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ABSTRACT

Microfluidics is an emerging technology enabling the development of Lab-on-a-chip (LOC) systems for clinical diagnostics, drug discovery and screening, food safety and environmental analysis. Currently, available nucleic acid diagnostic tests take advantage of Polymerase Chain Reaction (PCR) that allows exponential amplification of portions of nucleic acid sequences that can be used as indicators for the identification of various diseases. At the same time, isothermal methods for DNA amplification are being developed and are preferred for their simplified protocols and the elimination of thermocycling. Here, we present a low-cost and fast DNA amplification device for isothermal Helicase Dependent Amplification (HDA) implemented in the detection of mutations related to breast cancer as well as the detection of Salmonella pathogens. The device is fabricated by mass production amenable technologies on printed circuit board (PCB) substrates, where copper facilitates the incorporation of on-chip microheaters, defining the thermal zone necessary for isothermal amplification methods.

Keywords: Lab on a Chip, Microfluidic Platform, DNA diagnostics, HDA, PCR, breast cancer mutations, Salmonella, PCB

1. INTRODUCTION

The interest in microfluidic devices has been continuously growing for the last two decades. Thus, large amount of scientific papers and patents on this topic have been published and issued. As a result of the publication cascade and the strong involvement in this area, microfluidics is considered as a well-established arsenal both for industry and academia for developing not only new methods but also products related to life sciences. However, the commercialization of such microfluidic devices for bio-medical purposes or food safety is far behind. This mainly stems from the lack of appeal to end users since its operation is not completely automated providing an uncomplicated readout/result. Nevertheless, microfluidic platforms offer new opportunities compared to conventional systems such as higher sensitivity, shorter analysis time, lower cost per test (low reagent and power consumption), wearability, and portability. Furthermore, the reduction of the footprint improves the degree of parallelization and, as a result, it enhances the throughput of the analysis performed.

Miniaturization of conventional laboratory processes in microfluidic platforms using well-established microfabrication technology has drawn great attention, and led to the development of the so-called Lab-on-a-Chip (LOC) devices. LOC aims at integration, miniaturization, parallelization, and automation of biochemical processes, performed into a small chip of a few square millimeters to a few square centimeters in size (Erickson and Li 2004). The higher degree of

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automation as well as the reduced energy consumption renders such microfluidic devices excellent candidates for point of care (PoC) diagnostics.

In a plethora of cases, nucleic acid amplification is an indispensable tool for the development of diagnostics serving not only the detection but also the identification of genetic disorders, mutations, infectious diseases, and other purposes. Currently, there are numerous nucleic acid amplification methods available which are used in analyzing and detecting a small quantity of nucleic acids. The most widely used method is the Polymerase Chain Reaction (PCR). Miniaturized PCR (μPCR) devices efficiently amplifying DNA can be categorized into static chamber (SC) and continuous flow (CF) devices. The SC devices resemble the conventional thermocyclers at their operation; the sample is static in a chamber (well) and both the device and the sample undergo thermal cycling (Shen et al. 2005); temperature ramping is required during the operation. On the other hand, in continuous flow (fixed-loop or closed-loop) devices (Kopp et al. 1998), no temperature ramping is required: The sample moves through fixed temperature zones to achieve the required thermal cycling, leading generally to faster DNA amplification and lower power consumption. Moreover, the use of thin (flexible) polymeric films for the fabrication of μPCR devices or other heated devices (Moschou et al. 2014; Papadopoulos et al. 2014), and the evolution of the heating elements from external to integrated on chip at a small distance from the DNA sample, allow further reduction in the thermal mass of the devices and as a consequence in rapid heating/cooling rates (Ahmad and Hashsham 2012). Fast equilibration time is an additional auxiliary property for microfluidic devices which in turn is extremely crucial for applications that need thermocycling such as PCR (Haeberle and Zengerle 2007; Mark et al. 2010).

Despite these advantages, a shift towards isothermal methods in microfabricated diagnostics is observed, mainly stemming from their simplicity and reduced thermal budget. Recent advancements in molecular biology have broadened the archery, offering different alternative isothermal methods for DNA amplification (Asiello and Baeumner 2011; Gill and Ghaemi 2008). For example, Loop-Mediated Isothermal Amplification (LAMP)(Fang et al. 2010), Rolling Circle Amplification (RCA)(Mahmoudian et al. 2008) , Strand Displacement Amplification (SDA), Recombinase Polymerase Amplification (RPA) (Lutz et al. 2010), and Helicase Dependent Amplification (HDA)(Mahalanabis et al. 2010) are some of the isothermal amplification methods that have already been miniaturized. All the aforementioned methods offer exponential amplification of DNA. The main advantage of isothermal methods over PCR is the elimination of the need for thermocycling, as the isothermal methods are realized under a stable and constant temperature over time.

In detail, HDA (Vincent et al. 2004) relies on the ability of a DNA helicase (enzyme) to unwind DNA without heat for the separation of the double stranded DNA (dsDNA) to single stranded DNA (ssDNA), which is subsequently used as a template in the amplification process. Similarly to PCR, sequence specific primers bind to the ssDNA (hybridization/annealing) and afterwards, DNA polymerase starts the polymerization (extension) of the target sequence. Advantageously, in HDA all the aforementioned steps can be performed at the same temperature (approximately 65°C).

In this work, we develop low-cost devices for fast DNA amplification (Kaprou et al. 2015), focusing on HDA, and we implement them in the detection of mutations related to breast cancer as well as Salmonella detection. Genetic analysis of mutations in BRCA1 gene that are associated with breast and ovarian cancer is routine in many molecular biology laboratories and most of the detection methods employed are based on DNA amplification combined with fluorescent labels or gel electrophoresis. A new concept for mutation screening and detection based on acoustic wave sensing was recently proposed (Papadakis and Gizeli 2014). The proposed method is suitable for a LOC concept through integration of miniaturized acoustic devices with low-cost microfluidic devices that can be used for DNA amplification and further manipulations, such as restriction digestion (Papadopoulos et al. 2014) (Kefala et al. 2015). Towards the goal of the development of such an integrated platform for mutation detection, we have used microfluidic devices to amplify a DNA fragment of the exon 20 of the BRCA1 gene that contains at least two positions where significant mutations occur (Vorkas et al. 2010). The same integration strategy can be applied for a LOC addressing food related concerns for in situ detection of harmful pathogens such as Salmonella, thus enabling and ameliorating public safety and health.

The devices presented in this paper are fabricated by low cost, mass production amenable technologies on printed circuit board (PCB) substrates, where copper facilitates the incorporation of on-chip resistive microheaters, defining the thermal zones necessary for DNA amplification methods. The proposed technology has allowed so far the use of copper as structural material for the microfluidic network (Wego et al. 2001) as well as the integration of microfluidic devices with heterogeneous components such as electronic circuits (Wu et al. 2010), sensors (Moschou et al. 2013), and microheaters, following the current trend in LOC technology (Aracil et al. 2015). To guide our device design, SC and CF μ-PCR devices fabricated on polymeric substrates with integrated microheaters are simulated. Comparison is made in terms of energy consumption of devices fabricated on the same material stack, with identical sample volume and channel dimensions, and is implemented by a computational study coupling heat transfer in both solid and fluid, mass conservation of species, and joule heating (Papadopoulos et al. 2015). The pros and cons of each configuration are compared and discussed and the conclusions drawn are used as a guide for the devices to be realized and implemented for efficient DNA amplification.

2. COMPUTATIONAL STUDY-SIMULATIONS

The objective of the computational study is the investigation of the role of the substrate thickness on the energy consumption of microfluidic devices with and without temperature ramping during their operation. Temperature ramping is required in a SC μPCR device, while it is not required in a CF μPCR device or a device implementing isothermal HDA amplification. Two devices are evaluated in terms of energy consumption through the computational study: The first is a fixed-loop CF μPCR device, where no temperature ramping is required, and the second is a SC μPCR device, where temperature ramping is necessary. The comparison is performed for the same material stack, i.e. polymeric films (thin or thicker, from 100 to 1000 μm) with metal layers for integration of microheaters, the same PCR protocol (time duration at each zone), and the same volume of PCR mixture. The mathematical model utilized for the computational study consists of the continuity equation, the momentum conservation equation, the heat transfer equation in both solid and fluid, and the joule heating equations on both devices, coupled with a Proportional-Integral-Derivative (PID) temperature controller for the case of SC device. More details on the devices and the mathematical model can be found in (Papadopoulos et al. 2015).

The results of the comparison are summarized in Fig. 2, where the effect of the substrate thickness on the energy consumption of both SC and CF μPCR devices is shown. The increase of the substrate thickness induces an increase of the energy consumption for both devices. However, the energy consumption for the SC device is more sensitive to the substrate thickness. The energy consumption for the SC device is significantly lower than that of the CF device, for substrate thickness less than 500 μm. If the substrate is thicker than ~800 μm, the energy consumption at the SC device exceeds the energy consumption at the CF device.

The difference in the energy consumption can be explained by the heat losses to the ambient at each device; the SC device has lower heat losses due to the smaller area in contact to the ambient compared to the CF device (Papadopoulos et al. 2015). At low substrate thickness, the constant heat losses at the CF device exceed the energy required for the thermal cycling of the SC device. At greater substrate thickness, the thermal inertia of the SC device plays a role due to the necessary temperature ramping: The inertia increases due to the increase of the thermal mass, and as a consequence, the time required for the thermal cycling and the energy consumption for the SC device increase. This increase for substrate thickness greater than ~800 μm is enough to exceed the small consumption increase for the CF device. The latter is attributed to the increase of the distance between the microheaters and the microchannel and as a consequence to the slightly greater temperature required at the microheaters to achieve the set point temperatures in the microchannel.
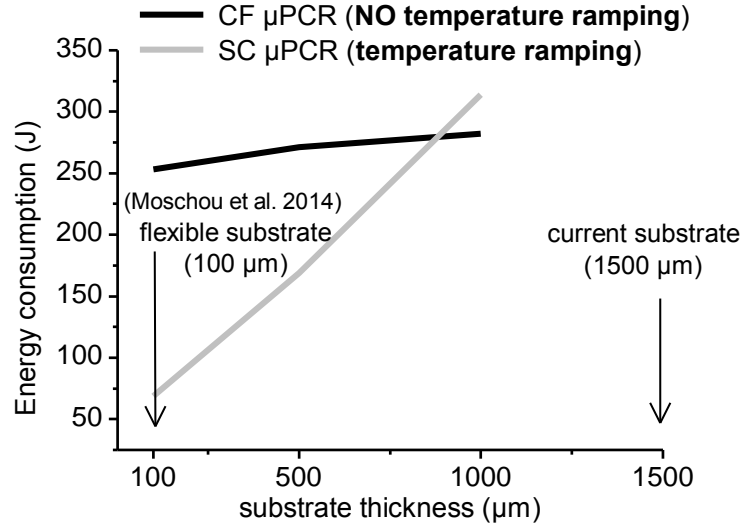
Figure 1. Energy consumption in CF and SC µPCR devices as a function of substrate thickness

The computational study shows that SC µPCR devices or devices which require temperature ramping are advantageous regarding the energy consumption, when they are realized on very thin polymeric substrates; at substrate thickness greater than 800 µm, it is beneficial to avoid temperature ramping and to use CF devices for the PCR. The conclusion of the computational study, although it refers to µPCR devices, can verify that the HDA isothermal DNA amplification is beneficial on the substrate utilized (1.5 mm PCB) compared to a PCR taking place in a SC device (where temperature ramping is required). The energy consumption of a device for HDA will be close (or even lower) to that of CF µPCR device where no temperature ramping occurs.

3.   MICRO-DEVICE FABRICATION

Herein we present the fabrication of a microdevice for isothermal DNA amplification. The device consists of the microfluidic network where DNA amplification takes place and resistive microheaters embedded on the same substrate to facilitate the fabrication. For this reason, commercially available and low cost PCB substrate is chosen, where the copper layer is the structural material of on-chip microheaters, defining the thermal zone necessary for DNA amplification, and serving as temperature sensors for temperature control. The process flow followed for the fabrication of such a device is depicted in Fig. 2. First, the copper resistive microheaters are designed with a PCB design software and realized by major PCB vendors according to our design specifications. They are fabricated in a large number of replicas at a low cost and excellent reproducibility in terms of resistor value accuracy (±1 Ohm). They have a meandering shape, to maximize their electrical resistance in the space allocated for each thermal zone. Then, the microfluidic network is patterned by means of computer numerical control (CNC) micromilling, expanding the fabrication capability made possible by photolithography of thin dry films (Kaprou et al. 2015) to higher microchannel depths. The design of the fluidic network is introduced using a Gerber file. A meandering microchannel with a total channel length of 65 cm crosses the thermal zone. The width of the meandering microchannel is 500 µm, while its depth is 100 µm, thus the microchannel volume is 35 µl. The chip area is 5.5 cm x 2.5 cm [Figs. 3 b and c].
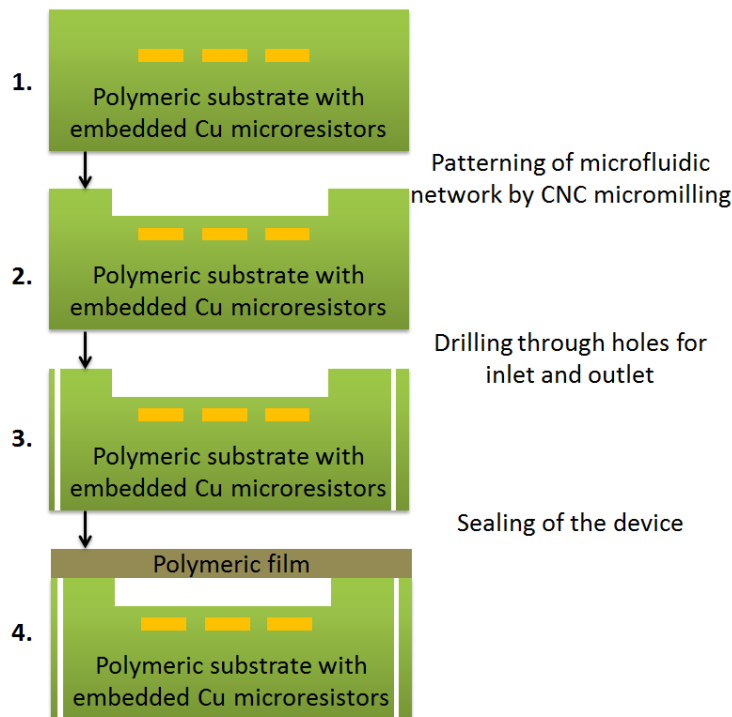
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Figure 2. Process flow followed for the fabrication of the DNA amplification devices

A schematic inclined view of the device is depicted in Fig. 3a. In more detail, the device has one meandering microheater, embedded within the 1.5 mm-thick PCB, at a distance of 300 μm from the top side, where the microchannel is to be fabricated. Once the channels are formed by CNC machining (Fig. 3b), through holes are drilled for the inlet and outlet and subsequently the device is rinsed with acetone, propanol, and water, and dried with nitrogen. Immediately after, the device is sealed with a polymeric film (with adhesive layer on one side) using a heat press. The yield of fabricated devices reaches 95% after the patterning process, a percentage reduced to 80% after the sealing. In some cases, clogging of the fluidic network was observed in some areas due to the adhesive layer of the sealing film, thus rendering such devices non-functional. The successful final devices (a representative image is shown in Fig. 3 c) have proven to be very reliable and robust, while at operation under elevated temperatures (T>95 ℃) and flow rates up to 20 μl/min.

The control of the microheater temperature is performed independently using a temperature control unit providing the voltage needed across each resistive microheater, whilst a small resistor is used to measure the current flowing through. Thus, the controller measures in real time the resistance of the microheater to derive its operating temperature through the temperature coefficient of resistance for copper (i.e. using the microheaters as temperature sensors), and the resulting value is used in a proportional-integral (PI) feedback control loop to stabilize the temperature of the microheater at a certain set value.
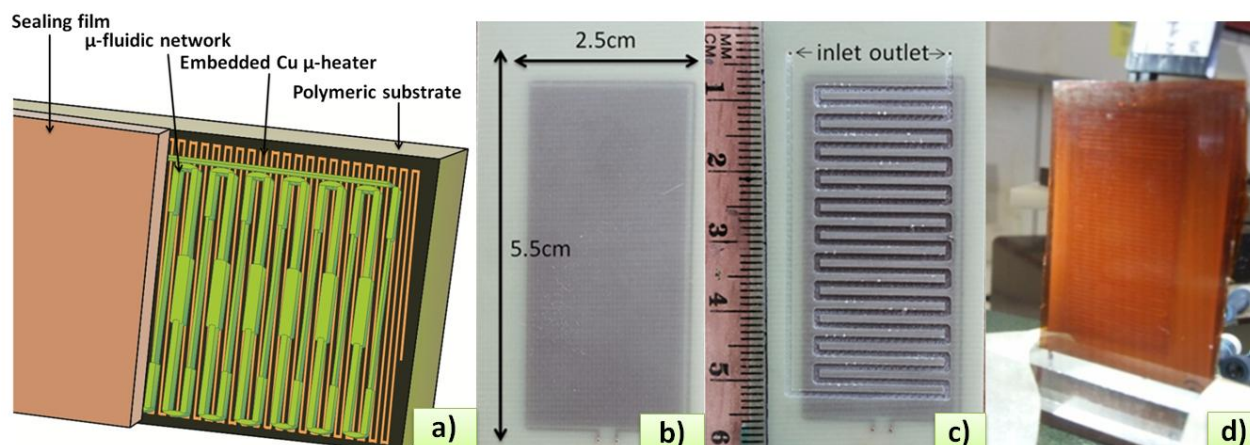
Figure 3. a) Schematic of an inclined view of the isothermal DNA amplification device, showing the discrete layers of the device, b) polymeric substrate with embedded Cu microheaters (before microfluidic patterning), c) fabricated device before sealing (meandering microfluidic network and meandering microheaters), d) fabricated device after sealing

4. EVALUATION OF THE DEVICES FOR DNA AMPLIFICATION

For the performance evaluation of the fabricated device, we employed the HDA amplification strategy to amplify a particular gene sequence, i.e., exon 20 of the BRCA1 gene that contains at least two positions where significant mutations occur. The HDA reactions were supplied with a set of primers (Forward: 5'-TGA TGG GTT GTG TTT GGT TTC TTT CAG -3' and Reverse: 5'- ACC TTT CTG TCC TGG GAT TCT CTT GCT -3') that produce a 113 bp fragment and a 157 bp PCR fragment containing the sequence of interest (Vorkas et al. 2010) used as template.

In addition to the work in (Kaprou et al. 2015), an evaluation was performed employing the microdevice with the HDA method using Salmonella genomic DNA in order to detect Salmonella strains stemming from a 500 bacteria culture after cell lysis and purification step (Tsougeni et al. 2016). This is a common procedure used in food safety controls. A different set of primers was used in the second HDA reaction (Forward: 5'-TCCTTTTCCAGATTACGCAACAGATACT-3' and Reverse: 5'-TTGGGTTCTGGATTTTTGATTATCCTGC-3') producing a 88bp amplicon characteristic of Salmonella bacteria.

25 μl of an HDA isothermal amplification reaction prepared according to the manufacturer's instructions (Biohelix, IsoAmp® II Universal tHDA Kit) were loaded in the device and incubated for 15 min (BRCA1) and 90 min (Salmonella) at 65°C. A similar reaction was placed in a bench-top thermocycler under the same conditions. Figure 4 shows the amplified products derived from the microfluidic device and the thermocycler, for BRCA1. Both reactions were performed for 15 min with approximately similar amplification efficiency, as is indicated on Fig. 4. In Fig. 5 the amplified products are depicted, which were received from the microdevice after the HDA amplification reaction of the genomic Salmonella DNA.

These results demonstrate that this amplification strategy, i.e., isothermal HDA, can be performed within the developed microfluidic devices that are suitable for integration with additional elements such as acoustic sensors for the development of integrated platforms for DNA diagnostic applications.
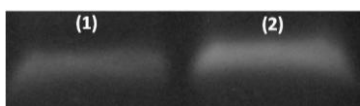
Figure 4. Gel electrophoresis image depicting the 113 bp fragments amplified in a microfluidic device (lane 1) and in an eppendorf tube placed in a thermocycler (lane 2) using the HDA isothermal method

Figure 5. Gel electrophoresis image depicting the 100 bp ladder (lane 1) and the 88 bp fragments amplified in a microfluidic device (lane 2) using the HDA isothermal method

5. CONCLUSIONS

Taking full advantage of the potential of PCB technology in the development of LOC, microfluidic devices for DNA amplification with integrated resistive microheaters were developed, expanding our previous work on flexible (flexible printed circuit, FPC) substrates (Moschou et al. 2014) . A computational study showed that a device without temperature ramping is more advantageous in terms of energy consumption for the thickness specification (1.5 mm) imposed by a typical PCB substrate on which the device was fabricated. Following the conclusion of the study, a device without requirements for temperature ramping was realized to implement isothermal DNA amplification (HDA). In the device, rapid (within few min) DNA amplification was demonstrated for a fragment of BRCA1 gene where significant mutations occur. In addition, Salmonella strain was detected from genomic DNA which is a useful application for safety controlling of various dairy products from food-borne pathogens. In the near future, a novel functional platform is planned with a surface acoustic biosensor for the detection of amplified DNA products.

AKCNOLEDGEMENTS

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