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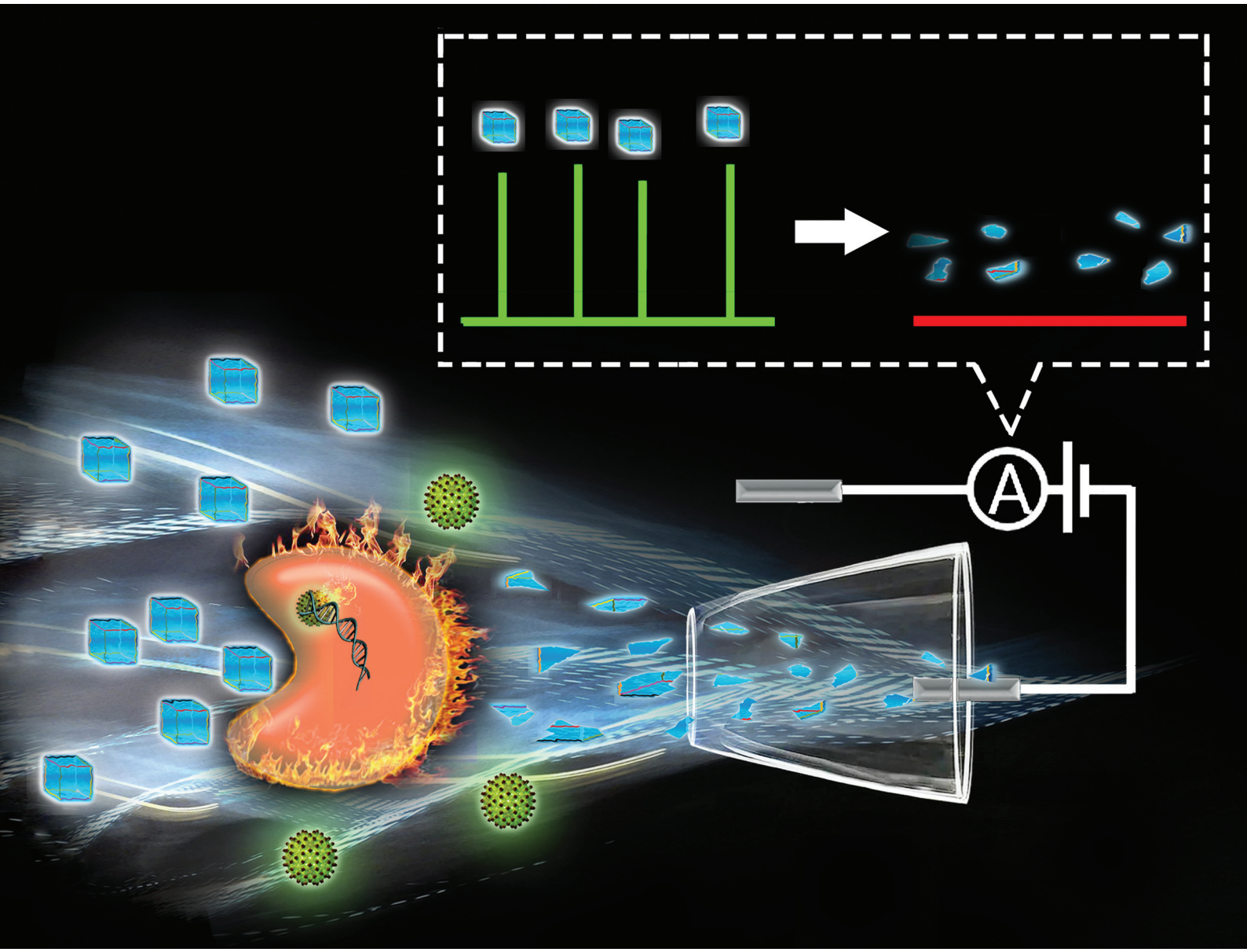

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A signal on–off strategy based on the digestion of DNA cubes assisted by the CRISPR–Cas12a system for ultrasensitive HBV detection in solid-state nanopores†

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Solid-state nanopores have been proven as a powerful platform for label-free single-molecule analysis. However, due to its relatively low resolution and selectivity, developing biosensors with good translocation signals faces two significant challenges: (1) small-sized chemical or biological targets show difficulty in producing recognizable translocation signals because of their weak interaction with the nanopore and (2) protein interferents that widely exist in biological samples or buffers would considerably deteriorate the noise level of the nanopore, submerging the translocation signal. Herein, we demonstrate an effective way to overcome both the challenges. DNA cubes were used as signal transducers that can achieve an ultra-high (>50 : 1) signal-to-noise ratio (SNR) translocation signal, which is maintained even in protein interferent-rich buffers. A sensing strategy was constructed *via* hepatitis B virus (HBV) target-triggered cleavage of the component elements of the DNA cube with the assistance of the CRISPR–Cas12a technology, which caused a great drop in the translocation rate. The elements to cleave were optimized, and the sensor performance was tested in different protein stabilizer-rich buffers and human serum. Coupling with the polymerase chain reaction (PCR) pre-amplification technology, HBV-positive or -negative classification was achieved with the detection limit reaching 5 aM. It is worth noting that in our method, all reaction buffers were directly used without further optimization, which is of great help for the practical application of solid-state nanopores.

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Introduction

Single-molecule analysis, which only investigates one individual molecule per time, has drawn much research attention due to its extensive application prospects in biosensing and biomedicine.[1,2] As one of the most promising single-molecule sensing technologies, nanopores hold unique advantages of ultrasensitivity, easy operation, and label-free analysis, which have been widely applied in DNA sequencing, biomolecular interaction exploration, and biomolecular detection in recent years.[3–10] Among the family of nanopores, glass nanopores have attracted increasing research interest for their ultra-low fabrication cost, mechanical stability, and adjustable pore size, which make them ideal candidates for clinical and commercial applications.[11–13] The working principle of glass nanopores is through continuously monitoring the ionic current of the nanopore to measure and record the temporal blockage current associated with the translocation of a target molecule, so as to obtain the concentration of the target molecule.[14] However, due to the relatively low resolution and selectivity of glass nanopores, to develop a sensor with good translocation signals, two important challenges need to be faced: (1) most biochemical analytes are not standard enough to produce good enough translocation signals. In particular, for analytes that are of small dimension such as oligonucleotide fragments, peptides, and other small-scale molecules, due to their scale mismatch and weak interaction with the glass tube, it is still hard or impossible to directly measure them using glass nanopores.[15] (2) The widespread presence of protein interferents such as bovine serum albumin (BSA) and recombinant albumin in the commercial reaction buffer of enzyme-involved biochemical reactions that are often used to establish sensing or signal amplification strategies will greatly deteriorate the noise level of glass nanopores, drowning the translocation signals that should be generated by the analyte, leading to an experiment failure.[16,17]

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Valuable works have been done to overcome these two challenges. For the first challenge, researchers have tried to use signal transducers or amplification methods to increase the size of the analyte, enhancing the interaction between target molecules and the glass nanopore to produce better translocation signals.[11,18] These methods are known as indirect detection methods, which achieved to solve the first challenge to some extent. However, the signal transducer or amplification method they selected was still not good enough owing to the unevenness in size and the relatively low SNR translocation signal, which increased the difficulty of subsequent analyses. For the second challenge, although several attempts have been tried, including optimizing the buffer component to replace BSA,[16,17,19,20] introducing purification steps to remove BSA,[21] or designing sensing strategies with BSA-free methods, such as hybridization chain reaction (HCR)[22,23] or loop-mediated isothermal amplification (LAMP),[24,25] they are still not good enough to overcome the second challenge because these methods consume additional time to make changes to commercial reaction buffers only especially for nanopore applications, require additional experimental steps and cost, and can only be compatible with limited biochemical reactions. These defects hamper the practical use of glass nanopores, which greatly limits their application. Therefore, it is of great significance to develop methods that not only can amplify the translocation signal of the target molecules, but can also generate stable signals in various buffers.

Through a large number of literature searches and experiments, we found that using a DNA cube[26] as a signal transducer and designing a target-triggered damage of its 3D structure as a sensing strategy, both the challenges can be ideally solved. The DNA cube is a kind of nucleic acid nanostructure with a fine 3D spatial morphology, which has strong interaction with glass nanopores and can generate ultra-high (>50 : 1) signal-to-noise ratio (SNR) translocation signals. Even in the buffer containing a high concentration of BSA, the translocation signal is maintained owing to its powerful anti-interference ability. Moreover, due to the strictly folded spatial structure of the DNA cube, when damaging the elements in the synthesis process of the DNA cube, the translocation signal of the DNA cube will be significantly affected due to its incorrect spatial folding, which weakens their interaction with the nanopore. Owing to this good property, sensing strategies can be designed *via* associating target molecules to element breakdown of DNA cubes.

How to achieve a target-triggered breakdown of elements in the synthesis of DNA cubes is the key issue to realize the sensing strategy. Recently, the development of the CRISPR–Cas technology has provided a new way of sequence-specific signal translation.[27,28] Particularly, with the unique trans-cleavage property of Cas12a, single-stranded DNA (ssDNA) can be indiscriminately cleaved when target DNA is present in the solution.[29,30] Extensive novel sensing mechanisms of fluorescence, bioluminescence, colorimetric signals, or current blockage in nanopore sensing have been designed based on this property.[31–35] Since the constituent elements of the DNA cube are all ssDNA, they can be easily cleaved by target-induced Cas12a activation.

In this work, we developed an ultrasensitive hepatitis B virus (HBV) detection method that can generate large translocation signals and work in protein interferent rich buffers based on the digestion of component elements of DNA cubes assisted by CRISPR–Cas12a in glass nanopores. HBV is a virus that causes hepatitis B infection, a global public health problem.[36] In this strategy, the DNA cube was applied as the signal transducer to amplify the translocation signal of target HBV fragments. The CRISPR–Cas12a system was used in designing the HBV-triggered sensing strategy. When target HBV is present in the analyte, the trans-cleavage property of Cas12a will be activated to cleave the elements of the DNA cube, causing the synthesis failure of the DNA cube and thus decreasing the event rate. Polymerase chain reaction (PCR) was applied to improve the overall sensitivity. The results indicated high selectivity and sensitivity of the sensor, with the detection limit reaching 5 aM. Moreover, good compatibility of the sensing strategy was achieved because commercial buffer without further optimization can be directly used during all steps of the experiment, which will be of great advantage for the clinical application of the glass nanopore due to the great improvement of compatibility and anti-interference ability of our method.

Results and discussion

Working principle

As shown in Fig. 1a and (ESI Fig. S1†), the DNA cube was used as a signal transducer in this work, which consisted of six elements, namely, A, B, C, D, E, and F. The working principle of our HBV sensing strategy based on the digestion of DNA cubes assisted by CRISPR–Cas12a is illustrated in Fig. 1b. First, Cas12a and its sequence-specific crRNA were mixed together with two elements of the DNA cube (E and F) to form a reaction buffer. Then, the analyte solution was added into the reaction buffer, mixed, and incubated. There are two situations here: (1) if target HBV was present in the analyte solution, it would be amplified by PCR and specifically bound to the crRNA. Therefore, the trans-cleavage activity of Cas12a would be activated to cleave elements E and F in the solution and (2) if no target HBV existed, the Cas12a remained inactive, thus no degradation of element E and F would happen. Afterward, the other four elements of the DNA cube (A, B, C, and D) were added, and the solution went through an annealing process before it was finally analyzed using a glass nanopore sensor. Since DNA cubes could only be correctly synthesized and produce translocation when elements E and F were not cleaved, the event rate could be "turned off" with the existence of the target HBV in the analyte. Thus, we have achieved a signal on–off strategy based on the element changes of the DNA cube assisted by CRISPR–Cas12a with a glass nanopore sensor, which can be applied in HBV detection.

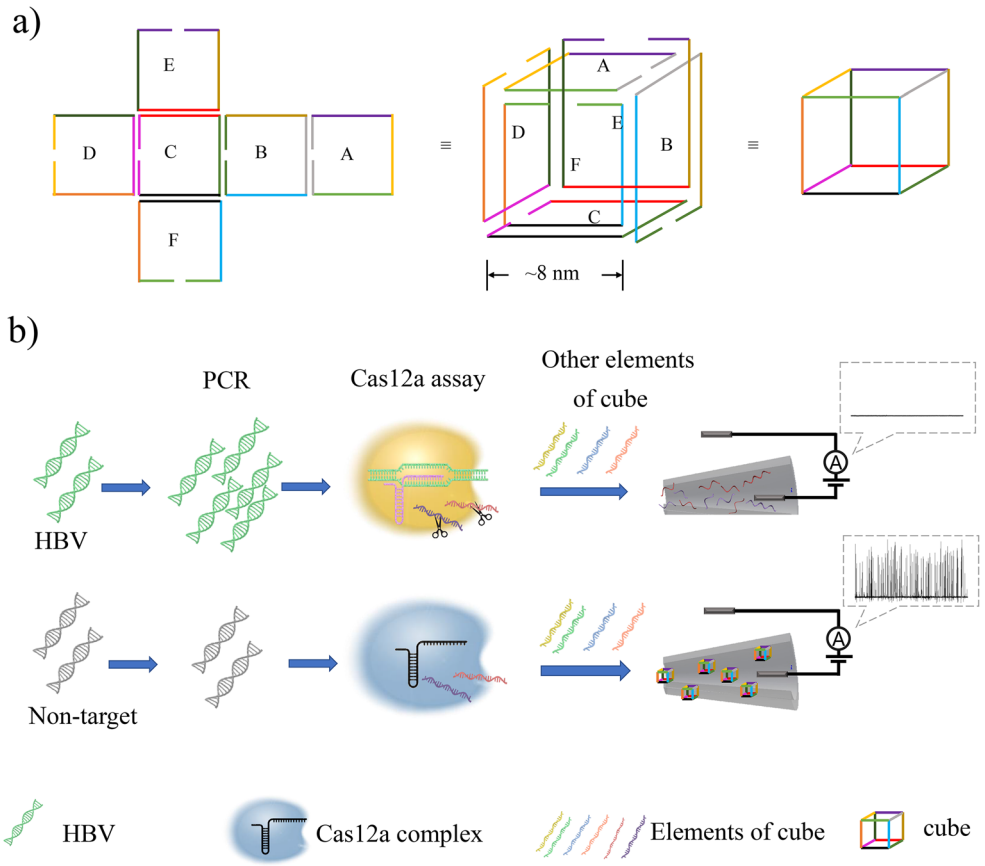

Fig. 1 (a) Structure of DNA cubes (each strand colored differently). (b) Schematic illustration of the signal on–off strategy based on element changes of the DNA cube assisted by CRISPR–Cas12a for HBV detection. With target HBV, the trans-cleavage activity of the Cas12a will be activated, which causes degradation of elements E and F, resulting in the digestion of the elements of the DNA cube, thus the signal is turned off. Without target HBV, Cas12a will remain inactivated, thus elements E and F remain in the solution and the complete cube can be synthesized; therefore, the signal is turned on.

To confirm the translocation signal was indeed caused by the translocation of correctly synthesized complete DNA cube rather than other components or incomplete DNA cube in the reaction solution, we carried out several experiments. As shown in Fig. 2a & b, the diameter of the glass nanopores used in our experiment was typically about 12 nm, and the current was between −13 and 14 nA when −1 to 1 V voltage was applied. In fact, nanopores with a pore size varying from 10 to 20 nm could be applied in this work. According to Fig. 2c, there was no translocation signal when samples contained only HBV target or Cas12a complex, indicating that the nanopore used in our experiment was inert to these small-sized biomolecules. Besides, the incomplete DNA cube (missing one or more elements) was also unable to cause any translocation signals. This is mainly because the spatial structure of the DNA cube cannot correctly form with one or more elements missing, causing a great reduction of the interaction with the nanopore. When complete DNA cubes existed in the solution, distinct translocation events can be observed, suggesting that the signal can only be caused by the translocation of complete DNA cubes. Moreover, the translocation signal of the DNA cube was relatively large, and the amplitude was mainly distributed between 1.5 and 2 nA.

Optimization of the signal transducer

Previous studies have shown that if BSA was present in the reaction buffer, the noise level of a glass nanopore would severely deteriorate due to the strong adsorption of BSA on the surface of the glass nanopore.[16,17] Unfortunately, for many kinds of enzyme-involved commercial reaction buffers, such as the commercial Cas12a reaction buffer and PCR reaction buffer applied in this experiment, BSA is an essential component that can help to improve the stability of the enzyme during the reaction. Previous works chose to seek an alternative reaction buffer, carry out extra purification steps or apply BSA-free reactions to design sensing strategies. However, these methods need delicate experiment design and have strong uncertainty. It is a better choice to develop a sensing strategy that can work in various buffer situations, including buffers containing BSA.

To verify that performance of our proposed sensing strategy with the DNA cube better than others in protein interferent-

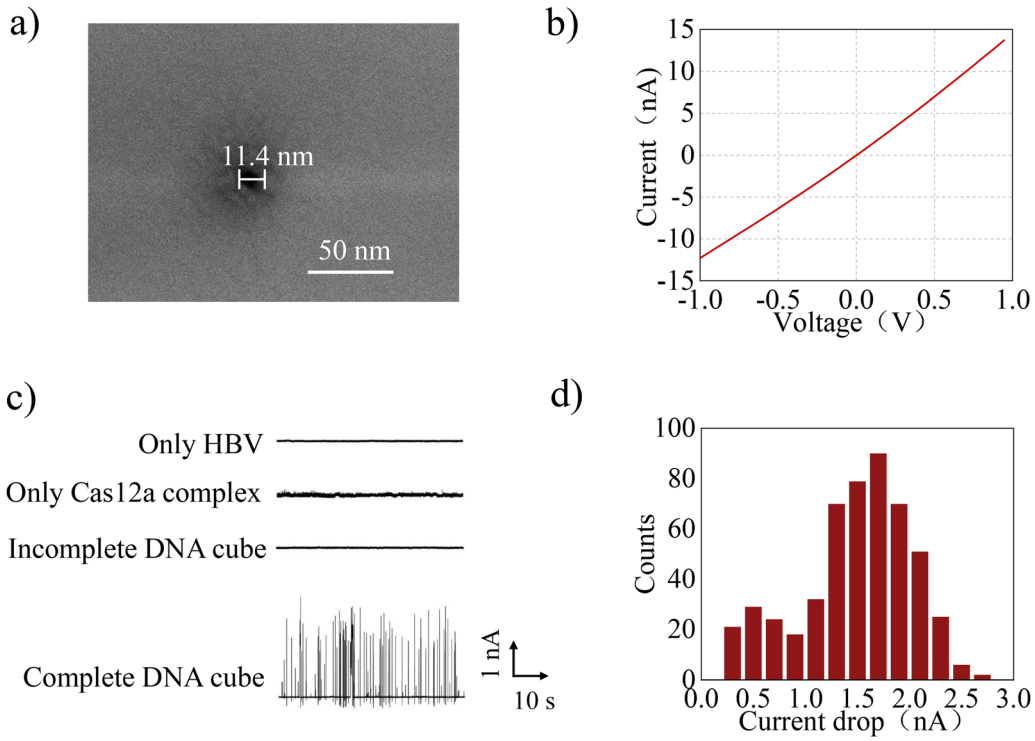

Fig. 2 Verification of the current pulse. (a) Top view of the SEM image of a typical glass nanopore. (b) *I*–*V* curve of a typical glass nanopore in the test buffer. (c) Current traces of the glass nanopore in the presence of different components with the bias of 1000 mV. (d) Histogram of signal amplitude distribution.

rich buffer situations, we compared the translocation signal of the DNA cube and three other signal transducers used in other works (circular M13mp18 ssDNA used by Guan, lambda DNA that simulated HCR product used by Li and DNA tetrahedron used by Jin and us before) in buffers with or without BSA. As shown in Fig. 3a, all nanostructures can produce visible translocation signals in the buffer without BSA. Among them, the SNR of the DNA cube was the highest. However, when BSA is contained in the buffer, only the translocation signal of the DNA cube is maintained, and that of the other three signal transducers was submerged to varying degrees in the enhanced noise of the glass nanopore sensor due to the strong adsorption of BSA. In detail, the signal of M13mp18 ssDNA and lambda DNA are completely submerged, and the signals of DNA tetrahedron were partly submerged, leaving only part of the large signals. Thus, the DNA cube was finally chosen to develop our sensing strategy for its extremely high SNR and strong anti-interference ability.

After proving that the translocation signal of DNA cube as a signal transducer is stable in buffer with or without BSA, we explored the effect of concentration of BSA on the event rate of the DNA cube. Since NEBuffer 2.1, which contains 100 μg mL^{-1} BSA, is commercially used as the reaction buffer for Cas12a, and with the progress of other reaction steps, BSA concentration will be diluted gradually. Therefore, we tested the event rate of DNA cube in buffers that contained a BSA concentration ranging from 0 to 100 μg mL^{-1}. As shown in Fig. 3b, when the solution did not contain BSA, the event rate was about 1 s^{-1}. The event rate increased with the increase in concentration of BSA in the solution and stabilized at about 1.8 s^{-1} after the concentration of BSA exceeded 20 μg mL^{-1}. This may be due to the existence of BSA that can help to stabilize the fine 3D nanostructure of the DNA cube.[37] Although the event rate of DNA cube varies with different concentrations of BSA, once the reaction system is determined, the concentration of BSA is constant, with the event rate of the same concentration of DNA cube fixed. Owing to the stable event rate of the DNA cube under a large concentration range of BSA, we can directly apply commercial NEBuffer 2.1 as the reaction buffer without further optimization in the experiment.

In addition, we also tested the translocation signal of the DNA cube in NEBuffer r2.1 that contained recombinant albumin. A similar result has been obtained, which is shown in Fig. S2.† Therefore, using the DNA cube as a signal transducer, the reaction buffer can be selected more freely, making the sensing strategy more compatible, which is of great significance in practical application.

Optimization of the cleavage elements of the DNA cube

As stated above, the HBV-triggered signal on–off strategy was achieved by cleaving part of the elements of DNA with the assistance of Cas12a, causing less complete DNA cubes to synthesize and thus decrease the event rate. Therefore, how to select the elements to be cleaved will have the main effect on

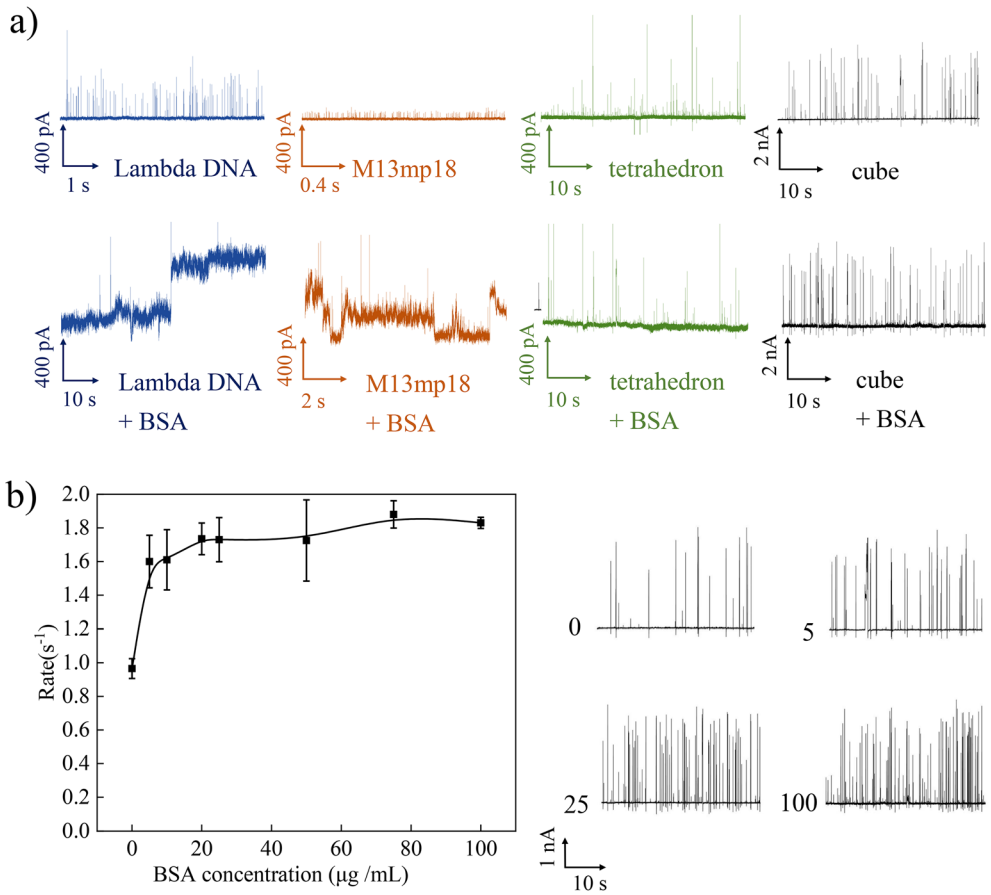

Fig. 3 Performance of the DNA cube as a signal transducer in different buffer situations. (a) Translocation signal of circular M13mp18 ssDNA, lambda DNA, DNA tetrahedron, and DNA cube in buffer with or without BSA. (b) Event rate of the DNA cube in a buffer containing different concentrations of BSA. The concentration of the DNA cube is all 30 nM.

the performance of this strategy. The event rate change of cleaving one element, two elements, and six elements of the DNA cube to different extents was tested. As shown in Fig. 4, the event rate decreased with a higher element cleavage percentage, whether cleaving one element, two elements, or six elements. However, when cleaving only one element, even if it was all cleaved, the event rate cannot fully reach 0 s^{-1}, resulting in an incomplete off state. While cleaving all six elements, the event rate can reach 0 s^{-1}, but the decrease was too slow, meaning that the amount of HBV target required will increase to cleave more elements of the DNA cube and thus reduce the sensitivity of the sensor. Since the event rate decreased the fastest to 0 s^{-1} when two elements were selected to be cleaved, two elements were selected to be cleaved in this work.

We also tested whether there were any differences in the event rate change in choosing to cleave elements A, B, C, D, E, or F, and the results of event rate change when cleaving elements A, B, C, D, E, or F are shown in Fig. S3.† We found that all elements were equivalent except element A. The event rate when cleaving element A decreased linearly but decreased exponentially when cleaving other elements. This may be due to the element A has the most starting and ending points (3) when complementary pairing with other elements than other elements (B:1, C:1, D:1, E:0, F:0), causing easier damage to the 3D structure of DNA cube even when cleaved in small amounts.

Target quantification based on nanopore event rate

To evaluate the performance of our strategy, the event rate of the glass nanopore with different concentrations of the HBV target (range from 0 nM to 1 nM) was tested three times per concentration. As illustrated in Fig. 5a, the event rate keeps stable at around 1.75 s^{-1} when the HBV concentration is below 0.3 nM, indicating that the target with a low concentration was not enough to change the "on" state of the signal switch. When the concentration of HBV exceeds 0.3 nM, the event rate decreases rapidly and reaches 0 s^{-1} after the concentration of HBV exceeds 0.5 nM, which means the signal switch turns to an "off" state. This "off" state maintains with the continuous increased concentration of HBV. It is clear to see that our strategy implements a signal switch that can identify the presence of HBV or not in the analyte, with a detection limit of 0.5 nM.

Then, PCR was applied to improve the overall sensitivity of the signal switch. In detail, before adding the analyte to the

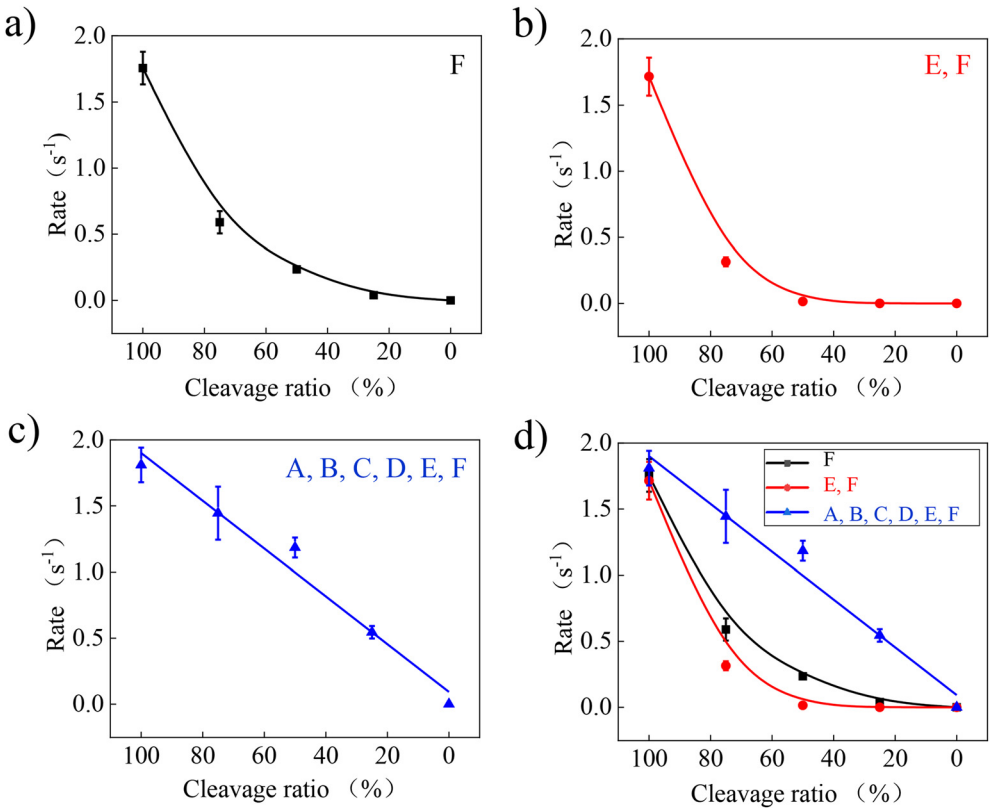

Fig. 4 Event rate change of cleaving different ratios of the DNA cube: (a) one element; (b) two elements; and (c) six elements. (d) Simultaneous display. When two elements were chosen to cleave, the event rate decreased the fastest to 0 s^{-1}.

reaction buffer, a PCR pre-amplification step of HBV was conducted to boost the signal. With this step, the concentration of target HBV in the analyte would increase significantly, while that of other interferences would remain the same. Moreover, in order to make the test results better understood and to eliminate the influence on the test results by the fluctuation of event rate caused by the different pore diameters from batch-to-batch of the glass nanopores, we used the cleavage ratio (CR) to illustrate the test results. CR $= \frac{r_0 - r_1}{r_0}$, where r_0 is the event rate of a certain concentration (30 nM in this experiment) of the original DNA cube as an internal reference for every single glass nanopore, and r_1 is the event rate of the test result. Therefore, CR is a decimal between 0 and 1. If the value of CR is 0, it means that the DNA cube was not cleaved at all, demonstrating that no target HBV was in the analyte, while the value of 1 indicates that the DNA cube was completely cleaved and the analyte was HBV positive. Since our strategy is a turn-off method, it may suffer from the background noise due to the fluctuation of the DNA cube signal rate when the analyte concentration was low. Therefore, in order to ensure high confidence levels, we consider HBV positive only when the CR value exceeded 0.9. As shown in Fig. 5b, after adding a pre-amplification step, the detection limit of the sensor was raised to about 5 aM.

In addition, a problem to be considered is that once the DNA cube was degraded with time, a false-positive result would have occurred. Therefore, we have studied the degradation of the DNA cube with time. As shown in Fig. S4,† the event rate of the DNA cube can maintain stable within 1–3 days. When the DNA cube was synthesized for over a week, the event rate would drop to about 65%. For such samples, we can reanneal them, and the event rate can be almost recovered.

Sequence-specific test of the strategy

To further determine the selectivity of this strategy, other two targets, HPV and HIV, were applied as interferences. First, 10 aM HBV, 1 fM HPV18, and HIV were respectively added to the analyte and then analyzed with the glass nanopore. It is clear in Fig. 6 that the CR only increases when the HBV target was present, and no change in the CR was observed with other nonspecific targets even if their concentration was 10 000 times higher than that of HBV. This result indicated excellent specificity of our sensor with no cross-reactivity to other human dsDNA viruses, which is high enough for clinic HBV-positive or -negative classification.

Analysis of HBV in human serum

The commercial human serum was diluted 10 times in enzyme-free water prior to use. To simulate the real physiological conditions, different concentrations of HBV target (0, 1, 3, 5, 7, 10, 20, 50 aM and 1 fM), 1 fM HPV target, and 1 fM HIV target were added to the diluted serum respectively, and tested

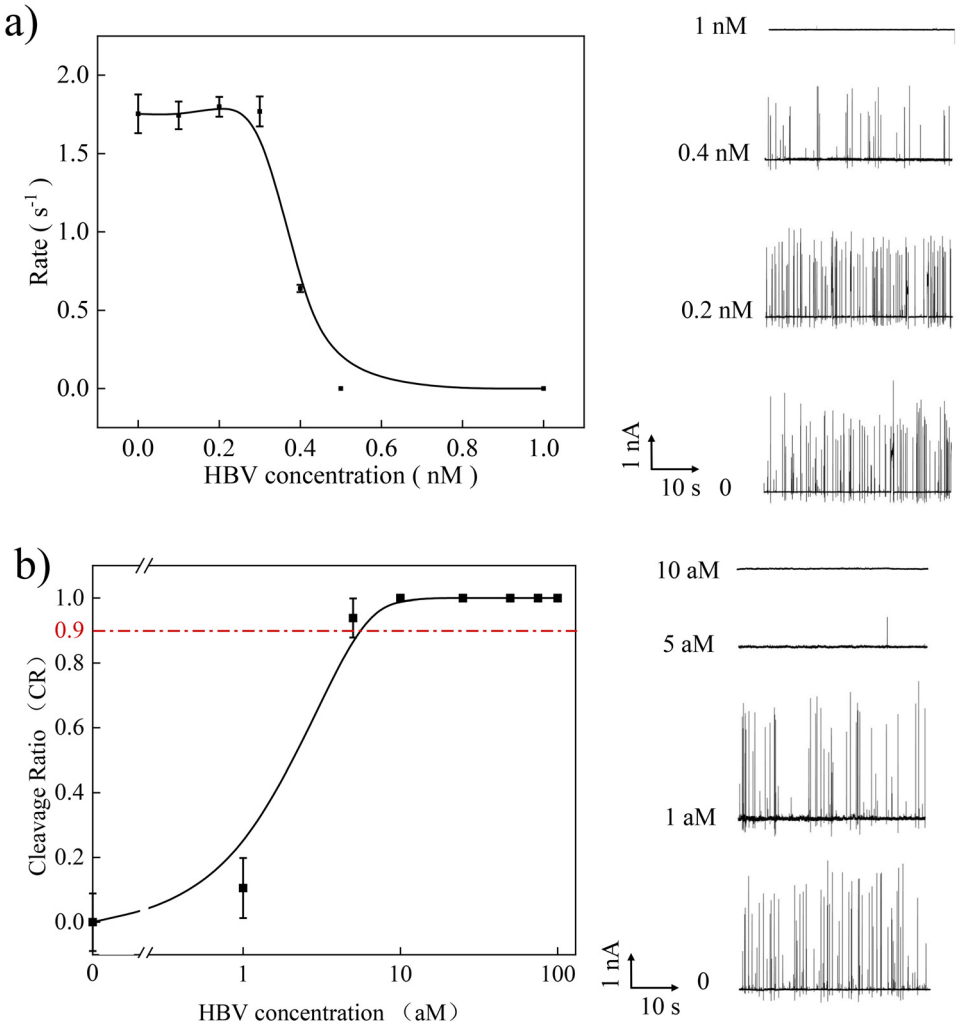

Fig. 5 Quantification test of the nanopore sensor. (a) Calibration curve of the sensor when the HBV concentration ranges from 0 nM to 1 nM. (b) Calibration curve of the sensor after adding the PCR step with HBV ranging from 0 to 100 aM.

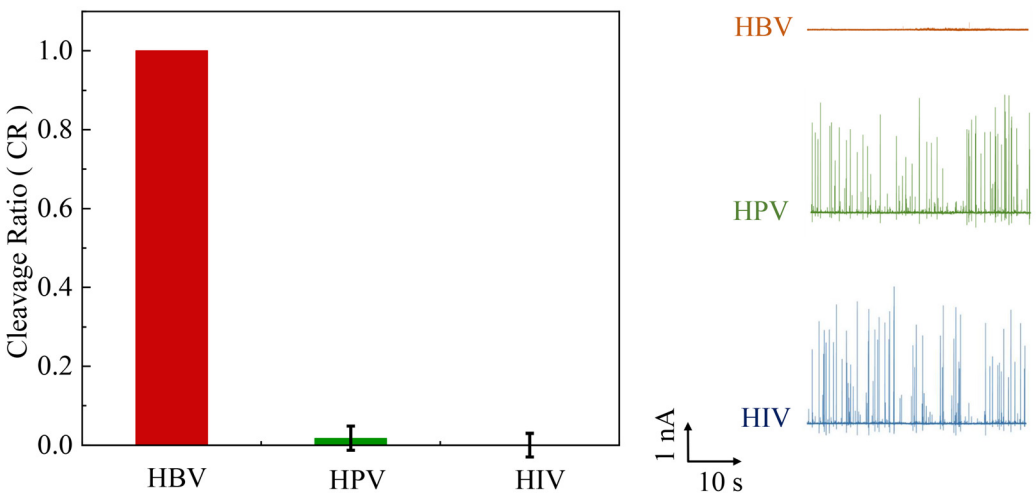

Fig. 6 Specificity test of this strategy. The event rate of the sensor was tested with 10 aM of HBV, 100 fM of HPV, and HIV. Although other interferents were 10 000 times in concentration, a significant decrease in translocation events can only be seen when the HBV target is presented.

Table 1 Sensor performance in human serum

	HIV	HPV	HBV								
Concentration (aM)	1000	1000	0	1	3	5	7	10	20	50	1000
Sample 1	–	–	–	–	+	+	+	+	+	+	+
Sample 2	–	–	–	–	+	+	+	+	+	+	+
Sample 3	–	–	–	–	+	+	+	+	+	+	+

"–" means a negative test result, while "+" means a positive test result.

with our proposed strategy to evaluate the analytical sensitivity and specificity. Three repeats were conducted for each concentration. As listed in Table 1 and Fig. S5,† the results were negative for both nontarget viruses (HPV and HIV), even though their concentrations were high (1 fM). As for the analyte containing HBV targets, when the concentration was below 3 aM, they were classified as negative, whereas the results turned positive when the concentration of HBV exceeded 3 aM. False-negative judgment occurs, when HBV was 1 aM, indicating that the detection limit of the sensor to HBV in human serum was around 3 aM. These results were consistent with those in the buffer. Therefore, the performance of the sensor in real samples is valid.

Experimental

Materials and reagents

All DNAs and RNAs were purchased from Sangon Biotechnology Co., Ltd (Shanghai, China). Their sequences are listed in Table S1 (ESI†). HBV, HIV, and HPV18 plasmids were synthesized by GenScript (Nanjing, China) by inserting part of their genes into separate pUC57 plasmids. M13mp18, lambda DNA, EnGen® Lba Cas12a (Cpf1), BSA, NEBuffer 2.1 and NEBuffer r2.1 were obtained from NEW ENGLAND Biolabs Inc. (NEB). The Platinum SuperFi II DNA polymerase master mix was obtained from Thermo Fisher Scientific Co., Ltd (Shanghai, China). TAEMg buffer (25 mM $Mg(OAc)_2{\cdot}4H_2O$, 45 mM Tris and 2 mM EDTA, pH 8.0 @ 25 °C) and test buffer (1 M KCl, 10 mM Tris and 0.1 mM EDTA, pH 8.0 @ 25 °C) were prepared in lab. Ultrapure water (>18.25 MΩ cm^{-1}) obtained from a Millipore Milli-Q water purification system was used for all solution preparation in the experiment. DNA Marker and 4S GelRed (10 000×) for gel electrophoresis were purchased from Sangon Biotechnology Co., Ltd (Shanghai, China) and 6× loading buffer was from Takara Bio Inc. (Dalian, China). Agarose was purchased from Aladdin Chemistry Co. Ltd (Shanghai, China). Piranha solution was freshly prepared in lab by mixing hydrogen peroxide (H_2O_2) with sulfuric acid (H_2SO_4) in the ratio of 3 : 7. Quartz capillaries (O.D.: 1 mm; I.D.: 0.5 mm; QF100-50-10) were purchased from Sutter Instrument Co.

Apparatus

A UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan) was used to quantify the concentrations of DNA solutions at 260 nm. The preparation of DNA cubes, PCR and CRISPR–Cas12a reaction were carried out using a Bio-Rad T100 thermal cycler (Bio-Rad, USA) with temperature gradients. An Automatic Gel Imaging Analysis System (Peiqing Science and Technology Co., Ltd) was used for the gels electrophoresis analysis. Glass nanopores were fabricated using a CO_2-laser-actuated pipette puller (model P2000, Sutter Instrument Co.).

Fabrication of glass nanopores

All glass pipes used for fabricating nanopores were cleaned thoroughly by soaking in a freshly prepared piranha solution for at least 2 hours followed by cleaning with deionized water. The purpose of this step is to remove residual organic impurities from the glass pipes. After that, they were dried at 80 °C for 20 minutes before the pulling process in a vacuum drying oven. The glass capillaries used in this experiment were pulled using a pipette puller with a two-line program including the following settings: (1) heat 850, filament 5, velocity 50, delay 140, and pull 50 and (2) heat 850, filament 4, velocity 30, delay 155, and pull 255.

Self-assembly of the DNA cube

The DNA cube consisted of six different ssDNA, the sequences of which are listed in Table S1 (ESI†). For the assembly of DNA cubes, six DNA strands at a concentration of 100 nM were heated to 95 °C for 5 minutes, rapidly cooled to 45 °C and then held for 20 minutes in a TAEMg buffer. The DNA cube was stored at 4 °C for more than 2 hours before use.[26]

Gel electrophoresis

The self-assembly of DNA cubes was loaded onto 8% PAGE in a TBE running buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA). The gels were run at 4 °C for 70 minutes under a constant voltage of 120 V.[38]. The PCR products were loaded onto 2% agarose gel in a TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and run at 120 V constant voltages for 30 minutes. Afterwards, all gels were stained with GelRed, visualized under UV light and finally photographed using an Automatic Gel Imaging Analysis System (Peiqing Science and Technology Co., Ltd).

Polymerase chain reactions

Double-stranded DNA (dsDNA) production was amplified by primer sets (Table S1†) and PCR with the Platinum SuperFi II DNA polymerase. The polymerase was used according to the

manufacturer's protocol for the standard PCR. In detail, 50 μL PCR master mix consists of 10 μL primer mix (500 nM forward primer, 500 nM reverse primer), 12.5 μL H_2O, 25 μL Platinum SuperFi II DNA polymerase master mix and 2.5 μL HBV plasmid (HBV-Cas12a assay validation 1 fM, and for analytical sensitivity test different concentrations ranging from 1 aM to 200 fM were used). The PCR protocol using the Platinum SuperFi II DNA polymerase consisted of an initial denaturation step at 98 °C for 30 s, followed by 30 cycles of denaturation at 98 °C for 10 s, 60 °C for 10 s, and 72 °C for 25 s. Finally, the dsDNA production was held at 4 °C.

Experimental procedures

First, 0.7 μL Cas12a (35 nM), 3.6 μL crRNA (36 nM), 2 μL PCR products (from 100 aM to 0.1 aM), 2 μL 10× NEBuffer 2.1 and 10.7 μL ultrapure water were mixed, forming a 19 μL solution. For Cas12a activation, the solution was incubated at 37 °C for 10 min. Afterwards, 1 μL element E and F mix of DNA cube was added and incubated at 37 °C for 0.5 h for cleavage. After cleavage, 5 μL of other elements of DNA cube mix (A, B, C, D), 2 μL 10× TAEMg buffer and 13 μL ultrapure water were added, forming totally 40 μL of the solution for the self-assembly of the DNA cube, the concentration of which was 100 nM. The solution was processed following the above-mentioned self-assembly protocol and finally added to the test buffer before testing.

Nanopore sensing and data analysis

In our experiment, the translocation signal of the DNA cube was studied using a HEKA system. Specifically, one Ag/AgCl electrode, which serves as the working electrode, was inserted into the backside of a glass nanopore filled with the solution, while the other electrode was immersed in the test buffer outside the glass nanopore, serving as the reference electrode. A constant voltage of 1000 mV was applied across the glass nanopore to produce the translocation signal. Then, the signal was amplified, digitized and recorded using HEKA EPC 10, and the sampling rate was 50 kHz. For each data point, the sampling time was about 300 s, and repeated three times, with the total sampling time reaching 900 s. Transalyzer was used to analyze the current time trace and extract the single-molecule translocation information. Origin was used to analyze the data and plot the experimental results.

Conclusions

Overall, we have achieved a sensing strategy based on the digestion of DNA cubes assisted by CRISPR–Cas12a in glass nanopores for the detection of HBV. Translocation signals of several DNA nanostructures in buffer with and without BSA were tested, and the strong anti-interference ability of the DNA cube was proven. The number of the cleavage elements in the DNA cube was optimized to gain a better signal on–off performance. PCR was introduced to improve the overall sensitivity of the sensor. This sensor achieved a limit of detection of 5 aM of target HBV with no cross-reactivity with other human dsDNA viruses. Moreover, the reaction buffer in each step was directly used according to the commercial formula without worrying that the interference component in the buffer would affect the readout of the translocation signal. This method has strong compatibility and extensibility, which will be a good candidate for the practical diagnostic application.

Author contributions

Jiahai Wang put forward some valuable suggestions to the experiment and data analysis, modified the article and gave the final approval of the article. Le Luo carried out most of the data in the experiment, analyzed the data in this work. Yunhui Li, Lanfang Chen, Cenlin Gui and Jianji Zhu help to carried out part of the experiment. Huizhen Li and Wenlong Wang give some suggestions to the writing of the article. Daqi Chen designed the research concept and the experiment, collect and analyze the data, and wrote the article.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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