Screening and electrochemical detection of an antibiotic producing gene in bacteria on an integrated microchip

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We demonstrate the electrochemical determination of a secondary metabolite producing gene on an integrated microfluidic chip. The fabricated microchip was assembled with a continuous channel for polymerase chain reaction (PCR) and an electrochemical detector in order to achieve rapid and sensitive determination of the valC gene. valC is a gene responsible for producing antibiotic validamycin A in Streptomyces hygroscopicus. Biotin-conjugated primers amplified the valC gene. After the PCR, a DNA amplicon was analyzed in the electrochemical cell containing a streptavidin functionalized Au working electrode. The guanosine present in the DNA amplicon released electrons upon electrochemical oxidation at 0.93 V and the peak current linearly increased with the concentration of the captured DNA amplicon. The fabricated chip successfully amplified and detected the valC gene as low as 30 pg mL⁻¹ resulting in a sensitive, portable and integrated DNA analysis for a secondary metabolite.

1. Introduction

Validamycin A is an antifungal antibiotic produced by Streptomyces hygroscopicus and used in East Asia as a crop protectant for the treatment of sheath blight disease of rice plants and damping-off of vegetables.¹ Validamycin A is a representative antibiotic of the C7:N aminocyclitol family of natural products.² It is a non-systemic antibiotic that controls the spread of the pathogen by reducing the hyphal growth rather than degrading the fungi.³ Interest in such biological control of plant pathogens has increased considerably to minimize the use of hazardous chemical pesticides and also because it provides control of diseases that cannot or can only partially be managed by other control strategies. The biosynthesis pathway of validamycin A involves the valC gene, functioning as a C7-cyclitol kinase.⁴

Over the past few decades, numerous strains of such antibiotic producing organisms have been isolated from diverse geographical regions.⁵,⁶ The classical culture and biochemical methods have been the conventional approaches used in the screening of antibiotic producing bacteria. The major limitations of these assays are that the test methods repeatedly use similar target organisms. Expensive and time consuming taxonomic studies are often required for identifying and characterizing the microorganism, after the biochemical confirmation. Rational selection of microorganisms by chemical or genetic fingerprinting provides a way to exclude previously isolated organisms from screening programs and thus, overcomes the problems of repetition.

Recently, molecular techniques have been used to detect these functional genes present within bacterial communities.⁷ After screening of an antibiotic producing bacterium and the responsible gene, various molecular studies and genetic manipulations can be performed to improve the activity. The isolated gene can be re-engineered and cloned to increase the antibiotic production. Genetic information of such genes is also important to study the antibiotic resistance.

In this regard, we designed and fabricated a microfluidic platform which is capable of screening and amplifying a target gene based on a continuous flow polymerase chain reaction (PCR) phenomenon using specific primers. The amplified gene can later be detected and quantified using on-chip electrochemical methods thereby leading to a self-sustained lab-on-a-chip (LOC). This on-chip PCR consumes much less sample and time to amplify and detect the gene than a conventional thermal cycler. A typical gene amplification and detection take about 1–2 days, but it has been performed in 70 min on our micro-analytical system. The fabricated microchip was employed to isolate, amplify and detect the validamycin A producing gene. For specific detection of the amplified gene, several step-modified gold electrodes with streptavidin at the terminal and biotin-tagged primers were utilized to produce an enhanced signal.

2. Materials and methods

2.1 Reagents and materials

The target DNA was extracted from transgenic Escherichia coli cloned with the valC gene. Biotinylated primers for valC gene amplification with the following base sequences: 5'-biotin-GTA
CGT CCG CAT ACC CAC GAC CCT GAT C-3’ (forward primer) and 5’-biotin-CAT CCC CAC GCT GGG TGA GAA GGA GTG-3’ (reverse primer) were used. Primers for valC were designed to amplify an 800 bp genomic region in the template DNA. 1 kb plus step DNA ladder and 100 bp step DNA ladder were used as DNA rulers (Bionics, Korea). Cystamine dihydrochloride, glutaraldehyde, streptavidin (from *Streptomyces avidinii*), bovine serum albumin (BSA) and potassium ferrocyanide [K₄Fe(CN)₆] were of analytical grade. Deionized water (DI water) and phosphate buffer (PBS) (0.2 M, pH 6.6) were used throughout the experiment.

2.2 Device fabrication

The integrated microchip was composed of a serpentine microchannel and an electrochemical cell enclosed in a polydimethylsiloxane (PDMS) layer. The detection electrodes were deposited on the indium tin oxide (ITO) coated glass substrate (Fine chemicals, Korea). ITO was used as a microheater for thermal heating due to its property of showing linear variation in its temperature by application of DC power.

To fabricate the ITO microheater, a positive photoresist (PR) (AZ-1512, Micro-Chem, USA) was spin-coated on the ITO side of the glass and then patterned using photolithography to cover the regions meant for different temperatures. The remaining ITO film was then etched using the FeCl₃/HCl solution and the photoresist was removed using acetone. Au electrodes for cyclic voltammetry were designed and deposited over the non-ITO side of glass, using photolithography and the evaporation method. A positive PR layer was first spin coated on the opposite side of a cleaned ITO patterned glass wafer and exposed to UV light through a photomask containing the electrode design. Subsequently, a 50 nm Ti layer and a 320 nm thick Au layer were deposited on this glass wafer using a vacuum thermal evaporator. Then, the patterned positive photoresist was removed by ultrasonication in acetone.

The microchannels used in this study were molded in the PDMS (Dow corning, USA) polymer using the negative molding method. For this purpose, a 120 μm thick negative photoresist (SU-8 2075, Micro-Chem, USA) was spin-coated and patterned on a silicon wafer. A degassed mixture of a Sylgard 184 silicone elastomer and a curing agent (in 10 : 1 v/v ratio) was poured on the SU-8 patterned wafer and cured for 4 h at 75 °C. The thus formed PDMS mold was peeled off manually and drilled to make access holes of 3 mm diameter each. For finalizing the device fabrication steps, the PDMS mold and glass substrate containing ITO/Au electrodes were bonded with each other by UV-ozone treatment, keeping the Au electrode side of the glass in contact with PDMS.

2.3 Temperature control for the PCR microchip

The temperature zones for PCR of the valC gene were set to 94, 55 and 72 °C for denaturation, annealing and extension, respectively. To achieve the desired temperatures, a calibrated DC power was applied to the ITO heaters. The temperature was optimized by applying different sets of DC power to the ITO electrode while DI water was flowed at the rate of 3 μL min⁻¹ using a syringe pump. The temperatures of the zones were measured using the thermocouple (CHAL-001, Omega, USA) specially bonded between the microchannels and the glass substrate. The power at separate electrodes had a synergistic effect on temperatures of different zones and was fixed to be 3640, 884 and 2940 mW for 94, 55 and 72 °C, respectively.

2.4 Microchannel treatment and electrode functionalization

Prior to PCR, the inner surface of the PDMS microchannel was treated by flowing 0.1 N HCl and 0.1 N NaOH for 5 minutes, respectively. After the acid/base treatment, DI water was pumped to clean the channel and then BSA was injected to coat the surface of the microchannel. Finally, the channel was cleaned again with DI water to remove the levorotatory BSA.

After the microchannel treatment, the Au electrode in a triangle shaped microchannel was cleaned by repetitive potential cycles between −0.2 and 1.2 V in 50 mM KOH (scan rate, 0.05 V s⁻¹) until a stable cyclic voltammogram was obtained. After electrochemical cleaning, 50 mM cystamine dihydrochloride was injected through the modification inlet and incubated for 24 h in the dark at room temperature (RT) and subsequently rinsed with phosphate buffer. Following the cystamine dihydrochloride binding on the Au electrode, 10% glutaraldehyde was introduced and incubated in the same way for 12 h at RT. The surface of the glutaraldehyde–cystamine/Au electrode, now containing exposed aldehyde moieties, was reacted with streptavidin for 4 h at 4 ºC to obtain a self-assembled streptavidin modified electrode (streptavidin–glutaraldehyde–cystamine/Au).

2.5 Electrochemical measurements

In this study, cyclic voltammetry (CV) measurement and electrochemical impedance spectroscopy (EIS) were carried out with CHI 800B and CHI 660B electrochemical analyzers (CH Instruments Inc., USA), respectively. The sequentially modified working Au electrodes and Au counter and reference electrodes were used for the measurement. Each step of electrode modification was confirmed and characterized using CV and EIS. CV after each modification step was performed in 5 mM K₄Fe(CN)₆ prepared in PBS buffer at a scan rate of 20 mV s⁻¹ within a potential range of −0.4 to 0.6 V. The EIS measurement was performed under similar conditions within a frequency range of 10⁴ to 1 Hz.

The final electrochemical detection of the streptavidin/biotin–DNA conjugate was performed in PBS buffer using CV. This CV was carried out within a scan range of 0 to 1.2 V at a scan rate of 10 mV s⁻¹.

2.6 Microchip operation

The overall experimental procedure was carried out using the following pathway. After the microchannel surface treatment and Au electrode modification, a calibrated DC bias was applied to the ITO electrodes laid on the backside of the glass substrate for heating the denaturation, annealing and extension PCR zones. To start the PCR, a mixture of template DNA, biotinylated primers and a pre-PCR mixture containing dNTPs, DNA polymerase, reaction buffer, and MgCl₂ (total volume: 50 μL) was injected into the inlet through a siliconic tube using a syringe.
pump at the flow rate of 3 μL min⁻¹. In this zone, the amplification of the target gene took place with the help of the enzyme and cyclic change in temperature. Finally after amplification, the amplicon was pumped into the electrochemical cell and the extra sample was allowed to pass to the outlet reservoir, which can be used for further analysis. Inside the electrochemical cell, the biotin conjugated DNA amplicon was left to bind with streptavidin for 30 min. After the designated time, the leftover DNA sample was sucked out using a syringe and the cell was filled with PBS buffer. Finally the CV was carried out to detect the amplified DNA.

3. Results and discussion

3.1 Microchip design

The image of the integrated microchip is shown in Fig. 1. The microchip was fabricated using standard photolithographic procedures on a 96 mm × 96 mm × 0.7 mm glass substrate. The microchip was disposable and meant for single use only. Microchannels were cast in the PDMS layer by a negative molding technique. The width and depth of the microchannel created were 250 μm and 120 μm, respectively, with 2950 mm length for 30 cycles. A continuous flow system transports the PCR mixture through different pre-fixed temperature zones for PCR. Compared to a stationary chamber based format, the continuous system approach provides faster thermal cycling because thermal inertia depends on the thermal mass of the sample, rather than the chip. In this system, the required heating and cooling sequence and the residence time were controlled by channel routing and flow speed. Each zone for denaturation, extension and annealing measured an area of 1150, 1250 and 1150 mm², respectively. The length of the channel for extension in each cycle was double those of the other zones. A larger extension zone assured longer residence time of the sample within this zone. The PCR module also contained provision for pre-PCR denaturation and final extension. The gaps between the temperature zones ensured efficient cooling of the circulating liquid in between them. Recently Peham et al. reported the use of Peltier devices attached with metal blocks to fabricate PCR. Such types of devices are rather far from miniaturization, and make the process tedious. In contrast, the patterned ITO heater used in this work enables the realization of a handheld device on a monolithic chip. PDMS is also cost effective compared to other reports, in which a SU-8 based microchannel or a silicon based device was used. The electrochemical cell had a total volume of ~10 μL. The dimensions of the Au working electrode were 3 mm × 3 mm while counter and reference electrodes had a length and a width of 4 mm × 1 mm.

3.2 Microchannel treatment and electrode functionalization

The PDMS surface has an affinity to bind with biomolecules. Thus, PCR within an untreated microchannel will lead to an insufficient amplification due to binding of polymerase and other biomolecules to the inner surface of the PDMS microchannel. Acid/base and BSA treatment of the microchannel

![Fig. 1](image1.png)

Integrated microchip. (a) Photograph of the integrated chip. The dotted line marks the ITO based heating zone (i) denaturation, (ii) annealing and (iii) extension; (iv) electrochemical cell showing working (W), counter (C) and reference (R) electrodes. (b) Cross sectional schematic of the device.

![Fig. 2](image2.png)

Schematic image of the Au electrode modification process. (i) 50 mM cystamine dihydrochloride deposition for 24 h in the dark at RT; (ii) 10% glutaraldehyde deposition for 12 h at RT; (iii) streptavidin deposition for 4 h at 4 °C; (iv) biotinylated DNA amplicon bound on the modified Au electrode by streptavidin–biotin interaction.
helped in preventing this erroneous amplification.\textsuperscript{16,17} Ionic treatment of the channel neutralized the inner charge of the PDMS microchannel while the injected BSA coated the microchannel surface. With these modifications, no free PDMS surface was left to bind with polymerase and other biomolecules.

The functionalization scheme of the Au electrode is summarized in Fig. 2. In short, the thiol group of cystamine dihydrochloride reacted with the Au surface to form a self-aligned monolayer.\textsuperscript{19} Later, one end of the glutaraldehyde bonded with the amine group of cystamine dihydrochloride while the other end bonded with streptavidin. The rinsing of excess cystamine dihydrochloride prevented its reaction with glutaraldehyde, resulting in solid mass formation. Glutaraldehyde easily binds with any protein through its amine group. To avoid the contamination of the PCR microchannel, glutaraldehyde was carefully introduced into the electrochemical cell. Finally with these modifications, the Au electrode had a streptavidin terminal end to bind specifically with the biotin conjugated DNA amplicon.\textsuperscript{19} Recently Moscovici \textit{et al.} also reported a similar technique for the detection of prostate cancer cells.\textsuperscript{20} Alternatively, Feng Li and coworkers proposed a gold surface modification protocol based on the self-assembly of gold nanoparticles on a mercapto-diazoaminobenzene monolayer modified electrode for the electrochemical sensing of DNA. Although this modification increased the sensing efficiency, the protocol is excessively complicated and time consuming.\textsuperscript{21}

Confirmation of each modification was performed using CV and EIS. Fig. 3(a) shows the cyclic voltammograms of the Au electrode in 5 mM K\textsubscript{4}Fe(CN)\textsubscript{6}. CV of K\textsubscript{4}Fe(CN)\textsubscript{6} using the Au electrode after each modification gave different electrochemical responses. The current signal from K\textsubscript{4}Fe(CN)\textsubscript{6} reduced consequently due to the binding of molecules on the Au electrode and less availability of free Au which confirms the functionalization of working electrodes. To further investigate the feasibility of the electrochemical assay, the EIS of electrodes was carried out under the same conditions. As shown in Fig. 3(b), the similar results were obtained using EIS. The bare Au surface exhibited a relatively straight line, attributing to a free electron transfer process. After the surface was treated with cystamine dihydrochloride, a significant increase of the electron transfer resistance was seen leading to a larger semicircle.

3.3 On-chip PCR

At the desired zonal temperature level, the mixture of template DNA, biotinylated primers and the pre-PCR mixture containing dNTPs, DNA polymerase, reaction buffer, and MgCl\textsubscript{2} was subjected to PCR amplification on the microchip. The reaction mixture was injected at an optimized flow rate of 3 \muL min\textsuperscript{-1} using a syringe pump, which took about 40 min for 30 cycles. The primer set used, amplified an 800 bp target of the \textit{valC} gene responsible for the synthesis of the antifungal antibiotic validamycin A.

Different initial concentrations of the template DNA were used to check the efficacy of our PCR microchip. The DNA amplicon obtained in this way was verified by conventional slab agarose gel electrophoresis for the presence of the desired product. A conventional PCR amplification of the target DNA was also performed for verification. Fig. 4 shows the gel-doc image of agarose gel electrophoresis for different amplified products. Lane 1 of Fig. 4(a) represents the target amplicon obtained using the microchip from a 30 ng \muL\textsuperscript{-1} initial template DNA concentration whereas lane 2 shows the amplicon from conventional PCR amplification. As clearly seen, the microchip produced a higher amount of amplified DNA as a result of continuous dynamic mixing of the reaction mixture, which is not available in a typical thermal cycler. Lanes 3, 4 and 5 represent the amplicon obtained using the microchip from 3 ng \muL\textsuperscript{-1}, 300 and 30 pg \muL\textsuperscript{-1} of initial template DNA concentration. For the negative control, amplification of lambda phage DNA and \textit{E. coli} was performed using the primers meant for the \textit{valC} gene. As anticipated, no product was obtained from lambda phage DNA, which is confirmed in lane 6 of Fig. 4(a) and lane 1
of Fig. 4(b). This result proved the efficacy of the device showing no non-specific amplification. The amplified product was later analyzed using a voltammetric electrode for verification and quantification.

3.4 Electrochemical detection of DNA
As mentioned in Section 3.2, the modification of the Au electrode surface was carried out to capture specifically the amplified target gene. Electrochemical measurement of each amplicon resulting from the different initial template concentrations was carried out within a scan range of 0 to 1.2 V at a rate of 10 mV s\(^{-1}\) in PBS buffer. The complete experimental flow of DNA amplification and detection of each concentration was performed on fresh devices and at least thrice to check the reproducibility of the proposed method. The guanosine present in the amplicon DNA released electrons upon electrochemical oxidation, and they passed to the gold working electrode through the linkers. The oxidation of guanosine is a two-step mechanism involving the loss of two electrons in each step. The oxidation, and they passed to the gold working electrode through the linkers. The oxidation of guanosine is a two-step mechanism involving the loss of two electrons in each step. The oxidation peak obtained was in accordance with the previously reported value. This obvious fact was also confirmed during our experiment. Continuous voltammetry for multiple cycles produced no change in oxidation peak or current response, which makes our system a very stable platform.

Typically in most of the integrated PCR chips, post-PCR processes carry out optical detection based on the fluorescence scanning of the biomolecule tagged with the reporter dye. In many cases, the fluorescent dye used for detection inhibits the PCR either by preferentially binding at certain regions of template DNA or altering their melting curve or inhibiting the activity of the enzyme, resulting in decreased amplification efficiency. Moreover the optical detection system consists of several submodules such as the laser source, lenses and the photomultiplier tube, which make the system hefty and highly expensive.

Alternatively, the electrochemical detection method relies on a relatively simple technique with use of only 3 electrodes, and can be fabricated (or miniaturized) on a chip. Few other groups

![Image](https://example.com/image.png)
have also reported the integration of electrochemical detection for PCR amplicons, which proved to be advantageous. For the determination, Yeung et al. microfabricated ITO electrodes while Ferguson et al. used gold as working electrode, and similarly Yamanaka and co-workers used a disposable printed electrode chip for amplicon detection.

The voltammetric peak obtained was only due to the binding of the amplified DNA product and not because of the binding of unused biotinylated primers. To demonstrate this, we performed a control voltammetry of the pre-PCR mixture along with primers, sans the template DNA. The mixture resulted in a noticeable voltammetric peak. The curve (▲) in Fig. 6 represents blank voltammetry of only PBS buffer while the curve (*) shows the control voltammetry of PCR primers. As a negative control, PCR amplicons of lambda phage DNA using non-biotinylated primers from a bench top thermal cycler showed no current response, which was well expected (Fig. 6 curve (●)).

4. Conclusion

We developed a microchip integrating two useful systems i.e. molecular analysis based on PCR and electrochemical detection for the screening of genes. The device was validated by amplifying and detecting the valC gene responsible for validamycin A biosynthesis. Effective integration of DNA amplification and electrochemical detection was successfully demonstrated here. Specific modification of the electrode proved essential for the detection. The fabricated chip exhibited high sensitivity and good selectivity for valC gene analysis. The ability to screen and detect the antibiotic producing gene can be useful to obtain a novel antibiotic, to tackle the ever-growing problem of multidrug resistance in pathogens.

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