Supplementary Information

In vitro selection of electrochemical peptide probes using bioorthogonal tRNA for influenza virus detection

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Material and Methods

Synthesis of 3,4-ethylenedioxythiophene (EDOT)-aminophenylalanyl-tRNA

Boc-ε-aminophenylalanine was coupled to 5′-*O*-phosphoryl-2′-deoxycytidylyl-(3′-5′) adenosine (pdCpA) to give the corresponding aminophenylalanine-pdCpA, (AF-pdCpA). Then, a DMSO solution of EDOT-succinimidyl ester was treated with a DMSO solution of AF-pdCpA (5 mM, 40 μL) in aqueous pyridine-HCl (5M, pH 5.0, 80 μL), and the resulting mixture was incubated at 37 °C for 3 h. The EDOT-AF-pdCpA product was purified by reversed-phase HPLC using an XTerra C18 column (4.6 × 20 mm, 2.5 μm particle size; Waters, Milford, MA, USA), which was eluted at a flow rate of 1.5 mL min^{-1} with a linear gradient of 0–100% acetonitrile in 0.1% trifluoroacetic acid over a period of 10 min. The identity of the product was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Voyager, Applied Biosystems, Foster City, CA, USA). The resulting EDOT-AF-pdCpA was ligated to an amber-suppressor tRNA, which was derived from *Mycoplasma capricolum* Trp_1 tRNA without the 3′ dinucleotide, using a previously reported chemical ligation method.[1] The purified EDOT-AF-tRNA molecules were lyophilized and stored at –80 °C.

In vitro selection of an electrosensitive peptide ligand against the influenza virus

The selection protocol is shown schematically in Fig 2. A 13Trx plasmid was employed in the current study carrying a promoter sequence for the T7 RNA polymerase, an *Escherichia coli* ribosome-binding sequence, an *Sfi*I restriction enzyme sequence, a protein-linker sequence, and a ribosome-arrest sequence. Random double-stranded DNA (dsDNA) was also prepared in parallel. The library sequences were obtained from Eurofins Genomics (Tokyo, Japan). These sequences were based on the general sequence 5′-ATATGGCCATGCAGGCC$(VVN)_3$TAG$(VVN)_7$GGCCAGCTAGGCCAGTT-3′,

where V represents G, C, or A; and N represents G, C, T, or A. The VVN sequence covers only 10 of the naturally-occurring amino acids, and was selected to exclude hydrophobic amino acids, such as leucine, valine, and tryptophan, and stop codons (e.g. TAG, TAA, and TGA). This design strategy was selected for peptides with high solubility in an aqueous buffer, and to allow for the incorporation of one EDOT molecule at the same position in each library sequence, whilst avoiding the unintentional incorporation of additional EDOT molecules. To run selection, dsDNA was prepared by one cycle of a polymerase chain reaction (PCR) using Takara Ex taq DNA polymerase (Takara Bio Inc., Otsu, Shiga, Japan), and the crude material was purified with a QIAquick PCR Purification kit (Qiagen, Valencia, CA, USA). The resulting dsDNA and 13Trx plasmid were digested with a restriction enzyme (*Sfi*I; New England Biolabs, Ipswich, MA, USA) and fused (DNA Ligation Kit; Takara Bio). Finally, a dsDNA library was prepared by PCR with new primers (New-T7-fp-rec-1: 5′-GTAATACGACTCACTATAGGCCGCGTCGACAATAA-3′ and New-rp-fp-M13-NS: 5′-GATTACGCCAAGCTGAGTGAGA-3′). Transcription was performed at 37 °C for 3 h, and the product was then treated with DNase. The mRNA was purified using a RNeasy kit (Qiagen). *In vitro* translation was performed using a PURESYSTEM Classic II kit (Wako Pure Chemical Industries Ltd., Osaka, Japan) with EDOT-AF-tRNAs. Given that the mRNA did not contain any stop codons, it was not possible for the ribosome to release the mRNA or the translated peptide, and the mRNA, ribosome, and peptide became coupled as a ternary complex (the PRM complex, see Fig. 2). The reaction was stopped by placing the mixture on ice for 10 min. The translated solution was then incubated with inactivated influenza virus A/California/07/2009 (H1N1, kindly provided as a gift from Denka Seiken Co., Ltd., Tokyo, Japan), which was immobilized on silica affinity beads

(Sumitomo Bakelite Co., Ltd., Tokyo, Japan) in the selection buffer (0.1% Tween 20, 50 mM Tris-acetate, 150 mM NaCl, 50 mM magnesium acetate, pH 7.4) at 4 °C for 1 h. The virus-immobilized beads were collected by centrifugation at 10,000 × g for 5 min and washed with buffer (50 mM Tris-acetate, 150 mM NaCl, 50 mM magnesium acetate, pH 7.4) at 4 °C to remove any free mRNA-ribosome-peptide complexes. The mRNA was recovered from the bound mRNA-ribosome-peptide complex following a 30-min period of incubation with ethylenediaminetetraacetatic acid at room temperature, which allowed for the removal of the Mg^{2+} ions and resulted in the detachment of the ribosomes from the mRNA. The isolated mRNA was purified using an RNeasy kit. Preparative PCR was performed to amplify the reverse transcription products, and the DNA product was purified using a QIAquick PCR purification kit (Qiagen). The isolated DNA was used as the template for the next round of selection.

After six rounds, the sequences of the selected DNAs were analyzed by the next-generation sequencing service provided by Takara-bio (Otsu, Japan) using a MiSeq sequencer (Illumina, San Diego, CA, USA). The obtained sequences (approximately 200,000) were trimmed to use only the random library sequences and converted from nucleotide sequences to amino acid sequences. The trimmed and translated reads were aggregated, and those with 90% sequence identity were clustered using Cd-hit.[2] The clusters representing singletons (with a multiplicity of one) were discarded, and the remaining clusters were ranked based on the sum of multiplicities within each cluster. Representative sequences, which are defined as the reads with highest multiplicities, were determined for the clusters. Representative sequences and multiplicities for each cluster were tabulated, and the top 100 clusters were identified as the enriched sequences mediated by the selection process (Table S1). The analysis indicates the enrichment of

selected peptide sequences against the influenza virus, and the three peptide sequences with the highest enrichment (Sequence 1, Sequence 2, and Sequence 3) were selected for interaction analysis (Table S2).

Synthesis and purification of peptide sequences.

Selected peptides sequences from the Next Generation Sequence Analysis (Sequence 1, Sequence 2, and Sequence 3) were synthesized using a conventional Fmoc-solid phase peptide synthesis method on preloaded Wang resin (Watanabe Chemical Industries, Hiroshima, Japan). Peptide coupling and deprotection reactions were performed with a Discover microwave (CEM Corporation, Matthews, NC, USA) using standard CEM protocols, with mild N_2 bubbling, at a 0.1 mmol scale (at 70°C, 5 and 3min for coupling and deprotection, respectively). For the incorporation of EDOT into the selected peptide sequences, a non-natural amino acid, Fmoc-aminophenylalanyl EDOT (EDOTaa), was prepared according to the scheme shown in Fig S1. EDOTaa was attached using optimized conditions for coupling (10 min at 50 °C and 25 min at 50 °C) and deprotection (5 min at 50 °C). After coupling EDOTaa, elongation with other amino acids was also carried out at reduced temperature but for prolonged times (at 50 °C, 7 and 5 min for coupling and deprotection, respectively) to prevent the degradation of the non-natural amino acid. The elongation of the peptide sequence was confirmed by checking the MALDI MS at various steps, and after final cleavage from the resin. For cleavage, a ΔTIS cocktail consisting of an 85:5:4:4:2 (v/v) mixture of trifluoroacetic acid: phenol: thioanisole: ddH_2O: ethane dithiol was used to avoid reducing the thiophene moiety. The cleaved peptides were precipitated via chilled diethyl ether and centrifuged, and the peptide pellet was washed and lyophilized for further purification. For the peptide-

binding assay, the *N*-terminal primary amine group of the peptide was modified with fluorescein at the RIKEN Brain Science Institute, Japan. The fluorescein-labeled reaction was carried out on a solid support using a fluorescein-NHS ester (Thermo Fisher Scientific Inc., Rockford, IL, USA). After confirming the reaction, the resin was thoroughly washed, and the peptide was cleaved as described above.

Peptide purification and analysis was performed using an Extrema HPLC (JASCO, Tokyo, Japan). Purification was performed with a linear gradient using 0.1 % trifluoroacetic acid in water, and acetonitrile in a COSMOSIL 5C18-AR-II column (Nacalai Tesque, Kyoto, Japan). Finally, the peptide was characterized by HPLC, as well as MALDI-TOF MS (Fig. S2a and S2b) prior to the interaction analysis.

Evaluation of peptide binding affinity and target selectivity

To determine the biding affinity using immobilized microbeads, first, the inactivated influenza virus (1 μg) was immobilized on 20 mg of silica microbeads (Sumitomo Bakelite, Tokyo, Japan) following the manufacturer's protocol. Then, the peptides labeled with fluorescein were incubated with 300 μg of the virus-immobilized silica microbeads containing approximately 15 ng of inactivated influenza viral particles, maintaining the volume at 200 μL in the selection buffer at 25 °C for 1 h. During the incubation process, the beads and peptide solution were continuously mixed using a microtube mixer (ThermoMixer C; Eppendorf, Hamburg, Germany) at 1000 rpm. After incubation, the beads and peptide solution were centrifuged at 10,000 rpm for 5 min, and the solution containing unbound peptide was discarded. The peptide-bound beads were washed three times with 300 μL washing buffer (50 mM Tris-acetate, 150 mM NaCl, 50 mM magnesium acetate, pH 7.4) and were protected from light during the experiment. For the

fluorescence measurements, the bead volume was adjusted to 100 μL, and the suspension were transferred to a 96-well black microplate (PerkinElmer, Waltham, MA, USA), and the fluorescence intensities were quantified at 530 nm using a microplate reader (Enspire 2300; PerkinElmer, Hamburg, Germany). All data are presented as the mean values ± SD (n = 3).

Biological specificity

The biological specificity of the binding of the Sequence 2 peptide to the influenza virus was studied using a dot blot analysis. A membrane (Immobilon-P Transfer Membrane, pore size: 0.2 μm; Millipore, Bedford, MA, USA) was immersed in methanol followed by blotting with a buffer solution [25 mM Tris, 192 mM glycine, 20% (v/v) methanol]. The membrane was then placed on wet filter paper to avoid excessive drying. A 2-μL portion of 1 mg mL^{-1} inactivated influenza virus in PBS buffer was then dropped onto the membrane. Similar samples of Epstein–Barr virus, gelatine, and bovine serum albumin (Sigma-Aldrich) were also dropped onto the membrane as negative controls. After it had dried out, the membrane was immersed in methanol, followed by the blotting buffer. The membrane was then immersed in a blocking buffer (5% w/v of ECL Advance blocking agent) and a TBS-T buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.4), and incubated for 1 h at room temperature to block any nonspecific binding. The membrane was briefly washed with TBS-T buffer and put on parafilm to keep it wet. Fluorescein-labeled Sequence 2 peptide (500 nM) was dropped on to the membrane to cover the entire area. After being incubated for 1 h at room temperature in the dark, the membrane was washed three times with TBS-T buffer for 10 min each time, and an image of the surface of the membrane was recorded using a Molecular Imager FX system (Bio-Rad Laboratories, Hercules, CA, USA) at the RIKEN Brain Science Institute, Japan. The

image was analyzed using the ImageJ64 software package to find the integrated densities of the spots.

Electropolymerization of EDOT-conjugated peptide ligand

Electrochemical experiments were carried out using an Autolab potentiostat system (ALS/CH, electrochemical analyzer, 700C, CH Instruments Inc. Austin, TX, USA). A gold electrode (ALS Au 6 × 3.0 mm; ALS Co., Ltd., Tokyo, Japan) was used as the working electrode, and a platinum wire and an Ag/AgCl electrode (RE-1S, ALS Co., Ltd., Tokyo, Japan) were used as the counter and reference electrodes, respectively. After setup, the distance between the counter/reference electrodes and the working electrode was kept at 1 mm. Twelve microliters of the Sequence 2 peptide solution were dropped onto the working electrode, ensuring that the counter and reference electrodes were submerged in the solution. Cyclic voltammetry (−0.6 to 0.95 V vs. Ag/AgCl, scanning at 0.1 V s^{-1}) was conducted during the polymerization reaction in 0.1 M Tris-HCl buffer (pH 7.0) supporting electrolyte. After the polymerization reaction, the gold electrode was gently washed with Milli-Q water to remove unpolymerized peptide. For the optimization of the electropolymerizing conditions, Sequence 2 peptide polymerization was performed at various oxidative potentials (−0.6 and +0.70 to +1.4 V). At lower potentials (below +0.75 V) the peptide did not polymerize, because no current inhibition was detected on the gold electrode even after 15 cycles of polymerization. At higher potentials, the peptide polymerized after fewer cycles and started to be destroyed, and this observed phenomenon was similar to a reported EDOT polymerization.[3] Thus, we selected the parameters of –0.6 to 0.95 V at 0.1 V s^{-1} scanning speed for 12 cycles for further analysis.

For the FT-IR measurements, electropolymerization of the peptide was performed on a gold-coated chip (5 × 10 mm) cut from a gold-coated silicon wafer (Sigma-Aldrich).

The experimental setup was similar to the peptide electropolymerization experiment described above, but a gold-coated silicon chip was used instead of the gold working electrode. A 20 μL portion of a 2 mg mL^{-1} solution of Sequence 2 peptide in 0.1 M Tris-HCl buffer (pH 7.0) was dropped onto the gold-coated silicon chip, and cyclic voltammetry (−0.6 to 0.95 V vs. Ag/AgCl, at a scanning speed of 0.1 V s^{-1} for 12 cycles) was performed using a potentiostat system. The gold-coated chip was dipped in Milli-Q water to remove any unpolymerized peptide. After drying, the sample was analyzed by FT-IR spectroscopy using an FT-IR 4100 system (Jasco, Tokyo, Japan). The FT-IR spectra were recorded in the range of 4500–600 cm^{-1} using an average of 80 scans to increase the signal to noise ratio, and the spectral resolution was 4 cm^{-1}. The FT-IR spectrum of the Sequence 2 peptide monomer was also measured for comparison.

Electrochemical detection of influenza virus

Three electrodes were set up as described above for the peptide polymerization. First, the effective concentration of the peptide was determined by running the polymerization reaction on the gold electrode (−0.6 to 0.95 V vs. Ag/AgCl, at a scanning speed of 0.1 V s^{-1} for 12 cycles) using various concentrations, and then the current was measured. The cathodic current was effectively suppressed at 0.1 mg mL^{-1} of the peptide (Fig. S3), so this concentration of peptide was used. For the detection system, 12 μL of Sequence 2 peptide was dropped on to the gold electrode, ensuring that the entire surface of the electrode was covered. Cyclic voltammetry was performed for the polymerization reaction, as described above. After cleaning, the working electrodes were set up in the same manner as before, and 12 μL of 10 mM $K_3[Fe(CN)_6]$ was dropped onto the surface of the gold electrode. Then, cyclic voltammetry (−0.3 to 0.65 V vs. Ag/AgCl, at 0.1 V s^{-1}) was performed, and the current at the gold electrode was recorded. In the same way,

12 μL of the mixture of Sequence 2 peptide (final concentration, 0.1 mg mL^{-1}) and various concentrations of influenza virus were applied to the detection system, and the current at the gold electrode was recorded as a function of the influenza virus concentration. The spectrum of the second cycle is shown in the figures because the spectra stabilized after the second cycle. To determine the limit of detection (LOD) of the system, the anodic current obtained after the electrochemical analysis of inactivated influenza virus at different concentrations in the presence of a fixed amount of Sequence 2 Peptide was analyzed statistically at the 95% confidence interval. The p-values of the different concentrations were calculated by comparing the results with those recorded in the absence of influenza virus using Student's t-test.

Interference of microorganisms on the electrochemical detection system.

The possible interference of other microorganisms in the electrochemical detection system was examined using bacteria commonly found in the oral cavity (*Staphylococcus aureus* and *Strepotococcus pneumonia*). They were kindly provided by Dr. Suresh Panthee at Teikyo University Institute of Medical Mycology, Japan, after heat treatment at 121oC for 15 min by an autoclave. For this measurement, we used a much larger amount of microorganisms (1×10^6 CFU mL^{-1}) than the amount of staphylococci species usually found in saliva (10^2–10^4 CFU mL^{-1}).[4] Specifically, all three electrodes were set up as described above for the peptide polymerization and then the current on a clean working electrode was recorded by cyclic voltammetry (−0.3 to 0.65 V vs. Ag/AgCl, at 0.1 V s^{-1}) using 12 μL of 10 mM $K_3[Fe(CN)_6]$ in 0.1 M Tris-HCl buffer (pH 7.0). Cyclic voltammetry was performed during the polymerization cycle (−0.6 to 0.95 V vs. Ag/AgCl, at a scanning speed of 0.1 V s^{-1} for 12 cycles) using 12 μL of the Sequence 2

peptide (0.1 mg mL^{-1}) in the presence of 1×10^6 CFU mL^{-1} of microorganism, only the Sequence 2 peptide (0.1 mg mL^{-1}), and only the microorganism [1×10^6 CFU mL^{-1} in 0.1 M Tris-HCl buffer (pH 7.0)]. Then, the current at the gold electrode was recorded again, as described above, and the obtained cyclic voltammograms were compared (Fig S5). The results showed that the microorganisms did not interfere with our influenza detection system. Neither species of microorganism remarkably suppressed the CV current measured after the polymerization cycle (blue and red lines in Fig S5) nor did their presence affect the peptide polymerization (green and purple lines in Fig S5).

Table S1. Sequence list of the selected peptides from the Next Generation Sequencing analysis. “B” represents EDOT-aminophenylalanine.

Peptide Sequences	Amino Acids	Identical /209177	Peptide Sequence	Amino Acids	Identical /209177
1	AAPBKAGKGAP	5546	51	QRPBADSPKKP	536
2	ARRBGHRKPRR	5278	52	TQRBGGTGAPK	523
3	AGRBRRGAHDT	4042	53	ERDBEPRPQRR	517
4	GPRBTTAANRR	3045	54	GGGBSPQGAGG	521
5	AAABTGENPTT	2692	55	TGGBPGEATTP	508
6	RTPBNGPRADA	2518	56	GRPBRNGARQT	506
7	NRABGDPTGNR	2499	57	PGGBRDDHRDQ	497
8	NERBSADAGAH	2413	58	HRTBDHSPPDR	486
9	EDPBGGANTGR	2480	59	SRTBHPAQKDS	501
10	HAABHATPTSG	2116	60	RGQBADREHDT	505
11	TEPBGDRSRRR	2106	61	TEHBKKGNGTR	497
12	TSDBSRPRQDE	1970	62	TQTBPERAAGR	487
13	TRTBDTKNTTT	1810	63	DGHBDGAEPAR	486
14	AREBPAAGEQG	1689	64	AQRBRGATQEN	457
15	NPRBGRQQREG	1682	65	APRBHPGPADK	464
16	EAHBKPSPPHE	1478	66	AREBAANGKGP	452
17	GKEBTPRPEPP	1440	67	DKDBGPQRPSP	435
18	TTRBTGQEEQE	1309	68	RRRBQGGGRGH	422
19	TSRBTPGANKR	1180	69	GAHBRREGGRR	415
20	GPGBAHRRGDP	1167	70	KGTBHEARTKP	425
21	NAKBGGSTRRR	1068	71	KGRBRTHPETG	402
22	TKEBERARRGQ	1043	72	RPABSHHDRGP	403
23	APNBEGEDTDP	1069	73	QQPBGGAATPR	411
24	PPDBPDTTRKR	1026	74	PRGBRAAGAHP	384
25	SNGBTREAQHT	1016	75	QTGBANPEPQE	409
26	SNGBEPTREKP	949	76	ATTBAAGTDTT	405
27	PRNBGRTGAAK	893	77	GGEBGNGADGR	393
28	SRRBARHPRRG	879	78	KPGBAPGKAGA	390
29	RERBESSRSDS	855	79	RSQBDQRNGDG	389
30	EDRBQQGARAP	830	80	GKABSRTASAG	389
31	REPBRDDATDQ	812	81	RTGBPPRNNRR	386
32	RGQBNAPQEEA	801	82	GPHBRRAHAER	388
33	SRABPQSRKGP	796	83	AGPBKSAGPGG	380
34	DQSBRGRRARP	765	84	RRGBNGGEDGQ	383
35	GAGBPGTTRKT	750	85	PGHBKGQKHST	377
36	GQEBDRARKTR	723	86	DGTBPREETAS	377
37	GPGBGGNRPRG	698	87	ERABGPGGATA	386
38	ARGBGPGGNQR	722	88	AGTBTGGTPHR	372
39	TRABGANRRAG	697	89	QGEBTGPGSHA	351
40	HGGBTGPHERP	687	90	EGEBTPQTTTQ	367
41	GNSBQANNRTR	666	91	TDGBRGSRNRP	364
42	PDQBRAGGRAG	684	92	PSTBDRRGNPK	362
43	GQTBEENERRS	632	93	STQBGARGTAP	360
44	RGNBERRSDPT	617	94	SDRBSQDPAGG	340
45	TGABRTRATRH	373	95	DRQBTAKGNNR	351
46	DRABPPRGGGA	595	96	GGPBSDGRAGA	339
47	NRGBARNAGAR	592	97	QRGBGTHTTDA	334
48	PPTBKGKAPPT	580	98	EKPBRPGDQEP	337
49	RGEBPDHTAGT	552	99	RGNBGDNRAGS	345
50	ATRBPGGKGEG	540	100	DESBAETESGD	347

Table S2: Nominated peptide sequences for the interaction analysis.

Peptide Name	Amino acid sequences[a]	Ratio[b]
Sequence 1	AAPBKAGKGAP	2.65
Sequence 2	ARRBGHRKPRR	2.52
Sequence 3	AGRBRRGAHDT	1.93
Sequence 2 (ΔEDOT)	ARRFGHRKPRR	–

[a] B stands for EDOT-coupled aminophenylalanine, which is a non-natural amino acid. F indicates where phenylalanine was used in place of B to synthesize the Sequence 2 peptide without EDOT (ΔEDOT).

[b] Sequence repetition percentage out of the total number of sequences.

Table S3: *P*-values calculated using the Student's t-test for the detection limit of the influenza virus.

Influenza Virus	6.25 μg mL^{-1}	12.5 μg mL^{-1}	25 μg mL^{-1}
P-Value	0.27	0.02	0.001

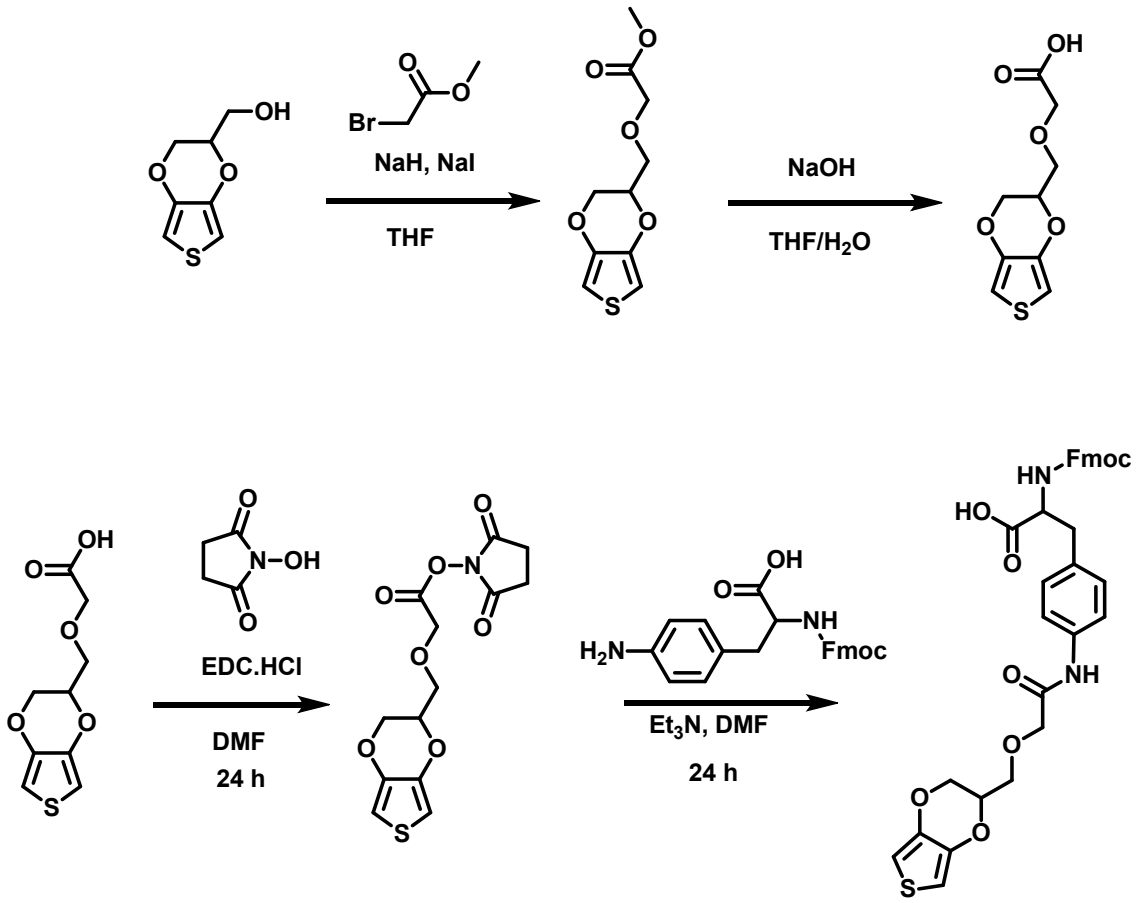

Fig. S1 Synthesis of the non-natural amino acid Fmoc protected aminophenylalanine coupled to an EDOT molecule (EDOTaa).

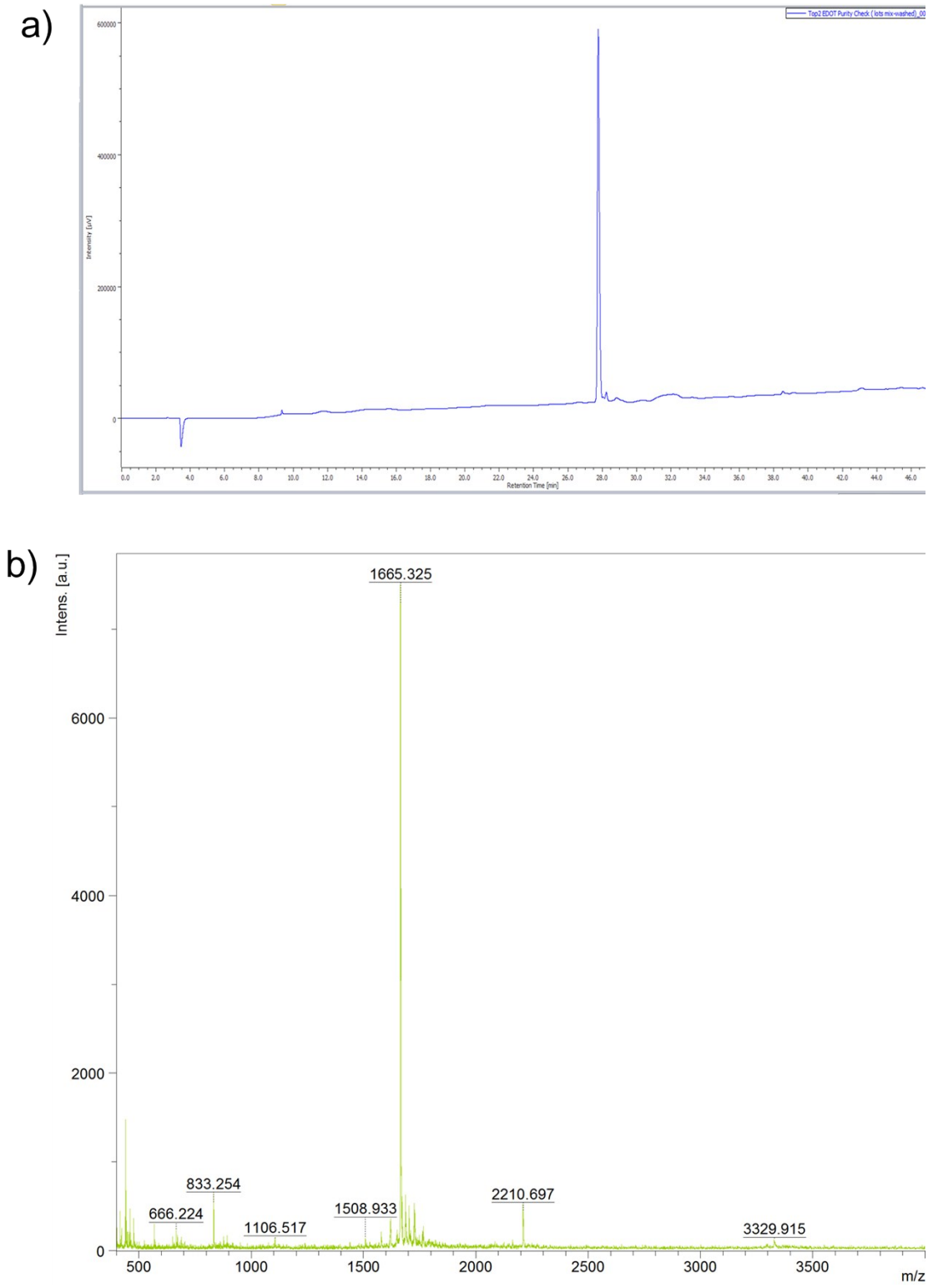

Fig. S2 Characterization of Sequence 2 peptide a) HPLC analysis showing the absorbance at 220 nm. b) MALDI MS analysis (Calculated M+H = 1663.99, experimental monoisotopic mass M+H = 1664.32)

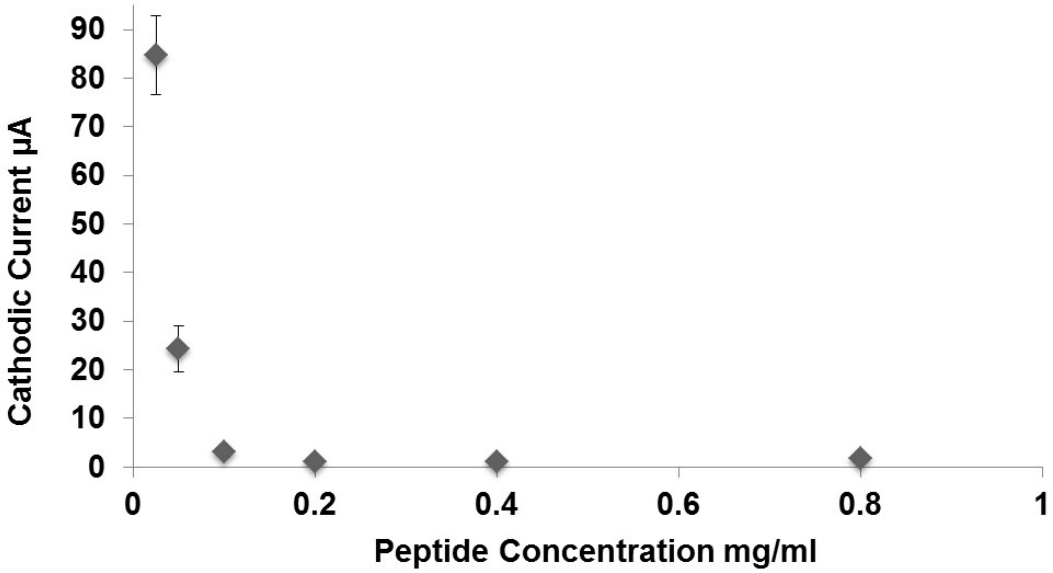

Fig. S3 Averaged cathodic current after the polymerization of various concentrations of Sequence 2 peptide on a gold electrode. The cathodic current was effectively suppressed above a concentration of 0.1 mg mL^{-1} (effective peptide concentration).

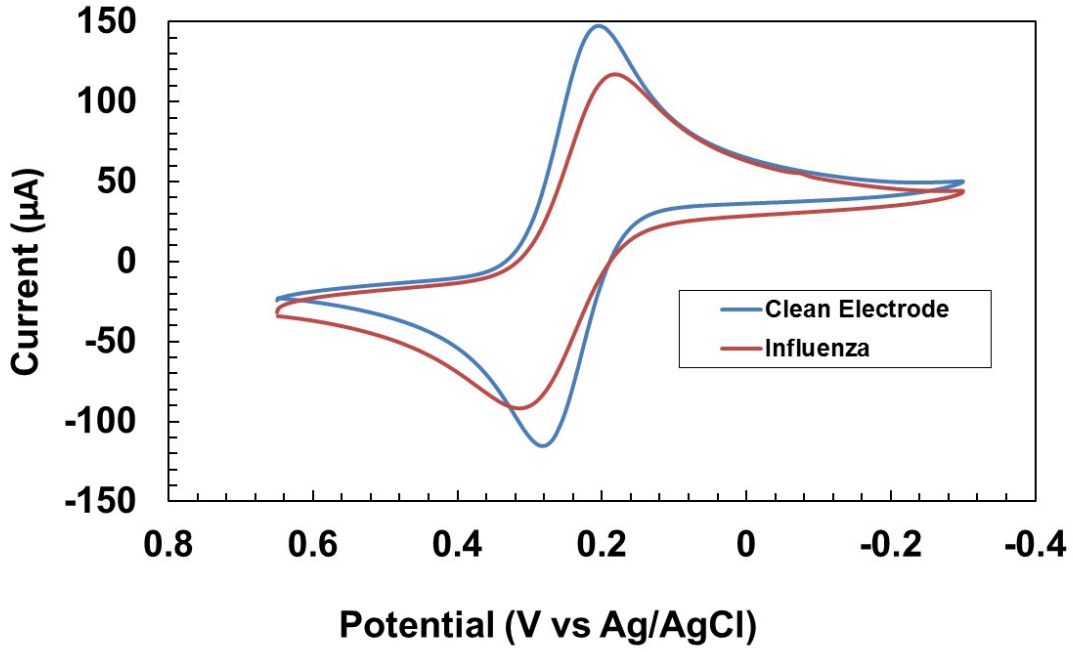

Fig S4. Cyclic voltammogram changes caused by the influenza virus protein. Cyclic voltammograms were measured on a clean electrode, after running the polymerization cycle with 0.15 µg mL-1 of influenza virus.

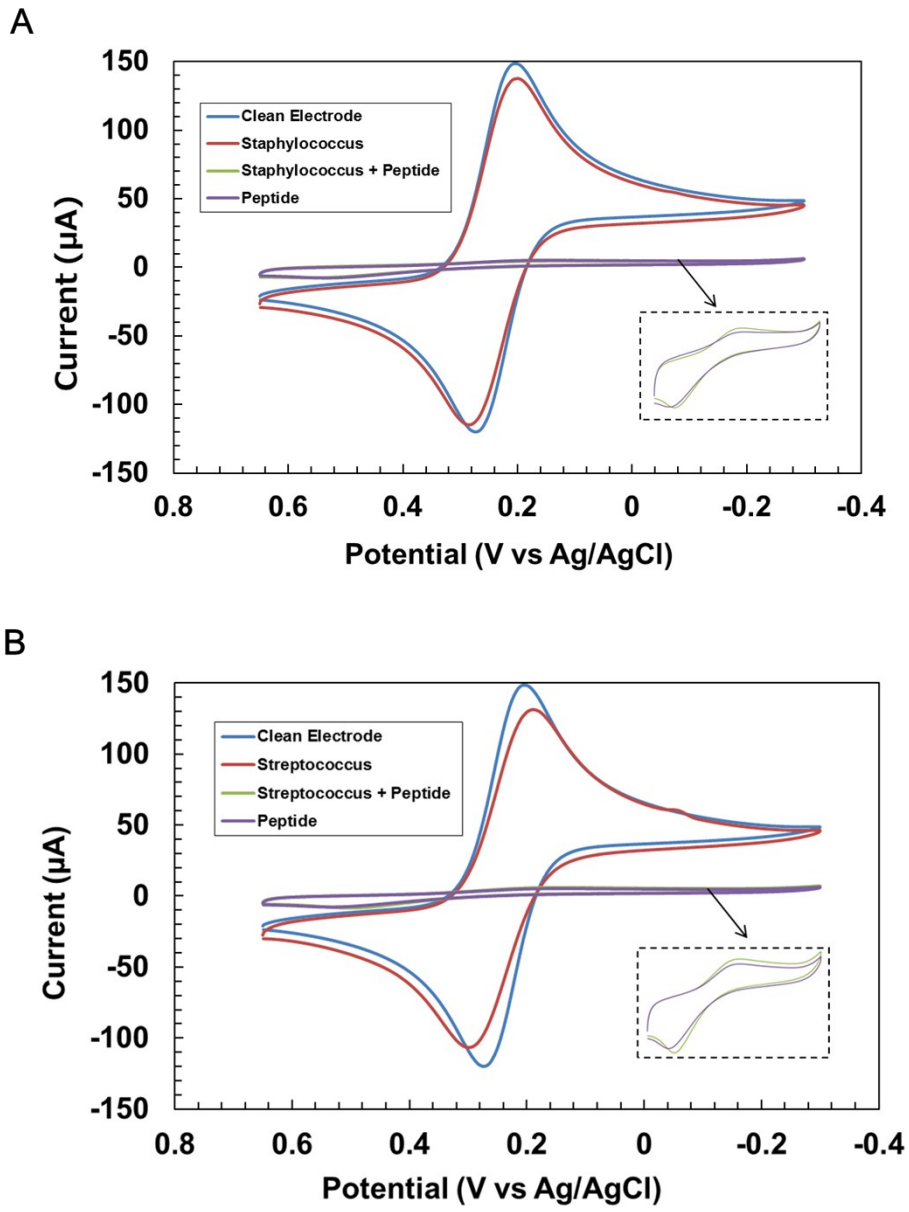

Fig S5. Comparison of cyclic voltammogram (CV) current on a working electrode. CV current on a clean electrode (blue), after polymerization of Sequence 2 peptide in the presence of microorganism (green), only microorganism (red), and only Sequence 2 peptide (purple). (A) *Staphylococcus aureus* and (B) *Strepotococcus pneumonia*.

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