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Feasibility of On-chip Detection of Endotoxin by LAL Test

Chang Woo Suh[1], Sang Youn Hwang[1], Hyo Jin Choi[1], Gi Hoon Seong[2], Yoo Min Ahn[3], Yang Sun Kim[4], and Eun Kyu Lee[1]*

[1] Department of Chemical Engineering,
[2] Department of Applied Chemistry,
[3] Department of Mechanical Engineering,
[4] Micro Biochip Center, Hanyang University, Ansan 425-791, Korea

Abstract The LAL (Limulus amebocyte lysate) test for the detection and quantification of endotoxin is based on the gelation reaction between endotoxin and LAL from a blood extract of *Limulus polyphemus*. The test is labor intensive, requiring dedicated personnel, a relatively long reaction time (approximately 1 h), relatively large volumes of samples and reagents and the detection of the end-point is rather subjective. To solve these problems, a miniaturized LOC (lab-on-a-chip) prototype, 62 mm (L) × 18 mm (W), was fabricated using PDMS (polydimethylsiloxane) bonded to glass. Using this prototype, in which 2 mm (W) × 44.3 mm (L) × 100 μm (D) microfluidic channel was constructed, turbidometric and chromogenic assay detection methods were compared, and the chromogenic method was found the most suitable for a small volume assay. In this assay, the kinetic-point method was more accurate than the end-point method. The PDMS chip thickness was found to be minimized to around 2 mm to allow sufficient light transmittance, which necessitated the use of a glass slide bonding for chip rigidity. Due to this miniaturization, the test time was reduced from 1 h to less than 10 min, and the sample volume could be reduced from 100 to *ca.* 4.4 μL. In summation, this study suggested that the LOC using the LAL test principle could be an alternative as a semi-automated and reliable method for the detection of endotoxin.

Keywords: LAL test, endotoxin, lab-on-a-chip, PDMS, kinetic chromogenic assay, microfluidic chip

INTRODUCTION

Recently, the development of assays using microfluidic chips is one of the active research areas in life sciences, particularly because they could reduce the sample volume, shorten the turn-around time and allow several independent assays to be performed at the same time, *i.e.*, multiplexing [1]. Microfluidic chips are fabricated by applying semiconductor fabrication techniques. Since a silicon substrate is semi-transparent and unsuited for biochemical reactions, glass is more frequently used. However, a glass substrate is harder and more expensive to fabricate. To overcome these drawbacks, various polymeric materials have been developed for mass production using various techniques, such as REM (replica molding), μTM (micro transfer molding), MIMIC (micro molding in capillaries) and hot embossing [2]. Especially, PDMS (polydimethylsiloxane) is inexpensive, easy to fabricate and non-toxic, and thus widely used as a molding substrate [3]. LOC (lab-on-a-chip) based on microfluidics, are devices that allow continuous pretreatment, reaction, separation, detection, and analysis procedures on a chip. The advantages of a LOC over conventional assays in clude: shortened assay times, reduced sample volumes and simultaneous multiplexing [4].

Endotoxin is a lipopolysaccharide substance in the outer wall of Gram-negative bacteria, which is released when the cell wall is disrupted during cell division and decaying [5]. LAL (Limulus amebocyte lysate), a blood extract from *Limulus polyphemus* found on the Atlantic shores of the American continent, forms a gel from a gelation reaction with endotoxin [6]. This principle, called the LAL test, is used as an assay method for the detection of endotoxin in a variety of samples, such as pharmaceutical water, including water for injection, pharmaceutical intermediates and medical devices, *etc.* [8]. Several detection methods are available for the LAL test; gel clot, KTA (kinetic turbidometric assay) and KCA (kinetic chromogenic assay). The gel clot method looks at the hardness of the gel formed by a series of reactions involving several clotting factors, as shown in Fig. 1, by simply inverting a test tube containing the reaction mixture. The KTA measures the turbidity change at 340 nm when coagulogen finally gels into coaguline [9]. The KCA replaces the coagulogen with an artificial chromogenic peptide [10]. This method measures the color intensity at

***Corresponding author**
Tel: +82-31-400-5275 Fax: +82-31-408-3779
e-mail: eklee@hanyang.ac.kr

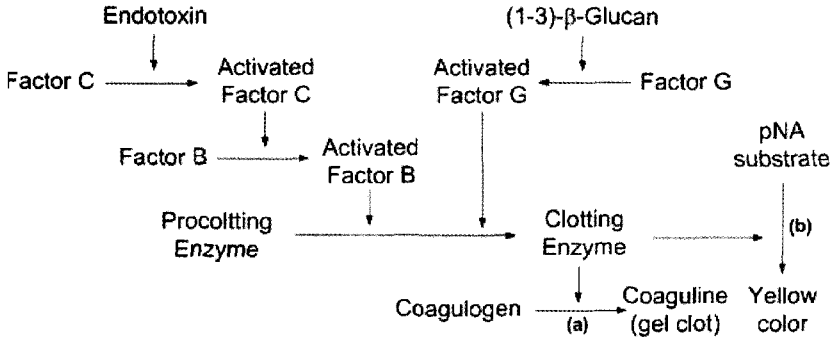


Fig. 1. Principle of the LAL assay. Endotoxin activates the Limulus amoebocyte lysate (LAL) pro-enzyme, resulting in gel formation (from [11]). (a) gel clot and kinetic turbidimetric assay, (b) kinetic chromogenic assay. pNA is p-nitroaniline.

405 nm (see Fig 1(b)), which is proportional to the endotoxin concentration in a sample. The current LAL test requires a 1 h reaction time and a 100 μL sample volume [11].

In this study, attempts were made to develop a LOC based on the LAL test principle, and the following feasibility experiments performed: First, the optimal on-chip detection method was investigated and, the minimal sample volume was then experimentally determined. Finally, the LAL test was performed on a PDMS-based chip to evaluate the accuracy and reproducibility of the on-chip detection method.

MATERIALS AND METHODS

Materials

The LAL test reagent was composed of LAL, LRW (LAL reagent water), containing less than 0.001 EU/mL, and CSE (control standard endotoxin). Endotoxin-free glassware and plasticware were used to prepare endotoxin and sample dilutions. All the regents for the KTA and KCA methods were purchased from Endosafe, Inc., USA. The LAL test was performed at 37 ± 1°C. An ELISA reader (Power Wave X340, Bio-Tec Instruments, USA) and 96-well plate were used for the KTA and KCA methods.

Analysis of Endotoxin

To confirm the validity and effectiveness of the LAL test method, a confirmation experiment was initially performed by certifying the sensitivity of the reagents and the non-inhibition of the sample [12]. The confirmation experiment was followed by negative, positive and positive product controls in the usual order and in duplicate. The negative control was carried to confirm the LRW used to prepare the diluted and standard endotoxin was not contaminated. The positive control was performed to identify the sensitivity of the reagents and the interference of the negative control, which used twice the endotoxin concentration of the standard CSE solution. Positive product and negative product control tests were not performed. After the confirmation experiment, a standard curve was constructed using 0.05, 0.1, 0.5, 1, 2, and 10 EU/mL CSE. Standard curves are needed for the KTA and KCA methods to quantify the LAL test following a predetermination [13]. The end-point method detected the absorbance at 405 nm after a predetermined time, and the kinetic-point method detected the time to reach the predetermined absorbance at 405 nm. A standard UV spectrophotometer was used to measure the absorbance.

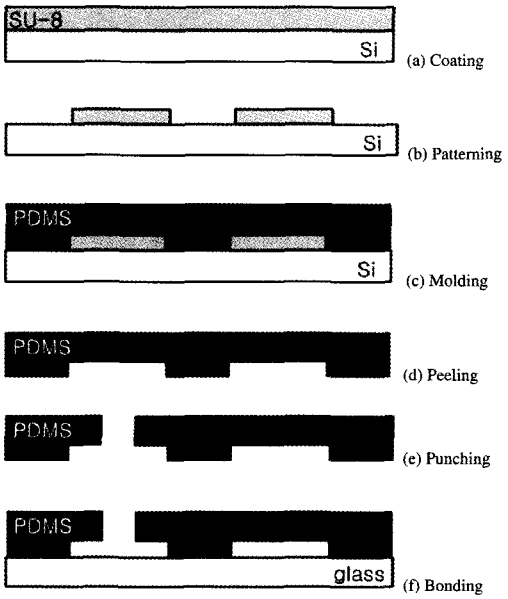


Fig. 2. Fabrication process of the PDMS chip.

Fabrication Process of PDMS Microchip

A PDMS LOC was constructed using the replica molding method. The top layer of a LOC was molded with PDMS, with a permanent seal formed between the slid glass and PDMS layer, which contained microchannels. Quartz and film masks were initially made using a photolithography process. Negative-photoresist, SU-8 (Nano™, SU-8, Microchem, USA), was spin coated onto a silicon circuit with a 100 μm thickness (Fig. 2(a)). To remove

the solvent from the coated photoresister it was heat treated at 65°C for 10 min and at 96°C for 30 min. The mold was created using photolithography (Fig. 2(c)). The molded PDMS was kept in a vacuum oven for 1 h to remove air bubbles. The PDMS replica was hardened by heat treatment at 65°C for 4 h, in a controlled temperature and humidity chamber (MTH2200, Sanyo, Japan). The PDMS replica was separated from the mold, and then punched to make a tubing hole (Fig. 2(e)). To seal the PDMS to the slide glass, the replica surface was treated with O_2 plasma in a RIE (reactive ion etching) machine (Fig. 2(f)). Lastly, a sample injection tube was inserted into the hole in the PDMS LOC.

Both LAL and CSE were dissolved separately with LRW to the predetermined concentrations. At time zero, equal volumes of LAL and CSE solutions were mixed and then injected to the chip through the sample hole using a syringe pump. Each sample volume was enlarged to cover the entire fluid channel. The spectrophotometer was situated to measure the absorbance at the middle of the channel. After 1 h, the absorbance was measured.

RESULTS AND DISCUSSION

Scouting of Optimal Detection Method

Since the gel clot method is unsuited for on-chip detection, the KTA and KCA methods were compared at low CSE concentrations (0.05~0.06 EU/mL) and a 50 μL sample volume. For the first 5 min of the reaction, both methods showed similar rates of change; however after 10 min the color intensity change in the KCA was much stronger. After 60 min, the absorbance at 405 nm in the KCA was 0.52, whereas that at 340 nm in the KTA was only 0.05. This result showed the KCA was more sensitive as a detection method than the KTA. Therefore, the following experiments employed the KCA method.

Effect of Sample Volume Reduction on Detection in KCA

When the sample volume was varied from 200 to 50 μL, the changes in the absorbance profile were nearly identical, and the gelation was completed in all the samples in *ca.* 50 min (see Fig. 3(a)). The final optical density was dependent on the volume, but when it was divided by the sample height, which was directly proportional to its volume on a 96-well plate, it was independent of the sample volume (see Table 1) In other words, the LAL reaction was dependent only on the CSE concentration. This implied that the depth of the detection was an important factor on the chip.

An effect of the CSE concentration (0.05, 0.5 and 5 EU/mL) on the absorbance change kinetics was observed with a 50 μL sample volume. As shown in Fig. 3(b), the initial changes depended on the concentration, but all assumed similar values after 50 min. To construct a calibration curve of the endotoxin concentrations against the absorbance values, the end-point and kinetic-point detections were compared. The end-point method used the absorbance value after 20 min, and the kinetic-point method used the time to reach an absorbance value of 0.1. Fig. 4 shows the kinetic–point method to be much more accurate (r^2 = 0.99) than the end-point method (r^2 = 0.84). The Korean Pharmacopoeia also specifies the linear regression coefficient in the LAL test to be higher than 0.98 [11,12]. Therefore, the kinetic-point was selected as the detection method.

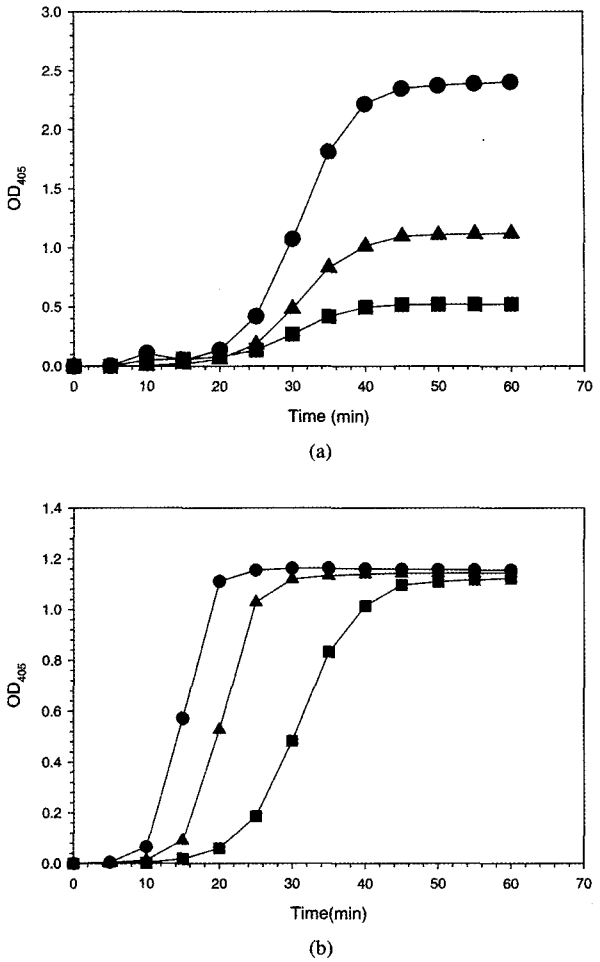
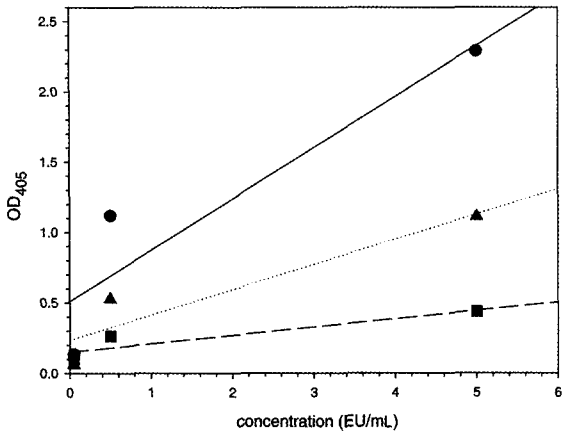


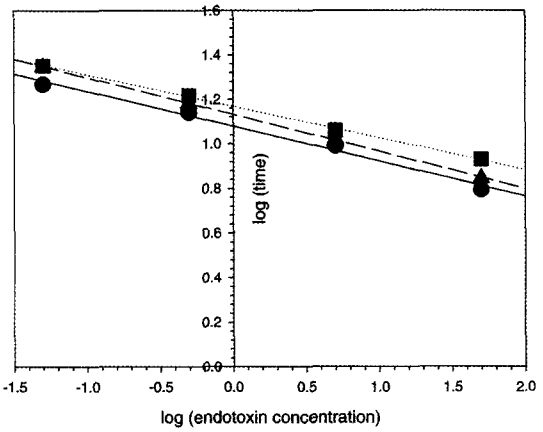
Fig. 3. (a) Effect of reaction volume; 50 (■), 100 (▲) and 200 μL (●), with 0.05 EU/mL CSE on the KCA results, (b) Effect of endotoxin concentration on the KCA with 50 μL. 0.05 (■), 0.5 (▲), 5 (●) EU/mL CSE.

Table 1. Relationship between the volume and optical density per unit depth

Volume (μL)	OD_{405nm} (-)	Depth (mm)	OD/Depth (mm^{-1})
50	0.523	1.0	0.52
100	1.123	2.5	0.45
200	2.404	5.0	0.48



(a)



(b)

Fig. 4. Standard curve for the KCA; 50 (■), 100 (▲) and 200 μL (●). (a) On set time. Correlation coefficient (r^2) is lower than 0.98, (b) On set OD_{405}, r^2 is higher than 0.98.

LAL Test Using the PDMS Chip

While the PDMS is easier to fabricate, obtaining the correct light absorbance value may be problematic due to its intrinsic light absorption property. Therefore, it was decided to determine the optimal thickness of the PDMS chip by measuring the light transmittance, with respect to the chip thickness. Fig. 5 shows the transmittance decreased with increasing thickness, and at a thickness of 2 mm, or greater, more than 15% was lost to absorption. However, because the PDMS was too soft a 2 mm-thick PDMS chip was bonded to a glass slide. A mold was made for mass production, as shown in Fig. 6(a). A simple lab-chip, consisting of a sample and reagent injection port, a linear fluid channel and a detection well, was constructed (see Fig. 6(b)). The chip length and width were 62 and 18 mm, respectively, and the width, length and depth of the fluid channel were 2 and 44.3 mm and 100 μm, respectively.

Employing this chip, the color intensity changes were measured using the KCA method at CSE concentrations

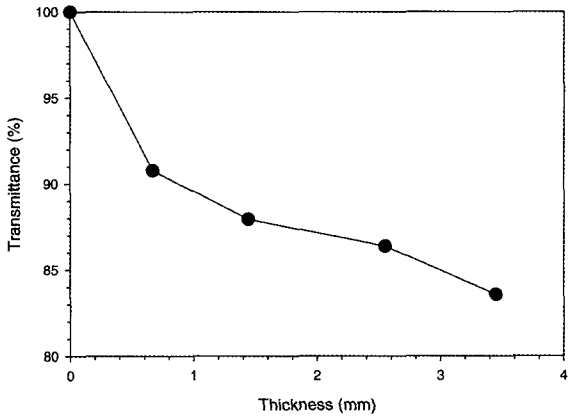
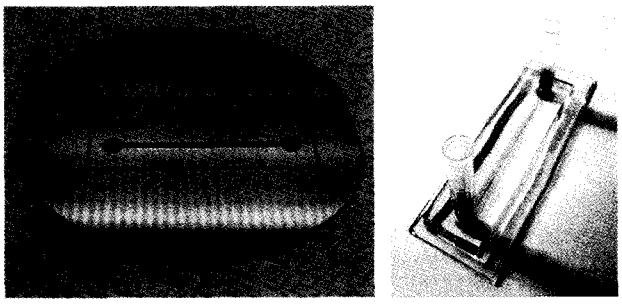


Fig. 5. Relationship between the PDMS chip thickness and light transmittance.



(a) (b)

Fig. 6. (a) Mold of LOC and (b) photograph of LOC.

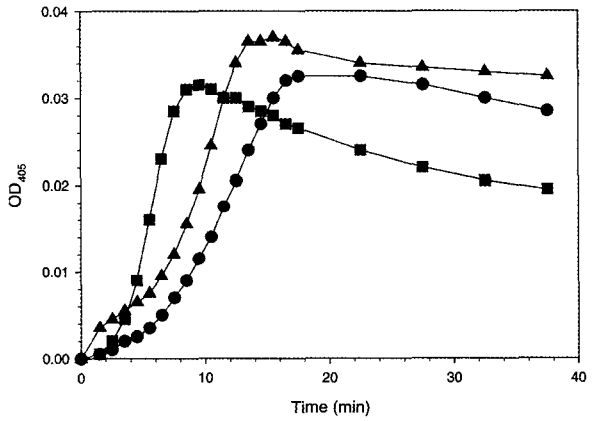


Fig. 7. Effect of endotoxin concentration on the optical density of the LOC. 0.05 (●), 0.5 (▲) and 5 (■) EU/mL CSE.

of 0.05, 0.5 and 5 EU/mL, with a 4.43 μL sample volume. As shown in Fig. 7, the absorbance changed rather rapidly during the early stage, depending on the endotoxin concentration, but plateaued or even decreased after 10~20 min. When a standard calibration curve was constructed, taking the time to reach an absorbance value of 0.01 at 405 nm in to consideration, a very accurate

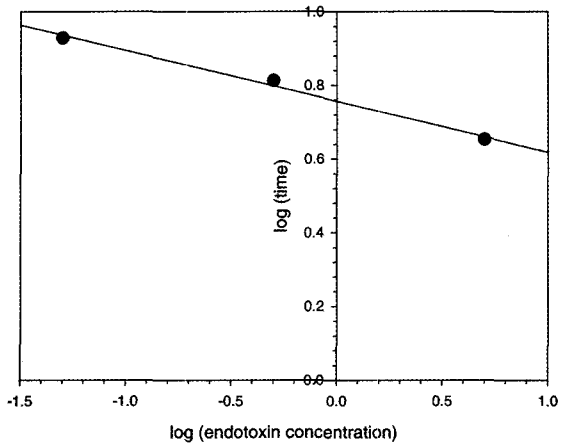


Fig. 8. Kinetic point standard curve from the LOC (on set OD_{405} = 0.01).

plot ($r^2 > 0.99$) resulted (see Fig. 8). The detection time was also reduced to less than 10 min. The accuracy and reliability of the chip test results were comparable with those from the conventional LAL test. Furthermore, this simple chip could be improved; having separate ports for sample injection and reagent introduction, and multi channels for multiple sample handling. This work is in progress.

CONCLUSION

The KCA was selected as the on-chip detection method of choice for two reasons; its sensitivity was higher at a lower sample volume, and the same absorbance value was obtained at the same CSE concentration, regardless of the sample volume. The kinetic-point method was also superior to the end-point method in terms of data accuracy. When the PDMS chip was fabricated and tested with the LAL assay, the assay time and sample volume could both be reduced, without sacrificing the data reliability. This feasibility study suggested the possibility of using a lab-chip for the detection of endotoxin using the LAL test principle.

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