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Sample Pretreatment and Nucleic Acid-Based Detection for Fast Diagnosis Utilizing Microfluidic Systems

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Abstract—Recently, micro-electro-mechanical-systems (MEMS) technology and micromachining techniques have enabled miniaturization of biomedical devices and systems. Not only do these techniques facilitate the development of miniaturized instrumentation for biomedical analysis, but they also open a new era for integration of microdevices for performing accurate and sensitive diagnostic assays. A so-called "micro-total-analysis-system", which integrates sample pretreatment, transport, reaction, and detection on a small chip in an automatic format, can be realized by combining functional microfluidic components manufactured by specific MEMS technologies. Among the promising applications using microfluidic technologies, nucleic acid-based detection has shown considerable potential recently. For instance, micro-polymerase chain reaction chips for rapid DNA amplification have attracted considerable interest. In addition, microfluidic devices for rapid sample pretreatment prior to nucleic acid-based detection have also achieved significant progress in the recent years. In this review paper, microfluidic systems for sample preparation, nucleic acid amplification and detection for fast diagnosis will be reviewed. These microfluidic devices and systems have several advantages over their large-scale counterparts, including lower sample/reagent consumption, lower power consumption, compact size, faster analysis, and lower per unit cost. The development of these microfluidic devices and systems may provide a revolutionary platform technology for fast sample pretreatment and accurate, sensitive diagnosis.

Keywords—MEMS, Microfluidics, Micro-TAS, PCR, Sample pretreatment, Nucleic acid.

ABBREVIATIONS

CCD	Charge-coupled device
CE	Capillary electrophoresis
CGE	Capillary gel electrophoresis
DEP	Dielectrophoretic
DNA	Deoxyribonucleic acid
DNase	Inhibit deoxyribonuclease
EOF	Electroosmotic flow
E. coli	*Escherichia coli*
HAD	Helicase-dependent amplification
LAMP	Loop-mediated isothermal amplification
LIF	Laser-induced fluorescence
LOC	Lab-on-a-chip
MEMS	Micro-electro-mechanical-systems
MGE	Microchip gel electrophoresis
Micro-TAS	Micro-total-analysis-systems
NASBA	Nucleic acid sequence-based amplification
OD	Optical density
ODEP	Optically induced dielectrophoresis
PCR	Polymerase chain reaction
QD	Quantum dot
Q-PCR	Quantitative PCR
RBC	Red blood cell
RCA	Rolling circle amplification
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase PCR
SDA	Strand displacement amplification
SMAP	SMart-amplification process
SMART	Signal-mediated amplification of RNA technology
SPIA	Single primer isothermal amplification
SPR	Surface plasmon resonance
TMA	Transcription-mediation amplification
WBC	White blood cell
cDNA	Complementary DNA
ssDNA	Single-stranded DNA
dsDNA	Double-stranded DNA
micro-TAS	Micro-total-analysis-system
mHDA	Mesophilic HDA
tHDA	Thermophilic HDA

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INTRODUCTION

Infectious diseases, especially emerging or re-emerging infectious agents, such as avian influenza, severe acute respiratory syndrome (SARS), and the human immunodeficiency virus (HIV), have become a global health concern recently. It is a challenge to any health authority or center for disease control (CDC) to make an accurate diagnosis within a short period of time to prevent the pandemic spread of an infection. Many molecular diagnostic platforms, which usually involve real-time polymerase chain reaction (PCR) or reverse transcriptase PCR (RT-PCR), have become popular and powerful methods to specifically detect and identify infectious microorganisms with a high accuracy. In addition, these molecular diagnosis methods can be also used for preventive medicine and the rapid diagnosis of genetic diseases, which have attracted substantial interest recently. People who have defective genes may be screened and pretreated in advance if they can be diagnosed early enough. As a result, there is a great need to develop a compact tool for the rapid detection of genetic mutation and genetic diseases.

Most microbial and animal cells share physical similarities. A cell mostly consists of a jelly-like cytoplasm (70–80% water in weight) in which the cell constituents are suspended. The remainder of the cell is composed of cell membranes or walls, proteins, lipids, and nucleic acids. Approximately 5–30% of the weight of a dry cell is attributed to nucleic acids, depending on the genome size, surrounding environment and the cell's current growth phase. The nucleic acids, including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), play an important role in terms of storing genetic information, and maintaining normal metabolism and growth. DNA/RNA extraction is a routine procedure that collects DNA/RNA from various types of organisms.[133] There are three basic steps involved in DNA/RNA extraction including (1) cell disruption, (2) removal of sub-cellular components, and (3) DNA/RNA extraction. In addition, purification and enrichment of a complex bio-sample at an extremely low concentration is crucial in many biomedical assays. These steps can efficiently increase the detection limit of the subsequent detection system. However, clinical samples are usually contained in a biological medium that would normally inhibit the subsequent DNA/RNA amplification. Therefore, the extraction of the target DNA or RNA from a complex bio-sample is usually inevitable in clinical practice for most existing nucleic acid-based detection systems. These existing DNA/RNA extraction processes are usually time-consuming and labor-intensive though. Therefore, there is a great need to develop microsystems to perform these processes in an automatic fashion. Recently, several sample pretreatment process performed in microfluidic chips have been developed.[12,48,124] This paper will review the recent progress in the development of these miniaturized devices and systems for sample pretreatment.

After extraction of target DNA or RNA, nucleic acid amplification techniques, including PCR, RT-PCR, and isothermal DNA amplification, are commonly used for genetic identification and disease diagnosis.[3,61] PCR is a common and often indispensable technique used in medical and biological research. It is a well-developed method for nucleic acid amplification in the fields of genetic identification and diagnosis.[92,115,116] Based on the proper selection of specific primers, the PCR technique can be used to perform nucleic acid amplification *in vitro*, resulting in the production of a large quantity of a target nucleic acid sequence. The specific primers are single-stranded DNA (ssDNA) molecules of about 20–30 nucleotides, which are specifically designed to flank the two ends of the target genome.[117] A specific segment of double-stranded DNA (dsDNA) can be replicated during a thermal cycling process involving three different temperatures for denaturation, annealing, and extension, respectively. These denaturation–annealing–extension steps comprise one complete reaction cycle, which can rapidly duplicate DNA segments,[103] and are crucial in the PCR process. Thus, PCR provides a sensitive and selective means of detecting low numbers of, or slow-growing, pathogens in clinical specimens, and hence has had considerable impact in the field of diagnostic microbiology. Table 1 shows a simplified process for molecular diagnosis. However, traditional PCR machines are usually bulky and relatively expensive. Besides, the PCR process is relatively time-consuming. Therefore, miniaturized devices for performing PCR processes rapidly are in crucial need.

Alternatively, isothermal methods can be used for DNA amplification instead of using three different temperatures in the PCR process. These methods include strand displacement amplification (SDA),[134] a Qb replicase system,[82] nucleic acid sequence-based amplification (NASBA),[83] transcription-mediation amplification (TMA),[119] signal-mediated amplification of RNA technology (SMART),[36] isothermal multiple displacement amplification (IMDA),[25] single primer isothermal amplification (SPIA),[58] rolling circle amplification (RCA),[20] loop-mediated isothermal amplification (LAMP),[91] SMart-amplification process (SMAP),[41] helicase-dependent amplification (HDA),[132] and circular helicase-dependent amplification (cHDA).[146] Similarly, these isothermal processes are usually performed in large-scale machines, which usually take a relatively long period of time for DNA amplification.

TABLE 1. Basic steps and methods in sample pretreatment, nucleic acid extraction and amplification, product measurement, and observation in a microfluidic system.

Part A. Tested sample pretreatment	Step I. Separation of cells, viruses, and bacteria from clinical samples	1. Hydrodynamic approach[46,114,143,147,151,152] 2. Microfiltering approach[21,50,122,131,140,144,148] 3. Acoustic approach[60,145] 4. Electrokinetic approach[17] 5. Dielectrophoretic approach[6,7,32,37,75,101,106] 6. Magnetic approach[68,78,113]
	Step II. Cell disruption/lysis	1. Chemical treatment[14] 2. Physical treatment: a. Thermal approach[66,112] b. Ultrasonic approach[89,108] c. Mechanical approach[53,128] d. Electrical approach[4,26,62,63,84,85,96,111,135] e. Optically induced dielectrophoretic approach[76]
Part B. Nucleic acid extraction and amplification	Step III. Remove cellular components	
	Step IV. Nucleic acid extraction	1. H-filter approach[39] 2. Dielectrophoretic force approach[109] 3. Magnetic beads a. Positive-charge approach[67] b. Nucleotide-probe approach[137,138]
	Step V. Nucleic acid amplification	1. Micro PCR chip a. Stationary PCR chips[27–30,34,59,71,88,90,97,107,123,125,142,150,157] b. Continuous-flow PCR chips[13,19,22,31,56,79,94,100,102,121,126,139,156] 2. Micro-isothermal amplification a. LAMP[98,137,138] b. RCA[5] c. HDA[132]
Part C. Product measurement and observation	Step VI. Product measurement and observation	1. Slab gel electrophoresis[10] 2. Capillary gel electrophoresis[44,57,155] 3. Fluorescence detection[15,51,65,69,70,81,95]

In the past decade, bio-MEMS technology has been used to miniaturize biomedical devices and systems.[16,49,110,120,158] A microchip fabricated using this technology is usually referred to as a ''lab-on-a-chip'' (LOC) or a micro-total-analysis-system (micro-TAS). The LOC or micro-TAS can perform the basic functions of sample preparation, mixing, reaction, transport, collection, separation, and detection on a single chip automatically. The advantages of the LOC or micro-TAS include smaller amounts of samples and reagents are required, and faster analysis with a higher sensitivity. Furthermore, the functionality and reliability of a LOC or micro-TAS could be significantly enhanced by the integration of other functional miniaturized components. Among these biomedical devices, PCR devices are one of the most extensively studied biological and chemical analytical devices and have been miniaturized using the MEMS technology for medical diagnostics, microbial detection and other bio-analysis applications. This review paper will summarize the recent progress in these miniaturized PCR or RT-PCR devices and systems. Furthermore, several approaches to perform isothermal DNA amplification in microfluidic systems have been reported recently. We will also review the progress in developing these miniature systems. Measurements of amplified DNA have been performed using several different microfluidic approaches, which will be also reviewed in the paper. The ultimate aim of a microfluidic system which has integrated sample pretreatment, nucleic acid amplification and on-line detection is to provide a compact platform for fast and accurate analysis of biological samples obtained from suspected aberrant tissues, body fluids, or nature environment.[45,54,86,87] In the following sections, traditional processes for sample preparation, nucleic acid amplification and detection schemes will be first reviewed. Then microfluidic devices and systems that can perform these corresponding processes will be assessed and discussed.

SEPARATION OF CELLS, VIRUSES, AND BACTERIA FROM CLINICAL SAMPLES IN MICROFLUIDIC SYSTEMS

Several approaches for the extraction of cells, viruses, and bacteria from clinical samples in microfluidic systems have been reported recently. Typical mechanisms

include hydrodynamic,[46,114,143,147,151,152] microfiltering,[21,50,122,131,140,144,148] acoustic,[60,145] electrokinetic,[17] dielectrophoretic,[6,7,32,37,75,101,106] and magnetic[68,78,113] approaches. These approaches are summarized in Table 1 and will be reviewed in the following sections.

Hydrodynamic Approach

Several methods using hydrodynamic forces to separate cells, viruses, or bacteria from clinical samples have been realized in microfluidic systems *via* the use of microflow cytometry,[151] pinched flow fraction,[147] a combination of pinched flow fraction and electroosmotic flow (EOF),[143] and a combination of gravity and flow fraction.[46] One of the most popular methods to hydrodynamically separate biosamples at a cellular level is to use flow cytometry.[151] For microfluidic-based flow cytometers, neighboring sheath flows with a higher velocity hydrodynamically squeeze a central flow containing fluorescence-labeled cells into a narrow stream. When test samples pass through an optical detection area, fluorescence emission due to laser excitation from the test samples are then detected and then the test samples are sorted and separated to the appropriate subsequent collectors.[151,152] With this hydrodynamic approach, cells, viruses, and bacteria can be separated for many subsequent applications.

Recently, simple hydrodynamic methods using the characteristics of laminar flow have been also reported for cell sample separation.[114,143,147] For instance, based on the concept of a pinched flow fraction for continuous sample separation, red blood cells (RBCs) were successfully separated from human whole blood utilizing microfluidic devices.[114,147] In this approach, the cell alignment method was similar to the technique used in flow-field flow fractionation, in the sense that RBCs were forced into uniform positions by another liquid flow. Then, at the boundary between the pinched and the following expanding microchannel segments, a force toward the center of the microchannel was exerted by the expanding flow profile, whereas a force toward the sidewall was exerted mainly on the RBCs. Since this method utilizes only the laminar flow profile inside a microchannel, complicated flow control mechanisms could be eliminated, which is usually required for other types of particle separation methods such as field flow fractionation. Also, this method can be applied both for particle size analysis and for preparation of monodispersed particles, since separation can be rapidly and continuously performed.

In addition, a microfluidic device using a combination of an EOF and a pinched flow for separation of *Escherichia coli* (*E. coli*) and yeast cells was also demonstrated.[143] It operates in a similar manner to the hydrodynamic spreading mechanism described above. With the same mechanical pressure control, a sample flow containing various particles and a carrier flow without particles converge in a narrow channel. In this channel, where the flow is laminar, the carrier flow occupies most of the channel width. When an electric potential is applied on the carrier flow, the dynamic behavior of the fluids changes with the magnitude of the applied voltage. Electroosmosis occurs when there is an electric double layer at the solid–liquid interface and it arises from the electrostatic attraction between the surface charge of the solid channel wall and the ions in the fluid. When an external electric field is applied along the length of the channel, the mobile positive ions in the diffusion layer move towards the cathode. The ions will couple to, and hence induce a dragging force on the bulk fluid, resulting in a bulk fluid movement along the channel. Generally, the pressure attainable with the EOF is weaker than that with a pressure-driven flow. Therefore, the EOF usually has a very limited effect on a pressure-driven flow. However, when the magnitudes of the two such generated forces are similar, this effect becomes significant. As a result, the hydrodynamic spreading of fluids can be tuned arbitrarily with an adjustable power supply. With this approach, separation of *E. coli* and yeast cells has been demonstrated successfully.

Alternatively, a microfluidic sorter utilizing a combination of gravity and hydrodynamic forces was demonstrated for continuous mass-dependent separation of samples and cells.[46] Gravity was used to drive fluid flows through microchannels as well as to induce differential sedimentation of particles according to their masses. The difference in positions between particles initiated by sedimentation was further amplified hydrodynamically by laminar flows with expanding flow streamlines, facilitating direct monitoring and isolation of fractionated subpopulations within short distances.

Microfiltering Approach

In addition, sample separation can be also performed by hydrodynamic filtration.[21,148] Even though these devices can be used for sample separation, they require precise fluid control to attain higher separation efficiencies. In order to solve this problem, microfilters with different geometries have been integrated into the microchannels.[140] A weir-type filter, in which a narrow gap between a silicon dam and a glass top acted as the filter, proved to be effective for cell separation. Another study also reported that leukocytes could be isolated from blood by using a microfluidic diffusive filter.[122] Similarly, a microfilter separator for isolating white blood cells (WBCs) from human whole blood was designed using a cross-flow method in an array of microchannels.[131] Another silicon-based, cross-flow

microfilter for WBCs separation was also presented.[50] An average separation efficiency of 70–80% for trapping WBCs can be achieved. Furthermore, a new microfluidic device utilizing a combination of T-junction focusing and tilted louver-like structures was demonstrated for bead and stem cell separation.[144] This two-step separation process was performed with a high separation efficiency of 97.1%.

Acoustic Approach

Acoustic forces generated from ultrasonic waves can be also used to sort cells.[60] An acoustic standing wave causes a band or multiple bands of particles to form according to the positions of the nodes or antinodes in the flow-through resonator. By using proper flow balancing of the outlets and the spatial positioning of the standing wave, a band of particles can be directed into a selected channel while a cleared medium is collected in other channels. This mode of operation represents an attractive way of implementing acoustically controlled continuous flow separators. Fractionation of suspended particles in a standing wave field utilizing the fact that particles with dissimilar sizes or physical properties are affected differently by acoustic radiation has also been reported. Larger particles will, for example, experience a larger acoustic radiation force than smaller ones, which means that they will move faster towards a node or an antinode than the smaller ones. By using flow splitters at the outlet and balanced flows, it is possible to separate larger particles from the smaller ones since they have travelled different distances. In addition, bio-particles can also be concentrated by using an asymmetric surface acoustic wave method.[145] However, such systems usually require complicated fabrication processes or costly equipment. Furthermore, cell samples may be damaged when applying external forces.

Electrical Approach

Cells can be also electrokinetically sorted and separated. For example, a digital image projection (DIP) technique using optical tweezers capable of catching and switching the target cells under a microfluidic configuration has been reported to electrokinetically sort and to separate beads or cells.[17] Besides, a variety of sorting devices using dielectrophoretic (DEP) forces were reported to be promising for cell separation.[32,37,106] When an inhomogeneous electric field exists, polarized cells will move due to the induced dipole. Obstacles such as ridges and wedges embedded in the microchannel can be used to generate the inhomogeneous electric field. However, these obstacles may result in serious clogging of the channel. Recently, a microfluidic device was presented using a virtual, projected pillar array to induce a negative DEP force, thus eliminating the clogging issue.[6,7]

Alternatively, an optically induced dielectrophoresis (ODEP) device can be used to generate various types of virtual microelectrodes to manipulate beads and cells.[101] With this approach, there is no need to fabricate physical microelectrodes and beads or cells can be manipulated and separated. For example, an optically induced flow cytometer for continuous counting and sorting of beads and cells using ODEP has been reported by our group recently.[75]

Magnetic Approach

Alternatively, a micromachined magnetic separator for cell sorting in microfluidic systems has been presented.[113] Its flexible design utilized fully integrated electromagnetic inductors that were placed underneath the chip. Before excitation, the magnetic bead solution flows from the inlet through the microchannel and is evenly distributed between outlets. When a driving signal is applied to one of the pairs of the inductors, the magnetic beads will be sorted to a specific channel due to the magnetic forces exerted on the magnetic beads. However, the flow rate of the beads has a large effect on the separation efficiency. Furthermore, heating issues caused by the current driving scheme of the device should be considered. Similarly, a microdevice using an inhomogeneous magnetic field perpendicular to the direction of the flow such that beads/cells of different sizes can be separated was also reported.[78] Similarly, living *E. coli* bacteria bound onto magnetic nanoparticles can be separated in a continuous laminar flow by applying a local magnetic field gradient.[68] Briefly, magnetic beads coated with antibodies specific to surface markers of the cells, viruses, or bacteria have been one of the popular approaches to separate biosamples. This technique have been extensively used in microfluidic devices for fast diagnosis of dengue virus,[72] influenza virus,[74] cancer cells,[73] and methicillin-resistant *Staphylococcus aureus* (MRSA).[137]

ON-LINE CELL LYSIS USING MICROFLUIDIC SYSTEMS

Cell lysis is a basic and crucial technique to extract proteins and nucleic acids for a variety of research applications. There are several methods reported in the literature recently using microfluidic systems to lyse cells including chemical,[14] thermal,[66] laser,[112] ultrasonic,[89,108] mechanical,[53,128] electric,[4,62,63,84,85,111,135] electrochemical,[26,96] and ODEP approaches.[76]

Chemical Treatment

For instance, cells can be easily lysed by using chemical reagents such as detergents. Although the cell lysis process using chemical reagents is simple and straightforward, it cannot lyse a specific cell within a group of cells, which is necessary for single cell study.[14]

Physical Treatment

Thermal and Pressure Wave Approaches

A microfluidic thermal reactor can be easily used for cell lysis by simply heating up the cells to a high temperature (about 100 °C).[66] In addition, cells can also be lysed by using both laser-induced[112] and ultra sound-induced[89,108] pressure waves. Microchannels or microchambers can be easily fabricated and then cell lysis can be performed by using connected large-scale apparatus to generate required pressure waves such that cell lysis can be achieved. Nonetheless, it may be difficult to control the cell lysis process since this involves precise control of the pressure wave propagation. Alternatively, mechanical forces can also be used to disrupt the cells. For example, the interaction between beads and cells were used to disrupt the cells in a compact disc chamber.[53] Two flat plates were also used to squeeze the cells, thus generating the cell lysis.[128] In addition, the pressure wave generated by collapsing bubbles is another method to lyse cells.

Electrical Approach

Another popular method for cell lysis is to apply an electric field on the cells. Figure 1a is a schematic illustration showing the working principle of the cell lysis chip. The lipid bi-layer of a cell membrane is known to have a dielectric property. When it is exposed to an electric field, a trans-membrane potential, $\Delta\varphi$, is then induced. The trans-membrane potential can be regarded as opposite charges induced on the inner and outer membranes. This attraction of opposite charges causes a compressive pressure on the cell membrane which makes it thinner and permeable.[33] The electroporation of the cell membrane can be reversible or irreversible depending on the strength of the external electric field. When the trans-membrane potential is higher than about 1 Volt (V),[63] it can cause irreversible electroporation to the cell membrane and thus disrupt the cell.

Recently, microfluidic technologies have enabled a variety of biomedical applications, especially for cell-based assays. Compared with their large-scale counterparts, these microfluidic devices are compact in size and may be more efficient for performing cell-based assays. More importantly, these microfluidic devices allow for automation of the entire cell lysis process. For instance, in-plane micro-electrodes were fabricated on a microfluidic chip for electrical cell lysis.[63,111] In order to improve the cell lysis rate and to reduce heat and bubble generation, three-dimensional micro-electrodes were also reported.[84,85] The specific geometry of the microchannel was also reported to provide a focused electric field to disrupt the cells.[62,135] Bacteria cells can also be disrupted by using a similar method.[4] In addition, an electrochemical approach has been demonstrated on a chip for cell lysis. For instance, on-chip cell lysis based on local hydroxide electro-generation was also reported.[26,96] Hydroxide ions can porate the cell membranes, thus causing cell lysis.

Optically Induced Cell Lysis Approach

Even though these microfluidic devices can be used for cell lysis, it is still challenging to lyse a specific cell within a group of cells. Besides, it is also difficult to only disrupt the cell membrane without damaging the nucleus, which is especially useful in studies of mitochondria. Therefore, an optically induced cell lysis device was reported to selectively disrupt a specific cell or only lyse the cell membrane without damaging the nucleus.[76] In addition, two types of human cells, including a fibroblast cell and an oral cancer cell, were used to demonstrate the capabilities of the developed device. The operating parameters including the beam spot diameter and the illumination power density were investigated to explore the cell lysis rate.

RNA/DNA EXTRACTION IN MICROFLUIDIC SYSTEMS

Recently, several microfluidic devices have been reported for RNA/DNA extraction, which play an important role for molecular diagnosis.[24] Two approaches including DEP force[109] and magnetic beads[1,38,67,137,138,153] have been demonstrated in microfluidic systems for RNA/DNA extraction.

Alternatively, DEP forces has been demonstrated for DNA extraction.[109] DEP induces the movement of a polar or polarizable object in the direction of the electric field gradient. DEP trapping is the trapping of such objects in high electric field regions, and a charged polymer like DNA has a strong length-dependent dielectric response at extremely low frequencies. Therefore, by using shaped insulating barriers, very high electric field gradients can be created to selectively trap the chromatin from a lysed cell. The idea for DNA purification is to trap the chromosome with an alternating current (AC) electric field while applying a small direct current (DC) electric field to remove the

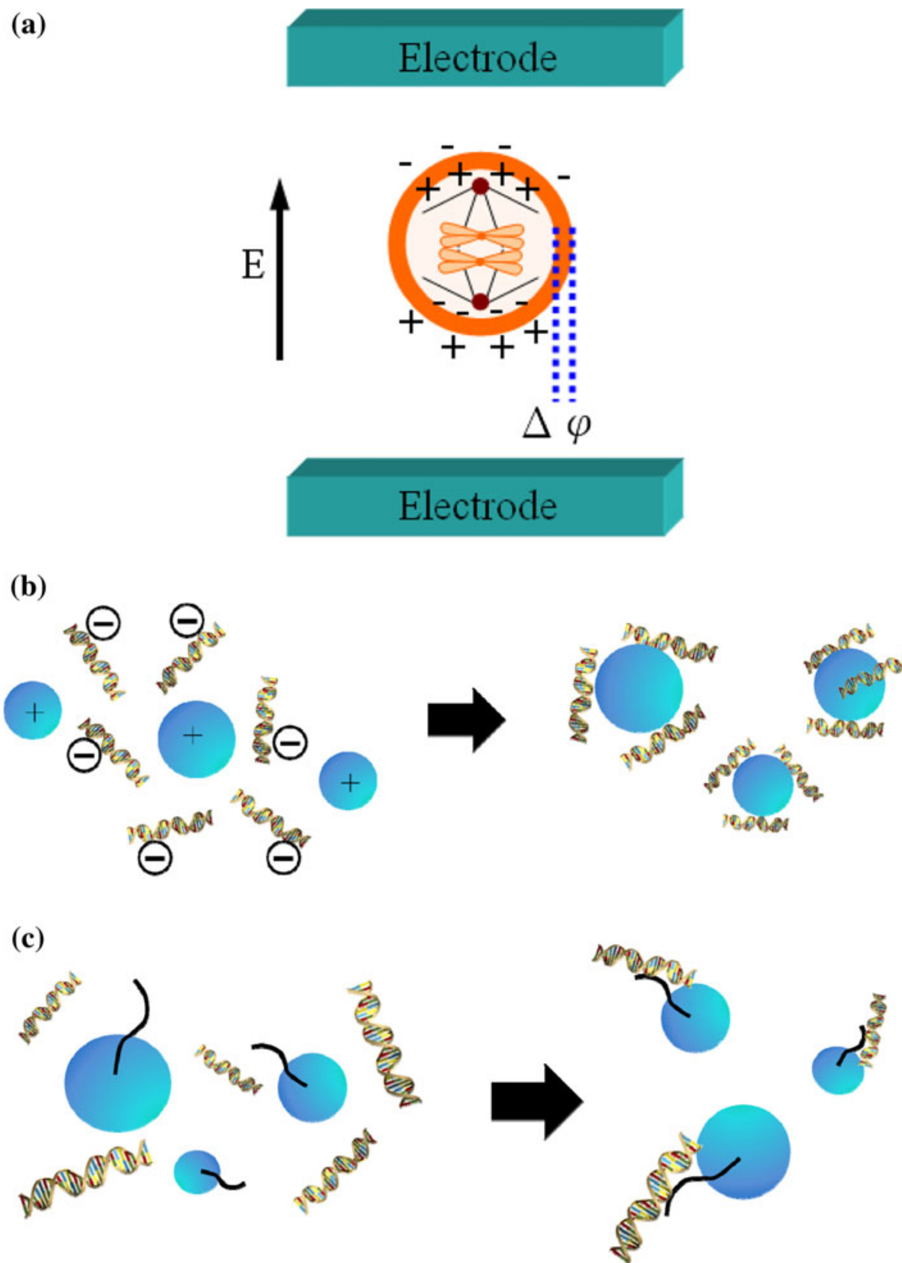

FIGURE 1. (a) The operating principle of cell lysis by applying an electric field. When a cell is exposed to a high electric field, the trans-membrane potential (opposite charges on the inner and outer membranes) can be induced. When the induced trans-membrane potential is higher than a certain value (about 1 V), it may cause the cells to be disrupted. After the cell lysis process, the magnetic beads either with (b) positive charges or (c) nucleotides probes are used in the microfluidic system to perform the nucleic acid extraction for molecular diagnosis.

smaller components of the lysate such as proteins, RNA and membrane fragments.

The most popular method for RNA/DNA extraction in microfluidic systems is using magnetic beads. Magnetic beads, either with positive charges[67] or nucleotide probes[137,138] have been used for RNA/DNA extraction, as schematically shown in Figs. 1b and 1c. Typically, large-scale electromagnets or permanent magnets were used to collect the magnetic beads so that the bound DNA or RNA can be extracted. A similar method for extracting genomic DNA materials inside living cells utilizing functionalized magnetic particles was demonstrated.[153] When incorporated with microfluidic technology, the magnetic beads can be collected from a dilute solution near a large-scale rare-earth magnet to form a packed bed within a microfluidic channel.[38] More importantly, the magnetic beads can be separated and manipulated by

using meandering-type inductors (microcoils) inside microchannels. With this approach, a compact system to perform magnetic bead manipulation and collection can be realized, and DNA extraction as well.[1]

NUCLEIC ACID AMPLIFICATION

The nucleic acid techniques (NAT) that enable the amplification of a few target molecules have provided new tools for specific and sensitive detection. The commercially available nucleic acid amplification technique that is most widely used in molecular diagnostics is PCR. Furthermore, several isothermal nucleic acid amplification techniques have been reported. Recent advancements in microfluidic-based PCR and isothermal amplification processes for nucleic acids will be reviewed in this section.

Micro-PCR Chip

Several approaches to realize PCR and isothermal amplification of DNA or RNA have been demonstrated on microfluidic systems recently. For instance, miniature PCR systems using MEMS technology have attracted considerable interest.[3,52,115,118] Typically, micro-PCR chips are classified into two major categories: microstationary PCR chips[27–30,34,59,71,88,90,97,107,123,125,142,150,157] and continuous-flow PCR chips.[13,19,22,31,56,79,94,100,102,121,126,139,156]

Microstationary PCR Chips

By modulating the thermal cycling program (including cycle numbers and reaction temperatures), DNA amplification can be performed in microstationary PCR chips, similar to the manner in which conventional PCR instruments work. Due to the relatively small dimensions of the microstationary PCR chip, it can perform faster thermal cycling as compared with its conventional large-scale counterparts, with a heating rate of 10–30 °C/s and a cooling rate of 2.5–4 °C/s.

One of the critical issues associated with low duplication efficiency for DNA is an appreciable non-uniformity of the temperature field inside the PCR chamber. Therefore, challenges remain in maintaining a uniform temperature distribution inside a micro-PCR chamber and avoiding cross-talk issues for neighboring microheaters if multiple reaction chambers are required.[59,64] An M-shape microheater was thus fabricated inside a micro-PCR chamber to increase the temperature uniformity.[64] In addition, microthermal cyclers with different microheater patterns such as blocks,[71,79] serpentine shapes,[23,107,127,149,154] or fence-like forms[28] have been reported in the literature to increase the temperature uniformity.

Silicon-based thermal cyclers are typically used for micro-PCR applications. Not only do they provide high heating and cooling rates, but they can also be easily fabricated using compatible micromachining techniques. Furthermore, glass-based thermal cyclers are commonly used for micro-PCR applications. In order to precisely control the operating temperature during the DNA amplification process, a microthermal cycler is used, which typically consists of microheaters and a built-in temperature sensor. The microheaters are used to precisely heat up a specific area inside the reaction chamber without the need for external heating equipment. The temperature sensor is then used to detect the temperature inside the reaction area and to feed back a precise signal to the microheaters.

The edge regions inside the PCR chamber exhibit a significant temperature gradient caused by the ambient environment, which is at a lower temperature. Thus, edge heaters or suspended structures have been used to improve the thermal uniformity of the PCR chip.[95,159] Recently, a new design of array-type microheater with active compensation units was used to enhance the thermal uniformity in the reaction region of the PCR chip.[42,43] The new microthermal cycler was composed of main heaters and active compensation heaters, as schematically shown in Fig. 2.

Microcontinuous-Flow/Flow-Through PCR Chips

In addition to stationary PCR chambers, the development of continuous-flow (also referred to as flow-through) micro-PCR devices has also attracted considerable interest recently. Typically, nucleic acid amplification has been achieved by driving the mixture of DNA templates and PCR reagents to continuously flow through a capillary or a microchannel that incorporates three thermally isolated reaction zones. With this approach, the time needed for the heating and cooling of samples can be greatly reduced. For example, a continuous-flow PCR chip using three sets of oil-baths and thermostats with different temperatures for annealing, extension and denaturing has been reported.[94] DNA samples flowing through a capillary tube were successfully amplified rapidly. Similarly, DNA samples were rapidly amplified by flowing them through a serpentine-shape microchannel on three copper blocks with different temperatures set for PCR thermal cycling.[56]

Furthermore, different forces including hydrodynamic,[22,56,94,100,102,121,126] magnetohydrodynamic,[139] electrokinetic,[13] dielectrophoresis,[31] and pneumatic[19,79] forces have been used to continuously drive samples through the microchannel for continuous

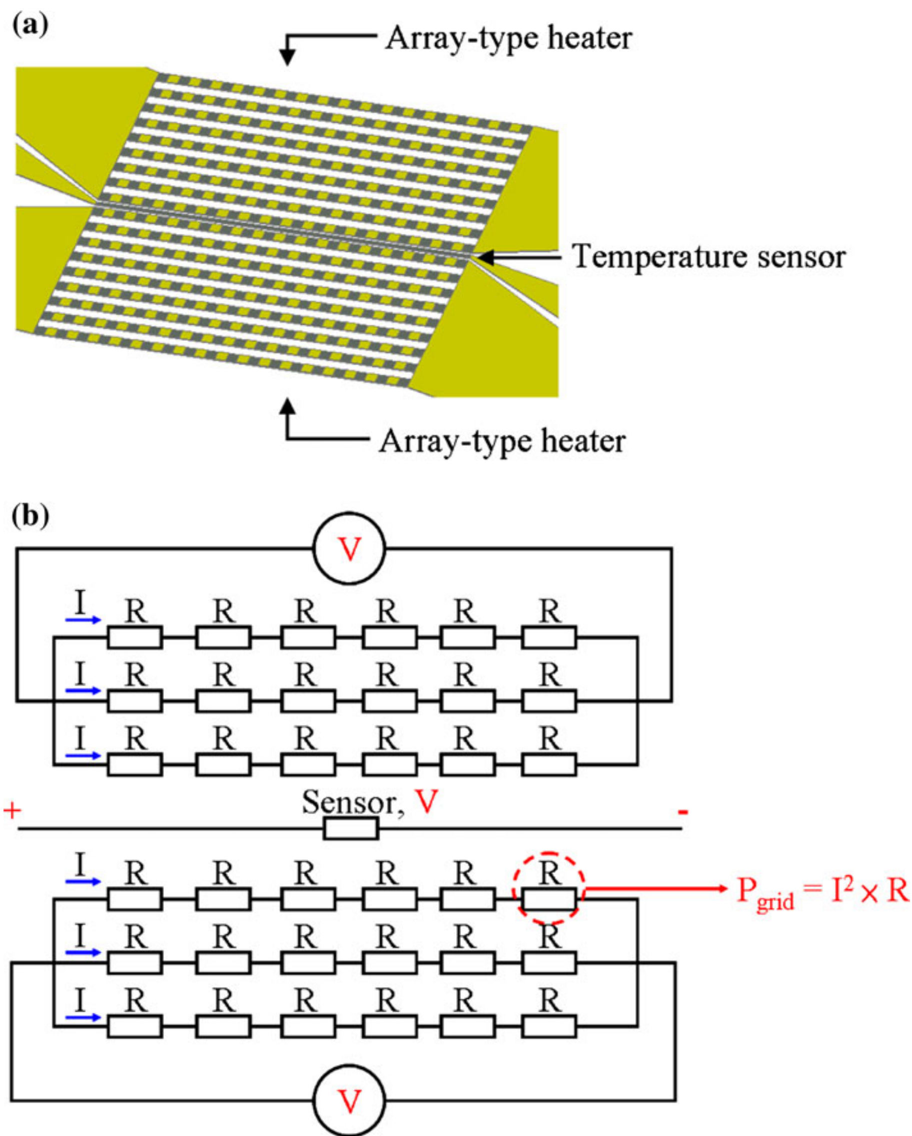

FIGURE 2. Concepts and equivalent circuit of array-type heaters. (a) The grid pattern of array-type thermocycler is the heating power of each array-type heating grid. (b) Equivalent circuit for the grid array pattern. *I* is current, *V* is supplying voltage, and *R* is resistance of the original heating grid.

PCR applications. For instance, DNA amplification utilizing a continuous-flow PCR chip integrated with a rotary pneumatically driven micropump was reported.[79] In this ingenious work, a flow-through rotary PCR chip was designed in which samples were continuously driven at a fixed flow rate along an annular channel using a pneumatically driven micropump through three heating regions. As the dimensions of the three heating regions were fixed, the time ratio for the three thermal processes remained constant, accordingly.

Even though successful DNA amplification has been performed using continuous-flow PCR devices, they still suffer from several drawbacks. For example, the layout of the capillary usually determines the duration of the annealing, extension, and denaturing processes, resulting in a constant time ratio. It may be difficult to optimize the PCR process if the time ratios of the three temperature processes need to be adjusted. In order to solve this problem, a serpentine channel that had controlled outlets at different fractions of a complete cycle number, for controlling selection of the desired cycle ratios, has been used for continuous PCR applications.[100] Furthermore, large-scale syringe pumps or external power supplies are usually required for hydrodynamic-driven or electrokinetically driven flow-through PCR systems. Moreover, the size of flow-through PCR chips is relatively larger than that of

stationary PCR chips due to the layout area of the serpentine-shape channel or the capillary tube.

In addition to the above mentioned two types of micro-PCR chips, the use of convective PCR chips has also been reported recently.[130] The convective flow generated in a cavity with the top and bottom surfaces maintained at the annealing–extension and denaturation temperatures was used to carry out a two-temperature PCR process. However, the efficiency of the convective PCR chip may not be as high as that of the other two types of PCR chips.

Recently, microcontinuous PCR chips integrated with other microfluidic control devices have been extensively investigated.[9,11,34,40,47,51,77,90,93] For instance, micromachined flow-through PCR chips consisted of a microfluidic control module and a temperature control module have been demonstrated for fast DNA amplification. The microfluidic control module, which used two different motions of multiple membranes[136] and suction-type membranes[18] for flow transport, as shown in Fig. 3, was used to rapidly transport the DNA samples through the three heating sections. The microfluidic control module was used to allow adjustment of the cycle numbers and resident times of the sample in the three temperature control zones in which the PCR thermal cycles were performed. Three individual array-type heating[42,43] and temperature-sensing sections were integrated to modulate the specific temperature field for the three thermal steps of a PCR process.

Isothermal Amplification in Microfluidic Systems

An ideal microfluidic device for isothermal amplification should have (1) a high operating (incubation) temperature, as this reduces the incidence of non-specific target amplification; and (2) a low number of enzymes, since multiple enzymes in the reaction will increase the probability of adsorption of these enzymes onto the surface of the microchip and disrupt the desired coordinated catalytic activity of the enzymes. Recently, three new isothermal DNA amplification methods including LAMP,[98,137,138] RCA,[5] and HDA[132] have been extensively explored. The LAMP technology uses four primers that recognize six distinct sequences of the target DNA to generate 10^9 copies in less than 60 min.[98] Although LAMP is highly sensitive, the design of the primers is more challenging and requires dedicated software (PrimerExplorer V4).

Another isothermal DNA amplification method, RCA, which is similar to LAMP, was also reported recently.[5] A glass coverslip with the primer array was

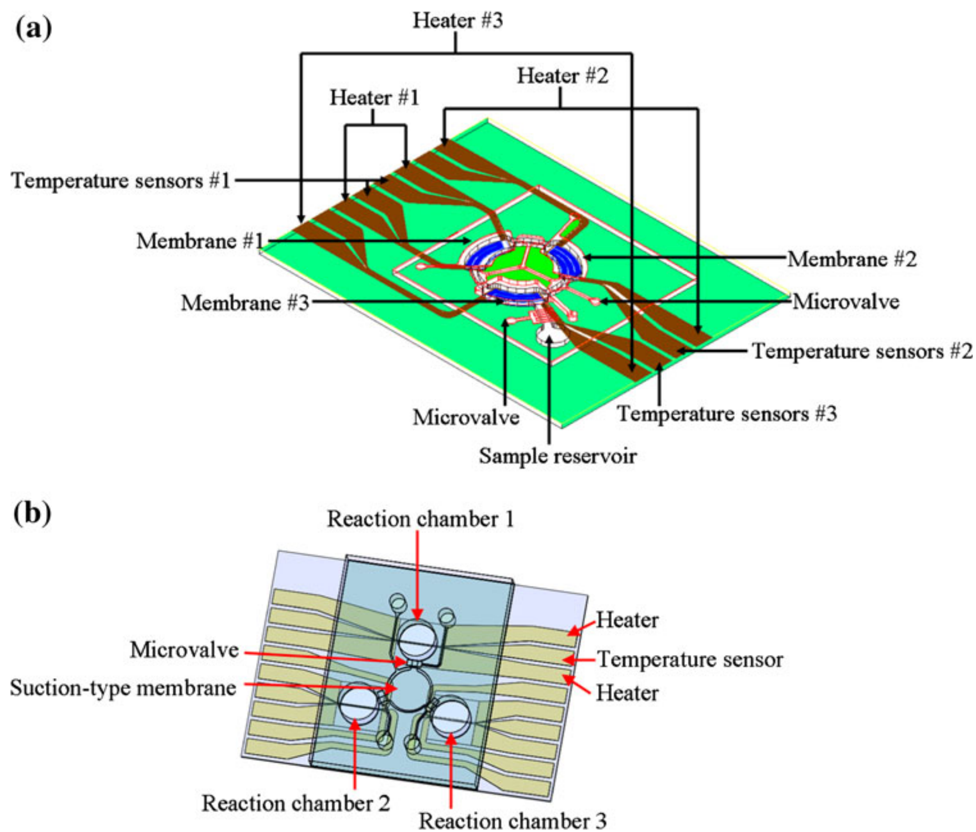

FIGURE 3. Schematic illustration of the flow-through PCR chip with multiple membrane activation (Wang *et al.*,[136] Copyright 2007 IOP Science) and suction-type membrane activation (Chien *et al.*,[18] Copyright 2009 Springer).

assembled into a microfluidic chip and DNA polymer brushes were synthesized on the oligonucleotide array by rolling-circle DNA amplification. Similarly, mesophilic HDA (mHDA), a true isothermal DNA amplification technology, which does not require initial denaturation of the dsDNA was reported.[132] Subsequently, a thermophilic HDA (tHDA) platform was reported.[2] Although SDA, NASBA, TMA, LAMP, and tHDA have been successfully commercialized, uses of these methods in conventional instruments are limited by their capability to analyze multiple genes in parallel. Furthermore, these methods are usually performed using large-scale apparatus. Hence, it is desirable to develop microfluidic devices as a high-throughput platform for isothermal nucleic acid amplification methods.

DNA MEASUREMENT IN MICROFLUIDIC SYSTEMS

There are two techniques including slab gel electrophoresis[10] and capillary gel electrophoresis,[44,57,155] and fluorescence detection[15,51,65,69,70,81,95] that have been frequently reported in the literature to measure amplified DNA products in microfluidic systems. This section will briefly review the advancements in these techniques.

Slab Gel and Capillary Gel Electrophoresis in Microfluidic Systems

Slab gel electrophoresis is one of the most popular methods used for the analysis and detection of PCR products. Traditionally, these PCR products are transported to the electrophoresis gels for electrokinetic separation by using pipettes and then detected using a bulky imaging system. To avoid the risk of sample contamination during transportation of samples/reagents and to reduce the size of the imaging system, a compact system comprising of a liquid injector, a mixer, heaters, temperature sensors, electrophoresis gel channel and electrodes, as well as photodetectors for fast amplification, analysis, and detection of PCR products was reported in a single chip.[10] This device demonstrates the capability of detecting DNA samples (detection limit about 10 ng/μL) in a polyacrylamide gel within a distance of 0.5–3 mm.

Although slab gel electrophoresis is commonly used for the analysis and detection of PCR products, this technique is time-consuming, labor-intensive, and produces only semi-quantitative results. High electric fields cannot be applied due to Joule heating effects and hence hinders the possibility for reducing the time required for analysis. In order to improve the separation efficiency, miniaturized formats for slab gel electrophoresis were developed, which are now well known as capillary gel electrophoresis (CGE) and microchip gel electrophoresis (MGE). They are alternatives to conventional slab gel electrophoresis for fast DNA separation and detection due to their excellent separation performance. Moreover, a laser-induced fluorescence (LIF) system is commonly applied in these PCR–CGE or PCR–MGE systems due to its superior sensitivity and capability for quantification of the results.[155] However, a traditional LIF system comprising a laser source, a photodetector, filters, and mounting components is relatively bulky and somewhat mitigates the advantages of these miniature microdevices. Therefore, combing a PCR device and a CGE microchip as well as the detection system with integrated on-line detection in a PCR–CGE system is attractive for practical DNA/PCR products analysis.[57]

In order to realized this system, an integrated microfluidic chip capable of performing DNA/RNA amplification, separation, and on-line optical detection in an automatic mode was presented, as shown in Fig. 4.[44] In this system, DNA/RNA samples were first amplified in a micro-PCR or RT-PCR module and then transported to a sample reservoir through an on-chip pneumatic micropump followed by being electrokinetically driven into a microchannel for separation and finally optically detected by a buried optical fiber. However, when amplified DNA samples and reagents were transported to capillary electrophoresis (CE) reservoirs by using electrokinetic forces, they will move at different speeds due to their different charge-to-mass ratios. This may affect the subsequent injection and separation process. The developed design overcomes this problem by using a series of pneumatic micropumps to manipulate the DNA/RNA samples prior to the electrophoretic separation process, and then uses conventional electrokinetic forces to perform sample injection and separation once the DNA/RNA samples have been amplified. Additionally, the high temperature field ($>$95 °C) in the PCR chamber can cause a drying of the CE buffer, and hence can affect the injection of the amplified DNA samples into the CE channel. Therefore, the pneumatic micropumps also provide a micro-valving function such that the PCR chambers and the CE buffer reservoir can be effectively isolated.

Fluorescence Detection for DNA Analysis in Microfluidic Systems

Two common methods are used for the quantification of DNA samples *via* fluorescence detection. The first one is the use of fluorescent dyes to intercalate

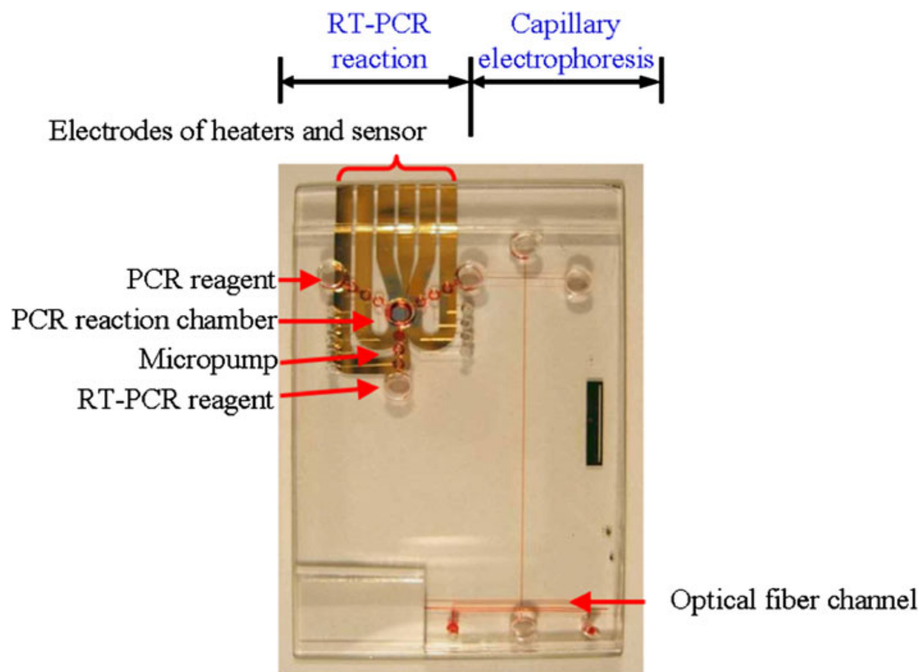

FIGURE 4. A photograph of the microfluidic chips capable of DNA/RNA amplification, electrophoretic injection and separation and on-line detection of DNA samples. First, the integrated microfluidic chip can perform two-step RT-PCR using pneumatic micropumps to transport RT-PCR/PCR reagents (reservoirs 2 and 3) to the PCR chamber (reservoir 1). The RT-PCR chamber performs the reverse transcription reaction on mRNA. Precise amounts of RNA reagents/templates can be first transported to the neighboring ''PCR chamber'' from the ''RT-PCR reagent chamber'' for reverse transcription of RNA templates using pneumatic micropumps. After synthesis, complimentary DNA (cDNA) samples can be further amplified after pumping PCR reagents from the neighboring ''PCR reagent chamber''. The two-step RT-PCR amplification process provides a more reliable method for genetic identification. If only DNA samples are to be amplified, then the reverse transcription process can be omitted. Secondly, these pneumatic micropumps could also act as microvalves such that PCR reagents and CE buffers could be properly separated (Huang *et al.*,[44] Copyright 2006 Wiley-Blackwell).

with dsDNA.[15,65,69,95] A DNA-binding dye intercalates with amplified dsDNA in a PCR to emit fluorescence. As the amount of the DNA product increases over each thermal cycle, the measured fluorescence intensity also increases, and hence the DNA concentration can be simultaneously quantified. Another method is the use of modified DNA oligonucleotide probes which fluoresce upon hybridization with complementary DNA.[51,70,81] The amplified DNA products containing a sequence-specific DNA-based probe can be specifically increased and quantified even in the presence of non-specific DNA amplification. Compared with the detection of traditional PCR products by gel electrophoresis, this real-time fluorescence detection method is highly sensitive and therefore has attracted considerable interest.

For a multiplexed assay capable of detecting several genes, the use of specific probes with different-colored labels provides a similar amplification efficiency in the same reaction for all genes. In order to detect different fluorescence wavelengths emitted from the different amplified target DNA, commercial real-time PCR machines with discrete channels of photodiodes, and a corresponding set of specific narrow-band filters and dichromatic mirrors were used to allow fluorescence with the desired wavelength to reach the respective channel. Biochips[65,126] and a multi-channel fluorescence detection method[141] have been proposed to realize the capability of multiplexed real-time PCR. To increase the fluorescence collection efficiency, one fluorescence input towards the corresponding photodiode detector can be separated into many optical wavelengths utilizing a photo multiplexer tube, and the fluorescence collection efficiency can exceed 70% for each optical channel using this sequential optical separation technique.[55,80,129]

Another method to measure the fluorescence is to utilize a spectrometer with continuous spectral dispersion and a linear charge-coupled-device (CCD) array detector during thermal cycling in a real-time PCR machine.[65] However, a spectrometer with a concave holographic grating can only normally achieve a 25–35% collection efficiency for fluorescence wavelengths ranging from 300 to 800 nm, and the linear CCD array detector has the highest sensitivity for the spectral distribution corresponding to fluorescence only at −70 °C; thus, the whole system including the cryogenic cooler is relative bulky and expensive for practical applications. Table 2 shows a

TABLE 2. Comparison between a commercial Q-PCR machine and a MEMS-based Q-PCR system integrated with a fluorescence detection scheme utilizing a linear CCD and spectrometer[65].

	Fluorimeter	Roche equipment
Detector type	Linear CCD+ spectrometer	Photodiode
Detection wavelength	400–800 nm continuous	530, 640, 710 nm discrete
Sensitivity@ 530 nm, 10 μL sample volume	1 femto mol fluorescein	10 femto mol fluorescein
Resolution	24 bit	12 bit
Range of detection sensitivity	1–10,000	10–1000

comparison between a commercial quantitative PCR (Q-PCR) machine and the fluorescence detection utilizing a linear CCD and spectrometer. Recently, miniaturized systems with both integrated fluorescence detection and on-chip heaters/sensors have been reported.[8,99,105] PCR enables the detection of trace levels of DNA/RNA after a large number of amplification cycles. The quantitative applications of this method, however, may suffer from complications due to background amplification and variances in amplification efficiency.[35,104] Hence, a great deal of effort is still needed to enhance the sensitivity and speed of nucleic acid detection and quantification, while at the same time, help to validate the quantitative reliability of the available techniques. The development of lab-on-a-chip PCR systems has been considered a promising approach to overcome the difficulties associated with the clinical utilization of PCR assays.

CONCLUSIONS

Many molecular diagnostic platforms based on PCR or RT-PCR in microfluidic systems have been developed, which are powerful methods to specifically detect infectious microorganisms with a high sensitivity. MEMS technology and micromachining techniques have enabled the miniaturization of biomedical and chemical analysis devices and systems. The combination of these two technologies has enabled the realization of a micro-TAS. Various fluidic operations in microfluidic systems, such as sample preparation, sample injection, sample transport, filtration, reaction, separation, and detection, have been successfully demonstrated. The advantage of using microfluidic systems as a diagnostic platform offers many advantages including a smaller sample/reagent requirement, a shorter analysis time, a lower per unit cost, disposability and automation.

Biological samples usually contain a mixture of bioactive substances that may interfere with the subsequent DNA/RNA amplification. Therefore, the purification and enrichment of a complex bio-sample, especially those with extremely low initial concentrations of targets become crucial in many biomedical assays. The sample pretreatment process can efficiently increase the detection limit of the sensing system and is crucial for the success of the entire nucleic acid-based detection method. Miniaturized devices for sample pretreatment have shown great potential for reduction of the required sample volume and processing time, as well.

The nucleic acid amplification process can be performed in a micro-PCR or micro-RT-PCR chip. The miniaturized PCR or RT-PCR chip has significantly shortened the time required for the nucleic acid amplification process. It also requires less sample and reagent volumes and exhibits a higher amplification efficiency. Therefore, it is extremely conducive for fast diagnosis. Finally, it is envisioned that an integrated system, including sample pretreatment, PCR or RT-PCR, and on-line detection, can perform the entire process for molecular diagnosis and can become a powerful platform for the detection of viruses, bacteria, or other microorganisms.

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