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Chapter 19

Protocol for the Use of a Silica Nanoparticle-Enhanced Microcantilever Sensor-Based Method to Detect HBV at Femtomolar Concentrations

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Abstract

DNA sensors that are capable of detecting specific DNA sequences in a bio-sample have recently been highlighted as a powerful and sensitive approach to detect infectious diseases caused by pathogens such as viruses and bacteria. Generally, DNA samples extracted from biological fluids are amplified by PCR prior to analysis by DNA sensors or directly analyzed by DNA sensors equipped with a signal amplification process. Nanoparticles have recently been used to amplify the sensor signal and have been shown to play an important role in improving the sensitivity of mechanical resonating sensors. This is because the weight of the nanoparticle can increase the change in the resonance response of the mechanical sensor since this signal change is closely related to mass. Here, we introduce an experimental method to detect HBV at femtomolar concentrations using a silica nanoparticle-enhanced microcantilever resonating sensor. This method includes the preparation of detection probe-conjugated silica nanoparticles, immobilization of capture probe on the microcantilever sensor and sandwich type detection of HBV DNA.

Key words: Microcantilever sensor, Silica nanoparticles, HBV DNA, Sensitivity enhancement, Sandwich assay

1. Introduction

Hepatitis B virus (HBV) infection is one of the most severe viral infectious diseases worldwide, with an estimated 400 million people chronically infected. Approximately, 70 % of hepatocellular carcinoma cases develop from the chronic hepatitis type B. HBV also has the capacity to escape immune surveillance by mutating structural genes encoding epitopes recognized by the immune system, resulting in a quasi-species population (1, 2). Because of the clinical reasons described above, it is highly important to diagnose HBV at the

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early stage before chronicity and fatal complications occur (3, 4). The diagnosis of viral infectious diseases has been mainly performed by quantitative DNA analysis methods including the use of polymerase chain reaction (PCR) amplification, transcription-mediated amplification (TMA) and branched DNA (bDNA) amplification (5–8). These techniques are based on detecting the multiplied DNA molecules or applying specific amplification probes to DNA captured on probe DNA. Here, we developed an experimental method to detect HBV at femtomolar concentrations using a silica nanoparticle-enhanced microcantilever sensor (9, 10). A 243 bp sequence of HBV DNA precore/core region was used as the target DNA. In this assay, the capture probe on the microcantilever surface and the detection probe conjugated to silica nanoparticles were specifically designed for the target DNA. The applied nanoparticle concentrations and the resonant frequency shifts of the microcantilever were strongly correlated and the quantitative relationship between the mass and resonant frequency shift was validated.

2. Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a resistance of 18 MΩ cm at 25 °C), analytical grade solvents, and reagents with purity of >99 %. Prepare reagents at room temperature and store all products in the dark at 4 °C (unless indicated otherwise).

2.1. Silica Nanoparticle Components

1. Rhodamine B isothiocyanate (RITC), 3-aminopropyltriethyoxysilane (APTS), *N*,*N*′-dimethylsulfoxide (DMSO), tetraethoxyorthosilicate (TEOS), succinic anhydride, *N*,*N*-diisopropylethylamine (DIEA), ammonium hydroxide (NH_4OH, 25 %), ethanol (EtOH, 99.0 %), and water can be purchased from Sigma-Aldrich.
2. 1.5 mL conical tubes (e.g., Eppendorf Safe-Lock Microcentrifuge Tubes).
3. Magnetic stirrer.
4. Triangular and egg-shaped magnetic bars can be purchased from Thermo Fisher Scientific.
5. 50 mL conical tubes (e.g., BD Falcon™ 50 mL conical tubes).
6. Microcentrifuge (e.g., Eppendorf Refrigerated Microcentrifuge Model 5417 R).
7. 2-[Methoxy(polyethyleneoxy)propyl]trimethoxysilane (MPPTS): 9–12 C_2H_4O groups (e.g., Gelest, Morrisville, PA, USA).

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2.2. Components for Detection Probe Conjugation

1. MES buffer (pH 6.0, 50 mM): 50 mM 2-(*N*-morpholino) ethanesulfonic acid. Dissolve 195.2 g of MES in 0.9 L water and adjust pH with 1 N NaOH. Adjust volume to 1 L with additional water.
2. Detection probe (100 μM): Dissolve 0.05 μmol of the detection probe (5′-ATCTGGCCACCTGGGTGGGAAGTAAT$_{10}$-(NH_2)-3′) in 500 μL of water and keep it in a freezer (−20 °C). Thaw immediately before use.
3. 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinmide (NHS) can be purchased from Sigma-Aldrich.
4. Phosphate buffered saline (PBS): 150 mM PBS, pH 7.4. Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 0.9 L H_2O and adjust pH with 1 N NaOH and 1 N HCl. Adjust volume to 1 L with additional water.

2.3. Components for Capture Probe Immobilization

1. Lead zirconate titanate (PZT)-embedded microcantilever: Use fabricated piezoelectric (PZT) thin layer-embedded microcantilevers (see Note 1). Sequentially deposit the chrome and gold layer with thickness of 10 and 50 nm using an e-beam evaporator (SME-200E, ULVAC Corporation, Japan) (see Note 2).
2. 48-Well plate (e.g., BD Falcon™ 48-well Multiwell Plate).
3. Capture probe (1 μM): Dissolve 0.05 mol of capture probe (5′-(SH)-(CH_3)$_{18}$-T$_{10}$TGGAGCTTCCGTGG AGTTACTCTCT-3′) in 500 μL of water and keep it in a freezer (−20 °C). Before use, thaw the solution (1 mM) immediately and mix 1 μL with 990 μL of TE buffer (see Note 3).
4. TE buffer (pH 8.0, 10 mM): Dissolve 1.58 g of Tris–HCl and 0.292 g of *N*,*N*,*N*′,*N*′-ethylenediaminetetraacetate (EDTA) in 0.9 L water and adjust pH with 0.1 N NaOH. Adjust volume to 1 L with additional water.
5. Orbital shaker: Compact open air shakers (Eppendorf Inc., Hamburg, Germany).
6. Backfiller (5 mM): Dissolve 16.8 mg of (11-mercaptoundecyl) tri(ethylene glycol) (e.g., Sigma-Aldrich) in 10 mL of EtOH freshly before use.
7. Ethanol 95 %.
8. Water.
9. Nitrogen gas: 99.9 %.
10. Impedance analyzer (e.g., Agilent Technologies Model 4294A).

2.4. HBV Target DNA for Standard Curve

1. HBV PCR product (243 bp) of 2.31 nM (see Note 4).
2. TE buffer (pH 8.0, 10 mM): Dissolve 1.58 g of Tris–HCl and 0.292 g of *N*,*N*,*N*′,*N*′-EDTA in 0.9 L water and adjust pH with 0.1 N NaOH. Adjust volume to 1 L with additional water.

3. Orbital shaker: Compact open air shakers (Eppendorf Inc., Hamburg, Germany).
4. Impedance analyzer (e.g., Agilent Technologies Model 4294A).

2.5. HBV DNA Samples

1. HBV PCR products of clinical sera with suspected HBV infection (see Note 4).

3. Methods

All procedures are performed under room temperature and room humidity if not specified.

3.1. Preparation of Dye-Incorporated Silica Nanoparticle

1. Dissolve 2.2 mg (4 μmol) of RITC and 1.88 μL (8 μmol) of APTS (RITC:APTS = 1:2) with 0.5 mL DMSO in a 1.5 mL conical tube and stir the solution with egg-shaped magnetic bar on the magnetic stirrer for 12 h at room temperature.
2. Transfer this solution to a round-bottomed glass flask containing 5 mL of TEOS, 1 mL of NH_4OH, 10 mL water, and 78 mL of EtOH. Stir the solution with egg-shaped magnetic bar on the magnetic stirrer for 12 h at room temperature (see Note 5).
3. Transfer the final solution into the 50 mL conical tube, centrifuge the solution at 24,000 × *g* for 10 min, and discard the supernatant. Pour 50 mL of EtOH into the tube and re-disperse the pink-colored pellet of RITC-incorporated silica nanoparticles (RITC-SiNPs) (see Note 6). Repeat the centrifugation and dispersion steps five times (~100 nm silica nanoparticles).
4. Transfer 55 mg of RITC-SiNPs dispersed in 1 mL ETOH to a 1.5 mL conical tube (see Note 6) and add 55 mg of MPPTS and 4.4 mg of APTS. Stir the solution using a triangular magnetic bar on the magnetic stirrer for 12 h. Centrifuge the solution at 24,000 × *g* for 10 min and discard the supernatant. Pour 1 mL of EtOH into the tube and re-disperse the pellet of nanoparticles (RITC-SiNP-PEG600/NH_2) (see Note 6). Repeat the centrifugation and dispersion steps five times.
5. Transfer 18 μL of the RITC-SiNP-PEG600 solution into a new 1.5 mL conical tube and add 482 μL EtOH. Add 2.5 mg of succinic anhydride and 4.1 μL of DIEA into the solution. Stir the solution with triangular magnetic bar on the magnetic stirrer for 12 h at room temperature. Centrifuge the solution at 24,000 × *g* for 10 min and discard the supernatant. Pour 1 mL of EtOH into the tube and re-disperse the pellet of silica nanoparticles (RITC-SiNP-PEG600/COOH). Repeat the centrifugation and dispersion steps five times.

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3.2. Conjugation of the Detection Probe to Silica Nanoparticles (See Fig. 1a)

1. Disperse 1 mg of RITC-SiNP-PEG600/COOH in 1 mL of MES buffer.
2. Add 50 μL of 50 mg/mL EDC and 50 μL of 50 mg/mL NHS into the solution to activate carboxylic acid group on of RITC-SiNP-PEG600/COOH.
3. Stir the solution using triangular magnetic bar on the magnetic stirrer for 1 h at room temperature.
4. Add 50 μL of 100 μM detection probe and stir for 2 h at room temperature (see Table 1, detection probe).
5. Centrifuge the solution at 24,000 × *g* for 10 min and discard the supernatant. Pipette 1 mL of 150 mM PBS into the tube and re-disperse the cake of silica nanoparticles. Repeat the centrifugation and dispersion steps five times (see Note 7).

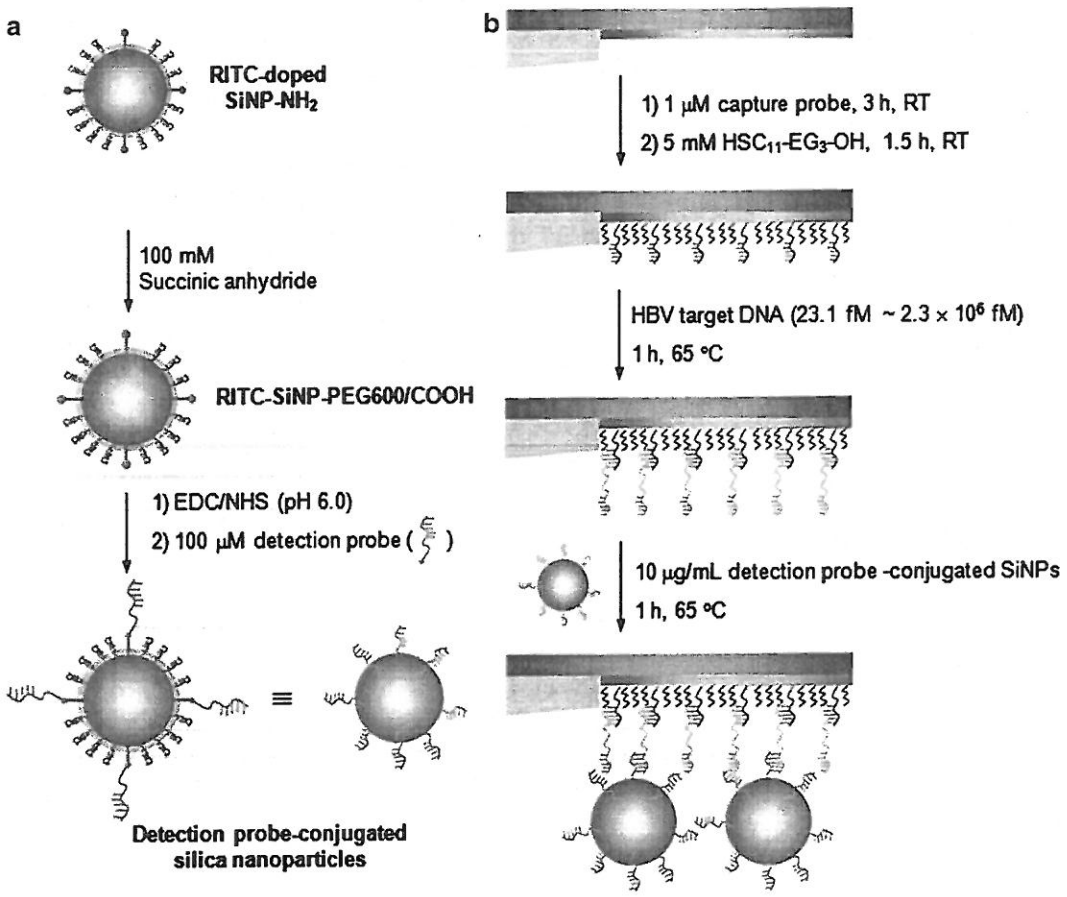

Fig. 1. (**a**) Preparation of detection probe-conjugated silica nanoparticles and (**b**) detection protocol of HBV target DNA using silica nanoparticle-enhanced microcantilevers. Reproduced from (10) with permission from Elsevier.

Table 1
Sequences of probe DNAs and target DNA

Probes	Sequences	Length (mer)
Capture probe	5′-(SH)-$(CH_3)_{18}$-T_{10} TGGAGCTTCCGTGGAGTTACTCTCT-3′	35
HBV target DNA	TCCCTGGATGCTGGGTCTTCCAAATTACTTCCCACCCAGG TGGCCAGATTCATCAACTCACCCCAACACAGAATAGCTT GCCTGAGTGCTGTATGGTGAGGTGAACAATGTTCCGGA GACTCTAAGGCCTCCCGATACAAAGCAGAGGCGGTGTC GAGGAGATCTCGAATAGAAGGAAAGAAGTCAGAAGGC AAAAAAGAGAGTAACTCCACGGAAGCTCCAAATTCTTT ATACGGGTCAATG	243
Detection probe	5′-ATCTGGCCACCTGGGTGGGAAGTAA T_{10}-NH_2-3′	35

All DNA probes are HPLC grade and over 1 OD

3.3. Immobilization of the Capture Probe on Microcantilever (See Fig. 1b)

1. Place the microcantilever device with fresh gold layer in a well of a 48-well plate.
2. Spot 100 μL of a 1 μM capture probe solution on the device and incubate it for 3 h at room temperature (see Note 8).
3. (Washing) Transfer the device to another well containing 500 μL of fresh TE buffer and shake the plate at 600 rpm for 5 min using the orbital shaker (1×). Transfer the device to another well containing 500 μL of fresh water and shake the plate at 600 rpm for 5 min using the orbital shaker (2×).
4. (Backfilling) Transfer the washed device to another well containing 100 μL of 5 mM backfiller solution and incubate for 1.5 h at room temperature.
5. (Washing) Transfer the device to another well containing 500 μL of 95 % ethanol and shake the plate at 600 rpm for 5 min using the orbital shaker (2×). Transfer the device to another well containing 500 μL of water and shake the plate at 600 rpm for 5 min using the orbital shaker (2×).
6. Dry the washed device under flow of nitrogen (see Note 9).
7. To measure the resonant frequency, connect between two electrodes the microcantilever (30 μm×90 μm) and impedance analyzer. Enter the input signal condition (0.5 V_{pp}) of impedance analyzer. Enter the frequency range with condition of ±1.5 kHz span from center frequency. Press the button with auto-scaling function. Find the frequency at the peak value of impedance phase signal.

3.4. Plot of Standard Curves

1. Prepare 100 μL HBV Target DNA (in TE buffer) of the following concentrations: 23.1, 231 fM, 2.31, 23.1, 231 pM, and 2.31 nM for each microcantilever (see Table 1, HBV Target DNA and Note 4).

2. Place six devices with the capture probe-immobilized microcantilever in well of a 48-well plate.
3. Carefully spot 100 μL HBV DNA solution on each microcantilever to immerse the microcantilever completely.
4. Incubate the device at 65 °C for 1 h (see Note 8).
5. (Washing) Transfer the device to another well containing 500 μL of fresh TE buffer and shake the plate at 600 rpm for 5 min using the orbital shaker (2×). Transfer the device to another well containing 500 μL of fresh water and shake the plate at 600 rpm for 5 min using the orbital shaker (2×).
6. Dry the devices under flow of nitrogen.
7. Measure the resonant frequency of microcantilevers (30 μm×90 μm) using impedance analyzer (see step 8 of Subheading 3.3).
8. (Signal processing) Plot the differences between the resonant frequencies from these measurements and those from step 8 of Subheading 3.3 at each concentration (see Fig. 2, filled square).
9. Place the device treated with HBV DNA solution to another well and carefully spot 100 μL of 10 μg/mL detection probe-conjugated silica nanoparticles in TE buffer to immerse microcantilever completely.
10. Incubate 65 °C for 1 h (see Note 8).
11. (Washing) Transfer the device to another well containing 500 μL of fresh TE buffer and shake the plate at 600 rpm for 5 min using the orbital shaker (2×). Transfer the device to

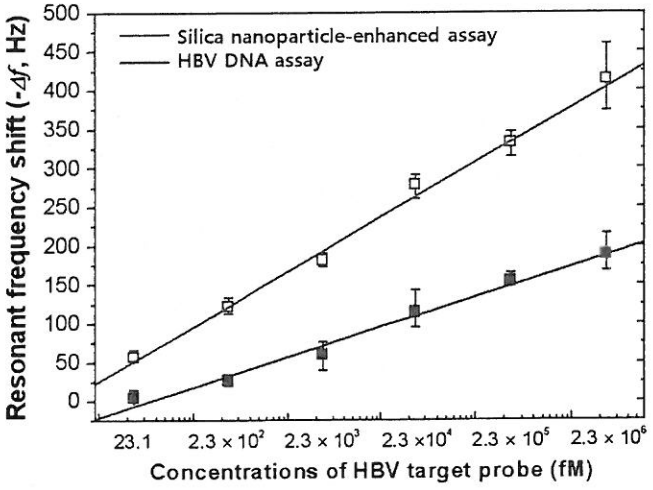

Fig. 2. Plots of the resonant frequency shifts acquired from the HBV DNA assay (*filled square*) and the silica nanoparticle-enhanced HBV DNA assay (*empty square*) in the concentration range from 23.1 fM to 2.3 nM. Reproduced from (10) with permission from Elsevier.

another well containing 500 μL of fresh water and shake the plate at 600 rpm for 5 min using the orbital shaker (2×).

12. Dry the devices under flow of nitrogen (see Note 9).
13. Measure the resonant frequency of microcantilevers (30 μm×90 μm) using impedance analyzer (see step 8 of Subheading 3.3).
14. (Signal processing) Plot the differences between the resonant frequencies from these measurements and those from step 8 of Subheading 3.3 for each concentration (see Fig. 2, empty square).

3.5. HBV DNA Assay on Microcantilever

1. Perform HBV DNA assay using HBV PCR product of clinical serum by following method of steps 3–14 of Subheading 3.4. Obtain the difference of the resonant frequencies between the capture probe-immobilized microcantilever (before assay) and silica nanoparticles-enhanced microcantilever (after assay) (see Note 10). Match the difference with the concentration of HBV DNA using the graph of Fig. 2.

4. Notes

1. The procedure used to fabricate the piezoelectric layer-embedded microcantilever is as follows (see Fig. 3a): the piezoelectric (PZT) thin layer-embedded microcantilever structures with silicon nitride (SiNx) supporting layer are fabricated by a surface and bulk micromachining process. The substrates are 100 mm-diameter p-doped Si (100) wafers (525±20 μm thickness) covered with a 1 μm-thick low stress SiNx layer deposited by low pressure chemical vapor deposition (LPCVD). Then, the platinum layer with thickness of 150 nm, which functions as the bottom electrode is prepared by sputtering on a thin tantalum (Ta) adhesion layer of 30 nm. The PZT films are deposited with a thickness of 1 μm using the diol-based sol–gel method. The PZT films are deposited by spin coating of a mixed PZT solution at 1,536×g for 30 s. The films are then heated at 400 °C for 5 min and annealed at 650 °C for 10 min. To assemble the metal-ferroelectric-metal (MFM) capacitor structure, a platinum layer is deposited as the top electrode by sputtering on the PZT layer. Using a multilayer deposited substrate, the microcantilevers are fabricated through photolithography and etching by repeating the patterning using masks for each layer. The platinum layer for the top electrode and PZT layers are etched using an advanced oxide etcher (AOE). After the top electrode and PZT layer are etched, a silicon dioxide (SiO_2) thin film with thickness of 200 nm is deposited by plasma enhanced chemical vapor

Fig. 3. (a) Phc microcantilev (the impedan quency obtai

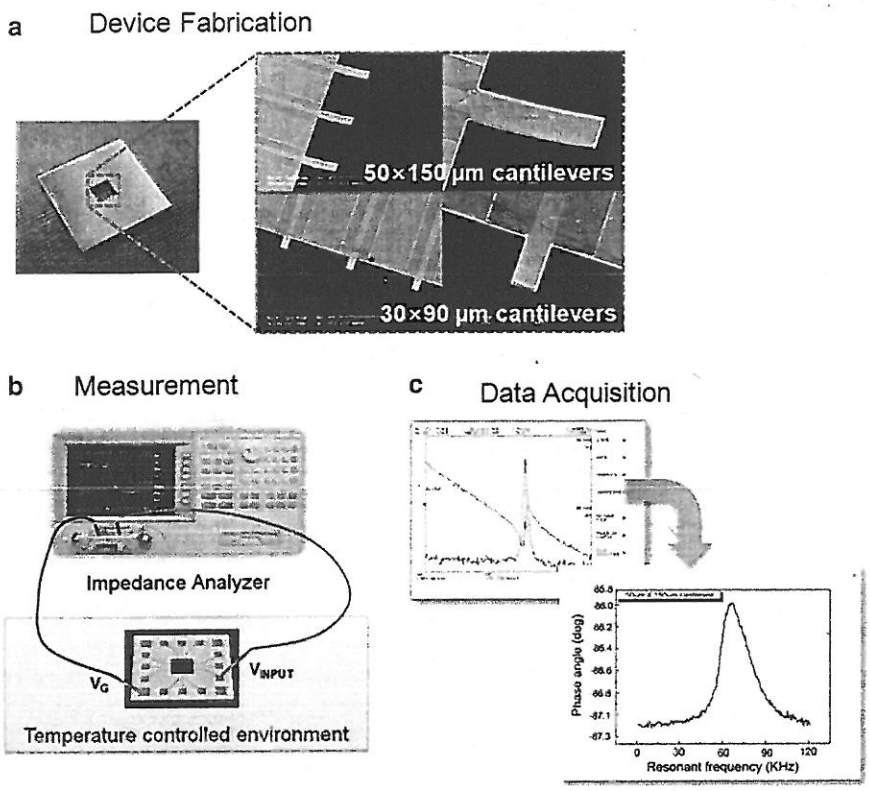

Fig. 3. (**a**) Photograph of the single device with 12 microcantilevers and SEM images of two types of microcantilevers (six microcantilevers of 30 μm × 90 μm and six microcantilevers of 50 μm × 150 μm), (**b**) schematic diagram of the equipment (the impedance analyzer) used to measure the resonant frequency of the microcantilevers and (**c**) peak of resonant frequency obtained from the impedance signal. Reproduced from (10) with permission from Elsevier.

deposition (PECVD) to provide electrical insulation in the buffer solutions. The contact holes on the top and bottom electrode are then etched and connected through a gold lift-off process. Patterning of the bottom electrode is carried out with AOE. The back SiN*x* window is patterned by reactive ion etching (RIE) and the bulk silicon is wet-etched using a KOH silicon etchant at a concentration of 30 %. Finally, the upper side of the SiN*x* is etched by RIE to release the cantilever. A single device contains six cantilevers of 30 μm × 90 μm and six cantilevers of 50 μm × 150 μm.

2. Deposit a gold film freshly before capture probe immobilization. Otherwise, use the piranha solution: mix 15 mL of concentrated H_2SO_4 and 5 mL of 30 % H_2O_2 in a 50 mL Pyrex beaker. Dip the gold-coated microcantilever devices into the solution for 5 min. Transfer the device to a Petri dish containing 2 mL water and shake it for 1 min on the orbital shaker (3×). Dry the device under a flow of nitrogen gas and transfer it to the capture probe solution as soon as possible.

3. The concentration of silica nanoparticles can be measured by weighing the solid pellet which is dried from 1 mL of the nanoparticles solution. The sample should be weighed at least five times and the average value should be regarded as the weight of the silica nanoparticles in 1 mL EtOH.
4. DNA of an HBV positive serum was extracted from 140 μL serum using the QIAamp DNA Mini-Kit, and 4.5 μL DNA sample was amplified in a 20 μL reaction containing 0.5 U Taq polymerase and 1× PCR buffer with 1.5 mM $MgCl_2$, 0.2 mM dNTP and 10 pmol of each primer (anti-sense: 5′-TCC CTG GAT GCT GG(G/A) TCT TCC AAA-3′ and sense: 5′-CAT TGA CCC (C/T)AT AAA GAAT T-3′). 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) with a final extension step of 72 °C for 5 min. PCR product was purified using the QIAquick PCR purification kit and DNA concentration measured photometrically (1 OD = 50 μg/mL DNA). Preparation of genomic DNA of clinical sera and subsequent HBV PCR were carried out accordingly.
5. The reaction should be performed in the dark place or the glass flask should be wrapped with aluminum foil.
6. Use a bath-type ultrasonicator to disperse the pellet well.
7. Keep the detection probe-conjugated silica nanoparticles in the dark at 4 °C.
8. Confirm the devices are fully dipped in the solution and the lid of the 48-well plate must be firmly sealed before incubation at 65 °C.
9. Store the devices in a drying desiccator before measuring the resonant frequency.
10. General measurement of resonant frequency: Measure the resonant frequency of the microcantilevers (30 μm × 90 μm) using an impedance analyzer (4294A, Agilent Technologies, CA, USA), which monitors the phase angle change of impedance in the frequency domain. Induce an ac signal of 0.5 V_{pp} (peak to peak) on the top and bottom electrode of the microcantilever. The scan range should be 3 kHz and the regime should be ±1.5 kHz from the resonant frequency (see Fig. 3b, c).

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