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Microfluidic electrochemical assay for rapid detection and quantification of *Escherichia coli*

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ABSTRACT

Microfluidic electrochemical biosensor for performing Loop-mediated isothermal amplification (LAMP) was developed for the detection and quantification of *Escherichia coli*. The electrochemical detection for detecting the DNA amplification was achieved using Hoechst 33258 redox molecule and linear sweep voltametry (LSV). The DNA aggregation and minor groove binding with redox molecule cause a significant drop in the anodic oxidation of LSV. Unlike other electrochemical techniques, this method does not require the probe immobilization and the detection of the bacteria can be accomplished in a single chamber without DNA extraction and purification steps. The isothermal amplification time has a major role in the quantification of the bacteria. We have shown that we could detect and quantify 24 CFU/ml of bacteria and 8.6 fg/μl DNA in 60 min and 48 CFU/ml of bacteria in 35 min in LB media and urine samples. We believe that this microfluidic chip has great potential to be used as a point of care diagnostic (POC) device in the clinical/hospital application.

1. Introduction

Escherichia coli, one of the most versatile bacteria around the world (Kaper et al., 2004), has a number of unique features. It can exist as beneficial probiotics in the commensal of the digestive tract (Clarke, 2001) as well as a poisonous pathogen present in food and the environment. This bacteria is the major cause of many nosocomial diseases such as food poisoning, Meningitis (Bonacorsi and Bingen, 2005) and urinary tract infection (UTI) (Marrs et al., 2005; Johnson, 1991; Norrby and Stamm, 2001).

The conventional method for the detection and identification of bacterial cells is culturing techniques. However, due to the time-consuming (few days to several weeks) and labor-intensive protocol, it is not practical for rapid point of care (POC) diagnostics. Enzyme-linked immunosorbent assay (ELISA) is another technique that has widely been used (Su et al., 2011). However, it is semi-quantitative, requires multiple steps and has poor sensitivity which limits its reliability and accuracy (Zourob et al., 2008). Rapidity, accuracy and sensitivity are the foremost elements for POC diagnostic devices. Recently, DNA hybridization and gene amplification techniques have widely been investigated for the rapid detection of *E. coli* with higher sensitivity and specificity (Arnheim and Eldrich, 1992). Among them, molecular diagnostic based on gene amplification techniques such as polymerase chain reaction (PCR) has become a well-developed method for accurate detection of bacteria. The sensitivity of PCR for *E. coli* DNA detection is 1 ng/μl (4 h) with high specificity (Pollard et al., 1990). Nevertheless, PCR requires demanding hardware capabilities such as precise temperature control, temperature ramping and cycling (Arnheim and Eldrich, 1992). In addition, time-consuming process for sample preparation, nucleic acid extraction and post-processing fluorescence imaging make it difficult and complicated to be applied as a convenient technique in point of care (Asiello and Baeumner, 2011) diagnostic device.

Consequently, isothermal amplification techniques, which implement the amplification of the target nucleic acids in a constant temperature, have attracted significant interest for the rapid detection of bacteria (Asiello and Baeumner, 2011). However, all these amplification techniques have limitations. They need either multiple enzymes for strand displacement of the DNA template or precision instruments or elaborative methods for amplification, due to low specificity (Notomi et al., 2000).

Loop-mediated isothermal amplification (LAMP) is an alternative isothermal amplification technique that is accurate, fast, cost-effective with high sensitivity and high specificity (Notomi et al., 2000). It can amplify a few copies of DNA to 10^9 copies in less than an hour at 60–66 °C (Parida et al., 2009). LAMP process has been used for detection of various pathogens such as *H1N1 Influenza* (Arnheim and Eldrich, 1992), *Staphylococcus aureus* (Misawa et al., 2007), *E. coli* (Hill et al., 2008), *West Nile* Virus (Parida et al., 2004), *Salmonella enterica* (Ohtsuka et al., 2005). Moreover, LAMP amplicons can be measured optically or colorimetrically

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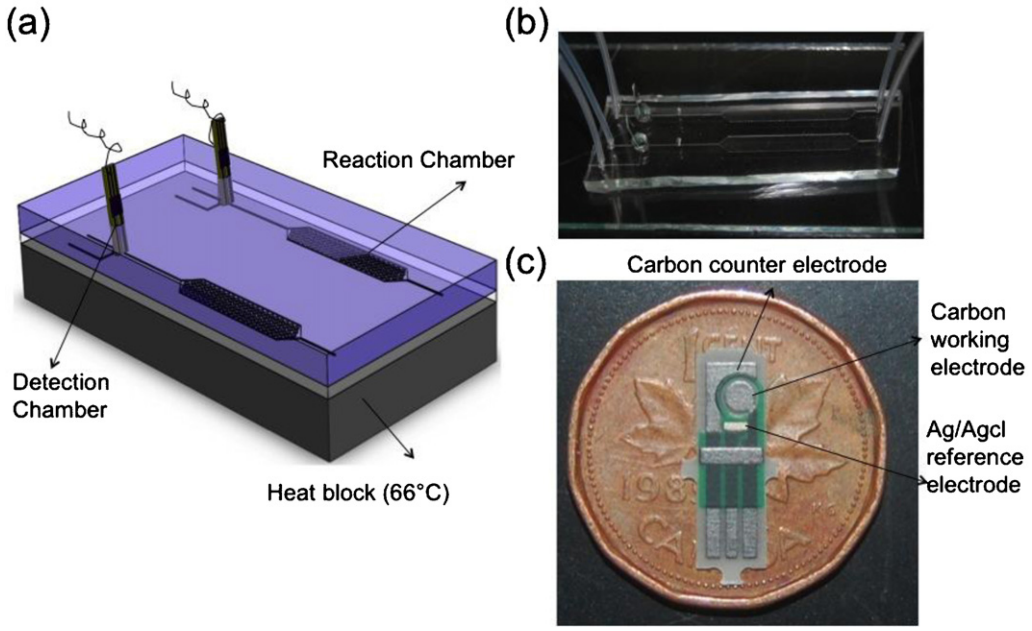

Fig. 1. Schematic representation of the microfluidic chip for *E. coli* detection. (a) Microfluidic chip composed of a heat block as the heat source to provide 66 °C, glass slide as the substrate and PDMS chip. The PDMS chip contained two parallel microfluidic chips for negative control and DNA detection. The microfluidic chip composed of a reaction chamber, an active valve (are not shown here) and an electrode chamber. (b) Image of the chip composed of two parallel microfluidic chip and capillary tubes, which connect the chip to the syringe pump, (c) micrograph of the disposable electrochemical printed (DEP) chip used for electrochemical detection.

using DNA-interacting dye (Gill and Ghaemi, 2008). However, conventional florescent-based optical measurements need flurophore labeling and expensive fluorescence analysis equipments. Colorimetric assays are less sensitive and they only provide qualitative result and analyze the existence of the DNA template in the samples (Su et al., 2011; Goto et al., 2009).

Despite the attractiveness of LAMP technique, there are still some problems and drawbacks, hindering its application in routine POC diagnostics. The sample preparation and nucleic acid extraction need precise protocol and highly skilled personnel and is a time-consuming procedure. In addition, DNA amplification is labor-intensive and requires laboratory based equipment and bulky controllers. Therefore, there is an immense demand for a compact device that can accomplish all the processes in a short period of time. This has led to the advance of microfluidics, which has been widely applied in the molecular diagnostics and gene amplification in the past years (Arora et al., 2010; Yager et al., 2008). Microfluidics refers to the manipulation of minute amount of liquid, which takes place in closed micro-scale channels (Manz et al., 1992; Whitesides, 2006). It reduces the consumption of the costly reagents. By increasing the surface to volume ratio, it enhances the undergoing reactions. Microfluidic is a reliable tool for the development and application of miniaturized biosensors. Yet, it requires a robust sensing technique in order to be implemented for the labeling rules (Lee, 2008). Over the past decade, many sensing techniques such as optical, colorimetric or electrochemical methods have been developed to provide new approaches to monitor biorecognition. Among them, electrochemical-based techniques provide sensitivity, selectivity, and low cost detection of the amplified DNA sequences. In fact, the advantages of the electrochemical detection over optical detection techniques include not only the inherent miniaturization and portability, but also the independence from sample turbidity, extremely low-cost/low-power requirements and compatibility with microfabrication technology (Lee, 2008; Drummond et al., 2003).

Here, we integrate the merit of the LAMP, microfluidic and electrochemical detection to construct an inexpensive portable device for the rapid detection of *E. coli*. First, the design and fabrication of the microfluidic device is demonstrated. Then, the electrochemical detection of *E. coli* on chip is presented, followed by an analysis of the sensitivity and specificity of the chip. The amplification time is optimized and the chip is calibrated and established as a robust, consistent diagnostic device.

2. Materials and methods

2.1. Bacteria preparation and DNA extraction

E. coli was freshly prepared and grown overnight (12 h) in 2% LB broth media. *E. coli* DNA was extracted using GenElute™ DNA extraction kit from Sigma–Aldrich (MO,USA). The final extracted DNA solution has 35.3 ng/μl concentration. Hoechst 33258 ([20(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,50-bi (1Hbenzimidazole)]) was purchased from Sigma–Aldrich (MO, USA). The Hoechst solution was prepared with a concentration of 100 μM and was kept in the dark at 4 °C.

2.2. Microfluidic chip

We have fabricated a microfluidic chip made of poly (dimethyl siloxhane) (PDMS), using soft lithography techniques (Duffy et al., 1998). The PDMS was purchased from Dow Corning Corporation (Michigan, USA). The heat block was made from aluminum and the controller unit was purchased from Omega Engineering Inc. (USA). The glass slides were obtained from VWR International (Quebec, Canada). Disposable electrochemical printed (DEP) chips contained a carbon working electrode, carbon counter electrode and Ag/AgCl reference electrode and were purchased from Bio-device Technology (Ishikawa, Japan). The chip was connected to syringe pumps (New Era Pump Systems Inc., New York, USA) by capillary tubes (Upchurch Scientific, USA).

2.3. LAMP reaction

We used the modified protocol as mentioned previously (Hill et al., 2008) to detect the *malB* gene in *E. coli*. The primers were

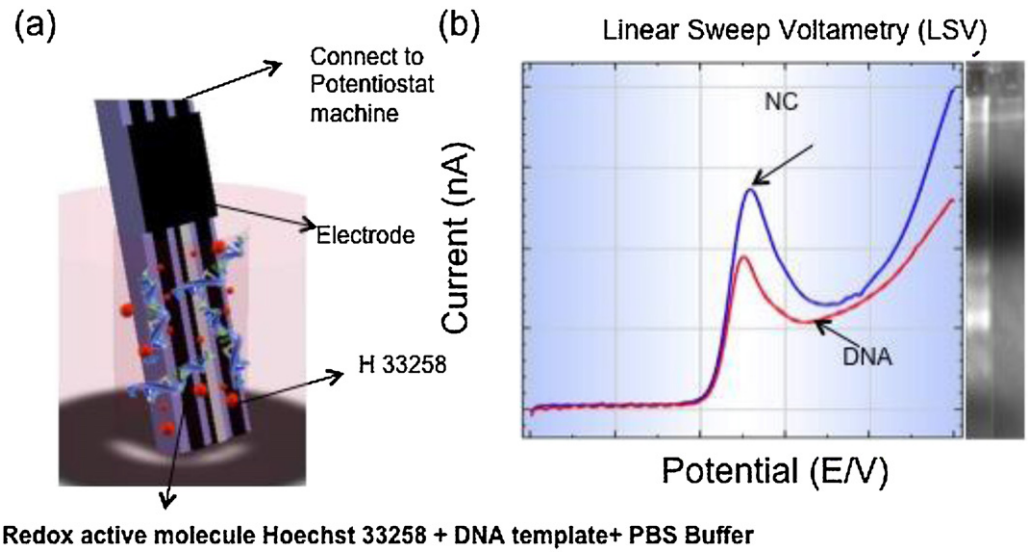

Fig. 2. Schematic of the electrochemical detection in the electrode chamber on microfluidic chip. (a) The redox active molecule Hoechst 33258 binds to the DNA minor groove, which causes the major reduction of electron on the surface of the electrode. (b) Linear sweep voltametry (LSV) as an electrochemical method demonstrating major drop in DNA detection in comparison with negative control sample. Gel electrophoresis image of negative sample, and positive sample, confirms the DNA amplification.

purchased from IDT (USA). 25 μl of reaction master mix contained 3.0 μl of 0.6 M Betain (Sigma–Aldrich, USA), 2.5 μl of 10× Thermopol buffer, 0.75 μl of 3 mM $MgSO_4$, 2.0 μl of *Bst Polymerase* (1600 units), 8000 U/ml (New England BioLab, Ontario, Canada), 0.4 mM concentration 0.4 μl dNTP (BioShop Canada Inc., Ontario, Canada), 5.75 μl of distilled water, 0.2 μl of 0.2 μM outer primers (F3, B3), 1.8 μl of 1.6 μM inner primers (FIP, BIP) and 0.8 μl of 0.8 μM loop primers (Loop F, Loop B).

2.4. Electrochemical detection

All electrochemical experiments were implemented using the linear sweep voltametry (LSV) method. We used the Autolab PGSTAT 101 (Eco Chemie, The Netherlands). The potentiostate was connected to the computer using the NOVA 1.6 software. The measurements were repeated at least three times and all the experiments were run at room temperature (22–27 °C). The step potential and the scan rate were 0.00244 and 0.1 V/s, respectively. DNA was detected by using the mixture of the LAMP solution with the PBS buffer and 20 μM of the Hoechst 33258 molecule.

3. Results and discussion

3.1. DNA detection

In the developed microfluidic assay, electrochemical detection of the amplified DNA was first implemented. This was followed by analysis of the extracted DNA detection on a chip. The chip is composed of an aluminum heat block as a source of thermal energy, glass substrate and PDMS chip. Each PDMS chip is composed of two parallel microfluidics to detect the negative control and sample with DNA template. The microfluidic chip contains channels with 200-μm in width and 200-μm in height, a reaction chamber and a detection chamber for electrochemical measurement. The reaction chamber has 35-μl volume and contains many cylindrical shape outgrowth with 2-μm diameter in width to increase surface to volume ratio. The detection chamber has 3 mm diameter width. Fig. 1 shows the schematic of the microfluidic chip. Each 30 μl of the LAMP consisted of 25 μl master mix and 5 μl DNA template (Ahmed et al., 2007).

Based on this ratio, 35 μl of LAMP solution was prepared and was injected into the reaction chamber using a syringe pump with a flow rate of 15 μl/min. The solution was kept in the reaction chamber using an active valve. The active valve was made of a piece of PDMS, which was set vertically to block the channel. The sample remained in the reaction chamber to amplify the DNA target and subsequently released to the detection chamber for electrochemical measurements. Meanwhile, the PBS buffer and redox solution from another entrance of the electrode chamber were added to the LAMP mixture. Each 6 μl of the LAMP product was mixed with 12 μl

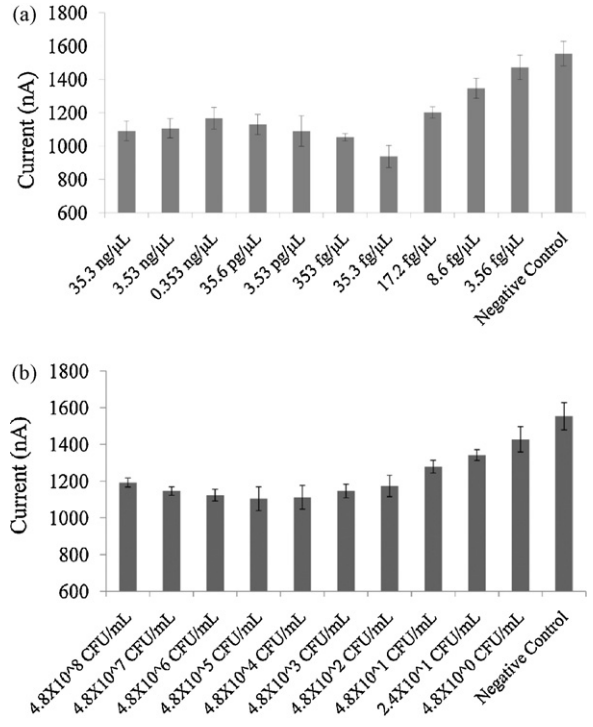

Fig. 3. Electrochemical detection of *E. coli* in the microfluidic chip. (a) Maximum peak current in LSV for different extracted *E. coli* DNA concentration. The detection limit on the chip is 8.6 fg/μl. (b) Maximum peak current in LSV for different bacteria concentration. The detection limit for the bacteria detection on chip is 24 CFU/ml.

of PBS buffer and 12 μl of the redox solution (Ahmed et al., 2007), produced, 30 μl of amplicon from the reaction chamber. Based on this mixing ratio, we added 120 μl of PBS buffer and redox solution to the detection chamber. This amount of solution covered the surface area of the electrode in the detection chamber. Then LSV measurement was performed and illustrated the anodic oxidation of the bio-sample (Ahmed et al., 2008). The difference in the maximum peak of the anodic oxidation between the negative control and the DNA sample form the basis for the detection and quantification of the amplified DNA. Fig. 2a shows the detection mechanism using the Hoechst 33258 redox molecule. When DNA was amplified, the more redox molecules are bound to the DNA minor groove causing a significant drop in the anodic oxidation of LSV as shown in Fig. 2b.

The drop in the peak maxima in the LSV with respect to the negative control for different concentrations of DNA were plotted for clarity as bars as shown in Fig. 3. It was found that the high concentration of the DNA has almost the same current peak (bar height) while the DNA concentration decreases the current peak

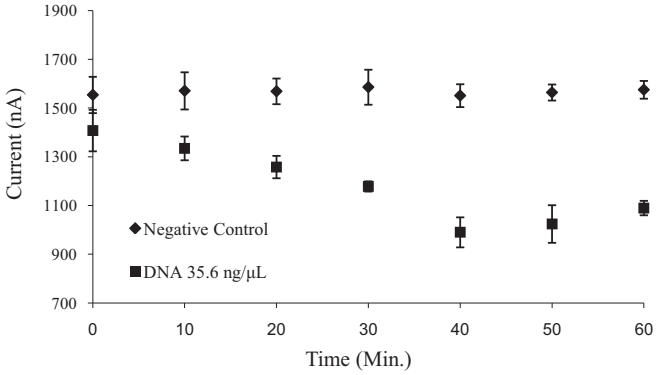

Fig. 4. Amplification time optimization on the chip. (♦) illustrates the negative control and (■) data represents the DNA sample. The chip detects the target DNA in 20 min. The DNA concentration is 35.6 ng/μl. Each measurement is carried out in separate chips and error bar has been included to reflect the reproducibility of the data.

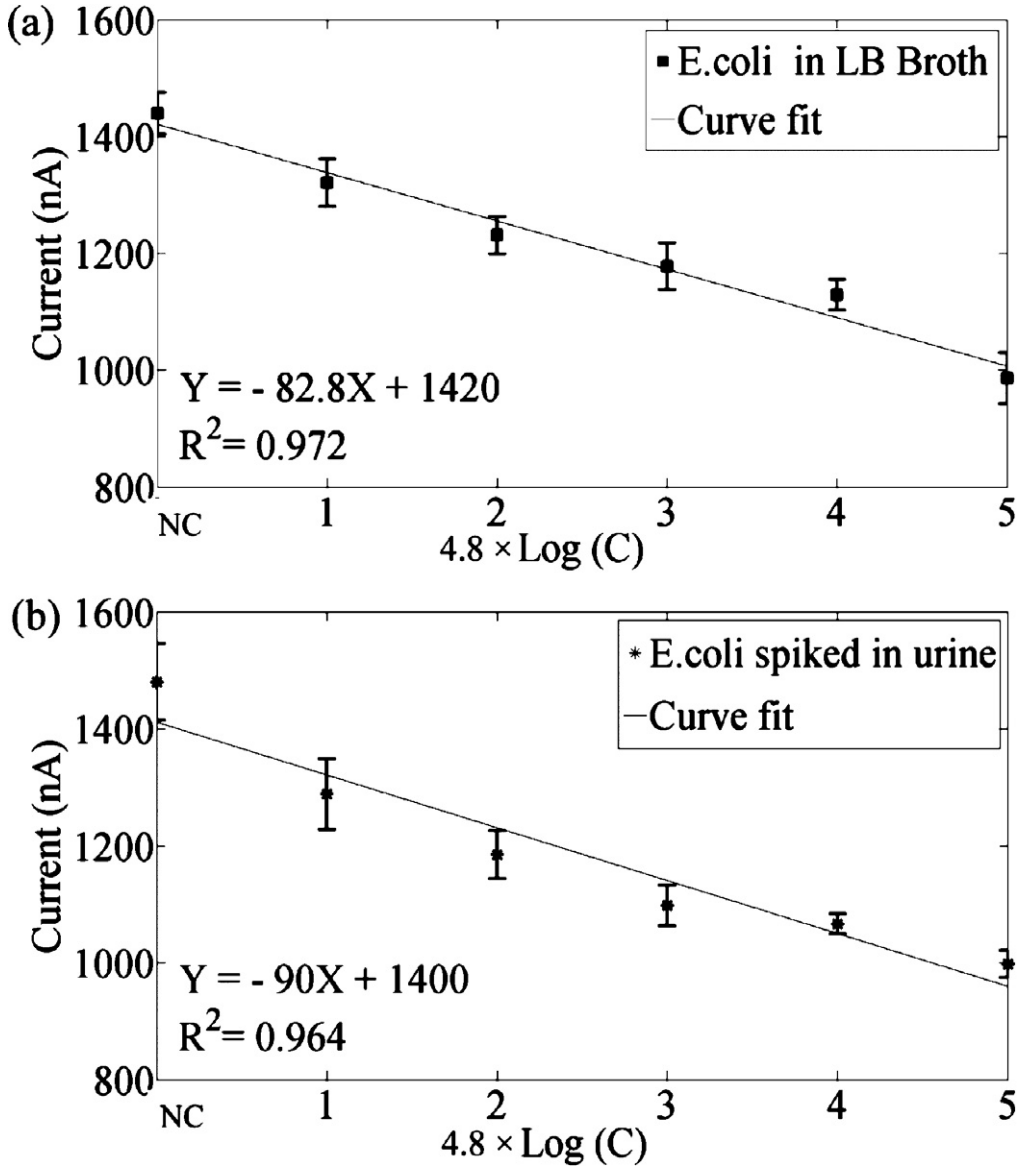

Fig. 5. Quantification of *E. coli* bacteria in different media. Microfluidic chip is calibrated with different samples of bacteria spiked in (a) LB broth, (b) urine samples using 35 min amplification time. Linear relationship exists between the logarithmic concentration and the anodic oxidation current. The R^2 for the LB broth and urine media are 0.97 and 0.94 respectively.

(bar height) increases (Fig. 3a). This behavior for the drop in the current with varying DNA concentration has been reported previously (Ahmed et al., 2007, 2009). For high concentration of DNA, the aggregation of the redox solution and amplicons reaches to saturation level. This aggregation causes a major reduction of the electrons on the surface of the electrode, which results in a significant drop of the peak maxima of the anodic oxidation. On the other hand, decreasing the DNA concentration causes less redox molecules to aggregate with the amplicons and accordingly, more electrons exist on the surface of the electrode. In turn, the anodic oxidation peak value increases (Fig. 3a and b).

3.2. Bacteria detection

The detection of bacteria at different concentrations was also assessed (Fig. 3b). Many diagnostic and theranostic devices have different sections/steps on the chip for bacteria lysis, DNA extraction and immobilization before the detection step. This makes the structure and design of the chip complicated and increases the fabrication cost. For example, Wang et al., used a microfluidic platform to lyse *S. aureus,* extract and purify the DNA from the cell component. The target DNA was amplified using the LAMP method, followed by optical detection (Wang et al., 2011). Nakamura et al., used the LAMP to detect six different nucleotides for gene analysis using electrochemical detection with Hoechst 33258 redox molecule (Nakamura et al., 2007). The detection was based on the immobilization of probe sequence on the electrode surface, which needs multi-steps treatment, as well as time-consuming/costly process. Furthermore, the detection took 2 h and required hybridization and washing steps. Conversely, the microfluidic chip presented here does not need any pretreatment step(s) for bio-samples. Isothermal temperature at 66 °C provides enough thermal shock to lyse the *E. coli* bacterial target in this study. Subsequently, the DNA from the bacteria was amplified and was detected using LSV without any pretreatment steps. Fig. 3b shows the detection of various concentrations of *E. coli* without any pretreatment steps and reagents. The detection limit for the *E. coli* bacterial cells is 24 colony-forming units (CFU/ml) in 1 h.

3.3. Time optimization

A series of experiments has been done to optimize the *E. coli* LAMP products under isothermal amplification for different time periods (0,10, 20, 30, 40, 50, 60 min), using the LSV electrochemical detection technique (Fig. 4). 35.3 ng/μl of DNA template was used in the experiment while water containing no DNA is used in the negative control. It was found that LAMP product can be detected in 20 min and the LAMP amplicons reached the saturation level after 40 min (Fig. 4). Based on Fig. 4 the DNA template can be detected in less than 20 min without immobilization and purification step. However, it is expected a longer amplification time is required for lower concentrations of DNA, since more time is needed to amplify the DNA to reach a detectable level.

3.4. Chip calibration

E. coli has been widely reported as the major cause for gastrointestinal disease around the world (Clarke, 2001). More importantly, pathological *E. coli* cells can be found in the human body fluids such as blood or urine. Therefore, detection of *E. coli* in the biological sample is necessary. A major improvement in disease control can be made if the target bacteria are rapidly detected and quantified accurately with high sensitivity by this microfluidic assay. In order to apply this device as a POC diagnostic device, calibration of the bacteria detection in the chip is required. We have reduced the amplification time to 35 min and measured the LAMP product at different concentrations of *E. coli*. The urine sample was filtered off-chip using 0.2 μm filter to purify and remove the impurities from the sample prior spiking with bacteria. It was reported (Kaneko et al., 2007; Hill et al., 2008) that the level of the urine sample dilution plays a critical role in the success of the LAMP amplification since certain urine concentrations in the sample could inhibit the amplification reaction. For example, Kaneko et al., showed that existence of the 2% urine in the sample inhibit LAMP amplification (Kaneko et al., 2007). Our LAMP primer's reactions are not inhibited in the presence of 20% of urine in the sample (Hill et al., 2008). 5 ml of the urine in 25 ml of the master mix was used, which is lower than 20% of urine inhibition limit. Fig. 5 shows a linear relationship between the current (anodic peak maxima for each bacterial concentration) and the logarithmic scale of the bacteria concentration in different media.

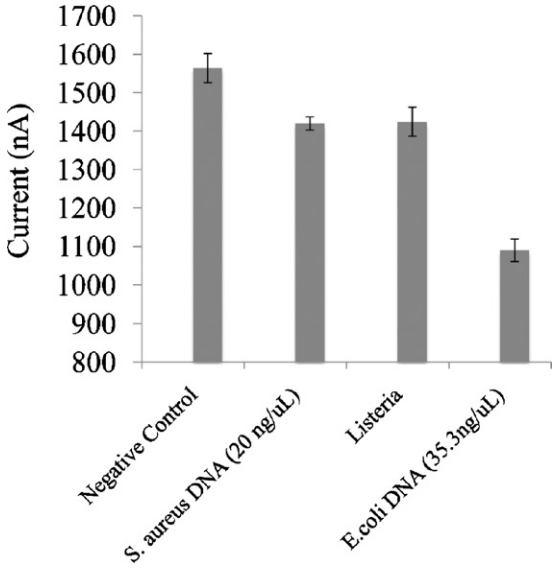

Fig. 6. Cross reactivity test for the *E. coli* primes in the chip. The negative control contained water with no *E. coli* DNA template. Due to existence of DNA and binding to redox molecule with its minor groove there is a small drop in the anodic oxidation peak current. The major drop is distinguishable between the *E. coli* DNA sample and other bacteria.

Fig. 5a and b show the results for bacteria samples in LB broth media and filtered urine respectively. The bacteria concentration varies from 48 (CFU)/ml to 4.8×10^5 (CFU)/ml. For accurate calibration the urine samples are freshly prepared from healthy people and sterilized by autoclave for 30 min at 121 °C.

3.5. Cross reactivity tests

Identification of the bacteria with high specificity on the chip is very important in the detection process. The specificity of the microfluidic chip was established by cross reactivity test using *E. coli*, *Listeria* and extracted DNA of *S. aureus*. The LAMP reactions were carried out using the primer sequences specific for the *E. coli* bacteria with *S. aureus DNA* and *Listeria* for 1 h. Water without DNA template was used as the negative control. No major anodic oxidation drop was observed for non-*E. coli* bacteria, suggesting that the *E. coli* primers cannot amplify other types of bacteria (Fig. 6).

4. Conclusion

In this paper, we demonstrated an isothermal amplification of DNA in a simple microfluidic chip for detection and quantification of *E. coli*. The microfluidic assay detected the bacteria electrochemically using LSV method with high sensitivity and specificity. The

sensitivity of the assay was 48 CFU/ml in 35 min. The specificity of this device was examined by using different bacteria such as *Listeria* and *S. aureus* DNA. This device has a high potential for POC applications. It is much faster and more sensitive and consuming a much smaller amount of reagents in comparison with the other commercially available devices such as Galacto-Light Plus™ and TaqMan®. Future work will include the improvement of the bacterial lysis efficiency to enhance the amplification efficiency and shorten the detection time. We plan to employ the developed approach for detecting various bacterial analytes without using costly and complicated methods.

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