Electronic Supplementary Material (ESI) for Analyst.

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

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Experimental Method

Instrumentation. Milli-Q (MQ) water (18.2 MΩ·cm) was obtained from a Millipore Synthesis A10 Milli-Q water system. pH measurements were carried out on a Fisherbrand pH probe. DRP-C220AT DropSens screen printed electrodes and DRP-CAC (Model code) connectors were purchased from Metrohm AG (Herisau, Switzerland). Each DRP-C220AT DropSens chip contains a gold working electrode, a gold counter electrode, and Ag pseudo-reference electrode. The surface area of working electrode is 12.6 mm^2. Electrochemical measurements were performed on a CHI6055E potentiostat purchased from CH Instruments (Austin, TX). All experiments were performed at room temperature under ambient conditions.

Preparation of TLR 2/6 hybridized Sensor. TLR sensors were prepared using DropSens gold screen printed electrode (SPE). 1-lipoic acid n-hydroxysuccinimide ester (LPA) was synthesized following a published protocol.[1] LPA solution at a concentration of 2 mM was prepared using ethanol/MQ water (volume ratio of 1:1). A clean plastic petri dish was lined with a filter paper which was dampened with a 1:1 solution of ethanol and MQ. The DropSens SPE was placed on the filter paper and 50 µL of the 2 mM LPA solution was dropped onto the working electrode. The petri dish was sealed with parafilm and placed in a fridge at 4 ℃ for 24 hours. An additional 50 µL of the 2 mM LPA solution was cast onto the working electrode and returned to the fridge for another 24 hours. After a total of 48 hours the SPE was rinsed thoroughly with ethanol and MQ water before being blown dry with nitrogen. TLR2 and TLR6 proteins were individually reconstituted into solutions at a concentration of 200 µg/mL using PBS buffer (pH ~7.4). The TLR2/6 protein mixture was prepared by mixing 100 µL of TLR2 and 100 µL of TLR6 and vortexing for 30 seconds in a 1.5 mL microcentrifuge tube. The SPEs were placed in petri dishes with filter papers dampened with PBS buffer (pH ~7.4). A small drop of TLR 2/6 mixture (5 µL) was dispensed onto each working electrode and then the petri dishes were sealed with parafilm and left in the fridge at 4 ℃ for 72 hours. The electrodes were rinsed with MQ water after the immersion and blown dried with nitrogen. An ethanolamine-tris buffer solution was prepared by dissolving 1.2 g of ethanolamine, 0.121 g tris(hydroxymethyl)aminomethane in 20 mL of MQ water. The pH of the solution was adjusted to 8.4 using concentrated hydrochloric acid. After the step of immobilizing TLR2/6 proteins, the electrode surfaces were immersed in ethanolamine solutions for 1 hour to deactivate the unreacted ester groups on the LPA monolayer.

Electrochemical Measurements. All electrochemical measurements were preformed in Faraday cages using DRP-CAC adapters for the SPE's being measured. CV, SWV, and EIS measurements were completed using CHI6055E potentiostats. The measurements were carried out in 10 mM HEPES buffer solutions with 5 mM $K_4Fe(CN)_6$ / 5 mM $K_3Fe(CN)_6$ redox couple and 1 M $NaClO_4$ as the supporting electrolyte. CV measurements were carried out in a window between -350 and 450 mV using a scan rate of 0.1 V/s. SWV was performed by scanning from -100 mV to 700 mV with a frequency of 15 Hz. Open-circuit potentials were always used for EIS measurement, which were conducted in the frequency range of 100000 to 0.1 Hz with an amplitude of 5 mV. The experimental EIS curves were evaluated to determine the film resistance using ZSimpWin 2.0 software. The exposure times for Pam2CSK4 and bacterial whole-cell samples were 5 and 15 minutes respectively. CV, SWV and EIS were always collected before and after exposure the sensors to the analytes.

Supporting Experimental Data

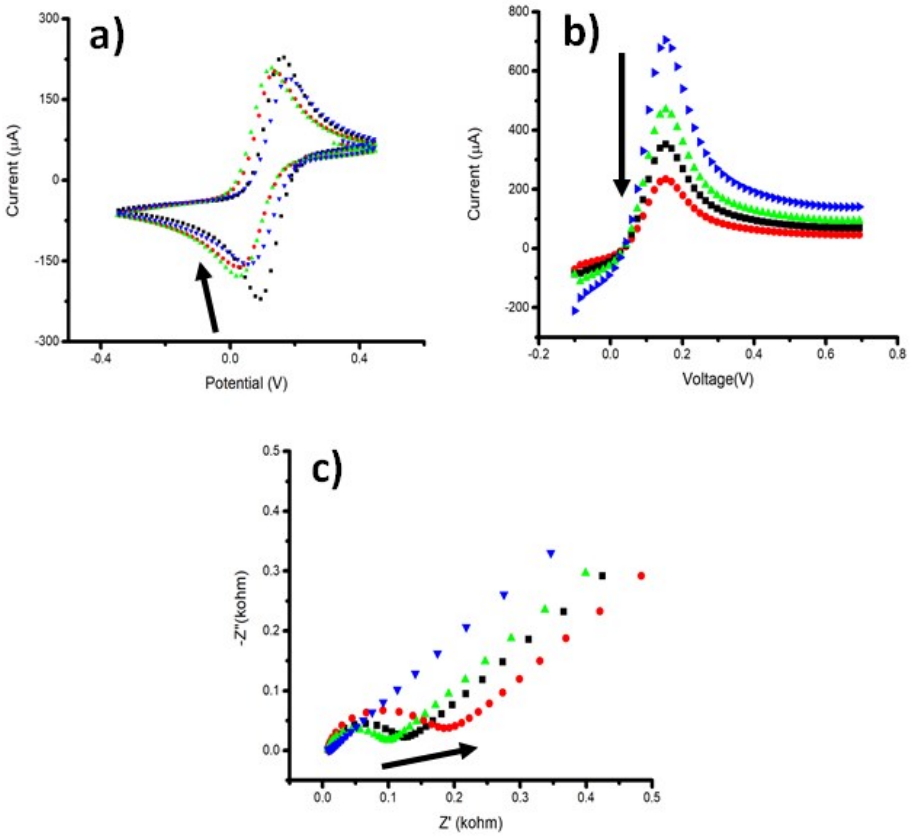

Figure S1. Characterization of sensor surfaces during preparation steps by (a) Cyclic voltammetry measuring bare gold surface (■, black), surface after LPA modification((▲, green), surface after TLR modification (●, red), surface after blocking with ethanolamine (▼, blue) (b) Square wave voltammetry measuring bare gold surface (►, blue), surface after LPA modification(▲, green), surface after TLR modification (■, black), surface after blocking with ethanolamine (●, red), (c) Nyquist plot measuring bare gold surface (▼, blue), surface after LPA modification(▲, green), surface after TLR modification (■, black), surface after blocking with ethanolamine (●, red). The arrow insets show- the trend of the changes in voltammograms and impedance plots.

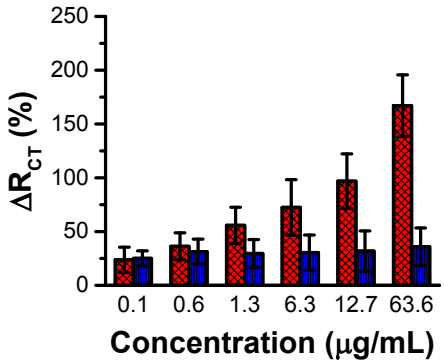

Figure S2. Comparison of TLR2/6 sensor responses to different concentrations of Pam2CSK4 (red, left bars), a Gram-positive bacterial PAMP, and *Salmonella* lipopolysaccharide (blue, right bars), a Gram-negative bacterial PAMP. The values are listed in **Table S1**.

Concentration (μg/ml)	ΔRct (%) Pam2CSK4	ΔRct (%) LPS
0.1	24 ± 12 %	25 ± 7 %
0.6	36 ± 13 %	31 ± 12 %
1.3	56 ± 17 %	30 ± 13 %
6.3	72 ± 26 %	30 ± 16 %
12.7	97 ± 25 %	32 ± 19 %
63.6	167 ± 29 %	36 ± 7 %

Table S1. TLR2/6 sensor responses to different concentrations of Pam2CSK4, a Gram-positive bacterial PAMP, and *Salmonella* lipopolysaccharide, a Gram-negative bacterial PAMP.

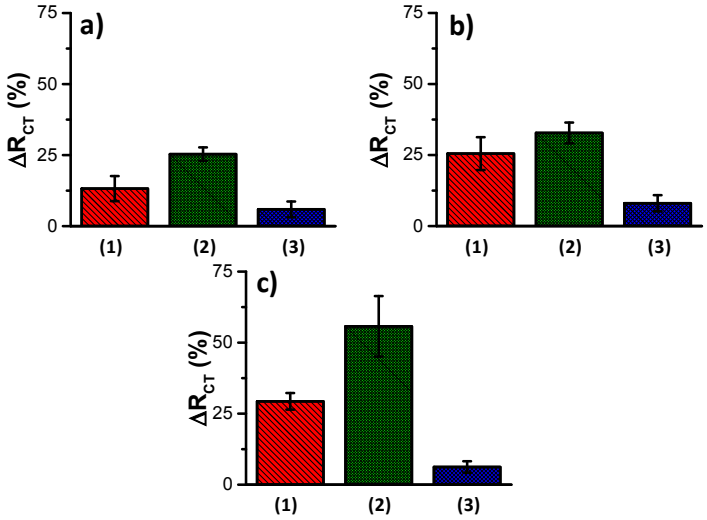

Figure S3. Plots of responses of TLR2/6 sensors to different strains of bacterial whole-cell cultures (1. *E. hirae* (ATCC 8043); 2. *B. licheniformis* (ATCC 12759); 3. *E. coli* (ATCC 25922)) with increasing concentration at a) 10^2 CFU/mL, b) 10^4 CFU/mL and c) 10^6 CFU/mL.

Targeted PAMP and Analyte	LOD	Bio-recognition element	Electrode type	Readout method	Reference
Triacylated lipopeptide	7.5 µg/ml	TLR1/TLR2	Gold	Electrochemical impedance spectroscopy	Ref. 2
Poly(I:C)	60 ng/ml	TLR3	Gold	Electrochemical impedance spectroscopy	Ref. 3
Lipopolysaccharide	0.0002 EU/mL	TLR4	Gold	Electrochemical impedance spectroscopy	Ref. 4
Lipopolysaccharide	1 ng/mL	TLR4	Gold	Electrochemical impedance spectroscopy	Ref. 5
Salmonella typhimurium (Lysed cell)	1 lysed cell/ml	TLR4	Gold	Electrochemical impedance spectroscopy	Ref. 6
Lipopolysaccharide	2 ng/ml	TLR4	Indium Tin Oxide	Electrochemical impedance spectroscopy	Ref. 7
Flagellin	3 ng/ml	TLR5	Indium Tin Oxide	Electrochemical impedance spectroscopy	Ref. 7
Diacylated lipopeptide	100 nM	TLR2/TLR6	Gold	Electrochemical impedance spectroscopy	This work
Bacillus licheniformis (whole-cell culture)	100 cell/ml	TLR2/TLR6	Gold	Electrochemical impedance spectroscopy	This work

Table S2. Comparison of different electrochemical TLR sensors and their corresponding analytes.

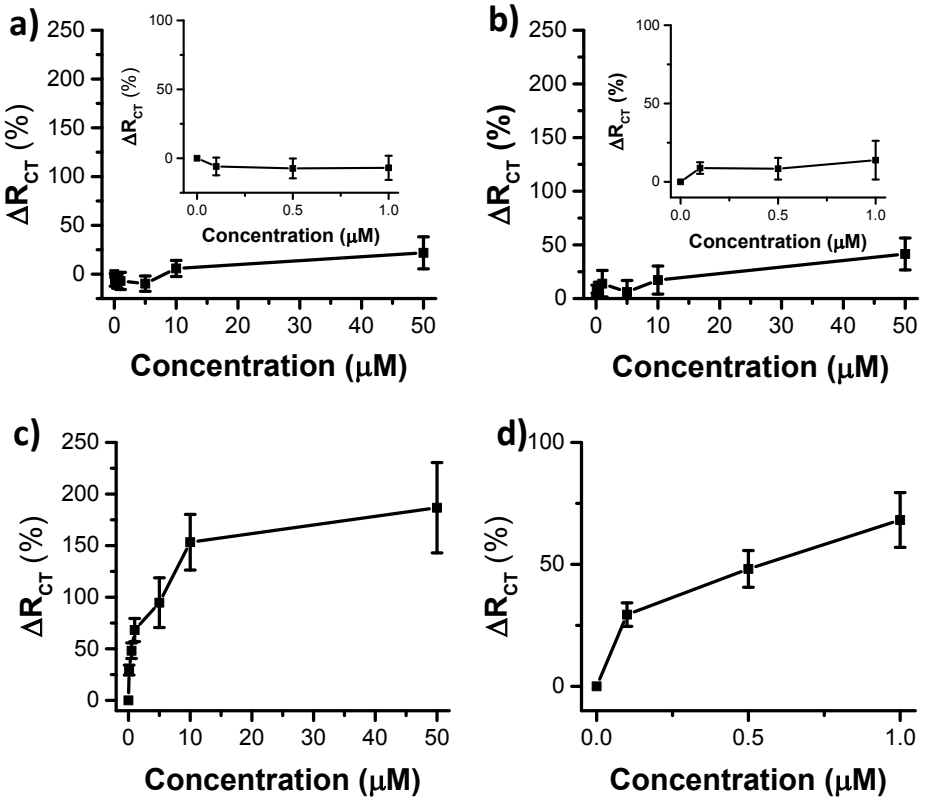

Figure S4. Calibration curves against different concentration of Pam2CSK4 obtained from TLR2/6 sensors stored under different conditions for 2 weeks: (a) 4 ℃ in PBS buffer, (b) -33 ℃ in 50 % v/v PBS/glycerol, (c & d) -80 ℃ PBS.

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