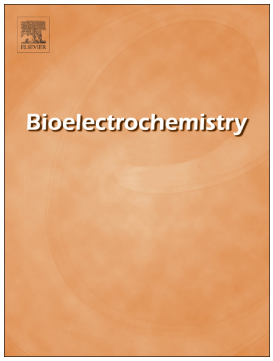

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Amplified Detection of Hepatitis B Virus using an Electrochemical DNA Biosensor on a Nanoporous Gold Platform

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ABSTRACT

In the present study, a nanoporous gold platform was applied for the amplification detection of Hepatitis B virus (HBV) by an electrochemical DNA biosensor. Ferrocene as a redox reporter was covalently attached to the DNA probe and its electrochemical signal was recorded as biosensor response. For real samples, DNA was firstly extracted from patient blood and then amplified with polymerase chain reaction (PCR) for 5 cycles. Sensitivity of this biosensor was enhanced by using nanoporous gold electrode, therefore this sensor can discriminate the genome of HBV in real sample with low PCR cycles. By this strategy and signal amplification using nanoporous platform and covalently attached electroactive label, the biosensor can distinguish between healthy and HBV patient with limited PCR cycles. Moreover, the errors of PCR with large cycles can be disregarded. With PCR, a linear dynamic range of 0.4 to 10 nmol of mutant DNA was achieved, with reliable reproducibility (RSD) 8.9 %.

Keywords: Nanoporous Gold Electrode; Electrochemical DNA Hybridization Biosensor; Polymerase Chain Reaction (PCR); Ferrocene Carboxylic Acid; Hepatitis B Virus.

1. Introduction

Sodality of Nano- and bio-technology suggest good potential for the surface functionalization in disease diagnosis. The constructed biosensors using these technologies are widely used for drug screening and clinical diagnostics especially for contagious disease [1]. Early Diagnosis of some viral disease is very important. For achieving this purpose, an acceptable level of selectivity and sensitivity is necessary [2]. Electrochemical DNA biosensors by combination the specificity of biological identification with the selectivity of physicochemical transducer offer a good strategy for in-vitro and in-vivo analysis of nucleic acid [2]

Hepatitis B is a viral infection of the liver and could develop a chronic infection. Infection of HBV is a public health problem of worldwide importance with acute and chronic clinical consequences. Persons infected with HBV are at high risks of developing liver cancers and liver failures [3,4]. This disease spread by the transmission of the virus through blood and body fluids. For screening of this virus, the Hepatitis B surface antigen (HBsAg) and a soluble antigen Hepatitis B e antigen (HBeAg) are mainly used [1,5,6]. Sometimes in the first stages of infection, HBsAg does not present or is not detectable. Therefore, development of ultrasensitive methods is necessary for the detection of the virus in the early stages. The detection of HBV genomic DNA is more reliable than HBsAg for the detection of this virus [7]. In recent year, different strategies have been developed based on the DNA analysis of this virus such as optical [7,8,9], piezoelectric [10] and electrochemical methods [6,11,12,13]. Also enzyme linked immunosorbent assay (ELISA) witch utilized enzymatic reaction to amplify the output signals for HBV assay has been developed [14,15].

Bonino and coworkers detected the infection by following DNA of HBV and compared the results with the detection based on HBsAg and HBeAg [16]. Similar strategy was used by

Baker's groups [17]. Ozsoz and his coworkers [11] applied cobalt phenanthroline as an indicator for the detection of short DNA sequences related to HBV . This research group also used methylene blue [18] and guanine oxidation signal [19] for the electrochemical detection of this virus in PCR samples. Also, Ju group studied the interaction of Di (2,2'-bipyridine osmium III) with DNA for detection of HBV [20].

Several compounds such as 2,9-dimethyl 1-1,10- phenonthroline copper complex [21], hydroxyl aniline, cobalt complexes [22] and bis(benzimidazol)cadmium(II) dinitrate [23] have been applied as redox probes. Zhu and coworkers used CuO_2 hollow microspheres for the construction of DNA biosensors for HBV and methylene blue as an indicator [24,25].

For the detection of special DNA sequences in real samples and body fluids, a very sensitive DNA biosensor is needed. Also, for the discrimination of a special sequence by other similar sequences, it is necessary to amplify the desired DNA target using several strategies including: polymerase chain reaction (PCR), electrocatalytic and enzymatic amplifications of the analytical signals, application of nanoparticles and combination of covalent binding of redox reporters on the top of target DNA and charge-transfer (CT) through DNA. [25,26]

In the present manuscript a new DNA hybridization biosensor for ultrasensitive detection of Hepatitis B virus in human serum plasma is investigated. By applying nanoporous platforms for the amplification of signals and covalent binding of the redox probe to reduce the baseline, the biosensor demonstrates the capability of ultrasensitive detection of HBV genome at low PCR cycles. Herein, the DNA hybridization biosensor has been constructed based on a sandwich assay by immobilization of amino-labeled probe on a nanoporous gold platform, hybridization with target and finally, hybridization of unhybridized section of target with covalently ferrocene labeled capture. The redox reporter could transfer electron with electrode surface through double

stranded DNA in the present of HBV genome and produce electrochemical signal. The reversible behavior of ferrocene and its amenability to derivatization and stability makes it a good redox reporter for electrochemical biosensor.

2. Experimental

2.1. Chemicals and Reagents

Ferrocene carboxylicacid (FCA), 6-mercaptohexanol(MCH) N-hydroxylsuccinimide (NHS), N-(3-imethylaminopropyl)-n-ethyl-carbodimide hydro- chloride (EDC), tris(2-carboxyethyl) phosphine HCl (TCEP), sodium dihydrogen phosphate, nitric acid, sulfuric acid, disodium hydrogen phosphate, sodium chloride, magnesium chloride and ascorbic acid were purchased from Merck (Germany). The distilled-deionized water was used in all solution preparations (18MΩ).

2.2. Apparatus

Electrochemical experiments were carried out using an Autolab electrochemical system PGSTAT 30 [Eco Chemie, The Netherlands] driven by GPES software. A conventional three-electrode system, consisting of small parts of gold recordable compact disks (CD-R) as working electrode[17], a platinum wire as an auxiliary electrode and an Ag/AgCl/3.0M KCl reference electrode were used for all electrochemical measurements. The differential pulse voltammograms have been recorded at the scan rate of 10 mVs^{-1} and the pulse amplitude of 25 mV. EIS experiments were accomplished at a constant DC potential of +0.23 V and the applied AC potential was 5mV. All experiments were performed at room temperature. Scanning electron microscopy images were captured using AIS-2100 (Seron technology, Korea).

The stock solution of the oligonucleotides (6 μM) were prepared in 1X PBS buffer solution and kept frozen at -20^{o}C. DNA oligonucleotides were obtained from Eurofins MWG/Operon Co. with the following sequence (From 5′ to 3′):

HS-C_6-AGGCTGTAGG: Capture

Probe: CATAAATTGG-C6-NH_2

Complementary: CCAATTTATGCCTACAGCCT

TGCCTCGCACCTACATAGTA: Non-complementary

Primer 1: TAGCAGGCTGTAGGCATAAATTGGT

Primer 2: GGCGAGGGAGTTCTTCTTCTAGGGG

2.3. Preparation of the nanoporous gold electrode (NPGE):

The gold electrode was according our previous article [26]. Briefly prepared from small pieces of recordable compact disk (CDtrode), a small cut of CD was placed in nitric acid for removing the protective layer of the CD. Then it secured to the bottom of a Teflon cell with o-ring and was used as a working electrode.

The NPGE was prepared according to previous methods [27,28], briefly; the NP-GE was constructed in two steps. In first step, the gold surface was anodized 3 min in a solution of phosphate buffer (pH 7.4) by applying a potential of 3.6 V vs. Ag/AgCl. In second step the anodized gold surface was covered with a fresh solution of 1.0 M of ascorbic acid for 5 min. the color of the electrode surface changed to dark.

The surface area of the gold electrode was calculated by following its cyclic voltammogram in 0.5 M of sulfuric acid, by presumption a specific charge of 386 μC/cm, for gold electrode

reduction peak. The surface area before and after porosity were 0.19±0.01 and 0.66±0.02 cm^2 respectively. To allow the formation of self-assemble monolayer of thiolated probe DNA.

The surface morphology of the gold electrode before and after becoming nanoporous was characterized by using SEM. The parallel grooves on the electrode are the grooves of CD and the size of porosity of gold electrode was about 70 nm. Fig. S1 shows SEM images of bare and nanoporous gold electrodes and the EDX analysis confirming high purity of gold as the reflective layer of CD.

2.2. PCR amplification of real sample

DNA samples were extracted from the patient's blood suspicious to infect by HBV using extraction kit (Qiagen, Germany). The amplification mixture, in a final volume of 50 μL, contains: 2.5 μL PCR buffer 10X, 0.75 μL 25 mmol l:1 MgCl2, 0.5 μL 20 mmol dNTPs, 1 μL of each primer, and 0.25 μL of DNA polymerase enzyme and 17 μL of double distillate water. PCR amplification of extracted DNA was performed in a Mastercycle gradient PCR (Eppendorf, Hamburg), in a final volume of 50 μL. PCR process was done according Merci method [20]. Briefly, the following temperature program was applied: a) initiation step, 95ºC for 5 min. b) second step 25, 30 and 35 cycles amplification consist of 1 min of 95 ºC, then 1 min of 60 ºC for annealing, 2 min of 70 ºC for extension and c) final step: cool down to room temperature The obtained amplified product was detected using electrophoresis in 1% agarose gel and 100 bp ladder. Samples were obtained from negative and positive patient.

2.3. Fabrication of ferrocene-modified DNA biosensor

At first, the prepared NPGE was electrochemically treated in 0.01 M of NaOH and 0.5 M of H_2SO_4 (using cyclic voltammetry until to achieve constant voltammograms). Then 10 µL of 4 µM thiolated-capture DNA 5µL of 2 M $MgCl_2$ and also 2 µL of 0.5 M NaH_2PO_4 were dropped on the NPGE surface,60 min, for reducing negative charge of single strand repulsion. After 1 hour, 2 µL of 4 µM MCH, as a back filler, was added to same solution on the electrode surface for 15 min at room temperature. After washing the electrode surface, 10µL of 4 µM target DNA or 4 µL of thermal stress denatured PCR product and 5 µL of 2 M magnesium chloride were placed on the electrode surface for 70 min at room temperature. In the next step after washing the electrode, 10 µL of 4 µM amine-labeled probe DNA and 5 µL of 2 M magnesium chloride were dropped on NPGE for 70 min at room temperature. The probe is complementary to non-hybridized section of the target and would be hybridized with this part. In the last step of the modification 60 µL of a solution containing 1 mM of FCA, 10 mM EDC and 16 mM NHS was put on the electrode surface in a dark place at room temperature for 7 hours. The resulting electrode was ready for the electrochemical detection. Schematic 1 is showing biosensor fabrication process.

Schematic 1.

Differential pulse voltammetry (DPV) was used for the hybridization detection of different targets and real samples. After 3 mL of 0.05 M phosphate buffer (pH 5.5) was placed into the Teflon cell, containing the modified Au electrode, then DPV determination of FCA, attached on DNA probe, was performed by scanning the potential from 0V to +0.65V. Each step of modification was monitored by EIS. For EIS analysis, a solution of 0.5 mM potassium

ferricyanide and potassium ferrocyanide (1:1) in 0.15 M KCl and 0.02 M of phosphate buffer with pH 5.5 was used.

2.4. ***Quantitation of surface density of DNA immobilization:***

The surface coverage of the immobilized DNA on the nanoporous gold electrode was determined by method. Briefly, the modified NPGE was subjected in hexamine ruthenium (III) (RuHex) solution. The amount of cationic redox marker, RuHex, was measured by chronocoulometry. The integrated charge, as the integrated Cottrell expression explains a function of time t, by using Cottrell Equation at t0.

$$\Gamma = Q / nFA_W \qquad \textbf{\textit{Equation 1}}$$

Γ :(surface concentration of RuHex), Q: charge at t0, n: the number of electrons per molecule reduction (that here n is 1), Aw: the electrode area (cm^2), F: Faraday constant. The real surface area of nanoporous gold electrode was determined with cyclic voltammogram in 0.5 M of sulfuric acid, it was about 0.66±0.02 cm^2. chronocoulometric experiment was done in 50µM RuHex and electrolyte solution (0.02m KCl).

The following equation is showing the relation between numbers of adsorbed RuHex on DNA strands:

$$\Gamma_{DNA} = \Gamma_o \left(\frac{z}{m}\right)(N_A) \qquad \textbf{\textit{Equation 2}}$$

The surface coverage of DNA on the smooth and nanoporous gold electrode were obtained equal to 4.3±0.2 $\times 10^{-13}$ $mole.cm^{-2}$ and 1.8±0.1$\times 10^{-12}$ $mole.cm^{-2}$, respectively.

3. Results and discussion

Using the ability of DNA-mediated charge transport as a signaling plan increases the selectivity of constructed biosensor. Also, using nanoporous electrodes make a good sensitivity for biological detection. Covalent attachment of the redox reporter molecules at the end of DNA probe can resolve the problems of DNA biosensor including: first, direct electron-transfer between the redox reporter at the electrode surface second, positioning of the reporter between the base mismatches and the electrode surface. Here we constructed a new DNA biosensor by employing tree above strategy and detect the HBV in real samples. Probe DNA self-assembled monolayers bind to the nanoporous gold surface via thiol linkage. This binding way is tightly enough due to the high standard Gibbs free energy.

3.1. Behavior of electrode surface in modification steps

Modification of the electrode surface was monitored using EIS experiment. Fig. 1 shows the impedance features of the electrode interfaces for each steps of the modifications. Upon self-assemble of thiolated DNA capture, hybridization with single strand target DNA and then hybridization with DNA probe were done respectively. At last step ferrocene with activated carboxylic acid was chemically attached at top of DNA layer by carbamide bonding (Fig. 1. Plots a, b, c, d and e), the electron transfer resistance (R_{ct}) enhance. These vary stemming from the blocking of the electrode surface and electrostatic revulsion of $[Fe(CN)_6]^{3-/4-}$ probe due to negative charge and spatial ceiling of DNA. The kinetics of the electron transfer on the DNA modified gold electrode is retarded by electrostatic repulsion between phosphate backbone of DNA and negative charges of ferri/ferrocyanide.

Preferred position for Fig. 1

PCR amplification of extracted DNA was performed in different cycles, 25, 30 and 35. In 35 cycles the results show a high background and in 25 and 30 cycles, the peaks were sharp with low background. Therefore, PCR with 30 cycles was used as optimum amount. Fig. 2 (A) shows gel electrophoresis of PCR amplified real samples related to the: a) DNA molecular marker for HBV (b) positive real sample 1, (c) Positive real sample 2 d) negative real sample.

The Ferrocene oxidation peak height (with baseline correction) was used as the analytical signal of the diagnosis. The procedure of hybridization detection of the real samples consisted of the following steps: probe immobilization, hybridization with DNA fragments obtained from PCR amplification, indicator binding and voltammetric transduction of the surface. The ability of duplex DNA layer to charge transfer has been accomplished using the electrochemical signal of covalently immobilized FCA on top of monolayer. Electrochemical behavior of immobilized FCA was explored by using differential pulse voltammetry. As Fig. 3 represents the voltammograms for the complementary, negative real sample, HBV real samples and PCR amplified real samples. Negative patient sample is not hybridized; or if hybridized a hybridization of a DNA double strand with several mismatches could be occurred. Therefore, as a result, the non-complementary and mismatches targets cannot transfer charge properly and the electrochemical signal of FCA decreased considerably. For mismatch targets, there is a small tunneling current in comparison to non-complementary sample. The highest signals for directly used sample and amplified real sample were obtained for real sample number 1. The positive patient sample that shows the concentration of HBV in sample 1 was more than sample 2. The electrophoresis analysis for PCR product confirmed this result. The peak current (I_p) of positive sample on nanoporous electrode was 2.3 µA and for smooth gold electrode was0.53 ±0.5µA. The peak current for non-complementary target was 0.16±0.4µA and for target containing a single

base mismatche was 0.31±0.3 μA. For relative standard deviation (RSD) determination the three subsequent experiments were done for the detection of target DNA hybridization from the positive real samples. The RDS was 8.9 % for real samples that means reproducible results.

Preferred position for Fig.3

3.2. Voltammetric transductions:

The analytical performance of the biosensor was explored using the different amounts of the complementary target in a 12 μL aliquot of the target solution, according to the described procedure. The dynamic range was from 4×10^{-10} to 1×10^{-8} moles of target (3×10^{-5} to 1×10^{-3} M) showing a linear range with increasing the Target DNA. The LOD was determined as 1×10^{-11} mole of target DNA (0.8 μM of target DNA).

Preferred position for Fig. 4

4. Conclusion

This electrochemical DNA biosensor introduces a new assay for HBV detection. The major advantages of this biosensor are its accuracy, sensitivity, and its selectivity due to using nanoporous gold electrode and using CT protocol through double stranded DNA respectively. Its sensitivity causes it capable to detect the HBV gene sequence with few cycles of PCR and its selectivity make it able to HBV in complicate real sample. The developed protocol can be used as a clinical identification method. In Table 1, a comparison between previously reported assays and the present biosensor is listed. The HBV diagnosis method by detection virus genome could

deleted false-positive or false- negative diagnosis with Hepatitis B surface antigen in real samples. Covalent attachment of Ferrocene Carboxylic Acid at top of DNA, for hybridization detection in electrochemical DNA biosensors, has eliminate high background signal, in comparison to well-known complexes such as methylene blue, ferrocenium hexafluorophosphate, $[Co(phen)_3]^{2+}$, $[Ru(bpy)_3]^{2+}$, as soluble markers. This gold platform showed good stability property due to covalent attachment of probe DNA, thus it is ideal for commercialization and mass production. By more effort on improving the sensitivity of biosensor the detection of target sequences directly from the plasma samples without any prior PCR amplification step. The RDS of this biosensor was about 8.9 % for real samples that means reproducible results for this method.

Preferred position for Table 1.

Acknowledgment

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Table 1. A Comparison between the proposed assay and other reported techniques for the Hepatitis B detection.

Method	Analytical method	%RSD	Detection Limit	Reference
Hepatitis B virus (HBV) DNA fragment (181 bps)/PCR	Electrochemical/ferrocenium hexafluorophosphate ($FcPF_6$)	3	40 cyles PCR	[29]
DNA /PCR	Electrochemical/DVP/ Di(2,2'-bipyridine) osmium(III)	3	8.3×10^{-21}mol of original genomic HBV DNA (40 cyles)	[20]
DNA /PCR	Electrochemical/SWV/methylene blue	10.8	35 cyles PCR	[18]
Antigen/antibody	Optical method/ localized surface plasmon resonance(LSPR) & SPR	11	$1fgmL^{-1}$	[30]
Antibody/antigen biosensor	SPR	9.8	10 pg/ml	[31]
DNA /PCR	Electrochemical/DPV/Ag-nanoparticle	5	5 aM	[32]
DNA /Without and with PCR	DNA hybridization biosensor/Nanoporous Au electrode/ Ferrocene carboxylic acid	**8.9**	5 cycle/10^{-14}mol	This study

Figure captions:

Schematic 1. Schematic presentation of biosensor fabrication steps

Fig. 1. The Nyquist plots obtained in the presence of $[Fe(CN)_6^{3-/4-}]$ (5×10^{-4} M; 1:1) as a redox probe at a constant DC potential of O.C.P. for a) bare smooth gold CDtrode, b) NPGE, c) capture immobilization, d) target hybridization and e) probe hybridization.

Fig. 2. A) Gel electrophoresis of PCR amplified real samples related to the: a) DNA molecular marker for HBV (b) positive real sample 1, (c) Positive real sample2 d) negative real sample at 35 cycles of PCR. B) a) DNA molecular marker for HBV (b) positive real sample 1, d) negative real sample at 25 cycle of PCR.

Fig. 3. Comparison of current of DPV at a scan rate of 10 mVs^{-1} and amplitude 25 mV in for a) complementary target b) negative real sample c) real sample 1, d) real sample 2, e) PCR amplified real sample 1 and f) PCR amplified real sample 2 in 0.02 M of $NaClO_4$

Fig. 4. The current response of biosensor for the target at different concentration in DPV analysis at a scan rate of 10 mVs^{-1} and amplitude 50 mV in potential in 0.02 M of $NaClO_4$. Inset shows DPV for different concentration of complementary target

Schematic 1.

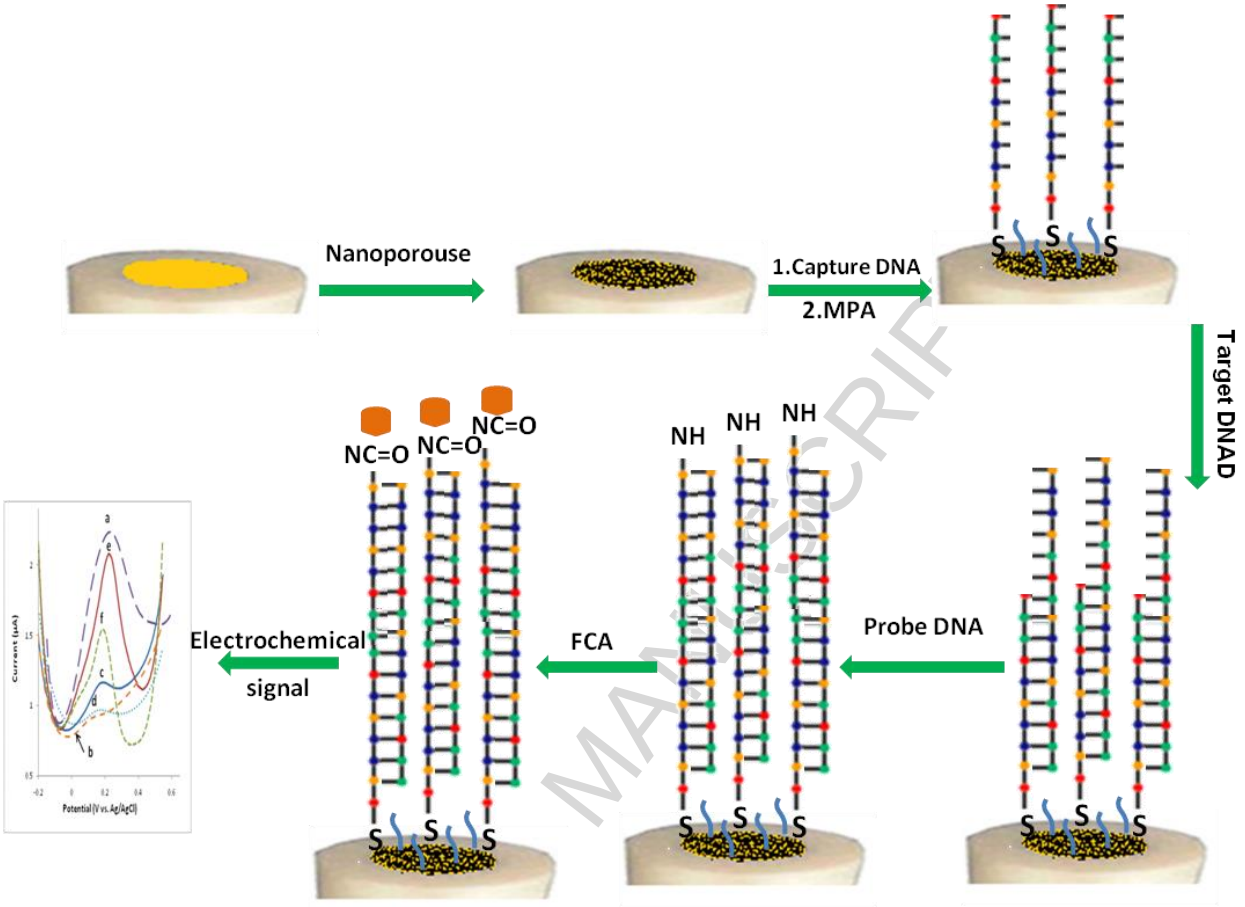


Fig. 1

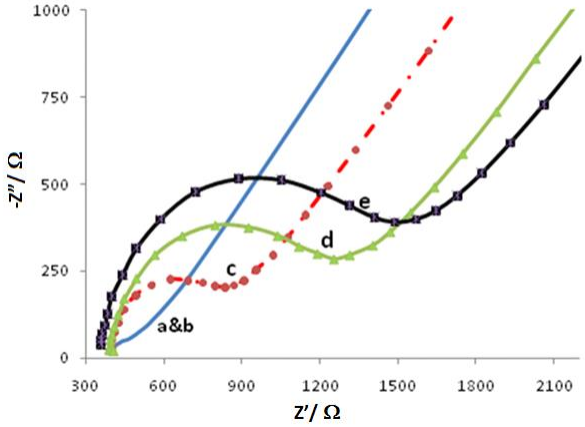


Fig. 2

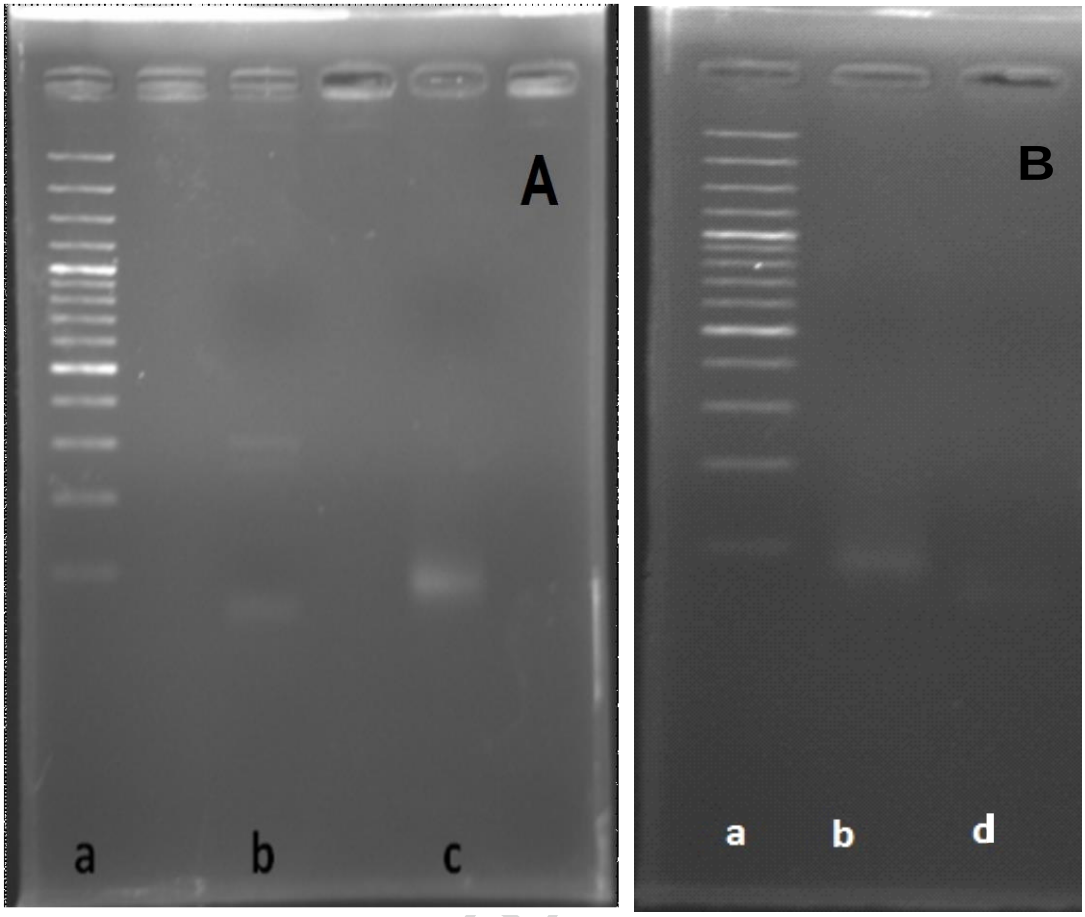


Fig. 3

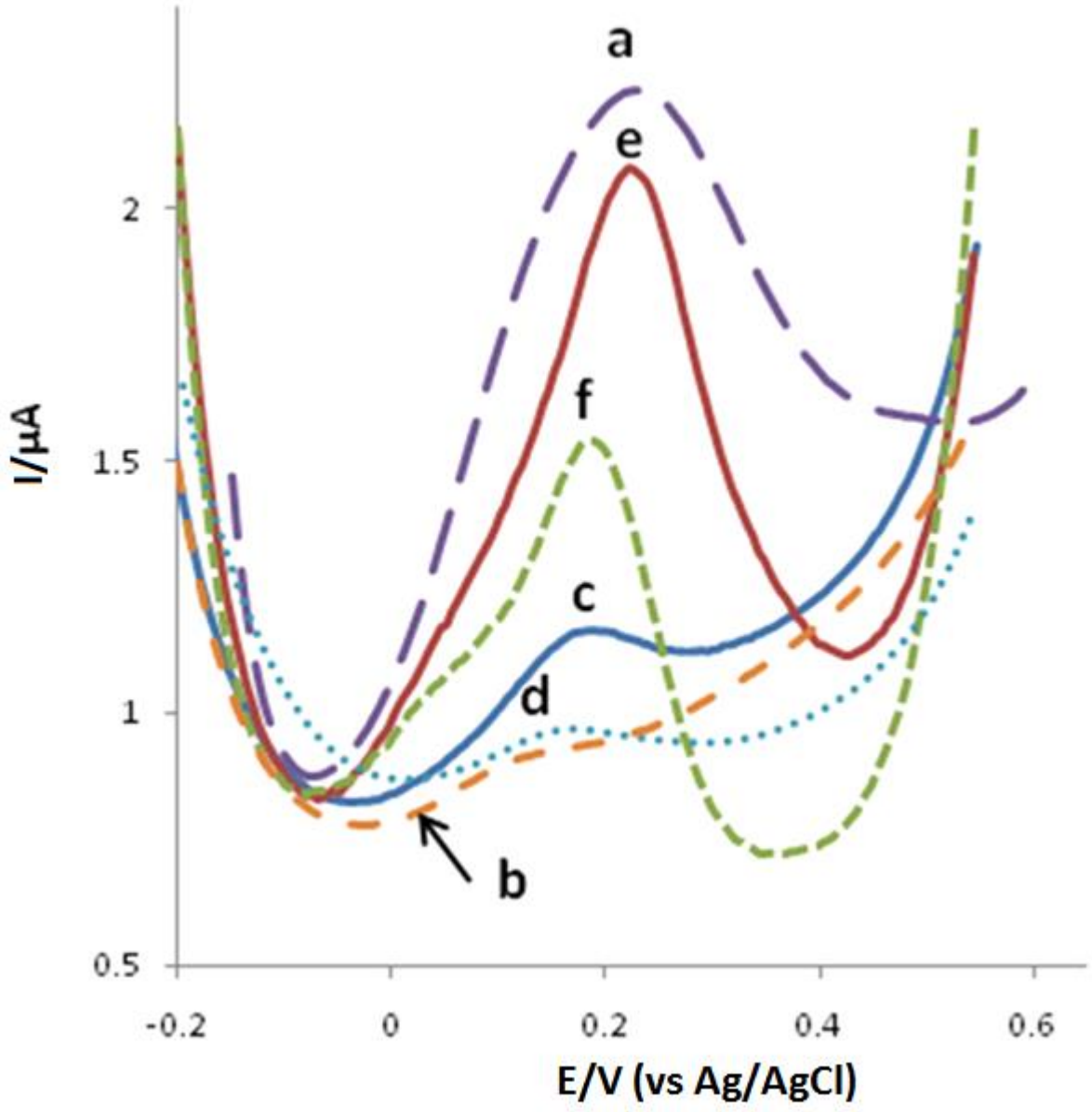
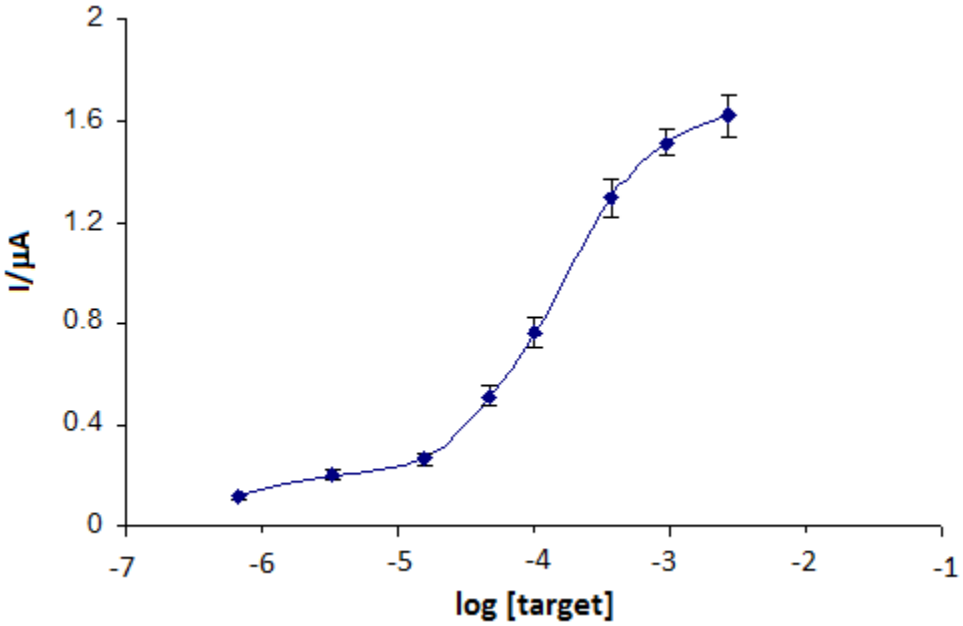


Fig. 4



Highlights:

- Amplified detection of hepatitis B using nanoporous gold electrode was introduced.
- DNA of virus was detected in blood samples by applying just 5 PCR cycles.
- The dynamic range was from 0.4 to 10 nmol of target with detection limit of 10 pmol.