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Research Paper

The detection of HBV DNA with gold nanoparticle gene probes

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Abstract

Objective: Gold nanoparticle Hepatitis B virus (HBV) DNA probes were prepared, and their application for HBV DNA measurement was studied. **Methods**: Alkanethiol modified oligonucleotide was bound with self-made Au nanoparticles to form nanoparticle HBV DNA gene probes, through covalent binding of Au-S. By using a fluorescence-based method, the number of thiol-derivatized, single-stranded oligonucleotides and their hybridization efficiency with complementary oligonucleotides in solution was determined. With the aid of Au nanoparticle-supported mercapto-modified oligonucleotides serving as detection probes, and oligonucleotides immobilized on a nylon membrane surface acting as capturing probes, HBV DNA was detected visually by sandwich hybridization based on highly sensitive aggregation and silver staining. The modified nanoparticle HBV DNA gene probes were also used to detect the HBV DNA extracted from serum in patients with hepatitis B. **Results**: Compared with bare Au nanoparticles, oligonucleotide modified nanoparticles had a higher stability in NaCl solution or under high temperature environment and the absorbance peak of modified Au nanoparticles shifted from 520nm to 524nm. For Au nanoparticles, the maximal oligonucleotide surface coverage of hexaethiol 30-mer oligonucleotide was (132 ± 10) oligonucleotides per nanoparticle, and the percentage of hybridization strands on nanoparticles was (22 ± 3%). Based on a two-probe sandwich hybridization/nanoparticle amplification/silver staining enhancement method, Au nanoparticle gene probes could detect as low as 10^{-11} mol/L composite HBV DNA molecules on a nylon membrane and the PCR products of HBV DNA visually. As made evident by transmission electron microscopy, the nanoparticles assembled into large network aggregates when nanoparticle HBV DNA gene probes were applied to detect HBV DNA molecules in liquid. **Conclusion**: Our results showed that successfully prepared Au nanoparticle HBV DNA gene probes could be used to detect HBV DNA directly. The detection-visuallized method has many advantages, including high sensitivity, simple operation and low cost. This technique has potential applications in many fields, especially in multi-gene detection chips.

Keywords: nanoparticle; Hepatitis B virus; Au

INTRODUCTION

Sequence-specific methods for detecting polynucleotides are critical to the diagnosis of genetic and pathogenic diseases. Most detection systems make use of the hybridization of a target polynucleotide with oligo- or polynucleotide probes containing covalently linked reporter groups [1-3]. Radioactive labels in polynucleotide detection offer exquisite sensitivity and are commonly used. However, radioactive probes create disposal problems requiring specially trained personne, and have a short shelf life. Increasingly, radioactive labels are being replaced by nonradioactive methods like fluorescent labels and chemiluminescence schemes[1,2]. Each of these strategies has advantages and disadvantages and no single method has gained supremacy.

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Nanotechnology is a fairly new research field that has been developing rapidly in recent years and produces an enormous influence in several research fields [4,5]. These advances in the research and application of nanotechnology in medical science, gives rise to a whole new scope for diagnosing many diseases. Particularly of interest during the last decade, has been the concept of using nanoparticles for DNA detection [6-12]. Mirkin for the first time reported a method for constructing DNA by way of a synthetically programmable assembler to guide the assembly of nanoparticles modified with complementary oligonucleotides into aggregates, which could be clearly observed by transmission electron microscopy (TEM)[13]. Elghanian *et al*[14] further reported a novel method for DNA detection based on a two-probe sandwich hybridization/nanoparticle amplification coloring technique. The above methods have become the foundation of the application of Au nanoparticle in the detection of composite oligonucleotides. However, DNA extracted from clinical samples (for example, serum) have not as yet been successfully detected with the above methods. It is necessary to further improve the sensitivity of detecting system to meet clinical requirements. More recent studies by Huber *et al* [15] have revealed that DNA and RNA samples can be detected by microarray-based methods, having their signal amplified by autometallography and then being measured via Au nanoparticle-mediated light scattering. However, high-cost equipment, complicated procedure and time-consuming limits it's further application.

In the present paper, by using Au nanoparticle gene probes, we established a method to detect the HBV DNA molecule extracted from hepatitis B patient on a nylon membrane by blot-hybridization or in liquid media by TEM.

MATERIALS AND METHODS

Reagents and instruments

$HAuCl_4$, trisodium citrate, mercaptoethanol and proteinase K were purchased from Sigma Chemical Co. (Milwankee, WI). Taq DNA polymerase, dNTPs and phenol were purchased from Promega Co. (Medison.WI). Other chemicals and biological reagents were of analytical reagent (AR) grade. The Nylon membrane was purchased from S&S Co. (Germany). Instruments include Beckman Coulter 21R centrifuge (USA), Philips FEI Tecnai $G^2$12 TEM (Netherlands), Beckman DU650 spectrophotometer (USA) and Shimadu RF-5301 fluorometer (Japan).

Serum samples

Serum samples were received from informed patients with hepatitis B from Tongji Hospital. The research protocol for clinical samples' collection was reviewed and approved by the hospital institutional review board of Tongji Hospital.

Primers and oligonucleotides

The PCR primers were designed with the aid of PRIMER software. The composite target was one part of HBV DNA PCR products. Oligo.1 and oligo.2 synthesized by Shanghai Sangon Bioengineering Co. Ltd were designed to be complementary to the composite target(***Tab 1***).

Tab 1 **Oligonucleitide sequence**

Name	Sequence (5′-3′)	Characteristic
oligonucleotide1(oligo.1)	5′ HS-$(CH_2)_6$-O-ttt ttt ttt tgt caa tgt cca tgc ccc aaa 3′	
oligonucleotide2(oligo.2)	5′ gcc acc caa ggc aca gct tgt ttt ttt ttt-O-$(CH_2)_3$-SH 3′	
Composite target DNA	5′ caa gct gtg cct tgg gtg gct ttg ggg cat gga cat tga c 3′	Complemetary with oligo.1 and oligo.2
FAM modified oligo.1(oligo.1-FAM)	5′ HS-$(CH_2)_6$-O-ttt ttt ttt tgt caa tgt cca tgc ccc aaa FAM 3′	The same sequence as oligo.1
FITC modified composite target DNA	5′ FITC caa gct gtg cct tgg gtg gct ttg ggg cat gga cat tga c 3′	The same sequence as composite arget DNA
Primer1	5′-tag gag gct gta ggc ata aat tgg t-3′	1774~1798
Primer2	5′-ggc gag gga gtt ctt ctt cta ggg g-3′	2364~2388

Extraction and PCR amplification of HBV DNA

Extraction of HBV DNA was carried out by mixing serum with proteinase K, and the mixture was then extracted with phenol/ chloroform/ isoamyl alcohol. 3 μl of solution containing desired HBV DNA (0.3 μg) was used as PCR template and added into the system containing 10 μM/each primer, 0.2 mM dNTPs, 2.5 mM Mg^{2+}, 0.4 u of Taq polymerase and 2 μl of PCR buffer (10×). Samples were denatured at 94℃ for 3 minutes followed by 31 cycles of 94℃ for 1 minute, 55℃ for 1 minute, and 72℃ for 2 minutes, and then a final extension for 10 minutes at 72℃. The PCR products were visualized following electrophoresis on 2%(w/v) agarose gel containing ethidium bromide.

Preparation of Au nanoparticle HBV DNA gene probes

Au nanoparticles were prepared by citrate reduc-

tion of $HAuCl_4$. Mirkin's method[13] was used for the preparation of nanogold-supported DNA detection probes, but ten T bases were inserted into mercaptohexyl or mercaptopropyl-derived oligonucleotide (oligo.1 or oligo.2) to avoid the steric hindrance which affects the hybridization of the probes with complementary oligonucleotides. The prepared probes were kept at 4℃. UV-vis spectra of these nanoparticle-supported DNA probes was assayed.

Fluorescence quantification of oligonucleotides loaded on nanoparticles and hybridization efficiency of DNA:nanoparticle conjugates

Oligo.1-FAM was first absorbed to the surface of Au nanoparticles following the protocol outlined above. DNA:nanoparticle conjugates were washed and centrifuged twice to ensure removal of any nonspecifically adsorbed molecules. The coverage of oligonucleotides loaded on nanoparticles was assayed according to Demer's method[16].

Oligo.2:nanoparticle conjugates were prepared as described above to get the maximal coverage. 5'6-FITC labeled targets, which were complementary to oligo.2, reacted for 24 h with oligo.2-modified surface under hybridization conditions. In this case, composite HBV DNA target concentration was adjusted to maintain the desired mole ratio of oligo.2 : target at 1 : 2,1 : 1,5 : 1 and 10 : 1, respectively. The hybridization efficiency was assayed according to the reference [17]. Hybridization efficiencies were calculated based on the concentration of the limiting DNA strands, for excess target:hybridization efficiency equals to the number of hybridized oligo.2/total oligo.2, while for limiting target, hybridization efficiency equals to the number of hybridization target/total target number.

Blot hybridization of nanoparticle-supported probes to detect HBV DNA

1 μl of oligo.1 (without-SH modified) was dropped onto a nylon membrane. The same amount of oligonucleotides with a random sequence(or PBS) was applied as the negative and blank control, respectively. The denaturation of oligo on the membrane was carried out at 120℃ for 30 minutes then blocked with prehybridization solution at 56℃ for 30 minutes. HBV PCR products were heated in a boiling water bath for 10 minutes and rapidly cooled in an ice bath for 90 seconds for denaturation. Subsequently,100 μl of nanoparticle-supported HBV-specific probes and 1 μl of composite target with different concentration ranging from 10^{-12} mol/L to 10^{-8} mol/L of pretreated HBV PCR products, were added to the hybridization solution. The membrane immersed in the solution was kept at 56℃ for 6 hours for hybridization. The membrane was washed two times(5 minutes each time) with 2 × SSC、0.1% SDS at room temperature and then washed two times (15 minutes each time) with 0.5 × SSC、0.1% SDS at 50℃. Lastly, the membrane was soaked in 0.2 M citric acid buffer (pH3.5) for 3 minutes and put in the developing solution for 10 minutes, then washed with de-ionized water and visually observed.

HBV DNA detection with nanoparticle-supported probes by TEM

20 μl of oligo.1 or oligo.2 modified Au nanoparticles were mixed, then 0.4 μl of composite target with different concentrations (ranging from 0.01 nmol/L to 0.1 μmol/L) of predenatured extracted HBV DNA was added into the mixture. Subsequently, the mixture was kept at 50℃ for 4 hours and then observed by TEM.

RESULTS

Characteristics of prepared nanoparticles and nanoparticle-supported probes

TEM showed that prepared Au nanoparticles were well-dispersed, having diameters of (10 ± 5)nm (***Fig 1***). Compared with bare Au nanoparticles, the absorbance peak of modified Au nanoparticles shifted from 520nm to 524nm(***Fig 2***) and oligonucleotide modified nanoparticles, had a higher stability in NaCl solution or under a high temperature environment. When heated or in solution of high salt concentration [(0.1~1)mM NaCl], bare nanoparticle colloids underwent irreversible particle growth reactions that resulted in their precipitation. In contrast, the DNA-modified nanoparticles were stable at an elevated temperature and in aqueous(0.1~1)M NaCl solutions for days. It is important to note that a high salt concentration is needed for DNA hybridization events.

Quantitation of surface coverage and hybridization efficiency of 30 base oligonucleotides on Au nanoparticles

Our experiment showed the quantitation of surface coverage,varied with the mole ratio of oligonucleotides/nanoparticles. On average, when the ratio was between 1 and 3000, the coverage increased as the ratio increased. There was no correlation however when the ratio was above 3000. The maximal coverage for Au nanoparticles was (132 ± 10) oligonucleotides per nanoparticle.

The coverage of hybridized complementary target

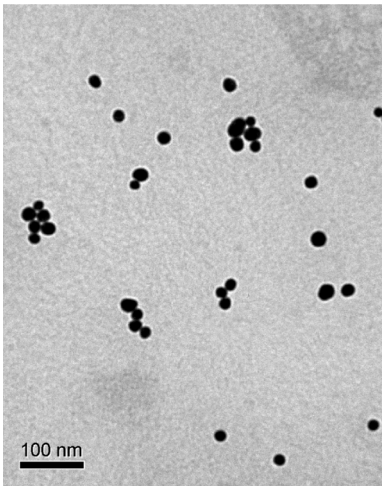
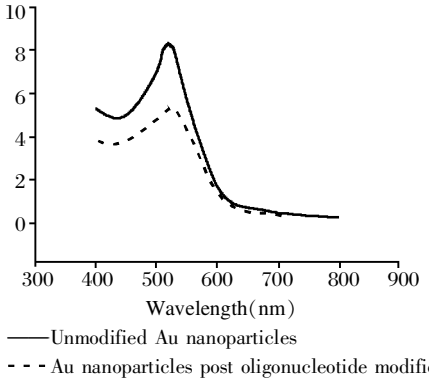


Fig 1 TEM image of Au nanoparticles



~10 nm diameter Au nanoparticles were suspended in water and Au nanoparticles in functionalized with 5′-hexanethiol 30-base oligonucleotides in 0.3 M NaCl, 10 mM PBS (pH 7). Abscissa and ordinate represent wavelenth and absorption, respectively.

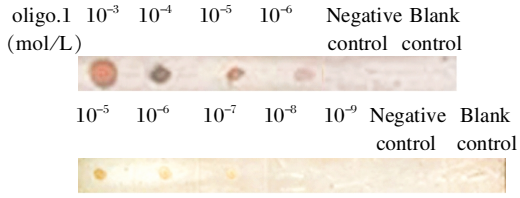
Fig 2 Comparison of visible spectra of Au nanoparticles before and post modification with oligonucleotides

was much lower for excess particle-bound oligo.1 (the mole ratio of target:oligo.1 was 1∶5 or 1∶10) as compared to experiments in which solution-phase complementary targets were in excess (the mole ratio of target:oligo.1 being 2∶1). The hybridization efficiency peaked with a mole ratio of 2∶1, which was (22 ± 3)% for Au nanoparticle. However, the quantitation of hybridization did not correlate with the increasing of the mole ratio. In the following hybridization tests, the maximal coverage and hybridization efficiency of nanoparticles was investigated.

HBV DNA detection by blot hybridization with nanoparticle-supported probes

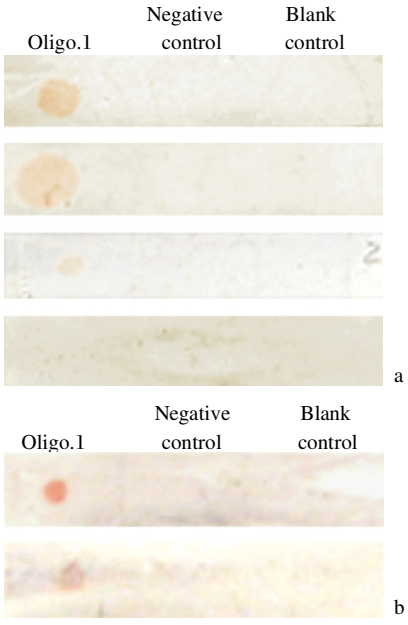
Different quantities of oligo.1, which worked as HBV capturing probes, were immobilized on a nylon membrane. 100 μl of nanoparticle-supported HBV-specific probes and 1 μl of 10^{-7} mol/L composite target were hybridized to the membrane in hybridization buffer with visual detection as shown in ***Fig 3***. It could be visually observed that the capturing probe focusing on the membrane displayed dark-colored spots against an un-dyed background, negative and blank control. It was found that when the capturing probe concentration was above 10^{-7} mol/L(1 μl). The intensity of silver-staining had a positive correlation with the quantity of the capturing probes on the nylon membrane, that is, the larger the quantity of the capturing probes, the darker the staining. The lowest allowable detection quantity of the capturing probes is 10^{-7}mol/L(1 μl)/spot. We choose 10^{-6}mol/L (1 μl)/ spot HBV capturing probe in the following hybridization tests on nylon membranes.

Fig 4a showed the lowest allowable detection concentration of composite targets for this nanogold amplification/silver staining(enhancing colouring method)



1 μl of different concentration of oligo.1, which worked as HBV capturing probes, was immobilized on nylon membrane.

Fig 3 Detection of HBV DNA with different concentration of capturing probes on nylon membrane

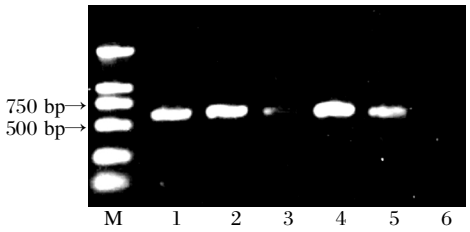


a: The allowable minimal detection concentration of composite target with Au nanoparticle-supported probes, the concentration of composite target from up to down was 10^{-9},10^{-10},10^{-11} and 10^{-12} mol/L, respectively; b:the detection of HBV DNA PCR products with Au nanoparticle-supported probes.

Fig 4 The detection of HBV DNA with nanoparticle-supported probes on nylon membrane

mentioned above. There was a direct correlation between the composite target concentration above 10^{-11} mol/L and the staining grade (the spot intensity) of spots. This implied that this method could be applied for the quantitative analysis of DNA.

614 bp of HBV DNA PCR products were obtained (***Fig 5***), which could also be detected with this method(***Fig 4b***).



M:DL-2000 Marker;1~5:PCR products of HBV DNA;6:Negative control.

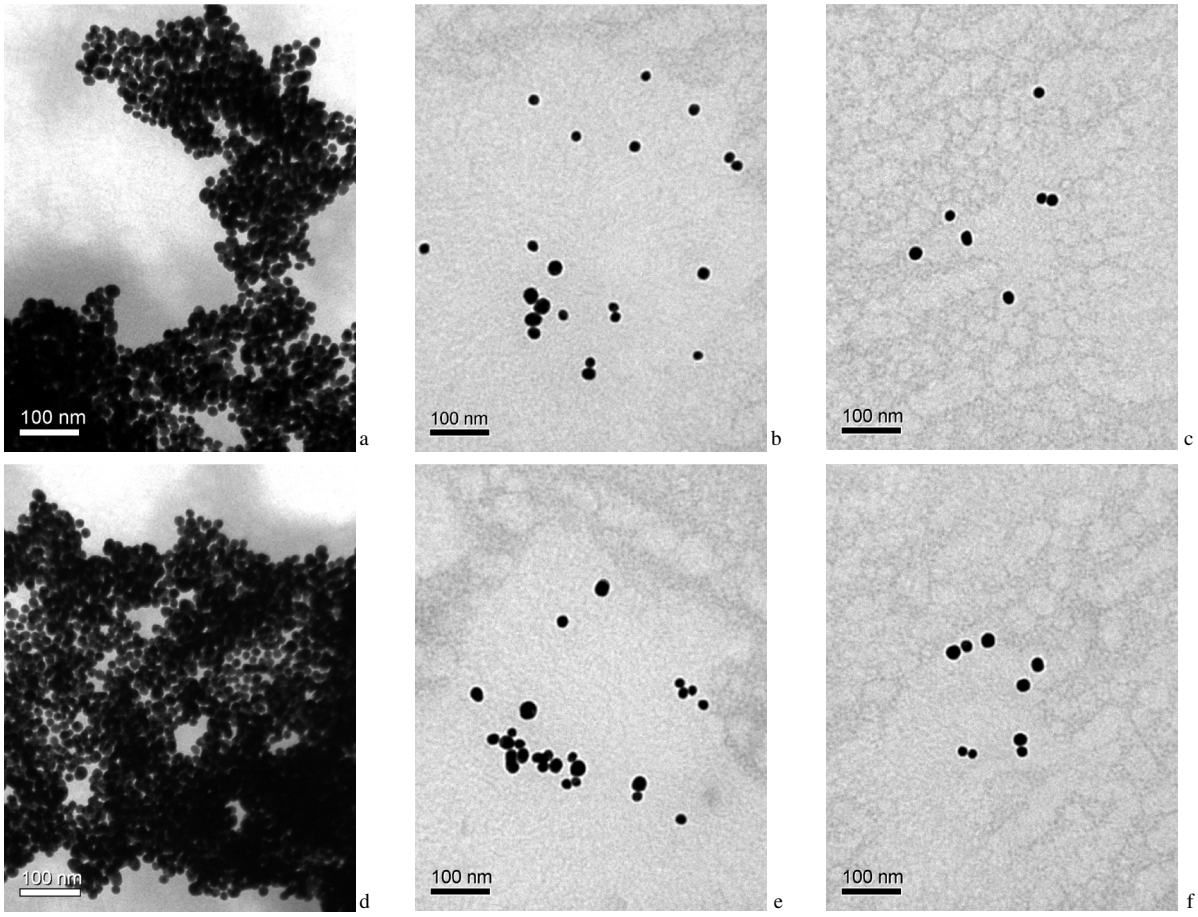
Fig 5 PCR products of HBV DNA extracted from the serum of hepatitis B patients

HBV DNA detection with nanoparticle-supported probes in liquid media by TEM

Composite targets as low as 10^{-16} mol/L for HBV DNA extracted from serum of hepatitis B patients were added to the detection system composed of nanoparticle-supported probes. TEM showed the nanoparticles self-assembled into massive aggregates (***Fig 6***). TEM also showed every particle within these aggregates was still intact and showed no evidence of fusing with other particles. The melting profiles of these aggregates illustrated were linked by DNA. There were only dispersed nanoparticles in the system of irrelevant negative control or blank control.

DISCUSSION

The study of molecular diagnostics has expanded rapidly in the past decade as it offers some highly sensitive methods for the detection of genetic disease[18]. Detection of specific DNA sequences plays a growing importance in gene diagnosis and evaluation of therapeutic effects of disease. In this study we focus on the analysis of DNA bases and genomic sequences. Techniques for DNA molecule detection



a:An assembly formed from Au nanoparticle HBV DNA probes with 0.1fmol composite DNA; b:Negative control of a, irrelevant DNA was added; c:Blank control of a; d:An assembly formed from Au nanoparticle HBV DNA probes with annealed HBV DNA extracted from serum of hepatitis B patient; e:Negative control of d, normal DNA was added; f:Blank control of d, normal serum was extracted with the same method as HBV DNA and the extraction was added.

Fig 6 TEM images of DNA-linked Au nanoparticle assemblies

usually include nucleic acid hybridization (Southern blot and Northern blot), restriction fragment length polymorphism, PCR and microarray technologies. These techniques have been adapted to meet many different needs. Advances in nanoscience are having a significant impact on many scientific fields, and are resulting in the development of a variety of important technologies. This impact is particularly great in the field of biodiagnostics, where a number of nanoparticle-based assays have been introduced for biomolecular detection, with DNA-functionalized nanoparticles being used as the target-specific probes.

Gold nanoparticles have long been used in biotechnology over the last 4 decades as immunocytochemical probes as well as biological tags, due to the small size and unique properties of these nanoparticles [19,20]. Oligonucleotides can be immobilized on gold nanoparticles through the Au-S bond to form a specific oligonucleotide probe, then through DNA hybridization we can explore disease-related gene mutations and the biomolecular recognition processes.

Nanoparticle-based assays provide an analysis of the unique biophysical properties displayed by nanoparticles, and have advantages over the above conventional detection methods. Some of the advantages include the assays' PCR-like sensitivity, their selectivity for target sequences and their time efficiency.

Our work has shown how oligonucleotides, and presumably other biomolecules(e.g.,proteins) can be used to modify the surface of Au nanoparticles, thereby imparting useful biorecognition properties to them. Au nanoparticle-supported probes detect HBV DNA on the nylon membrane by blot hybridization or in liquid media by TEM. TEM shows that nanoparticles assemble into large network aggregates, when nanoparticle HBV DNA gene probes are applied to detect HBV DNA molecules (extracted from a hepatitis B patient). While high-cost TEM limits its wide usage, this method reveals a PCR-like sensitivity. Based on a two-probe sandwich hybridization/nanoparticle amplification/ silver staining enhancement method, Au nanoparticle gene probes could detect as low as 10^{-11} mol/L composite HBV DNA molecules on a nylon membrane, and PCR products of HBV DNA visibly. However, the detection sensitivity of blot hybridization on nylon membrane with nanoparticle probes, is not sufficient to detect HBV DNA extracted from the hepatitis B patient directly. For this to occur, detection sensitivity needs to be further improved in order to detect clinical samples directly when using this method.

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