# Supporting Information for

# A pH-independent electrochemical aptamer-based biosensor supports quantitative, real-time measurement in vivo

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# Materials and Reagents.

General chemicals and solvents were purchased as reagent grade and used without further N-Hydroxysulfosuccinimide purification. sodium salt (Sulfo-NHS), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), and tris(2-carboethyl)phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich; kanamycin sulfate and 6-Mercapto-1-hexanol from Aladdin; all were used as received. The precursor exTTF-alkyne was prepared according to the previously reported procedure.1 The artificial urine samples were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China) and used as received. The composition of these samples are 72.1 mM of sodium chloride, 14.5 mM of sodium sulfate, 2.2 mM of sodium citrate, sodium oxalate 0.15 mM, sodium dihydrogen phosphate 18.7 mM, potassium chloride 19.3 mM, and ammonium chloride 9.3 mM, creatinine 9.3 mM. A variety of solution with different pH values are obtained via pH adjustment using HCl or NaOH. Real urine samples were collected from the volunteers. For column chromatography silica gel 60 (230-400 mesh, 0.040-0.063 mm) was purchased from E. Merck. Thin Layer Chromatography (TLC) was performed on aluminium sheets coated with silica gel 60 F254 purchased from E. Merck, visualization by UV light. NMR spectra were recorded on a Bruker AC 500 with solvent peaks as reference. <sup>1</sup>H and <sup>13</sup>C NMR spectra were obtained for solutions in DMSO-d<sub>6</sub>. MALDI-TOF-mass spectra were carried out on a Bruker BIFLEXTM

matrix-assisted laser desorption time-of-flight mass spectrometer using 2-[(E)-3-(4-tertbutylphenyl)-2methylprop-2-enylidene] propanedinitrile (DCTB) as matrix. 2 and 7/8" microcloth, 1 µm and 0.05 µm alumina powder were obtained from CH Instruments Ins. Gold wire (0.2 mm diameter), tungsten wire (0.2 mm diameter) and silver wire (0.1 mm) for electrode fabrication were purchased from Wuhan Shenshi Chemical Technology co. LTD. Teflon tubes used as insulating materials were purchased from Zeus Industrial Products. Gold electrodes (2 mm in diameter), fritted Ag/AgCl electrodes, and platinum wires were purchased from CH Instruments, Inc. (TX, USA). The relevant methylene blue, anthraguinone and thiol-modified DNA aptamer sequences (sequences as reported in references 1 and 2) were synthesized by Sangon Biotech (Shanghai) Co., Ltd., purified by C18 HPLC, confirmed by HPLC profile and spectrometry. These were dissolved in ΤE buffer (1×) (10 mass mМ tris(hydroxymethyl)aminomethane, 1 mM EDTA, pH 8.0) to a final concentration of 100 µM, aliquoted and stored at -20°C prior to use.

The sequences used in this study are:

Cocaine aptamer-NH<sub>2</sub>:

5'-HO-(CH<sub>2</sub>)<sub>6</sub>-S-S-(CH<sub>2</sub>)<sub>6</sub>-AGACAAGGAAAATCCTTCAATGAAGTGGGTCG-(CH<sub>2</sub>)<sub>7</sub>-NH<sub>2</sub>-3'

Kanamycin aptamer-NH<sub>2</sub>:

5'-HO-(CH<sub>2</sub>)<sub>6</sub>-S-S-(CH<sub>2</sub>)<sub>6</sub>-GGGACTTGGTTTAGGTAATGAGTCCC-(CH<sub>2</sub>)<sub>7</sub>-NH<sub>2</sub>-3'

# Sensor fabrication

We followed the standard procedure for sensor fabrication. Specifically, gold wire electrodes were made using the following procedure: the wire is composed of two parts: gold wire (5-6 mm, 0.2 mm diameter) and tungsten wire (8 cm, 0.2 mm diameter). These two components were first cold soldered using electrically conductive silver epoxy adhesive. The tungsten wire was then insulated using a heat shrinkable teflon tubing leaving the gold wire exposed for DNA

modification and 1 cm tungsten wire at the other end for connection to the potentiostat. Following the insulation step, the gold portion of these was electrochemically roughened in order to increase the surface area. Briefly, the sensors were immersed in 0.5 M sulfuric acid and rapidly pulsed between  $E_{initial} = 0.0$  V to  $E_{high} = 2.0$  V vs Ag/AgCl for 400,000 times with each pulse being of 2 ms duration. Immediately prior to sensor fabrication we prepared a solution of thiol-exTTF-modified DNA in phosphate buffered saline buffer (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.0) by incubating a solution of 100  $\mu$ M DNA and 20 mM tris-(2-carboxyethyl) phosphine hydrochloride (1 : 200) for 1 hour at room temperature followed by dilution with PBS to 200 nM as confirmed by UV-Vis spectroscopy. We then immersed freshly cleaned electrodes in this solution for 1 hour at room temperature. The resulting sensors were washed with deionized water and then incubated in 20 mM phosphatidyl choline solution in PBS overnight at 4°C before being rinsed with water prior to use.

#### Electrochemical measurements

Electrochemical measurements were performed at room temperature using a multichannel CHI1040C potentiostat (CH Instruments, Austin, TX) and a standard three-electrode cell containing a platinum counter electrode and an Ag/AgCl (3 M KCl) reference electrode. Square wave voltammetry (SWV) was performed using a potential window of -0.15 to 0.15 V, a potential step of 0.001 V and an amplitude of 0.05 V. We interrogated cocaine- and kanamycin-detecting sensors using 2 Hz and 20 Hz for the measurements, respectively. McIlvaine buffer (pH range from 4.5 to 8.5) was prepared according to a published work. Single donor human urine for in vitro research was collected from health volunteers.

#### Circular dichroism (CD) measurements

In order to determine whether the pH changes in the measurements conditions alters the binding conformation between aptamers and their respective targets, an unmodified (no methylene blue, no thiol) sequence was taken up at 5  $\mu$ M, in either its own solution in phosphor buffer (pH 4.5, pH 6.5, pH 7.5 and pH 8.5, 20 mM) or mixed with a target concentration of 0.5 mM or 0.1 mM for kanamycin and cocaine, respectively. These samples were characterized by circular dichroimsm spectroscopy (CD) on an Jasco model-J-810 spectrometer (JASCO International Co., Ltd, Japan) in a 10 mm pathlength quartz cell.

## In vivo Experiments

The animal ethics involved in the study were approved by Tongji Hospital, Tongji Medical College, Huazhong University of science and technology (TJH-201903002). For the anesthetized preparation, rats were treated with intraperitoneal injection of 5% chloral hydrate anesthesia according to the rat weight at 1 mL/100 g. After exposing both ventral jugular veins and bladder, a simple catheter made from a plastic tube of a vein detained needle fitted with a steel cannula was implanted into the bladder. The sensor was inserted into the bladder and secured in place with surgical suture. Kanamycin and NaHCO<sub>3</sub> was injected through left and right jugular veins respectively. Following drug infusions, animals were euthanized by overdose on anesthetic.

# Synthesis and characterization of exTTF derivatives and modified DNA



Scheme S1. Synthesis of exTTF derivatives and DNA-exTTF conjugates.

## exTTF-COOH derivative

To a solution of the exTTF-alkyne<sup>1</sup> precursor (500 mg, 1.24 mmol) and 14-azido-3,6,9,12tetraoxatetradecanoic acid (293 mg, 0.95 mmol) in THF (8 mL), were added sequentially a solution of CuSO<sub>4</sub>·5H<sub>2</sub>O (240 mg, 0.95 mmol) in water (4 mL) and sodium ascorbate (NaAsc, 577 mg, 2.86 mmol) in water (4 mL). The reaction mixture was refluxed overnight and the solvent was removed under reduced pressure. The crude product was purified by silica gel flash chromatography (MeOH/CH<sub>2</sub>Cl<sub>2</sub> 9:1  $\rightarrow$  5:1), providing the exTT-COOH compound (350 mg, 54%) as a yellow amorphous solid.

<sup>1</sup>H-NMR (500 MHz, DMSO-*d*<sub>6</sub>)  $\delta$ : 8.67 (s, 1H), 8.20 (d, *J* = 1.7 Hz, 1H), 7.79 (dd, *J* = 8.0, 1.7 Hz, 1H), 7.72 (d, *J* = 8.0 Hz, 1H), 7.70 – 7.64 (m, 2H), 7.39 – 7.35 (m, 2H), 6.77 (m, 4H), 4.62 (t, *J* = 5.2 Hz, 2H), 3.89 (t, *J* = 5.3 Hz, 2H), 3.71 (s, 2H), 3.56 (m, 2H), 3.53 – 3.49 (m, 4H), 3.48 – 3.45 (m, 6H); <sup>13</sup>C-NMR (125.8 MHz, DMSO-*d*<sub>6</sub> + CDCl<sub>3</sub>)  $\delta$ : 170.1, 146.7, 135.7, 135.4, 135.1, 134.5, 134.4, 134.2, 127.7, 125.3, 124.7, 124.2, 124.2, 122.4, 121.4, 121.0, 120.9, 120.4, 116.6, 116.5, 116.4, 70.1, 69.8, 69.8, 69.7, 68.8, 49.6; HRMS (MALDI-ToF): m/z calcd for C<sub>32</sub>H<sub>31</sub>N<sub>3</sub>O<sub>6</sub>S<sub>4</sub> [M]<sup>+</sup>: 681.1096; found: 681.1070.

# **DNA-exTTF conjugates**

The amine-modified oligonucleotides (50 nmol) were dissolved in 100  $\mu$ L of 1X PBS buffer (pH 7.4). To this solution were added exTTF-COOH (2.5 mg, 3.7 mmol) dissolved in 50  $\mu$ L DMSO, followed by EDC (7.3 mg, 3.8 mmol) and N-Hydroxysulfosuccinimide sodium (1 mg, 4.6 mmol) dissolved in 50  $\mu$ L PBS buffer. The resultant suspension stirred at room temperature for 4 hours. The solution was desalted and diluted to 600  $\mu$ L with water and purified by RP-HPLC under the following conditions (Figure S4): C18 column, room temperature; flow rate:

1.0 mL min<sup>-1</sup>; eluent A: 0.1 M TEAA (pH 7.0); eluent B: 80% acetonitrile, 20% TEAA; linear gradient, 5-100% B in 40 min; detection wavelength: 260 nm and 373 nm. The isolated oligonucleotides were investigated by LC-MS at negative mode, see Figure S5 to S6. Extinction coefficient 20000  $M^{-1}cm^{-1}$  (at 260 nm) for exTTF units was used for concentration determination.



Figure S1. The redox process of MB. It undergoes one-proton, two-electron transfer process.



**Figure S2.** Cyclic voltammograms of MB in McIlvaine buffer (pH range from 4.0 to 8.0, black curve: pH 4.0, and the curve shifts towards lower potential with the increase of pH value) at a concentration of 200 mM, with a scan rate of 0.1 V/s and active electrode surface area of 0.032  $\text{cm}^2$ .

$$E = E^{0'} + \frac{RT}{2F} ln \frac{[MB_{0x}][H^+]^2}{[MB_{Red}]}$$
  
=  $E^{0'} + 0.0296 lg \frac{[MB_{0x}]}{[MB_{Red}]} - 0.0592 pH$  Equation S1

where E0' is the formal potential of the electrode,  $[MB_{Ox}]$  and  $[MB_{Red}]$  are the concentration of oxidized and reduced methylene blue, respectively,  $[H^+]$  is the concentration of proton participating in the electrode process. From this equation, it is clear that the electrochemical behavior of MB on electrode is different at various pH because the number of  $[H^+]$  participating in the electrode process is related to the pH of the solution (Figure S2).



**Figure S3.** The redox process of exTTF. It undergoes a two-electron transfer process without proton participation.



**Figure S4.** Cyclic voltammograms of exTTF in McIlvaine buffer (pH range from 4.0 to 8.0) at a concentration of 200 mM, with a scan rate of 0.1 V/s and active electrode surface area of 0.035 cm<sup>2</sup>. We observed a small fluctuation in the anodic peak potentials (from 0.12 V at pH 4.0 to 0.05 V at pH 8.0) without a clear dependence on the pH value, due to the fact that the TTF-based oxidation is an example of an electron transfer reaction with significant inner reorganization energy accompanied by a drastic geometric change, from a highly distorted butterfly-like geometry in the neutral state, to a planar and aromatic hydrocarbon skeleton in the dicationic state. For this reason, small changes in the conditions of measurement (e.g., polarity by changing the pH) might cause subtle fluctuations in the peak potentials. Nevertheless, the cathodic peak potential remains invariant in all the pH range investigated. The calculated half-wave potentials are very similar in all cases (0.50 V-0.57 V) and differences are within the experimental error.

$$E = E^{0'} + \frac{RT}{2F} ln \frac{[exTTF_{0x}]}{[exTTF_{Red}]}$$
$$= E^{0'} + 0.0296 lg \frac{[exTTF_{0x}]}{[exTTF_{Red}]}$$
Equation S2

where E0' is the formal potential of the electrode,  $[exTTF_{Ox}]$  and  $[exTTF_{Red}]$  are the concentration of oxidized and reduced exTTFs, respectively. Equation 2 indicates that the electrochemical behavior of exTTF is insensitive to the local pH change.



**Figure S5.** <sup>1</sup>H NMR (500 MHz, DMSO- $d_6$ ) of exTTF-COOH.



Figure S6. <sup>13</sup>C NMR (125.8 MHz, DMSO- $d_6$  + CDCl<sub>3</sub>) of exTTF-COOH.



Figure S7. MS spectrum of the exTTF-COOH (MALDI-TOF). Matrix DCTB.



Figure S8. RP-HPLC method for DNA-exTTF conjugate purification.



Figure S9. LC chromatogram of the cocaine-apt-exTTF conjugate.



Figure S10. ESI mass spectrum of the cocaine-apt-exTTF conjugate (negative mode).



Figure S11. Deconvoluted mass spectrum of the cocaine-apt-exTTF conjugate.



Figure S12. LC chromatogram of the kanamycin-apt-exTTF conjugate.



Figure S13. ESI mass spectrum of the kanamycin-apt-exTTF conjugate (negative mode).



Figure S14. Deconvoluted mass spectrum of the kanamycin-apt-exTTF conjugate.



**Figure S15.** The voltammograms obtained from exTTF-based sensors in the absence and presence of the targets. (A) and (B) are the voltammograms recorded from cocaine- and kanamycin-detecting sensors with/without their targets.



**Figure S16.** Stability test of exTTF-based sensor in artificial urine. The pH-independent sensor performance likewise holds for sensors deploying in artificial urine samples at varied pH conditions. Cocaine-detecting sensors (A) and kanamycin-detecting sensors (B) exhibited excellent stability with less than 5% signal fluctuation during high-frequency measurement conditions (>1200 scans) over a duration of 12 hours.



**Figure S17.** Stability test in real urine samples. We interrogated these two sets of sensors directly in urine sample, observing a significant signal loss for (A) MB-based sensors and (B) a minimal signal fluctuation of exTTF-based sensors. Such instability is also observed when implanted MB-based sensors in the bladder in the living animals (the inset of panel A).



**Figure S18.** (A) and (B) cocaine-detecting sensors exhibited pH-independent target-dose responses in artificial urine over a wide range of pH 4.5~8.5 at ambient conditions, with negligible difference in their binding affinities and signal gains.



**Figure S19**. The pH-independent sensor performance at 37°C, which likewise holds for sensors when being interrogated in such elevated temperature. For example, kanamycin-detecting sensors exhibited pH-independent target-dose responses over a wide range of pH 4.5~8.5.



**Figure S20.** Circular dichroism studies for the characterization of the target-recognition aptamers. (A) CD spectra of kanamycin-recognizing aptamers in their bound and unbound states at pH 7.5, illustrating no significant conformational change and (B) CD spectra of these bound aptamers at various pH. (C) CD spectra of cocaine-recognizing aptamers in their bound and unbound states at pH 7.5, illustrating an obvious conformational change upon the target recognition and (D) CD spectra of these bound aptamers at different pH.



**Figure S21. Circular dichroism studies for the characterization of the target-recognition aptamers.** (A), (B) and (C) Shown are CD spectra of kanamycin-recognizing aptamers in their bound and unbound states at pH 4.5, 6.0 and 8.5, illustrating no significant conformational change at various pH values. (D), (E) and (F) Shown are CD spectra of cocaine-recognizing aptamers in their bound and unbound states at pH 4.5, 6.0 and 8.5.



**Figure S22.** The raw current from exTTF-based sensors in vivo. E-AB sensors with exTTF redox reporter exhibited a minimal fluctuation in signaling when they are implanted in the bladder of a living rat in the absence of target while with an injection of buffer at t = 1 h.



**Figure S23.** The HPLC-MS chromatogram of the kanamycin derivative in urine sample, which was collected from bladder in the living animals.



**Figure S24.** The mass spectrum of kanamycin. (A) and (B) The peak at elution time of 0.96 min is identified as two species with m/z values of 163.1 and 324, respectively. These two species are known to be two fragments of kanamycin molecules.<sup>4</sup>



**Figure S25.** The in-vivo E-AB sensor measurements in multiple rats. Our sensors exhibit a consistent pH-independent performance, with results in good accordance when deploying them in several individual rats: (A) The raw current of three individual sensors and (B) their respective concentration plots.

References

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