Chemiluminescence Analysis for HBV-DNA Hybridization Detection with Magnetic Nanoparticles Based DNA Extraction from Positive Whole Blood Samples

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Molecular detection of HBV has a significant impact on prognosis and therapy of the disease. In this paper, a sensitive nucleic acid detection method of HBV was established taking advantage of magnetic nanoparticles (MNPs), chemiluminescence (CL) and polymerase chain reaction (PCR). HBV-DNA was extracted from hepatitis B positive human blood samples using MNPs adsorption method and biotin was labeled on the DNA segment after base insertion of biotin-dUTP in PCR. The biotinylated DNA segment was captured by amino probe immobilized on carboxyl MNPs and was detected by the chemiluminescence system of alkaline phosphatase catalyzing 3-(2′-spiroadamantane)-4-methoxy-4-(3′-phosphoryloxy) phenyl-1, 2-dioxetane. Different concentrations of HBV-DNA were detected under the optimized experiment conditions and the relevant CL intensity were obtained, which provided a novel research or clinic diagnosis method for the quantification detection of HBV-DNA.

Keywords: Magnetic Nanoparticles, Chemiluminescence, HBV-DNA, Hybridization.

1. INTRODUCTION

Infection with Hepatitis B Virus (HBV) presents challenges worldwide. Molecular detection of HBV is especially important for early diagnosis of the disease.1–2 As one of the most important nanostructured materials, magnetic nanoparticles (MNPs) have been widely applied in biological scopes due to their large specific surface area and excellent separation efficiency.3–21 At present, chemiluminescence, which is safe, sensitive and has a wide linear range, is regarded as a powerful tool for viral detection.22 Combining the merits of magnetic separation, signal amplification by biotin and chemiluminescent analysis, a new method was developed and optimized to detect HBV-DNA quickly and sensitively.

In this paper, MNPs adsorption method which offered swift adsorption and separation of DNA and excellent separation efficiency was applied to DNA extraction. The whole extraction procedure avoided centrifugation and other trifles. After several washings, contaminations that restrained the subsequent amplification could be removed and the method was suitable for automation.

Recently, we reported a method of chemiluminescence detection of synthetic sequence-specific oligonucleotides of HBV-DNA based on the hybridization at the surface of magnetic nanoparticles.16 In this research, biotin was labeled to the PCR product using biotin-dUTP as one of monomer applied in amplification of the target strand which was a conserved region of the HBV genome overlapping the genes encoding the X-protein and DNA polymerase.21 A specific DNA segment was picked out as a HBV probe from this conserved region and was modified to the surface of MNPs to capture biotinylated PCR segment of HBV-DNA. A series of optimization of conditions which would influence the effect of detection were investigated.

The genome DNA was extracted from hepatitis B positive human blood samples using MNPs adsorption method. The principle of MNPs-based DNA hybridization with
CL detection was depicted in Scheme 1. Synthetic amino probe was immobilized onto the carboxylated MNPs, followed by a hybridization reaction of MNPs with the biotinilated target DNA segment. Afterwards, streptavidin-ALP (alkaline phosphatase) was added and bound to the hybrid-conjugated MNPs through biotin-streptavidin reaction. Finally, the CL signal was obtained by the ALP-catalyzed 3-(2′-spiroadamantane)-4-methoxy-4-(3′-phosphoryloxy) phenyl-1, 2-dioxetane (AMPPD) reaction system, and the results were displayed in CL intensity.

2. MATERIALS AND METHODS

2.1. Materials

Involved materials are as follows: (3-aminopropyl) triethoxysilane (APTES), succinic anhydride (SA), Tris-HCl, N, N-Dimethylformamide (DMF), bovine serum albumin (BSA), MgCl₂, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), 2-(N-morpholino)ethanesulfonic acid (MES), sodium dodecylsulfate (SDS), salt-sodium citrate (SSC), Tween 20, and streptavidin-alkaline phosphatase (streptavidin-ALP), hybridization solution, and 3-(2′-spiroadamantane)-4-methoxy-4-(3′-phosphoryloxy) phenyl-1, 2-dioxetane (AMPPD). AXYGEN™ PCR Gel Purification Kit. All of them were purchased from Sangon Shanghai, PRC. SiO₂@Fe₃O₄ MNPs was Laboratory homemade.²⁵

Amino probe: 5′-CCGTGTGCACCTGCCTCACCTGCG-3′ (Sangon Shanghai, PRC); HBV positive human blood (The Second People’s Hospital of Nanjing). Involved apparatus are microtiter plate, thermocycler (BIOER, PRC), Victor X3 (PerkinElmer, USA).

2.2. Methods

2.2.1. Extraction of Genomic DNA from HBV Positive Human Blood and Biotin Labeled PCR

100 μL of human whole blood, 10 μL of Proteinase K (20 mg/mL) and 100 μL of lysis buffer (4 M GuHCl, 12 mM EDTA, 10 mM NaCl, 4% Triton X-100) were mixed in a 1.5 mL Eppendorf tube and incubated at 56 °C for 20 min to lyse cell structures. 1.2 mg of the SiO₂@Fe₃O₄ MNPs were mixed with 300 μL of binding buffer (3 M NaCl, 2% (v/v) PEG-6000) to form a suspension, which was then transferred to the lysisate followed by gentle inversion for three times and incubated at room temperature for 5 min. The MNPs with the adsorbed DNA were separated by magnetic separation and washed with 400 μL of 70% (v/v) ethanol for three times. Finally, TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH = 8) was added into the complex and the mixture was incubated in a 65 °C water bath for 5 min to elute DNA, which was in the supernatant.

The extracted DNA was used as template for the PCR production of biotin-dUTP labeled DNA segments. HBV-DNA was amplified according to the published PCR protocol which consisted of an initial step at 50 °C for 10 min, followed by 45 cycles of 15 s denaturation at 95 °C and 60 s at 60 °C.²⁴ PCR reaction mixtures contained 1 × Taq buffer 5 μL, 1.5 mM MgCl₂, 250 μM dATPs, dCTPs, dGTPs, 210 μM dTTPs, 40 μM biotin-11-dUTP, 0.5 μM of Primers, and 5 μL of Taq DNA polymerase. The primer pairs were 5′-CCGTCTGTCCTCCTCATCTG-3′ (forward) and 5′-AGTCCCAAGAGTTCTTCTTATGCAAAGACCTT-3′ (Reverse).

The gene of GAPDH was amplified with the procedure consisted of an initial step at 95 °C for 3 min, followed by 35 cycles of 45 s denaturation at 95 °C, 40 s annealing at 56 °C and 60 s extension at 72 °C, and a final elongation step of 7 min at 72 °C. PCR reaction mixture contained 5 μL 1 × Taq buffer, 1.5 mM MgCl₂, 250 μM dATPs, dCTPs, dGTPs, 210 μM dTTPs, 40 μM biotin-11-dUTP, 0.5 μM of Primers, and 5 μL of Taq DNA polymerase. The primer pairs were 5′-CCGAGTCAACGGATTTGGTCGTAT-3′ (forward) and 5′-AGTCCCAAGAGTTCTTCTTATGCAAAGACCTT-3′ (Reverse).

Products were visualized on agarose gels after electrophoresis.

2.2.2. MNPs Based Hybridization and Detection of HBV-DNA

MNPs were modified with APTES to get MNPs with amino-functional groups (MNPs-NH₂) which were then mixed with SA in the solution of DMF (SA/DMF) for 24 h to get MNPs with carboxylic-functional groups at their surface (MNPs-COOH).²⁵ MNPs-COOH with amino probe were obtained by incubating MNPs-COOH with amino probe in the solution of MES and activated by EDC. 10 μL of the composites (10 mg/mL) were placed in a 0.2 mL PCR tube, followed by a hybridization procedure, with a mixture of 30 μL solution, including 10 μL of hybridization solution, 19 μL of deionized water, and

Scheme 1. Schematic representation of MNPs-based HBV-DNA hybridization detection with chemiluminescence analysis.
1 μL of biotinylated PCR segment of HBV-DNA which had a complementary segment with the amino probe. Negative and blank control groups were performed by adding biotinylated PCR segment of GAPDH gene (an enzyme of ∼37 kDa that serves to break down glucose for energy and carbon molecules), and deionized water instead of biotinylated HBV-DNA. The process of hybridization was as follows: 10 min at 95 °C, followed by 50 °C for 1 h. The hybridized MNPs were purged two times with 100 μL of 2 × SSC-0.1% SDS, 0.1 × SSC-0.1% SDS, and washing buffer (50 mM Tris, pH 7.5, 0.15 M NaCl), respectively, and re-suspended in 60 μL of the blocking buffer containing 0.25% BSA. After 30 min, streptavidin-ALP (diluted to 1:1000 in Tris buffer) was added. The MNPs were re-suspended by gentle vortexing for 30 min, followed by washing with Tris buffer for several times. Finally, AMPPD solution (0.25 mM) was added into the complex and CL signals were recorded every other 5 min from the ensuing chemiluminescent reaction. Each treatment was repeated three times.

2.2.3. Effect of the Amounts of MNPs

Six kinds of amounts of MNPs were applied (6, 8, 10, 12, 14, 16 μL) in the experiment. All experiments include negative and blank control groups and other conditions were the same as stated in Section 2.2.2. The effect on CL intensity was showed according to the sample signals at 40 min from which the relevant negative signals were substracted.

2.2.4. Optimization of Carboxyl Concentration to Amino MNPs

The MNPs-NH₂ suspended in DMF were added into the same volume of DMF, which had different concentrations of SA (0.01, 0.1, 1, 10 and 100 mM). The other experiments were carried out according to the section 2.2.2. The effect on CL intensity was showed according to the sample signals at 40 min from which the relevant negative signals were substracted.

2.2.5. Effect of the Concentration of Specific Amino-Probe

Five different concentrations of amino probe in the solution of MES (0.02, 0.2, 2, 20 and 100 μM) were set up when modifying them on carboxyl MNPs with SA/DMF 0.1 mM. The other experiments were carried out according to the section 2.2.2. The effect on CL intensity was showed according to the sample signals at 40 min from which the relevant negative signals were substracted.

2.2.6. Temperature of Hybridization

A temperature gradient (41, 45, 49, 53, 57, 61 °C) was set up to check the effect of hybridization temperature on CL intensity. The other experiments were carried out according to the section 2.2.2. The effect on CL intensity was showed according to the sample signals at 40 min from which the relevant negative signals were substracted.

2.2.7. Detection of Different Concentrations of PCR Products

PCR products of HBV-DNA and GAPDH gene were purified from gel by AXYGEN™ PCR gel purification kit and the biotinylated PCR product quantified with ultraviolet spectrophotometer. Dilute the diluent to a series of different concentrations and detect them with MNPs based CL hybridization method with the optimized conditions. The other experiments were carried out according to the section 2.2.2. The effect on CL intensity was showed according to the signals of HBV-DNA and GAPDH gene at 40 min from which the relevant blank signals were substracted.

3. RESULTS AND DISCUSSION

3.1. Extraction of Genomic DNA from HBV Positive Human Blood and Biotin Labeled PCR

Two specific segments labeled with biotin were amplified from viral DNA templates extracted from HBV positive human blood samples by MNPs adsorption method. Figure 1 showed the gel electrophoresis of PCR products. The bands were clear without other interfered bands due to the purity of the DNA template. Lane 2 showed the band of HBV-DNA which was a 120 bp DNA segment. Lane 3 showed the GAPDH gene of 350 bp.

3.2. MNPs Based Hybridization and Detection of HBV-DNA

The dynamic statistics in Figure 2 showed that the signal experienced a rising in the first 35 min, and became
stable at 40 min. Also, the chemiluminescent intensity of HBV-DNA sample was distinctly higher than those of the negative GAPDH gene and blank group. It was indicated that the hybridization method of HBV-DNA detection had high specificity.

3.3. Effect of the Amounts of MNPs

The effects of different amounts of MNPs were showed in Figure 3. The chemiluminescent signal experienced an increase when the amounts of MNPs were lower than 80 μg while it almost changed to a flat curve when the amounts were higher. This phenomenon can be easily understood. More MNPs would provide more surface groups for linking but it would at the same time screen CL signals due to their black color. However, too much MNPs would produce severe steric hindrance and thus impede hybridization. It was indicated that 80 μg was a proper amount for HBV-DNA chemiluminescence detection.

3.4. Optimization of Carboxyl Concentration to Amino MNPs

Figure 4 showed that when SA concentration was lower than 0.1 mM, CL intensity went through an evident rise as the increase in SA concentration. 0.1 mM and 1 mM gave a higher CL intensity, and it began to decline sharply afterwards. Higher SA concentration would provide more linking positions for amino probes as well as negative charge repulsive interaction. In theory, with a given amount of amino-functional groups modified on the surface of MNPs, lower or higher concentrations of SA would be insufficient or congested, there must be a medial one which can provide a proper space for the subsequent connection with amino probes and the hybridization. Since more elaborate division of concentrations between 0.01 and 0.1 mM showed a rise in the CL signal intensity with the increase of SA concentrations while higher concentrations between 0.1 and 1 mM revealed a plateau, 0.1 mM was selected as the optimal carboxyl concentration for the following research.

3.5. Effect of the Concentration of Specific Amino-Probe

The effects of different concentrations of amino probe were showed in Figure 5. With the increase in the concentration of amino probe, the signal intensity demonstrated no obvious effect variation between 0.02 and 0.2 and it increased slowly first and sharply later and reached a peak when the concentration of amino probe was 100 μM. It was indicated that higher concentrations of amino probe would give rise to a more crowded population of the probes at the surface of MNPs, and thus a steric hindrance was produced, which rendered it more difficult for the subsequent hybridization. 100 μM can make a more efficient
connection of amino probes to MNP-COOH, and would benefit to the following hybridization detection process. However, it would be a considerable reagent if 100 UM was employed in the experiment because it was the mother solution of amino probe. Since 2 \( \mu \text{M} \) and 20 \( \mu \text{M} \) demonstrated no obvious difference, 2 \( \mu \text{M} \) of amino probe in the solution of MES (25 mM, pH 6) was selected as the optimal concentration for the following research.

3.6. Temperature of Hybridization

The hybridization temperature played an essential role in the specificity of amino probe catching the biotinylated DNA segment. Experiment showed that 45 °C was the optimal temperature for the hybridization (Fig. 6). Actually, a large scope of hybridization temperatures were checked out before reaching this range (from 41 °C to 61 °C), because the sensitivity of the proposed CL method depended largely on the thermal stringency hybridization and washing procedure which markedly distinguished the complementary sequences from the negative sequences. Statistics showed that with temperatures lower than 30 °C and higher than 70 °C, the final CL signal intensity were extremely low.

3.7. Detection of Different Concentrations of PCR Product

Biotinylated PCR product had high purity after purification with a value of 1.83 for \( A_{260}/A_{280} \). A series of different concentrations of PCR product were diluted and detected. Figure 7 showed the CL intensity of seven different concentrations of HBV-DNA and GAPDH gene. It was indicated that all of those concentrations of HBV-DNA had higher CL intensity in comparison with GAPDH gene and when log concentrations were lower than 4, the result should be considered as a negative one because the ratio of sample and negative group was lower than 2.1 according to the investigation by Zhao.26

Genomic DNA was extracted from HBV positive human blood samples by MNPs adsorption method which was simple and easy to automate. The PCR products of HBV-DNA and GAPDH gene were labeled with biotin. In our experiment, the MNPs modified with specific probe were used to capture the target HBV-DNA. Then, the complexes were combined with streptavidin-ALP. Finally, the chemiluminescent signals were detected by adding the substrate AMPPD. The results showed that the proposed CL detection method can markedly distinguish the HBV-DNA from GAPDH gene. Conditions that influence the detection were examined and optimal CL detection conditions were as follows: 2 \( \mu \text{M} \) of amino probe concentration, 0.1 mM of carboxyl MNP-COOH concentration, and 45 °C of hybridization temperature. A concentration of 10000 copies/mL of HBV-DNA could be detectable with this method. More conditions will be investigated to ensure a high sensitivity. Recently, magnetic nanoparticles have found more and more application in biomedical fields.27–41 Owing to the advantages of chemiluminescence and magnetic nanoparticles, this method to identify HBV-DNA is a novel and simple method, which can also be applied to the field of diagnosis of other DNA virus related diseases.

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References and Notes


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