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ARTICLE

Developing A Toll-like Receptor Biosensor for Gram-Positive Bacterial Detection and its Storage Strategies

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The biorecognition ability of hybridized toll-like receptors (TLRs) 2 and 6 proteins on electrode surfaces has been studied. TLR biosensors have been designed to be non-specific to particular bacterial strains but rather to provide broad spectrum detection of cells and toxins containing relevant pathogen-associated molecular patterns (PAMPs). Our electrochemical TLR2/6 biosensors demonstrated selective detection towards Gram-positive bacterial whole-cells and a synthetic diacylated lipopeptide (Pam2CSK4), a PAMP. Responses towards *Bacillus licheniformis* (*B. licheniformis*) and *Enterococcus hirae* (*E. hirae*) were obtained. The biosensor was able to differentiate signals between *B. licheniformis* and a Gram-negative bacterial cell (control) as low as 100 CFU/mL. One challenge in developing protein-based biosensors is to improve the shelf-life of the biosensor chips and preserve the detection activity of the protein molecules, therefore we did our first exploration into storage conditions. The activity of stored biosensors was found to be strongly dependent on storage medium, and that effective 'shelf-life' was obtained makes an important step towards creating robust sensors for real-life applications.

Introduction

Bacteria detection and identification play important roles in environmental monitoring and clinical diagnostics. Detection and identification are currently done using traditional methods, which require the transportation of samples to a laboratory. Moreover, traditional methods often need 2 to 5 days to generate results. Many techniques are in development to improve bacteria diagnostics, such as enzyme linked immunosorbent assay[1, 2], polymerase chain reaction (PCR)[3-5] , and field-effect transistor-based sensors[6]. Although these methods produce reliable results, the drawbacks include time-consuming processes, cost, and the need for skilful operators working in a laboratory setting. Portable coliform detection systems for water analysis are available and within hours they provide total counts of bacterial cells without classifying or identifying specific strains.[7] Clearly, there is still a need to develop point-of-care diagnostic methods. Emerging methods using optical, electrochemical and piezoelectric transducers have been demonstrated. Pingarrón *et al.* have reported a piezoelectric biosensor for detecting *Escherichia coli* (*E. coli*) using Lectin-modified gold surfaces.[8] The detection limits have been commonly reported in the range of 10^2 to 10^5 CFU/mL.[9, 10] Fluorescence-based biosensors are very sensitive, as Mouffouk *et al.* have demonstrated a limit of detection for *E. coli* at 15 cells/mL.[11] The major drawback of using fluorescence-based techniques is that samples need to be labelled with fluorescent reagents, which adds additional cost and technical steps.[12] Surface plasmon resonance is a label-free technique and easy to use. Zourob's group showed how to detect *E. coli* as low as 10^3 CFU/mL using ultra-sensitive long-period fiber gratings.[13] Escobedo *et al.* demonstrated real-time detection of bacteria cells in human urine samples utilizing nanohole array-based biosensing chips.[14]

Inspired by the success of the glucose sensor, electrochemical transducers have been widely studied as portable methods for detecting bacterial cells and toxins.[15, 16] Electrochemical bacteria sensors, which commonly are built with transducer and bio-recognition components, are capable of a detection sensitivity of 1 cell/mL.[17] Using DNA sequences as the bio-recognition element and nanostructures on the electrode surface has been shown to be effective in detecting 1 CFU/mL by the Kelley group.[18] As the detection is highly dependent on the configuration of DNA molecules on surfaces, Bizzotto *et al.* have studied optimization strategies using *in situ* fluorescence microscopy.[19] Brosseau *et al.* have demonstrated a new platform to detect and differentiate bacterial strains by combining electrochemistry and Surface-Enhanced Raman Spectroscopy.[20] While DNA bacteria sensors offer flexibility in targeting genetic sequences in the cells, it requires additional steps to lyse the cell and make the DNA targets available for biosensors.[21] Antibody and antimicrobial peptide-based biosensors, which are highly specific, can detect cells without lysis steps.[22] Kraatz *et al.* have shown how a ferrocene moiety could be used in constructing a label-free electrochemical sensor for detecting *E. coli* O157:H7 cells down to 10^3 CFU/mL. Automated detection procedures were enabled using microfluidic devices and horseradish peroxidase labelling materials.[23] The challenge in creating a commercially viable electrochemical bacteria biosensor, is that the biosensor needs to be fully functional and meet the application requirements of detection and identification.[24] Specific pathogen detection and identification is very important for applications in clinical diagnosis, as medical professionals need to be able to provide

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corresponding treatment, such as prescribing correct antibiotics to patients. However, the limitation with specific recognition using monoclonal antibodies is that the sensor is only able to detect one strain of pathogens. This is not suitable for environmental monitoring. For an environmental setting, detecting multiple bacterial contaminants quickly from samples, such as beach water samples, is very important for reducing public exposure to bacterial contamination.[25] Bacteria such as non-specific *E. coli* are often applied as a water quality indicator. Since monoclonal antibody-based biosensors are specific to bacterial strains, they do not provide the broad-spectrum detection needed for early-warning at a setting such as beach water analysis. For example, the absence of *E. coli* O157 in lake or beach water does not mean it is free from other *E. coli* strains used as indicators. The total count of all *E. coli* is used by the regulators to assess beach water quality, therefore non-specific *E. coli* detection is necessary for these applications. Toll-like receptors (TLR) in non-specific (but partially selective) biosensors can provide the broad-spectrum response needed for early-warning detection. Other rapid broad-spectrum applications could include counter-terrorism response, where the near-real-time selection of personal protective equipment and decontamination strategies are essential.

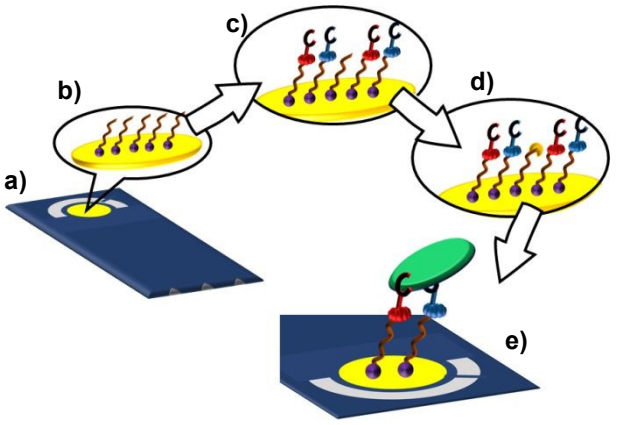
Toll-like receptors as bio-recognition elements selectively recognize pathogen-associated molecular patterns, which are not associated with any specific bacterial strains.[26] For example, TLR4 detects lipopolysaccharides (LPS) in the outer membrane of Gram-negative bacteria cells regardless of the strain.[27] Therefore TLR-based biosensors have attracted significant interest in providing broad-spectrum detection of bacterial contaminants in the past a few years. Cho *et al.* have reported using TLR4/MD-2 complexes on electrodes to detect endotoxin from *E. coli* O55:B5.[28] Birss *et al.* have furthered the TLR4 approach by improving the molecular designs in the sensor and dramatically enhancing the detection signals towards lipopolysaccharide and lysed bacterial cells.[29, 30] Kraatz *et al.* have demonstrated a TLR3-based biosensor which recognizes polyinosinic–polycytidylic acid as a dsRNA mimic, a molecular signature of viruses.[31] Nanostructured surfaces with increased surface areas are also very beneficial in improving the detection signal of TLR4 and TLR5 biosensors, which were demonstrated with detection of PAMPs extracted from *E. coli* O157:H7 and *Salmonella typhimurium*.[32] In our previous studies, we have shown how to incorporate multiple TLRs into the same sensor and use scanning electrochemical microscopy to perform multiplexed sensing of *E. coli* K12 whole-cells.[33] We also demonstrated how to enhance detection signals for triacylated lipopeptide (a PAMP) by using hybridized TLR1/2 biosensors.[34]

In this present study, we have prepared a toll-like receptor biosensor specifically for the detection of a PAMP from Gram-positive bacteria as shown in scheme 1. The sensor was constructed by immobilizing a mixture of TLR2 and TLR6 proteins on screen-printed electrode surfaces. These proteins play essential roles in providing bio-recognition capability. Unlike previous studies where isolated PAMPs and commercial bacterial cells were suspended as samples for testing, we have cultured batches of the whole-cell bacteria in broth solutions and used them in validating our sensors in this study. The intention was to investigate sensor responses to freshly prepared bacterial samples. Possible biological interference from LPS and Gram-negative bacterial cells were also examined and compared.

One of the challenges in making and using biosensors is the stability of the design and how to maintain performance of the sensor after storage. Plaxco *et al.* have reported how to improve biosensor stability by using the trithiol-anchoring groups to immobilize a DNA recognition element.[35] Crudden *et al.* have developed a new strategy of using n-heterocyclic carbene molecules to immobilize proteins.[36, 37] These studies emphasized the need to increase stability of the linker molecules for bio-recognition elements. In our study, we have focused on finding the conditions to stabilize TLR proteins while maintaining their binding affinities to the analytes. There are many variables to be considered when designing the storage conditions, therefore a series of studies are needed. In this initial study, we have explored conditions including different temperatures, humidity, and buffer solutions for the storage of TLR2/6 protein biosensors. Investigations of the stability of proteins immobilized on surfaces are needed because our study has shown that maintaining activities of these molecules on surfaces is different from maintaining these molecules in solutions. There is a lack of detailed understanding of protein stability on sensor surfaces. Finding suitable storage conditions is essential if laboratory studies are to transition to real-life applications.

Materials and Methods

Reagents. Anhydrous ethanol was purchased from Greenfield Global (Brampton, ON). HEPES (99 %, ionic strength ~0.01 M), phosphate buffered saline (PBS) buffer (pH ~7.4, ionic strength



Scheme 1. Illustration of the TLR2/6 biosensor preparation and detection. (a) Bare gold surface; (b) Modification with the linker molecule, 1-lipoic acid n-hydroxysuccinimide ester; (c) Immobilization of TLR2 and TLR6 proteins; (d) Blocking unreacted linker molecules; (e) Biosensor exposed to analytes.

~0.2 M), potassium ferrocyanide trihydrate (98.5 %), potassium ferricyanide (99 %) sodium perchlorate monohydrate (laboratory grade) and hydrochloric acid (37 %) were purchased from Fisher Scientific (Ottawa, ON). Tris base (99 %) was purchased from TCI America (Portland, OR). Ethanolamine (99 %) was purchased from Alfa-Aesar (Ward Hill, MA). Sodium hydroxide (98 %) was purchased from BioShop Canada Inc. (Burlington, ON). Toll-like receptor proteins, recombinant mouse TLR2 Fc chimera protein (1530-TR-050) and recombinant mouse TLR6 Fc Chimera protein (1533-TR-050) were purchased from R&D Systems Inc. (Minneapolis, MN). Pam2CSK4 was purchased from InvivoGen (San Diego, CA). Lipopolysaccharides from *Salmonella typhosa* were obtained from Sigma-Aldrich.

Bacteria Preparation. Whole-cell bacteria were cultured in sterile 15 mL test tubes. 5 mL of Tryptone Soya Broth (TSB) for *B. licheniformis* (ATCC 12759) and *E. coli* (ATCC 25922) and Todd Hewitt Broth (THB) for *E. hirae* (ATCC 8043) was added to each respective test tube. One bacterial colony from each respective strain was added to the appropriate test tube with a wooden inoculating stick, where the end containing the bacterial colony was broken off into the media. The test tubes were secured with a screw cap lid and placed in a shaking incubator at 37°C for 18 hours. After 18 hours the cultures were placed in a refrigerator to inhibit further bacterial growth. A 10-fold serial dilution was carried out in sterile test tubes. The bacterial cultures were taken from the refrigerator and vortexed 3 times for 3 seconds each. 1 mL of the bacterial culture was then added to a test tube containing 9 mL of reverse osmosis (RO) water and vortexed 3 times for 3 seconds. 1 mL of the newly diluted solution was placed in another test tube containing 9 mL of RO water and vortexed, and this was repeated a predetermined number of times depending on the bacteria strain and growth. A predetermined volume (bacteria dependent) of the lowest concentration was then dispensed onto a Tryptic Soy Agar (TSA) plate and spread over the surface with a sterile spreading instrument. The plate was then incubated overnight at 37 °C and the resulting colonies on the plate were counted allowing for the bacteria concentration to be calculated.

Analyte Dilution. Pam2CSK4 solutions were prepared by serial dilution. 1.5 mL of MQ water was added to a 1 mg container of Pam2CSK4 and vortexed for 2 minutes. A serial dilution was then carried out in HEPES buffer generating 0.1, 0.5, 1.0, 5.0, 10.0, and 50.0 µM solutions. *Salmonella* lipopolysaccharide (LPS) solutions were prepared by serial dilution. 3.0 mL of HEPES buffer were added to a 10 mg container of *Salmonella* LPS and vortexed for 2 minutes. A serial dilution was performed using HEPES buffer generating 63.6, 12.7, 6.4, 1.3, 0.6, and 0.1 µg/mL solutions matching the concentrations of Pam2CSK4 by mass. Bacteria solutions were prepared by a serial dilution in HEPES buffer. The cultured whole-cell bacteria was diluted in HEPES buffer generating solutions with 1 x 10^2, 1 x 10^4, and 1 x 10^6 CFU/mL.

Biosensor Storage. Detailed steps on surface preparations and electrochemical measurement are in the supporting information. The completed TLR2/6 biosensors were stored under different conditions shown in Table 1 for a period of 2 weeks. The responses from stored biosensors were obtained and compared with freshly made sensors (control). Batch F was dry stored in a container purged with nitrogen gas.

Batch	Environment	Temperature	Time
A	Reference. Fresh - no storage	N/A	0 week
B	PBS buffer	4 °C	2 weeks
C	50 % v/v PBS/glycerol	-33 °C	2 weeks
D	50 % v/v Tris/glycerol	-33 °C	2 weeks
E	PBS buffer	-80 °C	2 weeks
F	Container purged with N2(g)	-80 °C	2 weeks

Table 1. A list of storage conditions investigated in this study.

Results and Discussions

The TLR2/6 sensor was prepared following the steps shown in scheme 1. The gold surfaces and each modification were monitored using cyclic voltammetry, square wave voltammetry and electrochemical impedance spectroscopy. The oxidation and reduction peaks shown in Figure S1a are corresponding to the redox couple of $Fe(CN)_6^{3-/4-}$. The peak current is reduced as the gold surface of the electrode is modified with the linker and protein molecules. The molecules on the surface reduce the charge transfer between the gold electrode and the redox couple. This is also observed with impedance measurements. As shown in Figure S1c, surface modification leads to a larger arc in the Nyquist plots. Multiple batches of TLR2/6 sensors were prepared. Some were used instantly after preparation towards detecting different analytes, while the others were stored under different storage environments and subsequently tested.

The TLR2/6 sensor responses to different concentration of Pam2CSK4, a Gram-positive PAMP, were evaluated. Cyclic voltammograms collected for the TLR2/6 sensor before and after their exposures are shown in Figure 1a. As the concentration of the Pam2CSK4 increased from 0.1 to 0.5, 1.0, 5.0, 10.0 and 50.0 µM, the peak current was reduced from

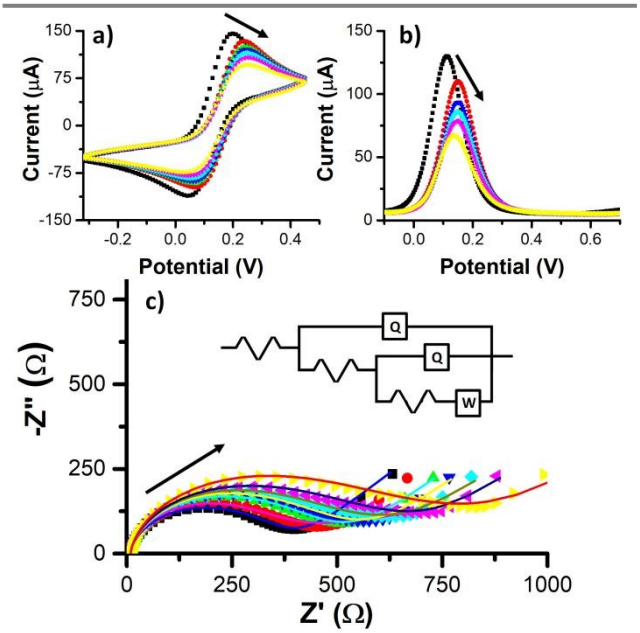


Figure 1. Electrochemical measurements of TLR2/6 sensors before (■) and after exposure to different concentrations of Pam2CSK4. The sensors were exposed to 0.1 (●), 0.5 (▲), 1.0 (▼), 5.0 (♦), 10.0 (◄), and 50.0 µM (►) of Pam2CSK4 for 15 minutes each. a) cyclic voltammogram b) square wave voltammogram c) Nyquist plots where the symbols indicate experimental data and solid line indicate simulated data. The CV was obtained using a scan rate of 0.1 V·s^{-1} and all measurements were obtained using a 10 mM HEPES aqueous buffer at pH 7.4, containing 5 mM/5 mM $Fe(CN)_6^{3-/4-}$ as a redox couple and 1 M $NaClO_4$ as the supporting electrolyte.

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147 μA to 96 μA. A similar trend was also observed using square wave voltammograms, as shown in Figure 1b. The current suppression from the voltammograms (Figure 1a, b) is indicative of surface coverage. Reduction in measured current correlates to the binding of Pam2CSK4 onto the TLR2/6 sensor. The binding increases the amount of molecules on the surface and blocks the electron transfer between the electrode and $Fe(CN)_6^{3-/4-}$. Electrochemical impedance spectroscopy was also applied to measure the resistance of the molecules on the surface. The Nyquist plots in Figure 1c show an increase in the size of the arcs. A simple model (Figure 1c inset) was used to produce simulated curves fitted in the experimental ones. The simulated and experimental curves are very similar, but not identical. This is due to intrinsic difference between simulated models and more complicated experimental samples. The interaction with the analyte on the surface is considered to be uniform in the simulation, while the interaction in the experimental samples is not perfectly uniform. Although the simplified model does not match the experimental situation exactly, it still allows a consistent evaluation of the impedance data, converting arcs into estimated values without requiring extensive computing power. The resistance for each concentration was evaluated.

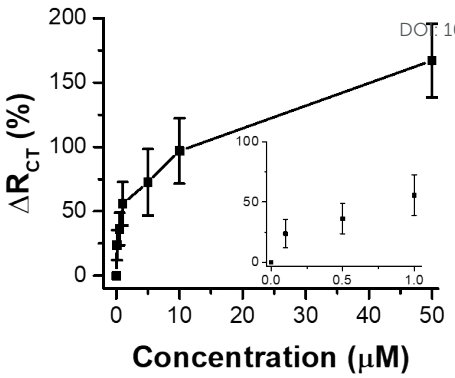

Figure 2. Plot of electrochemical responses to different concentrations of Pam2CSK4 at 0.1, 0.5, 1.0, 5.0, 10.0, 50.0 μM. ΔR_{CT}%= [R_{CT} (after) - R_{CT} (before)] / R_{CT} (before), the error bars are standard deviations based on 4 replicates. Each replicate represents one sensor prepared and tested independently.

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A normalized calibration of electrochemical response (ΔR_{CT}%) versus concentration of Pam2CSK4 was plotted as shown in Figure 2. The ΔRct(%) is the normalized difference in impedance response before and after exposure to the analyte. It is defined as: ΔR_{CT}%= [R_{CT} (after) - R_{CT} (before)] / R_{CT} (before). The average for each value was obtained from 4 replicates. Each replicate was carried out with one newly prepared TLR2/6 sensor. The TLR2/6 sensor provided responses from 0.1 to 50.0 µM of Pam2CSK4. As shown in Figure 2 inset, responses to 0.1, 0.5 and 1 µM of the PAMP were 24 ± 12 %, 36 ± 13 % and 58 ± 13 %, respectively. The increase from 0.1 to 1 µM shows a degree of linearity. The response factor to concentration was relatively less steep between 1 µM and 10 µM, suggesting the TLR2/6 binding sites on the sensor surfaces are getting saturated and require higher concentrations of Pam2CSK4 ligands to trigger binding to the remaining unoccupied sites. As the TLR2/6 combination is known to recognize PAMPs in Gram (+) bacteria rather than Gram (-) bacteria, a comparison experiment with *Salmonella* lipopolysaccharide, a Gram (-) PAMP, was carried out. A batch of TLR2/6 sensors was prepared by the same conditions. The sensors were then exposed to different concentrations of LPS from 0.1 to 63.6 mg/L. (Concentration by weight was used as the molecular weight of LPS varies.) Following the same experimental steps in electrochemical impedance spectroscopy and simulation using ZSimpWin, the responses were obtained and shown in Figure S2 and Table S1. The comparison between Pam2CSK4 and the Gram (-) agonist (*Salmonella* LPS) in Figure S2 demonstrates the specificity of this sensor towards Gram (+) strains of bacteria. The responses in ΔR_{CT}% from the *Salmonella* LPS and Pam2CSK4 at a concentration of 0.1 µg/mL are 25 ± 7 % and 24 ± 12 %. There is essentially no difference. However, when the TLR2/6 sensors were exposed to higher concentrations of Pam2CSK4 from 1.3 to 63.6 µg/mL, the sensor response increased to 167 ± 29 %. Responses from the *Salmonella* LPS did not increase with increasing concentration. The largest ΔR_{CT}% response from the *Salmonella* LPS was 36 ± 7 % at a concentration of 63.6 µg/mL. This experiment suggests that although there is some non-specific binding between PAMPs and sensor surfaces, TLR2/6 sensors are more selective towards Pam2CSK4, a Gram-positive PAMP.

After testing with isolated PAMPs as analytes, we have studied the responses of the TLR2/6 sensor to two strains of whole-cell Gram-positive bacteria seen in Figure 3. *B. licheniformis and E. hirae* are both Gram-positive bacterial strains, however *B. licheniformis* has flagella on cell surfaces while *E. hirae* does not. Since flagella is in the scope of TLR5[38] but not TLR2/6, while diacylated lipopeptide (e.g. Pam2CSK4) is recognized by TLR2/6, some responses from both strains was expected when using TLR5. One batch of TLR2/6 sensors was exposed to concentrations of *B. licheniformis* at 10^2, 10^4, and 10^6 CFU/mL. CV, SWV and electrochemical impedance were applied to monitor the changes on sensor surfaces as shown in Figure 3a b and c. After exposing the sensor surface to *B. licheniformis* solution, the current was reduced from 62 µA (black squares) gradually down to 43 µA (blue triangles). This indicates increases in blockage of heterogeneous charge transfer between the gold electrode surface and $Fe(CN)_6^{3-/4-}$ ions. The Nyquist plot in Figure 3c shows that the size of the arcs increased as the concentration of *B. licheniformis* was increased. Replicated preparation and analysis were performed to construct the calibration curve shown in Figure 3d. The responses (ΔR_{CT}%) for concentrations of 10^2, 10^4, and 10^6 CFU/mL were 25 ± 2 %, 33 ± 4 % and 56 ± 11 %, respectively. Another batch of sensors was used to detect different concentration of *E. hirae* whole-cells. A similar trend was observed as shown in Figure 3e f and g. The resistance of sensor surfaces increased with increasing concentration. However, the relative responses are slightly different for *E. hirae* whole-cells. The responses (ΔR_{CT}%) for concentrations of 10^2, 10^4, and 10^6 CFU/mL were 13 ± 4 %, 26 ± 6 % and 29 ± 3 %, respectively, which are consistently lower than the responses from *B. licheniformis*.

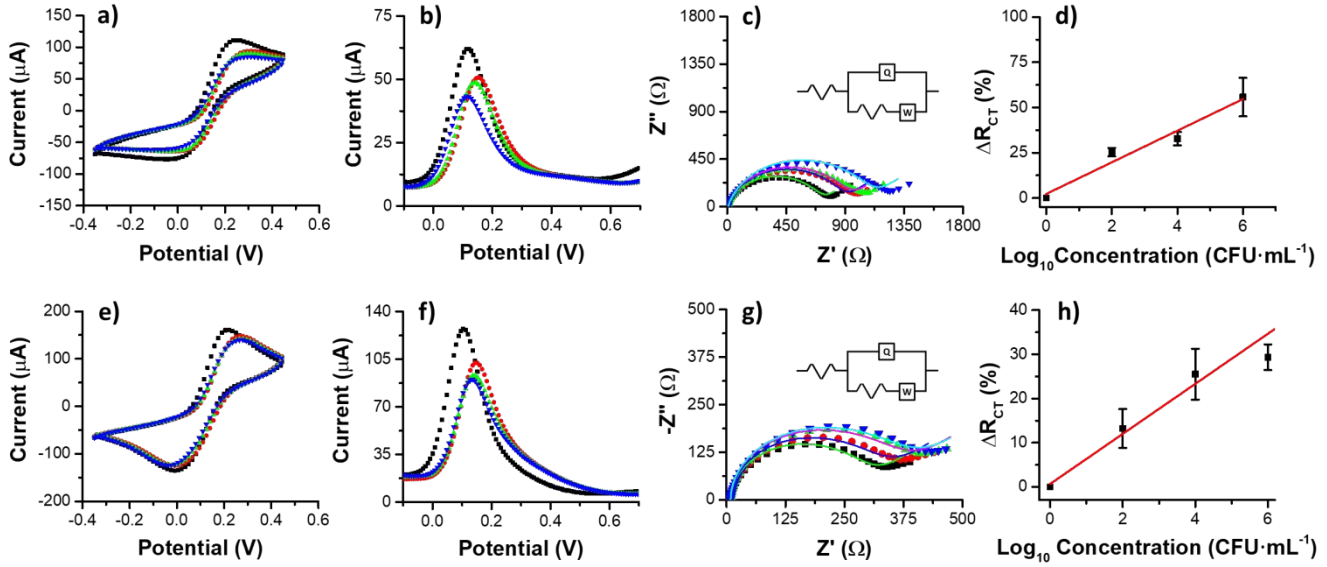

Figure 3. Electrochemical output from measuring different concentrations of Gram (+) bacteria. a) cyclic voltammogram b) square wave voltammogram c) Nyquist plot from electrochemical impedance measurements where curves plotted with (■), (●), (▲), and (▼) are for increasing concentrations of *B. licheniformis*. d) Response to different concentrations of *B. licheniformis* e) cyclic voltammogram f) square wave voltammogram g) Nyquist plot from electrochemical impedance measurements where curves plotted with (■), (●), (▲), and (▼) are for increasing concentrations of *E. hirae* h) Response to different concentrations of *E. hirae*. The average responses and error bars (standard deviations) were obtained from 3 repeats using 3 individually prepared sensors.

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A control experiment was carried out exposing TLR2/6 sensors to *E. coli* whole-cell cultures a Gram-negative bacterial strain. Figure 4 shows a comparison between the two Gram-positive bacterial strains and *E. coli* at consistent concentrations. As Gram-negative bacteria (e.g. *E. coli* 25922) is not in the scope of TLR2/6 recognition, it should not trigger any responses to TLR2/6 sensors in theory. Possibly due to nonspecific binding, 6 ± 3 % changes in ΔR_{CT}% are observed for 10^2 CFU/mL. The responses are not significantly changed when the concentrations of the *E. coli* were increased 100 and 10,000 fold, which were 8 ± 3 % and 6 ± 2 % for 10^4 and 10^6 CFU/mL, respectively. For each concentration, we have performed student *t*-tests on responses from Gram-negative bacterial samples and one of the Gram-positive bacterial samples. The responses from *E. hirae* and *E. coli* at 10^2 CFU/mL are not significantly different. (p-value is 0.07). For all the other concentrations, the responses from *E. coli* were significantly different from the Gram-positive bacteria strains at the same concentration (p-value ranged from 0.0004 to 0.01). This shows that detection by the TLR2/6 sensors is highly selective towards Gram-positive bacterial strains. We can differentiate the responses at 10^2 CFU/mL for *B. licheniformis* and at 10^4 CFU/mL for *E. hirae*. The limits of detection for TLR2/6 sensors is not as low as a reported study using TLR4, which was at 1 cell/mL. However, as TLR4 targets only Gram-negative PAMPs and bacteria, while TLR2/6 focuses on Gram-positive PAMPs and bacteria, the functionalities of these sensors complement each other. The reported studies of using TLRs as bio-recognition elements (explored a range of conditions) are shown in Table S2. Although analytical performance varies considerably, it is important to consider such studies in the context of their objectives which may have been the demonstration of ultimate sensitivity, or versatility, or proof of concept.

One topic of TLR sensor development which has not been well investigated is the robustness of the TLR sensors. While linker molecules and electrode materials could be altered to secure molecules better on sensor surfaces,[35, 37] it is also critical to preserve the bio-recognition ability of TLR proteins in the sensor designs. We performed an initial exploration on different storage conditions and temperatures using our TLR2/6 sensors as a model system. The goal was to screen for promising conditions for future microscopic studies. The sensors were stored under varying conditions for two weeks and then challenged by the PAMP (Pam2CSK4). The responses from freshly prepared (time zero) sensors was used as a reference point for the response of stored sensors. Comparisons are shown in Figure 5. (Calibration curves including more concentration points are shown in Figure S4.) Three storage temperatures were selected, 4 ℃, -33 ℃, and -80 ℃. Ionic salt solutions are commonly used to maintain protein folding and stability[39, 40], thus, to preserve the sensor during storage, varying buffer solutions were used. The first batch of sensors were stored in PBS buffer pH~7.4 at 4 ℃ (condition B). This is the condition recommended by the R&D system to preserve toll-like receptor proteins in solution.[41] The activities of the TLR proteins should remain 100 % after 1 month. However, this was not the case when we immobilized the TLR proteins onto our sensor surfaces. As shown in Figure 5, there were no significant responses from the TLR2/6 sensors after 2 weeks storage under condition B. The storage conditions for C and D are -33 ℃ in 50 % v/v PBS buffer pH ~7.4 and glycerol and -33 ℃ 50 % v/v Tris buffer pH 7.7 and glycerol respectively. Initially C and D had a very small response to Pam2CSK4 with no change in signal between the two smaller concentrations, 0.1 and 1.0 μM, although at the highest concentration (50 μM) there was a response. However, the activity was reduced to 30 % in comparison to the control sensor A. The storage conditions for E, -80 ℃ in PBS buffer pH ~7.4, showed the most promise. The response for all concentrations measured were within the uncertainty of the control sensor response, thus demonstrating no loss in sensor activity. This condition (E) was also compared with storage under -80 ℃ without immersing in any buffer (condition F). The results showed that the TLR2/6 sensors lost most of the responses without being frozen in PBS buffer environment.

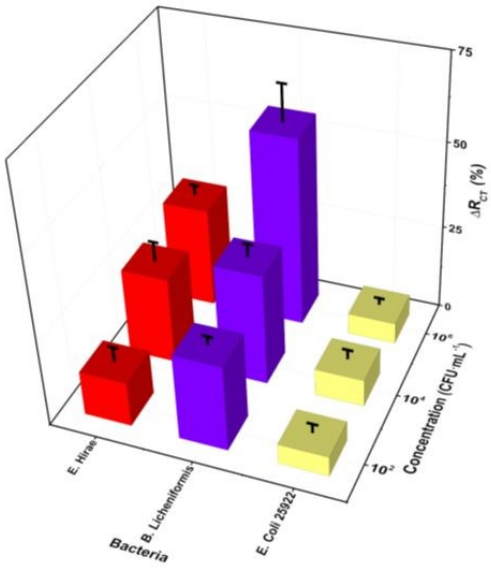

Figure 4. Three dimensional plots showing the TLR2/6 sensors' responses to different strains of bacterial whole-cell cultures with increasing concentration. 2-D plots at each concentration are shown in Figure S3. The average responses and error bars (standard deviations) were obtained from 3 replicates using 3 individually prepared sensors.

Conclusion

We have developed a new biosensor recognizing Gram-positive bacteria cells. The bio-recognition ability was created using TLR2 and TLR6 receptors and hybridizing them onto sensor electrode surfaces. Using such sensor chips, we would be able to reduce cost of the bacteria screening from $25 per sample[42] down to $5 per sample if the technology was brought to market. The detection was validated using Pam2CSK4, a Gram-positive pathogen-associated molecular pattern, and bacterial whole-cells. The detection limits for *B. licheniformis* and *E. hirae*, two Gram-positive cells, were 10^2 CFU/mL and 10^4 CFU/mL. The sensitivity of the sensor could potentially be increased if more complicated designs are adapted. (e.g. nanostructured surfaces and sandwich essays.[43, 44]). While studies have shown that our immune systems can cope with bacterial counts up to 10^5 CFU/mL without clinical complications,[45] the ability to detect lower counts of bacteria is still useful. It gives flexibility for either reducing the sample volume or implementing sample treatment steps that result in dilution. Regulatory levels may also be orders-of-magnitude lower than clinically significant counts. Drinking water tests require detection of a single organism in a 100 mL sample, the equivalent of 0.01 CFU/mL, which is below the likely range for all rapid devices unless they include a culture, amplification or preconcentration step.

The ability to store sensors and maintain their analytical ability is essential for future development of commercial detectors.

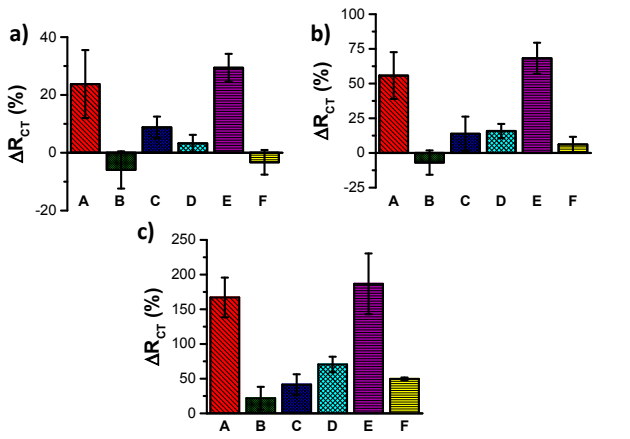

Figure 5. The detection responses obtained from the TLR2/6 sensors before storage (A), as a reference point, and with storage (B - F) under different conditions for 2 weeks: A. fresh sensors (time zero); B. 4 ℃ in PBS buffer; C. -33 ℃ in 50 % v/v PBS/glycerol; D. -33 ℃ in 50 % v/v Tris/glycerol; E. -80 ℃ in PBS F. -80 ℃ dry in a nitrogen atmosphere. Different concentrations of Pam2CSK4 at (a) 0.1 µM, (b) 1.0 µM and (c) 50 µM were used to trigger the responses. Each error bar represents the standard deviation of 4 replicates using 4 individually prepared sensors.

Simplified design in this study enabled us to investigate storage conditions for preserving (TLR) protein-based surfaces and maintain the responses after storage. Storing TLR2/6 sensor surfaces in PBS buffer under -80 ℃ was demonstrated to be the best out of the 5 conditions tested. The responses from these stored sensors towards Pam2CSK4 after 2 weeks were as strong as the responses from freshly prepared sensors. Conditions which did not maintain the detection capability will be investigated by scanning probe microscopic studies to uncover underlying mechanisms, thereby enabling better storage design strategies. The current detection protocol involves introducing samples and $Fe(CN)_6^{3-/4-}$ detection solution in two sequential steps, which could potentially be realized using devices such as microfluidic chips. However, it will be helpful in the longer term to have a single step design and real-time monitoring by TLR sensors that have a high degree of analytical and environmental robustness. This will require us to consider different molecular designs (e.g. using different linker molecules and electrochemical labels). Future work will apply these stabilized sensors in various environmental samples to examine the effects of various matrix components (such as different metal ions and varying pH) on sensor performance.

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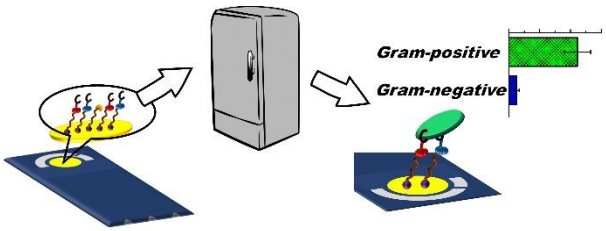

Conditions to store toll-like receptor2/6 sensors and use them to detect bacterial analytes, including pathogen-associated molecular patterns and bacterial cultures.