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Continuous flow polymerase chain reaction using a hybrid PMMA-PC microchip with improved heat tolerance

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Abstract

Recently, polymeric materials have been explored as more versatile alternatives for the fabrication of polymerase chain reaction (PCR) microchips. Poly(methyl methacrylate) (PMMA) is a popular substrate material due to its high mechanical stability, good chemical properties and most importantly, its suitability for cheap and simple CO_2 laser ablation. However, it has a low glass transition temperature (T_g) of 105 °C, which is just above the denaturation temperature for PCR, thus the bond integrity is compromised. Polycarbonate (PC) is preferred as a substrate for PCR microchip as it has a higher T_g of 150 °C; but since its thermal properties are not suitable for CO_2 laser light, the more expensive excimer laser has to be employed. Here we report a novel hybrid PMMA-PC microchip by bonding a PC cover plate with a PMMA substrate containing microchannel which is fabricated by CO_2 laser ablation. This hybrid microchip has improved heat tolerance, such that the bonding integrity is sustained at the denaturation temperature. DNA amplification is found to be more efficiently performed in a PMMA-PC microchip than in a PMMA-PMMA microchip.

Keywords: PCR microchip; PMMA; PC; Hybrid; CO_2 laser ablation

1. Introduction

Polymerase chain reaction (PCR) process is widely used as a molecular biological tool to replicate DNA, and can create copies of specific fragments of DNA by cycling through three temperature steps. Each temperature cycle can double the amount of DNA, and 20–35 cycles can produce millions of DNA copies. Recently, much attention has been paid to the development of miniaturized PCR devices [1–3]. All kinds of PCR microfluidic technologies have facilitated DNA amplification with much faster rates as a result of smaller thermal capacity and larger heat transfer rate between the PCR sample and temperature-controlled components [4]. Most PCR adopted silicon or glass as the substrates, but the fabrication progress of the standard micromachining techniques was complex and expensive. Polymeric materials have been explored as more versatile alternatives for the fabrication of PCR microfluidic devices [5].

As PCR involves three temperatures (95 °C, 60 °C and 72 °C for denaturation, annealing and extension, respectively), only polymers with glass transition temperature (T_g) greater than 100 °C can be utilized for this application. Polycarbonate (PC) [6] and poly(methyl methacrylate) (PMMA) [7], etc. have been used for the fabrication of PCR microchips. PMMA is a vinyl polymer, made by free radical vinyl polymerization of the monomer methyl methacrylate and the structure is shown in Fig. 1(a). It is a popular substrate material due to its high mechanical stability, good chemical properties and excellent optical clarity [8]. Most importantly, it is suitable for cheap and simple CO_2-laser ablation which has been proven to be rapid and effective for fabricating microfluidic devices especially for scientific trials and small-scale production [9]. PMMA is cut by melting and vaporization, leaving relatively unaffected material at the cut edge. The material on the edge solidifies smoothly, giving a "fire-polished" edge. However, PMMA has a low T_g of 105 °C, which is just above the temperature for denaturation. At 95 °C, PMMA softens and the bonding integrity deteriorates, causing air to leak into the microchannel. Furthermore, since the length of microchannel in a continuous flow microchip is

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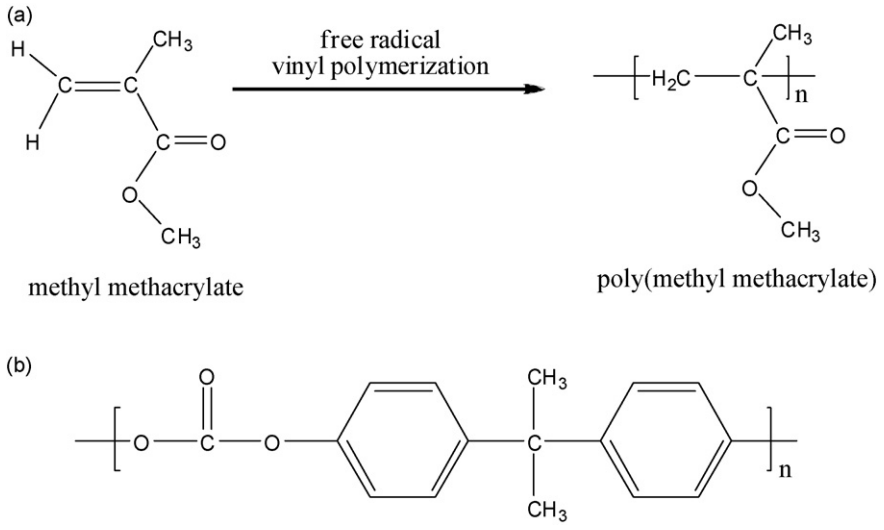

Fig. 1. (a) Structure of poly methyl methacrylate (PMMA). (b) Structure of polycarbonate (PC).

in excess of one hundred centimeter, syringe pumps are usually used for liquid actuation and pressures are often in excess of 0.1 MPa [10]. This leads to the bonding integrity being compromised. The appearance of air bubbles in the microchannel and leakage of fluids greatly reduce the PCR efficiency. As an alternative, we have previously used dry adhesive films for bonding between PMMA and other materials [11]. However, this bonding technique is only suitable for microreactors working at low temperatures and pressures such as micro fuel cells [12,13].

Hence, many researchers prefer PC to PMMA as PC exhibits outstanding heat tolerance [14]. PC gets its name from the presence of carbonate groups (–O–(C=O)–O–) in the long molecular chain, and the structure is shown in Fig. 1(b). Both PMMA and PC have compatible mechanical and thermal properties, as shown in Table 1 [15,16]. However, since PC has a higher thermal diffusivity and a higher glass transition temperature of 150 °C, it is not suitable for CO_2-laser ablation. The high diffusivity quickly dissipates the thermal energy of the laser beam, making it difficult to reach the relatively high glass temperature of PC. When PC is exposed to CO_2 laser light, it decomposes rather than melts, leaving a tarry brown residue, creating a rough edge [17]. For PC, excimer laser with wavelengths ranging from 157 nm to 351 nm has to be used, though it is more expensive due to the high cost of the excimer laser system and the corresponding masks.

Table 1
Common properties of polymethylmethacrylate (PMMA) and polycarbonate (PC)

Properties	PMMA [15]	PC [16]
Density (kg/m^3)	1190	1200
Yong's modulus (GPa)	2.4–3.3	2.3–2.4
Poisson's ratio	0.35–0.4	0.37
Thermal conductivity (W K^{-1} m^{-1})	0.17–0.19	0.19–0.22
Specific heat (J K^{-1} kg^{-1})	1400–1500	∼1200
Thermal diffusivity ($\times 10^{-8}$ m^2 s^{-1})	9.52–11.4	13.2–15.3
Thermal expansion coefficient ($\times 10^{-6}$ K^{-1})	70–77	66–70

In order to employ CO_2-laser ablation and make use of the heat tolerance of PC, we fabricated a novel continuous flow PCR chip by bonding a PMMA substrate containing microchannels with a PC cover plate. The serpentine microchannels were engraved by CO_2-laser micromachining which was also used to dice PMMA sheet and drill access holes. PC cover plate was affixed to PMMA substrate by direct thermal bonding. Thermal bonding is a process of joining two materials through solid-state diffusion, thus, the mixture of PMMA and PC were entangled and crosslinked at the interface. As glass transition temperature depends on the average chain length, this hybrid bonding provides higher T_g than the pure PMMA unity alone, allowing the bonding integrity to be sustained at the denaturation temperature. DNA amplification is found to be more efficiently performed in a PMMA-PC microchip than in a PMMA-PMMA microchip. Since the heat transfer between the heater blocks and the sample flow is based on heat conduction through the base plate made of PC and forced convection [18], the higher thermal conductivity and lower specific heat of PC would allow the sample to change its temperature faster while passing through the different temperature zones.

2. Experimental

2.1. Microchip design and fabrication

The layout of the microchip is shown in Fig. 2. It consisted of an inlet, an outlet, the denaturation zone, the extension zone and the annealing zone. The overall dimension of the CFPCR chip was 3 cm × 7 cm. The microchannel was 200 μm wide and 250 μm deep, with a total length of 166.4 cm, which was equivalent to a total volume of 41 μl and 26 cycles. The length ratio of denaturation, annealing and extension microchannels was 1:2:1 and the lengths of pre-denaturation and post-extention microchannels were 12 cm and 10.5 cm, respectively. The microfluidic pattern was designed using CorelDraw (Corel Co., Canada). The microchannel network was then sent

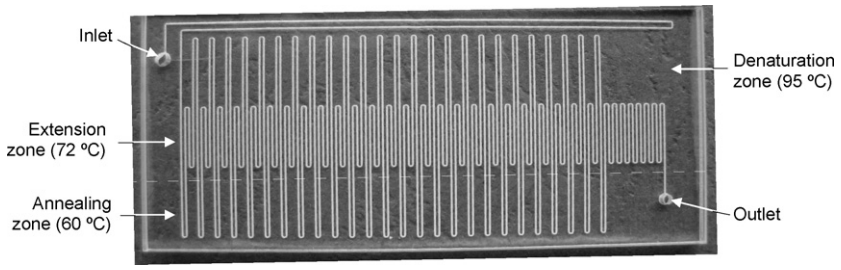

Fig. 2. The layout of the microchip which consisted of an inlet, an outlet, the denaturation zone, the extension zone and the annealing zone with 26 amplification cycles. The overall dimension of the continuous flow PCR microchip was 3 cm × 7 cm. The microchannel was 200 μm wide and 250 μm deep, with a total length of 166.4 cm, which was equivalent to a total volume of 41 μl and 26 cycles.

to a commercial CO_2 laser scriber (Universal M-300 Laser Machining Platform, Universal Laser Systems Inc., Arizona, USA) for direct micromachining on PMMA substrate (Goodfellow, England). Access holes were also drilled on the same PMMA substrate by CO_2 laser to allow fluid access to the microchannels. Two PMMA substrates were engraved. One of them was bonded with a PC cover plate (Goodfellow, England) and the other was bonded to a PMMA plate at 165 °C and 200 kPa for 30 min [9]. Microchannel widths and depths were measured using a scanning electron microscope (SEM) (N3500S, Hitachi Science Systems Ltd., Japan).

2.2. Bonding strength characterizaion

The tensile strength of the bond between PMMA-PC and PMMA-PMMA substrates were determined using an Instron 3369 tensile tester (Instron, Toronto, ON, CA). Two strips of substrates (75 mm × 25 mm × 1 mm) were overlapped (3 cm^2) and then thermally bonded to one another. The test specimen was clamped to the tensile tester with grips set 80 mm apart, and pulled away from each other at a rate of 1 mm/min. The force at which the bonding failed was measured. The shear bond strength of the specimen was calculated using the following equation:

$$\text{bond strength} = \frac{\text{break force}}{\text{bonding surface area}} \quad (1)$$

2.3. System set-up

The temperature control system (Tri-X Pte Ltd, Singapore) consists of a proportional-integral-derivative (PID) temperature controller, sensors and three copper heating blocks. Each copper block is 7 cm long, 0.8 cm wide and 3 cm high. Temperature can be set between 25 °C and 150 °C. The copper blocks are separated by small air gaps to ensure the formation of three distinct and steady temperature zones. Thermocouple sensors were attached to each copper block for temperature monitoring. The microchip was placed on top of the heating blocks. The temperature of the heater block at the denaturation zone was set at 95 °C, annealing zone at 60 °C and extension zone at 72 °C.

2.4. Preparation of PCR mixture

A 500 bp fragment of bacteriophage lambda DNA was amplified on both PMMA-PC and PMMA-PMMA chips. Forward primer is 23-mer (5′-GAT GAG TTC GTG TCC GTA CAA CT-3′) with a melting point of 64.1 °C. The primer coordinates are 7131–7153 on the lambda DNA template. Reverse primer is 23-mer (5′-GGT TAT CGA AAT CAG CCA CAG CG-3′) with a melting point of 70.3 °C. The primer coordinates are 7608–7630 on the template. The reaction mixture contained 10 mM Tris–HCl (pH 8.3), 2× BSA (0.5 μg/μl), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1 μM of each primer, 1 ng/μl lambda DNA as the PCR template, and 0.025 U/μl Taq DNA polymerase. All samples and reagents for PCR reaction were purchased from Research Biolabs (Singapore).

2.5. Procedure

Pre-mixed samples were transported to the microchip using a syringe pump (Cole-Parmer, Illinois, USA) through Teflon tube (Cole Parmer, Illinois, USA) fitted to the microchip inlet hole. After each run, the microchip was washed by deionized water and negative control of PCR was done to make sure that there was no carryover.

The amplified PCR products were analyzed using a 1.2% crosslinked agarose gel (Sigma Chemical Co., MO, USA) stained with Ethidium Bromide (EtBr) (Sigma Chemical Co., MO, USA). A 0.5 μg of 100 bp DNA ladder (500 μg/ml) was run together with PCR products in a slab gel electrophoresis apparatus (Hoefer, San Francisco, CA). The ladder can be used

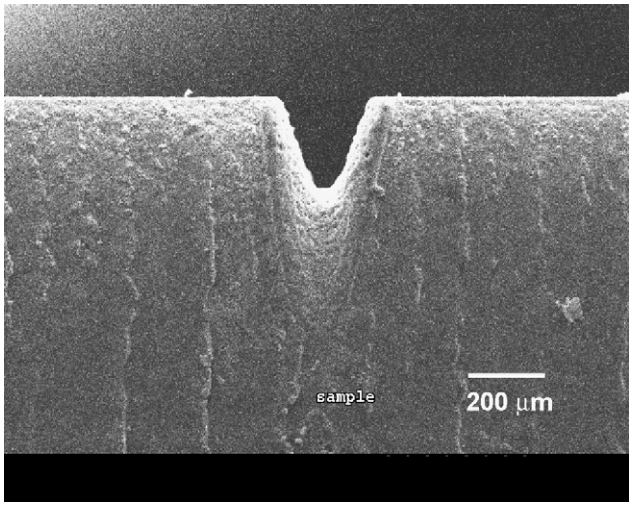

Fig. 3. SEM of the cross-section of a laser-machined microchannel in PMMA.

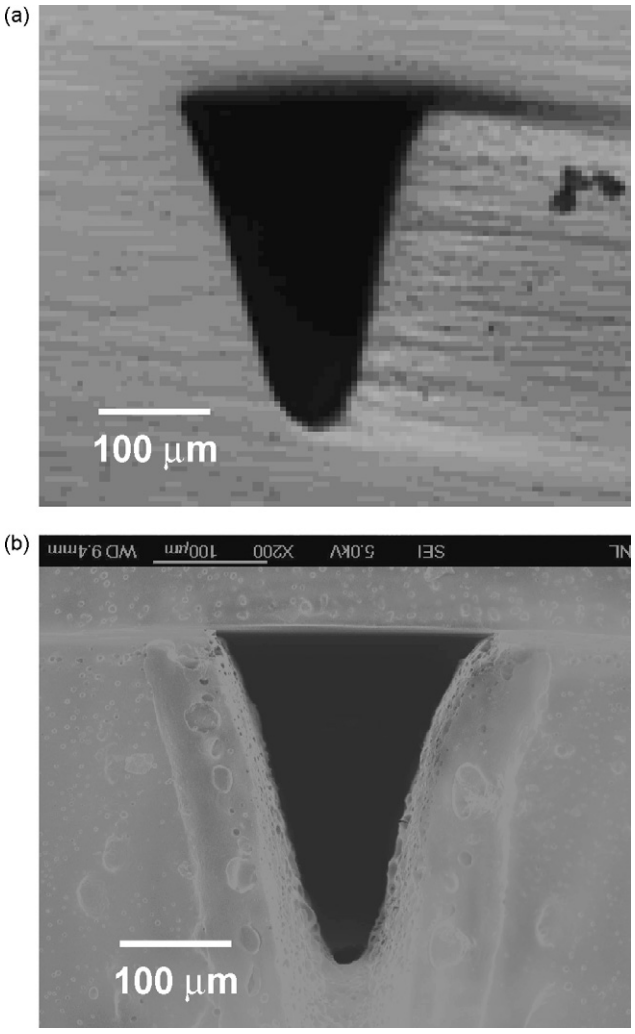

Fig. 4. Cross sections of the microchannels after thermal bonding. (a) PMMA-PMMA. (b) PMMA-PC.

for approximating the mass of DNA and the estimated mass of 500 bp DNA fragment in 0.5 μg loading is 97 ng. UV emission from the product was imaged by Genesnap software (Syngene Pte Ltd., UK) and integrated intensities of the product bands were calculated using image integration software Genetools (Syngene Pte Ltd., UK).

3. Results and discussions

3.1. Laser cutting

CO_2 laser has a relatively long characteristic wavelength of 10.6 μm and emits radiation continuously. It is very versatile and available with a wide range of output powers and at reasonable cost. Wherever the focused laser beam meets the PMMA substrate surface, the temperature of the irradiated spot will raise so rapidly that the material will first melt then vaporize, leaving a void in the substrate. By this means, the moving laser beam driven by stepper motors is able to cut structures such as microchannels and wells on the substrate. The cross section of the microchannel mainly depends on the shape of the laser beam, its scanning speed, the laser power and the thermal diffusivity of substrate material. The energy of the laser beam has a Gaussian distribution, resulting in a Gaussian shaped cross section. Fig. 3 shows the SEM of the cross section of the microchannel engraved in PMMA substrate. Engraved at laser power of 1.75 W and beam speed of 32 mm/s, the microchannel was measured to be 200 μm wide and 250 μm deep.

3.2. Thermal bonding

The two engraved PMMA substrates were bonded to PMMA and PC cover plate, respectively, by low pressure, high-temperature thermal bonding technique [9]. Thermal bonding is a process of joining two materials by the mechanism of solid-state diffusion; and unity of the materials is accomplished through the application of pressure at elevated temperature. When PMMA-PMMA or PMMA-PC substrates are put in close contact at 165 °C (the bonding temperature should be above the T_g of both materials), the long-chain molecules reciprocally diffuse into the other moiety. Fig. 4 shows the SEM of cross section of PMMA-PMMA and PMMA-PC microchannels. Almost no signs of bonding interface were observed. Despite the high bonding temperature, the microchannels engraved in PMMA retained their original Gaussian shapes and dimensions.

3.3. Bonding strength and heat tolerance

The bonding strength between PMMA-PC and PMMA-PMMA substrates were investigated at room temperature. Three specimens were prepared for each type of bonding. All specimens experienced bonding failure. The average break force for PMMA-PMMA chips was 645 N, corresponding to a bond failure pressure of 2.15 MPa. The bond failure pressure for PMMA-PC bonding was measured to be 2.28 MPa, slightly higher than that for PMMA-PMMA.

As the main aim is to create a microchip with higher heat tolerance, the temperature at which bonding integrity was overcome was measured. Deionized water was pumped into microchannel by syringe pump at the flow rate of 30 μl/min, and the temperature of the heating blocks was increased continuously by adjusting the PID controller. Formation of air bubbles and fluid leakage were observed in the PMMA-PMMA chip at 95 °C, flow became discontinuous and velocity could not be controlled. Whereas for PMMA-PC chip, bubbles formed and liquid started to leak at 114 °C. This shows that PMMA-PC bonding has better heat tolerance than PMMA-PMMA bonding. This is because for the PMMA-PC hybrid microchip, the mixture of PMMA and PC polymer chains were entangled and crosslinked at the interface. As glass transition temperature depends on the average chain length, this hybrid bonding can withstand higher temperature than the PMMA-PMMA microchip. The hybrid PMMA-PC microchip is thus better suited for high-temperature application, as in PCR.

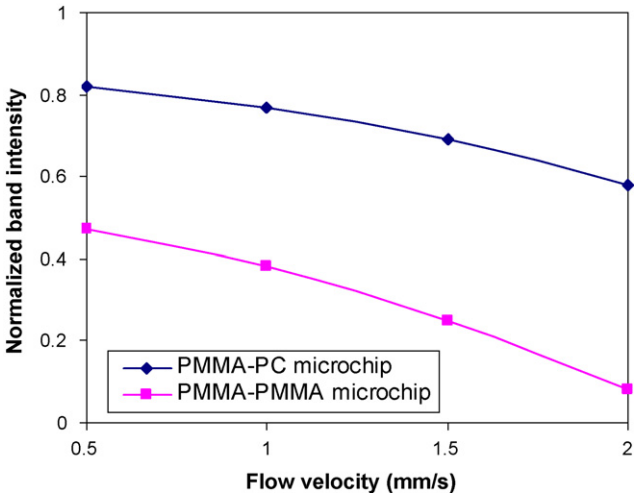

Fig. 5. Comparison of band intensities at different flow velocities. Values were normalized with respect to the intensity obtained from the 500 bp fragment of the 100 bp ladder. Each point was repeated three times.

3.4. PCR efficiency

DNA amplification was conducted on both PMMA-PC and PMMA-PMMA microchips with flow rate increases from 7.5 μl/min to 30 μl/min in step of 7.5 μl/min, corresponding to linear flow velocities of 0.5 mm/s, 1 mm/s, 1.5 mm/s and 2 mm/s, respectively. Twenty-six cycles were completed between 8.5 min and 34 min with cycle times from 20 s/cycle to 40 s/cycle. Fig. 5 shows the normalized gel band intensities of PCR products. It was found that the amount of PCR product from the hybrid PMMA-PC microchip was at least two times greater than that obtained from the PMMA-PMMA microchip. This is because as PMMA has a low glass transition temperature of 105 °C, at denaturation temperature, PMMA has started to change from glassy state to rubbery state, causing deterioration of the bonding integrity. Hence, the occurrence of air bubbles and fluid leakage greatly reduces the PCR efficiency. Unlike the PMMA chip, the hybrid PMMA-PC bonding has a higher glass transition temperature and is able to stay in glassy state at 95 °C; therefore, it can withstand high pressure and provide a much higher PCR efficiency.

With the increase of flow velocity, the PCR efficiency for both microchips was decreased. For the hybrid PMMA-PC microchip, the product yield at 2 mm/s was reduced 28% compared to that at 0.5 mm/s. Whereas, the amount of PCR product from the PMMA-PMMA microchip dropped more dramatically, and in this case, the yield at 2 mm/s was decreased 83% compared to the original amount at 0.5 mm/s. This decrease in PCR efficiency was partly due to the shorter residence time in the extension zone at higher flow speed, which led to insufficient time for DNA polymerase synthesis; hence, the PCR process could not be efficiently performed. However, it is obvious that the much larger drop of PCR efficiency, i.e. 83% decrease, for the PMMA-PMMA microchip was not solely caused by shorter extension time. With increase of pressure delivered by syringe pumps to increase the flow rate, the bonding was compromised and more bubbles were observed in the microchannel, resulting in discontinuous flow, which seriously affected the residence time of sample in each temperature zone. As a result, PCR efficiency was significantly reduced. On the contrary, the bonding integrity of hybrid PMMA-PC microchip could be sustained at 95 °C so that almost no bubbles appeared and flow inside the microchannel was well controlled, consequently it can endure higher flow rates to achieve rapid and high-efficiency PCR.

4. Conclusions

In this paper, we fabricated a novel hybrid PMMA-PC microchip for continuous flow PCR. The serpentine microchannels were engraved by cheap and simple CO_2-laser micromachining in PMMA. The substrate was thermally bonded to the PC plate to form the complete device. PCR efficiency obtained in the PMMA-PC microchip was higher than that in the PMMA-PMMA microchip. The hybrid PMMA-PC microchip exhibited improved heat tolerance, and was thus more suitable for high-temperature applications such as PCR or microreactors for reactions at elevated temperature. This new methodology for generation of microfluidic structures should facilitate the rapid fabrication of microdevices at low cost, and it could be widely used in biological, clinical and forensic fields.

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Nam-Trung Nguyen received his Dip-Ing, Dr Ing and Dr Ing Habil degrees from Chemnitz University of Technology, Germany, in 1993, 1997 and 2004, respectively. In 1998, he worked as a postdoctoral research engineer in the Berkeley Sensor and Actuator Center (UC Berkeley, USA). Currently, he is an associate professor with the School of Mechanical and Aerospace Engineering of the NTU in Singapore. His research is focused on microfluidics and instrumentation for biomedical applications. He published a number of research papers on microfluidics. The first and second editions of his bestseller "Fundamentals and Applications of Microfluidics" co-authored with S. Wereley were published in 2002 and 2006, respectively. His latest book "Micromixers: Fundamentals, Design and Fabrication" is published in 2008.

Yien Chian Kwok received his PhD in Chemistry in 2001 from Imperial College, London under the supervision of professor Andreas Manz. At Imperial College, he explored a novel detection methodology, named Shah Convolution Fourier transform (SCOFT), for ultra-sensitive detection in microfluidic devices. Now he is assistant professor of chemistry at National Institute of Education, NTU. His current research interests include the fabrication and use of plastic microfluidic devices for DNA and chemical analyses.