Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

Whole Blood Multiplex Measurements using Electrochemical Aptamer-Based Biosensors

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Materials and Reagents

Phosphate-buffered saline (PBS) tablets, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 6-mercapto-1-hexanol, dopamine hydrochloride, cocaine hydrochloride, serotonin hydrochloride, norepinephrine hydrochloride, primaquine biphosphate, chloroquine biphosphate and vancomycin hydrochloride were obtained from Sigma Aldrich (Ontario, Canada). Quinine monohydrochloride dihydrate was obtained from Fisher Scientific (Ontario, Canada).

Methylene blue-modified cocaine-binding and vancomycin-binding aptamers were obtained from Bio-Basic (Toronto, Canada) with the HPLC purification grade. Methylene blue-modified dopamine-binding aptamer was obtained from Biosearch Technologies (USA) with dual HPLC purification. ATTO 700-modified DNA was obtained from Integrated DNA Technologies Inc. (USA) as the HPLC purified. All sequences were used as received. The DNA was reconstituted in Milli-Q water to obtain a concentration of ~100 μM, which was verified using a Nano-Drop spectrophotometer (Implen NP80, Germany) and the molar absorption coefficient at 260 nm. The DNA was aliquoted in 50 μL portions and kept at -20 ˚C prior to use.

Sequence Name	**5' Modification**	**Sequence (5' to 3')**	**3' Modification**
ATTO 700 MN19	HS-SH C6	GAC AAG GAA AAT CCT TCA ACG AAG TGG GTC	ATTO 700N
MB MN19	HS-SH C6	GAC AAG GAA AAT CCT TCA ACG AAG TGG GTC	Methylene Blue
MB Dopamine	Thiol C6 SS	ACG CCA GTT TGA AGG TTC GTT CGC AGG TGT GGA GTG ACG T	Methylene Blue
ATTO 700 Vanco	HS-SH C6	CGA GGG TAC CGC AAT AGT ACT TAT TGT TCG CCT ATT GTG GGT CGG	ATTO 700N
MB Vanco	HS-SH C6	CGA GGG TAC CGC AAT AGT ACT TAT TGT TCG CCT ATT GTG GGT CGG	Methylene Blue

Electrochemical measurements

Electrochemical measurements were performed using an Ag|AgCl (saturated KCl) reference electrode, 2 mm diameter gold working electrodes and a platinum wire counter electrode in a three-electrode system interrogated with a CHI1040C potentiostat all acquired from CH Instruments (USA, Texas). The sensors were interrogated from -0.15 to -0.50 V using square-wave voltammetry with an amplitude of 25 mV, potential increment of 1 mV and a quiet time of 2 s with various frequencies. Peak potentials and peak currents were determined using an in-house Python script relying on Pandas, Numpy and Peakutils for baseline subtraction.

Electrode preparation

Electrodes were mechanically polished using a 0.1 μm diamond slurry solution on a polishing cloth (Buehler, USA) for 1 minute. The electrodes were rinsed and sonicated for 5 min in absolute ethanol. Then electrodes were polished using a 0.05 μm alumina slurry on a polishing cloth for 1 minute (Buehler, USA). The electrodes were sonicated for 5 min in Milli-Q water to remove adsorbed alumina. The polished electrodes were then electrochemically cleaned by cycling the potential from -1 to -1.6 V for 300 scans at a scan rate of 1 V s^{-1} in 0.5 M NaOH. The electrodes were then moved and cycled in 0.5 M H_2SO_4 from -0.35 to 1.5 V for 40 times at 4 V s^{-1} followed by cycling 8 times at a scan rate of 0.1 V s^{-1} in the same potential window. Finally, we determined the electroactive surface area of our electrodes with the gold oxide reduction peak obtained in 0.05 M H_2SO_4 when electrodes are cycled from -0.35 to 1.35 V at a scan rate of 0.1 V s^{-1}.

Singly-functionalized sensor fabrication

Reconstituted DNA (2 μL, 100 μM) in Milli-Q water was reduced using TCEP (6 μL, 10 mM in Milli-Q water) for 1 h in the dark. The obtained DNA was diluted to 250 nM using PBS 1X buffer prepared by dissolving 2 PBS tablets in 400 mL yielding 137 mM NaCl, 2.7 mM KCl and 10 mM phosphate buffer at pH 7.4. The cleaned electrodes were immersed for 1 h in 100 μL of the 250 nM solution DNA strands. The resulting electrodes were rinsed and immersed in 5 mM 6-mercaptohexan-1-ol in PBS 1X for 3 h at room temperature or 16 h at 4 ˚C to coat the surface with a self-assembled monolayer.

Co-functionalized sensor fabrication

Reconstituted ATTO labeled DNA and methylene blue labeled DNA (2 µL each, 100 µM) in Milli-Q water were reduced using TCEP (6 µL each,10 mM in Milli-Q water) for 1 h in the dark. The obtained DNA solutions were diluted to 500 nM using PBS 1X and 50 µL of each DNA solution were combined. The cleaned electrodes were immersed for 1 h in 100 µL of the resulting solution containing 250 nM ATTO labeled DNA and 250 nM methylene blue labeled DNA. The resulting electrodes were rinsed and immersed in 5 mM 6-mercaptohexan-1-ol in PBS 1X for 3 h at room temperature or 16 h at 4 ˚C to protect the surface with a self-assembled monolayer.

Titration experiment

Titration experiments were performed in PBS 1X while interrogating sensors at a square-wave frequency maximizing signal response. Stock solutions of cocaine or dopamine were prepared in degassed PBS 1X. Addition of target was performed, and the sensors were scanned once every 30 s. The solution was mixed and purged with argon with a BASI C3 Cell Stand in between interrogation to prevent dopamine oxidation.

Real-time experiment

The real time experiment was performed at an interrogating square wave frequency of 100 Hz to maximize signal response of our sensors. The experiment was performed in argon flushed PBS 1X to prevent dopamine oxidation. Stock solutions of 2 mM for dopamine and 20 mM cocaine in degassed PBS 1X were prepared prior to experiment. Our sensors were interrogated once every 20 s and the solution was mixed and purged with argon in between interrogation using a BASI C3 Cell Stand. Additions were performed sequentially (cocaine then dopamine) every 15-16 voltammograms.

Blood experiment

Undiluted bovine whole-blood was obtained bi-monthly from the Sherbrooke Research and Development Centre in Lennoxville, Québec that is part of the network of research centers from Agriculture and Agri-Food Canada (AAFC). The blood was collected directly from healthy cows into 10 mL BD vacutainer containing 158 USP units of lithium heparin to prevent coagulation. The tubes were kept at 4 ˚C and used within 1 day from collection. The blood was collected and

used according to the recommendation of an ethics committee. The blood was manipulated in a containment level 1 lab (CL1) and decontaminated according to the present legislation.

The real-time measurements in blood were performed at an interrogating frequency of 250 Hz to maximize the sensor response of our sensor. Stock solutions of dopamine were diluted in PBS 1X at a concentration of 20 mM and diluted with blood to 2 mM prior to addition to minimize dopamine degradation in the complex matrix. Cocaine solution was diluted to 20 mM in blood from a solution of 130 mM in PBS 1X. Our sensors were interrogated once every 22 s and the blood was mixed between each point using a BASI C3 Cell Stand. Additions were performed sequentially (cocaine then dopamine) every 15 voltammograms.

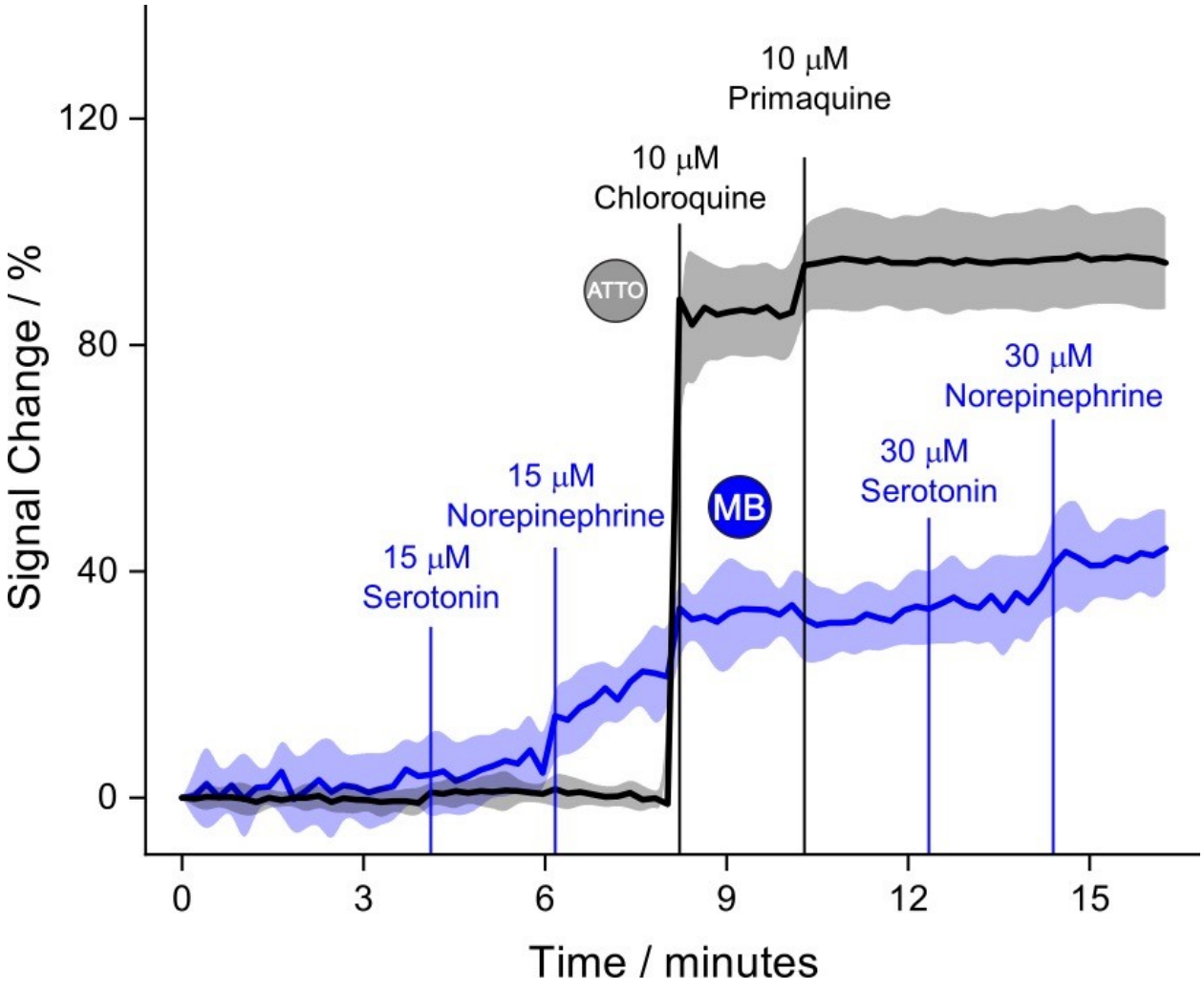

Fig S1. Co-functionalized ATTO labeled MN19 cocaine-binding and methylene blue labeled dopamine-binding aptamers respond to the addition of other molecules. We assess this by exposing our sensors to other neurotransmitters (serotonin and norepinephrine) and anti-malarial drugs (chloroquine and primaquine) previously found to interact with these aptamers. This experiment was conducted in PBS 1X while interrogating sensors at a square wave frequency of 100 Hz. Upon exposition to neurotransmitters serotonin and norepinephrine, we observed response of the methylene blue labeled dopamine-binding aptamer. Likewise, upon exposition to chloroquine and primaquine, the ATTO labeled cocaine-binding aptamer showed response. The shaded area represents standard deviation on 7 independently made sensors.

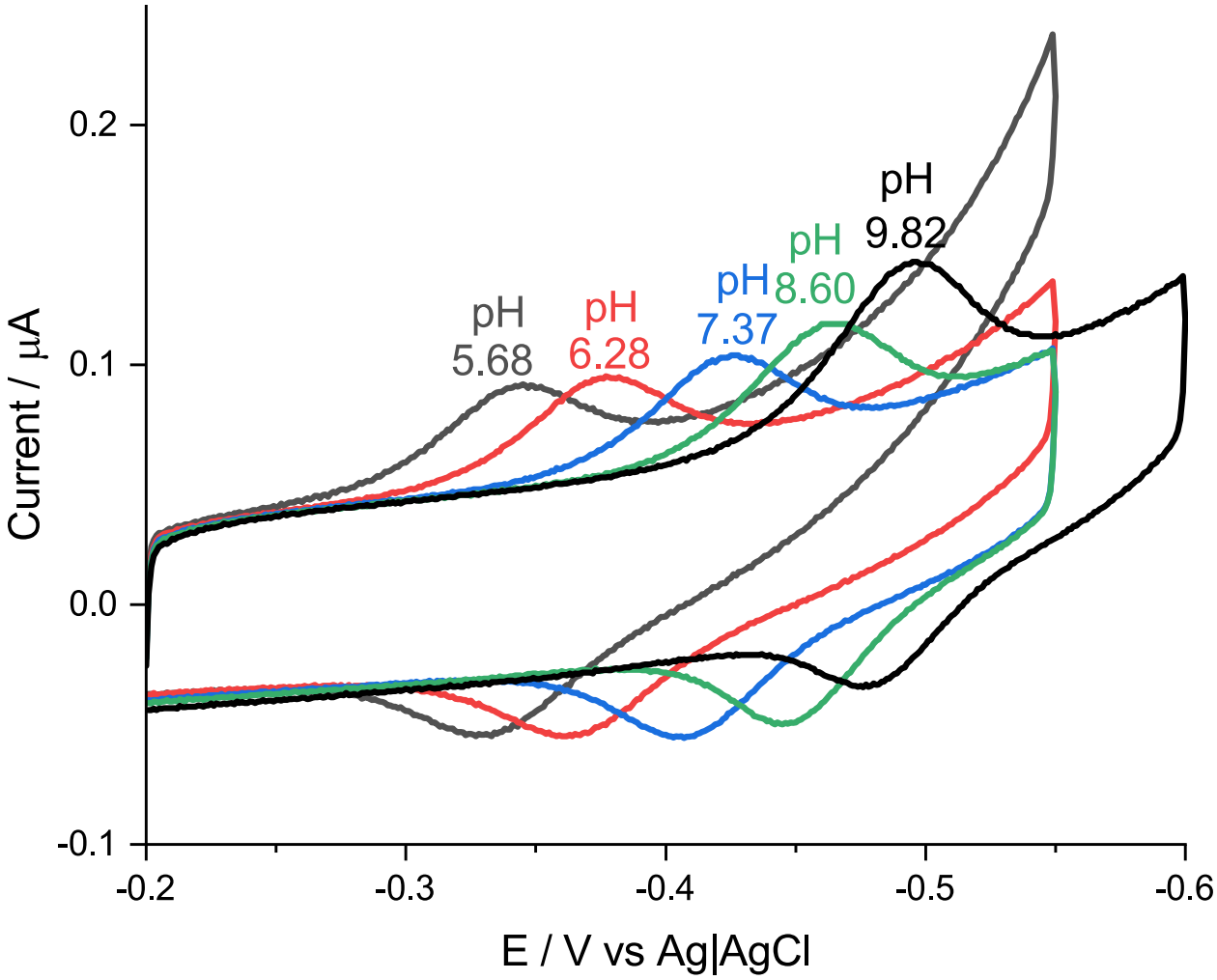

Fig S2. ATTO 700 exhibits a pH sensitive electrochemical response. We showed this by interrogating the MN19 ATTO labeled E-AB biosensors in PBS 1X adjusted at pHs ranging from 5.68 to 9.82. While the ATTO modified E-AB biosensors appeared to exhibit reversible electrochemistry at all pHs, its reduction potential shifted cathodically when moving in more alkaline solutions.

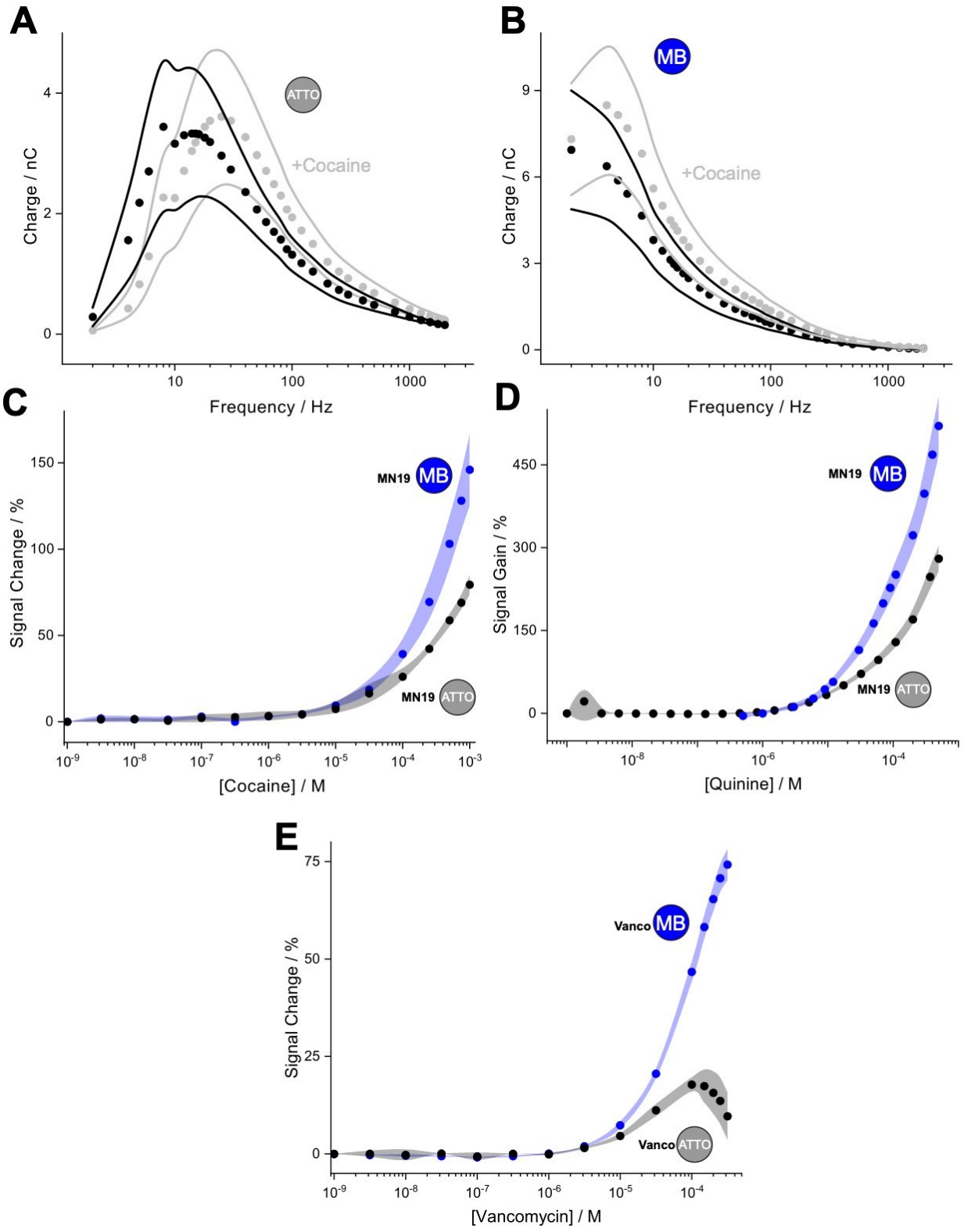

Fig S3. We compared the electrochemical properties and sensor performances of the same cocaine-binding or vancomycin-binding aptamer labeled with ATTO or methylene blue. (**A**) We started by interrogating sensors over a broad range of square-wave frequencies. In absence of cocaine, the ATTO labeled E-AB biosensors showed an electron transfer rate of ≈ 12 s^{-1}. When in presence of 200 µM of cocaine, we measured an increase in charge transfer

rate to ≈ 22 s^{-1}. This difference resulted in sensor signal change of 47%. (**B**) When using methylene blue as the redox reporter, in contrast, we obtained lower electron transfer rates of ≈ 2 s^{-1} and of ≈ 4 s^{-1} when in the absence or presence of the same concentration of cocaine which equally corresponded to a sensor signal change of 49%. (**C**) We then verified that both sensors produced comparable responses when undergoing calibration. For this, we incrementally increased the concentration of cocaine and interrogated using square wave voltammetry at a frequency of 100 Hz. We fitted the resulting response trace using a Hill equation to extract dissociation constants of 1.5 mM and 1.3 mM and maximum gain of 79% and 146% for the ATTO labeled aptamer and methylene blue sensors, respectively. (**D**) We further verified that our sensors showed comparable responses with the same aptamer interrogated with increasing amounts of a different target (here quinine). In doing so, we obtained maximum signal gains of 280% and 520% with estimated dissociation constants of 0.8 mM and 0.6 mM for the ATTO labeled and methylene blue labeled aptamer, respectively. (**E**) The same experiment was repeated with the vancomycin aptamer, which we labeled with ATTO 700 or methylene blue. We fitted the obtained binding traces to a Hill equation and obtained maximum responses of 17% and 74% and dissociation constants of 97 μM and 25 μM for the ATTO labeled and methylene blue labeled vancomycin-binding aptamer, respectively. Error bars represent standard deviation from at least 3 independently made sensors.

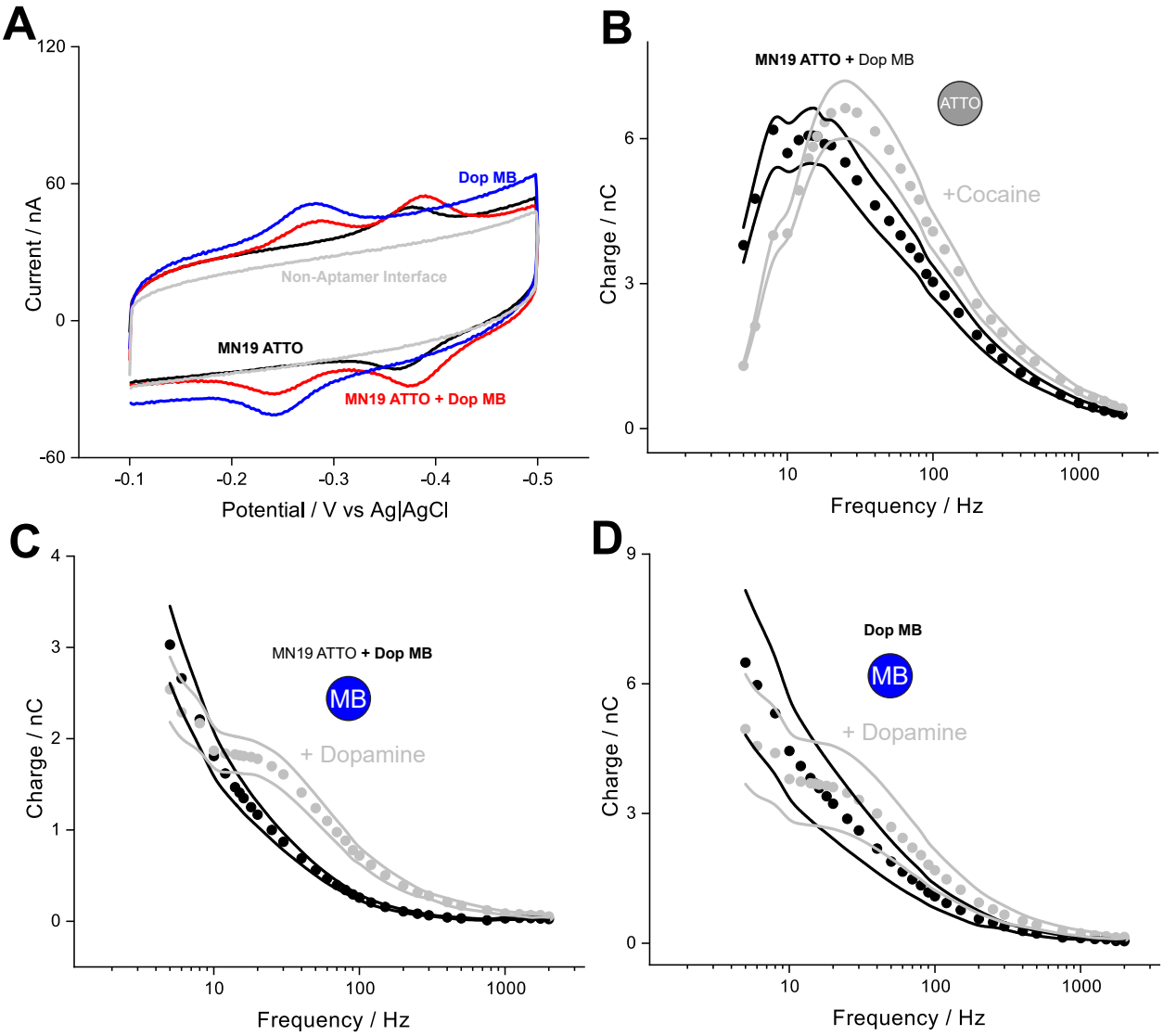

Fig S4. We compared the electrochemical properties of ATTO or methylene blue labeled cocaine-binding aptamer and dopamine-binding aptamer when singly or co-functionalized. (**A**) We started by performing cyclic voltammograms to measure the redox potentials of ATTO and methylene blue. In doing so, we observed that the co-functionalized electrode showed similar redox potentials than when singly functionalized. (**B**) When comparing the electron transfer rates of ATTO when singly or co-functionalized via square-wave voltammetric interrogations over a broad range of frequencies, we observed no significant change when in absence ($\approx 12\ s^{-1}$ *vs* $\approx 12\ s^{-1}$) or presence of cocaine ($\approx 22\ s^{-1}$ *vs* $\approx 22\ s^{-1}$) thus yielding comparable sensor signal gain (34 % *vs* 47%). (**C** and **D**) These conclusions also hold for the methylene blue modified dopamine aptamer (absence ($< 4\ s^{-1}$ *vs* $< 4\ s^{-1}$) or presence of dopamine ($\approx 18\ s^{-1}$ *vs* $\approx 18\ s^{-1}$) with comparable sensor signal gain (100% *vs* 80%)). Error bars represent standard deviation from at least 3 independently made sensors.

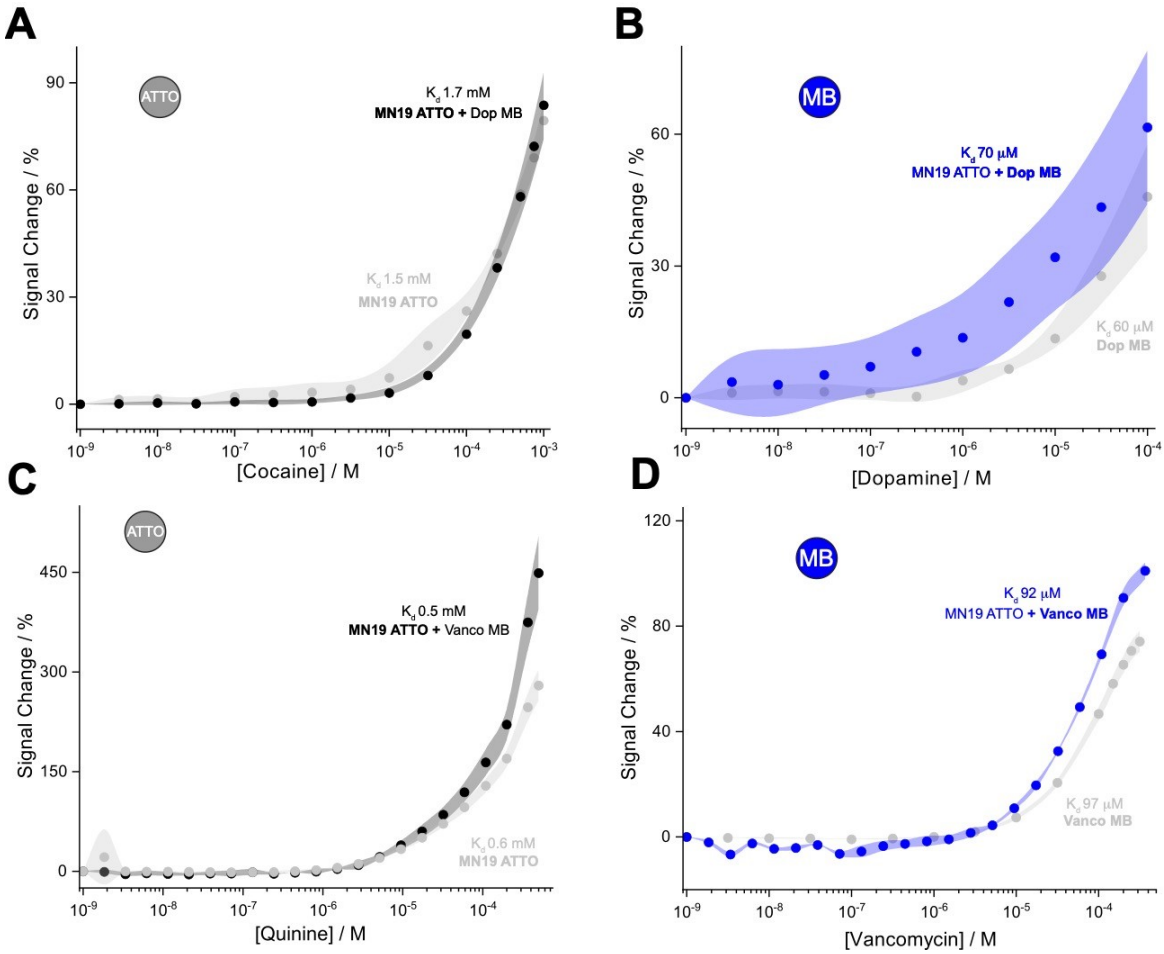

Fig S5. E-AB biosensor show similar analytical performances when singly or co-functionalized. (**A**) We show this by exposing the ATTO labeled cocaine-binding aptamer when either singly or co-functionalized to increasing amounts of cocaine in PBS 1X while monitoring the change in signal at an interrogating frequency of 100 Hz in square wave voltammetry. Using a Hill equation, we obtained similar dissociation constants and signal change (84% *vs* 79%) in the two sensor configurations. (**B**) We repeated the experiment, this time for the methylene blue labeled dopamine-binding aptamer when challenged with increasing amounts of dopamine. In doing so, we measured dissociation constants in the same order of magnitude and similar signal response for both electrode configurations. (**C**) We also interrogated another co-functionalized electrode modified with ATTO labeled cocaine-and-quinine-binding aptamer and a methylene blue labeled vancomycin-binding aptamer. When exposing our singly or co-functionalized electrodes, both modifications protocols retained the same response profile. Using a Hill equation, we obtained similar dissociation constants for singly or co-functionalized electrodes. (**D**) We repeated the same experiment with the methylene blue labeled vancomycin-binding aptamer and obtained similar response and dissociation constants. Error bars represent standard deviation from at least 3 independently made sensors.

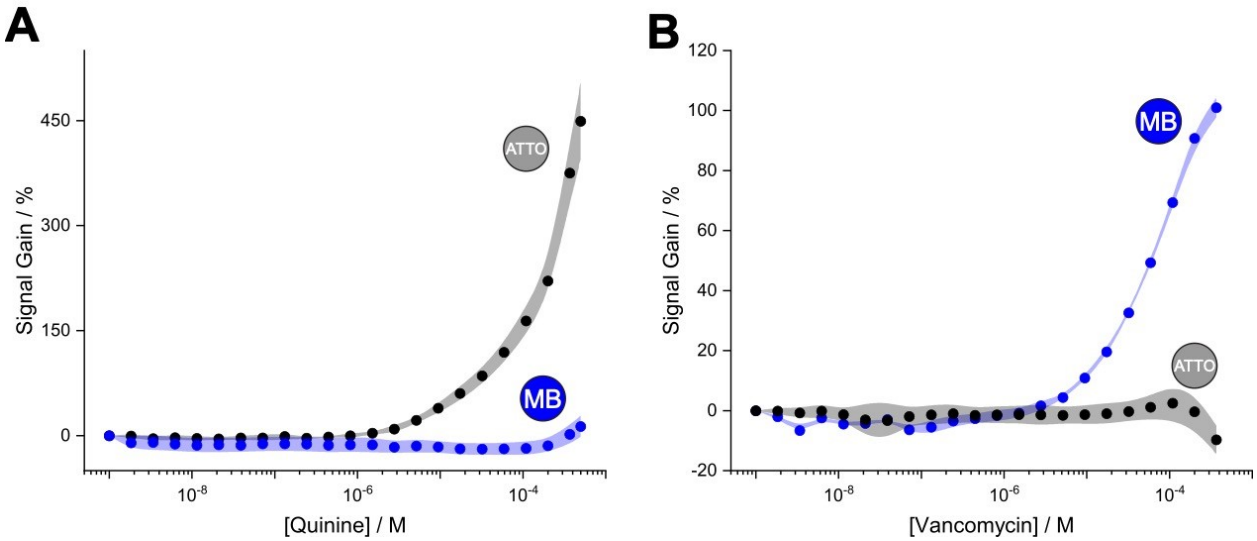

Fig S6. E-AB biosensors support multiplex measurements of multiple pairs of molecular targets. When aptamers for quinine and vancomycin are co-functionalized on the same electrode and when challenged with increasing amounts of (**A**) quinine or (**B**) vancomycin, we observed that only the (**A**) ATTO 700 or the (**B**) methylene blue signals associated with the quinine or the vancomycin aptamers, respectively, produced measurable responses. We attribute variations in responses at high target concentrations (similar to **Fig. S3E**) to confounding effects of targets that can affect the pH of the solution or change in the ionic strength[2]. Shadings represent the standard deviation of at least 3 independently fabricated sensors.

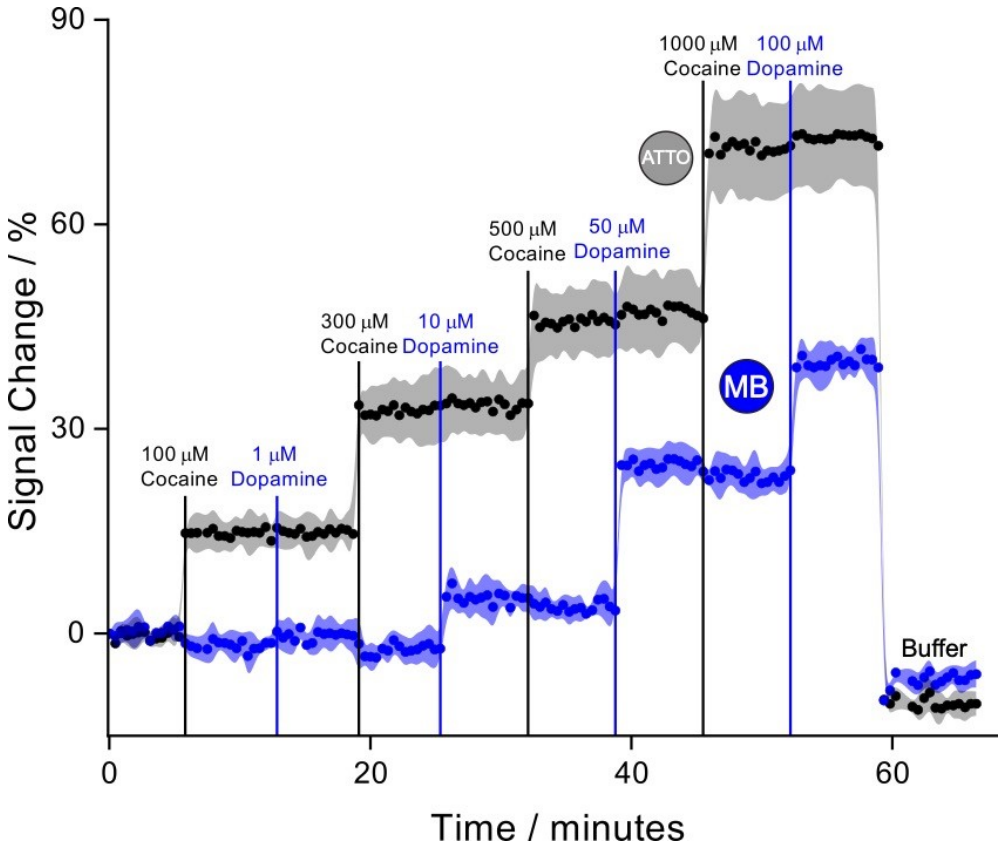

Fig S7. Our multiplex E-AB biosensors support continuous and real-time measurements of a pair of molecular targets directly in buffer. When deploying and electrochemically interrogating the co-immobilized E-AB biosensors in PBS 1X, only the corresponding electrochemical signal (methylene blue or ATTO 700) associated with either aptamer increased upon addition of cocaine or dopamine. Error bars represent standard deviation from at least 3 independently made sensors.

Fig S8. Photo of our electrochemical cell with 4 gold working electrodes, platinum wire and Ag|AgCl reference electrode.

REFERENCES

1 L. H. Negri and C. Vestri, lucashn/peakutils Zenodo 2017.
2 N. Fontaine and P. Dauphin-Ducharme, *Current Opinion in Electrochemistry*, 2023, **41**, 101361.