Research Article

A sensitive colorimetric DNA biosensor for specific detection of the HBV gene based on silver-coated glass slide and G-quadruplex–hemin DNAzyme†

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Abstract

A sensitive colorimetric DNA biosensor for specific detection of single stranded oligonucleotide (ssDNA) is proposed in this paper. The biosensor is based on silver-coated glass (SCGS) and G-quadruplex–hemin DNAzyme. Capture DNA is immobilized on the surface of SCGS by Ag-S bond. Signal DNA can be used to hybridize with the target DNA which is selected from the Hepatitis B virus(HBV) gene as target HBV DNA, and the HRP-mimicking G-quadruplex-hemin DNAzyme can be formed through the function of a guanine-rich fragment from signal DNA to catalyze the oxidation of 2,2-azinobis(3-ethylbenzothiozoline)-6-sulfonicacid ($ABTS^{2-}$) by H_2O_2. The reaction will be monitored along the side of absorbance changes at 418 nm and it can be viewed by naked eye with the change of color as well. Upon addition of target Hepatitis B virus(HBV) DNA, signal DNA could bind on the surface of SCGS, and the concentration of G-quadruplex-hemin DNAzyme immobilizing on the surface of SCGS is depended on that of target HBV DNA. Under the optimum conditions, the absorption was proportional to the concentration of target HBV DNA over the range from 0.5nM to 100nM, with a detection limit of 0.2nM. In addition, the biosensor is target specific and practicability. This assay might open a new avenue for applying in the diagnosis of HBV disease in the future.

Introduction

Genes as a part of oligonucleotides play a critical role in the genetic disorders, predisposition to diseases, and diversity to aim at drugs and therapy[1]. Human genome mutations always lead to human diseases or disorders[2]. For example, the specific genome mutations in the HBV gene sequence causes chronic infection, which is one of the highest risk factors for cirrhosis, hepatitis and hepatocellular carcinoma[3, 4]. Besides, specific DNA sequences were found to be closely associated with tumor development or progression. However, the analysis of specific DNA sequences can provide early diagnosis and risk assessment of malignancy[5]. Biosensors are attractive choice for the detection of specific DNA sequences of genes. They offer many advantages including high efficiency and sensitivity, low fabrication and measurement costs[6]. Different DNA sequences recognition strategies have been constructed depending on the detection method including colorimetry[7], fluorescent spectrometry[8], atomic absorption spectrometry[9], surface plasmon resonance[10], raman spectrometry[11], electrochemistry[12], quartz crystal microbalance[13], etc. Although these methods above provide precise validation, there are deficiencies for using complex procedures and employing expensive equipment. Thus, a nucleotide change directly detected by the naked eye without using instrument would be attractive.

The G-quadruplex–hemin DNAzymes can catalyze 3,3`,5,5`-tetramethylbenzidine (TMB) or $ABTS^{2-}$ into colored solution for their horseradish peroxidase

(HRP)-mimicking activity[14]. Colorimetric sensors with the quality of G-quadruplex–hemin DNAzyme catalyzed oxidations attract huge attention these days as a reason of their simplicity, label free, low cost and reliable analysis of multifarious biomarkers[15]. The G-quadruplex–hemin DNAzyme is suited to sensing application since it shows stable performance as the amplifying readout units of diverse biorecognition elements. This robust function is important in biosensors for detection of oligonucleotides, ions, proteins, small molecules and cells[16, 17, 18, 19].

Oligonucleotide immobilization can be used to separate bound biomolecules from free molecules, and several immobilization methods have been reported for the functionalization of surfaces such as glass, electrode, nano-magnetic microsphere and nanoparticles[20, 21, 22, 23, 24, 25]. An idea sensing platform is being developed by using the concept of silver coated glass slide(SCGS) which is an attractive separation material, because thiol-functionalized oligonucleotides can self-assemble on the silver surface through Ag–S bonds[26, 27, 28, 29], and SCGS is easily prepared by the silver mirror reaction[30, 31, 32, 33].

In this article, by using SCGS as an idea separation material, we describe a simple, sensitive colorimetric DNA biosensor for the determination of the target DNA which selected from the HBV gene sequence, based on G-quadruplex–hemin DNAzymes as an amplifying sensing platform. This assay might open a new avenue for applying in the diagnosis of HBV disease in the future.

Methods

Reagents and apparatus

$ABTS^{2-}$, Tri-(2-carboxyethyl) phosphine(TCEP), bovine serum albumin(BSA), DMSO and hemin were purchased from Sigma-Aldrich(The United States of America). Ammonia(25%), H_2O_2, potassium nitrate, silver nitrate and glucose were purchased from Sinopharm Chemical Reagent Co. Ltd.(Beijing, China). All Oligonucleotides (Table 1) were synthesized and purified by Sangon Bioengineering Technology and Services Co. Ltd. (Shanghai, China). The underlined sequence of signal DNA are guanine-rich fragment, and the $T_{(15)}$ in capture DNA and signal DNA are designed as spacer. All chemicals were analytical reagent grade. All solutions were prepared with ultrapure water. The quartz glass slides (10mm×10mm×1mm) were purchased from Guangliang High-tech Co. Ltd. (Jiangsu, China).

All Oligonucleotides stock solution were prepared by dissolving oligonucleotides in Tris–HAc buffer (20.0mM, pH 7.4, 200.0 mM $NaNO_3$, 1mM $Mg(NO_3)_2$), and were stored at 4 °C before use. The stock solution of hemin was prepared in DMSO, stored in the dark at -20 °C and diluted to the required concentration with Tris–HAc buffer when being used.

The pH values of the solutions were measured by pHS-3E digital pH meter (Shanghai Lei-ci Instrument Plant, China). UV–vis absorption spectra were obtained by a TU-1901 double-beam spectro-photometer (Beijing Purkingje General Instrument Co. Ltd, China).

Preparation of SCGS

As mentioned above, the traditional silver mirror reaction was used. For SCGS fabrication, the quartz glass slides was rinsed thoroughly. The Tollens' reagent was deserved by adding Ammonia (1%) into silver nitrate (3%) dropwise, and the ratio of glucose solution (10%) and Tollens' reagent was 2:1. Then, the quartz glass slides were dipped into the mixture at 25ºC for 5 minutes. Finally, shining silver film was formed on the surface of glass slide[8, 29].

Immobilization of capture DNA on the SCGS

Capture DNA(Oligo-1) can be immobilized on the SCGS according to the reported methods[34]. The SCGS was placed in Tris–HAc buffer which contained 1.0μM Oligo-1. The modified SCGS was washed twice by the same buffer in order to eliminate free Oligo-1.

Serum sample preparation

Human sera samples were collected from healthy volunteers with informed consent in Guangdong Ocean University Campus Hospital(Zhanjiang, China). All the experiments were performed following the relevant laws and institutional guidelines. Before the recovery experiment in human sera, each sample was removed small molecules by Amicon filtration device 10,000 [29, 35].

Fabrication of the sensor

The Scheme of the sensor is depicted in Figure 1. Firstly, the Oligo-1 modified SCGS was immersed in 3% BSA solution for 20 minutes to block possible remaining active sites, and avoid the nonspecific adsorption. Then the SCGS was submerged into different concentration of target HBV DNA(Oligo-3) solution for 1 hour. Then, the SCGS was immersed into 10nM signal DNA(Oligo-2) solution for another 1 hour. After washed twice by buffer, the SCGS was immersed into buffer containing 200mM potassium nitrate and 5μM hemin for 15 minutes. After that, 30mM H_2O_2 and 30mM $ABTS^{2-}$ were added into the above-mentioned solution at the same time, and kept the mixture for 10 minutes. Eventually, the absorbance spectra from 400 to 450nm were collected. The absorbance peak intensity at 418nm was employed to evaluate the performances of the proposed sensing system. The procedures mentioned above were carried out in Tris–HAc buffer at room temperature.

Results

Absorption spectra

The colorimetric signal was measured to prove the function of the proposed sensor. As seen in Figure 2, there was no colorimetric signal without signal DNA (curve c), and the colorimetric signal of the mixture was also weak lacking of target HBV DNA (curve b). However, the absorbance peak are maximum with addition of 100nM target HBV DNA (curve a), which means the colorimetric signal enhanced

effectively.

Optimization of the experimental conditions

The concentrations of capture DNA, signal DNA, potassium nitrate(K^+), hemin, H_2O_2 and $ABTS^{2-}$ are important for an optimal result. According to this research, a good detection performance was obtained under the condition of 1.0μM Capture DNA, 10nM Signal DNA, 200mM K^+, 5.0μM hemin, 30mM H_2O_2 and 30mM $ABTS^{2-}$ (Figure 3).

Calibration curve and absorption spectra for target HBV DNA

In this work, the relationships between the absorption and the concentration of target HBV DNA were studied under the optimum conditions. As shown in Figure 4, the absorption increased with the concentration of target HBV DNA over the range from 0.5nM to 100nM with a linear regression equation of:

$$A = 0.0054\,C + 0.165 \qquad (1)$$

Where *C*: nM, r = 0.992, *C* denotes the concentration of target HBV DNA, *A* represents the absorption, and a detection limit of 0.2nM, which was obtained from the equation of DL = 3б/slope.

Sequence selectivity of the biosensor

From the Figure 5, it can be seen that the absorption for target HBV DNA was about 0.750, which is highest among that of single-base mismatched strand M-1 (Oligo-4) (0.220) and two-base mismatched strand M-2 (Oligo-5) (0.190) and M-3

(Oligo-6) (0.175).

Detection of target HBV DNA in human serum

Addition and recovery experiment was executed to evaluate the application of the sensor. The measurement results are listed in Table 2. 1.0×10^{-9} to 5.0×10^{-8} M of target HBV DNA were added into each human serum, and the recovery rate were obtain over the range from 95.6% to 106.0 %, while the relative standard deviation values were in the range of 4.3% to 7.2%.

Discussion

Although the absorption depend on the concentration of G-quadruplex-hemin DNAzyme on the surface of SCGS, it is also affected by the concentration of capture DNA, signal DNA, potassium nitrate(K^+), hemin, H_2O_2 and $ABTS^{2-}$. Thus, this research considers the effect of each factor and selects the optimal condition to obtain the maximum change of absorption (ΔA).

$$\Delta A=A_{target} - A_{blank} \qquad (2)$$

Where A_{target} represents the absorption of the mixture containing 100nM target HBV DNA, while A_{blank} denotes the absorption in the absence of target HBV DNA.

For the effect of the concentration of capture DNA, it was found that the absorption increased with the concentration of capture DNA within a range of 0.1-1.0μM and reached a plateau at 1.0μM. Therefore, 1.0μM capture DNA was applied in this

research. Besides, 10nM of signal DNA was used in the research, because the absorption increased along with the concentration of signal DNA and reached a plateau when the concentration was 10nM. K^+ and hemin affect the formation of G-quadruplex-hemin DNAzyme resulting a change of ΔA. 200mM K^+ and 5μM hemin were used in the research after comparison testings. In addition, the $ABTS^{2-}$ can be oxidized by H_2O_2 in the presence of G-quadruplex-hemin DNAzyme. Thus, it is necessary to investigate the impacts of the concentrations of $ABTS^{2-}$ and H_2O_2 on ΔA. The ΔA increased with the addition of the $ABTS^{2-}$ concentration over the range of 0-30mM, and reached the maximum at 30mM. A similar phenomenon also occurs in H_2O_2, thus 30mM $ABTS^{2-}$ and 30mM H_2O_2 were used in the research.

To explore the sequence selectivity of the sensor, the sequence selectivity was studied through replacing the target HBV DNA by other three mismatched DNA. The measurement results illustrated that the proposed sensor had good sequence selectivity for target HBV DNA.

Moreover, some samples of human serum were used to detect target HBV DNA to assess the analytical application of the biosensor in clinical specimens. All the results of clinic sample analysis were verified by RT-qPCR, which showed that the recovery was ranged from 99.6% to 103.4 %, and the relative standard deviation values were in the ranges of 2.3%-4.2%. That meant the application of the proposed sensor in real sample was possible. These results can prove the successful application of the proposed sensor in real sample.

In conclusion, based on the amplifying property of G-quadruplex-hemin DNAzymea and the idea separation material SCGS, a simple and sensitive colorimetric DNA biosensor for sequence-specific recognition of target ssDNA was established. Under the optimum conditions, the absorption was proportional to the concentration of target HBV DNA over the range from 0.5nM to 100nM with a detection limit of 0.2nM. Some other methods were listed in Table 3 to make a comparison. The proposed concept promises a high sensitive and effective sensing. Owing to its simple fabrication and low price, this device might open a new avenue for the application of the diagnosis of HBV disease in the future.

Acknowledgements

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Figure legends

Figure 1. The Scheme for colorimetric biosensor for sequence specific recognition of target HBV DNA based on SCGC and G-quadruplex-hemin DNAzyme.

Figure 2. Absorption spectra of different mixture under the same process. (a) b + target HBV DNA; (b) Capture DNA + signal DNA; (c) Capture DNA + target HBV DNA.

Figure 3. (A) Effects of capture DNA concentrations; (B) Effects of signal DNA concentrations; (C) Effects of K^+ concentrations; (D) Effects of hemin concentrations; (E) Effects of H_2O_2 concentrations; (F) Effects of $ABTS^{2-}$ concentrations. 100nM of target HBV DNA were used in the whole experiments for optimal condition.

Figure 4. Calibration curve and absorption spectra for various concentrations of target HBV DNA.

Figure 5. Sequence selectivity of the biosensor. The concentration of target HBV DNA, M-1, M-2 and M-3 were 100nM. Every point is the mean of three measurements. The error bar was the standard deviation.

Table 1. Sequences of oligonucleotides

Probes	Name	Sequence
Oligo-1	Capture DNA	5'-HS-$(T)_{15}$-GC AGA CAC ATC CAG-3'
Oligo-2	Signal DNA	5'-CGA TAG CCA GGA CAA-$(T)_{15}$- GGG TAG GGC GGG TTG GG -3'
Oligo-3	Target ssDNA (Target HBV DNA)	5'- TTG TCC TGG CTA TCG CTG GAT GTG TCT GC-3'
Oligo-4	M-1 (Single-base mismatch HBV DNA)	5'- TTG TCC T**C**G CTA TCG CTG GAT GTG TCT GC -3'
Oligo-5	M-2 (Two-base mismatch HBV DNA)	5'- TTG TCC T**C**G CTA TCG CTG GAT G**A**G TCT GC -3'
Oligo-6	M-3 (Two-base mismatch HBV DNA)	5'- TTG TCC T**CC** CTA TCG CTG GAT GTG TCT GC -3'

Table 2. Recoveries of target HBV DNA from the spiked human serum samples. The values shown here are the average values from three measurements.

Serum samples	Added HBV DNA (M)	Founded HBV DNA (M)	Recovery(%)	Relative standard deviation (%)
1	1.00×10^{-9}	1.06×10^{-9}	106.0	7.2
2	5.00×10^{-9}	5.20×10^{-9}	104.0	6.6
3	1.0×10^{-8}	1.08×10^{-8}	108.0	6.1
4	5.0×10^{-8}	4.78×10^{-8}	95.6	4.3

Table 3. Comparison of our method with other methods for the determination of ssDNA

Method	Analytical range	LOD	Application to samples	Ref.
Photocatalytic electrochemical method	10aM-100nM	1.7aM	Yes	[1]
Hemin-graphene hybrid nanosheets method	5nM-100nM	2nM	No	[6]
Glucose oxidase amplification method	0.25nM-5.5nM	7pM	No	[7]
Atomic absorption spectrometry method	10nM-200nM.	0.23nM	No	[8]
G-quadruplex–hemin DNAzyme method	0.5nM-100nM	0.2nM	yes	This work

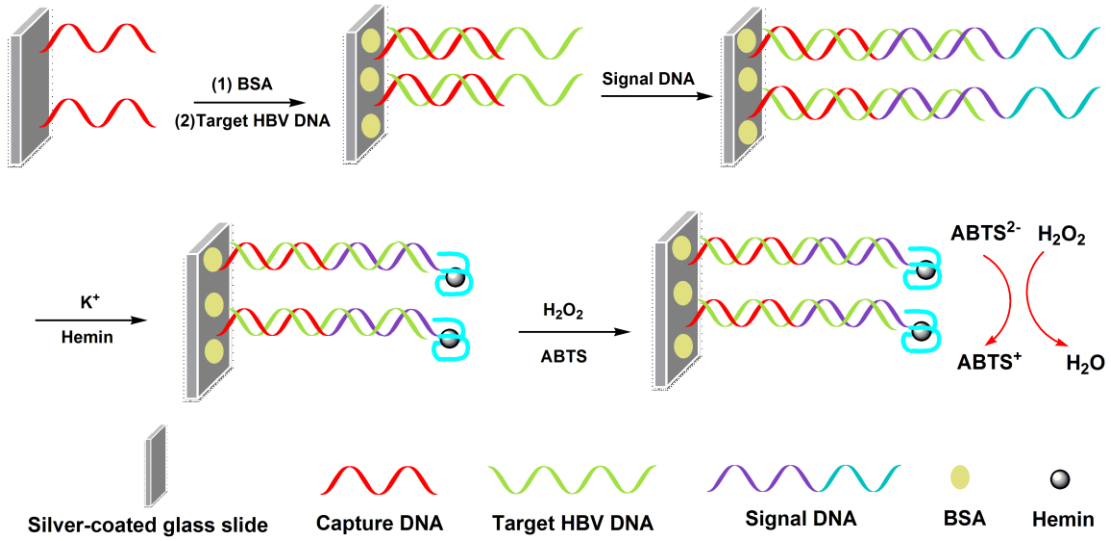

Figure 1

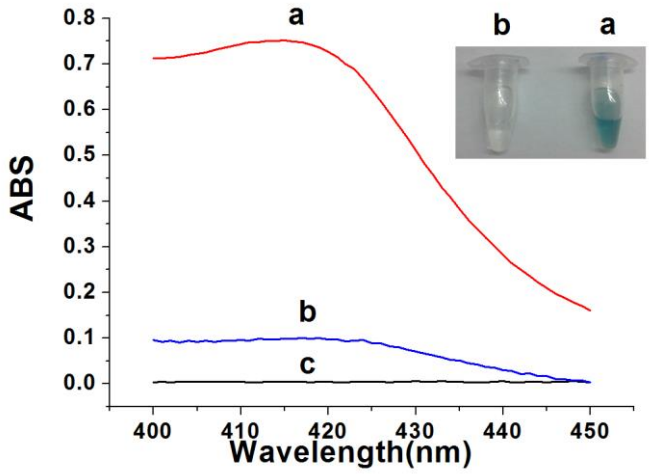

Figure 2

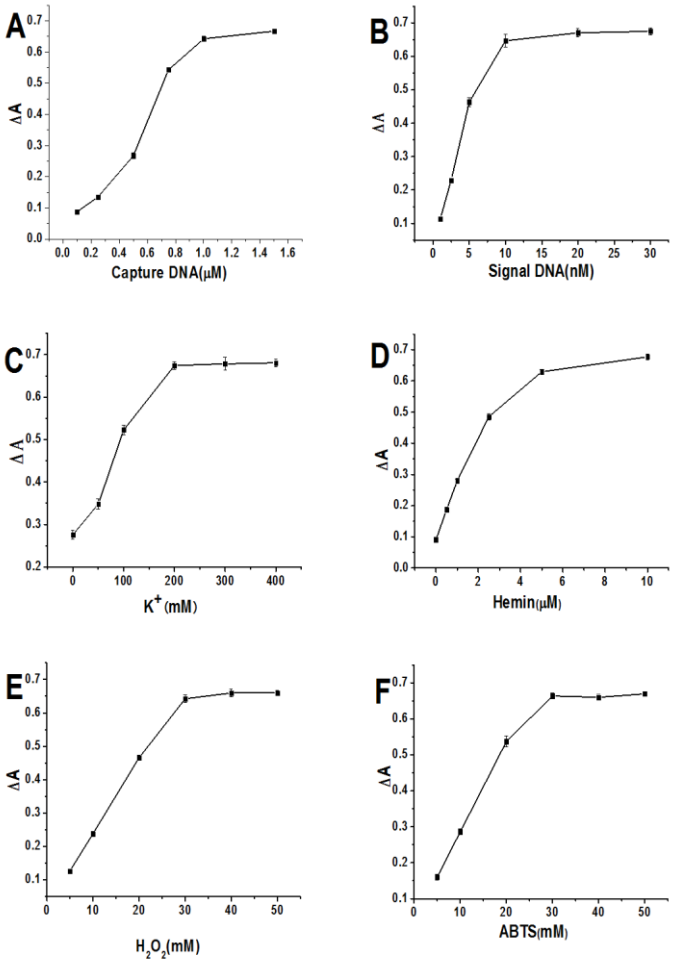

Figure 3

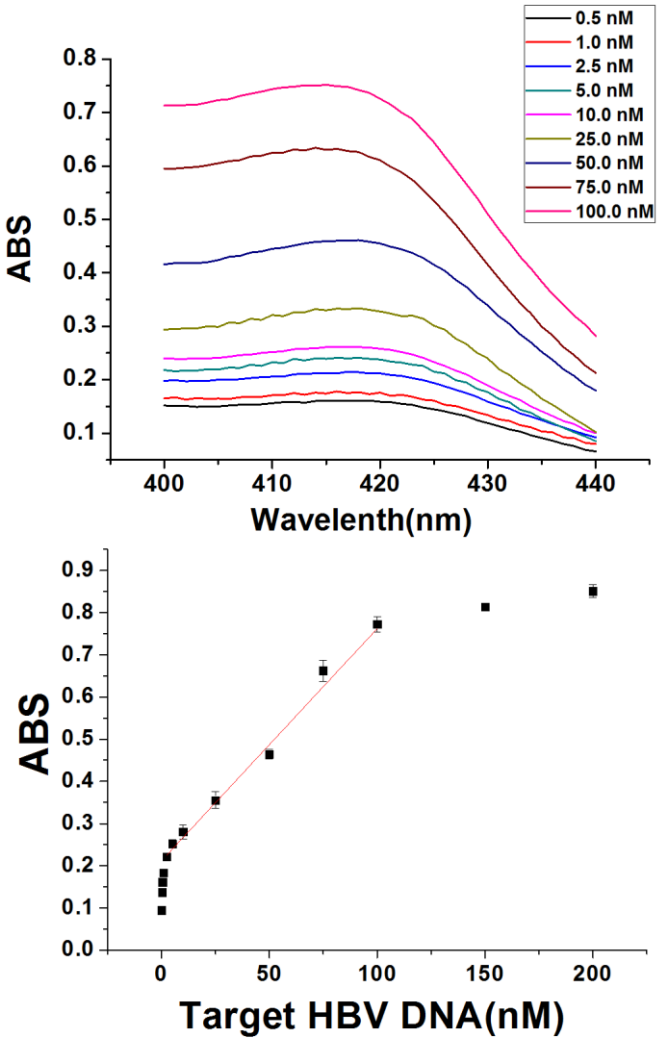

Figure 4

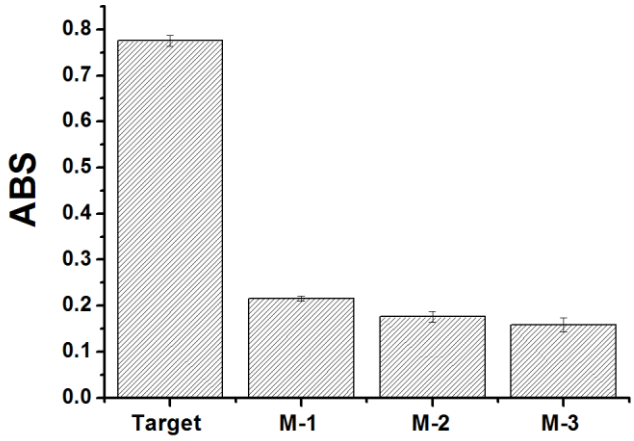

Figure 5