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A Transparent Microchannel Chip for Quantitative PCR

Kai Sun [1], Zheyu Li [2], Shigeki. Matsuo [2,4], Hiroaki Misawa [2,3,4]

[1] Satellite Venture Business Laboratory of Photonic Nano-Materials, The University of Tokushima, 2-1 Minamijyosanjima, Tokushima 770-8506, Japan
Fax: 81-88-656-7598 e-mail: ksun@svbl.tokushima-u.ac.jp

[2] Graduate School of Engineering, The University of Tokushima, 2-1 Minamijyosanjima, Tokushima 770-8506, Japan

[3] Research Institute for Electronic Science, Hokkaido University Kita 12 Nishi 6, Kita-ku, Sapporo 060-0812, Japan

[4] Core Research for Evolution Science & Technology, JST, Japan

A transparent microchannel chip for quantitative continuous-flow polymerase chain reaction (PCR) was developed. The microchannel was fabricated on a cover glass substrate using standard photolithography and wet-etching techniques, and sealed by another cover glass substrate. A heat source consisting of two indium-tin-oxide (ITO) films was deposited on the backside of the etched substrate. An insulation layer of SiO_2 was then deposited on the ITO heaters. Thin, patterned ITO films deposited on the insulation layer served as temperature sensors. The effects of substrate temperature in sputtering and annealing condition are described. Linear relationship between the sheet resistance of the thin ITO film and the temperature was confirmed and calibrated. Finally, a 97-bp segment of the mouse X gene was PCR-amplified from mouse tongue cDNA with the chip. A microscope and ICCD camera system was used to detect the accumulation of double-stranded DNA (dsDNA) in-situ using SYBR® Green I dye.

Key words: microchannel, PCR, ITO, heater, sensor

1. INTRODUCTION

Chip-based polymerase chain reaction (PCR) systems have been developed into a popular technique in these years because of their rapid reaction and saving of reagents [1-3]. Although plastic substrates, such as polymethylmethacrylate (PMMA), have been used as substrates in the microfabrication, their lower thermal conductivity makes them less efficient for thermal cycling. Borosilicate glass substrate still played very important role in these devices because of owning most of the advantages of quartz glass and being inexpensive, furthermore, it has lower soften point which allows us to use fusion bonding or high pressure bonding technique.

Integration of heaters and thermal sensors is another important part in the microfabrication, considering the need for high heat-transfer coefficient and accurate temperature control. A wide variety of investigations have been carried out to study integrated heaters and sensors for microreactors [1,4-7]. These heaters utilize metal [4] or polysilicon [1] thin film as a resistor to generate heat over a large surface area. Woolley and Northrup reported that the heating rate of ~10 ℃ /s was realized by using an integrated polysilicon heater (3000 Å thick by LPCVD and boron doped to 400 Ω/square) for their micro PCR and CE chip device [1]. ITO has been used as a material for heating small devices [7], because of its advanced features, including low resistivity (ρ=~$\times 10^{-4}\Omega$·cm), strong adhesion to glass substrates, and good optical transmittance in visible regions (> 80%). In addition, in contrast with polysilicon thin film, ITO can be more easily deposited by such as sputtering, thermal evaporation, spray pyrolysis and screen printing technique. Conventional thermal sensors consist of a thin metallic wire or a thermistor bead. Miniature thermal sensors usually involve a thin film and patterned using photolithography and deposition technique [4-6], thus it is smaller than conventional ones and is easily integrated. However, to avoid blocking the detection light, these sensors are always mounted at a small distance from the reactors. Thus the temperature of the reactors cannot be measured and controlled well.

Our work is aimed at developing a continuous flow PCR chip from transparent materials with low fabrication costs and high device performance. Borosilicate glass was used as the substrate of the chip, and indium tin oxide (ITO) films were employed as thermal sources and temperature sensors for the PCR respectively. To test its operation, a 97-bp segment of rat X gene was amplified using the developed microchannel chip, and the reaction was monitored by a microscope and ICCD camera system.

2. EXPERIMENTAL

2.1 Microfabrication

The fabrication of the microchannel has been described in detail previously [7]. Briefly, the channel incorporates 40 cycles, and was fabricated on the

borosilicate glass substrate (30×30×0.5 mm) by using standard photolithography and wet-etching techniques. To make effective heat transfer and thermal control, we formed two strips of ITO thin film heaters [7], and two ITO thin film thermal sensors on the chip. The ITO thin films, served as heaters, are sputtered on the back side of the etched substrate at substrate temperature 300℃ followed by sputtering deposition of 2000 Å of gold on top of 100 Å of chromium. The ITO target in using contains 10 wt% SnO_2. The thickness of the films are 400 nm, and their resistivity is 1.6×10^{-4} Ω·cm. The ITO films are fabricated photolithographicly, then etched in the solution of 6 M HCl and 0.2 M $FeCl_3$, the etching rate is 150 Å/min. A layer of SiO_2 is then deposited on the ITO heaters by electron beam (EB) evaporation to form an insulation layer. At last, a layer of ITO film is deposited on the top of the insulation layer, and is patterned by using the same method illustrated above. The substrate temperature of sputtering and annealing temperature are optimized in order to obtain more sensitive and stable temperature sensors.

2.2. Thermal control and fluorescence detection

The thermal control system is composed of three main units including microchip, data acquisition unit of temperature sensors and control unit of heaters. The schematic diagram of the whole setup is shown in Fig. 1. The temperature sensors are connected to a $6^1/_2$-digit high-performance digital multimeter (DMM: Model 2000, KEITHLEY), and the conversion from resistance to temperature is performed by a software based on the calibration curve of the temperature sensors after the data is transmitted to a computer through RS232 interface from the DMM. The heaters are driven by a DC power supply and are controlled by solid-state relays (SSR), a D/A board, and computer-controlled proportional/integral/derivative (PID) arithmetic.

The fluorescence detection system contains a microscope (Olympus IX70) and ICCD camera. The microscope is equipped with a mercury burner lamp and an excitation filter (BP460-490) to illuminate the sample. The filter (BA515-550) in front of CCD camera is for cutting reflected excitation light and passing only the desired fluorescence from the sample. A precision syringe pump (Harvard, Model 975) is used to deliver the solution into the microchannel, and fluorescence is measured under a continuous solution flow in-situ.

2.3. Quantitative PCR conditions

SYBR® Green PCR Master Mix (ABI), including SYBR® Green I dye, AmpliTaq Gold DNA Polymerase, dNTP with dUTP, passive reference and optimized buffer, was used. Sensitive experiments were performed using rat tongue cDNA as a template, and the primers designed to produce a 97-bp segment of mouse X gene. The primer concentration was optimized

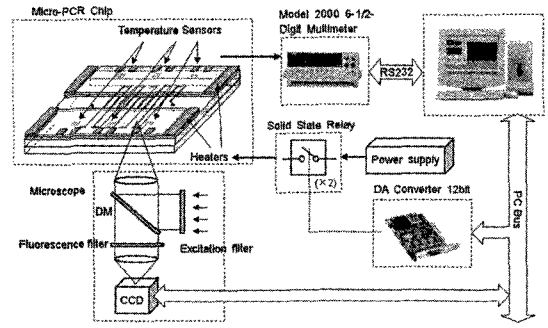

Fig.1 Schematic of the temperature control and the fluorescence measuring system.

using the ABI PRISM 7700 Sequence Detection System. All reactions were performed in 20 μl volumes. The template was diluted to the concentration of 50ng, 25ng, 10ng, 5ng, and 2.5ng per micro liter. The mixture flowed through the channel with 40 cycles at 95℃ 10 s for denaturation and 15 s annealing at 60℃. The flow rate was 83.5 nl/min. The quantitative PCR analysis was performed by measuring the fluorescence intensity of the SYBR® Green I dye during each annealing/extension phase.

3. RESULTS AND DISCUSSION

3.1. Calibration of temperature sensor

Two of the most important requirements for sensors are sensitivity and stability. All the experiments were finished in still air with the assumption that the surface temperature of samples is equal to the ambient gas temperature. And all the data were obtained by temperature cycling (TC) approach [6], which means that the sensor ambient temperature varies in an appropriate range, and a time period of about 30 min is required for each temperature. Fig. 3(a) shows the image of the temperature sensor, the width of which is 50 μm. The ITO films were sputtered at different substrate temperature, room temperature (hereafter called Sensor 1), 200℃ (hereafter called Sensor 2) and 300℃ (hereafter called Sensor 3). Fig. 2 shows the relative resistance change of them due to temperature change. Here, R_r is the resistance at room temperature (RT) 22℃, and T for ambient gas temperature. A linear rise is found in the curves of Sensor 2 and Sensor 3 with increased temperature. Nevertheless, after being heated to 110℃ the resistance of Sensor 2 needs even 30 min to lower several ohms to be smooth, the possibility of the effect from the hotplate was excluded by using a reference temperature sensor. This phenomenon did not happen below 100℃. Although the Sensor 1 after annealing at 200℃ for 1 h also shows a similar linear relationship, it needs long time to stabilize after being heated over 70℃ (about 1 h at 110℃), and could not be reproduced well. Annealing at 300℃ has an interesting influence on Sensor 1, its resistance decreases when temperature is above 70℃. Also,

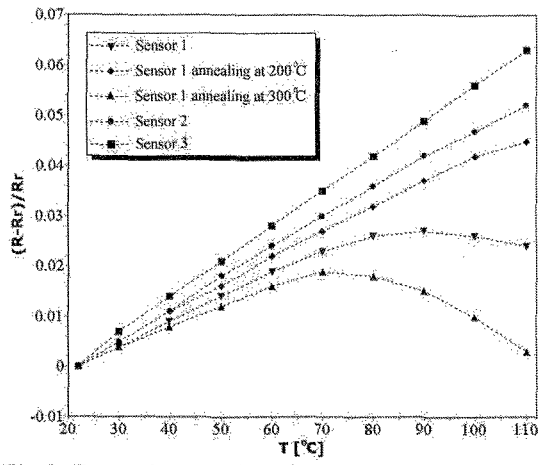

Fig.2 Dependence of resistance on temperature for different deposition and annealing conditions.

Sensor 1 shows about one percent of negative drift error in its repeatability. The surface topography of ITO thin films deposited on cover glass substrate at substrate temperature of RT, 200℃ and 300℃ observed by atomic force microscopy (AFM) are presented in Fig. 3(b-d). The mean roughness at RT, 200℃ and 300℃ are 4.4 nm, 7.6 nm and 9.1 nm. The ITO grain size becomes larger with the increasing of substrate temperature. In general, crystallinity increased with annealing. For all amorphous samples there was no evidence of crystallinity until annealed to at least 100 ℃. Some samples had no crystalline peaks until annealing at 200 ℃. From 200 ℃ -300 ℃, annealing increased crystallinity. For samples that were already crystalline, annealing at all temperatures increased their crystallinity slightly. Here, we think low substrate temperature during sputter deposition will result in amorphous or partially amorphous material. Moreover, it is clear that the crystallinity improves with increasing temperature. The improvement may be due to an increase in carrier density with increasing substrate temperature and this in turn can be attributed to the improvement of the crystallinity which results in decreasing donor sites trapped at the dislocations and grain boundaries [8].

It is well known that an important aspect of a sensor operation is its stability (drift) during operation and its repeatability over an extended period of time. The difference between calibration curves 24 h in operation and one week delay without operation of sensor 3 is less than 0.07%. Hence, we adopted the ITO thin film sputtered at substrate temperature of 300℃ as temperature sensor. Usually, the resistance of a resistor with positive temperature coefficient is represented by:

$R_T=R_0\, e^{A(T+273)}$

Where R_T is the resistance of the sensor at temperature (T, ℃), R_0 is a constant, which is decided by the size and physical characteristics of the material, and A is a constant, equal to the

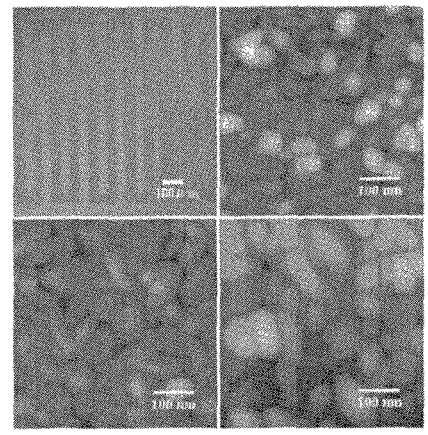

Fig.3 (a) The image of the ITO temperature sensor; and the surface topography of ITO thin films deposited on cover glass substrate at three substrate temperatures observed by AFM; (b) RT, (c) 200℃ and (d) 300℃.

temperature coefficient of resistance (TCR, /℃) of the sensor in value. Here, the TCR of ITO sensor is 0.7×10^{-3}/℃. To simplify the calibration, a linear temperature-resistance relationship is used, the linearity of correlation is represented by:

$$\boldsymbol{R_T} = 3.26 + 2.72\boldsymbol{T} \qquad (20<\boldsymbol{T}<110)$$

This relationship was used for the calibration of temperature sensors in PCR experiment.

3.2. PCR amplification

Optimizing primer concentration is very important and more complex for a SYBR® Green I assay. The optimization was completed by using the ABI PRISM 7700 Sequence Detection System. We contrasted the results using 100 nM and 400 nM primer concentrations. Both of the primer concentrations were challenged with no template controls (NTCs). We did not find strong amplification of the NTCs in dissociation curves and gel analysis. When using 400 nM primer concentration, the threshold cycle (Ct) values corresponding to 2.5 and 50 ng starting template quality were 38.1, and 33.6. While the primer concentration was lowered down to 100 nM, the

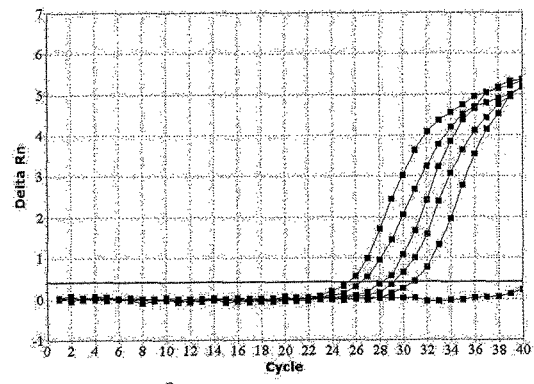

Fig.4 SYBR® Green I dye sensitivity amplification plot showing dilutions of starting template quantity.

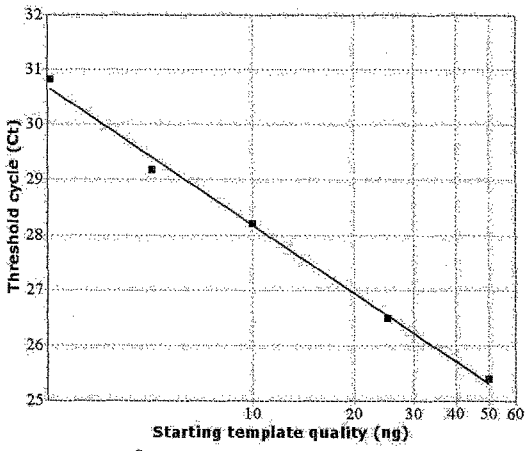

Fig.5 SYBR® Green I dye has been shown to produce results that are linear over 5 logs of starting template concentrations.

Ct values were 32.8 and 27.6 for 2.5 and 50 ng starting template quality. The Ct value is defined as the average standard deviation of the relative fluorescence intensity for the early cycles (3~20 here), multiplied by an adjustable factor (10 times here). In this case, the low primer concentration provided a lower Ct.

A representative experiment using the micro-chip and the optimal primer concentration (100 nM) is shown in Fig. 4. As expected, the more starting template quality in the reaction, the earlier such a rise in fluorescence occurs. Fig. 5 shows the linear relationship between the log of the starting template concentration and the number of cycles it takes for the amplifications in Fig. 5 to reach the Ct value. The line shown is regression fitted to the data points ($r^2 > 0.99$). The data point corresponding to 5 ng starting template quality has a big deviation from this line. Besides the data point at 5 ng, the data point most deviant from the regression line is at 2.5 ng. The Ct value corresponding to 2.5 and 50 ng starting template quality was 30.8 and 25.4. The Ct values are smaller than the values measured by ABI PRISM 7700 due to the micro scale. Fig. 6 shows the results of electrophoresis of PCR products. The gel was stained for 60 min with SYBR® Green I nucleic acid gel stain (using a 1:10,000 dilution of the stock reagent) and not destained. The SYBR® Green I dye-stained gel was then excited using 254 nm transillumination, and was photographed using a CCD camera and a SYBR® Green gel stain photographic filter. The 97-bp PCR products with different dilutions of templates were observed and non-specific products were not found. Thus, the microchannel chip performed the PCR amplification successfully.

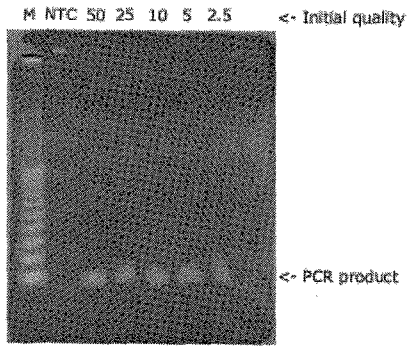

Fig.6 The expected mobility of the specific amplification product (97 bp) is indicated. The marker lane (M) contains 100, 200, 300, 400, 500, 700, 1200 bp DNA (170-8216, BIO-RAD).

4. CONCLUSIONS

A microchannel chip for quantitative PCR was developed by using transparent materials. So far there is no indication that a really transparent reactor has been developed and used for quantitative PCR. The difference between calibration curves 24 h in operation and one week delay without operation of the ITO temperature sensor is less than 0.07%. This result indicates that ITO film works well in measuring the temperature. The result of the quantitative PCR using our micro-chip shows that the Ct values are about 2 cycles smaller than the values measured by ABI PRISM 7700. The microchannel chip integrated with ITO films has high transmittance and thermal conductivity characteristics. This work demonstrated that the microchannel chip can be used to measure the fluorescence of the sample solution in the microchannel. Further improvements to this device will include multiple reaction (parallel channels), integration of purification unit and enhanced optical detection system.

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