Microfluidic devices for Loop mediated isothermal amplification

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Abstract. Isothermal amplifications of nucleic acid are promising amplification techniques, which simplify the temperature mode without compromising analytical sensitivity and specificity. The development of microfluidic devices for isothermal amplification, especially for digital amplification, allows a more reliable measurement of the amount of nucleic acids. The possibility of realization of a loops mediated isothermal amplification on microfluidic devices made by the method of soft lithography is considered in the paper. A prototype of a microfluidic device with a droplet generator was created, and conditions for high-throughput generation of droplets from the reaction solution were chosen. It was shown that the resulting emulsion remains relatively thermally stable under the required temperature conditions. The peculiarities of microfluidic devices made of silicone rubber materials for use under conditions of isothermal amplification are noticed.

1. Introduction

In medical diagnostics, environmental monitoring and quality control of food products, methods of nucleic acid amplification such as polymerase chain reaction (PCR) are in demand. High-throughput amplification techniques are advantageously realized in the micro-scale, in particular, in droplet-based microfluidics. The basic principle is the separation of the analyzed sample into a number of highly uniform micro-volumes and the possibility of manipulating of them. Each droplet is an analog of isolated reaction chamber which contained all the necessary components of the reaction. Carrying out the reaction simultaneously in thousands of droplets has led to the development of several novel applications that could not have been realized using other technologies. For example digital amplification methods allow a more reliable and sensitive measurement of nucleic acid amounts [1]. The method is useful for studying of point mutations, copy number variations, and it is used for clonal amplification in next-generation sequencing [2]. Loop mediated isothermal reaction (LAMP) has high amplification efficiency and is an alternative to PCR. It does not require high-speed precision heating devices and has advantages in ease of implementation [3]. The development of microfluidic devices for isothermal amplification especially based on droplet is a perspective approach.

In a microfluidic device, droplets can be formed using active and passive methods. Active methods require external energy sources (electromagnetic radiation, thermal fields, centrifugal forces, etc.) for generation and manipulation of droplets. In passive methods, the geometry of the flow intersection of two liquid phases leads to the formation of droplets. The method of flow focusing requires a system of intersecting channels. The dispersed (or discrete) phase flows through a central channel, and the continuous phase (transport flow of liquid) is fed through two channels located on either side of it. Compression of the flow of a dispersed phase under certain conditions leads to the formation of droplets in the outlet channel. The geometry of the intersection, wetting properties of channel surface, flow rates and fluid properties (viscosity, surface tension) determine the conditions for obtaining droplets. The selection of surfactants and the composition of the continuous phase make it possible to optimize the conditions for obtaining time-stable droplets with reproducible characteristics.

In the paper, the possibility of realization of isothermal amplification (LAMP) on microfluidic devices manufactured by the method of "soft" lithography is considered. Soft lithography based on master-forms and elastic polymers is widely used for rapid prototyping of microfluidic devices. The most common material for these purposes is polydimethylsiloxane (PDMS) of the brand Sylgard®-184 (Dow Corning, USA). It is a two-component silicone chemical-curing rubber. The advantages of the PDMS are: optical transparency, relative chemical resistance, as well as high adhesion to glass. There is the disadvantage of this material - gas permeability, which can lead to evaporation of liquid from PDMS structures during heating for amplification. Therefore, for prototyping of microfluidic devices for nucleic acid amplification the search for materials, without this disadvantage, is an actual problem. In the case of micro structures for droplets generating, the gas permeability of PDMS does not have a significant effect.

2. Experimental techniques and results

Two designs of microfluidic devices were developed: (1) with a droplet generator based on the principle of flow focusing; (2) with stationary reaction chambers for detection of amplification results at the end point.

The intersection of the channels for the droplet generator formed in the PDMS of the brand Sylgard®-184 (Dow Corning, USA) is shown in Fig. 1a, obtained with the aid of an optical microscope. The channel width is approximately 30 µm for dispersed phase (central channel) and 32 µm for continuous phase (two side channels). The aperture of the generator is 15 µm. This design of the droplet generator allows the formation of droplets in the size of 20 to 70 microns.

(a) (b)

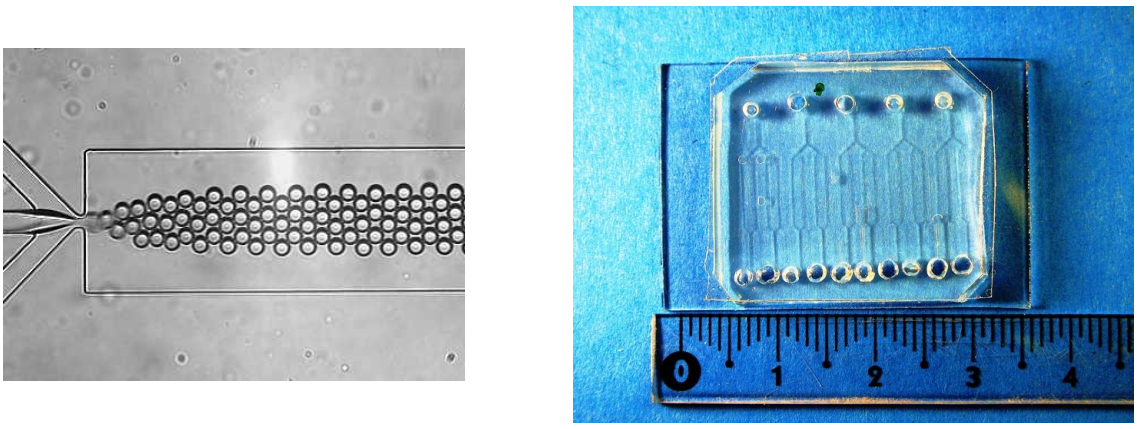

Figure 1. (a) The image of the droplet generator for the flow focusing method, obtained by means of an optical microscope; (b) The photo of microfluidic device with five pairs of sealed reaction chambers after isothermal amplification carried out.

The replica with the channels for droplet generation was sealed with a PDMS-film using high-frequency plasma treatment (Denier Zepto, Denier electronic GmbH, Germany) in oxygen medium at low pressure (1 mbar) for a minute. After plasma treatment, the hydrophobic properties of PDMS were restored by thermal annealing.

The second designed microfluidic device with five pairs of reaction chambers for isothermal amplification formed in LASIL T4 (Dow Corning, Germany) is shown in Fig. 1b. Compared to Sylgard®-184, this material is more viscous and less transparent. The width of the reaction chambers was approximately 1300 μm, the depth was 160 μm. Replicas with microstructures were sealed with a glass plate using the same plasma treatment. The glass bottom improved heat exchange in the reaction chambers. Preliminary studies have shown that such microfluidic devices from LASIL T4 material, filled by water, satisfactorily remained vapor resistance for one hour at a temperature of 65°C [4]. Consequently, these structures are suitable for carrying out isothermal amplification of nucleic acids.

For LAMP amplification the reagents with fluorescence dye and SD-Polymerase were provided by SYNTOL (Moscow, Russia). A synthetic DNA fragment with a concentration 10^5 copies/μl was used as the target template. Distilled water was used as a negative control.

BioXtra mineral oil (Sigma-Aldrich, USA) with the addition of 4% ABIL EM 180 (Evonik, Germany) surfactant was used as the continuous phase (of transport flow) for droplet generation. The flow rate of the continuous phase was 1.5 μl / min, and the discrete one was 0.4 μl / min. There was a capillary in the output hole of the droplet generator, which used for transfer the droplets from the microfluidic device to standard test tubes. The resulting emulsion was introduced into a glass well a diameter of 6 mm and a depth of 50 μm, covered with glass cover plates and heated according to isothermal amplification mode.

Ten microliters of pre-mixed LAMP amplification reagents are used for injection into a pair of reaction chambers. To avoid evaporation during heating, the inlet and outlet wells were sealed with a sticky film for PCR plates (Sarstedtag, Germany). Isothermal amplification was performed on a SwiftMaxPro Thermal Cycler (ESCO, Singapore). The temperature regime included a preliminary activation at a temperature of 86°C to 94°C for three minutes and subsequent amplification at 66°C for 45 minutes.

The results were recorded on a prototype of a fluorescent scanner of microchips (IAI RUS, Russia) with an excitation wavelength of 473 nm. The scanner enables to receive a set of image frames in the size 450 x 350 μm and to consider them both together and separately. In parallel with the experiments, control measurements were made in polypropylene tubes on the ANK-32 analyzer (IAI RAS, Russia).

Single frame images with droplets (macro-emulsion) of the LAMP reaction in oil medium before and after heating are shown in Fig. 2. The automatic analysis of frames was carried out with the help of the free software product ImageJ. It was found that the initial average droplet diameter is 36 ± 3 μm (n = 205). After heating, the average diameter varied a little – 34 ± 3 μm (n = 205). These results allow us to conclude that the droplets were thermally stable under isothermal amplification conditions.

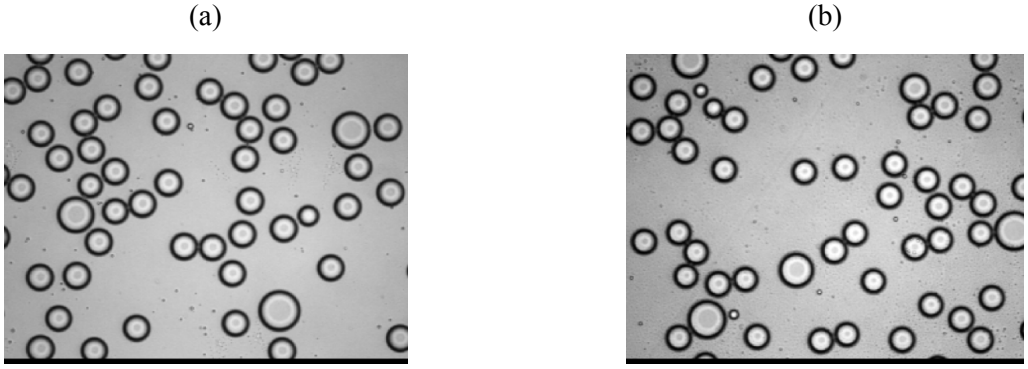

Figure 2. Single-frame images of the LAMP solution droplets in oil medium obtained in glass wells: (a) initial and (b) after heating according to isothermal amplification mode.

The full-frame image of three pairs of reaction chambers after LAMP amplification is shown in Fig. 3. Distilled water (negative control) was introduced into the middle pair of reactors, in the other two pairs - reagents for LAMP with a DNA target. Although the material of LASIL T4 has a relatively low transparency (about 80% of light transmission at 500 nm) and a small auto-fluorescence in the same range, the possibility of fluorescent detection of nucleic acids amplification in microfluidic device from this material was shown. In addition it should be noted that the reaction chambers remained sealed and no evaporation of the liquid occurred during heating or storage of the device.

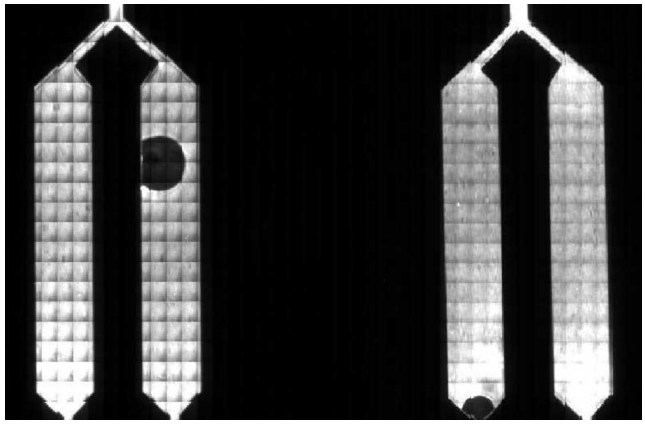

Figure 3. The full-frame image of fluorescent signal from three pairs of reaction chambers after carrying out isothermal amplification (negative control in the centre does not fluoresce).

3. Conclusion

The development of microfluidic devices for isothermal amplification in droplet-based microfluidics format allows the implementation of novel methods of digital amplification with a more reliable and sensitive measurement of the amount of nucleic acid. Using the method of soft lithography, a prototype microfluidic device with droplets generator was created and conditions for high-throughput generation of droplets of reaction solution for loop mediated isothermal amplification were chosen. It was shown that the macro-emulsion of reaction solution in an oil medium with addition of 4% surfactant ABIL EM 180 remains thermally stable under isothermal amplification conditions.

The method of soft lithography is relevant for the rapid prototyping of microfluidic devices. However, the application of this method for manufacturing of devices for nucleic acids amplification is limited by the vapor permeability of used polymeric materials. We applied the material of brand LASIL T4 for prototyping of microfluidic devices. In this material, it is possible to form microstructures that remain gas-tight under conditions of isothermal amplification. Since this material is not very transparent, the principal possibility of fluorescent detection of loop mediated isothermal amplification in reaction chambers from this material is shown.

4. Acknowledgements

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