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Detection of Hepatitis B Virus (HBV) DNA at femtomolar concentrations using a silica nanoparticle-enhanced microcantilever sensor

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ABSTRACT

We report Hepatitis B Virus (HBV) DNA detection using a silica nanoparticle-enhanced dynamic microcantilever biosensor. A 243-mer nucleotide of HBV DNA precore/core region was used as the target DNA. For this assay, the capture probe on the microcantilever surface and the detection probe conjugated with silica nanoparticles were designed specifically for the target DNA. For efficient detection of the HBV target DNA using silica nanoparticle-enhanced DNA assay, the size of silica nanoparticles and the dimension of microcantilever were optimized by directly binding the silica nanoparticles through DNA hybridization. In addition, the correlation between the applied nanoparticle concentrations and the resonant frequency shifts of the microcantilever was discussed clearly to validate the quantitative relationship between mass loading and resonant frequency shift.

HBV target DNAs of 23.1 fM to 2.31 nM which were obtained from the PCR product were detected using a silica nanoparticle-enhanced microcantilever. The HBV target DNA of 243-mer was detected up to the picomolar (pM) level without nanoparticle enhancement and up to the femtomolar (fM) level using a nanoparticle-based signal amplification process. In the above two cases, the resonant frequency shifts were found to be linearly correlated with the concentrations of HBV target DNAs. We believe that this linearity originated mainly from an increase in mass that resulted from binding between the probe DNA and HBV PCR product, and between HBV PCR product and silica nanoparticles for the signal enhancement, even though there is another potential factor such as the spring constant change that may have influenced on the resonant frequency of the microcantilever.

1. Introduction

Microcantilevers have emerged as viable biosensors demonstrating the prominent performance (Waggoner and Craighead, 2007; Fritz, 2008). Recently, the structures and materials of microcantilever have been improved due to advances in the micro/nano electromechanical system (MEMS/NEMS), and the applications of microcantilevers have been significantly expanded due to their utilization in the nano/biotechnology field. Microcantilevers have outstanding features such as high sensitivity and label-free detection, and have been used successfully for DNA hybridization (Wu et al., 2001a,b), immunoassays (Wu et al., 2001a,b) and particle detection including virus, bacteria and cell (Ilic et al., 2004). In particular, the static-mode microcantilevers have played a significant role in detecting DNA hybridization, and this mode of detection has been actively used for specific hybridization and single nucleotide polymorphisms (SNPs) detection (Hansen et al., 2001). Recently, dynamic-mode millimeter-sized cantilevers have been used for label-free detection of oligonucleotides at extremely low concentrations (Rijal and Mutharasan, 2007). In addition, there was a report on the sensitive DNA detection which used nanoparticle probe and silver enhancement to amplify the signal (Su et al., 2003).

Hepatitis B virus (HBV) infection is one of the most severe viral infectious diseases worldwide, with an estimated 400 million people chronically infected (Mao et al., 2006; Kim et al., 2007). Approximately, 70% of hepatocellular carcinoma cases are developed from the chronical hepatitis type B. HBV also has a capacity to escape immune surveillance by mutations of the structural genes, which encode epitopes that are recognized by the immune system, resulting in a quasi-species population. Because of the

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clinical reasons described above, it is very important to diagnose HBV at the early stage before chronicity and fatal mutations occur.

In this paper, we report Hepatitis B Virus (HBV) DNA detection using a silica nanoparticle-enhanced dynamic microcantilever biosensor. A 243-mer nucleotide of HBV DNA precore/core region was used as the target DNA. For this assay, the capture probe on the microcantilever surface and the detection probe conjugated with silica nanoparticles were designed specific for the target DNA. To efficiently detect the HBV target DNA using silica nanoparticle-enhanced DNA assay, the size of silica nanoparticles and the dimension of microcantilever were optimized by directly binding the silica nanoparticles through DNA hybridization. Finally, the correlation between the applied nanoparticle concentrations and the resonant frequency shifts of the microcantilever was discussed in order to validate the quantitative relationship between mass loading and resonant frequency shift.

2. Experimental

2.1. Device fabrication and resonant frequency measurement

The piezoelectric actuating layer (PZT) embedded microcantilevers were fabricated through a micro-machining process. PZT-microcantilevers do not require external actuators because of their direct signal transmission, which means that they have higher sensitivity than mono-layered microcantilevers that require an external actuator. Our microcantilevers consist of multi-layers with the following structure: Ta/Pt/PZT/Pt/SiO_2 on a SiN_x supporting layer. A process for the PZT microcantilever fabrication is reported earlier (Hwang et al., 2006, 2004; Lee et al., 2004, 2005). Through the micro-fabricated process, rectangular shaped microcantilevers with two different dimensions were fabricated. Their dimensions were $50\,\mu m \times 150\,\mu m \times 1.75\,\mu m$ and $30\,\mu m \times 90\,\mu m \times 1.75\,\mu m$ (width × length × thickness), respectively. To minimize the nonspecific binding of DNA, the microcantilevers were treated with PEG-Si (2-[methoxy(polyethyleneoxy)propyl]trimethoxysilane, Gelest, Inc., USA). The microcantilevers were dipped in a 100 mM PEG-Si solution (in 99.5% EtOH) for 6 h, and then they were cured for 3 min at 110 °C to firm the PEG layer on the microcantilever surface (Backmann et al., 2005).

The reference resonant frequency of the microcantilever was measured before and after hybridization using an impedance analyzer (4294A, Agilent, USA) in a temperature-controlled chamber with a relative humidity controlled environment of 20%. The resonant frequency of the microcantilevers was measured by monitoring the phase angle of the impedance (Hwang et al., 2007).

2.2. Preparation of HBV target DNA probes

The clinical serum, which was stored at −70 °C before using, was collected in Korea, and the HBV-positive serum was confirmed using a nested polymerase chain reaction (PCR). The DNA was extracted from 140 μL serum with QIAamp DNA Mini-Kit (Qiagen, Germany), as recommended by the manufacturer. The HBV target probe used for hybridizing the HBV pre C, C region was generated by nested PCR using the outer primer (sense: GGCATGGACATTGACCC(G/T)TATAA; anti-sense: CTAATTCCCTGGATGCTGG(G/A)TCT, 256 bp) and inner primer (sense: CATTGACCC(G/T)ATAAAGAATT; anti-sense: TCCCTGGATGCTGG(G/A)TCTTCCAAA, size: 243 bp). The 20 μL reaction mixture contained 0.5 U of Taq polymerase (Supertaq, England) and 10× PCR reaction buffer with 1.5 mM $MgCl_2$, 200 μM dNTP and 10 pmol of each primer. A 4.5 μL DNA sample was added to the PCR mixture. The PCR conditions were 5 min at 94 °C followed by 35 cycles of 45 s at 94 °C, 45 s at 56 °C, and 45 s at 72 °C in a 9600 thermal cycler (PerkinElmer, USA). A final extension step of 72 °C for 5 min was included. The first PCR product of 1.5 μL was amplified using nested PCR primers (sense: CATTGACCC(G/T)ATAAAGAATT; anti-sense: TCCCTGGATGCTGG(G/A)TCTTCCAAA, size: 243 bp) for an additional 25 cycles of 45 s at 94 °C, 45 s at 57 °C, and 45 s at 72 °C. The PCR product was purified using a QIAquick PCR purification kit (Qiagen, Germany), as recommended by the manufacturer.

Table 1
DNA sequences for the experiments.

cDNA1 (37mer)	5′-HS-T_{10} CTT TCC TTC TAT TCG AGA TCT CCT CGA-3′
dDNA1 (37mer)	3′-GAA AGG AAG ATA AGC TCT AGA GGA GCT T_{10}-NH_2-5′
cDNA2 (35mer)	5′-HS-T_{10} TGG AGC TTC CGT GGA GTT ACT CTC T-3′
HBV Target DNA (243mer)	5′-TCC CTG GAT GCT GGG TCT TCC AAA TTA CTT CCC ACC CAG GTG GCC AGA TTC ATC AAC TCA CCC CAA CAC AGA ATA GCT TGC CTG AGT GCT GTA TGG TGA GGT GAA CAA TGT TCC GGA GAC TCT AAG GCC TCC CGA TAC AAA GCA GAG GCG GTG TCG AGG AGA TCT CGA ATA GAA GGA AAG AAG TCA GAA GGC AAA AAA GAG AGT AAC TCC ACG GAA GCT CCA AAT TCT TTA TAC GGG TCA ATG-3′
dDNA2 (35mer)	5′-ATC TGG CCA CCT GGG TGG GAA GTA A T_{10}–NH_2-3′

All DNA probes are HPLC grade and over 1 OD.

2.3. Design of capture and detection DNA probes

All of the HPLC grade single-stranded DNA (ssDNA) probes were purchased from Bioneer (Korea). The details are shown in Table 1. To covalently immobilize the cDNA on the cantilever surface, the 5′-ends of two cDNAs (cDNA1 and cDNA2) were modified into thiol groups, and TTTTTTTTTT (T_{10}) was inserted between the thiol termini and cDNA sequence to increase the hybridization efficiency by creating space between the complementary DNA of cDNA and the surface (Herne and Tarlov, 1997). 35mer cDNA2 and 35mer dDNA2 were selected for hybridization with the HBV target DNA as presented in Table 1. Also, dDNA1 was designed as a complementary sequence of cDNA1. The 5′-end of two dDNAs (dDNA1 and dDNA2) were modified into amine groups for conjugation to the SiNPs.

2.4. Immobilization of capture probes

The following procedure was used to immobilize the thiolated cDNA. Cr/Au layers (10 nm/50 nm) were deposited on the bottom side of the microcantilever using an e-beam evaporator. The Cr layer was used as a adhesive layer between the SiN_x layer and the Au layer. Freshly Au-coated microcantilevers were immersed in 1 μM cDNA solution (TE buffer (Tris–EDTA buffer, pH 8.0)) for 3 h at RT, followed by spacer (5 mM HSC_{11}-EG_3-OH, Cos Biotech, Korea) backfilling for 90 min, which helps the formation of the self-assembled monolayer (SAM).

2.5. Preparation of silica nanoparticles–DNA conjugate

The detection probes were conjugated with 50 nm and 140 nm sized-SiNPs containing RITC (rhodamine B isothiocyanate) in their matrices. The prepared SiNPs were conjugated with the detection probes (called as dDNA-SiNPs). A 1 mg of SiNPs was dispersed in 1 mL of a MES buffer (2-(N-morpholino)ethanesulfonic acid) solution (pH 6.0). A 50 μL of 50 mg/mL EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) and 50 μL of 50 mg/mL NHS (*N*-hydroxysuccinmide) were then added to the