Supplementary information

Rapid and convenient electrochemical hemoglobin detection in mouse feces employing a DNA aptamer to evaluate the severity of colitis in a mouse model

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Materials and methods

Recombinant production of proteins

All the proteins including SpyCatcher (SC)-fused glucose dehydrogenase (GDH), SpyTag (ST)-fused UdgX, and SC-fused glucose oxidase (GOx) were recombinantly produced using *Escherichia coli* (*E. coli*) BL21(DE3) strain as an expression host. For SC-GDH-SC, the transformants were grown in the autoinduction Luria-Bertani (LB) medium containing 0.05% (w/v) glucose, 0.5% (v/v) glycerol, 0.2% (w/v) β-lactose, 25 mM $(NH_4)_2SO_4$, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, 1 mM $MgSO_4$, and 50 μg/mL kanamycin at 37 °C and 170 rpm for 24 h. Cells were disrupted using a French press (Ohtake Works, Tokyo, Japan) and the intracellular soluble fraction was obtained by a centrifuge at 13,000 × g for 20 min at 4 °C. The sample was purified using NGC Quest 10 Plus (Bio-Rad, Inc., Hercules, CA, USA) and HisTrap HP column (Cytiva, Tokyo, Japan), and the purified sample was dialyzed against 20 mM potassium phosphate buffer (pH 6.5).

GOx-SC was prepared from the intracellular insoluble fraction of *E. coli* BL21(DE3) as previously described with some modifications.[1] The transformants were cultured in 100 mL LB medium at 37 °C. When the OD reached 0.6, isopropyl-β-d-thiogalactopyranoside (IPTG) was added (0.5 mM) to induce protein expression, and the cells were cultured at 37 °C and 150 rpm for 24 h. The cells were collected via centrifugation at 4800 × g at 4 °C for 20 min and resuspended in 20 mM phosphate buffer (pH 7.0). Next, the refolded sample was concentrated using ultrafiltration with Amicon Ultra 30 K (30 kDa cutoff, Merck Millipore, Burlington, MA, USA) and purified using the NGC Quest 10 Plus (Bio-Rad) and HisTrap HP (Cytiva) and gel filtration chromatography using Superdex 200 Increase 10/300 GL (Cytiva).

UdgX-ST was prepared from the intracellular soluble fraction of E. coli BL21(DE3) LOBSTR strain as previously described.[2] Briefly, the transformants were cultured in the autoinduction LB medium containing 50 μg/mL kanamycin, 0.01% (w/v) $FeCl_3$ and twice the molar amount of citric acid at 28 °C and 170 rpm for 24 h. Cells were disrupted using a French press (Ohtake Works) and the intracellular soluble fraction was obtained by a centrifuge at 13,000 × g for 20 min at 4 °C. Then, the sample was purified using the NGC Quest 10 Plus (Bio-Rad) and HisTrap HP column (Cytiva). The purified sample was dialyzed against 50 mM Tris–HCl containing 500 mM NaCl and 10% (v/v) glycerol (pH 8.0).

The purity of all proteins was evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The concentrations of SC-GDH-SC

and GOx-SC were determined by measuring their absorbance at 280 nm, while the concentration of UdgX-ST was determined using the Bradford assay kit (Bio-Rad).

Fabrication of DNA aptamer-enzyme complexes

DNA aptamer used in this study was synthesized by Eurofins Genomics (Tokyo, Japan). DNA aptamer-enzyme complexes were prepared by mixing of 10 μM of UdgX-ST with 5 μM of SC-GDH-SC or GOx-SC and incubation at 4 °C for 24 h, followed by mixing with 5 μM UdgX recognition sequence-containing PEA3-01 in 10 mM sodium phosphate buffer containing 10 mM KCl (pH 6.0) and incubating at 4 °C for 18 h. To evaluate DNA aptamer-enzyme complexes formation, the reaction was terminated by addition of a buffer containing 125 mM Tris-HCl (pH 6.5), 20% (v/v) glycerol, 4% (w/v) sodium dodecyl sulfate, 3.1% (w/v) dithiothreitol, and 0.01% (w/v) bromophenol blue and by boiling at 95 °C for 10 min.

Dehydrogenase enzyme activity assay

The dehydrogenase activities of SC-GDH-SC, GDH-UdgX, aptamer-GDH were evaluated using phenazine methosulfate (PMS) and 2,6-dichlorophenolindophenol (DCIP). Each 20 μL of the samples was mixed with 160 μL of reaction solution containing 20 mM potassium phosphate (pH 6.5), 6 mM PMS, and 0.6 mM DCIP. Then, 20 μL of various concentrations of glucose was added. To calculate the kinetic parameters, the absorbance at 600 nm was monitored for 30 sec using a spectrometer (Shimadzu Corporation, Kyoto, Japan).

Evaluation of aptamer binding to mouse Hb

Enzyme-linked Oligonucleotide Assay (ELONA) was performed to confirm the binding ability of aptamer. 100 nM human Hb (Sigma-Aldrich, St. Louis, MO, USA) or mouse Hb (CUSABIO, Inc., Wuhan, China) diluted in carbonate–bicarbonate buffer (Sigma-Aldrich) was immobilized in a 96-well plate (Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 25 °C for 1 h. The wells were washed three times with wash buffer (50 mM Tris–HCl, 138 mM NaCl, 2.7 mM KCl, and 0.05% (v/v) Tween 20, pH 8.0) and blocked with wash buffer containing 2% (w/v) skim milk at 25 °C for 1 h. Various concentrations of fluorescein (FAM) -modified aptamer, aptamer-UdgX, aptamer-GDH were added, and the plate was incubated at 25 °C for 1 h. The plate was washed three times, and the fluorescence of FAM derived from aptamer was measured using a Varioskan Flash Microplate reader (Thermo Fisher Scientific).

Electrochemical mouse Hb detection in mouse feces

Anti-mouse Hb IgG (2.8 μg) was immobilized onto 40 μg of Protein A/G-coated magnetic beads (Thermo Fisher Scientific) by incubating at 25 °C for 1 h, and the beads were washed with 100 mM potassium phosphate buffer (pH 6.5) containing 0.05% (v/v) Tween-20 three times to remove unreacted antibodies. Then, the beads were blocked by incubation with 100 mM potassium phosphate buffer (pH 6.5) containing 0.05% (v/v) Tween-20 and 1% (w/v) bovine serum albumin (BSA) under gentle shaking at 25 °C for 18 h, and each washing step was repeated three times. Next, 40 μg of the anti-mouse Hb IgG-immobilized magnetic beads were mixed with 20 μL of 600 nM aptamer-GDH or aptamer-GOx diluted with 100 mM phosphate buffer (pH 6.5) and 20 μL of various concentrations of mouse Hb under shaking at 1,200 rpm at 25 °C for 15 min. Then, 35 μL of 15 mM mPMS was mixed, and 20 μL of the mixed solution was subsequently loaded onto a screen-printed carbon electrode (DEP-Chip EP-PP, BioDevice Technology Ltd., Ishikawa, Japan). Under the working electrode, a magnet was placed to accumulate the magnetic beads. Finally, chronoamperometry was performed by applying a potential of +150 mV vs. Ag/AgCl. After 2 min, 5 μL of 500 mM glucose was spiked and the current change was monitored, and the current increase after the glucose addition was plotted. To prepare the mouse Hb sample in mouse feces, 4 mg of mouse feces were ultrasonicated in 1 mL of phosphate-buffered saline (PBS). The homogenate was centrifuged at 3000 × g for 5 min, and the supernatant was used as the mouse fecal solution after spiking mouse Hb.

Statistical analysis

Data were presented as the mean ± standard deviation (SD) unless otherwise stated. Statistical analysis for each experiment was described in figure legends. The numbers of technical and biological replicates for each result were described in figure legends. The significance of the results was analyzed by Student's T-test (two-tailed) and $p < 0.05$ was considered to be a statistically significant difference. Each p-value was indicated in the results.

Collection of fecal samples from colitis model mice

Female BALB/c mice were selected in dextran sulfate sodium (DSS)-induced colitis mode. A total of twenty 5-week-old female BALB/c mice (Japan SLC, Inc., Shizuoka, Japan) were housed in a clean rack maintained at 23 ± 3 °C and 50 ± 20% humidity and were allowed free access to standard solid feed and drinking water under a 12-h light/12-h dark cycle. Polycarbonate circular enrichment items for mice and paper-

based enrichment materials were placed in the clean rack, and four mice were housed per cage. The mice were divided into two experiments: the preliminary study for dose selection of DSS (molecular weight 36,000–50,000, MP Biomedicals, Santa Ana, CA, USA). In the present study, we used feces from mice from the preliminary study. During the 5-day acclimation period, pure water was provided in the preliminary study. After 1, 2, 3, and 4% DSS was administered to each mouse (n = 1) via drinking water for 5 days, a 5-day recovery period was provided. Feces were collected on the fifth day after the start of the 4% DSS administration. When the animal caretaker restrained the mouse, defecation occurred; therefore, freshly excreted feces adhering around the anus were collected with tweezers, transferred to dedicated tubes, and stored at −70 °C. We also collected feces from the control mouse (n = 1). All animal experimental procedures were reviewed and approved by the Animal Experiment Committee of Tokyo University of Agriculture and Technology (Approval No.: R05-191). The study was conducted in compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Supplementary Figures

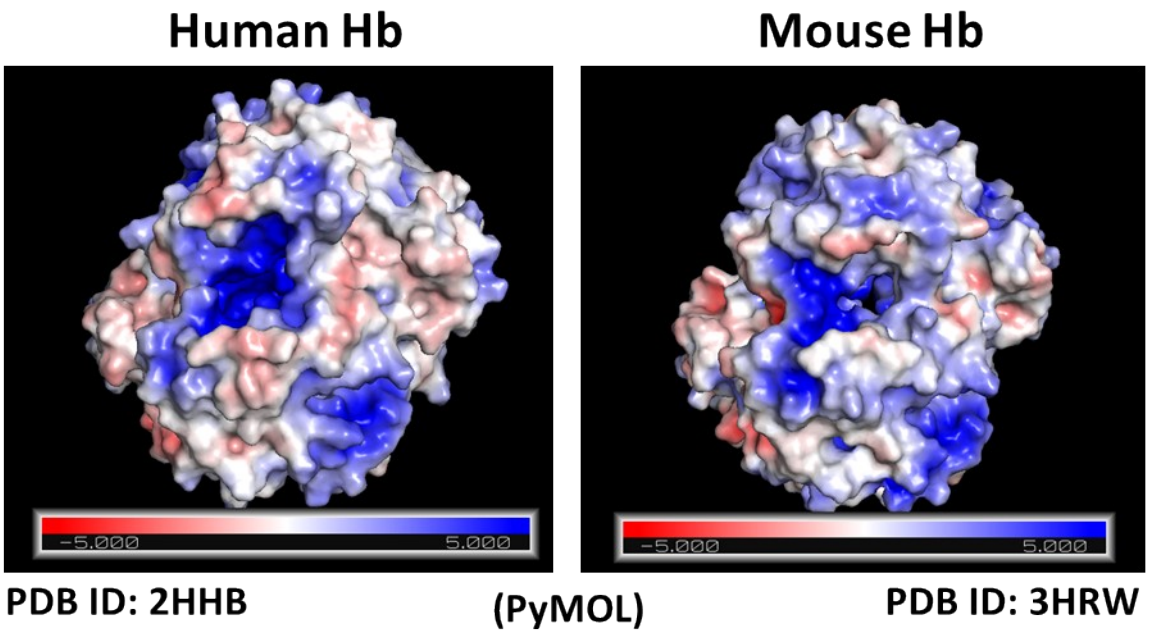

Figure S1. Results of charge calculation at pH 6.5 for human Hb and mouse Hb by PyMOL

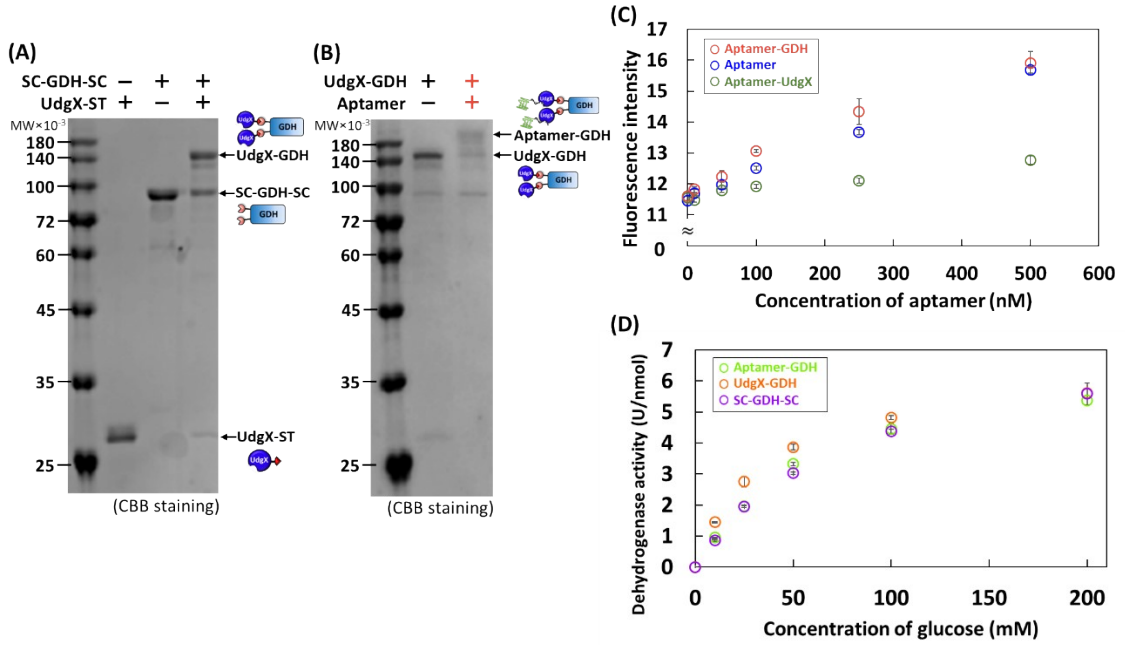

Figure S2. Preparation and functional evaluation of aptamer-GDH complex.
(A) The confirmation of the ST / SC reaction using 10 μM UdgX-ST and 5 μM SC-GDH-SC by SDS-PAGE stained with Coomassie brilliant blue (CBB). The incubation was performed at 4 °C for 24 h. (B) The Confirmation of the enzymatic DNA conjugation by UdgX using 2.5 μM UdgX-GDH and 5 μM DNA aptamer PEA3-01 with UdgX recognition sequence by SDS-PAGE stained with CBB. (C) Evaluation of binding ability of aptamer-GDH complex to mouse Hb (n = 3) by Enzyme-linked Oligonucleotide Assay (ELONA). Results are expressed as the mean ± SD (n = 3). (D) Evaluation of enzymatic activity assay using phenazine methosulfate (PMS) and dichlorophenolindophenol (DCIP). Glucose was added to Aptamer-GDH, UdgX-GDH, SC-GDH-SC at a final concentration of 0-200 mM. Enzyme activities were calculated from the reduction of DCIP at 600 nm. Results are expressed as the mean ± SD (n = 3).

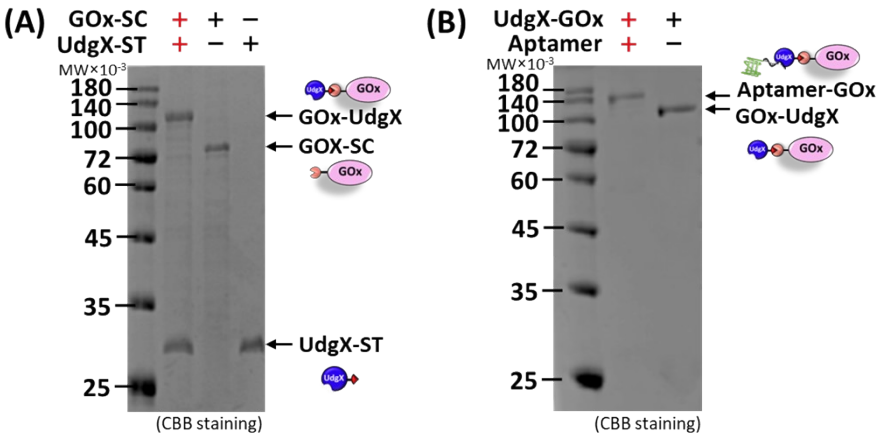

Figure S3. Preparation of Aptamer-GOx complex.

(A) The confirmation of the ST / SC reaction using 10 μM UdgX-ST and 10 μM GOx-SC by SDS-PAGE stained with Coomassie brilliant blue (CBB). The incubation was performed at 4 °C for 24 h. (B) The Confirmation of the enzymatic DNA conjugation by UdgX using 2.5 μM UdgX-GOx and 5 μM DNA aptamer PEA3-01 with UdgX recognition sequence by SDS-PAGE stained with CBB.

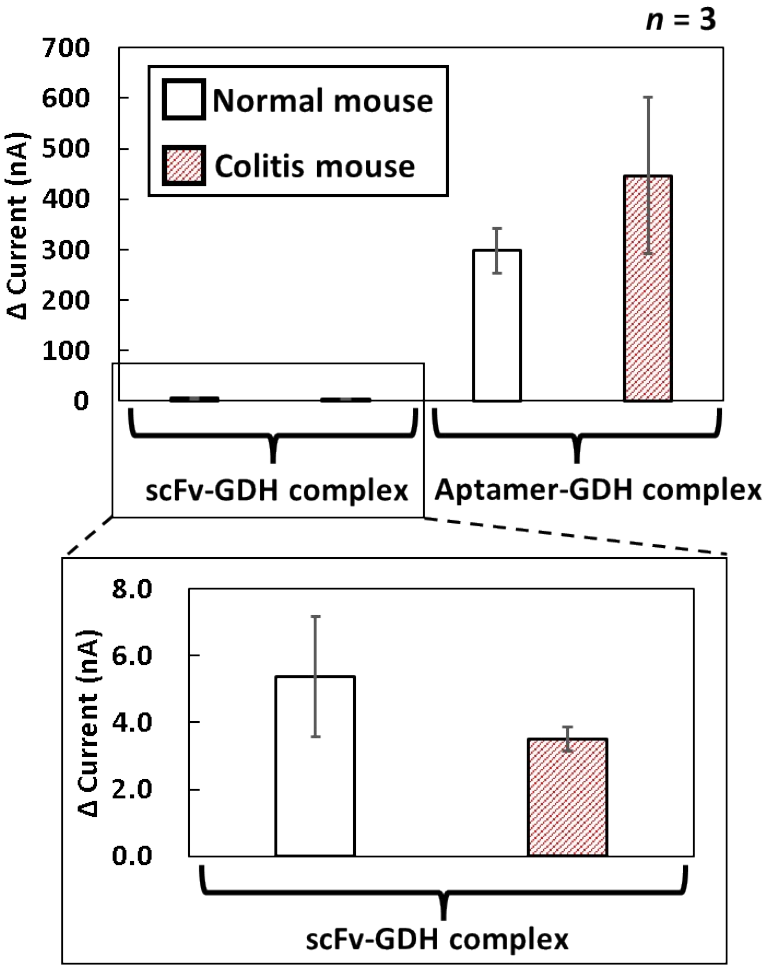

Figure S4. Comparison of electrochemical detection using human Hb-binding aptamers vs. anti-human Hb scFv

Table S1. Oligonucleotide sequences used in this study.

Name	Sequence (5' to 3')
PEA3-01-SSU9	**GGGCGGGTTGGGCTGGGTTTCTCAAGTGUAGGCATG CAAGAGCTTTTTCTACATCTCAGCATTCT**
PEA3-01-SSU9-FAM	**GGGCGGGTTGGGCTGGGTTTCTCAAGTGUAGGCATG CAAGAGCTTTTTCTACATCTCAGCATTCT [FAM]**

FAM: fluorescent compound (FAM) with excitation and emission peaks at 495 and 520 nm, Underline: sequences of aptamer; SSU9: UdgX recognition sequence (single-stranded DNA), respectively

Table S2. Protein sequences used in this study.

Sample	Sequence (N term – C term)
UdgX-ST	**MAGAQDFVPHTADLAELAAAAGECRGCGLYRDATQAVF GAGGRSARIMMIGEQPGDKEDLAGLPFVGPAGRLLDRAL EAADIDRDALYVTNAVKHFKFTRAAGGKRRIHKTPSRTEV VACRPWLIAEMTSVEPDVVVLLGATAAKALLGNDFRVTQ HRGEVLHVDDVPGDPALVATVHPSSLLRGPKEERESAFAG LVDDLRVAADVRPGSGAHIVMVDAYKPTKKLAAALEHHH HHH**
SC-GDH-SC	**MGSSHHHHHHSSGLVPRGSGAMVDTLSGLSSEQGQSGDM TIEEDSATHIKFSKRDEDGKELAGATMELRDSSGKTISTWI SDGQVKDFYLYPGKYTFVETAAPDGYEVATAITFTVNEQG QVTVNGKATKGDAHIGSGHMNTTTYDYIVVGGGTSGLVV ANRLSENPDVSVLLLEAGASVFNNPDVTNANGYGLAFGSAI DWQYQSINQSYAGGKQQVLRAGKALGGTSTINGMAYTRA EDVQIDVWQKLGNEGWTWKDLLPYYLKSENLTAPTSSQV AAGAAYNPACNGKEGPLKVGWSGSLASGNLSVALNRTFQ AAGVPWVEDVNCGKMRGFNIYPSTLDVDLNVREDAARAY YFPYDDRKNLHLLENTTANRLFWKNGSAEEAIADGVEITS ADGKVTRVHAKKEVIISAGALRSPLILELSGVGNPTILKKN NITPRVDLPTVGENLQDQFNNGMAGEGYGVLAGASTVTY PSISDVFGNETDSIVASLRSQLSDYAAATVKVSNGHMKQED LERLYQLQFDLIVKDKVPIAEILFHPGGGNAVSSEFWGLLP FARGNIHISSNDPTAPAAINPNYFMFEWDGKSQAGIAKYIR KILRSAPLNKLIAKETKPGLSEIPATAADEKWVEWLKANY**

	RSNFHPVGTAAMMPRSIGGVVDNRLRVYGTSNVRVVDAS VLPFQVCGHLVSTLYAVAERASDLIKEDAKSAGSGGAMVD TLSGLSSEQGQSGDMTIEEDSATHIKFSKRDEDGKELAGAT MELRDSSGKTISTWISDGQVKDFYLYPGKYTFVETAAPDG YEVATAITFTVNEQGQVTVNGKATKGDAHILEHHHHHH
GOx-SC	**MNGIEASLLTDPKDVSGRTVDYIIAGGGLTGLTTAARLTE NPNISVLVIESGSYESDRGPIIEDLNAYGDIFGSSVDHAYETV ELATNNQTALIRSGNGLGGSTLVNGGTWTRPHKAQVDSW ETVFGNEGWNWDNVAAYSLQAERARAPNAKQIAAGHYFN ASCHGVNGTVHAGPRDTGDDYSPIVKALMSAVEDRGVPT KKDFGCGDPHGVSMFPNTLHEDQVRSDAAREWLLPNYQR PNLQVLTGQYVGKVLLSQNGTTPRAVGVEFGTHKGNTHN VYAKHEVLLAAGSAVSPTILEYSGIGMKSILEPLGIDTVVD LPVGLNLQDQTTATVRSRITSAGAGQGQAAWFATFNETFG DYSEKAHELLNTKLEQWAEEAVARGGFHNTTALLIQYEN YRDWIVNHNVAYSELFLDTAGVASFDVWDLLPFTRGYVHI LDKDPYLHHFAYDPQYFLNELDLLGQAAATQLARNISNSG AMQTYFAGETIPGDNLAYDADLSAWTEYIPYHFRPNYHGV GTCSMMPKEMGGVVDNAARVYGVQGLRVIDGSIPPTQMS SHVMTVFYAMALKISDAILEDYASMQGSGGAMVDTLSGLS SEQGQSGDMTIEEDSATHIKFSKRDEDGKELAGATMELRD SSGKTISTWISDGQVKDFYLYPGKYTFVETAAPDGYEVAT AITFTVNEQGQVTVNGKATKGDAHIHHHHHH**

His: His-tag; ST: SpyTag; SC: SpyCatcher; GOx: glucose oxidase; GDH: glucose dehydrogenase, respectively

Table S3. Comparison of the performance of electrochemical detection of Hb.

	Method	Linear range (nM)	Sample Target	Ref.
Aptasensor	Square Wave Voltammetry	0.8-560	-	(Teniou et al., 2023)
scFv-enzyme complex	Chrono-amperometry	10-3.0×10^{2}	serum	(Miura et al., 2021)
Aptasensor	Difference Pulse Voltammetry	1.0×10^{-10}-1.0×10^{3}	buffer	(Shekari et al., 2017)
Ferromagnetic Fe@C Nps	Cyclic Voltammetry	1.0×10^{-3}-1.0×10^{2}	blood	(Matysiak et al., 2015)
Pencil Lead Electrode	Voltammetry	1.5×10^{2}-2.0×10^{3}	buffer	(Majidi et al., 2011)
Carbon nanotubes	Flow Injection Analysis	5.0-2.0×10^{3}	-	(Pakapongpan et al., 2011)
aptamer -enzyme complex	Chrono-amperometry	12.5-2.0×10^{2}	stool	This study

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