Concise Report

cocaine aptamer, biosensors, single-molecule junction, binding mechanism

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Ultrasensitive Detection and Binding Mechanism of Cocaine in an Aptamer-based Single-molecule Device

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Summary of main observation and conclusion Aptamer serves as a potential candidate for the micro-detection of cocaine due to its high specificity, high affinity and good stability. Although cocaine aptasensors have been extensively studied, the binding mechanism of cocaine-aptamer interactions is still unknown, which limits the structural refinement in the design of an aptamer to improve the performance of cocaine aptasensors. Herein, we report a label-free, ultrasensitive detection of single-molecule cocaine-aptamer interaction by using an electrical nanocircuit based on graphene-molecule-graphene single-molecule junctions (GMG-SMJs). Real-time recordings of cocaine-aptamer interactions have exhibited distinct current oscillations before and after cocaine treatment, revealing the dynamic mechanism of the conformational changes of aptamer upon binding with cocaine. Further concentration-dependent experiments have proved that these devices can act as a single-molecule biosensor with at least a limit of detection as low as 1 nmol·L^{-1}. The method demonstrated in this work provides a novel strategy for shedding light on the interaction mechanism of biomolecules as well as constructing new types of aptasensors toward practical applications.

Background and Originality Content

Much attention has been drawn to develop an ultrasensitive detection method for cocaine, as it is one of the most abused drugs around the world.[1] However, most of the current detection methods, including HPLC,[2] LC-MS,[3] TLC,[4] immunoassay,[5] *etc*., are time-consuming or suffer from high cost.[6] Hence, many researches focus on designing new molecules for the development of cocaine biosensors. Aptamer presents as an alternative for the biomolecular recognition of cocaine due to its high affinity, high specificity, low cost and reliability.[7-9] In general, aptamers are oligonucleotides[10] (DNAs or RNAs) that bind specifically to a large range of molecular targets with high affinity, such as small molecules, metal ions, proteins, and even whole cells selected by SELEX.[11] Cocaine aptamer, which was first developed by Stojanovic,[12] has been widely employed to build cocaine biosensors with easy fabrication and low production cost for cocaine detection.[13]

Till today, various aptasensors[14] have been developed for cocaine detection. According to the signal detected, they can be mainly divided into two types: i) optical method[15] and ii) electrical method.[16] The commonly-used optical methods contain fluorescence-based aptasensors,[12] colorimetric aptasensors,[17] chemiluminescence-based aptasensors[18] and SERS-based aptasensors.[19] In general, the cocaine aptamer is modified with fluorophores or nanomaterials such as quantum dots,[20,21] graphene oxide,[22] metal nanoparticle,[23,24] *etc*., which possess optical properties. The conformational changes of the aptamer before and after cocaine binding would produce different emission properties, which enables the differentiation of optical signals. However, the introduction of a chromophore on the aptamer may affect the intrinsic recognition process of the cocaine, and it often takes a long time (from minutes to hours) to obtain conclusive results. In contrast, the electrical methods, including the well-established electrochemical method and the new raised field-effect transistor (FET) method, are more feasible and reusable.[25] The electrochemical-based aptasensors, such as electrochemical impedance spectroscopy,[26] square wave voltammetry,[27] differential pulse voltammetry,[28] *etc*., often couple the aptamer with an electrode transducer. Similar to the optical methods, the addition of cocaine will trigger the formation of this cocaine-aptamer complex, leading to the change in the distance between the functional parts and the surface electrode. The FET-based aptasensors, as the name suggests, make use of the field effect to manipulate the source-drain current. A typical FET biosensor[16,29] contains two electrodes (source and drain) deposited on a semiconductor, an electrolyte medium, and a reference electrode linked to the source electrode to reflect the gate potentials, with the functional biomolecule often situated on the gate surface. The conformational changes in the biomolecule will cause a change in the gate potential, which will be detected by the equipment as the source-to-drain current. The FET biosensor has ultrafast response as it is sensitive to the environment stimuli. At present, both the optical and the electrical aptasensors can achieve a limit of detection (LOD) as low as ~nmol concentration.

Although the aptasensor technique is relatively mature, the functional moieties and the intrinsic properties of the aptamer are still unexplored. Only a few studies have reported the conformational changes of cocaine aptamers by NMR,[30] 2D PELDOR,[31] CD spectroscopy,[32] EPR,[33] *etc*. It is commonly assumed that the cocaine aptamer structure can be divided into three stems (Figure 1b), and the mechanism of the aptamer binding shifts with the change in the length of stem 1. When stem 1 is short, it follows a ligand-induced folding mechanism, while stem 1 becomes longer, it turns to a structure switch mechanism. The fact is that these techniques used for the cocaine binding kinetics are unable to do real-time detection, and the results only reflect the average properties of an ensemble of molecules, which conceals the details of conformational changes of cocaine-aptamer complexes at the single-molecule level.

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In this paper, we reported an electronic device which not only acted as an ultrasensitive aptasensor, but also put an intrinsic eye into the conformational changes during the binding process by real-time electrical signal recordings. We designed a partially complementary double stranded DNA containing the cocaine aptamer sequence on one strand. The cocaine aptamer was coupled onto the strand as a side chain molecule to maintain its flexibility for recognition and binding. This molecule was then immobilized on the graphene electrode by amide linkage. The use of graphene in the construction of aptamer-based electrical devices provides exceptional advantages in terms of high conductivity, stable interface, good biocompatibility and ease of device integration. Our single-molecule device allows for the improvement of the LOD. Hence, the current analysis would provide robust and precise evidence for the real-time conformational changes of aptamer upon binding with cocaine.

Results and Discussion

Device fabrication and molecule connection test

The nanogapped graphene electrode arrays were constructed by a dash-line lithographic (DLL) method,[34] with a gap size about 2—5 nm to match the length of the DNA strand. The DNA strand functionalized with amine groups on both ends was connected to the carboxylic acid group on the graphene via amide bond to form a graphene-molecule-graphene single molecule-junction (GMG-SMJ). The complementary strand containing the cocaine aptamer was held together by hydrogen bonds through complementary base pairing of ten base pairs in the DNA strand, forming a molecular bridge (Figure 1a, Figures S1—S3). The 39-base-cocaine aptamer was selected by SELEX and was most commonly used in studies (Figure 1b). To retain the native structure of the aptamer, the cocaine aptamer was designed as the side chain of the molecular bridge to maintain the flexibility of the aptamer for recognition and binding. More details of molecular synthesis and device fabrication can be found in Experimental and supporting information. The successful establishment of the GMG-SMJ was identified by electrical testing. Since the amide linkage was robust, the molecule showed a good conductivity (~nA) at 0.5 V with reproducible results (Figures 1c, S4). It was found that under optimized conditions, the connection rate of molecular junctions was ~2%, that is, ~6 of 338 devices on the same silicon chip exhibited similar increase in the conductance. Based on the statistical analysis,[35] we concluded that the connection rate of single-molecule junctions in all of the junctions was over 98%.

Real-time electrical measurements

The reaction was processed in a poly-dimethylsiloxane (PDMS) solvent reservoir with a hole of 3 mm diameter. The time-dependent electrical recordings were carried out in the solid-liquid interface at room temperature. We performed bias voltage-dependent test and concentration-dependent test to investigate the structural changes of the aptamer. The test was conducted at a zero-gate voltage with a sample rate of 500 Sa/s.

Voltage-dependent measurements

Since the applied voltage may have an impact on the biomolecule structure due to electrokinetic forces,[36] we conducted a voltage-dependent test to clarify the effects of an applied voltage on the kinetics of the cocaine conformational change. Low voltages (0.1 V, 0.2 V and 0.3 V) were chosen to avoid the occurrence of redox reactions in the buffer solution. Remarkably, two obvious states were observed under all voltages (Figure 2a). Control experiments by using devices which were not cut completely did not exhibit similar behaviors (Figures S5—S6). Moreover, we found that the proportion with higher conductance of single-molecule junctions increased with the increase of the applied voltage, thus confirming the impact of voltage on the stability of cocaine aptamer conformation. We proposed that the phenomenon of the bistable property was due to two kinds of conformational transitions. Based on the aptamer structure (Figure 1b), the binding aptamer adopts a three-way junction,[17] and the cocaine binding site was at the center of the three stems. Stem 1 and Stem 2 only contain Watson-Crick base pairs, but Stem 3 contains two putative non-canonical base pairs. In previous literatures,[30] it was reported that the mutation (for example, change from GA to GC) on Stem 3 would dramatically reduce the binding affinity. Hence, the mutation of Stem 3 will significantly alter the cocaine binding processes. Also, it has been shown[37-39] that the three-way structure of the aptamer provided a positive driving

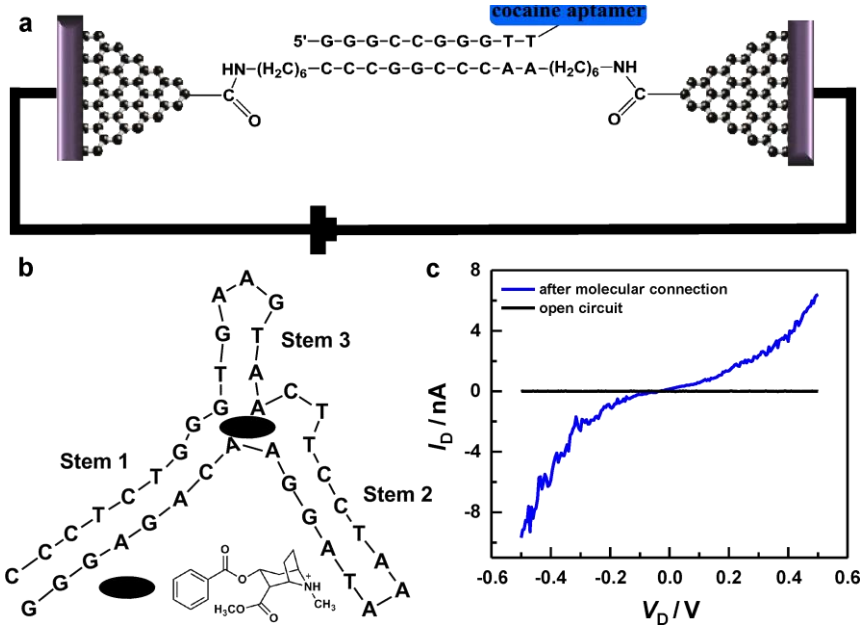

Figure 1 Device structure and molecule connection. (a) Schematic representation of the device structure. (b) The structure of aptamer for cocaine recognition and binding. (c) Typical *I-V* curves of open circuits with graphene point contacts (black) and single-molecule junction after molecular connection (blue).

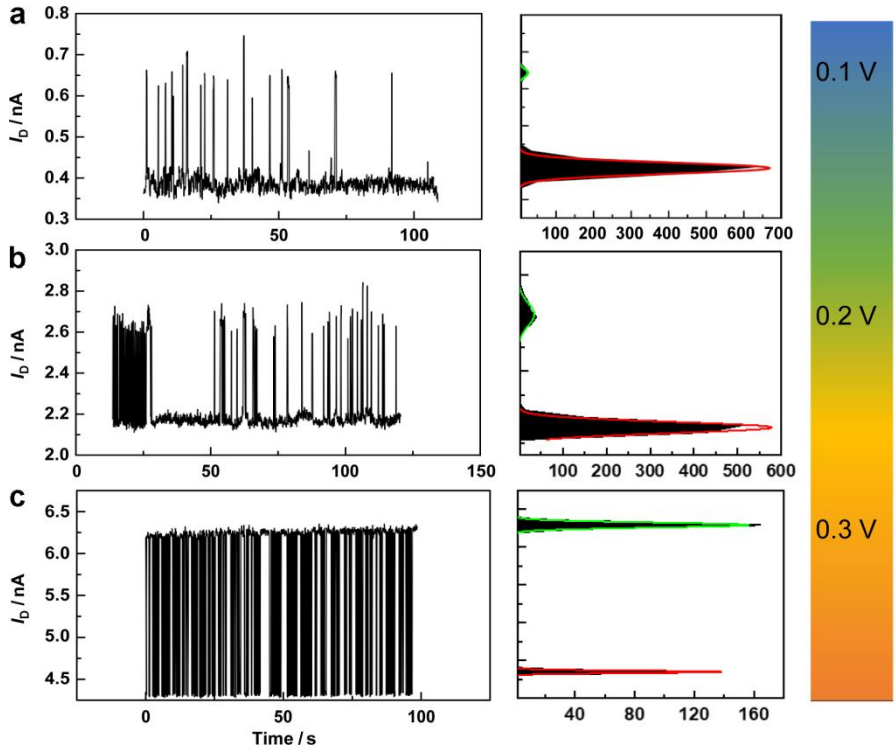

Figure 2 Real-time voltage-dependent measurements of cocaine aptamer. *I-t* curves, corresponding histograms of a single-molecule junction at (a) V_D = 0.1 V, (b) V_D = 0.2 V and (c) V_D = 0.3 V.

force for binding, as the binding affinity increased with the increase in the length of Stem 1. Moreover, Stem 1 with four base pairs or longer provided a better conformation for binding. Since the chosen length of Stem 1 in the research was long and Stem 3 contained no mutation, we concluded that the secondary structure did not change during the binding, and the addition of cocaine only changed the tertiary structure of the aptamer. Hence, the bistable property of the aptamer could be ascribed to two kinds of tertiary structures of the aptamer.

Concentration-dependent measurements

We added different concentrations of cocaine into the reaction reservoir to test the sensitivity of our devices (Figure 3). The device containing the aptamer was washed with deionized water three times after being treated with cocaine to elute cocaine completely. Both the *I-t* curves and the corresponding histograms of each state suggested that there was only one state after the addition of cocaine, indicating the highly stable tertiary structure of the aptamer-cocaine binding complex. Moreover, the current remained unchanged as the concentration of cocaine increased, confirming that our device was a single-molecule device without concentration dependence. In comparison with the magnitude of the current for the single aptamer tested previously (Figure 2), we found that the higher state of the aptamer has a similar current level at the same bias voltage after cocaine treatment. Thus, we can conclude that the addition of cocaine stabilizes the tertiary structure to a more conductive one. Combining with the previous theoretical calculation data,[31,33] we suggest that the aptamer can adopt two kinds of tertiary structure, "T" shape and "Y" shape. Although the aptamer structure did not affect the charge transport pathway, the transformation of the two conformations affected the electronic cloud distribution of the whole complex. After the treatment of cocaine, the complex adopted a "Y" shape and thus, only exhibited one state (Figure 4). Notably, our device allows ultrafast detection as it produced a response as soon as the cocaine was added. Moreover, the GMG-SMJ was very stable after ten days and the cocaine detection was reproducible for ten cycles (Figure S7).

Statistical analyses

To investigate the voltage-dependent properties in detail, we used a QuB software to analyze the time intervals of the high and low conductance states (τ_{on} and τ_{off}). The plots of the dwell times were obtained by frequency analysis (Figure 5a), and the data were fitted with a single-exponential probability distribution function, demonstrating that the conformational changes of the single-molecule aptamer were random events (Figures 5b, c). The average lifetimes of the *on* and *off* state at different bias voltages were obtained as shown in Table S1. When the bias voltage was increased, τ_{on} increased from ~2 ms to ~17 ms, while τ_{off} decreased from ~1358 ms to ~2 ms. We could simplify the aptamer conformational change as $T \rightleftharpoons Y$. Thus, the rate constant of the two states were $k_{on} = 1/\tau_{on}$, $k_{off} = 1/\tau_{off}$. To reveal the correlation analysis of the applied voltage and the transformation energy, based on the equation, $-\ln k \propto \Delta E_a/RT$ (E_a was the activation energy). we plotted ln *k*-voltage curves and found that both $-\ln k_{on}$ and $-\ln k_{off}$ showed a linear relationship with the bias voltage (Figure 5c). From the plots, we found that ΔE_a of the *on* state increased with increasing the voltage, while the ΔE_a of the *off* state decreased with increasing the voltage. Hence, we concluded that the electric field changed the energy of the transition state between the *on* and *off* forms. Moreover, the strong voltage dependence confirmed the role of electrical force applied on the molecule, which gives a guideline for the derivation of single-molecule dynamics obtained by the electrical method.

Conclusions

In summary, we have demonstrated an efficient single-molecule electrical detection by using an aptamer-based biosensor with high sensitivity to provide insights on the conformational changes of cocaine aptamer upon recognition and binding. The nanocircuit-based structure is label-free, ultrasensitive, time-saving, low-cost and reproducible,[40-42] which will be promising

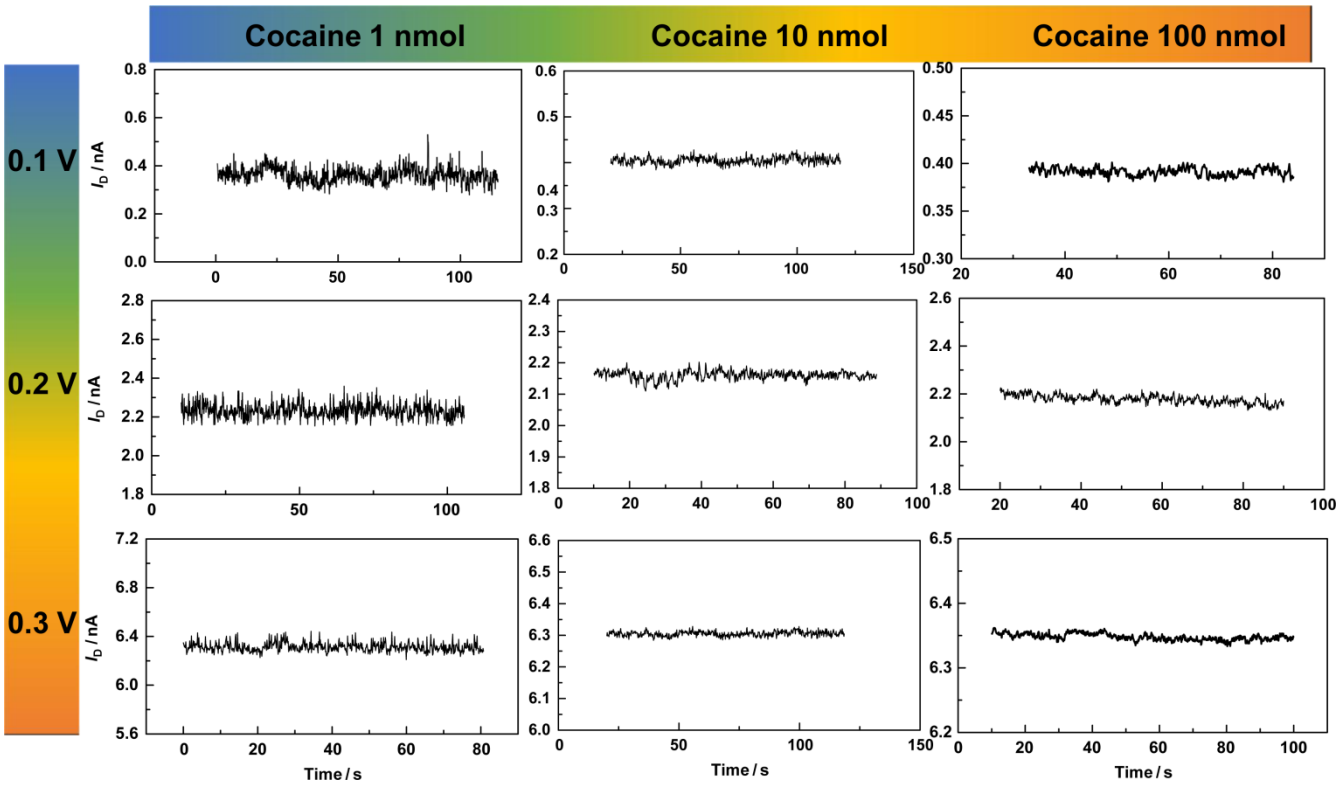

Figure 3 Voltage-dependent and concentration-dependent real-time recordings. The rows exhibit the concentration-dependent test and the columns exhibit the bias voltage dependent test.

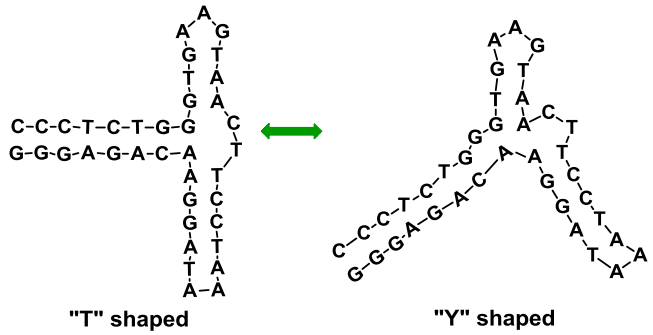

Figure 4 Proposed structural fluctuations of aptamer before cocaine binding.

for drug detection in clinical or forensic diagnostic applications. The ultrafast response of single-molecule kinetics reveals that the cocaine aptamer undergoes reciprocal conversion of two conformations before binding, and the binding of cocaine changes the tertiary structure to a state with a higher conductance. In addition, we have investigated the effect of the applied voltage on the aptamer behavior by analyzing the relationship between the rate constant and the applied voltage. The results provide a quantitative analysis for molecular kinetics and a qualitative analysis for the tunable activation energy between two conformations, offering a useful tool for intrinsic molecular dynamics detection. At present, the robust GMG-SMJ platform has yielded encouraging results in revealing the dynamic processes of chemical reactions,[43] molecule isomerizations,[44] DNA sequencing,[45] hydrolase dynamics,[46] *etc*. With further precise control of the molecular conformation within graphene gaps and simplification of device fabrication, we believe that the unique superiority of this platform will make it a powerful technique to have wide applications in bioanalytical systems with the potential to be commercialized in the near future.

Experimental

General information

All oligonucleotides used in this study were purchased from

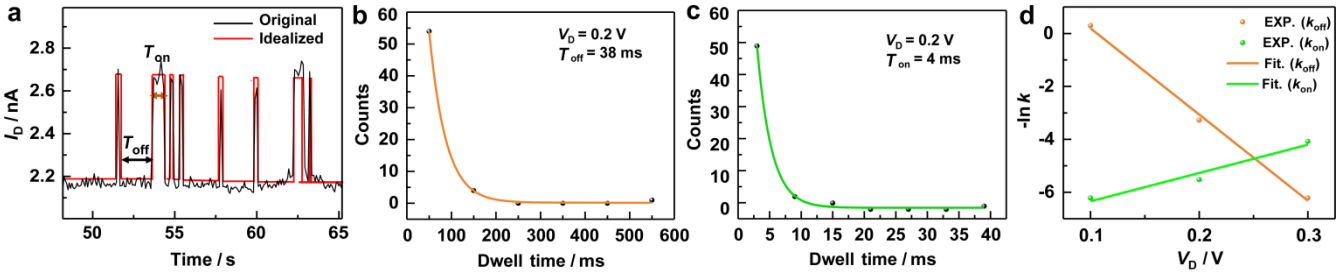

Figure 5 Statistical analyses of aptamer dynamics. (a) *I-t* curve of the cocaine aptamer (black) and the idealized fit (red) based on hidden Markov model analysis by using a QuB software at V_D = 0.2 V. (b) (c) Plots of intervals of the *off* state and *on* state through the fitting in (a). (d) The changes of the two rate constants as a function of the bias voltage.

Takara Biomedical Technology (Beijing) Co., Ltd. We designed the primer sequence as 5′-NH_2-$(CH_2)_6$-AACCCGGCCC-$(CH_2)_6$-NH_2-3′, and the complementary pair with aptamer sequence as 5′-GGGC-CGGGTTGGGAGACAAGGATAAATCCTTCAATGAAGTGGGTCTCCC-3′. Both strands were purified by HPLC and preheated in TE buffer (1 mol·L^{-1} Tris-HCl, 0.5 mol·L^{-1} EDTA, pH 8.0) at 90 °C for 5 min. The strands were annealed into a partially complementary double-stranded DNA containing the aptamer complex when cooled at room temperature for 1 h. The concentration of the solution was 10 μmol·L^{-1}.

Procedure

The single-layered graphene was grown by chemical vapour deposition (CVD) on copper foils. The graphene films were then transferred onto the Si/SiO_2 wafers (300 nm silicon oxide) by wet etching. The source/drain metal electrode arrays were patterned using three-step lithography, followed by thermal evaporation (8 nm Cr/60 nm Au) and electron beam evaporation (40 nm SiO_2). Later, dash-line lithography (DLL) was used to fabricate the nano-gapped graphene point contact arrays functionalized with carboxylic acid groups.[34] The partially complementary double-stranded DNA was coupled with the graphene electrode by two steps. Firstly, the carboxylic acid groups on the graphene point contacts were activated by 1-ethyl-3-(3-dimethylami-nopropyl) carbodiimide (EDCI) (20 mmol·L^{-1}) and *N*-hydroxysuccinimide (NHS) (10 mmol·L^{-1}) in a MES buffer (0.05 mol·L^{-1}, pH 6.5) for 2 h. The device was washed by deionized water three times and dried by N_2. Secondly, the –NH_2 modified DNA strand (30 μL, 10 μmol·L^{-1}) (TE buffer, pH 8.0) was coupled onto the activated carboxylic acid groups via amide linkages overnight.

Electrical characterization

The characterization of molecular connection and the real-time recordings were carried out by measuring the cyclic voltammetric curves through an Agilent 4155C semiconductor characterization system and a Karl Suss (PM5) manual probe station. For *I-V* measurements, the scanning interval was 5 mV per step. For real-time recordings, the sample rate was 500 Sa/s. During the *I-t* measurements, the molecular conductance fluctuated because of the conformational changes during the cocaine binding process.

Supporting Information

The supporting information for this article is available on the WWW under https://doi.org/10.1002/cjoc.201900225.

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