilayers by Vockenroth et al 2008.

3.4. Interaction of bacteria supernatant with pTBLM

Bacteria are living organisms, they respond to their environment in various ways – including down or up regulation of various biochemical processes which can lead to enzyme and toxin secretion, extra polysaccharide secretion, surface attachment, colonisation and biofilm formation. For this reason, in order to make analysis of the SPR data more straightforward, only the supernatant in which the bacteria had grown was studied. Bacteria secrete toxins either as they grow (in the case of *S. aureus*) or following growth in the stationary phase (for *P. aeruginosa*). Both bacteria were grown overnight and their optical density measured (see supplementary data). The bacteria took approximately 12 hours to reach their stationary phase, where they remained for a further 4 hours. The filtered growth medium (LB broth) will contain enzymes from the bacteria secreted both as they grow and in their stationary phase. In comparison with the experiments which studied the interaction of pure toxin with pTBLMs, the response of pTBLM to bacterial supernatant creates a more complex situation.

Non-specific toxins in the MSSA 476 genome include PFTs as α-hemolysins and leukocidins [Sanchez et al 2002]. Other toxins present in the supernatant of this strain of *S. aureus* include pyrogenic exotoxins which require the present of specific receptors on the cell surface to be able to activate. Although PFTS make up only a small part of the 'armory' of *S. aureus* MSSA 476, it is likely that will dominate interactions with the bilayer. The lower shift in SPR resonance angle compared with pure α-HL suggests a fairly low expression level of membrane-interactive toxins in the supernatant. However, as shown in the summary of impedances in figure 5, the trend on addition of supernatant from the two bacterial overnight cultures is broadly similar to that observed for pure toxins (figure 2).

Figure 5

The SPR experiments involved forming pTBLMs in standard lipid buffer and once bilayer formation had taken place exchanging the buffer for LB broth and warming the cuvette to 37 °C. The pTBLM films were left for at least 50 minutes to stabilise. On addition of supernatant from *S. aureus* MSSA 476, an increase in SPR resonance angle (figure 6a) of around 40 m° was observed, suggesting that material in the filtered supernatant was binding to the bilayer. In contrast, on addition of filtered supernatant from *P. aeruginosa* (figure 6b) a decrease in SPR resonance angle was observed of up to 150 m° (over 5 hours) suggesting significant loss of lipid from the surface and little adsorption of material in the supernatant. The low adsorption is possibly due to the fatty acid hydrolysis products of the lytic enzymes present in the supernatant helping to solubilise proteins and material in the supernatant. It is known that many virulence factors associated with the gram-negative bacteria *P. aeruginosa* are endotoxins, lipases and lipopolysaccharides [Winsor 2009]. The results in figure 6b show an apparent similarity with the effect of PLA_2 in figure 4, suggesting that the primary mode of virulence in the supernatant was lytic enzymes such as phospholipases.

Figure 6

4. Conclusions

In conclusion, we have implemented a putative pTBLM based SPR sensor able to discriminate the clinically important human pathogenic bacteria by their common mode of action against host cell membranes. Discrimination was based on interaction of lipid bilayer with the supernatant of toxins secreted by Methicillin suspected *S. aureus* (MSSA476) and *P. aeruginosa* (PAO1) and the detection and discrimination mechanism was either lipid loss or non-lipid loss from the pTBLM following interaction with relevant but common PFTs and lipid damaging enzymes. SPR measurements with

purified α-HL and PLA2 clearly indicated the resonance angle shift due to pore formation and lipid lysis. Supernatant of bacterial growth medium of *S. aureus* (MSSA476) and *P. aeruginosa* (PAO1) were tested against the pTBLM system and it was found out that the toxins mostly associated by *S. aureus* (MSSA476) were PFTs while *P. aeruginosa* (PAO1) secreted more lipid damaging lipolytic enzymes and lipopolysaccharides.

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Figures

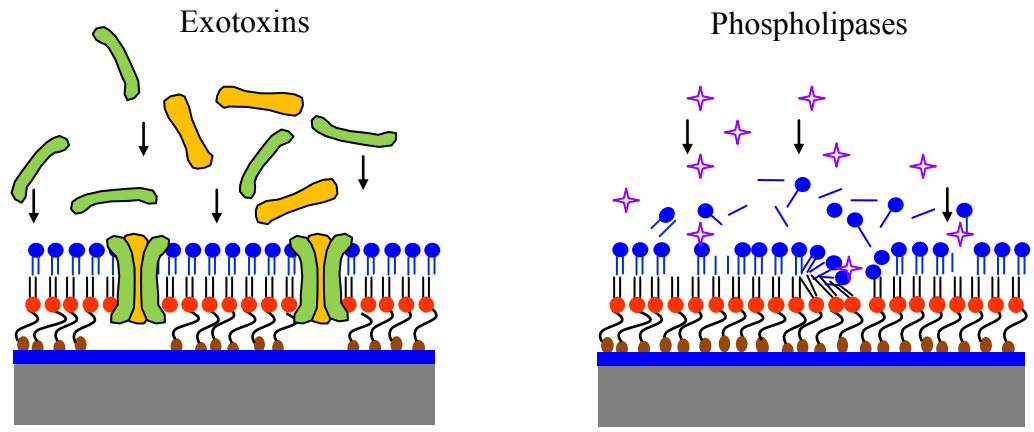

Figure 1. Schematic depictions of pTBLM with (a) the insertion of PFT monomers and subsequent pore formation, and (b) loss of lipids from the surface due to the hydrolysis of ester links phospholipases.

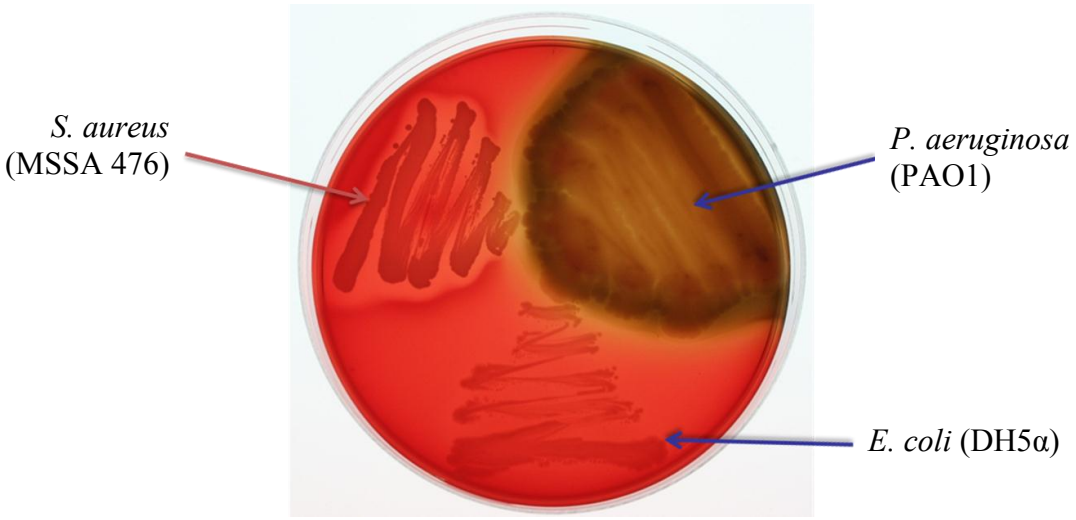

Figure 2. Direct observation of hemolysis of red blood cells on a blood agar plate by *S. aureus* MSSA 476 and *P. aeruginosa* PAO1. No activity was observed by the *E. coli* DH5α.

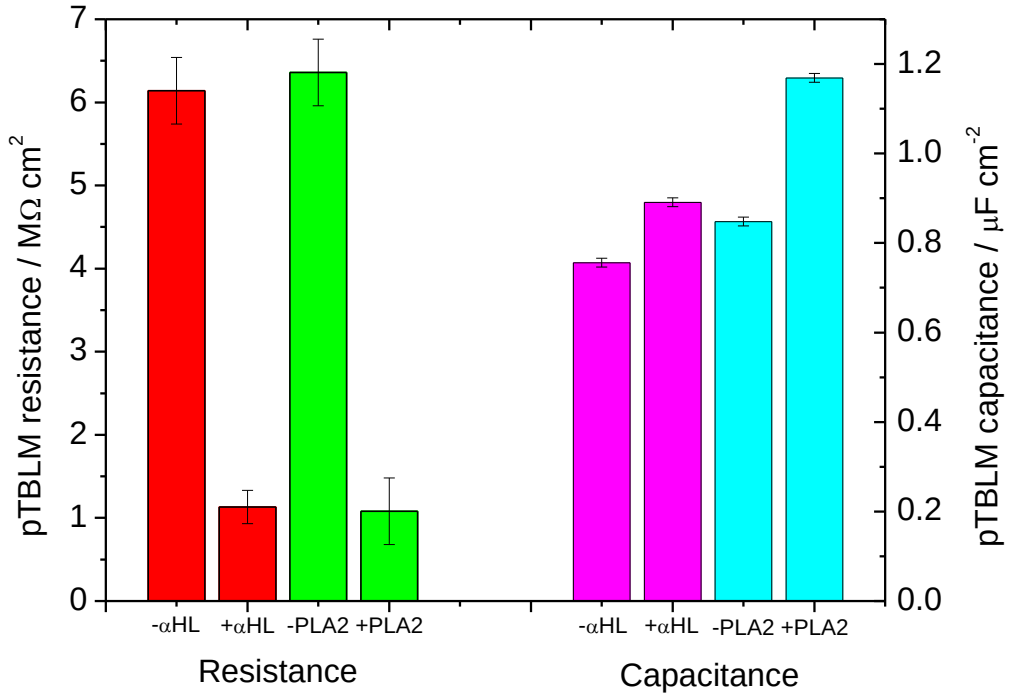

Figure 3. Change in impedance parameters: resistance and capacitance before and after the addition of 0.5 μg/ml α-hemolysin (α-HL) and 0.5 μg/ml phospholipase A_2 (PLA_2) (Data plotted are average resistance and capacitance of three separate measurements with standard deviation of data in term of error bars).

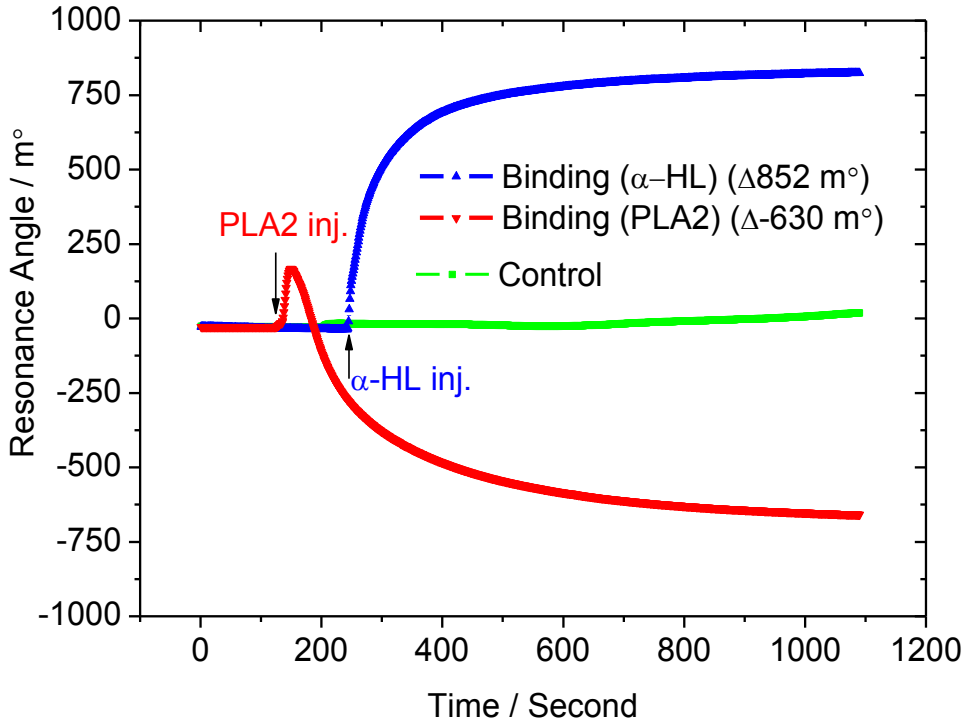

Figure 4. Kinetic response of pTBLM to purified α-HL and PLA_2 in NaCl + $CaCl_2$ at room temperature – binding of PFTs α-HL caused the upward shift of resonance angle indicating pore-formation without lipid loss, and adding of PLA_2 induced the downward shift of resonance angle due to lipid lysis by PLA_2.

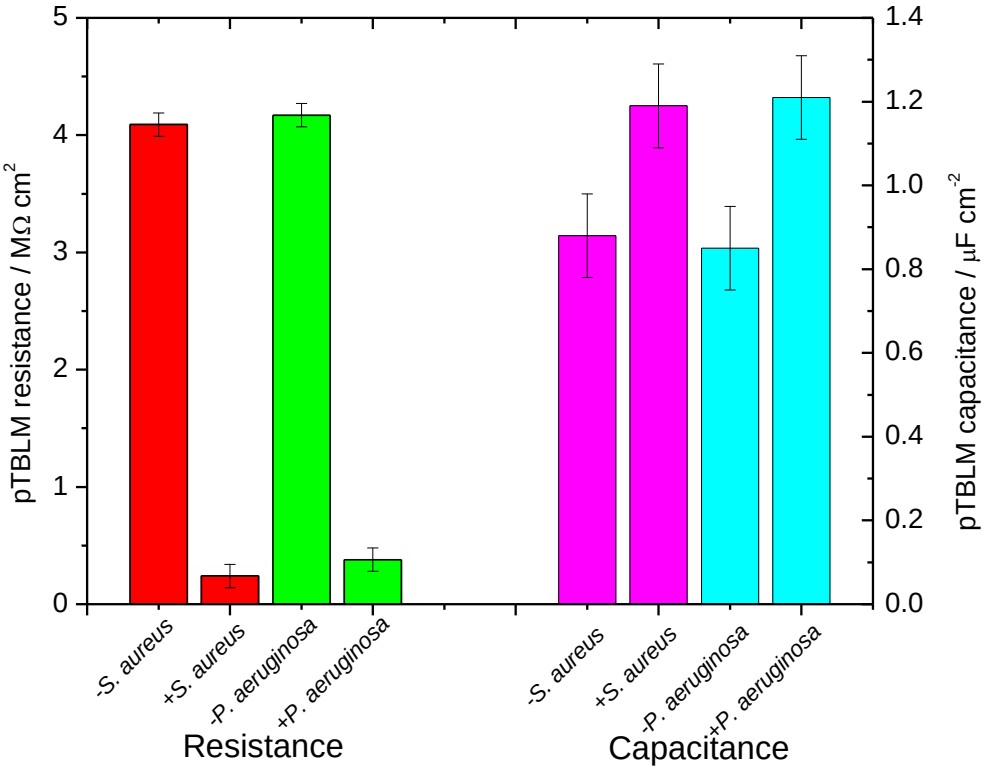

Figure 5. Impedance of pTBLMs before, and after addition of filtered supernatant from overnight growth of *P. aeruginosa* PAO1 and *S. aureus* MSSA 476 - significant decreases in bilayer resistance are observed and small increases in capacitance. (Resistance and capacitance data are average of three separate measurements tested – error bars indicated the standard deviation of three measurements with supernatants)

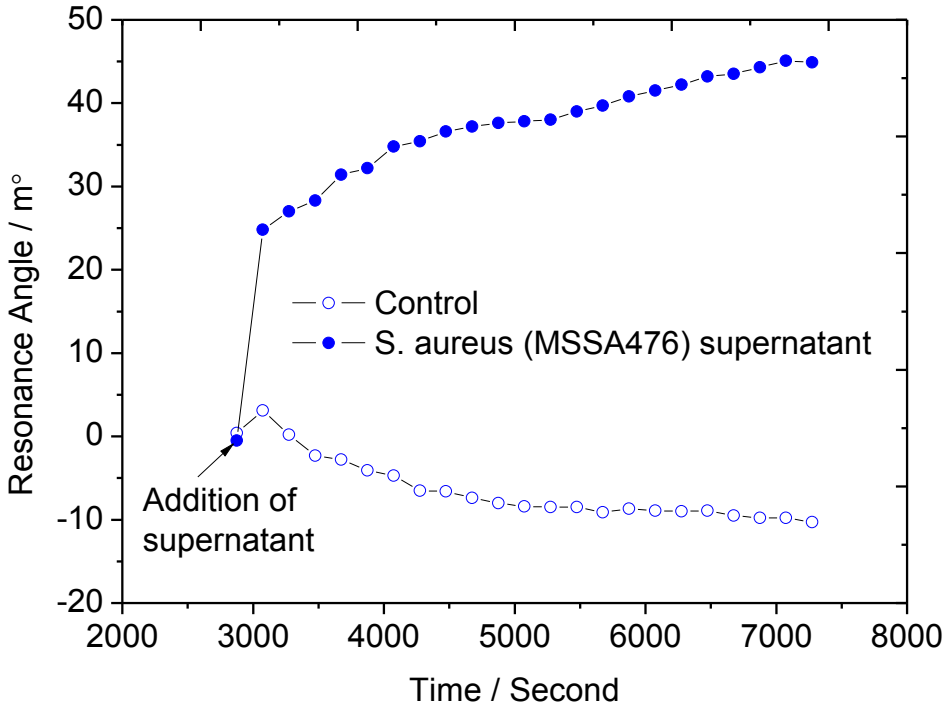

Figure 6a. SPR response on addition of filtered supernatant from overnight culture of *S. aureus* MSSA 476

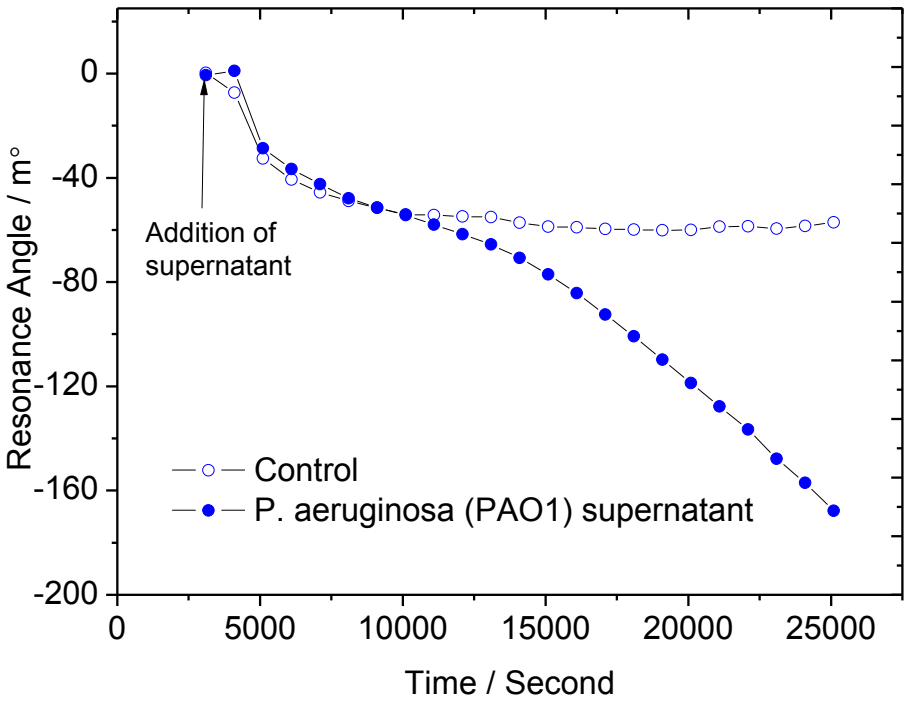

Figure 6b. SPR response on addition of filtered overnight supernatant from overnight culture of *P. aeruginosa* PAO1.