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Polymer-based microfluidic chips for isothermal amplification of nucleic acids

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Abstract. Creation of low-cost compact devices based on microfluidic platforms for biological and medical research depends on the degree of development and enhancement of prototyping technologies. Two designs of polymer and hybrid microfluidic devices fabricated by soft lithography and intended for isothermal amplification and polymerase chain reaction are presented in this paper. The digital helicase-dependent isothermal amplification was tested in the device containing a droplet generator. Polymerase chain reaction was carried out in the hybrid microfluidic device having ten reaction chambers. A synthesized cDNA fragment of GAPDH housekeeping gene was used as a target.

1. Introduction

Nucleic acid amplification methods serve as an important tool for biological and medical research, as they significantly increase the sensitivity of molecular genetic analysis. The implementation of these methods in microfluidic devices (MFD) is a promising approach to developing compact, relatively inexpensive, sensitive and effective diagnostic tools for clinical use, food safety control and environmental monitoring [1].

Technologies for isothermal amplification of nucleic acids operate at a single constant temperature, which allows the creation of simpler, cheaper and more reliable devices that are suitable for use in resource-limited conditions (point-of-care systems). For isothermal amplification, a water bath, resistive heaters or the heat of exothermic chemical reactions can be used as heating means. Isothermal amplification methods are becoming a promising alternative to polymerase chain reaction (PCR) and greatly simplify the implementation of amplification methods in medical diagnostic devices and analytical equipment. These methods are characterized by high productivity, specificity, promptness and simplified sample preparation. Peculiarities of the method include: requirement for specific pairs of primers, inability to use the amplification product in further studies, restriction on the length of fragments used, a probability of obtaining a false-positive result [2].

The development of microfluidic devices for PCR is associated with the need for complex thermal cycling equipment to amplify the target DNA. However, the enhancement of prototyping techniques for fabrication of microfluidic chips (MFC) allows for the development of new approaches, research

strategies and improvement of methods for nucleic acids analysis, in particular, the digital amplification method (digital isothermal/polymerase chain reaction). Digital methods allow to achieve high sensitivity (at the level of single molecules, which is necessary, for example, when diagnosing diseases at early stages and identifying residual signs of the disease) and productivity. However, in this cases complex devices are required to create stable fluid flows (in the case of droplet microfluidics) and thermocycling of the sample. When developing methods for droplet microfluidics, it is also necessary to select surfactants for ensure thermal stability of the emulsion.

At a present, disposable MFCs for amplification, which contain all the necessary reagents, enzymes, primers in a liquid or dehydrated state perform a special interest. These devices reduce user's errors, exclude potential sources of pollution and facilitate the analysis in the field conditions or at the site of medical care. In addition, holding the multiplex reactions if different pairs of primers are loaded into different reaction chambers on a chip becomes possible. In this case, several pathogens or genetic modifications of the organisms in the sample are simultaneously detected.

The development of MFDs for isothermal amplification based on droplet microfluidics is a perspective approach.

Soft lithography is applied for rapid fabrication of polydimethylsiloxane-based (PDMS) MFCs [3]. There are reveals the disadvantage of this material: porosity and high gas permeability, which leads to the evaporation of the reaction mixture during the thermocycling (in case of PCR). In the case of droplet-based amplification, the porosity of PDMS does not affect the reaction process, but certain requirements are imposed on the properties of the chip surface which provide the required conditions for droplet generation. Studies aimed at finding materials for prototyping, free from shortcomings of PDMS, are being carried out. Particularly, UV-curing polymer compositions and epoxy resins are considered promising [4].

Possible design of MFC prototypes for detection of nucleic acids are considered in this paper: 1) based on droplet microfluidics for isothermal amplification, 2) using the hybrid constructions for PCR.

2. Experimental setup

2.1. Material inhibition testing

A high surface-to-volume ratio of microstructures leads to an increase in the probability of interaction between the inner surface of the microchip and the molecules of the reaction mixture, which affects the efficiency of the reaction down to its termination (inhibition). Presumably, the mechanism of inhibition is determined by two major factors: physical adsorption and chemical interaction between the mixture components and the microchip material.

During the process of selecting materials for fabrication of microchip devices for amplification, preference should be given to those that do not affect the effectiveness of the reaction. Therefore, it is necessary to confirm that the materials do not inhibit the reaction.

The following materials were studied: PDMS Sylgard®-184 (Dow Corning, USA), single-component UV-curing adhesive Permabond UV630 (Permabond Engineering Adhesives Ltd, USA), adhesive compound PEO-221K (Saint-Petersburg State Institute of Technology (Technical University), Russia), cyclo-olefin polymer film Zeonor Film® ZF14-188 (Zeon Europe GmbH, Germany), polyethylene terephthalate film (DuPont, USA).

The tested materials were added to the tube during a real-time PCR, while a tube without additives was placed for comparison. ANK-32 thermocyclers (IAI RAS, Russia), as well as specific primers and probes (DNA-Synthesis, Russia) and reaction mixture M-428 «PCR mix» (Syntol, Russia) were employed for the experiments. A synthesized DNA fragment of GAPDH housekeeping gene 226 b.p. was used as a target in a concentration of 10^5 copies/μl. The value of the threshold cycle (C_t) was determined using the ANK-32 software for three to five repetitions. If the difference in the average threshold cycle values in the test tubes with a sample and the tubes without additives exceeded 1.0 cycle of real-time PCR, it was assumed that the samples inhibit the reaction.

The experimental results of polymers testing show that the materials do not inhibit the real-time PCR.

2.2. Amplification of nucleic acids in MFC prototypes

For experimental studies, MFCs of two configurations were fabricated: 1 – with a droplet generator for digital isothermal amplification; 2 – with ten reaction chambers for PCR.

For the fabrication of the MFCs, PDMS Sylgard®-184 and adhesive compound PEO-221K were used; and microstructures were formed by soft lithography. PDMS replicas with microstructures were sealed with a PDMS-film or a glass plate by high-frequency (13.6 MHz) plasma treatment at low pressure (1 mbar) in oxygen for 1 min (Denier Zepto, Denier electronic GmbH, Germany). When using PEO-221K, the sealing was carried out by a capillary method with UV-adhesive Permabond UV630.

The configuration of the MFC (figure 1, a) based on droplet microfluidics contained a droplet generator, which ensures the sample encapsulation by a focused flow of fluid into separated droplets with diameter of 10-40 μm in mineral oil. The width of the channel for the continuous phase is ~28 μm, for discrete – ~32 μm; the width of the generator is ~13 μm; the width of the reaction chamber is ~195 μm, its depth is ~31 μm.

a b

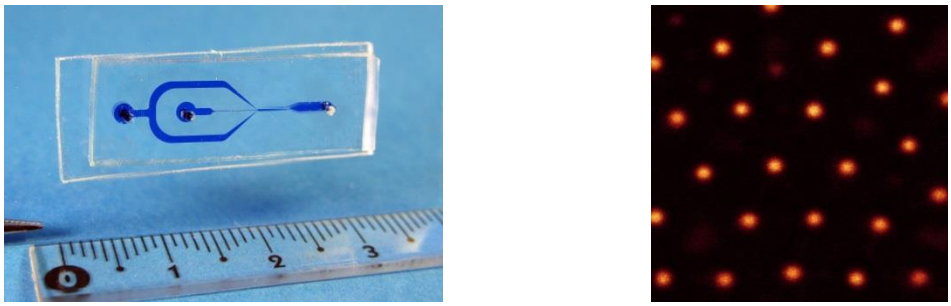

Figure 1. a – MFC with a droplet generator for digital isothermal amplification; b – image of emulsion flow after amplification, detected by CLSM in fluorescence mode. Size of the fragment is 100 x 100 μm^2.

BioXtra mineral oil (Sigma-Aldrich Corp., USA) with the addition of 5% Span® 80 surfactant and 0.5% Brij L4 (w/w) (Sigma-Aldrich Corp., USA) was used as the continuous phase (of transport flow); and, as the discrete phase, IsoAmp® II Universal tHDA Kit (BioHelix Corporation, USA) was used for helicase-dependent isothermal amplification. It was found that the combination of these surfactants ensures thermal stability of the droplets. The cDNA of GAPDH housekeeping gene was applied as a target, the amplification proceeded for 1 hour at 65 °C. The amplification results were registered by a fluorescence signal in the droplet flow (the volume of each droplet being ~29 nl); a confocal laser scanning microscope (CLSM) Leica TCS SL (Leica Microsystems, Germany) was used for this purpose. Detection conditions: excitation at 488 nm, emission at 510-530 nm (figure 1, b).

The obtained data: a) confirm the possibility of registration the results of amplification in the flow; b) indicate that the surfactant combination used does not affect the fluorescence of EvaGreen®.

The second configuration of the MFC had five pairs of reaction chambers (4 for reaction mixture, 1 for negative control) (figure 2, a), of ~160 μm depth and ~1.4 μl volume each. The design featured integration of the polyolefin film (Sarstedt AG & Co., Germany) into a PDMS-replica to prevent evaporation of fluid through the material when heated. Spectral measurements of MFC light transmittance showed that, in the spectral range of 500-850 nm, the transmission exceeds 94%, which ensures a reliable detection of the fluorescence signal of the most common fluorophores. Spectral characteristics of PDMS integrated with a cyclic olefin copolymer (COC) film (ZEONEX®, ZEON EUROPE GmbH, Germany) were also studied. The results of measuring the PDMS autofluorescence

with polyolefin and COC films showed that the constructions do not have intrinsic fluorescence when excited at 650 nm and recorded at 655-750 nm, which is necessary when using a Cy-5 probe.

a b

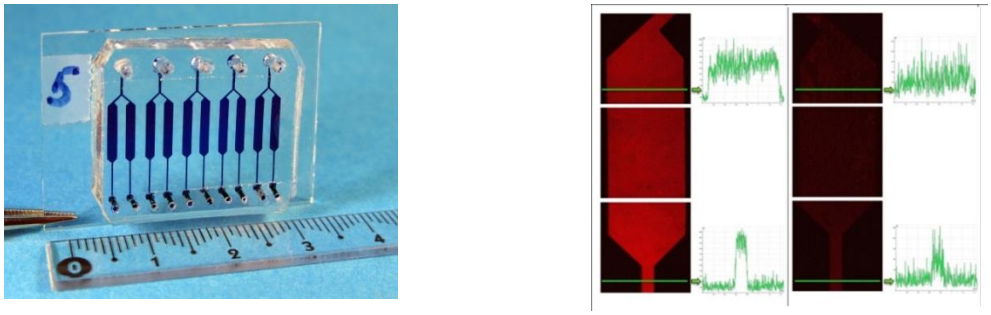

Figure 2. a – MFC for PCR with ten reaction chambers and an integrated polyolefin film; b – images of fragments of the reaction chambers with PCR-mixture and cDNA target after amplification (left); with negative control (right), detected with CLSM in the mode of measuring fluorescence. Intensities of fluorescence signal of reaction chamber and input channels are shown on the right side of the images. The size of each fragment is 1.5 x 1.5 mm^2.

After the chambers were filled with the reaction mixture, the input/output channels were sealed with silicone compound Pentelast®-712 A (Silikonovye Materialy, Russia), which cured during the first stage of MFC heating. PCR was carried out using TaqMan technology, specific primers and Cy-5 probe and reaction mixture M-428 «PCR-Mix». A synthesized DNA fragment of GAPDH housekeeping gene 226 b.p. was used as a target in a concentration of 10^5 copies/µl. The PCR results were registered by CLSM in the fluorescence detection mode at 650-670 nm with excitation at 633 nm (figure 2, b).

The obtained data show: a) the possibility of using Pentelast®-712 to seal the sample input/output channels; b) the effectiveness of the polyolefin film in preventing evaporation of the sample from the reaction chamber.

A hybrid construction was made of adhesive compound PEO-221K and a glass plate by soft lithography using the elastic PDMS-master mold. This combination of the materials also helps to prevent evaporation of the reaction mixture; however, it is necessary to select a compound for sealing of the sample reservoirs.

3. Conclusion

The results of the research showed that the selected materials for MFC prototyping: PDMS Sylgard®-184, single-component UV-curing adhesive Permabond UV630, adhesive compound PEO-221K, cyclo-olefin polymer film Zeonor Film® ZF14-188 and polyethylene terephthalate film have no significant effect on the amplification (do not inhibit the real-time PCR).

For the experimental studies, two microfluidic chip configurations were made: 1 – with a droplet generator for digital isothermal amplification; 2 – with ten reaction chambers for PCR. MFCs were made by soft lithography in the PDMS.

The results confirm the possibility of registration the results of droplet isothermal amplification in the transport flow, as well as in the reaction chambers of the MFC for PCR. The choice of the surfactants combination and their concentrations ensures thermal stability of the emulsion during isothermal amplification and does not affect the fluorescence of EvaGreen®.

A special feature of the MFC design for PCR was the integration of a polyolefin film to prevent evaporation of the sample, the effectiveness of which is shown by images of fragments of the reaction chambers. Pentelast®-712 A was used to seal the sample reservoirs.

Acknowledgments
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