Supporting information

Amplified Zinc Signal at a Nanocarbon Film Electrode for Lipopolysaccharide Detection

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MATERIALS AND METHODS

Materials

Chemicals

LPS-free water was purchased from Otsuka Pharmaceutical (Japan) (water for injection). The LPS used in this study was Japanese pharmacopoeia reference standard endotoxin purchased from the Pharmaceutical and Medical Device Regulatory Science Society of Japan. Zn standard stock solution (1000 ppm) and 25 % ammonia solution were obtained from Wako. Ammonium sulfate and Dulbecco's phosphate buffered saline (PBS) were purchased from Sigma-Aldrich. PBST (PBS containing 0.05 % Tween 20) was purchased from Thermo Scientific (USA). Block-Ace was purchased from DS Pharma Biomedical (Japan). All other chemicals were of analytical grade.

Chemicals for LPS probe

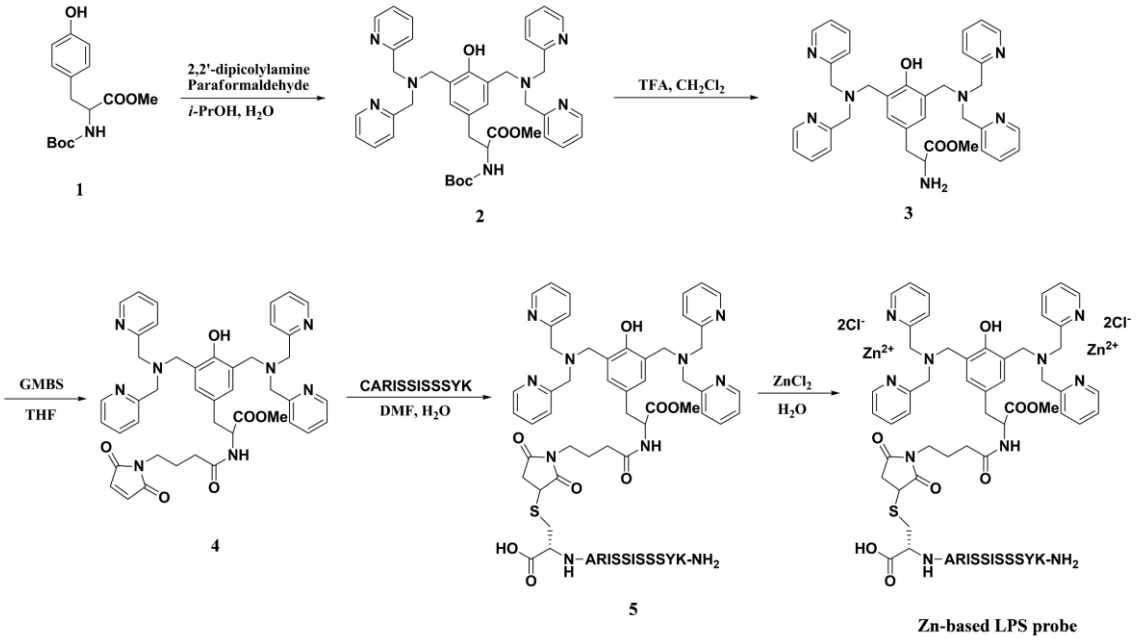
General information

All the chemicals used were of analytical reagent grade and were purchased from TCI (Tokyo, Japan), Wako (Osaka, Japan), and Aldrich. The peptides were purified using high-performance liquid chromatography. ^{1}H NMR spectra were recorded using a Bruker AV400M spectrometer.

Synthesis of Zn-based LPS probe.

The synthetic route of our Zn-based LPS probe is illustrated in Scheme S1.

Scheme S1. Synthetic method of Zn-based LPS probe.



Detailed methods are as follows;

Methyl 3-(3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxyphenyl)-2-(tert-butoxycarbonylamino)propanoate (2).

Paraformaldehyde (1.21 g, 40.4 mmol) was added to a solution of 2,2'-dipicolylamine (5.03 g, 25.3 mmol) in a mixture of H_2O (62.5 mL), *i*-PrOH (37.5 mL), and 2N HCl (2.0 mL) and heated at 80 °C for 30 min in an argon atmosphere. Boc-L-tyrosine-OMe (3.0 g, 10.1 mmol) was added, and the reaction mixture was refluxed for 24 hours. *i*-PrOH was then removed, the residue was cooled on an ice bath, and the solvent was removed by decantation. The precipitated viscous oil was dissolved in AcOEt, washed with saturated $NaHCO_3$, brine, and dried over Na_2SO_4. The solvent was evaporated *in vacuo*, and the product was purified by column chromatography (Al_2O_3, $CHCl_3$: MeOH = 10 : 1 v/v) to give an oil compound.

Yield: 52 %

^{1}H NMR ($CDCl_3$, 400MHz, r.t., TMS, δ/ppm) 1.38 (9H, s), 3.00 (2H, d, *J* = 7.0 Hz), 3.61 (3H, s), 3.77 (4H, s), 3.86 (8H, s), 4.52 – 4.50 (1H, m), 5.23 (1H, d, *J* = 7.0 Hz), 7.01 (2H, s) 7.13 (4H, d, *J* = 7.5 Hz) 7.46 (4H, t, *J* = 7.5 Hz) 7.61 (4H, t, *J* = 7.5 Hz), 8.53 (4H, d, *J* = 7.5 Hz), 11.06 (1H, br s).

Methyl 2-amino-3-(3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxyphenyl)propanoate (3).

To a solution of methyl 3-(3,5-bis((bis(pyridine-2-ylmethyl)amino)methyl)-4-hydroxyphenyl)-2-(tert-butoxycarbonylamino)propanoate (3.6 g, 5.1 mmol) in dichloromethane (25 mL) on an ice bath, trifluoroacetic acid (15 mL) was added, and the reaction mixture was stirred for 2 hours. After the removal of the solvent *in vacuo*, the residue was dissolved in H_2O, and alkalized with aq. NH_3. The resulting mixture was extracted with dichloromethane, and the combined organic phases were washed with brine and dried over Na_2SO_4. After removal of the solvent *in vacuo*, the product was obtained quantitatively as a viscous oil.

Yield: 97 %

^{1}H NMR ($CDCl_3$, 400MHz, r.t., TMS, δ/ppm) 3.01 (2H, d, *J* = 7.0 Hz), 3.62 (3H, s), 3.78 (4H, s), 3.85 (8H, s), 4.51-4.52 (1H, m), 7.02 (2H, s) 7.14 (4H, d, *J* = 7.5 Hz) 7.48 (4H, t, *J* = 7.5 Hz) 7.62 (4H, t, *J* = 7.5 Hz), 8.55 (4H, d, *J* = 7.5 Hz), 11.06 (1H, br s).

Methyl 3-(3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxyphenyl)-2-(5-(dimethylamino)naphthalene-1-sulfonamido)propanoate (4).

N-(4-maleimidobutyryloxy)succinimide (GMBS) (0.10 g, 0.36 mmol) was added to a solution of methyl 2-amino-3-(3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxyphenyl) propanoate (0.26 g, 0.36 mmol) in dry THF (10 mL), and the reaction mixture

was stirred for 12 hours at room temperature. The solvent was evaporated *in vacuo*, and the residue was dissolved in AcOEt, washed with water, and dried over Na_2SO_4. After the solvent was removed, the product was purified by column chromatography (Al_2O_3, AcOEt : MeOH = 20 : 1 v/v) to give a yellow solid.

Yield: 85%

^{1}H NMR ($CDCl_3$, 400MHz, r.t., TMS, δ/ppm) 1.25 (2H, t, *J* = 7.1 Hz), 1.78-1.83 (2H, m), 3.02 (2H, d, *J* = 7.0 Hz), 3.41-3.46 (2H, m), 3.65 (3H, s), 3.76 (4H, s), 3.84 (8H, s), 4.77-4.82 (1H, m), 6.55 (2H, s), 6.78 (1H, d, *J* = 7.0 Hz), 6.98 (2H, s), 7.12 (4H, d, *J* = 7.5 Hz), 7.47 (4H, t, *J* = 7.5 Hz) 7.62 (4H, t, *J* = 7.5 Hz), 8.52 (4H, d, *J* = 7.5 Hz), 11.04 (1H, br s).

Coupling reaction of methyl 3-(3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxyphenyl)-2-(5-(dimethylamino)naphthalene-1-sulfonamido)propanoate with peptide (5).

2-mercaptoethanol (1.5 μL) was added to a solution of peptide (5.0 mg) in H_2O (200 μL), and the reaction mixture was incubated for 90 min at 37 °C. After the removal of the solvent *in vacuo*, the residue was dissolved in H_2O (200 μL), and then methyl 3-(3,5-bis((bis(pyridin-2-ylmethyl)amino)methyl)-4-hydroxyphenyl)-2-(5-(dimethylamino) naphthalene-1-sulfonamido)propanoate (5.0 mg) in DMF (20 μL) was added, and the mixture stirred for 6 hours at 4 °C. The solvent was then removed, and the product was purified by HPLC (Eluent: 0.065 % TFA in 100 % water and 0.05 % TFA in 100 % MeCN, Flow rate: 1.0 mL min^{-1}, Column: TOSOH TSK-GEL ODS-100S 4.6 × 250 mm).

Yield: 92 %.

ESI-MS(+): $[M + H]^+$ = 2083.89

Zn-based LPS probe.

Aqueous $ZnCl_2$ solution (1.0 mM, 9.7 μL) was added to a solution of peptide (10.0 mg, 4.83 μmol) in water (500 μL). The reaction mixture was stirred for 1 h at 4 °C. The solvent including redundant Zn^{2+}ions was then removed, and the residue was washed with acetonitrile and finally dried in a vacuum to obtain the product.

Yield: 98 %

ESI-MS(+): $[M + 2Zn + 2Cl]^+$ = 2284.55

Methods

Electrochemical measurements of Zn^{2+} ions

Electrochemical measurements were performed using a three-electrode configuration with an electrochemical analyzer model ALS/CHI 1240B (CH Instruments, Inc.). In all the experiments, Pt wire and an Ag/AgCl electrode were used as auxiliary and reference electrodes, respectively. A nanocarbon film electrode (diameter = 2 mm) was used as a working electrode. A GC electrode (GC20SS, Tokai Carbon Co. Ltd, Tokyo, Japan) was used as a control electrode in the experiment.

Anodic stripping voltammetry (ASV) for Zn^{2+} ions was carried out as follows. First, the potential of the working carbon electrode was held at less than –1.0 V to preconcentrate the Zn metal on the carbon electrode surface, while the sample solution was being stirred (approximately 600 rpm). The sample solution was diluted from a standard stock solution with 0.1 M ammonium buffer (pH 8.0). The preconcentration potential was varied from –1.0 to –1.6 V to reveal the ASV performance. The preconcentration time was varied from 60 to 600 s. Then, we stopped stirring the sample, and carried out square-wave voltammetry (SWV). All the SWV measurements were performed with an amplitude of 25 mV and a ΔE of 2 mV at 40 Hz.

Figures S1 and S2 show the results for the ASV performance of Zn^{2+} ions. From these results, we determined optimized conditions of a preconcentration potential of -1.5 V (vs. Ag/AgCl) and a preconcentration time of 240 s.

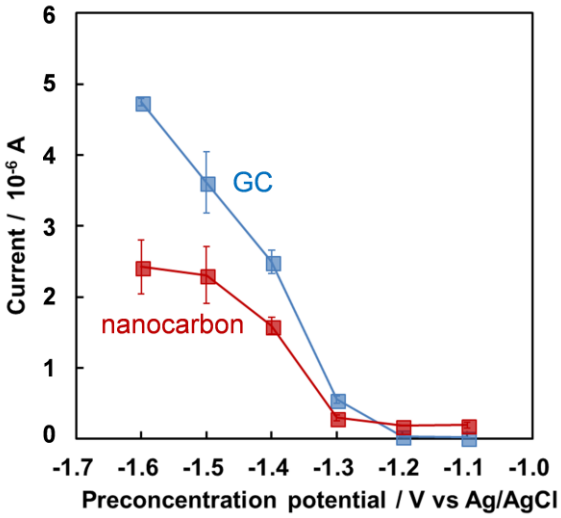


Figure S1. Dependence of preconcentration potential of Zn^{2+} ions (100 ng mL^{-1}) at nanocarbon film (red) and GC (blue) electrodes. The preconcentration time is 240 s. Amplitude = 25 mV, ΔE = 2 mV, f = 40 Hz.

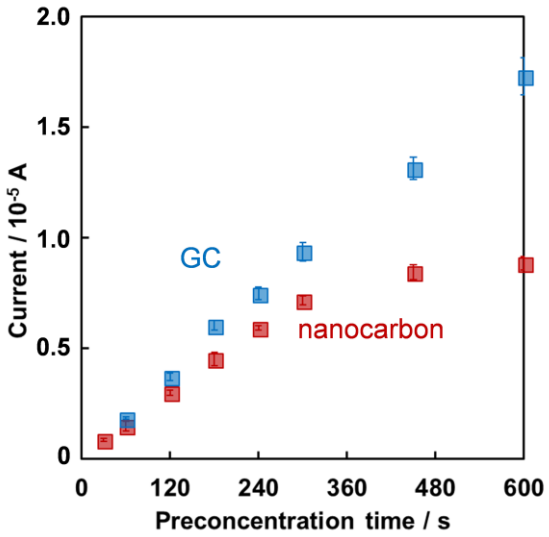


Figure S2. Dependence of preconcentration time of Zn^{2+} ions (100 ng mL^{-1}) at nanocarbon film (red) and GC (blue) electrodes. The preconcentration potential is –1.5 V. Amplitude = 25 mV, ΔE = 2 mV, f = 40 Hz.

Flow system for LPS measurements

A flow measurement system was constructed as shown in Figure S3. Briefly, a mini-column packed with LPS affinity microparticles (Cellufine® ET clean S, JNC corp., Japan) was constructed by introducing the microparticles (approximately 30 mg) into a PTFE tube (1/16" OD, 1.0 mm ID x 50 mm, GL Sciences Inc.). The mini-column consists of the PTFE tube, Supelco® ColumnSaver Precolumn Filter (2.0 μm, Sigma-Aldrich), and a PEEK fitting (Upchurch). The obtained mini-column was installed in the flow system. Next, the following solutions were flowed by using a syringe pump (model 100, BAS) at a flow rate of 20 μL min^{-1} (except for process 1 (100 μL min^{-1})), as summarized in Table S1. Finally, part (50 μL) of the obtained Zn^{2+}-containing acidic solutions (600 μL) was added to the 0.1 M ammonium buffer (0.1 M NH_3/0.1 M $NH_4(SO_4)_2$, pH 8.0) (final volume; 5000 μL), and ASV measurements were conducted.

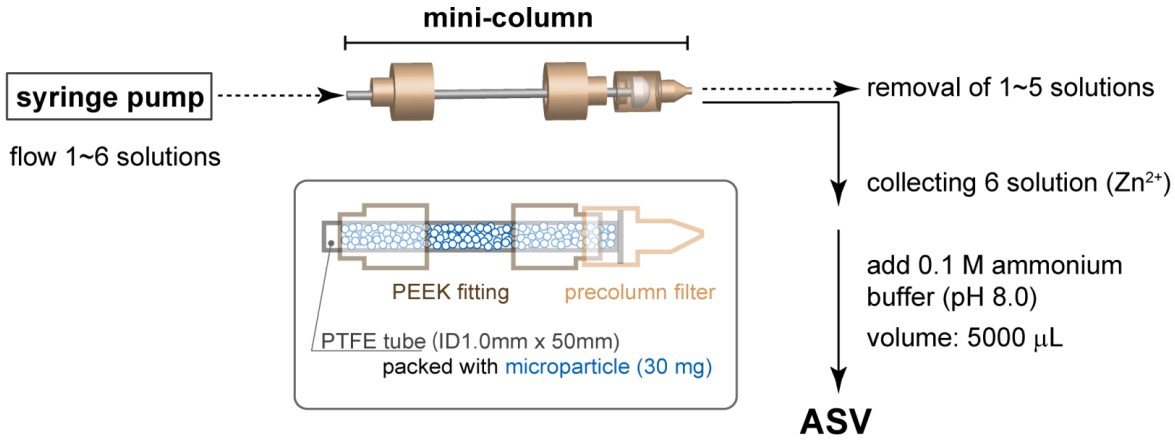


Figure S3. A flow system for LPS measurements. (Note: "remove solutions 1 – 5"; "collect solution 6 (Zn^{2+})")

Table S1. Flow process for LPS measurements

Process		Solvent	Time/min	Comments
1	LPS-free water	-	30	microparticles washed and neutralized
2	LPS	PBS	60	LPS capture
3	50 μM LPS probe	PBST+0.1% BSA	60	Probe capture
4	buffer 1	PBS+0.1% Block Ace	30	Removal of excess probe
5	buffer 2	PBS	30	Removal of excess Block Ace content
6	0.1M H_2SO_4	-	30	Extract Zn^{2+} ions

Calculation of amounts of the Zn adsorbed on the microparticles

The calculation method is as follows From Figure 3b, the obtained current value from LPS (2 ng mL^{-1}) was ca. 400 nA. This current value corresponded to 25 ng mL^{-1} of Zn^{2+} ions from revised Figure 2c (the calibration curve of Zn^{2+} ions at our nanocarbon film electrode). This measured solution (Zn^{2+}-containing acidic solution) was diluted 100 times as described in the SI, and therefore the original solution (acid treated) contains 2500 ng mL^{-1} of Zn^{2+} ions. The acid solution was allowed to flow for 30 min at 20 μL min^{-1}, so that the amounts of Zn adsorbed on the microparticles were 2500 n mL^{-1} x 30 min x 20 μL min^{-1}=1500 ng (22.9 nmol) of Zn^{2+} ions (11.45 nmol LPS probe).

LPS measurements by LAL method.

We used Endosafe®-PTS™ (Charles River Laboratories) to determine the LPS concentration based on the LAL method.

Selectivity tests

We estimated the selectivity of our method for glucose, peptidoglycan, and lipoteichoic acid (LTA). All the species had a concentration of 100 ng mL^{-1}, which was the same as that of β-D-glucan (Figure 4(a)). Figure S4 shows the obtained results.

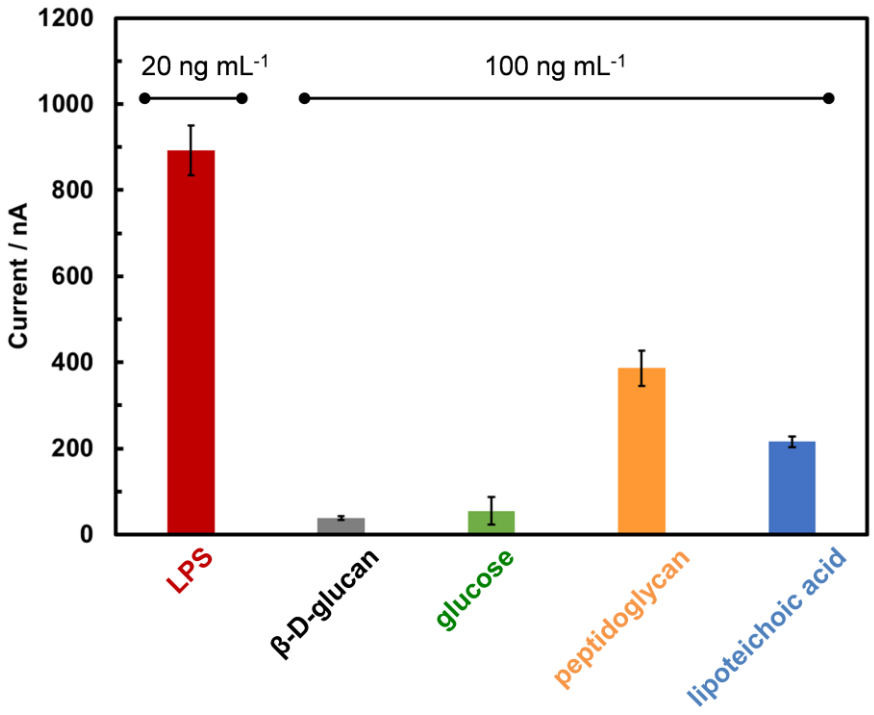


Figure S4. Selectivity tests for LPS performed with flow system measurements. (Note: The sample concentrations of β-D-glucan, glucose, peptidoglycan, and LTA were different from that of LPS)