Supplementary Information

A chimeric hairpin DNA aptamer-based biosensor for monitoring of the therapeutic drug bevacizumab

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1. Experimental

1.1. Instrumentation

A Microscale Thermophoresis (MST) Instrument Monolith NT.115 (NanoTemper Technologies, Munich, Germany) was recruited to investigate the dissociation constants (K_d) between DNA molecules and bevacizumab. A Monolith NT.115 capillary (NanoTemper Technologies) was used for MST measurements. Allelectrochemical experiments were performed using a CHI 660E electrochemical workstation (Shanghai, China) with a three-electrode system. A C220AT screen printed gold electrode (SPGE) purchased from Metrohm AG (Herisau, Switzerland) was utilized as a substitute for traditional disk electrode and independent three-electrode system. It consists of a gold working electrode (WE, 4 mm in diameter), a gold counter electrode (CE) and silver reference electrode (RE). A 5 mM $[Fe(CN)_6]^{4-/3-}$ (1:1) solution containing 0.5 M KCl was employed for CV and EIS analysis.

1.2. Temperature and time optimization for the binding between the DNA aptamer or hairpin DNAs and bevacizumab

The 6-FAM modified DNA (Table 1) powder was centrifuged at 4000 rpm for 30-60 s before opening to prevent spattering. Afterwards, 880 μL of PBS were added to a DNA solution (Optical Density, OD: 2) to prepare a 10 μM storage solution. Finally, serial dilutions of the DNA solution were prepared to test their fluorescence value with Monolith NT.115. According to the instructions of the instrument, the fluorescence value of the DNA solution should be between 200 and 1500 counts (the detection limits of the instrument). Before measuring the binding affinity, binding check experiments were carried out to determine whether there was an affinity between the receptor and the ligands. The effect of temperature on the binding was then investigated in the range between 22 °C and 35 °C. and finally the effect of incubation time (ranging from 15 to 60 min) on the binding was studied by MST. The steps for the binding affinity experiments were as follows: firstly, 16 microtubes (200 μL) numbered 1 to 16 were prepared, and then 20 μL DNA solution (100 nM) were pipetted in each microtube. Afterwards, 20 μL bevacizumab solution were added to these 16 microtubes, with

concentrations ranging from 0.256 nM to 8.4 μM. Subsequently, these 16 microtubes were incubated at room temperature for 30 min. Finally, 16 samples were withdrawn with 16 Monolith NT.115 capillaries for binding affinity testing by MST. The buffer solution used in the MST experiments was 10 mM PBS containing 0.05% tween-20 in all cases.

The K_d was estimated by fitting the following equation:

$$f(c) = Unbound + (Bound - Unbound) \times \frac{c + c_{target} + K_d - \sqrt{(c + c}}{2c_{ta}$$

where f(c) is the fraction bound at a given ligand concentration c; Unbound is the F_{norm} signal of the target alone; Bound is the F_{norm} signal of the complex; K_d is the dissociation constant of the complex; and C_{target} is the final concentration of the target in the assay.

1.3. Calculation of the density of the hairpin DNA on the gold electrode

The density (number of total molecules on the electrode surface) of the hairpin DNA on the gold electrode was determined according to the previously established relationship with ACV peak current described in the following equation: [1, 2]

$$I_{avg}(E_0) = 2nfFN_{tot}\frac{sinh(nFE_{ac}/RT)}{\cosh\left(nFE_{ac}/RT\right) + 1}$$

where $I_{avg}(E_0)$ is the average alternating current (AC) peak current in a voltammogram, n is the number of electrons transferred (eT) per redox event (MB label, n=2), F is the Faraday current, R is the universal gas constant, N_{tot} is the number of moles per unit area (cm^2) on the electrode, T is the temperature, E_{ac} and f are the amplitude and frequency of the applied AC voltage perturbation. A perfect transfer efficiency was assumed (i.e., that all MB molecules participate in electron transfer). Experimentally, four different frequencies were used (5, 10, 50, and 100 Hz), and the average current peak was calculated to derive the value of N_{tot}. To calculate the mean hairpin DNA density from N_{tot}, the apparent surface area (2 mm in diameter) was

employed.

1.4. Optimization of square wave voltammetry parameters

The response of the Ach probe labeled with the electrochemical active molecule methylene blue on the electrode surface is very sensitive, and the setting of electrochemical parameters has a very significant impact on the experimental results. Therefore, in these experiments, the optimization of electrochemical parameters is very necessary. Considering the fast response and high sensitivity of square-wave voltammetry, this technique was used in these experiments. The frequency, amplitude, and step potential of SWV were optimized. The frequency was first tested, and the other electrochemical parameters were set as follows: the initial potential was -0.1V, the termination potential was 0.5V, the step potential was at its default value of 0.004V, the amplitude (pulse height) was at its default value of 0.01V. Different frequencies ranging from 15 to 70 Hz, increased by 5 Hz increments, were tested, and the corresponding SWV signals were recorded. The frequency was then fixed at its optimized value and the step potential was set at the instrument default value of 0.004V. Then amplitudes ranging from 0.005 to 0.045 V, increased by 0.005 V increments, were tested and the SWV signals were recorded. Afterwards, the frequency and amplitude were fixed at their optimized values, and step potentials from 0.001 to 0.008 V, increased by 0.001 V increments, were tested and the SWV signals were recorded.

Table S1. Sequences of aptamer and hairpin DNAs.

Name	Sequence (5' to 3')
Aptamer	GCG GTT GGT GGT AGT TAC GTT CGC-FAM
H5	AAGCGGTTGGTGGTAGTTACGTTCGCTT-FAM
H6	AAAGCGGTTGGTGGTAGTTACGTTCGCTTT-FAM
H7	AAAAGCGGTTGGTGGTAGTTACGTTCGCTTTT-FAM
H8	AAAAAGCGGTTGGTGGTAGTTACGTTCGCTTTTT-FAM
H9	AAAAAAGCGGTTGGTGGTAGTTACGTTCGCTTTTTT-FAM
H10	AAAAAAAGCGGTTGGTGGTAGTTACGTTCGCTTTTTTT-FAM

Blue letters represent the aptamer sequence of bevacizumab; Underlined regions (in red) are stems of hairpin DNAs. The 6-FAM fluorophore is used for signal output in MST experiments.

2. Results and Discussion

2.1. Determination and optimization of the initial concentration of the DNA aptamer

Prior to conducting the hairpin DNA screening experiments, the concentration of DNA was first optimized so that the fluorescence intensity was between 200 and 1500 counts to meet the detection limits of the instrument (Figure S1). The fluorescence signal of 500 nM DNA at 2% excitation light intensity was first tested but it was found to lead to evident saturation. The DNA aptamer concentration was then diluted to 150 nM, the instrument automatically turned on 20% excitation light intensity but signal saturation still occurred. Finally, the DNA aptamer concentration was diluted to 100 nM, the excitation light intensity of the instrument was fixed at 20%, and the fluorescence intensity was found to be about 800 counts, which was within the detection limits of the instrument. Because the instrument produced low noise at low excitation light intensity, this value was not further increased, and a concentration of 100 nM was selected for the fluorophore labeled DNA probes customized by Shengong Company, which provided an important reference for the subsequent experiments.

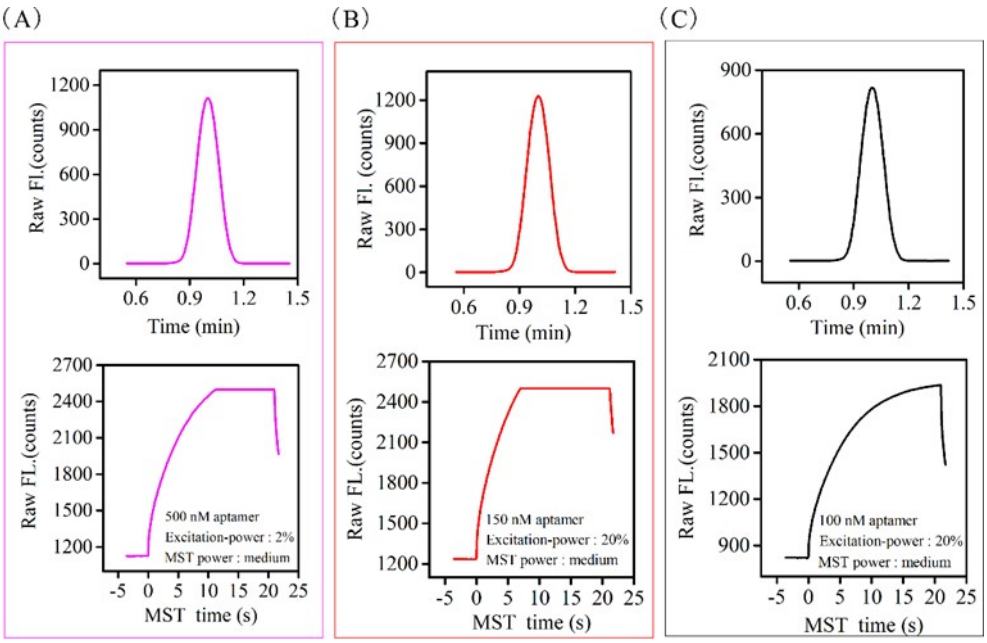

Figure S1. Initial concentration optimization of the DNA aptamer for the dissociation

constant measurements. The concentrations in A, B, and C were 500 nM, 150nM and 100nM, respectively. According to instrument requirements, the fluorescence values need to be between 200 and 1500 counts, and the MST traces cannot saturate.

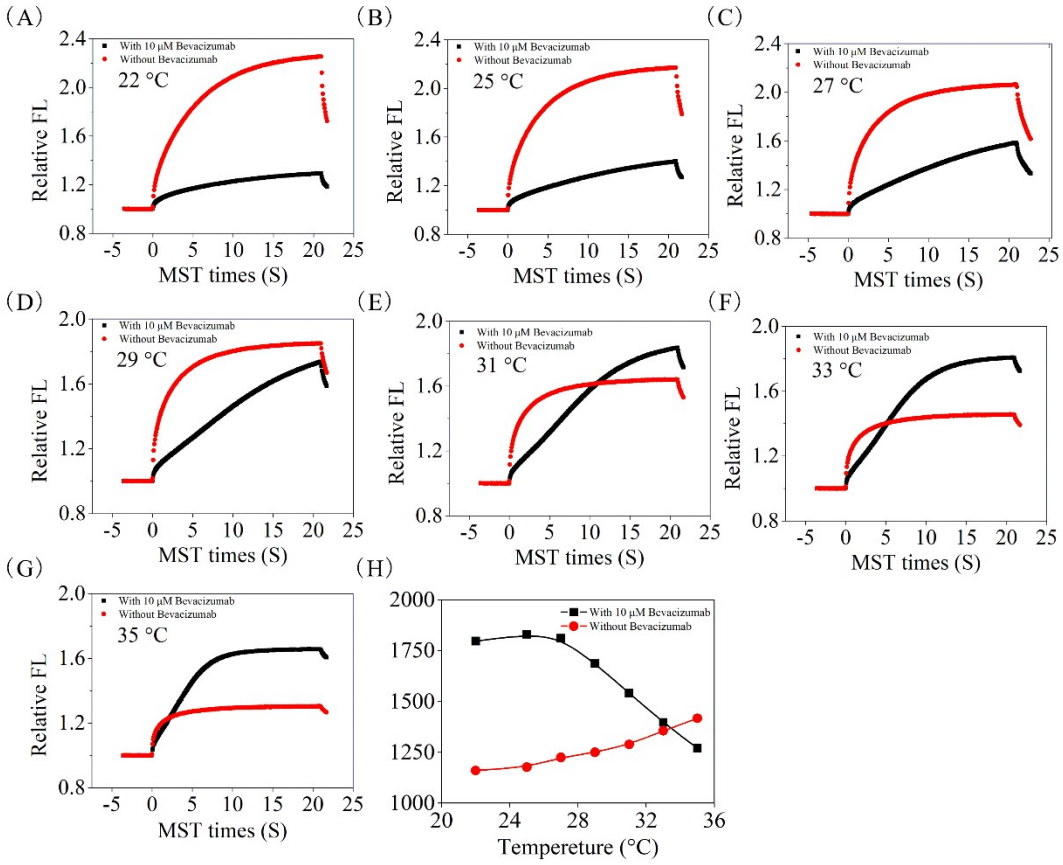

Figure S2. (A-G) Optimization of temperature for the binding between the DNA aptamer and bevacizumab using MST; (H) Comparison of the normalized fluorescence (Fnorm) at different temperatures ranging from 22 °C to 35 °C.

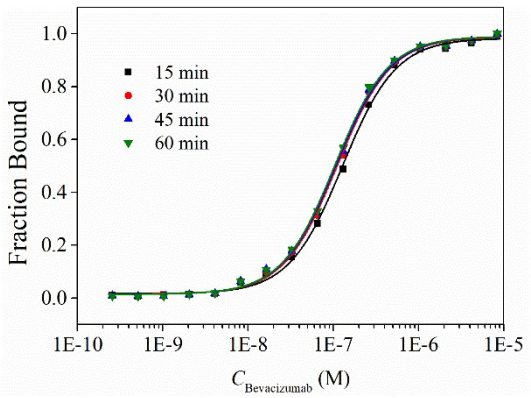

Figure S3. Optimization of the incubation time (ranging from 15 to 60 min) using the K_d fitting curves for the binding between the DNA aptamer and bevacizumab, the concentrations of bevacizumab were ranging from 0.256 nM to 8400 nM and the concentration of the DNA aptamer was fixed at 50 nM.

2.2. Optimization of the electrochemical parameters for SWV

Square wave voltammetry (SWV) is a differential technique of large amplitude and has wide applicability. It was originally proposed by Ramaley and Krause[3, 4] and further developed by Osteryoungs et al.[5] Square wave voltammetry combines the advantages of various pulse techniques and voltammetry, including the sensitivity and background suppression of differential pulse voltammetry, the qualitative judgment of conventional pulse voltammetry and the direct analysis of products by reverse pulse voltammetry, as well as the use of a wider time range. Figure S4 defines some key parameters of square wave voltammetry. These characteristic parameters are the pulse height ΔE_P relative to the step potential (corresponding to Amplitude in the instrument settings) and pulse width t_p, which can also be expressed as the frequency of square wave f=1/2 t_p. The step potential of each cycle is ΔE_S (Increment E), and the scanning speed of potential can be expressed as:

$$v = \frac{\Delta E_s}{2t_p} = f\Delta E_s$$

The current was sampled before the end of each pulse, with two pulses per cycle, for a total of two samples. The forward current i_f is collected from the first pulse of each

cycle, and the reverse current i_r is collected from the second pulse, and the current difference $\Delta i = i_f - i_r$. The forward and reverse currents are stored separately for qualitative judgment. In this way, each SWV experiment result will generate three voltammetry curves, the curves of the forward current, reverse current and differential current against the step potential.

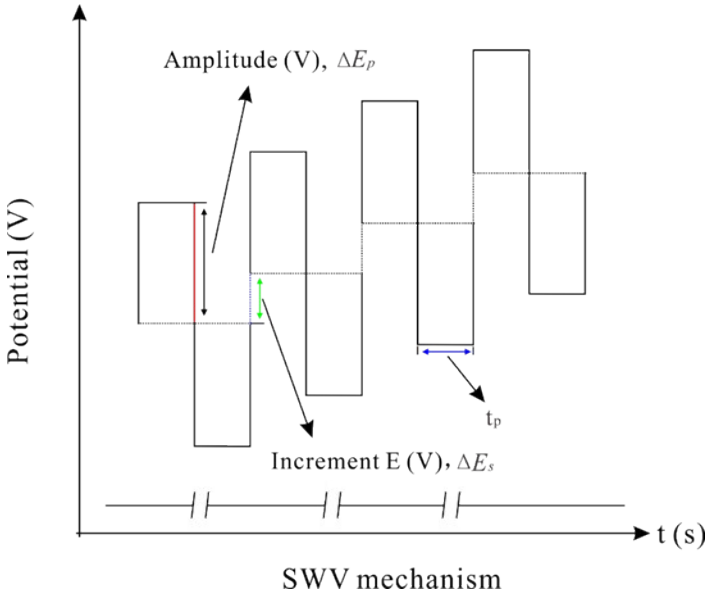

Figure S4. Schematic diagram of square wave voltammetry.

In general, t_p determines the time scale of the experiment, *Es* determines the interval between data points along the potential coordinates, and together they determine the time required for the overall potential scan (cf. Figure S4).E_P determines the potential range and potential resolution involved in each step of the cycle. In these experiments, several key parameters of square wave voltammetry were optimized. It mainly includes the three parameters of frequency, step potential (Incr E) and amplitude (pulse height, Amplitude). Firstly, the frequency of square wave voltammetry was optimized. Different frequencies from 15 to 70 Hz, increased by increments of 5 Hz, were tested. As shown in Figure S5, with the increase of frequency, the current signal is continuously enhanced, but the background noise of the current response is also increased. However, the background noise is particularly low at frequencies of 25 and 50 Hz. Considering both factors of the current intensity and background noise, the frequency of 50 Hz was selected as the optimal value for the next experiments.

Next, the amplitude, also called pulse width (*Ep*), was optimized. On the basis of a frequency of 50 Hz and other default parameters of the instrument, amplitudes were changed from 0.005 to 0.045 V by an increment of 0.005 V. As shown in Figure S6 A, when the amplitude is between 0.005 and 0.025 V, the current intensity keeps increasing; when the amplitude is between 0.025 and 0.030 V, the current intensity suddenly decreases and then slightly rises again at higher amplitudes. In this experiment, the amplitude of 0.025V leading to the maximum current intensity was chosen. In addition to the frequency and amplitude, the step potential is also a very critical parameter in SWV, as it determines the potential of each step and plays a dominant role in the total detection time and sensitivity. Therefore, the step potential was changed from 0.001 to 0.008 V by an increment of 0.001 V (Figure. S6 B). It was found that the current intensity decreases continuously with increasing the step potential, but at a step potential of 0.001 V, a distinct noise peak can be seen in the background signal. Therefore, a step potential of 0.002 V was selected as the optimal value.

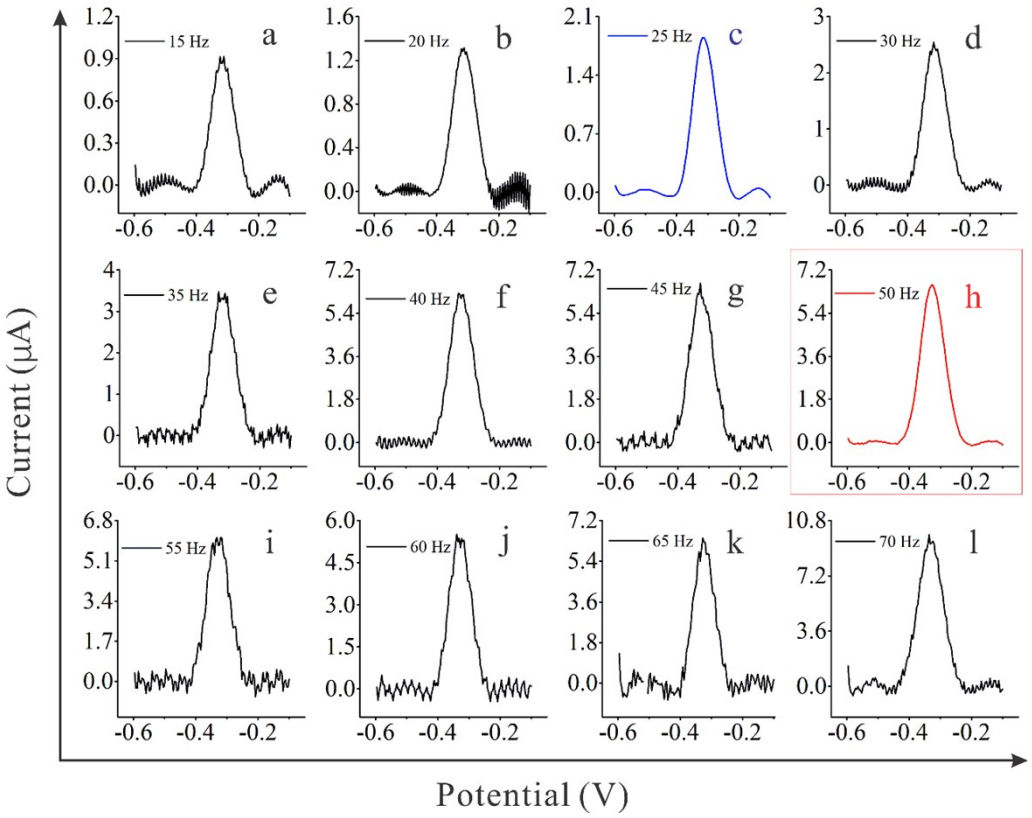

Figure S5. Frequency optimization of SWV for the biosensor (SPGE/Ach/MCH). Both the Incr E (0.004 V) and amplitude (0.025 V) are the default values of the instrument.

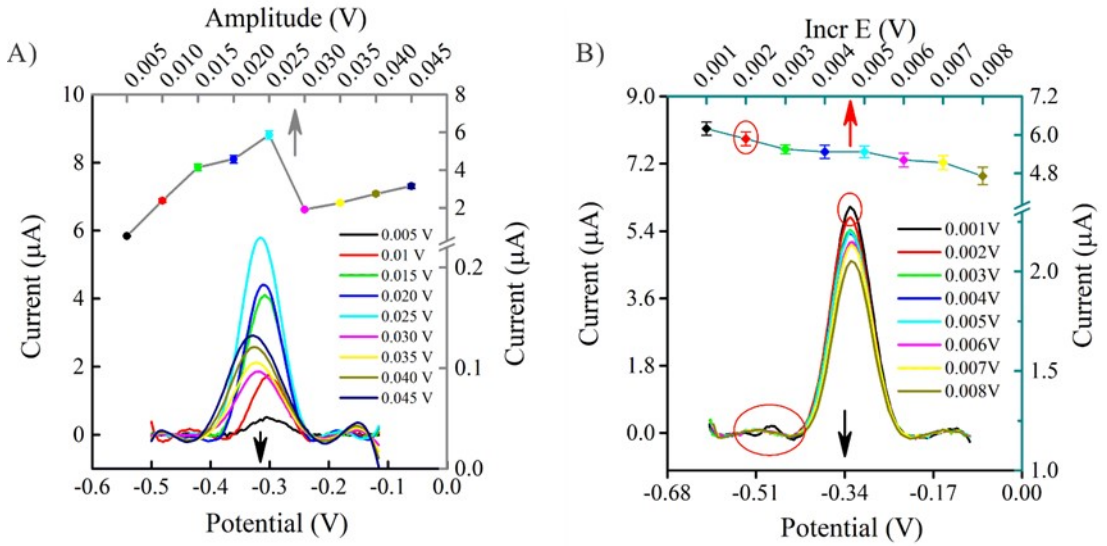

Figure S6. (A) Amplitude optimization of SWV for the biosensor (SPGE/Ach/MCH), the frequency (50 Hz) is the optimized value, and the Incr E is the default value (0.004 V); (B) Incr E optimization of SWV for the biosensor (SPGE/Ach/MCH). Both frequency (50 Hz) and amplitude (0.025 V) are pre-optimized values.

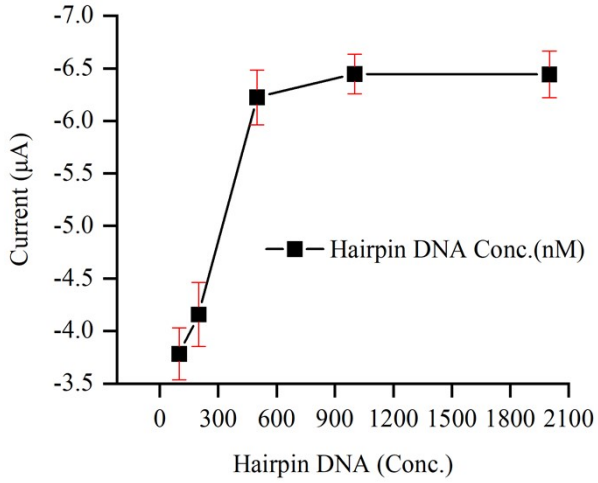

Figure S7. Optimization of hairpin DNA probe concentration in the range from 100 nM to 2 μM using SWV

References

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