**N-Heterocyclic Carbenes Meet Toll-Like Receptors**

Ishwar Singh®, Dianne S. Lee®, Shuaishuai Huang®, Hridaynath Bhattacharjee®, Wei Xu®, Jennifer McLeod®, Cathleen M. Crudden®, and Zhe She®§

®Department of Chemistry, Queen's University, Chernoff Hall, Kingston, Ontario, Canada, K7L 3N6

®School of Science and Engineering, The Chinese University of Hong Kong Shenzhen, 518172, China

®BeaY Water Research Centre, Queen's University, Mitchell Hall, Kingston, ON, K7L 3N6

®Institute of Transformative Bio-Molecules (WPI-ITbM), Nagoya University, Furo, Chikusa, Nagoya 464-8602, Japan

**Supporting Information**

**Table of Contents**

**General** ............................................................................................................................................................ 2

Materials ............................................................................................................................................................... 2

Methods ................................................................................................................................................................ 2

**Detailed Syntheses** ........................................................................................................................................... 3

**Surface Functionalization** ............................................................................................................................ 7

Self-Assembled Monolayer (SAM) Formation ................................................................................................... 7

Modification of gold electrode ........................................................................................................................... 8

Kinetics of self-assembly of 1H+•CF3SO3− on gold working electrode .............................................................. 9

Immobilization of TLR proteins ......................................................................................................................... 10

TLR functionalised Au electrodes ....................................................................................................................... 12

TLR5 functionalised sensors with thiol backfilling .......................................................................................... 14

NHC biosensor storage stability .......................................................................................................................... 14

Preparation of a bacteria spiked real world sample .......................................................................................... 14
General

Materials: Reactions were performed with reagent grade solvents, with exception of toluene, CH₂Cl₂ and pentanes, they were distilled from sodium benzophenone ketyl or calcium hydride under an argon atmosphere prior to use. 4-amino-3-nitrophenol, propargyl bromide, CuSO₄·5H₂O and sodium ascorbate were purchased from Alfa Aesar; trifluoromethanesulfonyl anhydride, maleimide, furan, chloroform-d, methylene chloride-d₂, acetonitrile-d₃, and methanol-d₄ were purchased from Sigma-Aldrich.

Methods: High-resolution mass spectrometric (HRMS) data were obtained from a Thermo Fisher Scientific Orbitrap Velos Pro with an electrospray ionization (ESI). ¹H, ¹³C, and ¹⁹F NMR spectra were recorded on Bruker Instruments (400, Neo-500, and Neo-700) operating at denoted spectrometer frequency given in megahertz (MHz) at 25 °C. ¹H chemical shifts are referenced to the residual protons of the deuterated solvents CDCl₃ (at d = 7.26 ppm) and CD₂OD (at d = 3.31 ppm); ¹³C chemical shifts are referenced to the CDCl₃ and CD₂OD signals at d = 77.16 and 49.00 ppm, respectively. ¹⁹F NMR spectra were calibrated using CFCl₃ (d = 0.0 ppm) as an external reference. The following abbreviations are used to describe NMR signals: s = singlet, d = doublet, t = triplet, q = quartet, sept = septet, and m = multiplet. Coupling constants obtained from ¹H NMR spectra are associated with an error and reported to the first decimal point (the digital resolution in ¹H NMR spectra and ¹³C NMR is 0.11 Hz and 0.64 Hz respectively). Assignments for newly synthesized compounds were supported by additional NMR experiments (COSY, HSQC, and HMBC). All data were processed using MestReNova 11.0 software. All electrochemical measurements were done with CHI6055E Electrochemical Analyzer potentiostat. All electrochemical experiments were performed using a three-electrode electrochemical cell set-up with a 2 mm diameter gold disk working electrode, Ag/AgCl in 3M KCl reference electrode and a platinum wire as a counter/auxiliary electrode. Salt bridge was used to allow free flow of ions between one cell to the other. The salt bridge was built using a 4 mm glass rod filled with a heated 2 % agar solution in 1 M KNO₃ (w-v⁻¹) and stored in 1 M KNO₃ solution. All electrochemical data was processed with OriginPro 2016 software. X-ray photoelectron spectra (XPS) were recorded on Kratos Nova AXIS spectrometer equipped with an AlN X-ray source and a MCP stack as well as a delay-line detector. The samples were mounted on an aluminum sample holder using a double-sided adhesive copper tape and kept under high vacuum (10⁻⁹ torr) overnight before analysis. Spectra were collected using Al Kα radiation at 1486.69 eV (150 W, 15 kV) with 10 mA emission current, a coaxial low energy electron source for charge neutralization and a delay-line detector containing three multichannel plates. The acquisition was done using ESCape software and processed in CasaXPS and MATLAB. Binding energy of spectra were charge corrected to Au 4f₇/₂ at 84 eV for gold samples. Shirley type background correction was applied to all high-resolution spectra.
Detailed Synthesis

Scheme S1: Detailed Syntheses of 6-(6-(4-((2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)methyl)-1H-1,2,3-triazol-1-yl)hexyl)oxy)-1,3-diisopropyl-1H-benzo[d]imidazol-3-ium trifluoromethanesulfonate (1H+•CF$_3$SO$_3$)

4-((6-bromohexyl)oxy)-2-nitroaniline (4)

Modified from a previously reported procedure.$^1$ 4-amino-3-nitrophenol (2, 3.091 g, 20.05 mmol) was taken in a dry round-bottomed flask and dissolved in reagent grade CH$_3$CN (60 mL). K$_2$CO$_3$ (8.354 g, 60.45 mmol) was added to the reaction mixture. This mixture was bubbled with argon for 10 min and stirred at r.t. for 30 min. After that, 1,6-dibromohexane (3, 9.2 mL, 59.81 mmol) was added dropwise to the reaction mixture and then stirred at 80 °C for 12 h. The reaction mixture was then cooled to r.t. and all volatiles were removed in vacuo. The crude product was then purified by flash column chromatography with a gradient solvent mixture of hexanes and ethyl acetate to yield 4-((6-bromohexyl)oxy)-2-nitroaniline (4) as an orange solid (4.012 g, 63%).

This mixture was cooled to r.t. and all volatiles were removed in vacuo. The crude product was then purified by flash column chromatography with a gradient solvent mixture of hexanes and ethyl acetate to yield 4-((6-bromohexyl)oxy)-2-nitroaniline (4) as an orange solid (4.012 g, 63%). H NMR (400 MHz, Chloroform-d) $\delta$ 7.53 (d, $J = 2.9$ Hz, 1H, CH$_3$(aromatic)), 7.06 (dd, $J = 9.1$, 2.9 Hz, 1H, CH$_3$(aromatic)), 6.75 (d, $J = 9.1$ Hz, 1H, CH$_3$(aromatic)), 6.57 (d, $J = 9.1$ Hz, 1H, CH$_3$(aromatic)), 5.88 (s, 2H, NH$_2$), 3.93 (t, $J = 6.4$ Hz, 2H, CH$_2$-CH$_2$-O), 3.43 (t, $J = 6.8$ Hz, 2H, -CH$_2$-CH$_2$-Br), 1.97 – 1.85 (m, 2H, -CH$_2$-CH$_2$-CH$_2$-), 1.79 (p, $J = 6.6$ Hz, 2H, -CH$_2$-CH$_2$-CH$_2$-), 1.56 – 1.43 (m, 5H, includes H$_2$O impurity from NMR solvent, -CH$_2$-CH$_2$-CH$_2$-). $^{13}$C NMR (101 MHz, Chloroform-d)
δ 150.31, 139.96, 131.66, 127.21, 120.17, 107.26, 68.64, 33.91, 32.79, 29.07, 28.03, 25.40. m/z calcd for C_{12}H_{17}BrN_{2}O [M+H]^+ 317.04226, found [M+H]^+ 317.05039.

5-((6-bromohexyl)oxy)-1H-benzo[d]imidazole (5)

Modified from a previously reported procedure. 1 4-((6-bromohexyl)oxy)-2-nitroaniline (4, 4.000 g, 12.61 mmol), iron powder (7.043 g, 126.1 mmol), NH_{4}Cl (6.745 g, 126.1 mmol), and iPrOH (100 mL) were taken in a flame dried round-bottomed flask. The mixture was then degassed by purging argon for ca. 15 min. After that, formic acid (90 mL, 88% aq. solution, 1.9 mol) was added to the reaction mixture and refluxed for 4 h. Iron powder was removed by vacuum filtration and the dark brown filtrate was concentrated and carefully neutralized by dropwise addition of saturated NaHCO_{3} solution (aq.). The aqueous phase was then extracted with CH_{2}Cl_{2} (3 ´ 50 mL) and the combined organic layer was dried over anhyd. Na_{2}SO_{4}, and concentrated to yield 5-((6-bromohexyl)oxy)-1H-benzo[d]imidazole (5) in a quantitative yield. This was used without any further purifications.

1H NMR (400 MHz, Methylene Chloride-d_{2}) δ 7.98 (s, 1H, CH(imidazole)), 7.51 (d, J = 8.8 Hz, 1H, -C-CH( aromatic)-CH-), 7.07 (d, J = 2.3 Hz, 1H, -C-CH( aromatic)-C), 6.89 (dd, J = 8.8, 2.4 Hz, 1H, -CH_{2}-CH_{2}-Br), 1.84 (m, 4H, -CH_{2}-CH_{2}-CH_{2}-), 1.62 – 1.40 (m, 6H, contains H_{2}O impurity from NMR solvent, -CH_{2}-CH_{2}-CH_{2}-).

13C NMR (101 MHz, Methylene Chloride-d_{2}) δ 156.67, 140.80, 116.97, 113.47, 98.84, 69.02, 45.79, 34.65, 33.35, 29.72, 28.50, 27.23, 25.99, 25.87. m/z calcd for C_{13}H_{17}BrN_{2}O [M+H]^+ 297.05243, found [M+H]^+ 297.05968.

5-((6-azidohexyl)oxy)-1H-benzo[d]imidazole (6)

Modified from a previously reported procedure. 1 5-((6-bromohexyl)oxy)-1H-benzo[d]imidazole (5, 3.748 g, 12.61 mmol) was taken in a round-bottomed flask and dissolved in DMSO (20 mL). NaN_{3} (2.463 g, 37.83 mmol) was then added to the solution and stirred at r.t. for 12 h. After that, the reaction mixture was quenched with saturated NaHCO_{3} solution (aq.) and extracted with CH_{2}Cl_{2} (3 ´ 50 mL). Combined organic layer was dried over anhyd. Na_{2}SO_{4}, and dried under reduced pressure producing 5-((6-azidohexyl)oxy)-1H-benzo[d]imidazole as a brown solid (6, 2.380 g, 73%). This compound was used without any further purification. 1H NMR (400 MHz, Chloroform-d) δ 7.98 (s, 1H, CH(imidazole)), 7.54 (d, J = 8.8 Hz, 1H, -CH-CH( aromatic)-C), 7.12 – 7.03 (m, 1H, -C-CH( aromatic)-CH-), 6.93 (dd, J = 8.8, 2.1 Hz, 1H, -C-CH( aromatic)-CH-), 5.30 (s, 1H, NH), 3.99 (t, J = 6.4 Hz, 2H, -CH_{2}-CH_{2}-O-), 3.28 (t, J = 6.8 Hz, 2H, -CH_{2}-CH_{2}-N_{3}), 2.62 (s, 2H, DMSO impurity), 1.92 – 1.35 (m, 12H, contains H_{2}O impurity from NMR solvent, -CH_{2}-CH_{2}-CH_{2}-). 13C NMR (101 MHz, Chloroform-d) δ 156.08, 140.65, 113.12, 68.53, 51.43, 45.10, 32.57, 29.25, 28.85, 26.71, 26.57, 25.78. m/z calcd for C_{13}H_{17}N_{5}O [M+H]^+ 260.14331, found [M+H]^+ 260.15047.

5-((6-azidohexyl)oxy)-1-isopropyl-1H-benzo[d]imidazole (7a) and 6-((6-azidohexyl)oxy)-1-isopropyl-1H-benzo[d]imidazole (7b)

Modified from a previously reported procedure. 2 5-((6-
azidoxy)-1H-benzo[d]imidazole (6, 2.360 g, 9.100 mmol) was taken in a round-bottomed flask and dissolved in DMSO (10 mL). KOH powder (1.532 g, 27.30 mmol) was added to the dark red solution followed by addition of 2-bromopropane (2.60 mL, 27.30 mmol). The resulting slurry was stirred at r.t. for 14 h. The reaction mixture was quenched by adding H₂O (100 mL) and extracted with CH₂Cl₂ (3 × 50 mL). Combined organic layer was then washed with H₂O (5 × 50 mL) and brine (1 × 50 mL), dried over anhyd. Na₂SO₄, and concentrated under reduced pressure obtaining a mixture of 5-((6-azidoxy)oxy)-1-isopropyl-1H-benzo[d]imidazole (7a) and 6-((6-azidoxy)oxy)-1-isopropyl-1H-benzo[d]imidazole (7b) as a red-brown oil (2.142 g, 78%). This compound was used further without purification.

To a 75 mL pressure vessel, maleimide (10, 2.420 g, 24.93 mmol) and furan (11, 3.403 g, 49.99 mmol) were added. The mixture was dissolved in 25.0 mL of dry toluene and heated at 90 °C overnight. After cooling, the product precipitated out as a white solid. This was vacuum filtered and washed with 3 × Chloroform-d₂.

Preparation of isopropyl triflate: Modified from a previously reported procedure.¹ Dry CH₂Cl₂ (10 mL) was taken in a flame dried round-bottomed flask and cooled to −78 °C followed by consecutive addition of 2-propanol (2.0 mL) and pyridine (2.0 mL). Triflic anhydride (3.6 mL, 21.32 mmol) was added to the reaction mixture at −78 °C dropwise over 30 min resulting in the gradual formation of a white precipitate. The resulting mixture was then stirred at that temperature for another 30 min and at r.t. for additional 30 min. After this time, 10 mL dry pentane was added to the slurry, stirred for ca. 5 min, and let the precipitate settle down.

Mixture of 5-((6-azidoxy)oxy)-1-isopropyl-1H-benzo[d]imidazole (7a) and 6-((6-azidoxy)oxy)-1-isopropyl-1H-benzo[d]imidazole (7b, 2.154 g, 7.15 mmol) was added into a separate flame dried round-bottomed flask and dissolved in dry CH₂Cl₂ (5.0 mL). Clear supernatant from freshly prepared isopropyl triflate was transferred with a syringe and resulting mixture was stirred at 35 °C for 14 h under argon atmosphere. Reaction mixture was quenched with saturated NaHCO₃ solution (aq.), extracted with CH₂Cl₂ (3 × 50 mL), dried over anhyd. Na₂SO₄, and concentrated resulting a dark red-brown oil as a crude. The crude product was then purified by flash column chromatography using gradient MeOH in CH₂Cl₂, producing 5-((6-azidoxy)oxy)-1,3-diisopropyl-1H-benzo[d]imidazol-3-ium trifluoromethanesulfonate (8H⁺·CF₃SO₃⁻) as a red-brown oil (2.73 g, 78%).¹ H NMR (400 MHz, Chloroform-d) δ 9.73 (s, 1H, CH₈imidazole), 7.63 (d, J = 9.2 Hz, 1H, CH₈(aromatic)), 7.22 (d, J = 9.2 Hz, 1H, CH₈(aromatic)), 7.11 (d, J = 2.1 Hz, 1H, CH₈(aromatic)), 4.95 (tt, J = 13.7, 6.8 Hz, 2H, -CH₂-(CH₃)₂), 4.07 (t, J = 6.3 Hz, 2H), 3.30 (t, J = 6.8 Hz, 2H), 1.95 – 1.40 (m, 24H, CH₈), contains H₂O impurity from NMR solvent, -CH₂-CH₂-CH₃ and -CH₃. ¹³C NMR (101 MHz, Chloroform-d) δ 158.79, 136.94, 132.21, 122.31, 119.12, 117.64, 114.35, 96.37, 68.91, 51.91, 51.38, 51.22, 28.73, 28.60, 26.28, 25.42, 21.69, 21.61. ¹⁹F NMR (376 MHz, Chloroform-d) δ −79.24. m/z calc for C₁₉H₂₅N₂O⁺ [M]⁺ 344.24449, found 344.24457.

3a,4,7a-tetrahydro-1H-4,7-epoxyisoindole-1,3(2H)-dione (11)

Modified from a previously reported procedure.¹ To a 75 mL pressure vessel, maleimide (10, 2.420 g, 24.93 mmol) and furan (11, 3.403 g, 49.99 mmol) were added. The mixture was dissolved in 25.0 mL of dry toluene and heated at 90 °C overnight. After cooling, the product precipitated out as a white solid. This was vacuum filtered and washed with 3 × 

---

5 | P a g e
5.0 mL of cold toluene. The product was air dried and subsequently dried on high vacuum to afford 3.715 g (90%) of 3a,4,7,7a-tetrahydro-1H-4,7-epoxyisoindole-1,3(2H)-dione (11). 1H NMR and 13C NMR were consistent with literature.3

**2-(prop-2-yn-1-yl)-3a,4,7,7a-tetrahydro-1H-4,7-epoxyisoindole-1,3(2H)-dione (13)**

The following synthesis was carried out according to literature with minor alterations.4 3a,4,7,7a-tetrahydro-1H-4,7-epoxyisoindole-1,3(2H)-dione (11, 1.663 g, 10.07 mmol) was taken in a 20 dram vial and dissolved in DMF (7.0 mL) followed by addition of K2CO3 (4.201 g, 30.40 mmol). Color changed to light pink upon addition of the base. It was then stirred at r.t. for 30 min followed by dropwise addition of propargyl bromide (12, 1.40 mL, 80 wt.% in toluene, 15.7 mmol). The resulting mixture was then stirred at 50 °C for 12 h. After that, it was cooled down to r.t., 50 mL H2O added to it, and extracted with CH2Cl2 (3 × 50 mL). Combined organic layer was then dried over anhyd. Na2SO4 and concentrated in vacuo to obtain a dark red oil. The crude product was then purified by flash column chromatography using a gradient solvent mixture of hexanes and ethyl acetate giving compound 2-(prop-2-yn-1-yl)-3a,4,7,7a-tetrahydro-1H-4,7-epoxyisoindole-1,3(2H)-dione (13) as a colourless oil (1.747 g, 85%). 1H NMR and 13C NMR were consistent with literature.4

**1-(prop-2-yn-1-yl)-1H-pyrrole-2,5-dione (14)**

Modified from a previously reported procedure.5 2-(prop-2-yn-1-yl)-3a,4,7,7a-tetrahydro-1H-4,7-epoxyisoindole-1,3(2H)-dione (13, 0.878 g, 4.32 mmol) and dry toluene (20 mL) were taken in a flame dried round-bottomed flask and refluxed under argon atmosphere for 14 h. Solvent was removed under reduced pressure and the obtained crude was purified by flash column chromatography using a gradient solvent mixture of hexanes and ethyl acetate. Pure compound 14 was isolated as a pale-yellow oil (0.519 g, 89%). 1H NMR and 13C NMR spectral data were consistent with literature.5

**6-((6-((2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)methyl)-1H-1,2,3-triazol-1-yl)hexyl)oxy)-1,3-diisopropyl-1H-benzo[d]imidazol-3-ium trifluoromethanesulfonate (1H+•CF3SO3–)**

Modified from a previously reported procedure.4 8H+•CF3SO3– (0.555 g, 1.13 mmol) and 14 (0.213 g, 1.58 mmol) were taken in a vial and dissolved in 18 mL degassed solvent mixture [THF/H2O (1/1)]. After that, CuSO4•5H2O (0.337 g, 1.35 mmol) and sodium ascorbate (0.267 g, 1.35 mmol) were added respectively to the reaction mixture, which was then stirred at r.t. for 14 h. 100 mL H2O was added to the clear brown reaction mixture and extracted with CH2Cl2 (3 × 75 mL). Combined organic phase was dried over anhyd. Na2SO4 and concentrated in vacuo to obtain a red brown oil. The crude product was then purified by flash column chromatography with gradient of MeOH in CH2Cl2 producing compound 1H+•CF3SO3– as a brown solid (0.218 g, 31%). When scaling up the reaction, column chromatography was detrimental to the final yield, therefore, the crude material was triturated in ethyl acetate to afford 1H+•CF3SO3– as a brown solid with 65% yield. 1H NMR (700 MHz, MeOD) δ 9.41 (s, 1H, CH[imidaole]), 7.93 (s, 1H, CH[triazele]), 7.89 (d, J = 8.8 Hz, 1H, CH[aromatic]), 7.44 (d, J = 2.4 Hz, 1H, CH[aromatic]), 7.28 (dd, J = 9.1, 2.3 Hz, 1H, CH[aromatic]), 6.84 (s, 2H, CH[maleimide]), 5.02 (m, 2H, -CH-(CH3)2), 4.74 (s, 2H, -N-CH2-C), 4.39 (t, J = 7.0 Hz, 2H, -CH2-CH2-N), 4.10 (t, J = 6.4 Hz, 2H, -O-CH2-CH2-), 2.01 – 1.31 (m, 24H, alkyl protons with HCl impurity from NMR solvent). 13C NMR (176 MHz, MeOD) δ 171.75, 160.31, 137.87, 135.59, 133.86, 126.60, 118.72, 115.64, 97.46, 69.98, 52.81, 52.24, 51.29, 49.41, 49.37, 49.24, 49.12, 49.04, 49.00, 48.88, 48.85, 48.84, 48.79, 48.76, 48.64, 33.46, 31.04, 29.83, 27.04, 26.79, 26.44, 26.41, 22.17, 22.13, 22.11. 19F NMR (376 MHz, MeOD) δ –79.81. m/z
calcd for $C_{26}H_{35}N_6O_3^+$ [M]$^+$ 479.27652, found 479.27649. Strong peaks for amide stretching observed at 1706.67 cm$^{-1}$ and a loss of stretching at 2090.48 cm$^{-1}$ indicating loss of -N$_3$ compared to $8H^+\cdot CF_3SO_3^-$. 

![Figure S1: Shows comparison of IR data before and after click reaction](image)

**Surface functionalization**

**Self-assembled monolayer (SAM) Preparation**

$N$-heterocyclic carbene (NHC) monolayer was prepared by dissolving $1H^+\cdot CF_3SO_3^-$ in 5 mL of HPLC-grade MeOH (10 mM solution). A gold substrate was immersed in this solution for 24 h at room temperature. Substrates were then rinsed with MeOH and dried under a stream of argon. Initial characterization of this substrate was done by performing a survey scan revealing the presence of Au, C, N, and O; thereafter, high resolution scans of these elements were performed. Furthermore, high resolution scans of F 1s and S 2p regions were performed to account for any physisorbed species on surface.
Modification of gold electrodes

The organic contaminants on gold electrodes were removed by immersion in piranha solution (H₂SO₄:H₂O₂ = 3:1 v/v) for 30 s. The electrodes were rinsed thoroughly with Milli-Q (MQ) water. Electrode surfaces were further cleaned by mechanical polishing using a figure-eight polishing motion. Different sizes of alumina powders (0.3 µm and 0.05 µm) were selected to prepare alumina suspensions in MQ water. This alumina powders on the gold electrode was removed by ultrasonicating in MQ water, absolute ethanol and again MQ water, respectively. Each ultrasonication step was done for 10 minutes. The electrodes were further electrochemically cleaned to remove absorbed species during the polishing procedure. Base and acid cleaning were performed in sequence with a pre-programmed CV cleaning method. The electrodes were then electrochemically cleaned by running 100 CV cycles in 0.5 M of NaOH solution (aq.) between 0 V and 2 V at a scan rate of 0.5 V·s⁻¹ and followed by 100 CV cycles in 0.5 M of H₂SO₄ solution (aq.) between 0 V and 1.5 V at the same scan rate.

Figure S2: High resolution XPS of 1@Au. (a) Au 4f, (b) C 1s, (c) N 1s, (d) O 1s and (e) widescan (Exp refers to the raw CPS)
Kinetics of self-assembly of $1\cdot CF_3SO_3H$ on gold working electrode

The gold working electrode was modified by immersion in 10 mM solutions of $1H^+\cdot CF_3SO_3^-$ in HPLC-grade MeOH for (0 s, 10 s, 30 s, 2 min, 5 min, 10 min, 15 min, 30 min, and 24 h) at room temperature. The samples were measured by CV in HEPES electrolyte buffer solution. Before each measurement, the electrodes were rinsed with methanol and dried under a stream of argon (4.8 Praxair).

Figure S3: Cyclic voltammograms obtained by immersing the freshly cleaned gold electrodes in a 10 mM MeOH solution of $1H^+\cdot CF_3SO_3^-$ after 0 s, 10 s, 30 s, 2 mins, 5 mins, 10 mins, 15 mins, and 30 mins. The CV was obtained in 10 mM HEPES aqueous buffer solution (pH 7.4) which contains 5 mM/5 mM Fe(CN)$_6^{3-/4-}$ as the redox couple and 1 M NaClO$_4$ as the supporting electrolyte. The scan rate was set to 0.1 V s$^{-1}$. Ag/AgCl/3 M was used as the reference electrode.

Figure S4: Cyclic voltammograms obtained by immersing the freshly cleaned gold electrodes in a 10 mM MeOH solution of $1H^+\cdot CF_3SO_3^-$ for 30 min and 24 h. The CV was obtained in 10 mM HEPES aqueous buffer solution (pH 7.4) which contains 5 mM/5 mM Fe(CN)$_6^{3-/4-}$ as the redox couple and 1 M NaClO$_4$ as the supporting electrolyte. The scan rate was set to 0.1 V s$^{-1}$. Ag/AgCl/3 M was used as the reference electrode.
Figure S5: Cyclic voltammogram of 1@Au electrode indicating that the SAM is completely stable over repeated cycling up to 150 cycles between -0.1 and 0.6 V. The CV was obtained in 10 mM HEPES aqueous buffer solution (pH 7.4) which contains 5 mM/5 mM Fe(CN)$_6^{3-/4-}$ as the redox couple and 1 M NaClO$_4$ as the supporting electrolyte. The scan rate was set to 0.1 V s$^{-1}$. Ag/AgCl/3 M was used as the reference electrode.

**Immobilization of TLR proteins**

The gold chips and working electrode was modified by immersing them in 10 mM solutions of 1H•CF$_3$SO$_3^-$ in HPLC-grade methanol for 24 h at room temperature. The electrodes were then rinsed thoroughly using HPLC-grade methanol and dried under a flow of nitrogen before incubation with TLR protein (TLR1-TLR5) in PBS buffer (pH = 7.4) for 72 h at 4 °C. Modified chips were used for measuring XPS, whereas cyclic voltammograms were measured in HEPES electrolyte buffer for the gold working electrode. Before each measurement, the electrodes were rinsed with MQ water and dried under a stream of nitrogen (5.0 Praxair).

Initial characterization was done by doing a survey scan, which revealed the presence of Au, S, C, N, and O corresponding to different elements present in our molecules. Therefore, high resolution scans of these elements were performed and are shown in the following figures. Four different regions were scanned on the same surface for 3 replicate chips and it was noted that shifts and elemental composition was consistent throughout. Diagnostic peaks were mainly observed in high-resolution scans for S 2p, C 1s, N 1s and O 1s. High-res XPS of various functionalised chips with annotations is shown in Figure S5. Peak fitted spectra for TLR5@Au are shown in Figure S6.
Figure S6: Summary of high-resolution XPS data collected from functionalised surfaces compared to TLRs@Au; (a) S 2p, (b) C 1s, (c) N 1s and (d) O 1s (Exp refers to the raw CPS)
Figure S7: Summary of peak fitted high-resolution XPS data for **TLR5@Au**; (a) O 1s, (b) N 1s, (c) C 1s, (d) S 2p and (e) Au 4f (Exp refers to the raw CPS)

**TLR Functionalised Au Electrodes**
Figure S8: Cyclic voltammograms of a gold modified with 1 and gold electrode immobilised with TLR1, TLR2, TLR3, TLR4 and TLR5. Reduction of the electrochemical currents are observed after immobilization for all TLRs. The CV was obtained in 10 mM HEPES aqueous buffer solution (pH 7.4) which contains 5 mM/5 mM Fe(CN)$_6^{3-}/4-$ as the redox couple and 1 M NaClO$_4$ as the supporting electrolyte. The scan rate was set to 0.1 V s$^{-1}$. Ag/AgCl/3 M was used as the reference electrode.

Figure S9: Cyclic voltammogram of TLR5@Au that shows repeated cycles for the reduction and oxidation of Fe(CN)$_6^{3-}/Fe(CN)$_6^{4-}$ in solution with different potential windows. (a) -0.1 V to 0.6 V (b) -0.2 V to 0.7 V (c) -0.3 V to 0.8 V (d) -0.4 V to 0.9 V (e) -0.5 V to 1.0 V (f) -0.6 V to 1.1 V. The CV was obtained in 10 mM HEPES aqueous buffer solution (pH 7.4) which contains 5 mM/5 mM Fe(CN)$_6^{3-}/4-$ as the redox couple and...
1 M NaClO₄ as the supporting electrolyte. The scan rate was set to 0.1 V s⁻¹. Ag/AgCl/3 M was used as the reference electrode.

**TLR5 Functionalised NHC biosensor with thiol backfilling**

To avoid any defects in the monolayer that could possibility lead to non-specific adsorption (NSA), TLR5@Au were backfilled with 1 mM of 6-mercapto-1-hexanol (MCH) in 10 mM HEPES buffer for 1 hour. For comparison, 1@Au were also immersed in 1mM of MCH in 10 mM HEPES buffer for 1 hour. TLR5@Au (with MCH) and 1@Au (with MCH) were exposed to varying concentrations of *E.coli* 25922 (10⁻²-10⁶ CFU mL⁻¹). Before each measurement, the electrodes were rinsed with MQ water and dried under a stream of nitrogen (5.0 Praxair). No significant difference was observed in the backfilled system between with and without TLR (*p*-value = 0.143 for 10⁻² CFU mL⁻¹ and 0.152 for 10⁴ CFU mL⁻¹).

Figure S10: Comparison of normalised responses of the *E.coli* 25922 between with or without TLR5 (with MCH) at a concentration of 1 x 10² & 1 x 10⁴ CFU mL⁻¹. Electrochemical measurements were done in 10 mM HEPES aqueous buffer solution (pH 7.4) which contains 5 mM/5 mM Fe(CN)₆³⁻/⁴⁻ as the redox couple and 1 M NaClO₄ as the supporting electrolyte. Ag/AgCl/3 M was used as the reference electrode.

NHC sensors functionalised with TLR5 were not backfilled with MCH due to the indifferent response between sensors with TLR and sensors without TLR when backfilled.

**NHC biosensor storage stability**

NHC biosensor was prepared according to the previously mentioned methodology with TLR5 (TLR5@Au). The gold disc electrode NHC biosensor was immersed and stored in a buffered amino acid solution (50 mM L-arginine and L-glutamine, 0.2 M NaCl, 1 M imidazole, and 50 mM Tris pH 8) for either 2 or 4 weeks at 4 °C. The stored sensors were taken out of the solution and rinsed with MQ water and dried a stream of nitrogen.

**Preparation of a bacteria spiked real world sample**

NHC biosensor was tested against a *E.coli* 25922 spiked lake water to observe the sensor’s response in a real world condition. Spiked lake water was prepared as follows. 1 L of water sample was collected from the shore of Lake Ontario when the temperature of the water was 2.1 °C. Collected water sample was boiled for 10 minutes before cooling it back down to room temperature. The sample was then filtered through a filter paper to remove any solid materials from the sample (ex. Dirt). The prepared water sample’s pH was measured to be 8.5. 25 mL of the autoclaved cultured *E.coli* 25922 was centrifuged down.
for 20 minutes to separate the bacteria from its cultured solution. The solution was decanted off carefully and the prepared lake water sample was added to the bacteria. The combined sample was vigorously vortexed for 5 minutes. This three-step procedure was repeated three times. The final sample was then diluted to make $1 \times 10^2$, $1 \times 10^4$ and $1 \times 10^6$ concentrations of \textit{E.coli} 25922 in lake water.

![Figure S11: Normalised responses of spiked lake water at various concentration of \textit{E.coli} 25922 CFU mL$^{-1}$ along with the background response from the lake water ($\Delta R_{ct} \%$).](image)

References


