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Supplementary Information

Ultrasensitive pathogen detection with a rolling circle amplification-empowered multiplex electrochemical DNA sensor

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Table S1. **The oligonucleotide sequences of circular DNA, primers and probes used in this study**.

Name	Sequence (5'→3')	Length (bases)
Salmonella typhi		
Capture probe (CP-ST)	Biotin-CGTTAACCCAGTCACAGC	18
Circular DNA (CDNA-ST)	TAACTTGTTCTTGATGTCGCTGTGACTGGGTTAAC GGCTAACTCCAGGAACGACTTTGTATCCA	64
Linear target (LT-ST)	TGGAGGAACAAGTTATGGATACAAA	25
Reporter probe (RP-ST)	Biotin-GCTAACTCCAGGAACGAC	18
Shigella flexneri		
Capture probe (CP-SF)	Biotin-CGTAAACTGGCGCTCTCA	18
Circular DNA (CDNA-SF)	AAAGGAAACCTTGATGTCTGAGAGCGCCAGTTTA CGAGACGGCAACCTGGTATTCCCAAATCGT	64
Linear target (LT -SF)	GCTACGGTTTCCTTTACGATTTGGG	25
Reporter probe (RP-SF)	Biotin-AGACGGCAACCTGGTATT	18
Vibrio cholerae		
Capture probe (CP-VC)	Biotin-CTCTCGATCGATGACTGA	18
Circular DNA (CDNA-VC)	GGAGTTAATATTGATGTCTCAGTCATCGATCGAG AGCGCTTCGTAAGGATCCAGCACAAGTATA	64
Linear target (LT -VC)	CTTTTTATTAACTCCTATACTTGTG	25
Reporter probe (RP-VC)	Biotin-CGCTTCGTAAGGATCCAG	18
Non-complementary target (NC-LT)	GTCACCCCAGAAACCACCGCCGG	23
Blocking probe (BP)	Biotin-AAAAAAAAAA	10

Preparation of silica microsphere with redox dye

One hundred µL of TEOS was added to 5 mL of 95% ethanol in an Erlenmeyer flask while cooling at 0 °C in an ice-cooled ultra-sonicating water bath. After that, 5 mL of ammonium hydroxide (28-30 %) was added to the solution during the sonication for 1 hour. This will produce the pure silica nanoparticles, which were then collected by centrifugation at 8,000 rpm for 10 min. The silica pellet was redispersed with water and acetone. The centrifugation and redispersion steps were repeated up to 6 times. The silica core particles were collected, air-dried and kept at room temperature until further use. The dye was incorporated within the cavities of the silica outer layer, 0.3 g of silica core were dissolved in 10.9 mL of 2-propanol containing 15 µmol of dye in an ultrasonic bath. Then, 0.55 mL of TEOS was added into solution followed by 1.5 mL of 25% ammonia solution, which was diluted with 12 mL of milli-Q water. The solution was mixed at 40 °C for 2 h until it formed a colloidal suspension. Finally, the resulting silica-redox dye particles were separated by centrifugation at 10,000 rpm for 5 min, washed 5x with milli-Q water and dried in a desiccator overnight. This method was used to produce silica microspheres incorporated with either MB, AO or $Ru(bpy)_3^{2+}$. The silica microspheres and silica-redox dye particles were characterized by scanning electron microscopy, SEM (JSM-6610LV, JEOL Ltd., Tokyo, Japan).

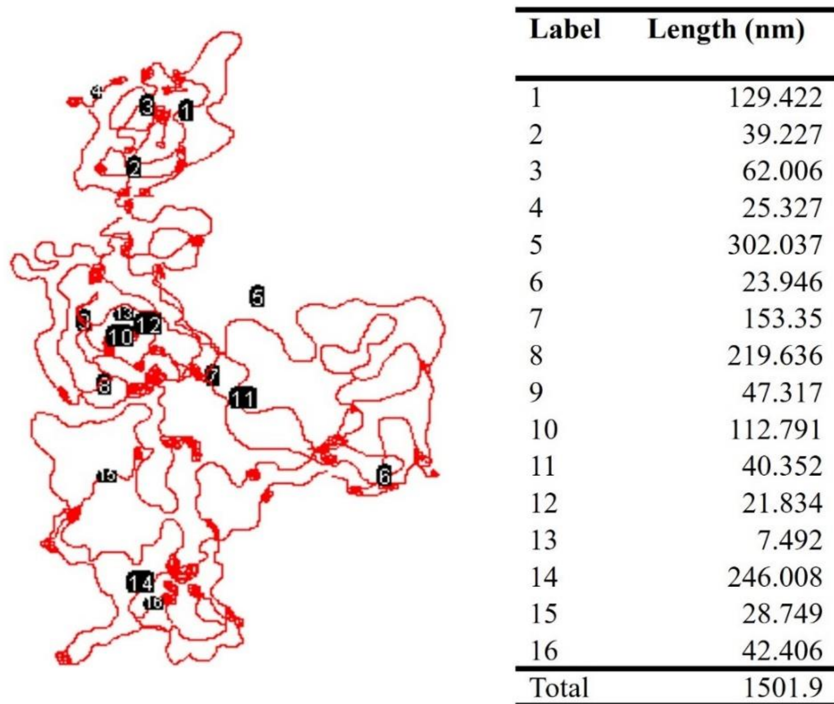
Conjugation of reporter probes to the silica-redox dye microspheres

Silica-redox dye particles (0.010 g) was sonicated in 1 mL of milli-Q water until a homogenous suspension. The silica-redox dye solution was mixed with 0.2 mL of PAA and PSS solution (10 mg mL^{-1} in 0.5 M NaCl). Each polyelectrolyte was allowed to adsorb for 30 min at room temperature, and three centrifugation/ redispersions steps were performed to remove excess polyelectrolyte and resuspended in 1 mL of 10 mM PB, pH 7.4. Then 10 µL of avidin (21.14 mg/mL) was added and incubated for 90 min with agitation at 37 °C. After that excess avidin was removed by 3 centrifugation/redispersion steps. Next, 300 µL of 5 mg/mL of avidin/silica-dye, 99 µL of 10 µM of blocking probe, 1 µL of 10 µM reporter probe were added to 100 µL of 0.1 M PBS and mixed well. The mixture was incubated for 30 min at room temperature with agitation. The DNA/silica-dye labels were then collected by three centrifugation/redispersion

steps. The resulting reporter probe/silica-dye label for each target were: *Salmonella typhi* (ST-RP/silica-MB), *Shigella flexneri* (SF-RP/silica-AO) and *Vibrio cholerae* (VC-RP/$Ru(bpy)_3^{2+}$). For multiplex detection, the 3-reporter probe/silica-dye labels were mixed in equimolar concentrations and added to the hybridization reaction.

Attachment of biotinylated capture probes to magnetic beads

One hundred µL of Dynabeads™ MyOne™ Streptavidin T1 (10 µg/µL) was washed 3x with 20 mM PBS containing 0.05% Tween 20, pH 7.4. The magnetic bead pellet was redispersed in 184 µL of 20 mM PBS, pH 7.4. Then, 4 µL of 100 µM biotinylated capture probe and 12 µL of 100 µM blocking probe were added and incubated at room temperature for 40 min with agitation. At the end of incubation, the magnetic beads with capture probe (MGB-CP) were washed 3x, and finally dispersed in 100 µL of 20 mM PBS, pH 7.4.



Label	Length (nm)
1	129.422
2	39.227
3	62.006
4	25.327
5	302.037
6	23.946
7	153.35
8	219.636
9	47.317
10	112.791
11	40.352
12	21.834
13	7.492
14	246.008
15	28.749
16	42.406
Total	1501.9

Fig. S1. The free hand schematic drawing of the 16 hours amplified RCA amplicon and estimation of its length as measured by ImageJ 1.52i (Fiji) software. The RCA amplicon was divided into 16 segments (red line drawing on the left) and the length of each segment was estimated and summed up (right table). The total length of the 16 hr RCA amplicon was approximately 1500 nm.

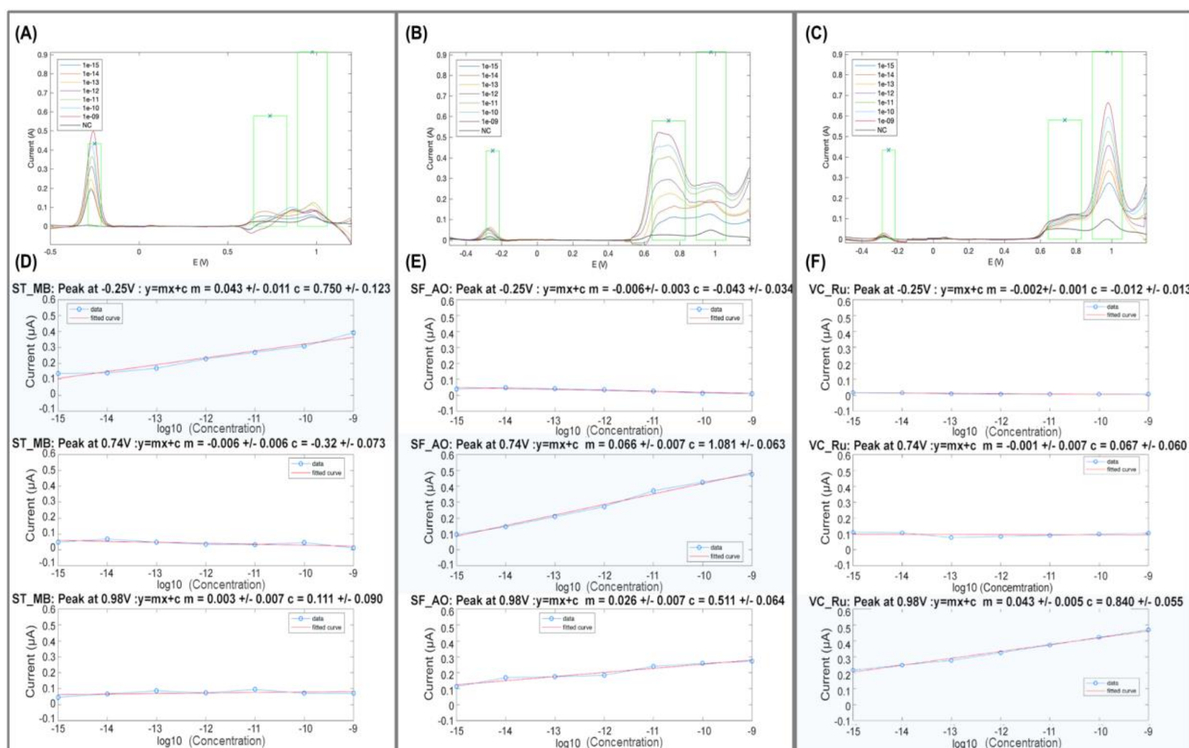


Fig. S2. Sensitivity of the monoplex RCA assay using different concentrations (1 fM to 1 nM) of linear target. In the DPV voltammograms (A, B, C), the peak current responses were observed at -0.25 V for *S. typhi* (A), +0.74 V for *S. flexneri* (B) and +0.98 V for *V. cholerae (C)*, respectively. Calibration curves of the monoplex RCA assay with *S. typhi* (D), *S. flexneri* (E) and *V. cholerae* (F) linear targets show a positive correlation in the current response to increasing concentration of linear target, only at their corresponding peak potential, and not at the other two non-corresponding peak potentials.

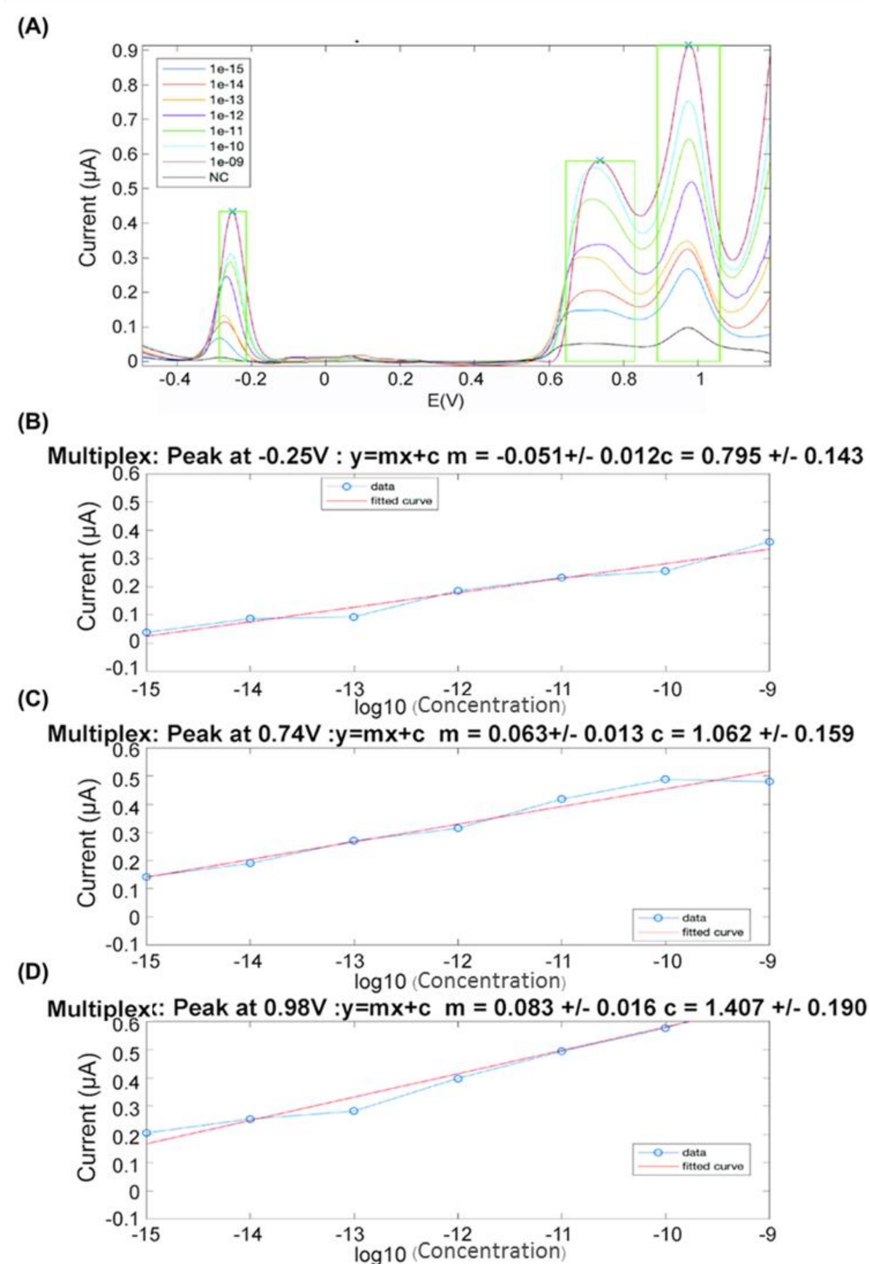


Fig. S3. Sensitivity of the multiplex RCA assay using different concentrations (1 fM to 1 nM) of each linear target. (A) In the DPV voltammogram, the peak current responses were observed at -0.25 V for *S. typhi*, +0.74 V for *S. flexneri* and +0.98 V for *V. cholerae*, respectively. Calibration curve of the multiplex RCA assay with *S. typhi* (B), *S. flexneri* (C) and *V. cholerae* (D) linear targets show a positive correlation in the current response to increasing concentration of linear targets.