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A polycarbonate based surface plasmon resonance sensing cartridge for high sensitivity HBV loop-mediated isothermal amplification

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ABSTRACT

In this study, we report a simple, low-cost surface plasmon resonance (SPR)-sensing cartridge based on a loop-mediated isothermal amplification (LAMP) method for the on-site detection of the hepatitis B virus (HBV). For LAMP detection, a SPR based LAMP sensing system (SPRLAMP) was constructed, including a novel SPRLAMP sensing cartridge integrating a polymethyl methacrylate (PMMA) micro-reactor with a polycarbonate (PC)-based prism coated with a 50 nm Au film.

First, we found that the change of refractive index of the bulk solution was approximately 0.0011 refractive index (RI) units after LAMP reaction. The PC-based prism's linearity and thermal responses were compared to those of a traditional glass prism to show that a PC-based prism can be used for SPR measurement. Finally, the HBV template mixed in the 10 μl LAMP solution could be detected by SPRLAMP system in 17 min even at the detection-limited concentration of 2 fg/ml. We also analyzed the correlation coefficients between the initial concentrations of HBV DNA templates and the system response (ΔRU) at varying amplification times to establish an optimal amplification time endpoint of 25 min (R^2 = 0.98). In conclusion, the LAMP reaction could be detected with the SPRLAMP sensing cartridge based on direct sensing of the bulk refractive index.

1. Introduction

Approximately 370 million people around the world suffer from chronic liver infection with the HBV, a kind of deoxyribonucleic acid (DNA) virus (WHO, 2011). Patients are at especially high risk when their serum viral load is higher than 100,000 copies/ml according to a report by the REVEALHBV (risk evaluation of viral load elevation and associated liver disease/cancer-hepatitis B virus) group (Iloeje et al., 2006; Lee et al., 2002). It is therefore clinically desirable to develop a quantitative diagnostic method to provide a high-sensitivity genetic assay for routine checkups or even for self-monitoring at home. Although polymer chain reaction (PCR) (Carrino and Lee, 1995) has been used in many areas of biotechnology and serves as the gold standard for clinical diagnosis, it requires efficient control of thermal cycles and thus has higher instrumentation costs. PCR is also known to have errors caused by its hybridization mechanism (Cadwell and Joyce, 1992). There are currently many research efforts devoted to miniaturizing the thermal cycler for point-of-care applications (Cooney et al., 2011; Zhang et al., 2011). However, PCR still requires hours of operating time to have enough end products for detection because of the limits of thermal transition rate and thermal conductivity (Zhang et al., 2004).

Compared to PCR, isothermal amplification methods have been reported to achieve higher efficiency and shorter amplification time for on-site measurement without a thermal cycling procedure. These methods include strand displacement amplification (SDA) (Walker et al., 1992), branched DNA (bDNA) amplification (Collins et al., 1997), invader (Kwiatkowski et al., 1999), NASBA (Compton, 1995), and rolling circle amplification (RCA) (Lizardi et al., 1998). As shown in Table 1, each method has particular benefits (Vrana, 1996) and drawbacks based on its amplification mechanism, sensitivity, and potential for multiple applications (Iqbal et al., 2000).

Loop-mediated isothermal amplification (Notomi et al., 2000) has become the preferred method for in-field screening (Harper et al., 2011) because of its isothermal condition, amplification efficiency and specificity. It has a higher DNA yield rate (10 μg/25 μl) than traditional PCR (0.2 μg/25 μl) (Mori et al., 2001). In addition, it has been demonstrated to have resistance to inhibitors in the blood (e.g., heme) because *Bacillus stearothermophilus* (Bst) DNA polymerase is used in the reaction (Poon et al., 2006). At present, LAMP has been applied for fast screening tests for several infectious

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Table 1
Comparison of characteristics of PCR and isothermal amplification reactions.

	LAMP	PCR	SDA	NASNA	RCA	bDNA	Invader
Target amplification	+	+	+	+	+		
Signal amplification	+				+	+	+
Isothermal	+		+	+	+	+	+
Sensitivity (copies/ml)	>10	>10	500	100	1	500	6000
Multiplex amplification	+	+	+	+			
Detection method	Turbidimetry and Fluorometry	Fluorometry	Fluorometry	Fluorometry	Fluorometry	Luminescence assay	Fluorometry Enzyme linked immunoassay

diseases, including *Mycobacterium tuberculosis* (Iwamoto et al., 2003), severe acute respiratory syndrome virus (Thai et al., 2004), human influenza viruses (Ito et al., 2006), avian influenza viruses (Imai et al., 2006) and herpes viruses (Iwata et al., 2006; Kaneko et al., 2005).

Currently, the LAMP reaction is detected in real time either by measuring the turbidity of its DNA polymerization by-product (magnesium pyrophosphate, $Mg_2P_2O_7$) or by using a fluorescent indicator (Tomita et al., 2008). Both methods are indirect detectors of amplification; both signals are proportional to the amount of produced DNA and the reaction time. Measurement of $Mg_2P_2O_7$ turbidity has one advantage: a simulated chemical reaction can be used to obtain $Mg_2P_2O_7$ for calibration purposes or internal quality verification without using real buffer medium and targeted DNA samples (Lee et al., 2008). However, LAMP detection by turbidimetry is limited by in-homogeneity of particle size, uneven spatial distribution, re-dissolution of $Mg_2P_2O_7$ particles and turbid samples; these factors result in relatively low sensitivity in short reaction time (approximately 20 min). In addition, this posts even greater challenge for the detection of smaller reaction volumes with smaller reactant amounts, e.g., polymerase, dNTP, buffer medium and extracted samples. One way to bypass this limitation is to use the surface sensing method rather than the traditional volume sensing approaches.

Most SPR biosensors use immobilized probes of nucleic acids or proteins on an Au surface for the detection of biomolecular interactions through hybridization processes; these sensors are known as affinity sensors (Bergström and Mandenius, 2011; Kim et al., 2011).

To develop a high-sensitivity, low-cost screening system for LAMP, we have demonstrated the detection of the LAMP reaction with the SPR analytical method without immobilizing probes. The SPR method was chosen because it allows label-free, high-sensitivity (Lin et al., 2010), real-time analysis and flexible system design, for example, the design of various orderly nanostructures has been shown to provide strong local electrical field enhancement (Chang et al., 2010), allowing higher sensitivity and wider dynamic range. Besides that, there is published report that demonstrated SPR based immunosensing amplified by enzymatically biocatalytic precipitates (Tang et al., 2007).

SPR method has been successfully demonstrated for a diverse range of applications, such as medical diagnosis, food industry safety (Terry et al., 2005; Van Dorst et al., 2010) and environmental and agricultural pollutants (Rodriguez Mozaz et al., 2006; Velasco-Garcia and Mottram, 2003; Van den Top et al., 2011). Kretschmann's configuration of the SPR technique has been extensively studied in the past few years because of its relatively simple optical setup (Lee et al., 2007a,b). It has been an important biosensor method in past biomedical research (Chien and Chen, 2004).

Because of the ongoing need for field-deployable applications, SPR sensing device design is trending toward miniaturization (Feltis et al., 2008), low cost and user friendliness (Chinowsky et al., 2007). We present a novel SPRLAMP cartridge design that replaces the glass prism with a miniature PC-based prism to investigate the possible solution of genetic testing based on SPR technique in a cost effective. To our knowledge, the use of a miniature PC-based prism for LAMP detection has not yet been demonstrated.

The objective of this study is to develop a high sensitivity SPR system for the real-time detection of LAMP amplification by surface sensing. This system includes a disposable SPRLAMP cartridge made of PMMA with a PC prism and a simple SPR imaging system with temperature control for LAMP amplification.

2. Materials and methods

2.1. Preparation of LAMP assay

The design of the HBV primers and the composition of the medium for the LAMP reaction were optimized and tested for turbidity measurement in a micro-reactor as reported previously (Lee et al., 2008). Briefly, we searched a public database for the DNA template of HBV (323 bps), and picked out the highly conserved fragments. We then considered the melting temperature (T_m) of each primer based on nearest-neighbor T_m theory, and designed the primer using Gene Runner software (Hastings Software, Inc., Hudson, NY, USA). Primers were produced by a contract service (Quality Systems, Inc., Taipei City, Taiwan, ROC). In addition, standard template DNA was prepared from a partial HBV polymerase gene inserted into a pGEM-T Easy vector (PROMEGA, Madison, WI, USA).

Assay mixtures for LAMP were premixed in a total volume of 25 μl. Assay mixtures contain 8 pmol each of IB-FIP (5′-TGGAATTAGAGGACAAACGGGTGCTGCTATGCCTCATCTT-3′) and IB-BIP (5′-GCTCAAGGAACCTCTATGTTTCGATGATGGGATGGGAATACA-3′), 2 pmol each of IB-F3 (5′-GGCGTTTTATCATCTTCCT-3′) and IB-B3 (5′-AGGTTACTTGCGAAAGCC-3′), 4 pmol each of IB-loopF (5′-TACCTTGATAGTCCAGAAGAACC-3′) and IB-loopB (5′-CTACGGACG-GAAACTGCAC-3_), 0.4 mM dNTPs, 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 6 mM $MgSO_4$, 0.1% Triton X-100, 1 M betaine, 5 units of the Bst DNA polymerase large fragment (NEW ENGLAND BioLabs, Ipswich, MA, USA), and 1 μl of DNA standard template, a partial HBV polymerase gene cloned into a pGEM-T easy vector.

2.2. Measurement of refractive index

To verify the feasibility of LAMP detection by SPR, we measured the refractive index of the bulk medium with a portable refractometer (RA130, KEN, Japan) before and after the LAMP reaction. We prepared 600 μl of a solution containing 20 ng/ml DNA templates and a set of six primers to recognize the selected sequences of HBV, which was divided two parts. The refractive index of a 300 μl aliquot was measured by the refractometer. The remaining solution (300 μl) was then incubated in a PCR machine (Mastercycler 5333, Eppendorf, Germany) at 65 °C for 1 h to allow the LAMP reaction to occur. After incubation, the rest of the LAMP products were then pipetted into the well of the refractometer to measure the refractive index. After above process, 5 μl aliquots of the LAMP solution were pipetted from both two parts and then electrophoresed in 2%

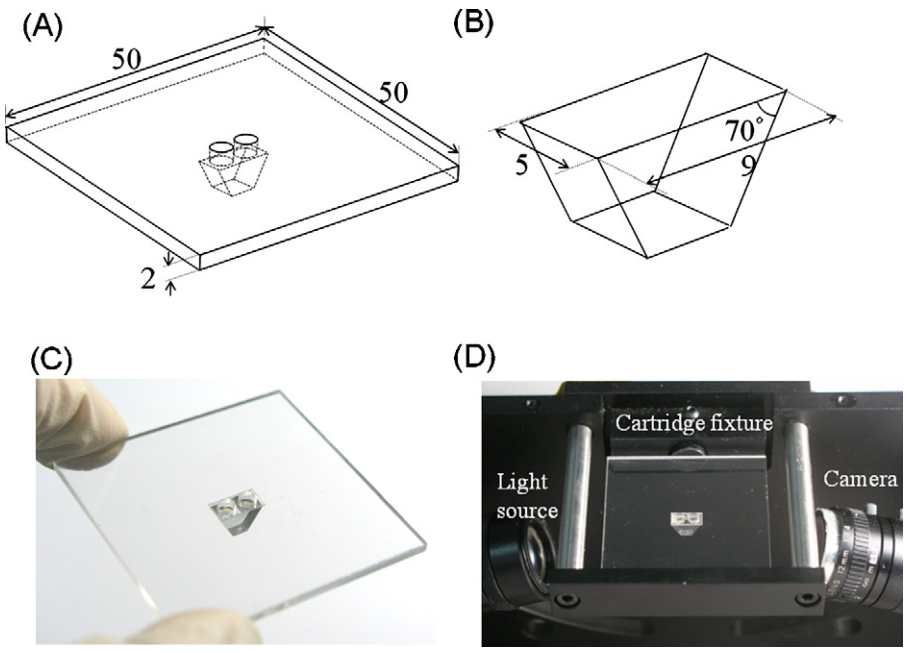

Fig. 1. Schematic diagram of disposable SPRLAMP sensing cartridge. (A) SPRLAMP cartridge contains PMMA micro-reaction chip and PC-based trapezoidal *prism* coupler. PMMA reaction chips were laser-cut to construct micro reaction chambers. The volume of one reaction chamber is 25 μl. (B) The SPR couplers were polycarbonate (PC)-based trapezoidal *prisms* produced by injection-molding. (C) Photograph of prism. The prism was coated with a 47 nm Au film using sputter at a pressure of 3.2×10^{-3} Torr. (D) Photograph of the base apparatus for holding the SPRLAMP cartridge. The disposable SPRLAMP sensing cartridge can be fixed on optical pathway by the apparatus and the HBV LAMP reaction can be started with the thermal control.

agarose gels (1× TBE). After electrophoresis, the gels were stained with SYBR Green I dye for visualization with a fluorescent imager (GelDoc-It Imaging System, UVP, Upland, CA, USA).

2.3. The SPRLAMP sensing cartridge

We have designed a disposable SPRLAMP sensing cartridge which consists of a polycarbonate (PC) miniature prism and a poly methyl methacrylate (PMMA) plate with two micro-wells as shown in Fig. 1A. The trapezoidal PC prism ($n = 1.51$) was designed and fabricated with precise angles and dimensions by injection molding. It functions as a coupling prism for the SPR sensor. To validate the performance of the PC prism, we also prepared a miniature BK-7 glass prism ($n = 1.51$). Both prisms were coated with 47 nm Au film with RF sputtering deposition at a working pressure of 3×10^{-3} Torr.

The micro-wells were fabricated by using a laser machining system (VLS2.3, Universal, USA) on a precision-cut PMMA plate of dimensions 50 mm × 50 mm × 2 mm. Each micro-well was of diameter approximately 3.9 mm so that each micro-reactor had volume approximately 25 μl. The micro-reactor plate was bonded to the miniature prism with UV adhesive (Ultrawide GN150, Everwide Chemical Company, Yunlin County, Taiwan, ROC), creating the finished SPRLAMP sensing cartridge. The micro-chambers of this cartridge were pre-filled with 10 μl of reagent and then sealed with an adhesive cover (Optical Adhesive Covers, Applied Biosystems, USA) to form a closed space as shown in Fig. 1C. The amplified targets were pipetted into the microreactors by poking through the cover.

2.4. SPRLAMP instrument

The SPRLAMP instrument was constructed based on the Kretschmann configuration. The instrument consisted of two sub-units: an SPR imaging system for real-time monitoring of reflectivity changes and a temperature controller to regulate the temperature for the LAMP reaction. Fig. 2 shows the schematic diagram of the SPR measurement system. The SPR optical system consists of a 850 nm near-infrared (NIR) LED light source collimated by an achromatic doublet and a CCD camera (FO124SB, NET, USA) connected to a computer through an IEEE 1394 interface. A plate holder is used to conveniently place the sensing plate in a designated position on the optical path. The SPR imaging system is used to measure the reflectivity changes of oblique light with an angle of incidence of approximately 72° in the current configuration. The surface plasmon wave is a collective electric charge oscillation and is extremely sensitive to the dielectric properties of the medium near the gold surface. LAMP amplification of DNA samples results in changes to the refractive index caused by the presence of LMAP production (e.g. chains of nucleic acids and $Mg_2P_2O_7$).

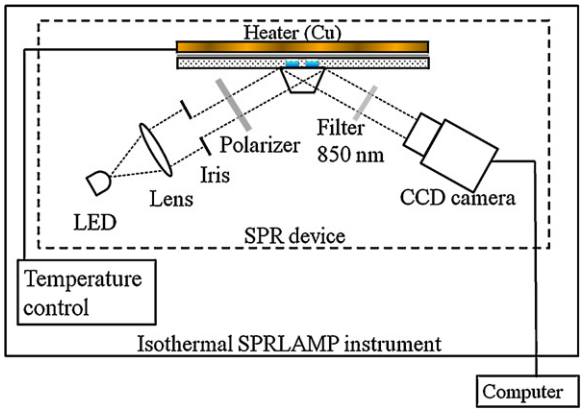

Fig. 2. Isothermal SPRLAMP system for LAMP amplification and detection. Isothermal SPRLAMP instrument. The isothermal SPR instrument consists of a light source module, CCD camera, and thermal control apparatus. The light source module employs an 850 nm light-emitting diode (LED) light source, lens and a polarizer to generate a parallel-polarized luminous flux. The thermal control apparatus consists of a 60 W power supply and a heater plane controlled by a proportional-integral-derivative (PID) controller with K-type thermal coupler feedback. The heater plane was constructed with a Cu plate (5 cm × 5 cm × 0.1 cm) bonded with two thin-film heaters and a thermocouple.

We defined θ_{max} and θ_{min} as the angles of incidence with maximum and minimum reflective intensity. Then, we fixed the parallel light beam at the angle of incidence θ_{in} between θ_{max} and θ_{min} with 2/3 maximum reflective intensity. The reflected light was captured by the CCD camera, which digitizes the reflective intensity into 12-bit gray scale images and then transfers these images by IEEE 1394 to a PC for visualization and further processing. For quantitative comparison, all gray-scale pixels in the regions of interest (ROIs) are averaged, yielding the response values (RUs). The proportional-integral-derivative (PID) temperature controller (ANLY Electronics, Taipei County, Taiwan, ROC) has a 60 W power supply and two 0.5 cm × 4 cm thin-film Kapton$_{TM}$ heaters (Minco, Minneapolis, MN, USA) with one k-type thermal coupler to provide feedback signal for closed-loop control. All the above sub-units were controlled by a program developed in LabVIEW 8.2 (National Instrument, TX, USA). The change in reflective intensity (ΔRU) was used as the measure of refractive index change for LAMP measurement.

2.5. Polycarbonate prism test

The performance of the PC-based miniature prism and the BK-7 prism was tested by using different concentrations of ethanol and repeated temperature cycles. To validate the performance of the miniature PC prism, we compared the linearity of refractive index changes and responses to thermal cycling of the PC prism with those of the BK7 prism. Graded ethanol solutions were applied to evaluate the linearity of both prisms at increasing concentrations (0.1–10%). Double distilled water (DD H_2O) was used to clean the micro-chambers between runs and as the reference solution. During testing, graded ethanol solution and DD H_2O were delivered by a syringe pump (KDS-200) to fill each micro-chamber and to fully immerse the Au film surface. For thermal response testing, each micro-chamber was filled with 10 μl of DDH_2O by micropipette and then sealed with an adhesive cover to prevent evaporation during the heating process. After placing the assembled SPRLAMP sensing cartridge onto the isothermal SPR instrument, we started the thermal cycling process according to the following protocol: heat to 65 °C, maintain at 65 °C for 15 min, and then we cut off the heating supply for 20 min to allow cooling to approximately 35 °C. These steps were repeated three times. After this process, the cartridge was allowed to cool to 25 °C.

2.6. Real-time measurement of LAMP reaction

Real-time measurement of the HBV LAMP reaction was demonstrated by using the previously described isothermal SPR instrument and testing procedures. First, micro-reaction chamber was pre-filled with 10 μl of assay mixture as described in Section 2.1. For continuous monitoring of reflective intensity changes, we first selected ROIs from the image shown on the monitor using a graphic user interface programmed with the imaging tool box of the LabVIEW software. Before data collection, the sensing cartridge was heated for 5–6 min until the temperature of the sealed micro-reactors became stable. We then acquired images of the amplification process for up to 30 min. To compare the change in reflectivity at different initial DNA concentration, all data were taken to be positive values. To find the optimal amplification time endpoint, the SPR response (ΔRU) was measured for various initial DNA concentrations with different amplification time endpoints, so the correlation coefficients of different endpoints could be obtained. The endpoint with the maximum correlation coefficient was taken as the optimal endpoint. Finally, the calibration curve (i.e., difference in response values (ΔRU) vs. HBV DNA template concentrations) was plotted at the endpoint time.

3. Results and discussion

3.1. HBV LAMP assay

3.1.1. HBV LAMP validation

After the LAMP reaction, the increase in precipitated magnesium pyrophosphate ($Mg_2P_2O_7$) is visible to the naked eye. Pyrophosphate ion is released from dNTP in LAMP reaction. With an increase of pyrophosphate ion, it reacts with magnesium ion pyrophosphate ion in the buffer yielding a precipitate. We demonstrated that the positive sample appears white and turbid (Fig. 3A). Furthermore, after LAMP, the products elongated to an average length of several kbps. The products consist of several inverted-repeat structures. Gel electrophoresis revealed bands of many different sizes of DNA (Fig. 3B).

3.1.2. Measurement of bulk refractive index of LAMP mixture

We measured the refractive index (n) of the buffer medium containing DNA templates (20 ng/ml) with and after the heating process. Refractive index values were 1.3492 before heating and 1.3481 after heating. The LAMP by-products of $Mg_2P_2O_7$ and elongated stem-loop DNA deplete the quantity of magnesium ions and dNTPs in the medium. Because the total mass in a sealed chamber should remain constant throughout the amplification process, it follows that in a sealed chamber, the mole numbers and molecular sizes of solute in the mixture will be changed by the LAMP reaction. Besides that, magnesium pyrophosphate was precipitated (diameter: 16.4 μm) (Sugiyama et al., 2005) and separated from the aqueous solution, resulting in a decrease of refractive index. Because the number and sizes of solute molecules change, the refractive index of the solution should also change. Our experimental results verify that the refractive index of the solution was altered by the LAMP reaction.

3.2. Isothermal SPRLAMP system

3.2.1. Isothermal SPRLAMP instrument

In this study, we constructed a simple and effective isothermal SPR system consisting of an SPRLAMP sensing cartridge and

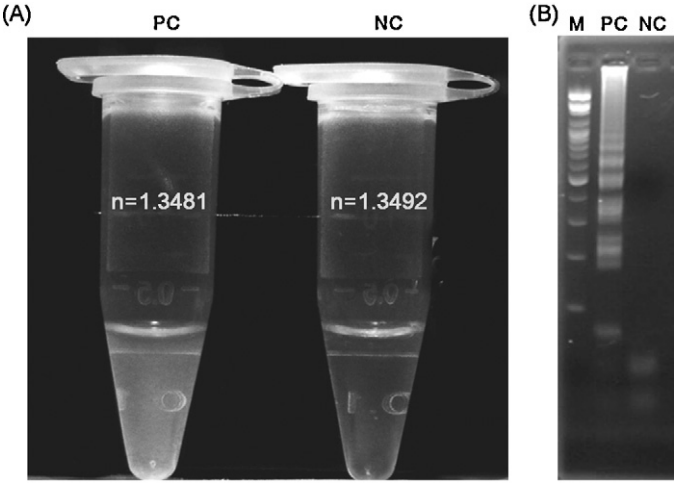

Fig. 3. Confirmation of LAMP reaction. M: 100 bp DNA ladder marker; NC (negative control): without heating; PC (positive control): with heating. (A) Aliquots (300 μl) of LAMP solution were heated in the thermal cycler at 65 °C for 60 min as the positive control. The white precipitate in the positive control tube is visible to the naked eye. The other aliquots (300 μl) were not heated by thermal cycler as the negative control. The refractive index of solution (PC and NC sets) was measured by a refract meter with 300 μl volume. The refractive index of the NC set is 1.3492 ± 0.001. The refractive index of the PC set is 1.3481 ± 0.001. (B) After the refractive index measurement, the amplified 5 μl LAMP solution from the PC and NC sets was analyzed by electrophoresis to validate the consistency of experimental results.

an isothermal SPR instrument as shown in Fig. 3. The isothermal SPR instrument has two sub-units: an optical system to both excite surface plasmon resonance and measure the resulting reflectivity changes and a PID temperature controller to maintain a temperature of 65 °C for HBV LAMP reactions. The system connects to a computer for user interface and interactive control. A narrowband (850 ± 50 nm) NIR LED (EDEI-1LA3, EDISON OPTO, Taiwan) was used as the excitation light source. Its beam was collimated and polarized by a plano-convex lens set and polarizer on the incident arm. This collimated beam was aligned toward the PC prism at a 72° angle of incidence. The reflected light intensity is affected by the dielectric property of the LAMP solution in the vicinity of the Au surface. Monitoring the changes in intensity of the reflected light gives a measure of the change in the refractive index and therefore a measure of the LAMP reaction. We have observed a difference of 3000 RU between the SPR signal maximum (3500 RU) and minimum (500 RU). A mechanical fixture with precision pins was constructed to hold the sensing cartridge at the desired position, allowing reflection and projection of the light to a CCD camera with a focused microlens (TV LENS 12MM 1: 1.4, Japan). The temperature controller supplied current to the flexible heating filament. The heating filament was coupled through a copper plate, which acted as a heat capacitor to minimize fluctuations and ripples in the temperature inside the SPRLAMP sensing cartridge. This heating block was brought in close contact with the top of the sealed membrane to ensure a homogenous temperature inside the reactor. The heating block also avoided evaporation and condensation of the LAMP solution on the membrane surface during heating.

3.2.2. Polycarbonate prism

To validate the function of a polycarbonate prism as an SPR coupler, we compared the relationships between response values (ΔRU) and ethanol concentration measured by a PC prism and by a glass prism, as shown in Fig. 3A. Results show that the linearity of the PC prism compares favorably with the linearity of the glass prism; both prisms have a good correlation coefficient ($R^2 > 0.99$). The response value (ΔRU = 540.984) of the glass prism at 10% ethanol is slightly less than the response value (ΔRU = 612.728) of the PC prism. This difference is caused by the amount of reflective light energy absorbed by Cr film, an adhesive layer between the glass and Au film. The LOD is approximately 0.08% with the glass prism and 0.07% with the PC prism. The refractive index of the ethanol solution was computed using the binary mixing rule (Tasic et al., 1992). We were able to calculate that 1 ΔRU was equivalent to a change in refractive index of 3×10^{-6} with the glass prism and 2.1×10^{-6} with the PC prism.

To validate the use of the PC prism for SPR measurement at the LAMP reaction temperature, we measured the dynamic response of SPR curves to repeated on-off heating cycles. Fig. 4B shows the results of three repeated cycles between 25 °C and 65 °C. These results demonstrate the reversibility of SPR responses to temperature cycling. The ΔRU changes are due to the temperature dependence of the refractive index of water. The difference of response values (ΔRU) between 65 °C and 25 °C was 178 ± 1.5 ΔRU for the PC prism and 177 ± 1.4 ΔRU for the glass prism. The 50 nm Au film coated onto the PC prism performed similarly to the 50 nm Au film coated on the glass prism; the film did not detach from the prism with temperature variance, even though the thermal expansion coefficient of PC substrate ($70.2 \times 10^{-6}\,°C^{-1}$) (Serini, 2000) is greater than that of glass substrate (approximately $7.1 \times 10^{-6}\,°C^{-1}$) (Brown, 2004). The difference of SPR responses between these two types of prisms was neglected. The time to increase temperature from 25 °C to 65 °C is approximately 5 min in our system. The 5-min temperature response time was used in a follow-up experiment. Since both prisms exhibit temperature-dependent changes in SPR measurements, it remains important to maintain a stable temperature for LAMP detection by SPR, stable temperature is necessary for LAMP detection by SPR.

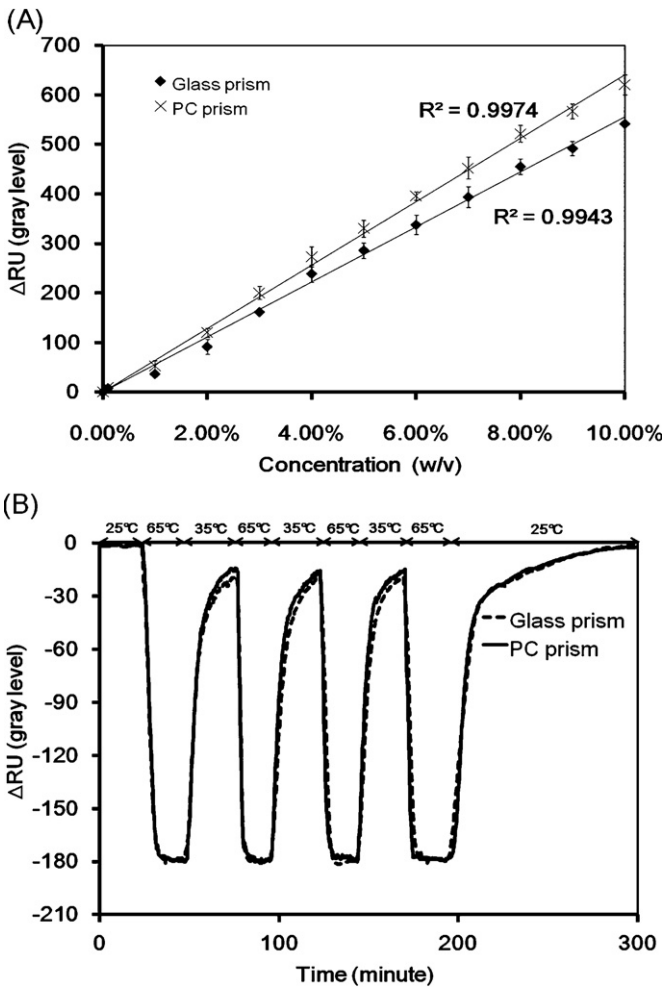

Fig. 4. Comparison between BK7 prism and polycarbonate prism. (A) Comparison of polycarbonate ($n = 1.49$) and glass prism ($n = 1.51$). Measured SPR response (reflected light intensity) versus refraction index of ethanol solutions. Ethanol concentrations are 0.1%, 1%, 2%, and 10%. The detection limits of ethanol solution are 0.08% and 0.07% with glass and PC prisms respectively. (B) Plot of the SPR response unit (RU) vs. time of thermal cycling with glass and PC prisms. The response value is 178 ± 1.5 ΔRU for the PC prism and 177 ± 1.4 ΔRU for the glass prism at 65 °C.

3.2.3. PMMA cartridge

We have designed a two-channel sensing cartridge to make differential measurements between a LAMP reaction channel and a negative control channel as a background reference. The SPR response is sensitive to temperature-induced refractive index changes in the micro-reactor. This approach can minimize fluctuations caused by environmental factors and thus provide better discrimination of the LAMP reaction. The traditional Kretcheman SPR chip uses matching oils for the coupling of an Au glass slide and a BK-7 prism. Our PC prism has the advantage of direct Au film deposition without the use of a glass slide. It thus provides easier and better integration with the PMMA cartridge and further reduces the cost of materials for disposable applications. We prefilled the micro-reactors with 10 μl of premixed LAMP solution to fully immerse the gold film. This volume does not cause the reagent to overflow when we seal the micro-reactors with adhesive film. Whether the reaction chamber is tightly sealed or not can be verified by the SPR response curve. We have noticed that if the seal is damaged, the LAMP reaction will be stopped, possibly due to

the evaporation of water changing the mixed volume ratio of the solution.

3.3. HBV LAMP analysis using SPR isothermal system

Although the dynamic response of the LAMP process has been successfully demonstrated by using both turbidity and fluorescent labeling methods, it has not yet been demonstrated using the SPR method. As shown in Section 3.1, the static refractive index of the LAMP medium decreased by approximately 0.0011 refractive index (RI) units. This change in refractive index might be due to amplified DNA, reaction by-products, or bulk medium. The change in refractive index provides a feasible mechanism for measurement of LAMP by the SPR sensor, which is known to have high sensitivity for refractive index changes. Fig. 5A shows the overlaid results of real-time SPR response versus different initial concentrations of DNA templates. The results are fitted to values measured with a refractometer. The detection range for initial DNA concentrations was 2 fg/ml to 20 ng/ml. Fig. 5 shows that as initial DNA template concentration increases, more prominent changes in SPR responses were recorded. The average response for 20 ng/ml is approximately 533 ΔRU, from which a refractive-index change of 1.1193×10^{-3} was obtained. These results demonstrate that the SPR technique is sensitive to the LAMP reaction, even at low initial DNA concentrations.

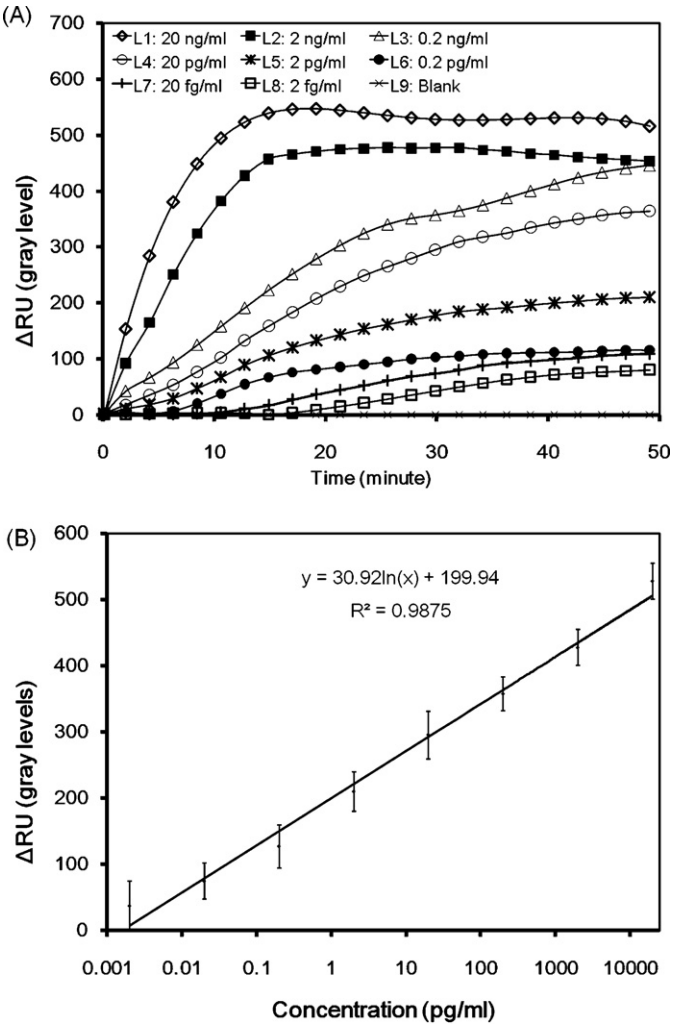

Fig. 5. Measurement of LAMP reaction by SPRLAMP system. (A) SPR validation of LAMP reaction with various initial HBV DNA template concentrations. System response change versus time. The serially 10-fold diluted HBV DNA plasmids are used to verify the feasibility of detecting the SPR response. (B) The initial DNA concentration is correlated with system response change in a logarithmic relationship with a good correlation coefficient ($R^2 = 0.9875$, $n = 3$).

The reaction time is also an important variable for quantitative measurement of the samples. According to the preliminary results, DNA templates at concentrations of 20 ng/ml, 2 ng/ml, 0.2 ng/ml, 20 pg/ml and 2 pg/ml can be detected in less than 3 min, and DNA templates at concentrations of 0.2 pg/ml, 20 fg/ml and 2 fg/ml showed obvious response changes (>10 ΔRU) in 6.5 min, 11.6 min and 17 min, respectively. Overall, a low number of HBV template copies could be detected in less than 20 min. In general, LAMP could be real-time detected by turbidimetry at the concentration of 2000 copies/ml (approximately 7.4 pg/ml) for 30 min (Lee et al., 2008). Therefore, the label-free SPR method is relative sensitive and less time consuming than turbidimetry.

For shorter reaction times (e.g., 5 min), the response for low-concentration DNA templates (2 fg/ml, 20 fg/ml, 0.2 pg/ml) is too small for our system to detect. However, if the amplification proceeds for too long (e.g., 50 min), responses for multiple DNA concentrations approach similar values (e.g., responses for 2 fg/ml, 20 fg/ml and 0.2 pg/ml are similar, and responses for 2 ng/ml and 0.2 ng/ml are similar). At very long reaction times, it is difficult to distinguish between two different initial DNA concentrations. Therefore, a careful choice of the reaction time endpoint is critical for LAMP analysis based on the SPR method.

To find the optimal reaction time endpoint, we used a simple method of finding the maximum correlation coefficients of the response curves. Linear curve fitting of the data with different endpoints (5, 10, . . ., 40 min) afforded the correlation coefficients listed in appendices (Table A.1). The correlation coefficient reached a maximum ($R^2 = 0.9688$) at a reaction time of around 25 min. From these results, we determined that the optimal endpoint was 25 min. These results demonstrate that SPRLAMP analysis can provide rapid and quantitative detection of amplification. The optimal endpoint (25 min) not only gives a clear cut-off value for qualitative analysis but also allows quantitative analysis of genetic detection by establishing a relationship between the system response value and initial DNA concentration.

We can then use the 25 min endpoint for the quantification of DNA template samples. All the DNA samples were measured three times and averaged for the calibration curve. Fig. 5(B) shows the logarithmic relationship $y = a \times \ln x + b$ with high correlation coefficient ($R^2 = 0.9875$) that denotes good sensor performance, where y is the system response (ΔRU) and x is the concentration of DNA templates. The proportional constants a and b were calculated to be 35.697 and 73.433. In our SPR setup, the LOD is determined to be 2 fg/ml with S/N = 3. The DNA templates in amounts well under the quantity recommended in the REVEAL-HBV study can be successfully detected with our system in 17 min. Our approach is different from the traditional SPR bio-sensing concept of immobilizing specific probes on the metal film as the transduction mechanism. Here, we demonstrated that refractive index changes of the LAMP bulk solution can be used for SPR detection without specific binding probes immobilized on the metal surface. Our results demonstrate that the refractive index of a bulk solution sealed in a closed chamber was changed by LAMP polymerization of nucleotides and the production of by-product magnesium pyrophosphate in a thermal-equilibrium system.

4. Conclusion

LAMP amplification can be detected directly by measuring the refractive-index difference of the bulk LAMP solution with SPR sensing methods with an inexpensive and disposable sensing cartridge. The advantages of SPR method compared to turbidimetry are relatively high sensitivity and rapid detection, and less

sample volume used. Using the SPRLAMP system, we have reported a novel flexible, low-cost rapid genetic detection system with high sensitivity and specificity. Research is on-going to improve the precision of the SPR based isothermal amplification system for analysis of other kinds of virus in complex real-world samples. In the future, SPRLAMP sensing cartridges can be manufactured precisely to decrease the cost of per test. It has potential future applications in genetic research and field diagnosis in third world countries.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.11.037.

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