Short communication

Rapid nested-PCR for tyrosinase gene detection on chip

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ABSTRACT

The availability of non-invasive, fast and sensitive technologies for detection of circulating cancer cells is still a critical need of clinical oncology, particularly for diagnosis of aggressive and highly metastatic tumors, like malignant melanoma. Here we present the first nested polymerase chain reaction process carried out by a microfabricated, hybrid plastic–glass microfluidic chip on the tyrosinase gene, a predictive marker for melanoma diagnosis. The device is a hybrid system consisting of a glass microchannel embedded in an elastomeric matrix, and operating in flow-oscillating modality on a droplet of biological sample. The convection heat transfer and the temperature distribution inside the carrier fluid in the device are investigated. The oil responds to temperature changes with a characteristic time around 53 s, and exhibits three different thermal gradients along the capillary, with temperature variations below 4 °C in correspondence of heater electrodes. The sample heating/cooling rates in the chip are as high as 16 °C/s, allowing rapid processes. The nested polymerase chain reaction process is performed in less than 50 min, namely more than four times faster than in a standard thermocycler. The rapidity of the analysis method, combined with the simple and low-cost fabrication, reduced sample evaporation, and flexibility of the overall microfluidic platform, make it promising for the detection of events of tumor spreading.

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1. Introduction

The malignant melanoma (MM) is characterized by highly frequent generated metastases, starting from the lymphatic system and eventually extending to many organs (Arenberger et al., 2008). Due to the small size of the most primary melanoma lesions, the detection of MM is particularly critical. Despite of screening techniques for melanoma prevention, the incidence of MM has grown by about 600% in 50 years (from 1950 to 2000), and this cancer remains the second most common type among people aged 20–39 years (Jennings and Murphy, 2009). Nowadays, the most of clinical practices for the melanoma diagnosis are based on imaging methods.

The molecular biology offers accurate and more sensitive routes to detect melanoma circulating cells in blood, representing a relevant prognostic tool for predicting metastatic spreading (Venditelli et al., 2009). Markers, such as tyrosinase (Smith et al., 1991), microphthalmia-associated transcription factor (Garraway et al., 2005), melanoma antigen recognized by T-cells-1 or Melan-A antibody (Koyanagi et al., 2005), have been used for diagnosis of metastatic melanoma cells, by reverse transcription-polymerase

chain reaction (RT-PCR). The possibility of detecting melanoma cells in the peripheral blood by RT-PCR is demonstrated by Smith et al. in 1991, using a nested-PCR on tyrosinase mature transcript (mRNA) (Smith et al., 1991). In fact, tyrosinase is the key enzyme in melanin biosynthesis, catalyzing both the tyrosine to dopa and the dopa to dopaquinone conversions (Smith et al., 1991). Many studies report on the frequent association between tyrosinase detection with various stages of melanoma (Medic et al., 2007). Though still debated (Mellado et al., 1999; Proebstle et al., 2000), tyrosinase remains therefore a relevant and useful candidate for clinical applications among melanoma-associated markers (Visus et al., 2007; Santonocito et al., 2005). In this framework, the development of fast, efficient and reproducible quantification methods for diagnostic PCR applications becomes important.

Nested-PCR involves two successive PCR reactions, working with two different pairs of primers. In the first process, a set of primers (outer primers) is used to generate the DNA product that is the template in the second amplification, where a second set of (inner or nested) primers, located internally to the first primer pair (Haff, 1994), is exploited. The amplification with nested primers shows enhanced specificity with respect to conventional PCR, since only specific amplicons from the first reaction are suitable templates for the second round of amplification (Cassinotti et al., 1993). In addition, the sensitivity of nested primer PCR is up to 10² times higher than amplifications with a single primer set, decreasing the

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Fig. 1. Thermal analysis of the PCR microfluidic chip in liquid static conditions. (a) Oil temperature response, with a single switched-on underlying heater, saturating at 55 °C (triangles), 72 °C (squares), and 95 °C (circles). Experimental data are fitted by the equation $T = A(1 - e^{t/\tau}) + T_{RT}$, where A and τ are constants and $T_{RT} \approx 25$ °C. Inset: Complete assembled device connected to microfluidic plastic tubes (bar: 0.5 cm). (b) Optical micrographs of the heaters. (c) Temperature distribution along the glass capillary with all the heaters on. Circles: experimental data. Dashed rectangles highlight the heaters areas, and dashed lines are linear fits in the different regions. Experimental errors (± 0.1 °C) are smaller than symbols.

threshold of molecular target detection and improving the accuracy of diagnostic predictions, compared to other traditionally available staging methods (Kotake et al., 1996; Grasso et al., 1998).

PCR systems based on microfluidics, including oscillatory, closed-loop and fixed-loop devices, attract increasing attention to guarantee short analysis time, small involved volumes of biological samples, together with high degree of miniaturization and portability (Zhang and Ozdemir, 2009; Zhang and Xing, 2007; Wang et al., 2006; Liu et al., 2002; Schaerli et al., 2009; Beer et al., 2008). However, nested-PCR on microfluidic devices is still unexplored notwithstanding the remarkable analytical importance of this class of reactions. Among microfluidic chips, systems based on oscillatory flow are especially interesting under this respect, because of their flexibility in the number of PCR cycles and potentialities for parallelization of multiple reaction processes (Wang et al., 2005; Cheng et al., 2005; Bu et al., 2003; Frey et al., 2007; Chiou et al., 2001).

Here we present the first nested-PCR developed on the tyrosinase gene by a microfluidic device. The system enables a nested-PCR in less than 50 min (vs. 3.5 h needed in a standard thermocycler), and it is based on a glass capillary core embedded into a polymeric matrix, and on microfabricated Ti/Pt heaters with integrated temperature sensors. The PCR master mix (1 μ L in volume) is moved back and forth, oscillating through three hydraulically connected reaction areas placed in correspondence of the heaters. The device can be used in fast assays of a wide range of samples of diagnostic interest.

2. Materials and methods

Photolithography and metal deposition are employed for the realization of three independent Ti/Pt electrodes, constituted by microheaters and temperature sensors. 20/200 nm of Ti/Pt are deposited onto the glass substrate by electron-beam evaporation (Maily et al., 2001; Matino et al., 2005, further details in Supporting Information). The electrodes are extended over 1.5×1.5 mm², with a periodic pattern of 50 μ m and 10 μ m-wide lines. The chip includes a glass capillary hosted by a matrix of polydimethylsiloxane (PDMS) and connected to an inlet and an outlet (inset of Fig. 1a). Before assembling the hybrid PDMS-glass device, the internal walls of the capillary are treated by 2% (v/v) of dimethyldichlorosilane in 1,1,1-trichloroethane (Sigma–Aldrich, St. Louis, MO), preventing surface adsorption of PCR mix components (Kopp et al., 1998; Obeid et al., 2003). The glass capillary is filled by 20 μ L of silanization solution and placed on an oscillating shaker for 1.5 h in air.

After the solution evaporation, the passivated capillary is dried at 60 °C for 30 min, and its terminations are then connected to plastic tubes for the fluid inlet and outlet. The PDMS prepolymer (Sylgard 184, Dow Corning, Midland, MI), obtained by mixing the base and the curing agent in a 20:1 (w/w) ratio, is cast onto the capillary and cured at 75 °C for 15 min.

Before nested-PCR experiments, we measure the temperature of the liquid phase into the glass capillary in static liquid conditions by a thermocouple with external diameter of 0.25 mm (Watlow mineral insulated thermocouple, St. Louis, MO). Nested-PCR on tyrosinase transcript is performed according to Smith et al. (1991) obtaining a final amplification product of 207 base pair length. Both PCR reactions are carried out on a tyrosinase positive control sample (MELANOMAS positive control, Nanogen Advanced Diagnostic, Turin, Italy), using the reagent mixture (MELANOMAS oligomix Alert kit, Nanogen Advanced Diagnostic), providing primer oligonucleotides for the first and the second amplification, the buffer solution, magnesium chloride and triphosphate nucleotides. After preparing both the reaction mixtures according to the manufacturer instructions, a 1 μ L droplet is employed in each amplification step. For comparison, 50 μ L of solution is employed in each step in a thermocycler (TC, Eppendorf Mastercycler ep). Ultrapure water is used as negative control.

For on-chip PCR, we use a two-phase liquid system consisting of a droplet of biological sample in a continuous phase of silicone PDMS oil with viscosity of 500 cP (by Sigma–Aldrich). The droplet oscillations are obtained by a twin syringe pump working in infusion and withdrawn mode (Harvard Apparatus, Holliston, MA, USA). The PCR thermal cycling profile in the chip (in TC) is as follows: for the first amplification, (i) hot start at 95 °C for 4 min (4 min in TC), (ii) 35 cycles, each composed by 10 s (1 min) at 95 °C, 10 s (1 min) at 55 °C, 10 s (1 min) at 72 °C, and (iii) final extension at 72 °C for 4 min (5 min). The amplified sample is then withdrawn into an Eppendorf tube and added to the mix reaction for reamplification by (i) starting denaturation at 95 °C for 2 min (2 min in TC), (ii) 30 cycles, each consisting of 10 s (1 min) at 95 °C, 10 s (1 min) at 55 °C, 10 s (1 min) at 72 °C, and (iii) final extension at 72 °C for 4 min (5 min). The PCR products are analyzed by electrophoresis on 1% agarose gel after ethidium bromide staining and visualized by UV transillumination.

3. Results and discussion

The heat transfer to the oil in static liquid conditions is analyzed by increasing the temperature of each single electrode from

Fig. 2. Schematics of the overall nested-PCR reaction (features not in scale). (a) Target DNA amplified with the first primer set (outer primers). (b) Primary products from the first reaction undergoing a second run with the second primer set (inner primers).

room temperature to the PCR operation temperature, and placing a thermocouple inside the glass capillary in correspondence to the electrode. The oil responds to the temperature changes according to convective heat transfer (Bejan and Kraus, 2003), with a characteristic time, $\tau = (53 \pm 2)$ s (value averaged on the three measurements, Fig. 1a). τ is higher than typical values of a few seconds reported for other PCR microfluidic chips (Bu et al., 2003; Hoang et al., 2008), due to the presence of intermediate PDMS and glass (Zhang and Ozdemir, 2009) between the heaters and oil. However, this does not affect the system operation, since the amplification reactions start after the oil phase above each heater stabilizes at the working temperature. Moreover, we measure the temperature distribution along the centerline of the capillary, keeping constant the temperature of the fluid above each heater (Fig. 1b) at 55, 72, and 95 °C, respectively. The temperature variations above each heater are below ± 4 °C (Fig. 1c). Along the capillary, the oil exhibits three different temperature gradients [(5.3 ± 0.3) °C/mm, (3.0 ± 0.1) °C/mm, and (6.9 ± 0.7) °C/mm], the temperature above each heater being also affected by heat diffusion from the neighbor regions. Therefore, the temperature gradient on the central electrode is smaller than

on the side heaters, because of the lower oil temperature externally to the heaters regions (i.e. in proximity of inlet and outlet). The smooth spatial thermal variations among the heaters are useful to maintain the temperature above 55 °C during the PCR, thus preventing unspecific annealing phenomena.

In fact, microfluidic chips operating by oscillating flows are characterized by a low thermal inertia, because only the biological sample cyclically undergoes heating and cooling, and consequently enables fast amplification processes. However, because of the continuous flow of the PCR mixture along the capillary, a major challenge of these systems is the possible adsorption of reactants to the channel walls and the consequent carryover contaminations (Markey et al., 2010). To overcome these problems, a static silanization of glass is used together with a double-phase fluidics, thus reducing the adsorption and the contact of PCR reagents on the sidewalls of the oscillatory device.

Another issue is limiting sample evaporation during the thermal cycles, whose related sample volume losses may determine local waste of reagents, solution concentration changes, and eventually ineffective nucleic acid amplifications. To this aim we introduce

Fig. 3. Optical micrographs of sample droplet during two consecutive amplification cycles. Droplet flowing through denaturation (a and d), annealing (b and e), and extension region (c and f). (g) Gel analysis of nested-PCR results for tyrosinase gene. Lanes showing 100 bp DNA ladder (M), negative control PCR containing all the reagents except the template (NC), PCR product obtained in the thermal cycler (TC) and in microfluidic device (Chip). The left arrow indicates the diagnostic fragment length of 207 bp. The different signal intensity between TC and Chip lines is mainly due to the different sample volumes.

a glass capillary as core element of the fluidic network, creating a vapor barrier that stops the diffusion of the biological water-based solution through the highly porous PDMS layer. This method preserves many advantages associated with using elastomers in microfluidics, such as structural flexibility and easy and low-cost manufacturing, overcoming the drawbacks related to PDMS significant vapor permeability (Prakash et al., 2006). The detailed characterization of the performance of this hybrid architecture in reducing sample evaporation are presented elsewhere (Polini et al., 2010).

The elastomeric layer (~500 μm thick) also promotes the reversible adhesion of the fluidic element to the substrate, without misalignments between the heaters and the fluidic network and without damages to the electrodes. Overall, differently from fixed-loop architectures, the entire fluidic platform is suitable to amplify molecular targets with different thermal profiles, simply by changing the sample flow rate and heaters temperature, as the number of PCR cycles and the residence time for each amplification step do not depend on the microchannel length.

As previously mentioned, nested-PCR involves two consecutive runs of reactions (schematics in Fig. 2a and b, respectively), using two different sets of primers, in order to reduce contamination events, and to avoid the amplification of unspecific primer binding sites. For the first reaction (Fig. 2a) we introduce a 1 μL droplet of PCR mix into the capillary, and then move the droplet oscillating into the oil phase. First, the biological sample undergoes denaturation for hot start and first denaturation (Fig. 3a). Then, the droplet is moved from heater #3 to heater #1 (Fig. 3b) and cooled at 55 °C for annealing. Subsequently, the liquid direction is reversed and the droplet is led on heater #2 (Fig. 3c) for extension. The droplet is placed again on heater #3 (Fig. 3d), and the second thermal cycle starts (Fig. 3e and f). The direction of the fluid motion changes after each denaturation and annealing step. After 35 cycles, a final extension process is carried out at 72 °C. In the second run (Fig. 2b), the primary products, exhibiting binding sites for the inner primers, are specifically amplified, ensuring the absence of unwanted products from primer dimers, hairpins, and alternative primer target sequences. This second PCR reaction is carried out in the same microfluidic system as for the first amplification, again by a 1 μL droplet oscillating in the oil phase.

The resulting electrophoretic gel of the reaction product is shown in Fig. 3g, evidencing an effective nested-PCR yield. The total reaction on chip takes 46.5 min, i.e. it is more than four times faster than nested-PCR of the tyrosinase gene as carried out by a standard thermocycler (211 min) (Standard protocols and tyrosinase transcript copies by Nanogen Advanced Diagnostic). We point out that, even using the same reaction thermal profile in the TC as in the chip, by the microfluidic device we achieve a significant reduction of the overall process time, because sample heating/cooling rates in TC are three times lower (5 vs. 16 °C/s) for the much larger thermal inertia. The value of the heating/cooling rate in the device is in agreement with reports on typical heating schemes of glass- and polymer-based microchips developed for rapid PCR (Hoang et al., 2008). The device offers therefore a fast and low cost method to analyze nucleic acid targets of diagnostic interest. Since 10^3 copies of tyrosinase transcript are present into 1 μL droplets of the used PCR mix, we estimate a sample concentration of one single copy of transcript per nanoliters. Our microfluidic platform, strongly reducing the sample evaporation from the chip, may offer a promising tool for downscaling the sample volume and detecting events of tumor spreading.

4. Conclusions

We demonstrate a microfluidic device based on oscillating flow for rapid nested-PCR reactions on tyrosinase gene. We obtain com-

plete amplification of the target, including the first and the second PCR reaction, in about 47 min, decreasing the time needed by a traditional benchtop thermocycler by more than four times. The method is effective and cheap, and adaptable to different molecular targets as well as thermal profiles. Next developments include tests based on parallel analysis on other tumor-associated antigens and performing real-time nested PCR, which offers the possibility to accurately quantify templates during the exponential stage of amplification (Max et al., 2002).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.09.008.

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