Supplementary Information (SI) for ChemComm.
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Supporting Information

High localized probe density greatly improves signaling stability of supramolecular electrochemical aptamer-based (Supra-EAB) sensors

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Materials and Methods

1 Materials

1.1 Oligonucleotides

The sequences used for the design of Capture are:

Target DNA: 5’-DBCO-TTT CTC CAT GGT GCT CAC-3’

Thrombin capture: 5’-MB-GGT TGG TGT GGT TGG CCC CGT GAG CAC CAT GGA G-3’

All oligonucleotides were custom synthesized by Integrated DNA Technologies, the relevant methylene blue and Dibenzocyclooctyne were synthesized by Sangon Biotech (Shanghai) Co., Ltd., purifified by C18 HPLC, confifirmed by HPLC profifile and mass spectrometry. These were dissolved in 1 M NaCl and stored at -20°C prior to use. Sodium chloride were prepared freshly prior to use.

1.2 Reagents

N_3-β- Cyclodextrin was purchased from Zhiyuan Biotechnology (Shandong) Co., Ltd; Ammonium persulfate (APS), N,N,N',N'-Tetramethylethylenediamine (TEMED), 6-Mercapto-1-hexanol (MCH) was purchased from Aladdin; Adamantane, Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) was purchased from Sigma-Aldrich; 25 DNA ladder, 250 DNA ladder, 4S Red Plus Nucleic Acid Stainall, DNase I enzyme was purchased from BBI (Shanghai) Co., Ltd; 6×loading Buffer was purchased from Takara (Beijing) Co., Ltd; Acry/Bis 40% Solition was purchased from Sangon Biotech (Shanghai) Co., Ltd and Thrombin was purchased from Shanghai Yuanye Bio-Technology (Shanghai) Co., Ltd; all were used as received.

1.3 Consumables

Dimethyl sulfoxide, Ethanol absolute, Isopropanol, Acetonitrile, Acetic acid, Sodium chloride, Triethylamine was purchased from Sinopharm Chemical Reagent (China) Co., Ltd; 20×PBS, 5×TBE buffer was purchased from Sangon Biotech (Shanghai) Co., Ltd. 2 and 7/8” microcloth, 1 μm and 0.05 μm alumina powder were obtained from CH Instruments Ins.(TX, USA). Gold electrodes, gold plate electron microscope electrode (2 mm in diameter), fritted Ag/AgCl electrodes, and platinum wires were purchased from CH Instruments, Inc. (TX, USA).

2 Equipment

Electronic balance (AL204) was purchased from Mettler Toledo Instrument (Shanghai) Co., Ltd; Vortex mixer (WH-861) was purchased from Hualida Equipment (Jiangsu) Co., Ltd; Blue Light Gel Imager (BD-BGC1), Adjustable speed Mini centrifuge (Super Mini Dancer)was purchased from Sangon Biotech (Shanghai) Co., Ltd; Eppendorf ThermoMixer (C) was purchased from Eppendorf (Hamburg, Germany); Chemiluminescence image analysis system (Tanon 4600) was purchased from Tianneng Technology (Zhejiang) Co., Ltd; Electrophoresis apparatus (DYY-8C) was purchased from Liuyi Biotechnology (Beijing) Co., Ltd; Vacuum centrifugal concentrator (RVC 2-25 CDplus) was purchased from Marin Christ (Osterode, Germany); Fluorescence spectrometer (FLS1000) was purchased from Tianmei Scientific Instrument (Beijing) Co., Ltd; Ultraviolet spectrophotometer (UV-2600) was purchased from Shimadzu instrument (Suzhou) Co., Ltd; Multichannel Potentiostat (CHI1040C) was purchased from CH Instruments, Inc. (TX, USA); High performance liquid chromatography (Agilent 1260) was purchased from Agilent Technologies, Inc. (California, USA).

3 Methods

3.1 Synthesis, purification and characterization

Synthesis and characterization of Ada-SH

1-Adamantyl methacrylate (2.2 g, 10 mmol) and 1,6-hexanedithiol (2.3 g, 15 mmol) were dissolved in 50 mL of chloroform. Michael-type addition proceeded by addition of diisopropylamine (70 μL, 0.5 mmol) for 24 h at room temperature. The solid product was collected by precipitating the solution into acetone. After being dried in vacuum, the Ada-SH compound was obtained as white powder with a yield of 72%. ^{1}H NMR (400 MHz, CD_3OD δ): 2.75-2.45 (m, 2H, CH_3CH-(C(=O))CH_2; m, 1H, CH3C*H*(C(=O))CH_2; m, 2H, HSCH_2-; m, 2H, CH_2SCH_2-), 2.18 (s, 3H, adamantane), 1.72 (s, 6H, adamantane), 1.65 (m, 6H, adamantane), 1.75-1.15 (m, 8H, HSCH_2-CH_2-; m, 3H, -CH_3). ESI-MS m/z: found 371.20 $[M]^+$ (calcd for Ada-SH $[C_{20}H_{34}O_2S_2]^+$ = 370.20).

Synthesis of β-CD-Mono-, Tri-, Penta-, Hepta-DNA. By optimizing the feed ratio of β-CD and DNA, we can achieve the optimal yield for each individual substitution of these DNA molecules. We employed a series of DNA and β-CD with relevant equivalence from 2:1 to 1: 18 for the optimization of reaction. Taking the N_3-β-CD/Capture DNA ratio of of 1:11 for example, we added 10 μL 5 mM capture DNA into 10 μL 0.46 mM N_3-β-CD solution in centrifuge tube, and placed it on the Thermo Cell. The reaction mixture was rotated at a speed of 1000 rpm under 25°C for 5 hours.

Polyacrylamide gel electrophoresis.

Preparation of polyacrylamide gel: 5 mL of H_2O, 3 mL of 40% Acryl/Bis solution, 2 mL of 5×TBE buffer, 100 μL of 10% APS solution and 10 μL of TEMED solution were mixed in a 15 mL centrifuge tube. The mixture was added into groove to form the gel.

To conduct the gel experiment, we first mixed 2 μL of 6×loading buffer into 5 μL of anti-HER2-seq and 5 μL of reaction mixture, respectively. Then we sampled DNA marker, DNA feedstock and reaction mixture sequentially, and the polyacrylamide gels

were run at 100 V for 1.5 h. The different substituted products were separately collected via the commercial polyacrylamide recovery kit to obtain the pure product.

Supramolecular assembly by FRET studies. 100 μL of 1 μM Hepta-DNA-Cy3 molecular nanoparticles was placed in a quartz dish to determine the initial fluorescence value. A certain amount of Ada-Cy5 solution is directly added to it, and the fluorescence spectrum was measured at an interval of 1, 3, 5, and 10 minutes.

3.2 Sensor fabrication

Hybridization of capture and aptamer. A 40 μL solution of 1 μM Hepta-DNA and 1 μL 40 μM Thrombin aptamer was mixed and placed on the Thermo Cell after fully mixing, first set at 25° C for one minute, and then at 90° C for 10 minutes, followed by at 25° C and shake for 16 hours。

Supramolecular assembly. We added a 37 μL solution of 0.87 μM Ada-SH (diluted by absolute ethanol) into 2.5 μL 20 mM tris-(2-carboxyethyl) phosphine hydrochloride (diluted by 1×PBS buffer) in Hybridized solution. The mixture was set at 25 ℃ for 2 hours .

Electrode cleaning. The gold disk electrode was first polished with Al_2O_3 powder with a diameter of 1.0 micron and cleaned in ultrapure water with ultrasonic for 5 minutes. Next, the electrodes was further polished with Al_2O_3 powder with a diameter of 0.05 micron, and cleaned in ultrapure water with ultrasonic for 5 minutes. As following, the electrodes were cleaned electrochemically with cyclic voltammetry for 10 cycles from 0 to -1.5 V at 0.1 V s^{-1} in 0.5 M NaOH solution via a three-electrode system (the platinum wire electrode as the counter electrode, Ag/AgCl as the reference electrode and gold as the working electrode). Then the gold electrodes were rinsed thoroughly with ultrapure water, transferred into 0.5 M H_2SO_4 and applied a chronoamperometry procedure with $E_{initial}$ = 0.0 V and E_{high} = 2.0 V vs Ag/AgCl for 320 steps and each pulse being of 0.02 s duration. Finally, the gold electrodes were transferred into 0.05 M H_2SO_4 using cyclic voltammetry at 0.1 V s^{-1} between 0 and 1.65 V to observe integrating the area under the curve of the gold oxide reduction peak,

which can be used for the determination of surface area.

Sensor fabrication. We immersed the freshly cleaned electrodes into a 100 μL solution of 400 nM aptamer at room temperature for 10 hours. Then the resulting sensors were rinsed gently with PBS buffer. After that, the functionalized sensors were immersed in 20 mM 6-mercapto-1-hexanol (diluted by 1×PBS buffer) solution and assembled at 4°C for another 10 hours.

Contact angle characterization. The contact angle measurements were performed at room temperature using a KRÜSS DSA100. In this experiment, the electrodes were cleaned via a standard cleaning protocol, and immersed in the solution of Ada-SH or the supramolecular assembly. Contact Angle test is carried out using 1 μL of H_2O.

3.3 Electrochemical measurements

The stability experiment. Electrochemical measurements were performed in 8 mL 1×PBS buffer using a CHI1040C potentiostat and a standard three-electrode cell containing a platinum counter electrode and an Ag/AgCl (Soak in 3 M KCl when not in use) reference electrode at room temperature. Set eight frequencies of 5 Hz-1000 Hz respectively, and delay 120 seconds for each test round, then continue the test for 12 hours.

The nuclease-resistant experiment. Electrochemical measurements were performed in 8 mL 1×PBS buffer using a CHI1040C potentiostat and a standard three-electrode cell containing a platinum counter electrode and an Ag/AgCl (Soak in 3 M KCl when not in use) reference electrode at room temperature. Set eight frequencies of 5 Hz-1000 Hz respectively, and delay 120 seconds for each test round. Add 8 μL 1000 units·mL^{-1} DNase I nuclease to the solution after 60 minutes of testing (about Twenty cycles), then continue the test.

The kinetics study. Electrochemical measurements were performed in 8 mL 1×PBS buffer using a CHI1040C potentiostat and a standard three-electrode cell containing a platinum counter electrode and an Ag/AgCl reference electrode at room

temperature. Set eight frequencies of 5 Hz-1000 Hz respectively, and delay 120 seconds for each test round. Add 6 μL 10 $\mu M \cdot L^{-1}$ Thrombin to the solution after 60 minutes of testing (about Twenty cycles), then continue the test.

The titration experiment. Electrochemical measurements were performed in 10 mL 1×PBS buffer using a CHI1040C potentiostat and a standard three-electrode cell containing a platinum counter electrode and an Ag/AgCl reference electrode at room temperature. Set eight frequencies of 5 Hz-1000 Hz respectively, and delay 3600 seconds for each test round. According to the calculation, the concentration and volume of thrombin protein in the **Table S2** are added after each round of scanning, continue the test for 12 rounds.

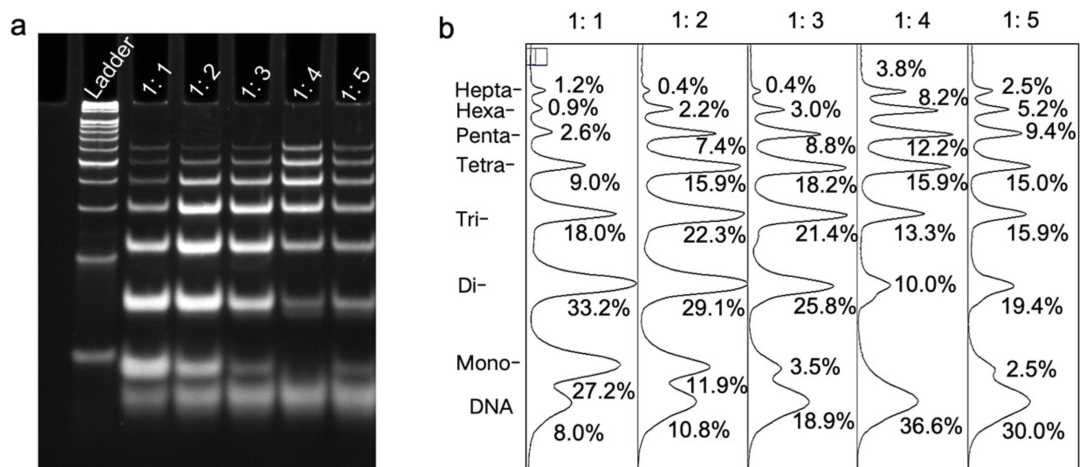


Fig. S1 The gel electropheris analysis of the reaction mixture using varied feed ratio of β-CD molecules and dibenzocyclooctyne (DBCO)-modified nucleic acids.

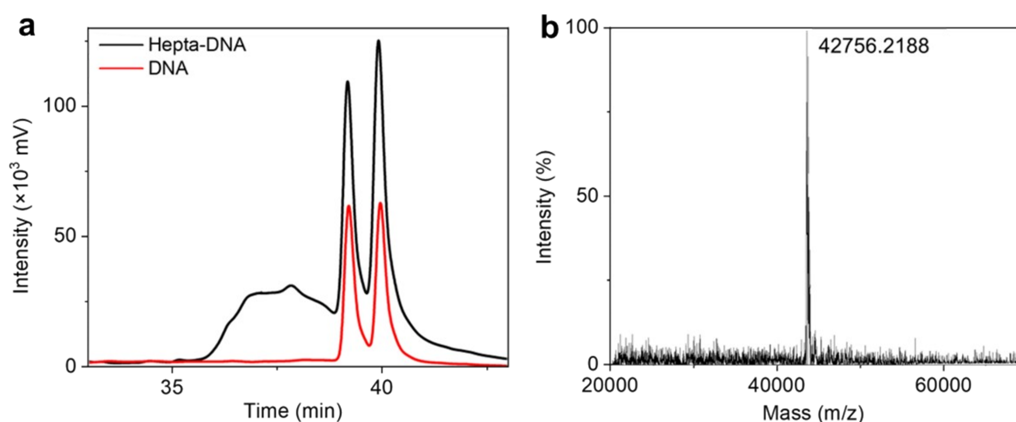
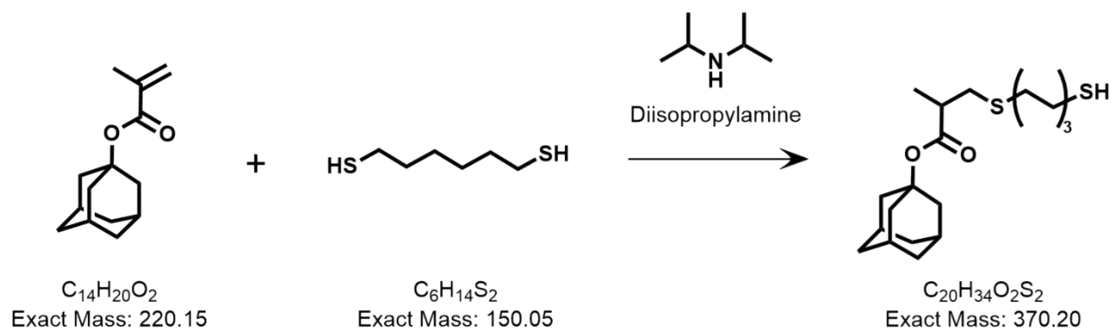


Fig. S2 (**a**) High performance liquid chromatography and (**b**) mass spectrum of Hepta-DNA.

It can be seen from the figure S2. (**a**) that free DNA may have two peaks due to the existence of secondary structure, and the liquid chromatography of the Hepta-DNA reaction mixture shows three peaks, two of which are consistent with the retention time and peak type of free DNA, indicating that these two peaks are redundant DNA raw materials in the Hepta-DNA mixture, and the other peak different from the raw material shows that the outflow time is earlier than that of the DNA raw material, indicating that its polarity is greater than that of free DNA, which is also consistent with β-cyclodextrin-based DNA molecular nanoparticles, we initially think it is our target β-cyclodextrin-based Chromatographic peak of cyclodextrin-based DNA molecular nanoparticles.

Dilute the purified Hepta-DNA product to less than 1 ppm for mass spectrometry sample detection. The test condition is to apply an electron. The test results are shown in figure S2. (**b**). It can be seen from the figure that the sample has a high density peak group, with the highest area peak at m/z=42756.2188, which is basically consistent with the theoretical calculation value of the seven-substituted product 42758.4, and it can be determined that the product synthesis is successful



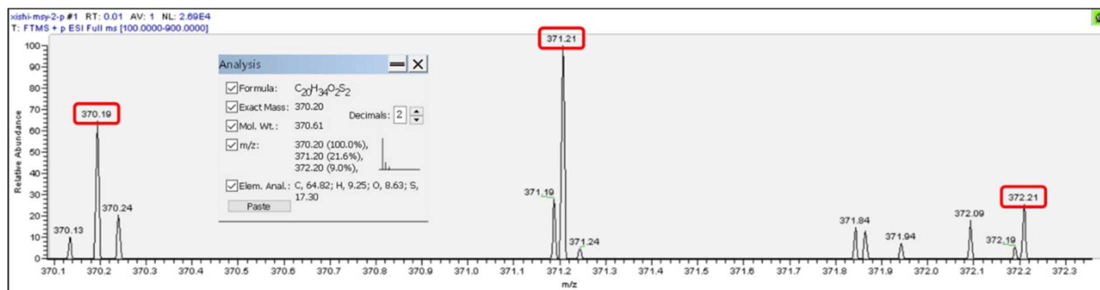


Fig. S3 Synthesis and mass spectrometry characterization of Ada-SH. The solvent is acetonitrile, dilute the sample concentration to less than 1 mg·L^{-1} before testing.

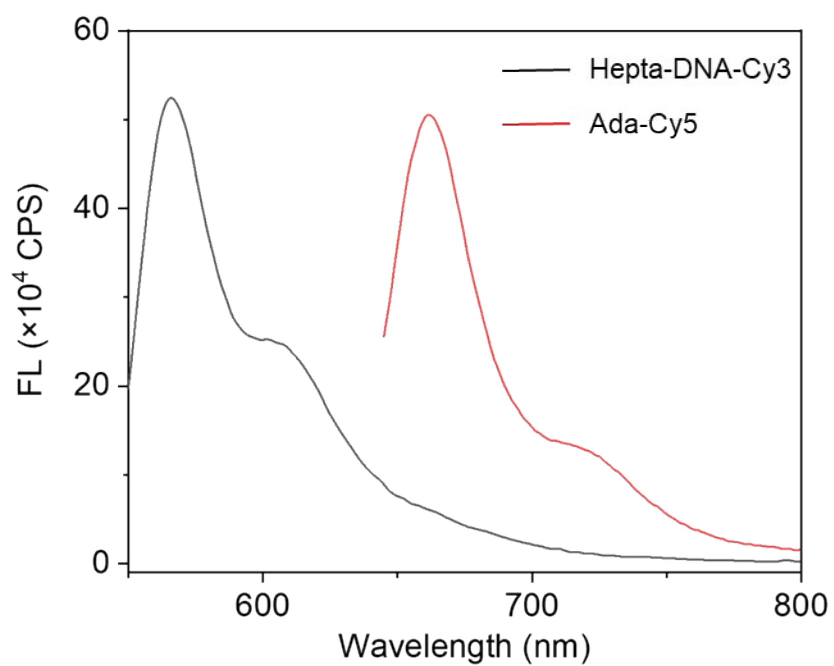


Fig. S4 Emission spectrum of Hepta-DNA-Cy3 and absorption spectrum of Ada-Cy5. The emission peak of Hepta-DNA-Cy3 and the absorption peak of Ada-Cy5 overlap, which can lead to fluorescence resonance energy transfer between them.

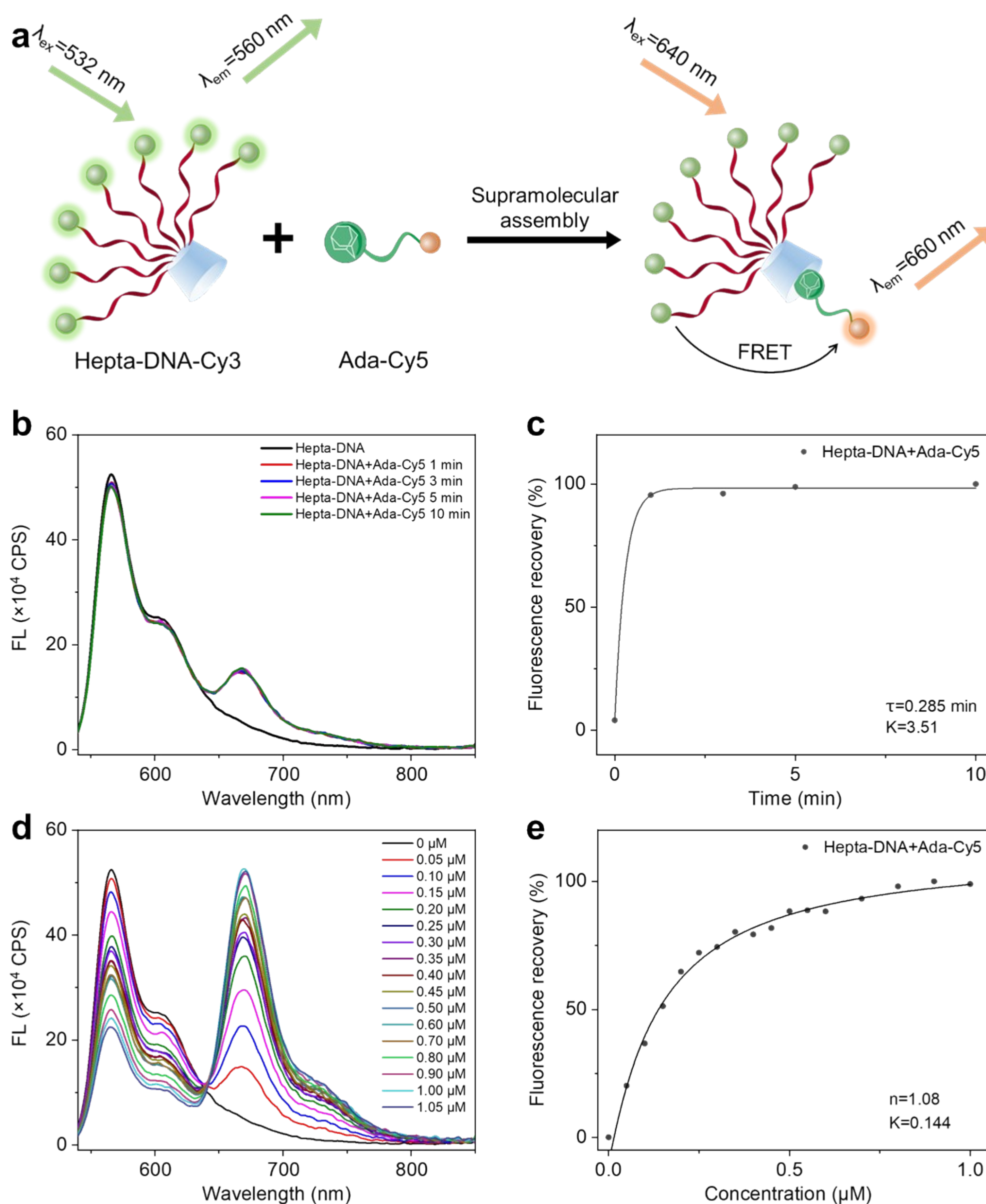


Fig. S5 (a) We employed a FRET mechanism to study the supramolecular self-assembly of Hepta-DNA-Cy3 and Ada-Cy5. (b) and (c) The assembly process was fast and reached its equalibrium within less than 2 mins. (d) and (e) Fluorescence titration spectrums of Hepta-DNA-Cy3 (1 μM) with different equivalent ratio of Ada-Cy5 from 0 μM to 1.05 μM.

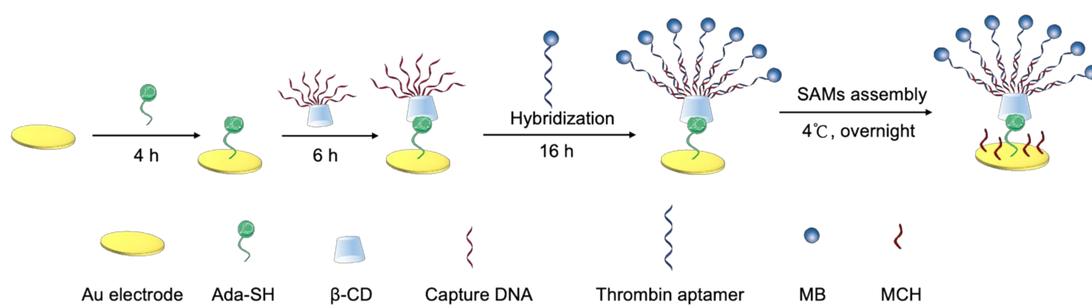


Fig. S6 We also tried a step-by-step assembly protocol of Supra-EAB sensors, which does not exhibit good electrochemical signals (data not shown).

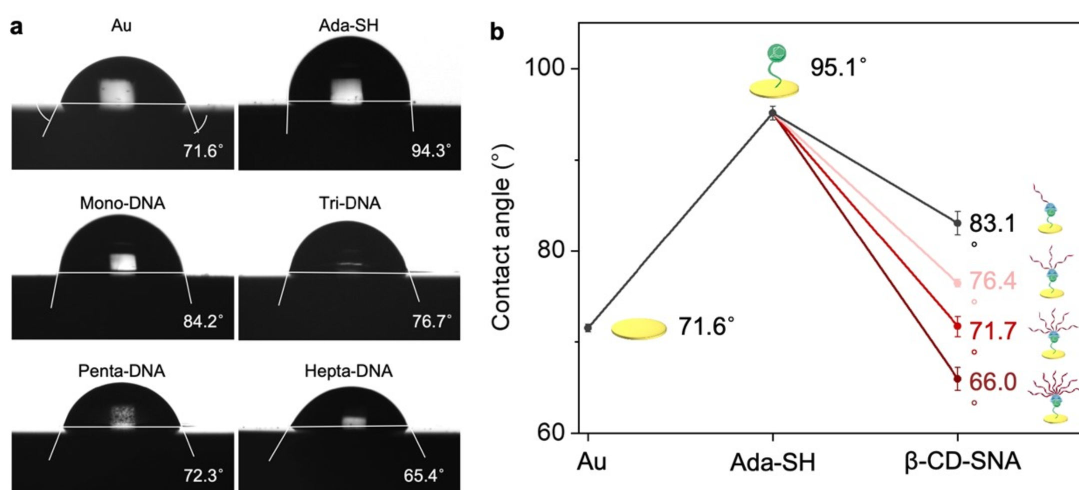


Fig. S7 We studied the wettability of these Supra-EAB sensors using contact angle measurements. (a) The contact angle images of Au electrodes, Ada-modified surfaces and Supra-EAB sensors are illustrated. (b) The statistic values of contact angles of these samples are summarized. The surface of Supra-EAB sensors are all hydrophilic.

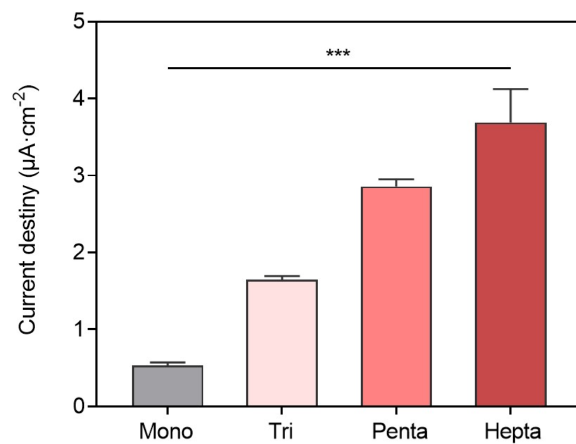


Fig. S8 The packing density of Supra-EAB sensors are illustrated (*p* values: $**p < 0.01$, $***p < 0.001$. Data are means ± SD, n = 3).

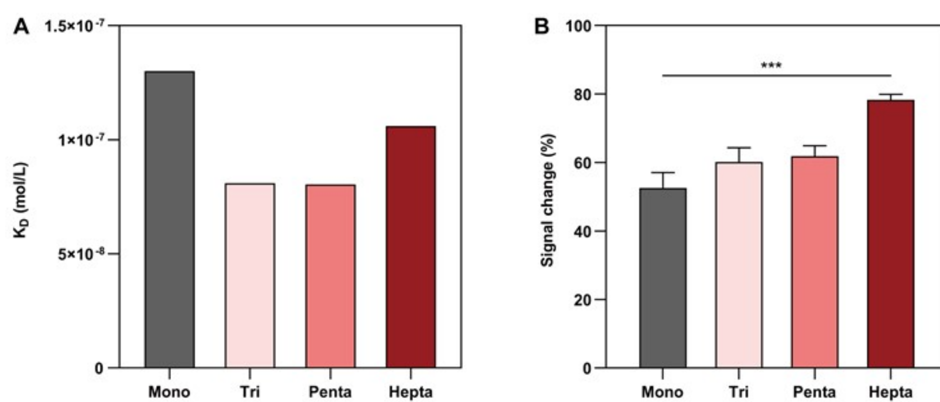


Fig. S9 Here we summarize the K_d values and the signaling response with the number of branches on the beta-CD for Supra-EAB sensors (p values: $**p < 0.01$, $***p < 0.001$. Data are means ± SD, n = 3).