Microfluidic chips for real-time PCR

A N Zubik, G E Rudnitskaya, A L Bulyanitsa, T A Lukashenko and A A Evstrapov
Laboratory of Bio-&Chemosensors microsystems, Institute for Analytical instrumentation RAS, Saint Petersburg 190103, Russia
tunix@yandex.ru

Abstract. The results of real-time PCR in single-chamber microfluidic chips made of silicon-glass materials and optically transparent polymethyl methacrylate are presented. Conditions for efficient thermal cycling in microchip devices with several reaction chambers are discussed. A simplified theoretical estimation of the duration of heating a liquid in a polymer microchip is proposed, the results of which correlate with experimental data.

1. Introduction
The development of micro-fabrication technologies and the improvement of bioanalytical methods have led to the emergence of various instruments and devices for studying biological samples at the "point-of-care" [1, 2], in particular, for the detection of nucleic acids by amplification methods (polymerase chain reaction (PCR), loop mediated isothermal amplification (LAMP), etc.). An efficient PCR requires a high accuracy of the set temperatures. Prolonged heating at the stage of denaturation leads to a decrease in the activity of the DNA polymerase enzyme and a decrease in the efficiency of the reaction [3]. Due to multiple (more than 35) heating-cooling cycles, the duration of the temperature change makes a significant contribution to the total duration of the analysis. The heating-cooling rate is determined by both the characteristics of the heater and the inertia of the reaction volume. Therefore, the use of planar systems (microfluidic chips) with a high surface area to volume ratio can increase the heat transfer rate, and therefore reduce the analysis time compared to traditional equipment and polymer tubes.

At present, the relatively high cost of microfluidic chips in comparison with traditional test tubes or microplates is a significant obstacle to their widespread use. The cost of microchips depends on the cost of the material and components, the manufacturing and sealing technologies used, in some cases – on the cost and laboriousness of additional technological operations (surface modification, integration of elements, lyophilization of reagents, etc.), serial production.

The high thermal conductivity of silicon (160 W (m·°C)) is an advantage of microfluidic chips for PCR. However, the silicon surface reduces the efficiency of PCR, and the opacity of the material in the UV and visible spectral (UV-VIS) ranges does not allow the implementation of optical methods for the analysis of PCR products on an all-silicon microchip. The combination of silicon with glass materials has become more widespread, allowing you to maintain high thermal conductivity and use the advantages of optical detection [4]. For silicon surfaces, various options for protective coatings have been proposed, for example, by applying inorganic layers (silicon oxide) or the formation of organic layers (silanizing agents, etc.) [5].
Manufacturing microfluidic chips from polymeric materials can reduce their cost. For polymers, many available technologies for the formation of microstructures have been developed. However, polymers have a low thermal conductivity \((0.1\div0.3 \text{ W} \cdot (\text{m}^2\cdot\text{°C})^{-1})\), therefore, they require more careful control of heating to carry out the PCR.

At the IAI RAS, microfluidic chips made of various materials were developed: glass, polymethyl methacrylate (PMMA), epoxy resins, etc. In this paper, a theoretical and experimental evaluation of the performance characteristics of microfluidic PCR chips with the same topology, but made of different materials (silicon-glass and PMMA) is carried out.

2. Materials and methods

Silicon-glass microfluidic chips were manufactured by "Svetlana-Electronpribor" LLC (St. Petersburg, Russia). Reaction chambers with a depth of 250 µm and a volume of 25 µL were formed in a silicon wafer 0.55 mm thick by photolithography and liquid etching. For bonding glass and silicon plates, an anodic bonding method was used.

Polymer microchips consisted of two plates glued together. The top plate (with inlet / outlet holes) was made of PMMA grade ACRYMA® 72 with a thickness of 1 mm; the bottom plate (with the reaction chambers) was 1 mm or 0.8 mm thick from PMMA grades TOSN or SO-120-K, respectively. Reaction chambers with a depth of 300 µm and a volume of about 30 µL were engraved by laser ablation at "Laser center" LLC (St. Petersburg, Russia).

In a polymer microchip, the heat transfer process under heating (from 30 to 95 °C) was described by a parabolic differential equation with the following simplifications:

- It was a spatially one-dimensional model with heat fluxes in the vertical plane.
- The temperature change was carried out by setting a temperature jump by 65 °C at the lower boundary of the microchip, at the other boundary, the condition of the Newtonian heat flux was set.
- Instead of jointly solving problems of thermal diffusivity for several media with different characteristics and subsequent smooth gluing of solutions, we considered an equivalent medium with averaged parameters: the coefficients of thermal diffusivity, thermal conductivity, heat transfer, and specific heat. The geometric mean is taken as the average value (by analogy with the models of averaging the dielectric constant for composite media).
- Based on the characteristics and dimensions of the polymer, the Bio criterion (which determines the ratio of heat transfer and thermal conductivity) for various microchip designs ranged from 0.4 to 0.8.
- The calculated value of the heating time was considered when the average temperature in the reaction chamber reached a value that differed from the steady-state (asymptotic) temperature value by no more than 1 °C.

Experimentally, the time required for the liquid to reach a predetermined temperature can be estimated using a test solution with fluorescent heat-sensitive probes [6]. When this solution is heated, the fluorescence signal increases, and the temperature dependence of fluorescence is nonlinear. When the fluorescence signal reaches a plateau, it can indicate that a predetermined temperature has been reached, which means uniform heating in the reaction chamber. Fluorescence measurement was performed in real time every 7 seconds. The heating time was determined in 3-5 repetitions by the output of the fluorescence signal of the test solution to the maximum value. The obtained values were averaged over all chambers of the microchip.

For real-time PCR, a kit of reagents was used to detect the DNA of the pathogen of potato ring rot (Cat. No. RN-001, "Syntol" LLC, Russia). Calibrators provided by "Syntol" LLC with known concentrations were used as the target DNA. Bovine serum albumin was added to the reaction mixture for these microchips to prevent the sorption of the DNA polymerase enzyme on the silicon surface. The reaction chambers were filled with a laboratory dispenser. After filling, mineral oil was
introduced into the microchip channels, and the inlets were sealed with adhesive films for PCR microplates. Real-time PCR was performed on devices developed at the IAI RAS: in test tubes – on ANK-32 and in microchips – on the prototype experimental device for microchips.

3. Results and discussion

The results of real-time PCR on microfluidic chips with a single chamber are shown in figure 1 (a). An increasing or decreasing background signal on the graphs can be corrected using mathematical processing. The thermal cycling mode for these microchips was the same: denaturation at 94 °C – 20 s, annealing and elongation at 65 °C – 70 s. The curve for a polymer microchip (with a 1 mm bottom plate) is comparable to that obtained in conventional test tubes. The curve for a silicon glass microchip has a steeper slope and an earlier plateau. This is probably due to better heat transfer through the silicon bottom plate and, as a consequence, an increase in the reaction efficiency.

![Amplification curves of real-time PCR](image)

Figure 1. (a) Amplification curves of real-time PCR: 1 – in test tubes on ANK-32 (solid line); 2 – in a PMMA microchip with a single chamber on the prototype device for microchips (dash line with dot); 3 – in a microchip made of silicon-glass materials with a single chamber on the prototype device for microchips (line with dot). The concentration of the target DNA was $5 \times 10^4$ copies/µl. (b) Amplification curves of real-time PCR in a multichamber chip from PMMA with sequential ten-fold dilution of the target DNA from $10^6$ to $10^2$ copies/µl.

For the transition from single-chamber microchips to multichamber chips, the heating conditions were studied in more detail. The overall dimensions of the five-chamber microfluidic chips were $(60 \times 25)$ mm. The rate of temperature change in the reaction chamber of the microchip, in addition to the characteristics of the heater, is influenced by the following factors: the thermal conductivity and the thickness of the bottom of the microchip, the mass of heated objects, and the thermal contact between the surface of heater and microchip. The latter depends on the design of the clamping device and the materials of the contacting surfaces. After PCR in microchips made of PMMA, an imprint of the heater was observed on the lower surface of the microchip, which indicates a tight fit of the microchip to the heater. A fragile (with such dimensions) multichamber silicon-glass chip with significant clamping force can break if random solid irregularities (or microparticles) are found on the contacting surfaces. The absence of a clamp can lead to insufficiently tight contact with the heater; therefore, earlier [7] we used a thin elastic substrate (0.2 mm thick, the thermal conductivity of the substrate is much lower than that of silicon) that provides contact with surfaces and is suitable for repeated use.

The duration of heating the fluorescent test solution in a silicon-glass multichamber chip on an elastic substrate was $(6 \pm 2)$ s, which is comparable with the measurement discreteness. Comparison of the calculated and experimental heating duration in polymer multichamber chips with different bottom thicknesses are presented in Table 1. The difference between the calculated values and the experimental ones can be caused by losses during heat transfer in a real heater-microchip system, as well as by uneven heating over the heater area. It is also possible that averaging the thermal diffusivity leads to some overestimation of the intensity of the heat transfer process, which leads to underestimated times. According to the calculated data, a further decrease in the thickness of the bottom of the microchip down to 0.1 mm results in a decrease in the heating duration to 8 seconds.
Table 1. Heating duration (30 – 95 °C) of fluorescent test solution in multichamber chips made of PMMA.

| Bottom thickness (mm) | Heat duration 30 – 95 °C (s) |
|-----------------------|-------------------------------|
|                       | calculated | experimental |
| 0.7                   | 29         | 35±3         |
| 0.5                   | 18         | 26±4         |

The obtained values can be used to determine the duration of the denaturation stage for PCR in polymer chips. It was taken into account that the temperature difference during thermal cycling is almost two times less than the temperature difference when using a test fluorescent solution (35 °C versus 65 °C). For multichamber polymer microchip with a 0.8 mm thick bottom plate, the following durations were set: the denaturation stage was 22 s, the annealing and elongation stage was 65 s. These times were consistent with the PCR program for tubes in ANK-32. The results of real-time PCR are shown in figure 1 (b). The systematic delay of the threshold cycles of PCR in the multichamber polymer microchip on the prototype of the experimental device was (0.76 ± 0.27) cycles in comparison with the test tubes on the ANK-32, while the definition of the threshold cycles was carried out according to various algorithms using the software of these devices. The value of the PCR efficiency determined by the calibration dependence for the microchip is comparable to the results in polymer tubes and amounted to more than 95%.

4. Conclusions

The advantage of microfluidic devices is the ability to create analytical systems that combine several stages of analysis on a single microfluidic chip. To implement a polymer chain reaction on a chip, it is necessary to carefully select the material, as this affects the reaction conditions. The use of silicon-glass microchips (with an oxide layer) provides a rapid temperature change in the reaction chamber. However, polymer materials are simpler and cheaper to manufacture, are suitable for one-time use, and the technologies used make it possible to quickly establish the serial production of chips.

For polymer microchips with a bottom thickness of 0.7 and 0.5 mm, the duration of heating up to 65 degrees (from 30 to 95 °C) is 18 to 35 s. The duration of the thermal cycling program for these microarrays is comparable to the duration of the analysis in polymer tubes, which is confirmed by the obtained PCR results. According to the calculated data, reducing the thickness of the bottom of the polymer microchip to 0.1 mm will reduce the heating duration to 8 s, which will provide the possibility of a faster temperature change and reduce the total duration of PCR analysis.

Acknowledgments

The research was carried out within the state assignment of Ministry of Science and Higher Education of the Russian Federation (theme No. 075-00280-21-00).

References

[1] Zhu H, Fohlerova Z, Pekarek J, Basova E and Neuzil P 2020 Biosens. Bioelectron. 153 112041
[2] Zhang L, Ding B, Chen Q, Feng Q, Lin L and Sun J 2017 TrAC 94 106–16
[3] Grunenwald H 2003 Optimization of Polymerase Chain Reactions PCR Protocols ed J M S Bartlett and D Stirling (Totowa: Humana Press) chapter 20 pp 89–99
[4] Shoffner M A, Cheng J, Hvichia G E, Kricka L J and Wilding P 1996 Nucleic Acids Res. 24 375–9
[5] Zhang C, Xu J, Ma W and Zheng W 2006 Biotechnol. Adv. 24 243–84
[6] Sochivko D, Varlamov D, Fedorov A and Kurochkin V 2016 Tech. Phys. Lett. 42 362–4
[7] Afonicheva P, Zubik A, Bulyanitsa A, Rudnitskaya G and Evstrapov A 2020 J. Phys. Conf. Ser. 1695 012060