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Microchip-based Systems for Molecular Genetic Analysis

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Abstract–Functionalities of microchip-based analytical systems for nucleic acids analysis are highlighted and driving forces for the future progress are reviewed. The review shows the main advantages of microchip-based systems and discusses up-to-date achievements in this area. Among the main advantages are a high speed of analysis, high rates of heating and cooling, a low reagents consumption, and a high potential to integrate several analytical processes in a single microchip device.

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INTRODUCTION

The progress in the manufacturing of microelectromechanical systems (MEMS) initiated active research on miniaturization of analytical devices. Over the past decades the MEMS technology has been used to develop miniaturized functional devices for biological and medical applications. These devices fulfill the same functions as traditional analytical devices but consume less reagents, thereby shortening analysis time and cost, as well as energy consumption. The key element of such a microanalytical system is a chemical microchip, specifically a miniaturized planar device with a branched network of microchannels and microreactors, which is manufactured by the MEMS technology from various materials (glass, quartz, silicon, as well as metals and alloys). The areas of microchips are generally a few square centimeters, and their linear dimensions in two dimensions vary from hundreds nanometers to hundreds micrometers.

The advantages of chemical microchips were demonstrated by the analyses combined with electrokinetic and chromatographic separation techniques, liquid−liquid and solid-phase extractions, electrochemical methods, as well as biochemical and immune ferment methods [1–3]. Various aspects of analytical applications of microchip-based systems have been described in a series of reviews and monographs [4–10].

Particular researcher's attention has been attached to microchips for molecular genetic analysis of nucleic acids (DNA and RNA) by means, in particular, of the polymerase chain reaction [11]. At present much effort is going to the development of new microchip-based analytical systems which offer radically new advantages [12, 13]. Such advantages include fast operation, high rates of heating and cooling of the reaction mixture, low consumption of high-cost reagents, and well as a high potential to integrate several stages of molecular genetic analysis in a single microchip device. The present review considers publications demonstrating the main advantages of microchip-based systems for molecular genetic analysis, as well as main up-to-date achievements in this field.

Polymerase Chain Reaction

The polymerase chain reaction (PCR), for whose discovery the 1993 Nobel Prize was awarded to K. Mullis, is a method of enzymatic synthesis of a peace of DNA with a particular nucleotide sequence.

The polymerase chain reaction involves repetitive thermal cycles resulting in the synthesis of a DNA fragment and production in a geometric progression fashion (amplification) of its copies (amplicons) [11, 14]. The attendant physicochemical processes are controlled by changing the temperature of the reaction mixture (temperature and time regime of amplification) in a certain order (Fig. 1). When the temperature is raised to 95°C, the double-stranded DNA molecule dissociates into two single strands (denaturation). These strands function as matrices for synthesis of complementary chains. When the temperature is lowered to 53−68°C (hybridization), the comple-

mentary fragments of single-stranded DNA add on short oligonucleotides (primers) which play the role of triggers for the synthesis of a new DNA chain. Further on the temperature of the reaction mixture is raised to 72°C, an optimal temperature for enzymatic synthesis of a complementary nucleotide sequence (elongation). One such cycle doubles the number of DNA fragments (amplicons are formed), whereas multiple repeated cycles increase their number in a geometric progression.

To accomplish PCR in solution, the following components, apart from DNA, are needed: DNA polymerase which synthesizes the complementary sequence, Mg^{2+} ions which act as a co-enzyme of DNA polymerase, deoxyribonucleoside triphosphates dATP, dGTP, dCTP, and dTTP hich act as building blocks for amplicons, and two oligo-nucleotide primers which flank the target fragment in opposite DNA strands [11, 14].

The amplicons synthesized by PCR are most commonly detected by fluorescent methods. These methods can be classed into several groups depending on the mechanism of fluorescence generation in solution [11]. The first group includes methods which make use of intercalating dyes (ethidium bromide, SYBR Green I, and BEBO) which do not fluoresce in the unbound state but start to fluoresce having intercalated with double-strand DNA molecules [11]. Analysis of PCR products using these dyes is performed either after separation of the post-reaction mixture by gel electrophoresis by the resulting fluorescence (endpoint method) or directly during reaction, i.e. the real-time method (Fig. 2). Of these

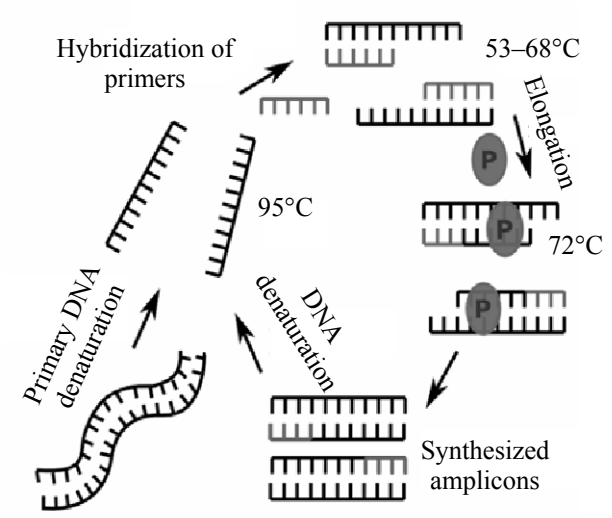

Fig. 1. Schematic presentation of the processes involved in a single temperature cycle of the polymerase chain reaction (P = polymerase).

methods, the real-time PCR monitoring (RT-PCR) is the only technique providing data on the quantity of DNA in the sample via the determination of the "threshold cycle" from the RT-PCR curve (Fig. 2a) and construction of the calibration plot [11].

The second group includes methods using fluorescence-labeled oligonucleotides: TaqMan probes, molecular beacons, or hybridized probes. Such probes detect DNA by measuring the change of fluorescence, which is associated with the quantity of synthesized DNA molecules. Fluorescence-labeled probes contain the nucleotide sequence complementary to the amplicon

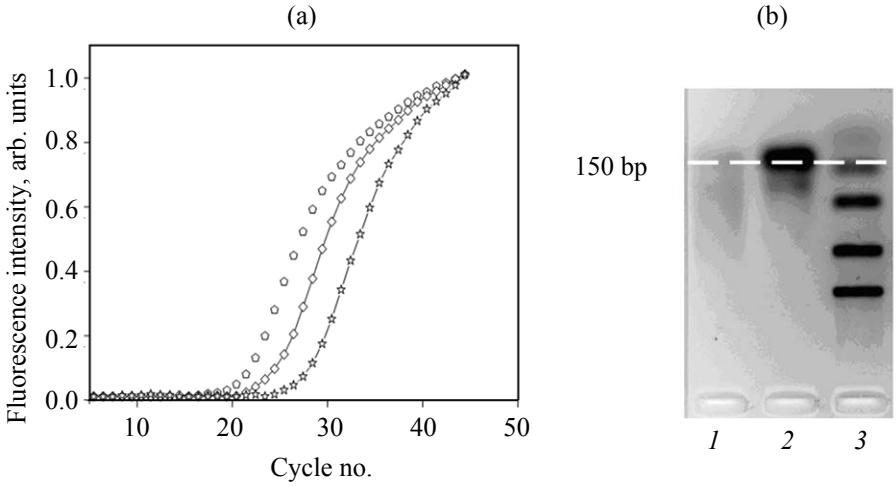

Fig. 2. Results of PCR analysis with different amplicons detection techniques: (a) real-time PCR at different starting DNA concentrations and (b) gel electrophoresis of PCR products: (trace *1*) negative sample, (trace *2*) positive sample, specific fragment, length 150 bp, and (trace *3*) DNA size standard solution.

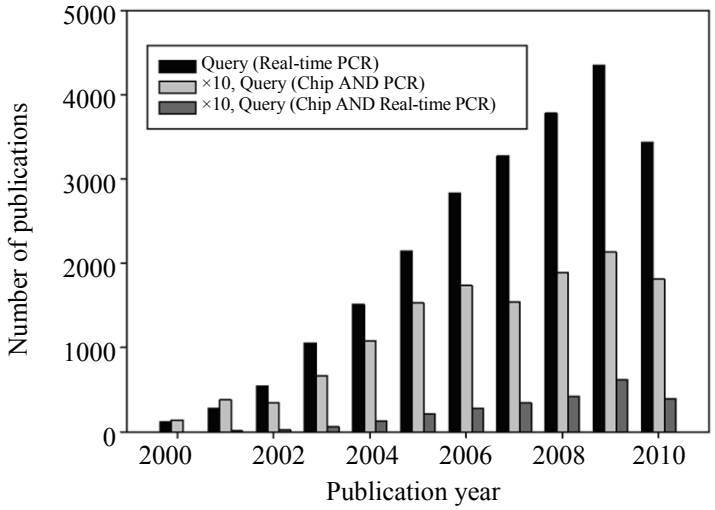

Fig. 3. Dynamics of growth of the number of publications on microchip PCR systems. Search for English language publications in PubMed, according to the queries in the legend. The publications in 2010 are covered until August 2010.

generated by PCR. Therefore, unlike intercalating dyes, they are selective indicators just for the nucleotide sequence of the amplified DNA fragment.

One of the key components of PCR analyzers is the thermal cycling block. Such characteristics as the rate of temperature change, uniformity of temperature distribution inside the reactor and rate of temperature equilibration, accuracy of temperature control, and uniformity of temperature distribution over several reactors define, in the long run, specificity of PCR, analysis time, and correctness and reproducibility of the results [14].

The improvements of the PCR which is traditionally performed in plastic tubes or in plates exhausted their potential but still do not meet the up-to-date requirements. The existing PCR analyzers feature low rates of thermal cycling (2−5°C/s) [11], non-uniform temperature distribution inside the reactor, long PCR time (1.5−2 h), and high reagent consumption (20−50 μl), and, as a result, high analysis

Table 1. Criteria for selection of components for microchip analytical PCR systems

Component	Options	Criteria
Microchip support	Material: Si, SiO_2, glass, metal, plastic Methods of fabrication, methods of material surface passivation	Thermal conductivity, electrical conductivity, optical properties, compatibility of material and coatings with PCR. Fabricability, cost of fabrication and passivation of microchip, possibility of large-scale production, microchip expendability
Microreactors	Size and shape, topology, mutual arrangement	Microreactor surface area-to-volume ratio. Mutual arrangement of microreactors: cross contamination, mixing, thermal isolation, ghost fluorescence signals
Cover layer	Material: glass, plastic, oil layer	Thermal conductivity, optical properties, compatibility of material with PCR, possibility of reagent immobilization, possibility of extraction of PCR products
Heating and cooling system	Operation principle: contact, noncontact, Peltier effect, Joule effect Location: external, integrated	Rate of heating and cooling, uniformity of temperature distribution. Fabricability, cost of microchips with integrated heaters and sensors, precision of temperature control
Thermal sensor	Operation principle: contact, noncontact. Material, dimensions, arrangement	Precision of temperature measurement, operation speed, compatibility of sensor material with PCR, fabricability
Detection system	Operation principle: fluorescence, electrochemical. Electrophoretic separation, endpoint detection, real time detection	Sensitivity, operation speed, possibility for miniaturization, fabricability of integrated detection systems

Table 2. Characteristic of materials for PCR microchips

Material	Thermal conductivity, $W\ m^{-1}\ K^{-1}$	Specific heat capacity, $J\ kg^{-1}\ K^{-1}$	Fabrication technology	Advantages	Disadvantages
Silicon	150	700	Photolithography, deep reactive ion etching, anisotropic and isotropic wet chemical etching Elecrochemical etching, laser ablation	High thermal conductivity Fabrication technologies are commercialized	Nontransparent in the UV and visible spectral ranges. High fabrication cost. Interaction with PCR components, reaction inhibition
Quartz	1.4	1000	Photolithography, isotropic wet chemical etching Elecrochemical etching, laser ablation	Optically transparent in the UV (quartz) and visible ranges. Dielectrics	Low thermal conductivity High fabrication cost Surface adsorption of charged macromolecules
Glass (pyrex)	1.1	750			
Polydimethylsiloxane (PDMS)	0.18	1460	Laser ablation, imprinting (PMMA, polycarbonate) Injection molding (PMMA) Matrix polymerization of liquid elastomers (PDMS)	Optically transparent in the visible range. Dielectrics. Compatible with PCR. Low fabrication cost; Possibility of single use	Low thermal conductivity Nonresistant to elevated temperatures, strong acids, and organic solvents
Polymethyl methacrylate (PMMA)	0.20	1446			
Polycarbonate	0.20	1350			
Stainless steel	16	500	Electrochemical etching	Low fabrication cost; Possibility of single use	Interaction with PCR components, reaction inhibition
Alumina	250	900	Stamping	Low fabrication cost; Possibility of single use	Interaction with PCR components, reaction inhibition

cost [12, 13]. Microchip analytical systems are, in principle, free of such disadvantages due to a low heat capacity of microchips (equal to the product of microchip mass by specific heat per unit mass of the microchip material) and small volume of microreactors (0.1−1 μl), which allows high rates of heating (175°C/s) and cooling (125°C/s) and realize forty PCR cycles for less than 6 min [15].

Wittwer et al. were the first to suggest in 1990 [16] a high-speed PCR analyzer with glass capillaries as microreactors. Northrup et al. in 1993 demonstrated a silicon PCR microchip [17]. These pioneering works gave rise to an exponential growth of publications devoted to microchip-based analytical systems (Fig. 3). By present a lot of scientific groups focusing on the development of miniaturized PCR systems have appeared. Diverse materials for chip supports have been suggested and microchip topologies have been developed; these systems were used for PCR analysis of various objects.

Table 1 lists the criteria for selection of the key components for microchip systems for PCR-based analysis.

Active R&D in the field of microchip-based analytical systems is prompted by the necessity of solving a complex of problems associated with transfer of the PCR methodology, especially real-time PCR, to a microchip format.

Materials and Technologies for Microchip Manufacturing. Surface Passivation

There is a great variety of materials for manufacturing PCR microchips (Table 2; for detailed review and analysis of materials and technologies for microchip manufacturing, see [5, 12, 13]).

Initially PCR microchip systems were most frequently manufactured from silicon [18–23] and quartz and glass [24–27]. The choice of these materials was first of all motivated by the availability of developed technologies of their production and processing in microelectronic industry. It is important that silicon has a good thermal conductivity, which makes possible fast heating and cooling of the reaction mixture during PCR. First PCR microchips were made of a glass-coated silicon, using external Peltier elements for heating and cooling [28, 29]. With the

advent of MEMS technologies, a possibility appeared to integrate into silicon microchips resistant metal heating elements and platinum film thermal sensors [18, 19]. Microchips with integrated heaters and thermal sensors are fairly costly, but the input power of such microchips decreases to a few watts per microreactor [30]. This makes it possible to create a portable battery-powered PCR analyzer suitable for field operation and for patient examination (in medical practice).

The low level of background radiation of silicon, too, favored its use in real-time PCR systems which measure fluorescence emission from microreactors through a glass cover plate [10, 31, 32] or a mineral oil film [33].

The use of quartz and various glasses in PCR microchips is primarily motivated by the need to ensure compatibility of microchips with the subsequent electrophoretic separation of PCR products, since these materials have a much higher electrical resistance than silicon. Ramsey and co-workers [34] demonstrated such glass microchips and applied them for PCR with electrophoretic separation of the resulting fragments [35].

The progress of MEMS technologies which made possible large-scale manufacturing of microstructures from other materials have raised recent interest in the R&D work on cheaper polymer materials [36] and metals [33, 37]. Thus, PCR microchips were successfully manufactured from polydimethylsiloxane [36, 38, 39], polycarbonate [40], and polymethyl methacrylate [41–43]. Giordano et al. [44] made use of laser ablation to form from 150-μm thick polyimide sheets two through-thick plates: the first with holes leading to channels and the second with channels and microchips. The two plates were stuck together, and then the third, solid plate was attached to this assembly on the side of the second plate to form a closed microvolume. The three-layer microchip thus obtained was thermally cycled under IR radiation [44]. Liu et al. [39] demonstrated a polydimethylsiloxane PCR chip with 12-nl microreactors [39]. Trung et al. [38] developed a polydimethylsiloxane multimicroreactor chip, where samples are introduction due to the gas permeability of the material; therewith, no pumps are needed for effectively filling microreactors without air bubbles. Such microchip was applied to success to amplify the human β-actin gene, and it holds promise for medical diagnostic applications.

Further improvement of the performance of PCR analyzers was reached due to on-chip microfluidic sample injection [45]. A chemical microchip with microchannels for sample injection and pneumatic channels for separation of microreactors was described; such microchip makes possible quantitative analysis of up to 48 samples for 770 genetic elements [46], which is especially demanded in genetic analysis. The microchip is manufactured from polydimethylsiloxane and does not allow high rates of thermal cycling, but this disadvantage is compensated for by the possibility to perform simultaneously a large number of reactions.

BioTrove produce microchips with 33-nl microreactors with through holes [37]. The microreactors are manufactured from N-18 stainless steel with a cross-linked polyethylene glycol coating for preventing PCR inhibition with the material of the microchip and imparting to it hydrophilic properties. Samples are injected through a standard plastic dispensing tip leant to the microreactor surface; the analyzed solution enters the microreactor due to capillary forces. Even though the microchip is manufactured from stainless steel which compares in thermal conductivity with silicon and potentially allows high rates of thermal cycling, the suggested hermetization method imparts to the heating element a high thermal capacity and does not allow high rates of heating and cooling.

The described system makes possible high-performance screening of several samples for a number of genetic elements. Thus, by real-time PCR one can analyze 48 samples for 64 DNA fragments and up to 192 samples for 16 DNA fragments within 2.5 h.

In [33] we described microchips with 1−2-μl open microreactors from aluminum alloy for RT-PCR. Such aluminum microchips can be manufactured by pressing, which reduces considerably their cost on large-scale production, but the thermal characteristics of the microchips remain very high, and the use of special coatings makes them PCR-compatible. In such a microchip, PCR was accomplished within 20 min with a high efficiency, which implies a lack of reaction inhibition [33].

At a larger ratio of the surface area of the microreactor to its volume, PCR is much affected by surface processes [12]. Because of the sorption of components of the PCR mixture and deactivation of polymerase, the concentrations and activities of the components in the solution decrease, thereby

decreasing the efficiency of the reaction or even its complete inhibition. Therefore, particular researcher's attention focuses on the development of simple and reliable methods for surface modification of materials for microchips.

Regardless of the low thermal conductivity which does not allow high rates of thermal cycling, polymer materials have found wide application in microchip engineering due to their inertness to PCR components (polymer microchips generally do not require surface modification) [47].

Microchips from silicon and silicon-containing materials require surface modification, since such materials tend to adsorb PCR components [48]. Two types of modifiers have been described [12, 47]: permanent and dynamic.

The mechanism of action of dynamic modifiers is based on competitive sorption of the components of the PCR mixture and the modifier which is present in the solution. The list of suggested dynamic modifiers includes bovine serum albumin [49], polyethylene glycol [50], glycerol [18, 51], formamide [18, 51], polyvinylpyrrolidone [28], and Twin 20 [28].

Permanent modifiers are applied on the surface before PCR, they form an inert coating which prevents sorption of PCR components on the surface of the microreactor [29]. The most commonly used permanent modifiers are organic compounds that are capable of forming hydrophobic coatings. Permanent modifiers for microchips with etched microchannels can be exemplified by dichlorodimethylsilane [52], SigmaCoat [28], and chlorotrimethylsilane [28]. In [15, 53], the surface of a glass plate was treated with the hydrophobic and oleophobic perfluoroalkoxysilane to fabricate a microchip with open virtual reaction chambers (VRC). The PCR in such a microchip occurs in a spherical water droplet encapsulated in a hemispherical oil droplet located on the surface of the modified glass plate.

In cases where a hydrophobic coating prevents sufficient thermal contact of the reaction system with the microreactor surface, the latter is covered with a hydrophilic film of SiO_2 [18, 54], bovine serum albumin [40, 55, 56], 3-glycidopropyltriethoxysilane [57], or polyethylene glycol [51] to ensure wetting of the surface with the PCR mixture [58]. Schmidt et al. [59] created a microreactor with hydrophobic and hydrophilic zones on the surface, which allowed PCR to be performed on a plane surface; therewith, spreading of the PCR mixture within the hydrophilic zone is restricted by the hydrophobic surface outside this zone.

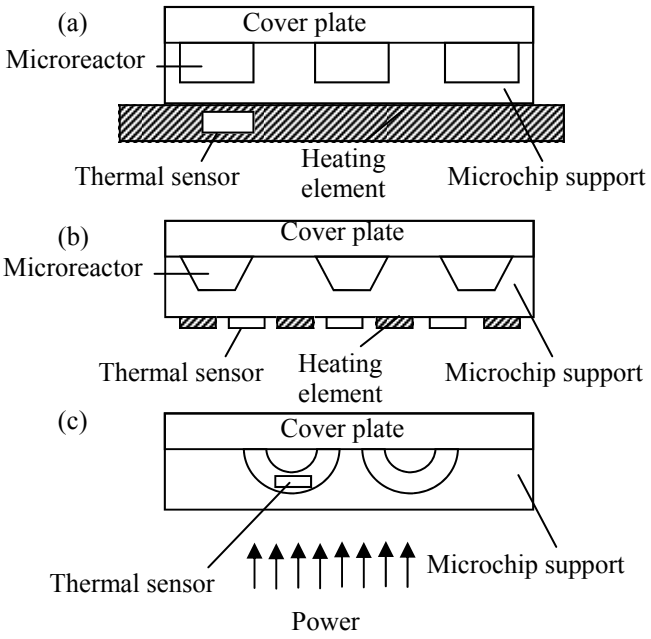

Fig. 4. Designs of stationary microchips with different modes of thermal cycling of the solution inside microreactors: (a) contact mode with an external heating element; (b) integrated thermoresistive elements; and (c) noncontact mode with external power supply.

Note that a great number of approaches to surface modification of microreactors have been reported, but researchers not always assessed the efficiency of real-time PCR, which hinders comparison the obtained coatings, and very rarely reported storage stability data for them. For this reason, the choice of techniques and optimization conditions for surface modification of microchips is still an urgent issue.

Types of Microchip Systems

The constructions of microchip-based PCR systems are fairly diverse. In terms of the way of performing PCR, microchips can be divided into two classes: continuous-flow and stationary. In a stationary PCR microchip, the solution is kept in a microreactor, and the temperature of the latter is cycled. The number of microreactors in such microchips can vary from one to several tens, hundreds, and even thousands. Microchips with a single microreactor provide more possibilities for optimizing operating speed, uniformity of heating, and mode of injection of a solution into the reaction, but the performance of such systems is quite low. Systems with a

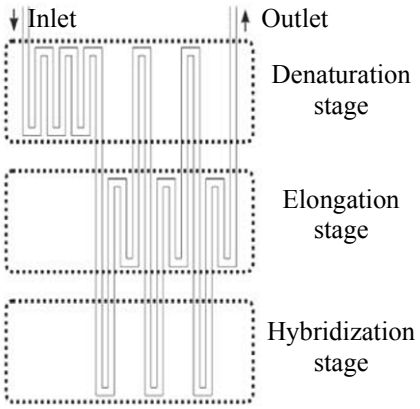

Fig. 5. Schematics of a continuous-flow PCR microchip.

great number of micrireactors exhibit enhanced performance, but in this case one should carefully optimize the rate and uniformity of heating, use a complicated injection mode, and sacrifice the operating speed.

The thermal cycling of solutions inside micrireactors can be effected in contact mode with an external element, by means of integrated thermoresistors, and in a noncontact mode with external energy sources, specifically IR radiation [24, 25], as well as inductive [60] or microwave [61] electromagnetic fields (Fig. 4). Microreactors in such microchips can look like as sealed cells which are quite difficult to manufacture but allow use of microfluidic channels for sample injection, as well as open cells which are much easier to manufacture but require additional dosing systems.

Over the past years a tendency is observed to develop stationary microchips with immobilized PCR reagents. This relates both to microchips with open cells, where PCR reagents can be immobilized fairly easily, and to microchips with sealed cells, where immobilization poses a series of problems associated with providing access of solution to sealed microreactors and evaporation of solvent, and well as with the lability of PCR components. These problems are solved in Fluidigm PDMS microchips: Primers and probes specific to various DNA fragments can be introduced and freeze-dried in their microreactors during the manufacture process [46]. Kim et al. [62] described the application of a microfluidic chip with dry PCR reagents which are isolated from the environment with paraffin and can be stored at room temperature. Paraffin plays a barrier function when aqueous solution of DNA to be analyzed is injected into the microreactor and melts at 95°C to let the PCR components to dissolve in the injected sample solut ion. The authors of [62] injected into a 30-μl microreactor 200 copies of the DNA of lambda phage and could register by gel electrophoresis amplification of a specific fragment. Note that the use of reagents immobilized in microreactors can drive introduction of chemical microchips into the analytical practice.

PCR microchips of the second type (Fig. 5) are designed for a dynamic flow PCR. The PCR mixture is successively pumped through 3 zones of a serpentine-shaped microchannel, with the temperature maintained constant in all the three zones. Such PCR technique in a glass microchip with copper heating elements was first suggested by Kopp et al. in 1998 [63]. The referees could accomplish PCR analysis of DNA in a microchip with 20 consecutive microchannel sections, within 1.5–18.7 min, depending on the rate of pumping the PCR solution through the microchannel.

The interest in systems with continuous-flow microchips is explained by the following their attractive features [12]: integration of pre- and post-treatment of PCR products; the possibility to control the thermal cycling dynamics exclusively by varying the flow rate of the solution; low thermal inertia which depends on the microchannel cross section; and variable sample volume (from 1 μl to several tens microliters). At the same time, wide use of such microchips is limited by certain disadvantages faced by researchers with first flow systems. These include the possibility of formation of air bubbles inside the microchannel on heating; the necessity of using fairly sizeable external syringe pumps; difficulty with varying PCR parameters depending on the microchannel design; and engineering problems in designing and constructing highly integrated systems for multisample analysis.

Further research was focused on solving the above problems. Thus, Nakayama et al. [64] suggested to fill the microchannel with a water-immiscible fluorinated oil and then to displace it by the PCR solution to avoid formation of air bubbles in the microchannel. This idea was developed in further research of this group and resulted in the creation of a flow-through PCR microchip for quantitative analysis of genetically modified organisms, such as the corn line MON810 [65].

Aiming at miniaturization of PCR systems with flow-through microchips, we suggested a polymethyl methacrylate microchip in which the reaction flow through the microchannel is created by means of a water-immiscible ferromagnetic fluid placed in this microchannel, rather than by means of an external syringe pump [42, 43]. An external (with respect to chip) miniature engine rotates constant magnet which causes the ferromagnetic fluid to move in the channel and thus drives the PCR solution through three temperature zones. This system was used to amplify the lambda phage DNA fragment 500 bp in length within 4 min. Forensic application of the developed system was also demonstrated.

Water-in-oil emulsions received original applications in flow PCR microchips. Thus, in 2006 Chabert et al. [66] suggested a technique in which the aqueous phase containing PCR components forms droplets in a water-immiscible oil, the resulting emulsion is injected into the microchannel of a PCR chip, and PCR products are detected by the end-point method. In the subsequent works [67, 68], various applications of PCR analysis with similar microchips and emulsions with picoliter droplets, including real-time PCR within 35 min (55 s/cycle), were demonstrated [69]. Low detection limits could be reached due to the possibility to detect even one DNA copy in a microdroplet, and digital PCR relying on random molecular distribution allowed detection of 1 DNA molecule per 167 droplets, which corresponded to an adenovirus DNA concentration of 0.003 pg/μl [69].

Methods of Heating and Measurements in Microreactors

Numerous systems for PCR thermal cycling in microchips have been developed. As seen from Table 1, selection criteria for systems of heating and cooling, as well as for thermal sensors depend on many factors, and, therewith, microchip characteristics and fabrication technology should be consistent with characteristics of the thermal cycling system.

All existing thermal cycling systems can be divided into two groups: contact and noncontact.

In contact systems, the surface of the heater and the surface of the thermal sensor are in a physical contact with the microreactor, whereas in noncontact systems there is no direct contact of the reaction system with heater and thermal sensor, the energy supply to the microreactor occurs through electromagnetic fields, and the temperature is also measured indirectly, for example, by optical methods.

Contact heating and cooling systems together with contact thermal sensors were in microchips and are up to now quite common in microchip tegrated into first PCR systems due to a high efficiency of heat transfer from the heater to microchip and easy temperature measurement and control.

In terms of the principle of operation of the heating element, contact heating systems can be classed into several types.

Microchip systems with resistive heating (the Joule effect) are the most common type of integrated devices. Modern MEMS technologies allow electroconducting coatings to be applied on diverse supports to obtain load resistors. Since one of the first applications of such heaters [17], several research groups could extend and supplement this technology, using platinum, gold, tungsten, and tin–indium oxide as resistor materials [18, 19]. Metal heaters are applied on supports by means of photolithography with thermal evaporation or plasma sputtering. Conducting contact spots for plugging-in the support to an external power source are generally positioned near the microchip edge. Quite important parameters of such heaters are the microstructural parameters of the resulting films, since any film defects developing on heating due to changes in the thermal resistance or thermal expansion coefficients can entail irreproducible temperature distribution during PCR. Heating elements can be formed on the outer side of the microchip for them not to contact with the PCR solution.

For a more precise temperature control Ke et al. [19] fabricated a chip with platinum heating and measuring resistors contacting the solution and coated these metal resistors with a thin film of silicon nitride to prevent the inhibition effect. In [70], microreactors were thermally isolated from the main body of the microchip by grooves, which provided high rates of heating (90°C/s) and cooling (74°C/s). Such grooves make it possible to decrease the power consumption for heating to a preset temperature. Erill et al. [30] implemented a heating technique involving a polysilicon resistive heater. Temperature is measured by changes in the resistance of this resistor, since its thermal resistance coefficient is 5.7×10^{-3} K^{-1}, and its response is linear in the working temperature range of PCR.

Most contact integrated heaters make use of a proportional integral derivative (PID) algorithm of temperature control. The temperature is maintained within ±0.5°C [71]. Due to numerous advantages of such integrated systems, they can be introduced to success into the practice of PCR analysis. This will become possible either when the costs of the microchip and the analytical systems as a whole are low enough for them to be competitive with classical PCR analysers or when the functionality of such devices provides the full cycle of the molecular genetic analysis in the sample-on-the-inlet and result-on-the-outlet mode. Up to now, such systems have found limited application.

In the course of research on the PCR technique, resistive Joule heating was applied not only to metal conductors but also to the PCR solution in microchannel, in view of the fact that the solution is an electrolyte and possesses ionic conductivity. Such approach was realized by Heap et al. [72]: PCR was performed in a capillary whose ends were connected to a source of alternating current (frequency 60 Hz, amplitude 0−1000 V; the PCR products were registered by gel electrophoresis. In [36], theoretical calculations were used to develop a polydimethylsiloxane microchip in which thermal cycling is performed by passing electric current through the PCR solution in the microchannel. Real-time PCR detection with fluorescent hybridization TaqMan probes was demonstrated; therewith, the consumed power was 1.3 W, but the heating and cooling rates were not too high (3 and 2°C/s, respectively).

Note that even though the above approach has found a number successful applications and holds promise in terms of further improvement of microchip-based analytical PCR systems, some problems still remain, associated with air bubble formation in the microchannel, complicated sample injection into the microchannel, necessity of stable temperature measurement by electrical resistance of the PCR solution, i.e. further steps are required to summarize the accumulated experience and introduce microchip systems into the practice of PCR analysis.

Many developers of microchips make use of external heating elements, since this allows one to simplify microchips, reduce their cost, and ensure economic efficiency of their disposability. External thermal cycling systems commonly include Peltier elements [10, 28, 31–33, 73, 74]. Peltier elements are less power-efficient than integrated resistive heaters [30] (power consumption 12.3 and 2.8 W, respectively) and demonstrate lower rates of thermal cycling (heating/cooling: 5/5 and 15/5°C s^{-1}, respectively). However, if the peak power output of the Peltier elements is increased to 56 W, fairly high heating:cooling cycling rates can be obtained: 9.5:10.3°C/s [33]. The advantage of external Peltier elements is that they allow to use simple disposable microchips, since all functions of the thermocycling system are realized in external (with respect to the chip) units of the PCR analyzer. The disadvantages include a slightly increased power consumption and limited thermal cycling rates. Note that most present commercially successful microchip-based PCR analyzers use external Peltier elements.

An interesting version of contact thermal cycling systems is presented by gradient convective microchips, in which the PCR solution circulates between several temperature zones due to a change in the density of the solution with temperature. Krishnan et al [75] described a microreactor 1.5 mm in diameter and 1.5 cm in height in a gradient temperature field: The temperatures of the top and bottom of the microreactor were maintained at 97 and 61°C, respectively. Such conditions induced convective motion which was visualized by means of fluorescent latex microspheres. This reactor was used to perform a PCR resulting in a 295 bp in length. An analogous approach was described by Chung et al. [76] who successfully amplified of a 125-bp DNA fragment for 10 min (starting DNA concentration 10 pg/μl) and a 470-bp genomic DNA fragment for 20 min, using a microchip with a three-zone temperature gradient.

An intermediate position between contact and noncontact thermal cycling systems is occupied by PCR thermal cyclers, systems in which the microreactor is heated with a stream of hot air generated by a heating element. Thermal cyclers have not attracted very much interest of developers of chemical microchips. This is explained by the fact that most researchers used glass capillary microreactors [16, 72], which is directly associated with the prevalence of commercial Roche LightCycler PCR analyzers. Even though such thermal cycling systems allow high heating and cooling rates, generating a stream of hot air is quite power consuming (300−1000 W), and this circumstance makes thermal cyclers poor candidates for application in portable systems.

Noncontact thermal cycling systems in PCR microchips use IR radiation, microwave radiation, or

induction heating. One of the first work in which aqueous solution in a capillary was heated with an IR lamp was published in 1998 [77]. Since water has strong IR absorption bands at 2.66, 2.78, and 6.2−8.5 μm, the researchers employed an incadescent lamp with the radiation range up to 4 μm. In 2001, the group of Prof. Landers [44] used such a noncontact heating system in polyimide microchips, where they performed PCR within 4 min. Proceeding with R&D in this direction, Landers and co-workers suggested an integrated microchip heated with an IR lamp. In the latter case, the heating and cooling rates were slightly lower (7.8 and 5°C/s, respectively), and PCR was complete in 12 min (30 cycles); the PCR products were subsequently separated by electrophoresis [24, 78]. This concept was further developed in [79], where successful amplification of a 211-bp fragment of *B. anthracis* genomic DMA within 18.8 min (30 cycles, three-stage temperature−time regime).

An analogous approach, potentially attractive in terms of development of PCR microchips, was used in [80] with the aim to control an enzymatic reaction by means of an IR laser (λ 1480 nm, 150 mW), whose radiation coincides with the absorption band of water at 1500 nm. Braun et al. [81] made use of an IR laser (λ 1480 nm, 75 mW) to develop a convective PCR device. Lasers can also be applied for heating absorbing microstructures in microchips [82].

An interesting version of noncontact heating is presented by a thermal cycling system [60] functioning due to high-frequency electromagnetic radiation (frequency 200 kHz, power 1.4 W) which ensures in a silicon−glass microchip the heating and cooling rates of 6.5 and 4.2°C/s, respectively. Shaw et al [61] reported a microchip in which aqueous PCR solutions were heated by microwave radiation; quite high heating and cooling rates (65 C/s) could be reached, and the accuracy of temperature control during thermal cycling was ±0.1°C.

Noncontact thermal cycling systems almost all hold much promise, since they demonstrate high heating and cooling rates and are compatible with fairly cheap microchips. However, such systems have still found limited application in view of the fact that devices with a large number of PCR microreactors have not yet developed, and problems associated with providing uniformity of heating and measuring the temperature in them have not yet been solved.

The choice of temperature measurement technique for microreactors depends on certain criteria associated with the specifics of PCR conditions (Table 1). Thermal sensors can arbitrarily be divided into two groups: contact and noncontact, by analogy with heaters. The role of contact thermal sensors in microchip-based PCR devices can be fulfilled by external thermocouples, thermoresistors, and semiconductor thermoelements [31–33, 74], miniaturized thermocouples built-in into microreactors [80], and film thermoresistors integrated into microchips [70]. Contact thermal sensors offer the advantages of relative simplicity of signal measurement and simplicity of calibration. The disadvantages are associated with the possible PCR inhibition with the sensor material and influence of the sensor on the measured temperature because of the comparable sizes of the sensor and microreactor, and, furthermore, on-chip thermal sensors require individual calibration.

Noncontact temperature measurements were reported to be performed by optical methods based on color change of liquid crystals [47] or on change of the fluorescence intensity of a dye due to the temperature-dependent quantum yield of the fluophofore or temperature-dependent shift of the absorption or fluorescence spectrum [83], as well as by direct IR thermometry [18] and Raman spectroscopy [20].

Thermotropic liquid crystals which are capable of changing their color on heating [47] were used by Noh et al. [22] for spatial mapping of the temperature distribution in 1-μl PCR microreactors and by Cheng et al. [41] for tracing the dynamics of temperature change in a PCR microchannel. This method allows quite precise temperature measurements (within ±0.1°C), but the measurement range is fairly narrow. Spherical capsules with thermotropic liquid crystals are tens of micrometers in diameter, and they can only be applied in microstructures of a larger size. Moreover, such microspheres, when introduced directly into a PCR solution, can inhibit reaction, and this entails the necessity of developing PCR-compatible capsules.

Mondal and Venkataraman [84] detected fluorescence quenching of the intercalating dye SYBR Green at the melting point of amplicons, whereas Neuzil et al. [15] revealed a temperature dependence of the 6-FAM dye. Over the past years the method based on the measurement of the fluorescence intensity of Rhodamin B has received the widest acceptance in microchips [36, 80]. The advantages of this method

include a wide dynamic measurement range, high measurement rate, and high temperature sensitivity. However, this method is not very precise. Aimed at improving the precision of this method, Shah et al. [85] suggested a new theoretical model for temperature calibration in the Rhodamine B method, which allowed correction of previously unaccounted mistakes. Samy et al. [86] made use of this approach for temperature measurements in the whole microchip in which Rhodamine B was introduced into a thin polymethyldisiloxane film, and the resulting polymer plate was contacted with a microchip to be studied.

Temperature measurements in PCR microchips by IR thermography were fulfilled in several works [18, 19, 41, 55]. It was found that a complex of investigations has to be undertaken for weighted analysis of such factors as radiation losses, background radiation, and uncertainty in the assessment of the radiating power of the microchip. Furthermore, optimization of the parameters of the IR thermometer and its careful calibration are required. The advantages of IR thermometry include fast response, sufficiently wide dynamic measurement range, high spatial resolution, and lack of interaction with PCR components.

One more noncontact thermometry method is a method [20] in which the temperature inside the microreactor is measured by means of Raman spectroscopy. Temperature measurements are based on employing the temperature dependence of the intensity ratio of H-bonded and free OH stretching vibration bands. The integration into a silicon microchip of a platinum resistive heater and the use of the developed noncontact temperature measurement system make possible a highly precise temperature control.

Note that, like noncontact heating systems, noncontact temperature measurement systems are potentially attractive. However, almost all developed thermal sensors necessitate further research for improving to precision and stability of measurements and reducing the cost of the devices.

Methods of DNA Detection in Microchips

In the initial period of the development of microchip-based PCR analytical systems most researchers relied on detection methods requiring withdrawal of the reaction products from microreactors and their transfer into an analyzer external with respect to the microchip. The most common analytical technique was gel electrophoresis with intercalating dyes [28, 29], but such analysis was time- and labor-consuming. Later the interest of researchers was focused on microchip capillarity electrophoresis for DNA separation, as a technique allowing one to accelerate and automate the analysis. In [18, 21, 57, 87] and [88], the application of, respectively, an Agilent 2100 Bioanalyzer and laboratory devices with laser fluorescence detection in microchip electrophoretic separation and identification of amplicons obtained in PCR microchips. Such a combination of two microchip systems, specifically PCR and electrophoresis, make it possible to simplify and accelerate PCR analysis of nucleic acids. However, this method involves manual sample transfer from PCR microchips to electrophoretic microchips, which entails a risk of cross-contamination and mistakes.

One of the first examples of an integrated analytical system with the functions of electrophoretic separation and fluorescent detection of products is provided by a microchip including a reactor connected with a cruciform separation microchannel [34, 89]. In this chip, the PCR mixture is injected into the microreactor for thermal cycling, after which the products are directed to the microchannel for electrophoresis. The microchip is fabricated of two glass plates: One of the plates contains etched hollows (channels, chambers, or communication holes), and the second plates covers the etched units. Thermal cycling is performed by means of external Peltier elements. Such microchip allows one to perform both PCR and quantitative determination of products, but it can process only one sample. Multiple sample analysis is feasible in a microchip accommodating several PCR chambers and several separation microchannels [35, 47, 90].

Increasing the number of microreactors in a single microchip was made possible by the introduction of fluorescence endpoint detection of PCR products [55, 66, 91]. The informative content was much enhanced by means of fluorescence hybridization analysis involving complementary binding of amplicons formed by PCR in microreactors with DNA fragments covalently grafted to the biochip surface, which allowed detection of mutations in the amplicons [92].

Electrophoretic DNA detection and fluorescent endpoint detection both do not allow quantitation of DNA in samples, and, therefore, further development of microchip systems for molecular genetic analysis was focused at real-time PCR (RT-PCR) analysis

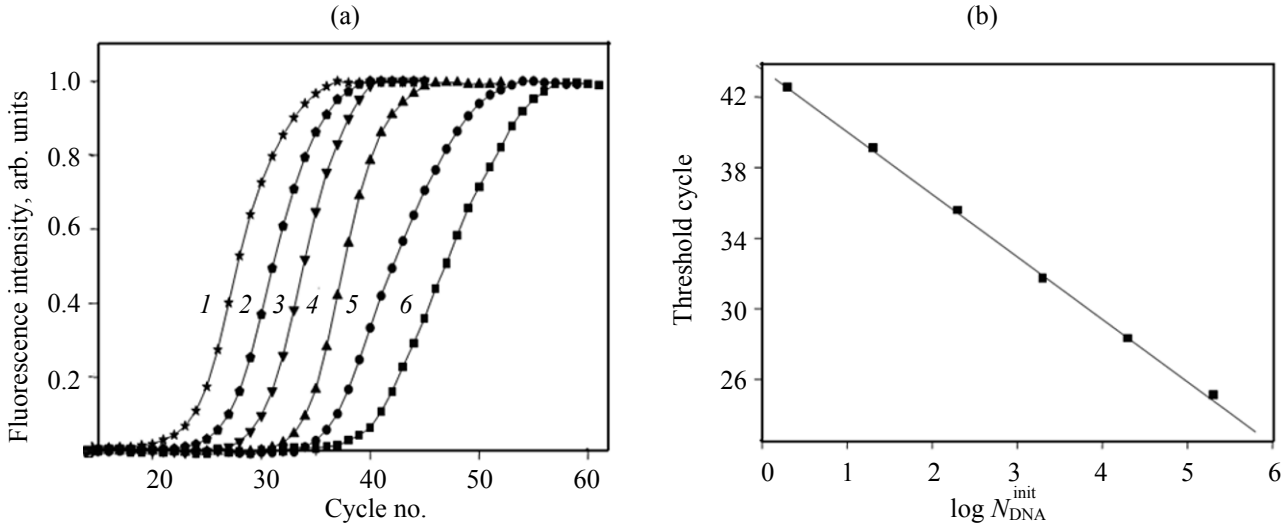

Fig. 6. Results of real-time PCR in a microchip at different DNA concentrations: (a) plasmid with a built-in Hepatitis C virus fragment and (b) calibration plot. Concentration, copies/μl: (*1*) 2×10^5; (*2*) 2×10^4; (*3*) 2×10^3, (*4*) 2×10^2; (*5*) 20; and (*6*) 2. The calibration plot was constructed for the PCR curves *1–6*.

which provides quantitative information. Microchip analytical systems for RT-PCR analysis with intercalating dyes and flurescence probes were reported in numerous publications (for example, cf. [18, 21, 36]). Some authors made use of melting on a microchip for identification of specific PCR products. The absolute detection limits of such systems approach the theoretical minimum: Fluorescent detection methods are able to detect single copies of DNA molecules, obtained in modern laboratories and commercial microchip RT-PCR analyzers. Such results are reached due to the optimized thermal cycling systems, microchips, and modifying coatings, which make possible highly efficient PCR reactions (up to 100% [32, 33, 74]).

Figure 6 presents the results of PCR with real-time analysis using hybridization probes [74]. The analytical system was based on fluorescent detection of PCR products formed in sixteen 1.3-μl reactorsof a silicon-glass microchip. The inner surface of the microreactors was modified by a polymethylmethoxy-siloxane film. The PCR time was 18 min. The high efficiency of the PCR (90%) implied lack of reaction inhibition.

In the framework of the research on miniaturization of detection systems in microchips, Defever et al. developed electrochemical methods for real-time DNA detection [93]. It was found that the consumption of one of the components of the PCR solution, specifically 7-deaza-dGTP (7-deaza-2'-deoxyguanosine 5'-triphosphate), can be followed using a tris(bi-pyridine)osmium(III) redox catalyst generated at the electrode. The reaction was performed in a microchip containing eight 50-μl microreactors with built-in electrodes. The detection limit of the electrochemical detector of 10^3 cytomegalovirus DMA molecules per microreactor is three orders of magnitude higher than that characteristic of fluorescence detectors. However, due to a much simpler construction, it allows development of portable devices. A similar approach was suggested by Fang et al. [94], who made use of the electroactive intercalator Methylene Blue (MB) and performed PCR in a continuous-flow microchip. After optimization of microchannel wall coatings and MB concentration, elimination of PCR inhibition by metal electrodes, and prevention of air bubble formation, the detection limit of the lambda phage DNA could be brought to a value compared to that of PCR systems with real-time fluorescence detection.

In [37, 46], microchips with a large number of microreactors (2500, 3000, and 9000) and digital real-time PCR for the determination of DNA in samples using the calibration curve in the coordinates DNA concentration–number of cells giving a PCR signal. This approach is based in the Poisson distribution of DNA molecules in microreactors and allows one to register minor variations in the concentration of the target DNA fragment [37, 46], which cannot be reached with log DNA concentration–threshold cycle calibration used in traditional RT-PCR. Such

microchips were used in commercial microchip analyzers produced by BioTrove and Fluidigm. It is to be noted that these analyzers fail to provide high rates of thermal cycling, since their microchips contain polymers with a low thermal conductivity. Furthermore, the dynamic range of digital RT-PCR measurements ranks much below that characteristics of traditional RT-PCR and does not exceed three orders of magnitude.

The trends in the development of microchip-based analytical PCR systems show that at present and in the near future the greatest demand will be for RT-PCR systems, in particular, digital RT-PCR ones, as well as systems based on hybridization analysis. Just such analytical microchip systems allow quantitation of DNA in the initial sample and analysis of mutations in the resulting amplicons.

Application of Microchip-based Systems for Molecular Genetic Analysis

The molecular genetic analysis of real samples involves isolation and purification of nucleic acids followed by PCR and determination of reaction products. Since the procedure of isolation of nucleic acids from various samples are fairly time- and labor-consuming, and the volumes of the initial and purified samples are hardly compatible with the microchip format of PCR, an urgent problem thus arises to develop microchip systems for sample preparation. An even MORE urgent problem is to developed a fully integrated system for complete molecular genetic analysis by the "sample at the entrance – answer at the exit" principle [78].

Many researchers attempt to adapt to the microchip scale existing procedures of isolation and purification of nucleic acids, for example, the fairly common procedure of solid-phase extraction of DNA on silica sorbents, followed by elution with buffer solutions. Breadmore et al. [95] made use of a microchip whose microchannel was filled with hybrid particles of a silica sorbent and sol−gel silica. A solution containing the lambda phage DNA (pH 6.1) was pumped through the microchannel at a rate of 250 μl/h. The recovery of the DNA was 67%; the isolation of DNA from whole blood was accomplished within 15 min.

An alternative to nucleic acid extraction is provided by methods based on filtration of various blood cell components. Wilding et al [96] developed a microchip with branched column- and slit-like microstructures in the microchannel. As blood is pumped through such microstructures, all blood components pass through, except for white blood cells. Since hemoglobin contained in red blood cells is the principal PCR inhibitor in whole blood, its removal favors successful amplification of the sample remaining on the filter, after its direct injection into the PCR mixture and destruction of wide blood cells in the course of initial heating. Similar techniques involving silica particles as sorbent for nucleic acids were also used in other works for isolation and purification of cellular DNA and performing PCR [97, 98].

The progress in knowledge and accumulation of experience in the development of microship devices for sample preparation and PCR made feasible a full analysis system [99]. This microfluidic system includes mixers, valves, pumps, a network of microchannels and microreactors, heating elements, and a hybridization matrix with an electrochemical detector. The system allows lysis of cells which enter the microchip with a biological fluid sample, by means of a piezoelectric vibrator, air bubbles, and magnetic sorbent particles adsorbing isolated DNA molecules. After that pumps whose operation principle is based on electrolysis of aqueous electrolyte solutions and electrokinetic phenomena direct the lysate flow to the PCR microreactor, where magnetic sorbent particles are trapped and washed. The PCR components are subjected to thermal cycling in the microreactor, and the PCR products are detected by a hybridization biochip equipped with several electrodes.

Automated complete molecular genetic analysis systems combining DNA isolation, purification, amplification, as well as amplicon separation and electrophoretic detection in a single microchip were described in a number of recent publications [100–102].

A unique procedure of cell destruction and DNA isolation was realized on a microchip with integrated electrodes which perform electrolysis producing hydroxide ions to induce cell lysis in the microreactor a few microliters in volume [103]. The developed device was used for RT-PCR of four types of bacteria, including gram-positive and gram-negative ones. Such advantages as the lack of need for heating the sample and adding chemical reagent, lack of inhibition, low DNA losses, as well as the ability of the device to function at low feed voltage and watt consumption were demonstrated.

Wang et al. [104] reported a prototype microchip-based flow analyzer for quantitative RT-PCR analysis of Hepatitis B virus DNA and Hepatitis C virus RNA. The device contains modules for controlling the microfluidic flows and temperatures of three stationary metal heating blocks, which allows flexible variation of PCR temperature and time parameters.

CONCLUSIONS

Our assessment of the dynamics of development of microchip-based analytical systems for molecular genetic analysis, as well as the review of recent publications in this field provide clear evidence to show that these systems have passed a major stage of their evolutionary progress and occupied firm positions in analytical practice. This was primarily contributed by the commercialization of microchip PCR analyzers and diverse microchips for important applied purposes. The original ideas implemented in the first microchip experimental devices for separate operations (fast PCR, fast electrophoresis, sample preparation) were step-by-step developed and implemented in the prototypes of real analyzers which combine all analytical functions in a single device.

All problems associated with surface modification of the most common materials for PCR microchips have already been practically solved. Various materials, including inexpensive polymers and metals have been tested, and their advantages have been demonstrated.

The growing number of publications concerning design, optimization, and application of new fully self-contained microchip analytical systems is evidence for the urgency of this task. The current trends are toward development of new cheap materials and fabrication technologies for PCR microchips, systems for non-contact thermal cycling and temperature measurement, further miniaturization of detection systems, and increasing the information content of such systems. Hybridization analysis, multiplex analysis, automated systems for sample injection into microchips, new methods of sample preparation in microchips, and microchips with immobilized reactants — this all reflect achievements in the field of microchip analytical systems.

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