An electrochemical impedance study of the effect of pathogenic bacterial toxins on tethered bilayer lipid membrane

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Abstract

Pathogenic bacteria secrete various virulence factors that can directly interact with the outer lipid bilayer membrane of eukaryotic cells, inducing cell death by apoptosis or necrosis. Such virulence factors account for much of the toxic action associated with bacterial infection; therefore the detection of such proteins could provide a methodology for sensing / detection of pathogenic bacteria in, for example, food or human tissue. Detection and identification of pathogenic bacteria by conventional methods such as plating and counting in laboratory is expensive and time consuming. With growing concerns over emergence and re-emergence of pathogenic bacteria with high resistant to current antibiotics, there is a potential need for effective detection of pathogenic toxins *in-vitro*. This paper presents the application of tethered bilayer lipid membrane (TBLM) as a sensing platform for the detection of the clinically relevant pathogenic bacterial, *Staphylococcus aureus* MSSA 476 and *Pseudomonas aeruginosa* PAO1 via their secreted virulence factors, using electrochemical impedance spectroscopy (EIS). A non-pathogenic strain of bacteria, *E. coli* DH5α was used as a control. A clear difference in the impedance of the TBLM for the pathogenic vs. non-pathogenic species was observed.

Keywords: TBLM, pathogenic bacteria, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, pore-forming toxins, electrochemical impedance spectroscopy

1. Introduction

Toxins produced by pathogenic bacteria interact the cell membrane of the host in different ways, depending on their type and mode of action. They can either damage the lipid of the membrane via enzymatic lipase action [1] or form heptameric pores in the membrane, disrupting the permeability of the host cell [2]. Other toxins are able to inhibit the synthesis of certain proteins by the host [3], disrupt intracellular signalling [4], inhibit the release of neurotransmitters [5] or activate the response of the host immune system [6]. Some toxins bind and activate receptors on the extracellular membrane, while others need to reach the intracellular targets to accomplish their tasks. Such intracellularly acting toxins must, therefore need to cross the extracellular cell membrane. In both cases, all toxins need to interact with the extracellular membrane. To date, there are approximately 300 bacterial protein toxins responsible for the various diseases in human and about one third are known to damage the cell membrane [7]. Many of these are pore-forming toxins (PFTs) secreted mostly by Gram-positive bacteria [8].

Some purified forms of PFTs are known to retain their structure and functions *in-vitro* and a various studies has been performed on the interaction of PFTs with the lipid bilayer membranes. The interaction of *S. aureus* α-toxin with lipid bilayer membranes [9] and pore-forming exotoxin A of *P. aeruginosa* with lipid vesicles [10] were studied by various analytical methods. Moreover, binding of clinically important toxins such as cholera, tetanus and pertussis toxins into lipid bilayer has been studied using fluorescence microscopy [11], quartz crystal microbalance [12] and scanning probe microscopy [13]. However, the bacterial toxins studied in above-mentioned works were specific and in their purified form. Therefore the broad and combined effect of various toxins produced by pathogens *in-vivo* with cell membrane has been largely neglected. Despite the fact that these purified bacterial toxins are only a fraction of the toxins produced by different strains of clinically significant bacteria such as *S. aureus* and *P. aeruginosa*, the effect of other major toxins produced by these pathogens causing several diseases are still not well understood. The potentially synergistic, multiple interaction of pathogenic bacterial toxins with lipid bilayer membrane *in-vitro* has been little explored. We examined the electrochemical response of the lipid bilayer membrane with the secreted toxins in the growth supernatant broth of two mammalian species of pathogenic bacteria *Staphylococcus aureus* MSSA 476 and *Pseudomonas aeruginosa* PAO1 and the results were compared with the supernatant solution of non-pathogenic bacteria, *E. coli*.

2 Experimental section

2.1 Materials

Thiol based 2,3-di-O-phytanyl-glycerol-l-tetraethylene glycol-D,L-lipoic acid ester lipid (DPTL) [14] and cholesterol-pentaethyleneglycol (CholPEG) [15] were previously synthesized and used as the monolayer. 1,2-di-o-phytanoyl-*sn*-glycero-2-phosphocholine (DPhyPC) lipid and cholesterol were directly purchased from Avantis Polar Lipids and used without further purification. Alpha Haemolysin (α-HL) from *S. aureus* (toxin protein in the form of lyophilized powder) and Phospholipase A2 (PLA2) was acquired from (Sigma-Aldrich) Lauria Broth (LB) base powder was purchased from Invitrogen. Ethanol used in preparation of monolayer solution was analytical grade and high electrical resistant water (18.2 MΩ) from Millipore, Amsterdam, Netherland was used in preparation of the lipid and buffer solutions. For extrusion of small unilamellar vesicles (SUVs), 50 nm diameter polycarbonate filter membrane was supplied from Avantis Polar and extruded 21 times using an Avestin mini-extruder. Diced polished silicon wafers were purchased from Crystal Technology GmbH, Berlin, Germany and commercially available epoxy resin and hardener from EPOTEK, MA, USA, were used for bonding of glass slabs onto Au evaporated silicon chips in fabrication of template stripped gold (TSG) substrates.

2.2 Preparation of Template Stripped Gold (TSG) substrates

Preparation of TSG substrates was previously described [16]. Polished silicon chips were first heated at 70°C for an hour in a mixture of water, hydrogen peroxide and ammonia with the ratio of 5:1:1 in volume. Then the chips were rinsed thoroughly in MilliQ water before blown dry with nitrogen gas. Balzers PLS 500 thermal evaporator was used to deposit 50 nm of Au onto the polished surface of the silicon chips. Diced microscopic glass slabs were clean in detergent and thoroughly rinsed in MilliQ water. Then glass slides were bonded onto Au surface of silicon chips using the epoxy glue and thermal curing of the glue was carried out in the oven at 150°C for an hour before the bonded chips were stored in a desiccator. The glass chip carrying Au surface was freshly stripped just before the surface assembly of monolayer. The surface roughness of TSG in root mean square (RMS) measured by atomic force microscopy (AFM) was less than 0.5 nm.

2.3 Tethered Bilayer Lipid Membrane (TBLM)

DPTL and CholPEG mixed-monolayer was assembled onto TSG substrate surface. Self assembly was carried out of a 1:1 mixture of DPTL and CholPEG at 0.2 mg/ml in ethanol for 24 hours. Monolayer formation was characterized by increase in surface hydrophobicity with

contact angle (CA) measurement. The upper leaflet of TBLM was formed by fusion of DPhyPC and cholesterol mixed vesicles. 67 mol% DPhyPC and 33 mol% cholesterol were hydrated in water, then vortexed. The mixture was then heated at 60°C for 30 minutes. SUVs were prepared by extruding the lipid-cholesterol mixture through the polycarbonate membrane filter. The extruded SUVs were directly fused onto DPTL + CholPEG mixed-monolayer in 100 mmol dm^{-3} NaCl buffer, resulting TBLM on TSG surface. NaCl buffer was replaced thoroughly with LB medium and the TBLM was subjected to 37°C in an incubator throughout the EIS measurement.

2.4 Bacterial cell culture

Pathogenic bacteria used in the experiment were clinically isolated strains of Methicillin-susceptible *S. aureus* (MSSA 476) and *P. aeruginosa* (PAO1), with a lab strain of *E. coli* (DH5α) with most virulence factors were removed and used as a control non-pathogenic bacterium. Bacterial cultured LB media was prepared in MilliQ water and autoclaved before usage. Bacteria were grown in 10 ml LB for 16 h on a shaker incubator at 37°C. The optical density (OD) of bacterial cultured LB medium was measured at 600 nm following growth. OD measurements for the bacteria after 16 h growth (in stationary phase) were: *S. aureus* = 1.970; *P. aeruginosa* = 1.466; *E. Coli* = 1.378. For the impedance measurements with supernatant bacterial solution, the cultured LB medium with bacteria was centrifuged for 3 minutes at 12000 rpm. The supernatant was extracted and filtered through 0.22 µm diameter standard filter to remove all bacteria from supernatant. All LB media used in impedance measurements was freshly filter sterilized. Preparation and handling of bacterial and toxin solution were conducted under a laminar flow hood in the laboratory.

2.5 Electrochemical Impedance Spectroscopy (EIS)

An Autolab potentiostat-frequency response analyzer (Ecochemie, Netherlands) was used to apply the 10 mV sinusoidal voltage signal in the frequency range of 5 kHz to 20 mHz, to measure the current response across the TBLM. The TSG was used as the working electrode (WE), a coiled-platinum as the counter electrode (CE) and Ag/AgCl as the reference electrode (RE). The measured area of TSG electrode was 0.28 cm^2 and the measurement cell was made of Teflon with enclosed volume of 1 ml. (For the impedance data representation with equivalent circuit, see the figure 3. Data was fitted using the Z-view program (Scribner associates).

3 Results and discussion

3. 1 Impedance spectroscopy data fitting

The complex impedance data (Nyquist plot) was fitted to the $R_{el}(R_{bil}C_{bil})C_{sc}$ equivalent circuit shown in figure 4 (inset). The complex admittance over frequency spectra for both the lipid monolayer, and lipid bilayer is shown in figure 1, together with the fitting to the same equivalent circuit. The complex admittance plot (y''/ω vs. y'/ω) allows straightforward visualization of the film capacitance (C_{bil}), which is seen as the diameter of high frequency semi-circle on the imaginary axis, and is a useful way to largely deconvolute the low frequency capacitive response (C_{sc}). The critical measurement here is R_{bil} and C_{bil}. R_{bil} can be understood in terms of the insulating properties of the TBLM on the TSG with values typically around 2 MΩ cm^2 (*vida-infra*). The capacitive response of the system was modelled as two capacitances in series in the equivalent circuit: one relating to the double layer capacitance / space charge of the TSG electrode, C_{sc}, seen at low frequencies and the high frequency capacitive response, C_{bil}, attributed to the dielectric properties of the lipid film. Both R_{bil} and C_{bil} were sensitive to the interaction of either pure bacterial toxins, or bacteria supernatant with the film. Although a constant phase element (CPE) could have been used to improve the quality of the data fit, it is the opinion of the authors that this does not increase understanding of the system as it introduces a further unknown variable (the exponent of ωC) into the fit.

Figure 1

3.2 Thermal stability of modified TBLM

Lipid bilayer membranes need to maintain the fluid phase in which they are optimized to interact with membrane proteins [17]. DPTL based DPhyPC TBLM was initially designed and synthesized to maintain the fluid phase at room temperature by incorporation of methyl side-branches in its phytanyl chains. However, when TBLM was subjected to 37°C, its resistance decreased rapidly, most probably due to a rapid increase in bilayer fluidity at the elevated temperature. To improve its thermal stability at 37°C, CholPEG and cholesterol were incorporated in lower and upper leaflets of TBLM respectively. A schematic of modified TBLM is depicted in figure 2. Cholesterol is one of the essential cell membrane components in animal cells and its percentage composition varies from cell to cell. In this work, 33 mol% of free cholesterol was present in the upper leaflet of modified TBLM, the CholPEG in lower leaflet was mixed with DPTL in various ratio to explore the effect of cholesterol on thermal

stability. It was noted that the greater the CholPEG composition, the higher the impedance / stability of the TBLM at 37°C (figure 3a). The presence of cholesterol increases the bilayer thickness, the packing and compressibility of the acyl chains. Moreover, calorimetric studies indicate that the melting transition of lipid broadens as the percent of cholesterol is increased in mixtures of cholesterol and pure phospholipids [18]. Therefore, lower fluidity in modified TBLM might be contributed to the better thermal stability in term of improved resistance at 37°C.

Figure 2

3.3 Sensitivity of modified TBLM to αHL

The incorporation of cholesterol lowered the fluidity of modified TBLM and consequently sensitivity to αHL decreased with the increase in CholPEG composition at 37°C (figure 3b). Cholesterol is known to be able to decrease the translational diffusion rates of phospholipids by slightly immobilizing the neighbouring lipids [18] and it might affected the binding and insertion of αHL to TBLM as we observed in our impedance measurement. Experiments showed that the optimum composition of CholPEG in which the bilayer membrane kept its sensitivity to αHL was 50% CholPEG in volume (figure 3b).

Figures 3a and b

3.4 TBLM interaction with pure α-HL and PLA2

Both α-HL and PLA2 are known to have different mode of actions against lipid bilayer *in-vivo* and *in-vitro*. Soluble α-HL monomers form the heptameric pores in lipid bilayer membranes [19]. The pore formation of α-HL was observed using EIS measurements when 150 nmol dm^{-3} purified α-HL monomer was added to the TBLM at 37°C (figure 4). As the αHL pores were formed, the ion permeation barrier of TBLM was partly broken and as a result, an immediate decrease in resistance and increase in capacitance of TBLM were observed (figure 4). PLA2 recognizes the acyl bond of phospholipids and catalyzes the release the fatty acid by hydrolysis [1]. This can damage the lipids and degrade the integrity of the bilayer membrane. The damage to TBLM was immediately observed when 150 nmol dm^{-3} purified PLA2 was injected into TBLM at 37°C. Similar impedance response of TBLM was noticed as in case of interaction with αHL (figure 4).

Figure 4

3.5 TBLM interaction with supernatant bacterial toxins

It is believed that most of the toxins and enzymes are produced by bacteria during their exponential growth phase, although this is species specific. Moreover, some bacteria secrete toxins in response to stress. In nutrient-rich media and at the optimum temperature, bacteria grow and reproduce well and a high secretion rate many toxic products can be expected [20]. To test this hypothesis, three supernatant LB solutions of overnight cultured bacteria containing: *S. aureus*, *P. aeruginosa* and *E. coli* were added to the TBLM and the membrane impedance measured with time (supernatant toxin preparation is described in section 2.4). Figure 5 shows the plot of resistance of TBLM with the measurement time at 37°C in LB medium with respective supernatant toxins of bacteria. An immediate decrease in resistance of TBLM was observed when the supernatant toxins of *S. aureus* and *P. aeruginosa* were added. A ca. 1 MΩ cm^2 decrease in resistance was attributed to the presence of toxins in supernatant solution after overnight culture of both pathogenic bacteria. Figure 6 is the same experimental result but the capacitance of TBLM is plotted with the time. It was noticed, however that the impedance responses of supernatant toxins varied with time for *S. aureus* and *P. aeruginosa*. The supernatant toxins of *S. aureus* induced steady but continuous decrease in resistance and increase in capacitance with time (figure 6). But in case of *P. aeruginosa*, after an initial decrease of 1 MΩ cm^2, the resistance stabilized for a few hours before a rapid decrease in resistance (shaded circles in figure 5). This rapid decrease was accompanied by a sudden linear increase in capacitance with no significant change before (blank circles in figure 6).

Figure 5

Figure 6

This observation could be explained by difference in two pathogenic strains of bacteria used in the experiment. The genome sequence of both *S. aureus* MSSA 476 and *P. aeruginosa* PAO1 has been published [21, 22] and genome mining for putative virulence factors reveals genes encoding toxins and membrane-damaging agents. *S. aureus* MSSA 476 genome carries several genes that encode mostly the PFTs, haemolysin and leukocidin with a few lipases. It is possible that the lipid bilayer damaged by PFTs exhibits steady change in impedance with time because the pores are more stable in their heptameric state rather than in monomeric forms (figure 5 and 6). On the other hand, *P. aeruginosa* PAO1 genome carries genes for secretion of exotoxin A and haemolytic phospholipases. The only PFT is the exotoxin A

which mainly acts as an inhibitor of intracellular protein synthesis. Therefore, the damage to the lipid bilayer is most likely due to the phospholipases and proteolytic enzymes secreted by *P. aeruginosa*. Lipids in bilayer are in high mobility and may be able to repair the damage sites hydrolyzed by phospholipases. Such instantaneous self-healing of lipids in bilayer might help maintain the bilayer to resist the uncontrolled flow of ions for a certain period, until the lipids falls apart and the sudden leaks of ions may attribute to an abrupt change in impedance (figure 5 and 6).

In contrast to the results above, there was no change in resistance of TBLM immediately when the supernatant solution of *E. coli* DH5α was added (shaded triangles in figure 5). This is probably due to DH5α strain of *E. coli* producing neither PFTs nor lipid damaging toxins. The impedance of TBLM did not changed significantly upon addition of supernatant solution of *E. coli* although slight decrease in resistance and increase in capacitance were observed with time. These results correlate well with recent work looking at the lysis, and dye release from lipid vesicles exposed to the three species of bacteria discussed in this communication. [23]

4 Conclusions

In this work, TBLM modified with cholesterol was used to detect and discriminate between the supernatant growth media of pathogenic and non-pathogenic bacteria. The damaging effect of bacterial toxins on the TBLMs was persistent, indicated by a continuous decrease in resistance and increase in capacitance with time. The change in impedance appeared quite linear when induced primarily (we believe) by pore-forming exotoxins. But the bilayer primarily subjected to endotoxic enzymes and phospholipases showed very different impedance – time responses. Therefore it may be possible to predict the major composition of supernatant toxins secreted a pathogen, by observing the impedance response of the lipid bilayer. The supernatant solution of non-pathogenic *E. coli* DH5α bacteria caused no significant change in TBLM impedance. This approach to detecting the lytic behaviour of pathogenic bacteria may point towards the future practical sensor based methods for sensing human pathogens.

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Figures

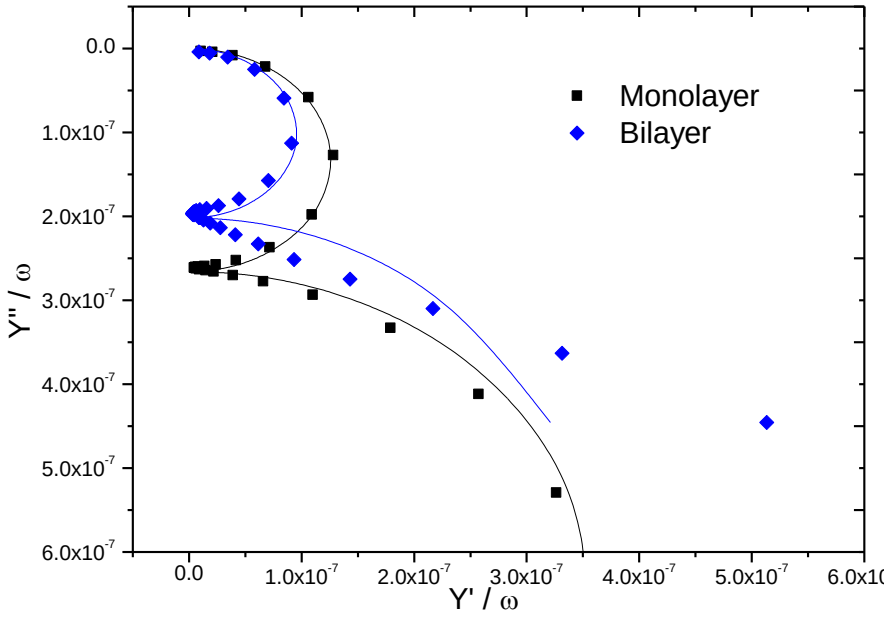


Figure 1 Complex plane fitting of real and imaginary admittance divided by frequency for monolayer, and following lipid adsorption, bilayer. Fitting of the R(RC)C equivalent circuit is shown in both plots as a line. Note, electrode areas are not corrected.

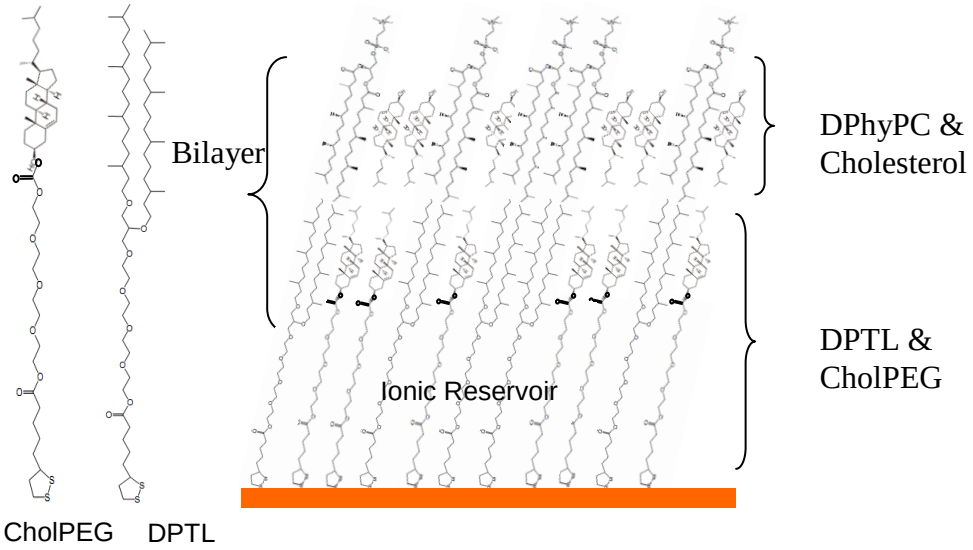
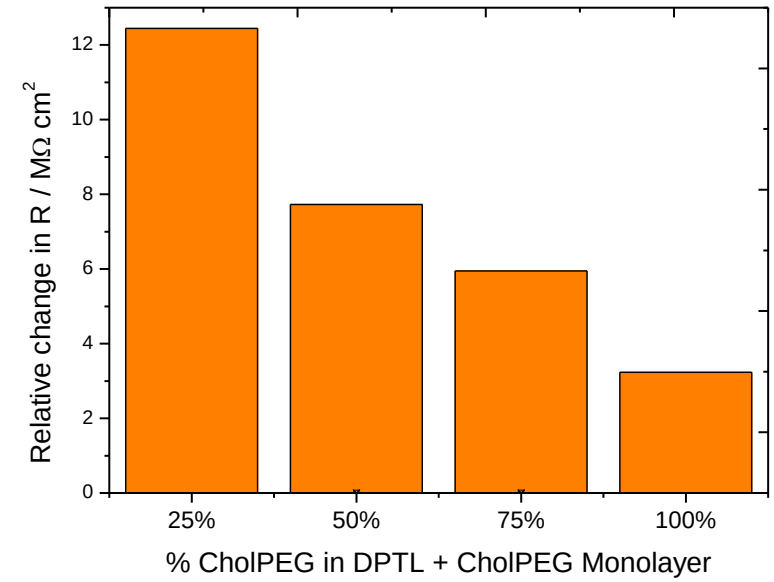
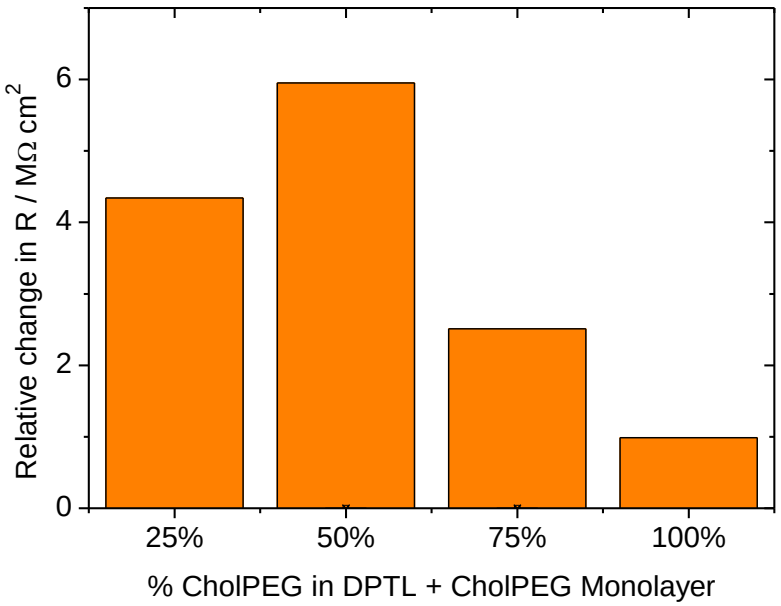


Figure 2 Schematic of modified TBLM: DPTL + CholPEG mixed-monolayer is self-assembled on TSG to form lower leaflet while DPhyPC + cholesterol mixed-vesicles are used to fuse onto the mixed-monolayer to form upper leaflet of TBLM (ionic reservoir below the bilayer provides the impedance measurement across TBLM).



(a)



(b)

Figure 3 Relative changes in resistance of modified TBLM with varying CholPEG composition (a) thermal stability after 15 hours at 37°C and (b) sensitivity of modified TBLM to 150 nmol dm^{-3} α-HL at 37°C (measurements are taken 3 hours after injection of α-HL)

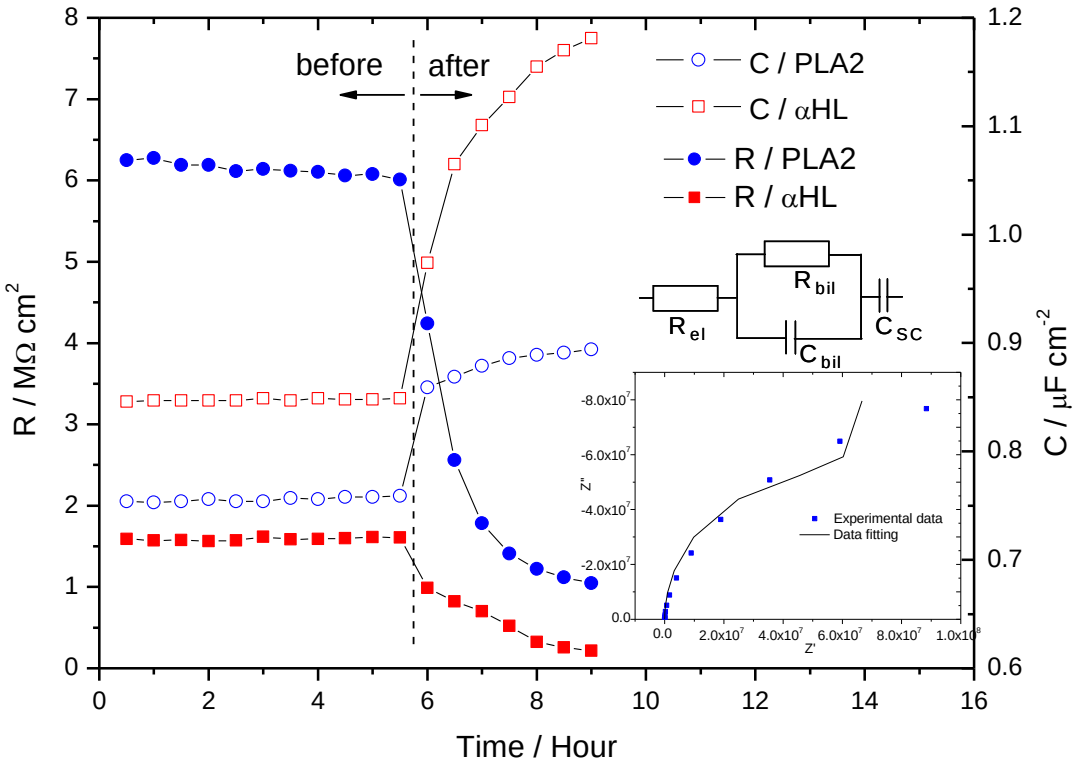


Figure 4 Effect of pore-forming α-HL toxin and lipid damaging PLA2 on resistance and capacitance of TBLM at 37°C (vertical dashed line indicates before and after injection of α-HL and PLA2). Inset is typical Nyquist impedance plot and data fitting of the bilayer prior to injection of PLA2 and the equivalent circuit model used to fit the experimental impedance data:

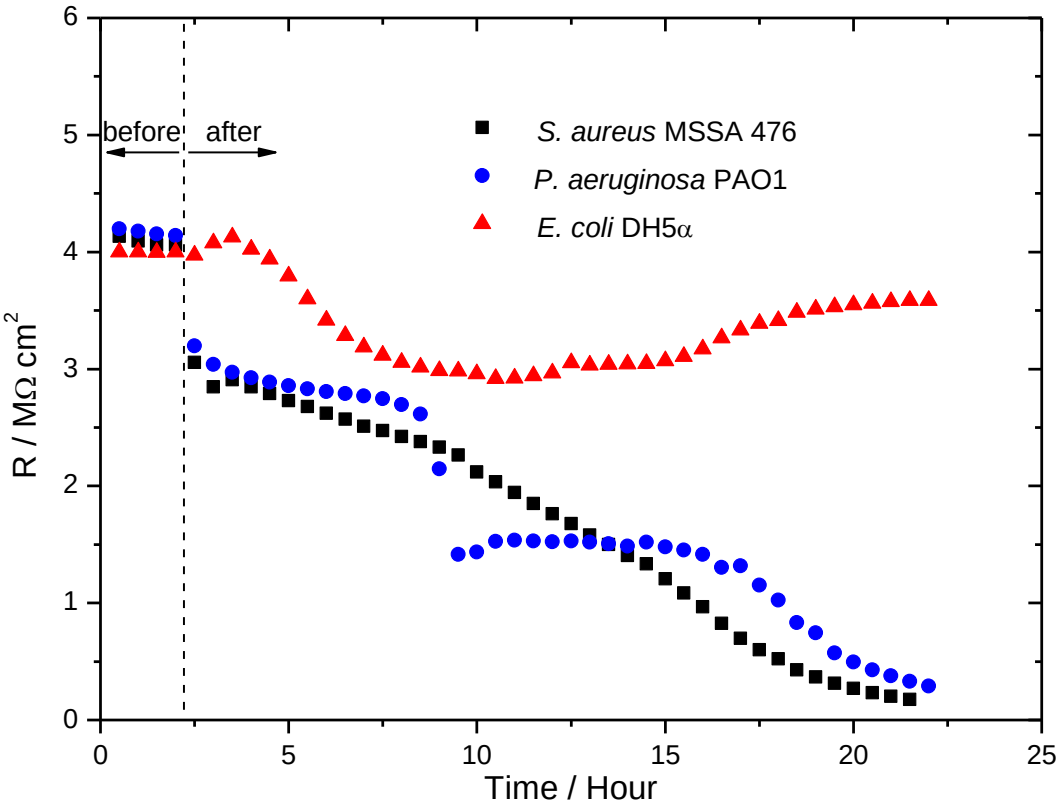


Figure 5 Effect of supernatant toxins of pathogenic and non-pathogenic bacteria on resistance of TBLM - MSSA 476 and PAO1 are pathogens with isolated strains while *E. coli* DH5α is lab strain of bacteria with most virulence factors removed (vertical dashed line indicates before and after injection of supernatant toxins at 37°C) – sudden decrease in resistance of TBLM (about 1MΩ cm^2) upon addition of supernatant toxins is most likely due to the damage of lipid bilayer.

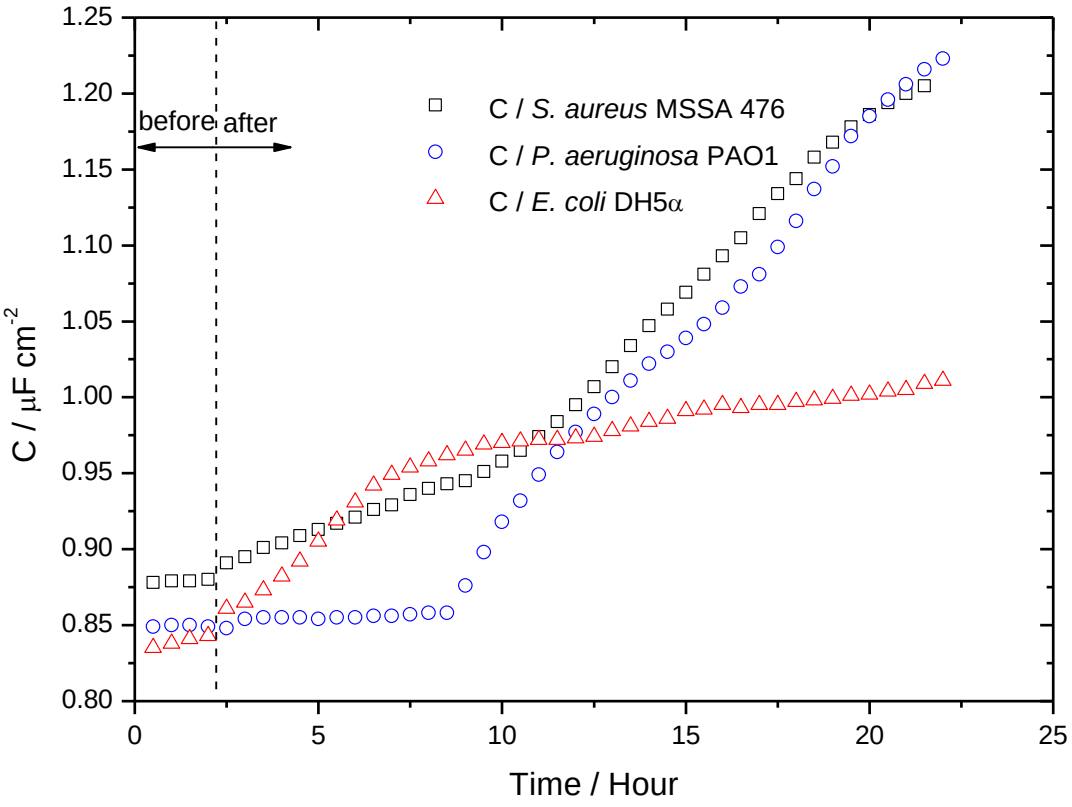


Figure 6 Effect of supernatant toxins of pathogenic and non-pathogenic bacteria on capacitance of TBLM (same experiment as described in figure 4) – capacitance due to the toxins of MSSA 476 increases continuously with time (squares) while capacitance due to the toxins of PAO1 remains almost unchanged until it is suddenly increased due to damage by phospholipase and enzymes (circles). *E. coli* DH5α exhibits a slight initial increase in capacitance, then stabilised. (triangles)