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Microplate chemiluminescent assay for HBV DNA detection using 3-(10′-phenothiazinyl)propionic acid/N-morpholinopyridine pair as enhancer of HRP-catalyzed chemiluminescence

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ABSTRACT

A sensitive sandwich assay for hepatitis B virus (HBV) DNA detection based on use of commercial CL-ELISA microplates was developed. To reveal the target the covalent conjugate of reporter oligonucleotide and horseradish peroxidase (HRP) was synthesized. An employment of enhanced chemiluminescence reaction, where 3-(10′-phenothiazinyl)propionic acid/N-morpholinopyridine pair was used as enhancer of HRP-catalyzed chemiluminescence, permitted to measure the enzyme activity of the conjugate with high sensitivity. Under the favorable conditions the limit of detection and a linear range of the assay were 3 pM and 0.07–2.0 nM, respectively. The coefficient of variation (CV) for determination of HBV DNA concentrations within the working range was lower than 4%. The obtained results demonstrated that the developed assay had high sensitivity and precision.

Introduction

Detection of DNA sequences plays an important role in the prognosis of cancer, pathogen detection and forensic analysis [1,2]. Sensitive and specific assays for determination of nucleic acids find fast growing applications in analytical practice [3–7]. “Gold standard” assay should be rapid, real-time, simple and sensitive for quantification.

Methods for the detection of DNA based on hybridization reaction can be divided on homogeneous and heterogeneous ones. The homogeneous methods are rapid and simple [8,9]. Fluorescence as a pioneering tool has wide applications in homogeneous DNA assays. Some fluorescent dyes and nanoparticles are used as labels of DNA probes [10]. Presently fluorescent molecular beacons and fluorescence transfer energy transfer are widely used in nucleic acids detection [11,12]. Drawbacks of this approach is that expensive instrumentation is required for fluorescence measurement and the fluorescent assays show high background. Also, homogeneous DNA assays based on DNAzymes use were developed [13–15]. It should be noted that in homogeneous methods a matrix of real samples affects a precision of determination of analytes.

Alternative strategy of DNA detection is based on heterogeneous methods [16–19]. Although hybridization of probes immobilized on solid surfaces proceeds slower and, hence, the heterogeneous methods are usually time-consuming, they are more sensitive than homogeneous ones. By this, the heterogeneous methods for DNA detection attract much attention by analytical biochemists.

For last years the heterogeneous methods are developed usually by using different nanoparticles (NPs) as a solid carrier (gold nanoparticles [20–22], magnetic beads [20,23,24], carbon nanotubes [25], silica beads [26] etc.). The researchers argue a choice of NPs by the fact that particles with nanoscale dimensions allow increasing surface of solid carriers used for the immobilization of capture biomolecules in comparison with surface of traditional microplate wells and, hence, increasing analytical signals [27]. However, this property of NPs is important only when the detectability of used label is low, for example, in the case of enzyme mimics. When highly sensitive detection systems are used, a limitation of the surface of microplate wells is not essential and allows the development of assays with high sensitivity.

Despite the increased binding area of NPs, the activity of capture molecules may be compromised due to steric hindrance effect and random orientation when bound to NPs. Moreover, practical issues of nanomaterial complexes, including their complicated bioconjugation and separation, relatively low yield, slow diffusion kinetics, poor redispersion, high cost and potential environmentally unfriendly impact, limit their commercialization [28]. In contrast, microplates are free from these shortcomings. As experience with ELISA shows, commercially available microplates are a highly standardized product that allows to perform bioanalytical studies in high accuracy and precision.

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Furthermore, use of microplates allows easily to automate such analyzes.

Here, we describe a microplate sandwich method for hepatitis B virus (HBV) DNA detection based on application of HRP-DNA reporter conjugate whose enzyme activity was measured towards luminol/H_2O_2 substrates by chemiluminescent method. To improve the sensitivity of the detection method 3-(10′-phenothiazinyl)propionic acid/N-morpholinopyridine pair, one of the most potent enhancers of HRP-catalyzed chemiluminescence [29,30], was used.

Materials and methods

Chemicals

Horseradish peroxidase (HRP, isoenzyme c, RZ 3.0) was purchased from Sigma (USA) and used without further purification. Streptavidin was from PrimeBioMed (Russia). Luminol, Tween-20, Tris-HCl, sodium periodate, $NaBH_4$ and EDTA were from Sigma (USA). Sodium 3-(10′-phenothiazinyl)propionic acid (PPA) was synthesized as described previously [31]. 4-Morpholinopyridine (MORP) was from Aldrich (USA); H_2O_2 (30%) and bovine serum albumin were from ChimMed (Russia). All salts were of analytical or chemical purity grade.

All DNA oligonucleotides (HBV DNA, 5′-TGG GAG GAG TTG GGG GAG GAG ATT AGG TTA AAG GT-3'; biotinylated capture, 5'-(biotin) TT TTT ACC TTT AAC CTA ATC TCC TC-3'; amino-containing reporter, 5′-CCC CAA CTC CTC CCA TTA GAA G (NH_2)-3′ were purchased from Sintol (Russia).

The concentration of HRP was measured by using ε_{402} = 102 000 $M^{-1}cm^{-1}$ [32]. The H_2O_2 concentration was determined by monitoring A_{240} using ε = 43.6 $M^{-1}cm^{-1}$ [33]. The required dilutions of H_2O_2 were prepared daily.

Synthesis of conjugate of HRP and reporter oligonucleotide

Reporter oligonucleotide was conjugated with HRP as follows: HRP (4 mg/mL) was oxidized with sodium periodate (17 mM) for 20 min in the dark at 25 °C [34]. The enzyme samples were dialyzed against a 1 mM acetic buffer, pH 4.5 at 4 °C overnight. The solutions of oxidized HRP (240 μL) were mixed with 30 μL of the reporter oligonucleotide soluble in distilled water and 30 μL of 1 M carbonate buffer, pH 9.6, and stirred for 3 h at ambient temperature. To reduce the azomethine bonds, 10 μL $NaBH_4$ solution (4 mg/mL) was added to the reaction mixture. The obtained solutions were incubated for 1 h in the dark at 25 °C and then were intensively dialyzed against a 10 mM Tris-HCl, pH 8.0. To separate the obtained conjugates and excess of the reporter oligonucleotide we used ultrafiltration on Amicon Ultra 0.5 (30 K). The purified conjugates were stored at 5 °C.

Determination of HBV DNA by microplate chemiluminescent assay

The determination of target DNA was carried out using 96-wells black polystyrene microplates (High Binding, Corning, USA). The plates were coated by adding to each well 50 μL of streptavidin (1 μg/mL) dissolved in 50 mM carbonate buffer, pH 9.5 and incubated at 4 °C overnight. The plates were then washed using 10 mM Tris-HCl, pH 7.2 with 300 mM NaCl and 0.05% Tween 20 (TBST) three times. Subsequently, to block the unoccupied surface of microplate wells 100 μL of milk casein (1 mg/mL) in 10 mM Tris-HCl, pH 7.2 with 300 mM NaCl (TBS) were added to wells and incubated for 1 h at 37 °C. The plates were then washed using TBST three times. Then, 50 μL of the biotinylated capture oligonucleotide (10 nM), annealed preliminarily at 88 °C for 15 min and cooled to room temperature for 60 min, in TBS were added to wells. The plates were incubated for 1 h at 37 °C and then were washed as described above. Later 50 μL of the target DNA (0–2.0 nM), annealed preliminarily at 88 °C for 15 min and cooled to room temperature for 60 min and diluted with TBS, were added to wells and incubated for 1 h at 37 °C. The plates were washed using TBST three times. Then 50 μL of the conjugate of HRP and reporter oligonucleotide (10 nM) in TBS with 1 mg/mL casein were added to wells. The plates were incubated for 1 h at 37 °C and then were washed as described above. Finally, 100 μL of freshly prepared substrate solution (100 mM Tris, pH 8.3, containing 1 mM luminol, 5.2 mM 3-(10′-phenothiazinyl)propionic acid, 9.3 mM MORP, and 3 mM H_2O_2 [29]) were added to each well and stirred. Intensities obtained were monitored at room temperature on a microplate luminometer SpectraMax L (Molecular Devices, USA).

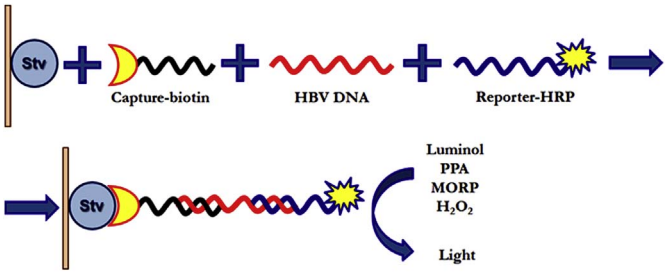

Fig. 1. Schematic illustration of the microplate assay for HBV DNA detection using chemiluminescent method of determination of HRP activity measured towards luminol and hydrogen peroxide in the presence of 3-(10′-phenothiazinyl)propionic acid (PPA) and N-morpholinopyridine (MORP).

Results and discussion

Design principle of DNA sensing

Scheme of the microplate sandwich assay for HBV DNA detection was presented in Fig. 1. Binding of capture oligonucleotide with microplate surface was performed in two steps. At the beginning streptavidin was adsorbed on surface of microplate wells and then the biotinylated oligonucleotide was bound with the immobilized streptavidin. To block unoccupied surface microplates were treated with 0.1% casein solution. Then the target DNA formed a duplex with the capture oligonucleotide.

To reveal the bound analyte in sandwich assays some researchers used streptavidin-biotin pair, despite the fact that they already used streptavidin-biotin interaction for coating of capture DNA [35–37]. In this case there is a possibility an interaction of biotinylated reporter not only with DNA target, but also with streptavidin immobilized on carrier surface. By this, such approach was abandoned by us. In our work we synthesized a covalent conjugate of HRP and DNA reporter which was partially complementary to the target DNA and used it as a revealing conjugate for HBV DNA detection.

Keeping in mind that microplate assays of DNA should use highly sensitive methods of label detection, to measure HRP activity of the revealing conjugate we applied an enhanced chemiluminescence reaction. In this method luminol and hydrogen peroxide were used as HRP substrates and 3-(10′-phenothiazinyl) propionic acid/N-morpholinopyridine pair as an enhancer. It should be noted that 3-(10′-phenothiazinyl) propionic acid/N-morpholinopyridine pair as well as 3-(10′-phenothiazinyl)propane-1-sulfonate/N-morpholinopyridine pair [29,30,38] are the most potent enhancers HRP-catalyzed chemiluminescence so far that makes this method extremely sensitive.

Optimization of microplate HBV DNA assay

To optimize the experimental conditions of performance of the proposed assay the concentration of biotinylated capture oligonucleotide varied in the reaction solution. As seen in Fig. 2, increasing the capture concentration increased the chemiluminescent intensity. Because at capture concentration higher 10 nM the change of the signal