Microfluid Nanofluid (2014) 16:965–987
DOI 10.1007/s10404-014-1386-y

RESEARCH PAPER

EWOD microfluidic systems for biomedical applications

Hsien-Hua Shen · Shih-Kang Fan · Chang-Jin Kim · Da-Jeng Yao

Received: 24 December 2013 / Accepted: 18 March 2014 / Published online: 30 March 2014

Abstract As the technology advances, a growing number of biomedical microelectromechanical systems (bio-MEMS) research involves development of lab-on-a-chip devices and micrototal analysis systems. For example, a portable instrument capable of biomedical analyses (e.g., blood sample analysis) and immediate recording, whether the patients are in the hospital or home, would be a considerable benefit to human health with an excellent commercial viability. Digital microfluidic (DMF) system based on the electrowetting-on-dielectric (EWOD) mechanism is an especially promising candidate for such point-of-care systems. The EWOD-based DMF system processes droplets in a thin space or on an open surface, unlike the usual microfluidic systems that process liquids by pumping them in microchannels. Droplets can be generated and manipulated on EWOD chip only with electric signals without the use of pumps or valves, simplifying the chip fabrication and the system construction. Microfluidic operations by EWOD actuation feature precise droplet actuation, less contamination risk, reduced reagents volume, better reagents mixing efficiency, shorter reaction time, and flexibility for integration with other elements. In addition, the simplicity and portability make the EWOD-based DMF system widely popular in biomedical or chemical fields as a powerful sample preparation platform. Many chemical and biomedical researches, such as DNA assays, proteomics, cell assays, and immunoassays, have been reported using the technology. In this paper, we have reviewed the recent developments and studies of EWOD-based DMF systems for biomedical applications published mostly during the last 5 years.

Keywords Digital microfluidic system · Electrowetting-on-dielectric · Biomedical application · Chemical application · Lab-on-a-chip device

H.-H. Shen · D.-J. Yao (✉)
Institute of NanoEngineering and MicroSystems, National Tsing Hua University, Hsinchu 30013, Taiwan, ROC
e-mail: djyao@mx.nthu.edu.tw

S.-K. Fan
Department of Mechanical Engineering, National Taiwan University, Hsinchu 30062, Taiwan, ROC

C.-J. Kim
Department of Aerospace and Mechanical Engineering, University of California at Los Angeles, Los Angeles, CA 90095, USA

1 Introduction

Microelectro mechanical systems (MEMS) for biological or biomedical applications, often called bio-MEMS (Folch i Folch 2013), has grown exponentially over the last two decades. Bio-MEMS devices such as biosensors (Hao et al. 2013; Tang et al. 2009), separators (Huang et al. 2013; Estes et al. 2009), neural microprobes (Chen et al. 2009, 2010, 2011, 2013), and diagnostic devices (Lo et al. 2013) have been extensively developed and aimed for rapid and accurate detection. The bio-MEMS started with the exploration to handle fluids on chip in mid-1990s and listing numerous application demonstrations today including some commercial success. The possibility of building and integrating multiple components, i.e., microstructures, sensors, and actuators, on one chip has brought about various system concepts for chemical and biomedical applications, such as lab on a chip (LOC), micrototal analysis system (μTAS), and point-of-care (POC) diagnostic devices. Populated with micrometer-sized components to process fluids, the miniature microfluidic devices

suggest some inherent advantages in performance, such as small reagent consumption and fast reaction time. Resembling the regular automated systems, which pump fluids through tubes, most of the usual microfluidic systems pump fluids through microchannels for the necessary biochemical reactions using an external pump (Huang et al. 2013; Jiang et al. 2013; Kise et al. 2013; Hsien-Hua Shen and Yao 2013). Such continuous-flow microfluidic systems face own challenges, such as low mixing efficiency, cross-contamination issue (ShaoNing et al. 2013; Ding et al. 2012; Shah et al. 2013), and complex flow regulation in multichannel systems. To solve the problems, it was proposed to manipulate the sample fluids in the form of droplets (Seemann et al. 2012; Clausell-Tormos et al. 2008; Tran et al. 2013; Chen et al. 2012) instead of the continuous flows, leading to the digital microfluidic (Cho et al. 2003; Lee et al. 2001) (DMF) systems.

In a DMF platform, discrete droplets of the sample fluids are individually processed. Instead of the conventional pumping, i.e., pressurizing one end of a fluid-filled channel to create the flows, different methods are employed to actuate individual droplets. Currently, electrowetting-on-dielectric (EWOD) (Seemann et al. 2012; Clausell-Tormos et al. 2008; Tran et al. 2013; Chen et al. 2012; Lee et al. 2002; Nelson and Kim 2012) is the most widely accepted actuation mechanism for the DMF system, employed to create, transport, separate, and merge micro- and nanoliter-sized droplets (Cho et al. 2003; Lee et al. 2001). Other actuation mechanisms applied for DMF systems include dielectrophoresis (DEP), thermal, and acoustic wave. In a hybrid approach lacking the control of individual droplets, some droplet microfluidic systems transport a stream of droplets by pumping an immiscible carrier fluid in a continuous-flow microfluidic system (Clausell-Tormos et al. 2008; Chokkalingam et al. 2013; Miller et al. 2010). In this paper, we focus on the DMF system for biomedical research based on the EWOD principle.

An EWOD surface is composed of actuation electrodes covered with a thin dielectric layer and a very thin hydrophobic topcoat. The electric field between the embedded electrodes and a conductive (e.g., aqueous liquid) droplet attracts the liquid to the surface. Since the attracted liquid appears to wet the surface more and its contact angle apparently decreases, the phenomenon is called electrowetting (Seemann et al. 2012; Clausell-Tormos et al. 2008; Tran et al. 2013; Chen et al. 2012; Beni and Hackwood 1981; Jones et al. 2004). If the electric field is applied at only one end, the droplet wets the surface asymmetrically and, if the wetting is strong enough, moves to the voltage-applied side. While the electrowetting was historically known with bare electrode (Seemann et al. 2012; Clausell-Tormos et al. 2008; Tran et al. 2013; Chen et al. 2012; Beni and Hackwood 1981), the device configuration of EWOD with the added dielectric layer allowed application of large voltages and stronger wetting (Berge 1993). The dielectric layer plays an important role of preventing the current leakage between the electrodes and the liquid at high voltages. The hydrophobic topcoat (Koo and Kim 2013) reduces the resistance of droplet against sliding so that the droplet can be easily moved by the EWOD actuation.

The magnitude of the EWOD attraction is observed by the degree of wetting, which is commonly quantified by the amount of contact angle reduction. The relation between applied voltages and contact angle reduction can be formulated by combining Lippmann's equation and Young's equation, as shown in Eq. 1, where $\theta(V)$ and θ_0 are corresponding contact angles at applied voltage V and 0 V, respectively, ε_r and ε_0 are the dielectric constant of the dielectric and the permittivity of vacuum, respectively, Y_{LG} is the liquid–gas interfacial energy, and t is the thickness of the dielectric layer (Berge 1993).

$$\cos\theta(V) - \cos\theta = \frac{\varepsilon_r \varepsilon_0}{2\Upsilon_{\mathrm{LG}} t} V^2 = Ew \tag{1}$$

See Seemann et al. (2012), Clausell-Tormos et al. (2008), Tran et al. (2013), Chen et al. (2012) and Nelson and Kim (2012) for the related equations in more general forms and the definition of the nondimensional electrowetting number Ew.

A typical EWOD device is composed of two plates—an actuation plate having patterned electrodes and a reference plate, forming a thin space in which droplets are squeezed into a disk shape. This parallel-plate configuration is popular because it simplifies the device fabrication and stabilizes the droplets against physical disturbances. Often, the space is filled with another liquid (instead of air) that is immiscible with the droplets to lubricate the movement (Pollack et al. 2000). The filler liquid also can prevent the droplet evaporation and help alleviating surface fouling, although it may limit the applications and compromise the droplet stability against physical disturbances (e.g., device orientation).

For a DMF system, the EWOD actuation can create unit-sized packets of droplets and transport, separate, and merge the droplets, using only electric signals. Sample manipulation with EWOD has many advantages compared with other microfluidic mechanisms, such as precise droplet actuation, less contamination risk, minimal dead volume, efficient reagent mixing, short reaction time, simple chip fabrication, and flexibility for integration. Furthermore, EWOD brings significant advantages to the system development, such as low-cost chip fabrication, simple system construction, low energy consumption, and system portability.

The above advantages of EWOD mechanism made the DMF systems a popular sample preparation tool for bio-MEMS (Lin and Yao 2012). For example, the possibility of performing a fast biochemical analysis on a disposable EWOD chip in a portable system is highly attractive for POC applications. After explaining various types of EWOD devices in terms of electrode design and chip fabrication, this paper reviews the EWOD-based DMF research for biomedical applications by dividing it into three assay targets and categorizing them with important processes: DNA (hybridization, amplification, cloning, and sequencing), protein (extraction, crystallization, mass spectrometry, and immunoassay), and cell.

2 System design and droplet actuation

2.1 Parallel-plate EWOD chip

The most commonly used EWOD system comprises two parallel plates. Addressable electrodes to actuate sample droplets are patterned on a bottom plate; the reference electrode is designed on a top plate. On both plates are deposited a dielectric layer and a hydrophobic layer. On providing the electric potential between top and bottom electrodes, the sample droplets can be manipulated by electrowetting phenomenon between two EWOD plates, which appears like a sandwich structure as shown in Fig. 1a (Yi and Kim 2006). The EWOD device is sometimes filled with silicone oil between the two plates, and the sample droplets are immersed in an oil bath. The oil environment decreases the rate of sample evaporation and probability of contamination and facilitates droplet operations (Sista et al. 2008; Chang et al. 2006; Hua et al. 2010).

The EWOD chips are generally fabricated from ITO glass because of the transparency of the ITO conducting layer. The ITO layer of bottom plate is then etched with lithography to pattern the EWOD electrodes. One layer of dielectric film and a hydrophobic layer are eventually deposited on the electrodes. Polymers, plasma-enhanced chemical vapor deposition (PECVD) of Si_3N_4 or SiO_2 are materials typically used as the dielectrics; fluoropolymers

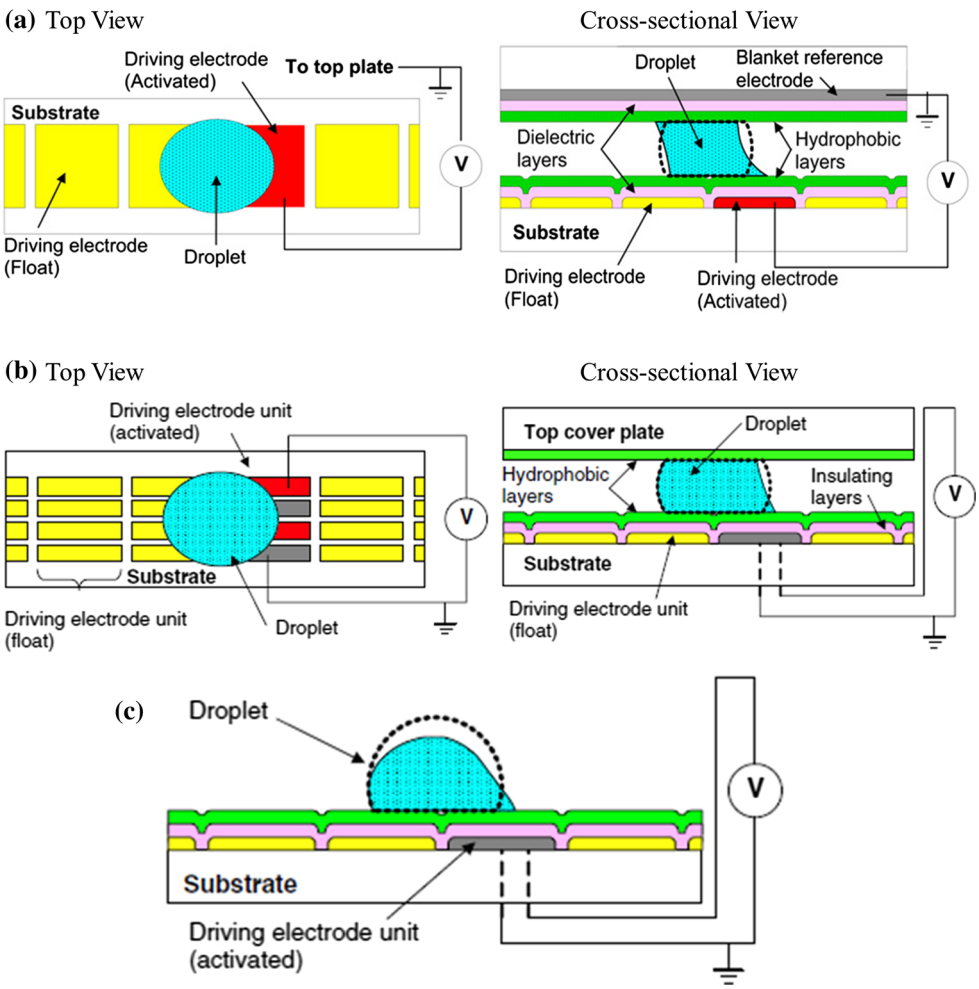

Fig. 1 Schematic diagrams of different kinds of EWOD devices. **a** A parallel-plate EWOD chip. **b** An EWOD chip with coplanar electrodes. **c** The cross-sectional view of an open-plate EWOD device with coplanar electrodes for droplet actuation

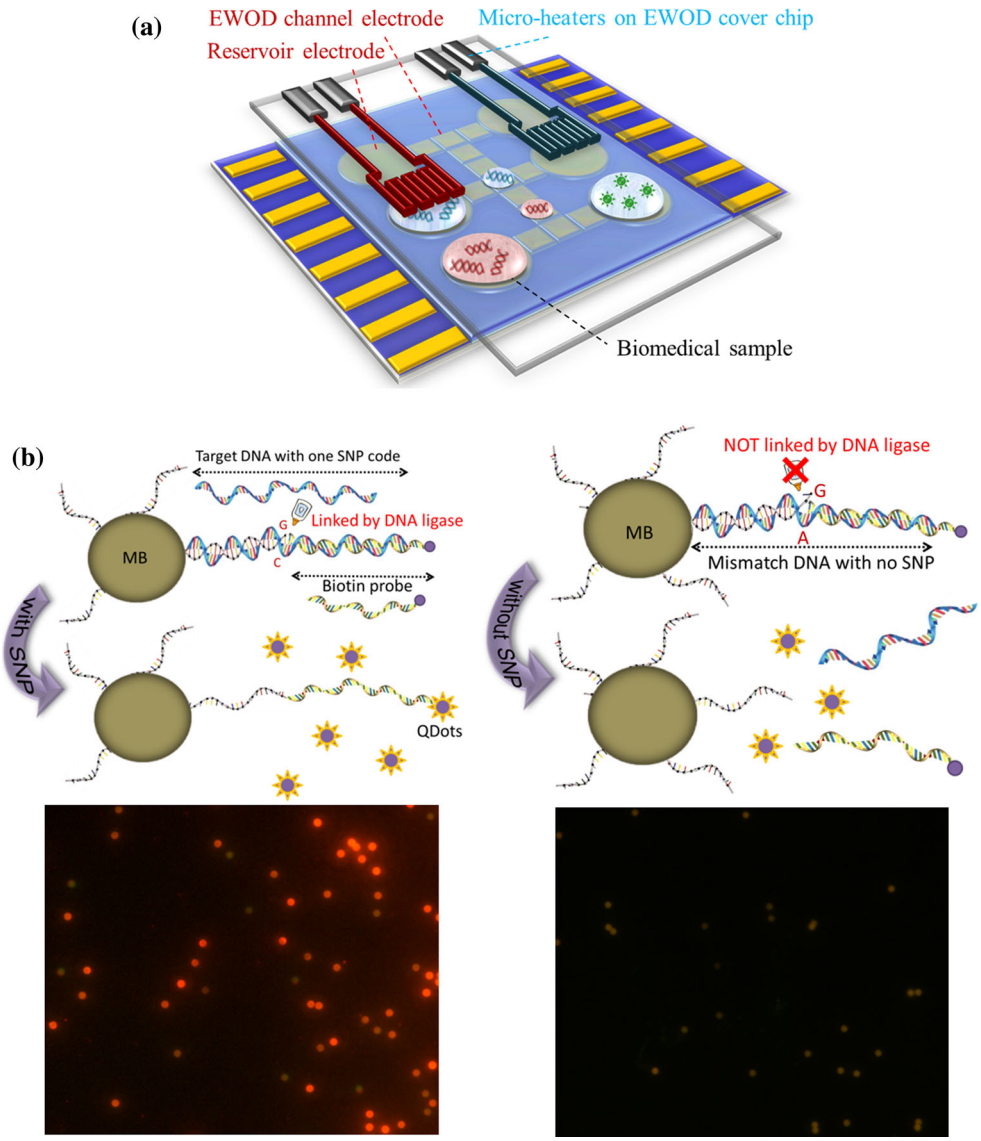

Fig. 2 Temperature-controllable DMF system for SNP detection. **a** Schematic diagram of coplanar electrodes DMF chips with microheaters design. **b** SNP detection mechanism, target DNA contained a SNP code in the DNA sequence, and the magnetic beads showed red fluorescent signal. **c** SNP detection mechanism, target DNA contained no SNP code, and the magnetic beads showed no fluorescent signal

such as fluoropolymers such as polytetrafluoroethylene (PTEF, commonly known as Teflon[@]) or amorphous fluoropolymers (CYTOP[@]) are common options for hydrophobic layers. The top plate is also made from an ITO glass, which requires no etching because there is only a ground electrode on the top plate. After the deposition of dielectric and hydrophobic layers, the top plate is packaged with the bottom plate for droplet manipulation.

2.2 EWOD chip with coplanar electrodes

Unlike a traditional parallel-plate configuration, electrodes are designed only on the bottom plate of a EWOD DMF chip (Yi and Kim 2006; Davoust et al. 2013) with coplanar electrodes for droplet actuation, as the top view shown in Fig. 1b. The top plate has a hydrophobic layer coated on a glass substrate according to the cross-sectional view in Fig. 1b, which is not responsible for droplet actuation. This feature provides much potential for integrating additional functions on the chip. The top plate of an EWOD device can be designed with sensing elements for biomedical reaction or biochemical detection. For example, the diagram in Fig. 2a shows coplanar electrode DMF chips with several heating circuits to control temperature (Shen et al. 2013a). Even without the top plate, the EWOD chip with coplanar electrodes can still manipulate a droplet; this arrangement is called the open configuration EWOD. Figure 1c shows the concept of an open-plate configuration EWOD for droplet actuation. EWOD droplet manipulation of this kind has both advantages and disadvantages. Without the top hydrophobic plate, the EWOD system becomes simpler, and a biomolecule has less chance to become adsorbed on the EWOD hydrophobic surface (Jiangang et al. 2006), but a μL-sized sample evaporates more rapidly in an open environment than if the droplet is covered with a top plate in an EWOD system.

2.3 EWOD chips of other types

Here, we introduce several variations on a typically prepared EWOD device, including fabrication of electrodes, substrates, interfaces and inlets, dielectric layers, and hydrophobic layers.

Watson et al. (2006) fabricated EWOD electrodes with microcontact printing (μCP) using poly(dimethyl siloxane) (PDMS) stamps for gold and chromium on glass, wet etching with a patterned self-assembled monolayer (SAM) of 1-hexadecanethiol (HDT), printing of palladium colloids on (3-aminopropyl) triethoxysilane (APTES)-treated glass for electrodeless deposition of copper, and patterning of SAM with mercaptosilane functionality ((3-mercaptopropyl) trimethoxysilane, MPTMS)) to trap gold colloids for subsequent electrodeless deposition of copper. The electrodes were coated with parelene-C (1 μm) and Teflon (50 nm).

For special substrates, cheap and rapid prototyping of an EWOD device was prepared with copper substrates of gold compact disks and rapid marker masking to replace photolithography (Abdelgawad and Wheeler 2008). Printed circuit boards (PCB) were used in EWOD devices to decrease the cost of mass production (Sista et al. 2008) and eased electric connection through the substrate holes (Gong and Kim 2008). Special post-PCB fabrication facilitating droplet manipulation was developed and reported. In addition to rigid substrates, Abdelgawad and Wheeler fabricated an EWOD device on industrial-grade flexible sheets with 9-μm copper and 50-μm polyimide using a laser printer toner as etching mask (Abdelgawad and Wheeler 2007); parylene-C and Teflon were then coated. Low-grade inflexible boards with copper (thickness 35 μm) were used to fabricate EWOD devices through conventional photolithography and wet etching, then spin-coated with PDMS and baked. Using flexible devices with copper-clad polyimide substrates coated with PDMS and Teflon, droplets were driven on curved and twisted surfaces (Abdelgawad et al. 2008). Moving droplets across an air–oil interface was demonstrated for oxygen sensing and DNA purification with liquid–liquid extraction. Fan et al. (2011a) demonstrated a droplet on a wristband in which droplets were continuously driven along an enclosed droplet track (length 204 mm) with in total 136 electrodes across four enclosed and connected flexible EWOD modules. Each module having appropriate connecting interfaces for droplet and electrowetting (EWOD) signal transfer was fabricated on polyethylene terephthalate (PET) with low-temperature processes. SU-8 (1 μm) and Teflon (66 nm) were spun on patterned ITO PET devices.

In addition to the interface between digital and digital microfluidic devices, Abdelgawad et al. (2009) demonstrated a digital-to-channel interface. A trimmed PDMS slab with microchannels was first bonded onto a glass substrate carrying patterned electrodes. The PDMS slab was carefully covered with low-tack dicing tape during deposition with parylene and coating with Teflon. The tape was eventually removed before device operation. Although the world-to-chip interface is crucial, its investigations are few. Simply punching a hole through the plastic (PET) substrate (Fan et al. 2011a) or machining a hole through the glass substrate (Shah et al. 2013) has been demonstrated. Capillaries or tubing was typically inserted through the inlet holes vertically or horizontally through custom-made in-plane ports (Kim et al. 2011).

Various dielectric layers have been tested. For example, polyethylene film, clerical adhesive tape, and stretched sheets of wax film were tested as a removable plastic "skin" to eliminate cross-contamination and to provide preloaded dried spots of enzymes (Yang et al. 2009). Fan et al. (2009) used a polymer-dispersed liquid crystal (PDLC) to achieve both electrowetting and electro-optical effects simultaneously.

Patterning Teflon is an important way to increase the functions of the EWOD device, especially in cell manipulation. With common structures of electrode or dielectric or hydrophobic layers, Fan et al. (2008) demonstrated cell concentration using EWOD and dielectrophoresis (DEP), and Barbulovic-Nad et al. (2008) demonstrated cell-based assays. For cell seeding and growth, the surface of a similar device with electrode or dielectric or hydrophobic layers was locally modified with islands of extracellular matrix (ECM) proteins, e.g., fibronectin, on manually pipetting aliquots (500 nL) of fibronectin and allowing them to dry (Barbulovic-Nad et al. 2010). Circular adhesion pads ($\sim$1 mm^2) were formed beside (4.8 mm^2) the actuation electrodes. The hydrophilic adhesion pad also assisted passive dispensing for media exchange. Patterning the top Teflon layer with a lift-off process to expose the underlying ITO for cell culture was demonstrated (Eydelnant et al. 2012), including for primary cells (Srigunapalan et al. 2012).

A modification involving patterning the bottom Teflon on parylene for biomolecules, e.g., poly-L-lysine (PLL-FITC), was demonstrated with wet lift-off (WLO) and dry lift-off (DLO) (Witters et al. 2011). In the WLO process, Teflon was briefly activated in by oxygen plasma and then patterned with photolithography and appropriate dry etching by oxygen plasma. After appropriate biomolecules were adsorbed on the surface, the sacrificial photoresist layer was ultrasonically stripped. The bottom plate was baked for 1 min at 200 °C to regain the hydrophobicity of Teflon. Alternatively, the DLO process used a removable parylene mask to pattern biomolecules on Teflon. A first parylene dielectric layer was deposited and briefly activated with the oxygen plasma before Teflon spin coating; a

second parylene layer (~1 μm) was then deposited. The subsequent photolithography and oxygen plasma dry etching patterned the second parylene and Teflon layers. After the photoresist was dissolved, biomolecules were modified on the surfaces including the first parylene layer. The second parylene was then detached from the bottom plate to realize micropatches (40 μm × 40 μm or 15 μm × 15 μm) on the bottom plate for the following cell clusters or single-cell attachment and culture. The DLO was modified to capture superparamagnetic beads (diameter 2.7 μm) in an array of microwells (diameter 4.5 μm and depth 3 μm) on the top plate (Witters et al. 2013). A thin aluminum ground electrode was first deposited on the top plate and then functionalized with a fluoroalkylsilane to enhance the subsequent Teflon adhesion (thickness 3 μm). A parylene layer (500 nm) was deposited, treated with the oxygen plasma, and covered with thin aluminum that worked as a hard mask after photolithography and wet etching. Oxygen plasma was applied to etch the parylene and thick Teflon layers, followed by peeling off the parylene. Dispensing fL droplets with the hydrophilic micropatches for metal–organic framework crystals was also demonstrated (Witters et al. 2012).

In another application, Cho et al. (2007) removed the top Teflon layer with lithography and RIE (reactive ion etching) in an oxygen plasma for particle separation using electrophoresis. A fluorocarbon surfactant was used to facilitate spin coating of a photoresist. The hydrophobicity of the Teflon surface remained without much degradation (contact angle decreased less than 5°). Removing the dielectric and Teflon layers on the electrodes was used for electrochemical measurements (Yu et al. 2013; Dryden et al. 2013). Patterning both dielectric and hydrophobic layers was reported to integrate EWOD and OET (optoelectronic tweezers) for droplet and particle manipulations (Shah et al. 2009). To position organic and nonpolar solvents, patterned photoresist structures were used on the top plate (Fan et al. 2011b) or bottom plate (Mousa et al. 2009) before deposition of Teflon to enhance the capillary forces.

3 DNA-based applications

3.1 DNA hybridization

DNA hybridization is the macromolecular interaction between two complementary single-stranded DNA (ssDNA) molecules, so that they bind with each other through hydrogen bonds at an annealing temperature to form a stable double-stranded DNA molecule. DNA hybridization has been applied in many MEMS devices such as biosensors, chemical sensors, and bead-based DNA probing system.

Tabrizian's research team brought the DNA hybridization to the DMF system (Malic et al. 2009); in their work, a dynamically configurable biochip platform with microarray surface plasmon resonance was proposed. Here, ssDNA probes of three kinds were used with 20–24-mer oligonucleotides; the 5′ end of the ssDNA probes were modified with a C6 linker and a thiol for covalent bonding with gold on the binding site of the DMF chip surface. After the sample droplets were manipulated with EWOD electrodes through the ssDNA binding site, the target complementary ssDNA was bound with the probes and detected with surface plasmon resonance imaging (SPRi). These authors also mentioned that the reason for used of SPRi to detect DNA hybridization is that the speed of DNA hybridization detection was greatly increased; this DMF system has the potential for other biomedical applications. Yao's research team designed ssDNA probes for a single nucleotide polymorphism (SNP) detection (Shen et al. 2013b). SNP is a DNA sequence variation when a single nucleotide base differs between individuals in the genome DNA. As the genome of a person comprising a specific SNP could result in response to drug therapies and sensitivity to particular disease, SNP identification is important in biomedical research to develop disease and to respond to pathogens, drugs and other agents. In that work, the ssDNA was anchored on the surface of magnetic beads (MB) as probes; the magnetic property of MB enabled the DNA probes to be collected and purified with an applied magnetic field. The DMF system used in that research is shown in Fig. 2a, which was composed of a coplanar electrode EWOD chip and a hydrophobic top plate with integrated microheaters. The top plate provided temperature control of the DNA ligation during SNP detection. Figure 2b and c shows the SNP detection mechanisms, while the detected target ssDNA strands contain a SNP code or no SNP code, respectively. The Taq DNA ligase would link the MB probe and the biotin probe together when the target DNA contained the SNP code in the sequence, and the MB showed a red fluorescent signal; in contrast, the MB showed no red fluorescent signal because the mismatched DNA used in ligation contained no SNP code in the sequence.

3.2 DNA amplification

In molecular biology, the polymerase chain reaction (PCR) is a common biochemical technique used to amplify DNA fragments (Bartlett and Stirling 2003; Saiki et al. 1985). DNA can be replicated in vitro through thermal cycling. Billions of copies of a desired DNA fragment can be replicated from a DNA template solution of small concentration. PCR is a useful tool applied in DNA cloning, DNA sequencing, immunoassay, pathogen analysis, proteomics,

and clinical diagnostics. Traditional PCR contains three main steps for DNA replication: the first involves melting of double-stranded DNA (dsDNA) of the template at 95 °C for 30 s; in the second step, the temperature is decreased to 50–65 °C for about 30 s to anneal the primers so that the ssDNA primer fragments hybridize with the template, and third Taq DNA polymerase is used for DNA extension about 72 °C, and the extension time depends on the length of the DNA template being amplified. The DNA polymerase elongates the primer in the 5′ to 3′ direction with the dNTP to form the complementary strand of the template. During one thermal cycle, the copy number of DNA is doubled, which means that, if the thermal cycle is repeated n times, 2^n DNA copies can be made through the PCR. PCR was demonstrated on the DMF platform by Lin et al. since 2006 (Chang et al. 2006). The system comprised the thermal sensor and heaters designed for the PCR on a chip, which performed precise thermal cycling for DNA amplification.

Real-time polymerase chain reaction (RT-PCR) is another technique used in molecular biology for DNA amplification (VanGuilder et al. 2008). Different from traditional PCR, fluorescent dyes are used to label the amplified DNA. By fluorescent detection during each thermal cycle, the amount of amplified DNA copies can be quantified in real time. RT-PCR provides an accurate and convenient method for DNA amplification. Pollack et al. (Advanced Liquid Logic Inc.) used the DMF system for RT-PCR research in 2010 (Hua et al. 2010). The PCR DMF chip was designed with electrodes to actuate droplets, two heaters, magnets, and regions of optical detection. During the experiment, the DMF chip was filled with silicone oil to prevent evaporation of reagents during the thermal cycles. The methicillin-resistant *Staphylococcus aureus* (MRSA) genomic DNA was used there as the target DNA for a parallel two-plex RT-PCR on a chip. The reaction began with template dsDNA melting at temperature 95 °C for 60 s, followed by 40 thermal cycles with 10 s at 95 °C for dsDNA melting, 30 s at 60 °C for annealing and extension. This DMF system was designed with a rapid thermocycling module, which decreased the duration of RT-PCR without compromising the reaction yield. Those authors mentioned that the RT-PCR attained an amplification efficiency of 94.7 %, and multiple DNA samples could be processed for high-throughput PCR applications. In 2012, Mitchell et al. used their DMF system for rapid and automated research on biomedical diagnostics (Schell et al. 2012). RT-PCR is a standard technique of analysis to detect the DNA of *Candida albicans* (*C. albicans*) in specimens of infected patient blood. Their DMF RT-PCR platform was capable for the Candida DNA detection from a small volume (1 mL) of blood specimen. Sixteen blood specimens from separate patients with culture-proven candidemia that tested positive for *C. albicans* DNA were analyzed with conventional and DMF RT-PCR in this research. The results showed that the sensitivity was 69 % (11/16) and 56 % (9/16), respectively; the combined sensitivity was 94 % (15/16). The authors concluded that their DMF platform is a portable and easy to use system for POC, which decreases both the duration of reaction and the cost when applied in RT-PCR.

3.3 Cloning

In molecular biology, the interactions among DNA, RNA, and protein macromolecules of various types are investigated to reveal the cell structures and functions. Cloning is a common technique and powerful tool to study genes in molecular biology since its proposal before 1980. After inserting a specific gene into a bacterial plasmid or yeasty vector, the target gene can be self-replicated and expressed by the host. The first step of the cloning technique is to build a DNA construct, which begins with amplification of a target gene with a polymerase chain reaction (PCR); the host vector DNA is then restricted, purified, and linked with a target gene by DNA ligase. Secondly, the construct is transformed into the host for gene expression. Figure 3a shows the protocol of DNA cloning using *Escherichia coli* (*E. coli*) as the host, but traditional DNA cloning requires much time and effort; several DMF researchers have hence tried to bring the cloning reaction to the nL-sized microfluidic channel to facilitate the reaction and to decrease the reagent volume and cost.

DNA ligation was demonstrated on the DMF by Yao's research group; this work tried to use an EWOD chip with coplanar electrodes as a platform to make a DNA construct (Liu et al. 2008). The insert DNA fragment was obtained from a lambda DNA by digestion with *Hind*III restriction enzyme, which was then inserted into the pUC19 plasmid vector with the same restriction recognition sites. During the ligation, insert and vector DNA sample droplets were created from the reservoir electrodes; all droplets were moved into a reaction buffer droplet with DNA ligase. The function of DNA ligase is to join the two DNA molecules from their sticky ends. After reaction, the droplet with the DNA construct was transformed into the host bacteria cell—here *E. coli*—for plasmid replication. The transformed bacteria were selected and cultured on a Luria-Bertani (LB) agar plate with a specific antibiotic. The bacterial DNA was then examined with standard plasmid extraction, digestion, and electrophoresis to prove that the DNA ligation was achieved on the DMF platform. After 2 years, Yao's research team improved the EWOD system of coplanar electrodes by designing electrode arrays for multisample reaction (Lin et al. 2010). DNA samples in four sets were actuated on a DMF chip at the same time for

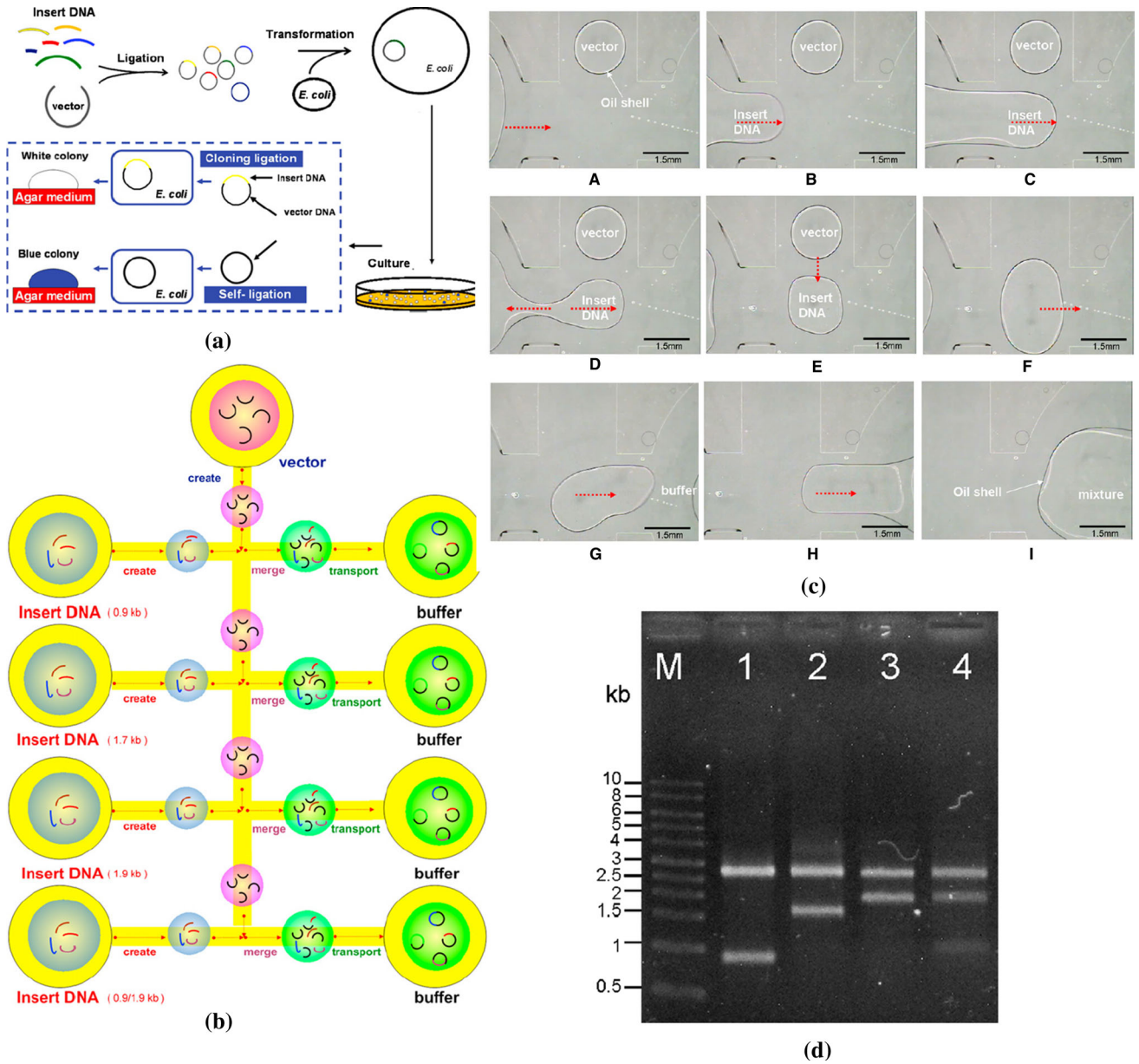

Fig. 3 Coplanar electrodes EWOD chip for DNA ligation. **a** The protocol of DNA cloning. **b** Diagram of four parallel DNA ligations on the DMF platform. **c** Sequential images of the DNA ligation on a DMF chip, insert DNA and vector DNA droplets were created and mixed with each other, and then the DNA mixture droplet was transported and mixed with reaction buffer containing DNA ligase. **d** The electrophoresis result of DNA ligation on a DMF system, four trials were presented and all of the plasmids contained the pUC19 vector (2.6 kb) and insert DNA. *Lanes 1–3* show the insert DNA 0.9, 1.7, and 1.9 kb, respectively, and *lane 4* shows both 0.9 and 1.9 kb insert DNA

DNA ligation. Figure 3b shows a diagram of four parallel DNA ligations. Through actuation with DMF electrodes, insert DNA and vector DNA solutions were created into droplets and mixed with each other; the DNA mixture droplet was transported and mixed with a reaction buffer containing DNA ligase. Figure 3c shows sequential images of the generation of the sample droplets, transport, and mixing on the DMF chip. To decrease the rate of droplet evaporation and to make the droplets easier to actuate, droplets manipulated in this research were all covered with silicone oil in a thin layer called core–shell droplets, as seen in Fig. 3c. After DNA ligation, the DNA construct mixture was transformed into the *E. coli* and cultured in the LB plate with ampicillin. The *E. coli* colonies on the LB plate were selected and incubated in a medium to increase the amount of bacterial plasmid. The plasmid was extracted and digested with a restriction enzyme to verify the vector DNA and insert DNA of the correct length. The

electrophoresis result in Fig. 3d shows that the size of the insert and vector DNA fragments was correct, which means that the DMF system achieved the DNA ligation. In general, a cloning requires reagent volume (15–20 μL) in a standard ligation protocol, but of the reagent volume about 85 % is wasted. Yao's DMF system required reagent volume only 2.1 μL to complete a cloning. The decreased volume of reagents in the DNA ligation meant that the cost of reaction was decreased, and also proved the potential of the DMF platform for biomedical application.

3.4 DNA sequencing

DNA sequencing is another important technique for DNA analysis used in molecular biology, which determines the precise order of nucleotides of four kinds within a DNA molecule. DNA sequencing has been a useful and precise examining tool for mapping the genome of various species for an evolutionist to assess the relation between independent but similar species. Sequencing enables scientists using information about the human genome for parental testing, forensic testing, study of genetic disorder and gene expression, development of crop strains, and other biomedical research. Pyrosequencing is the next-generation DNA sequencing technique with high throughput, accuracy, and repeatability (Ronaghi 2001; Elahi and Ronaghi 2004). In contrast with Sanger sequencing (Sanger and Coulson 1975; Sanger et al. 1977), pyrosequencing requires no fluorescent labeling, fluorescence detector, and electrophoresis during the testing. Of three steps in DNA pyrosequencing, the first is hybridization of the primer fragment with a ssDNA template with the reaction solution containing DNA polymerase, ATP sulfurylase, luciferase, luciferin, apyrase, and substrates adenosine 5′ phosphosulfate (APS). Second, one kind of dNTP is added for primer elongation. Individual dNTP complementary to the template is incorporated onto the template with DNA polymerase and releases one molecule of pyrophosphate (PPi). In the third step, the PPi is converted to ATP with APS by ATP sulfurylase; the ATP is used to convert the luciferin to oxyluciferin by luciferase with the generation of visible light. The intensity of light generated from luciferin is detected and analyzed. If the dNTP is mismatched with the template, which cannot be incorporated onto the template ssDNA, no light-emitting reaction can be induced. With no visible light signal, the dNTP or ATP is decomposed by apyrase, and the reaction can restart with another nucleotide. Research has been undertaken to improve the pyrosequencing accuracy and reaction rate with decreased cost (Zhou et al. 2001). The DMF system is considered an effective platform for DNA pyrosequencing to minimize the reaction volume and to automate the sample process.

A company (Advanced Liquid Logic, Inc.) has cooperated with Duke and Stanford Universities in USA and invested much effort to develop a DMF system for biomedical applications. In 2008, Pamula and Pollack's research team designed a DMF chip and controlling system for testing research (Sista et al. 2008) at a point of care, which demonstrated a droplet-based magnetic bead immunoassay on a DMF system for processing whole blood samples. Boles, Pollack et al. proposed a DNA pyrosequencing technique using the DMF system (Boles et al. 2011). The DMF chip and the controller used in this study are shown in Fig. 4a. The bottom plate was fabricated with a standard printed circuit board (PCB) with electrodes and contact pads; on the DMF chip was deposited a polyimide layer as a dielectric layer that was coated with a hydrophobic layer. The bottom plate was packaged with the top plate made of polycarbonate (PC) with sample and reagent reservoirs. An integrated multifunction controller with control electronics, magnets, and a PMT detector was used to drive the DMF chip. After connection, the DMF device was filled with silicone oil for actuation of sample droplets in an oil bath. The oil-filled environment decreased the rate of evaporation of the droplets, which is important because evaporation is a serious problem during manipulation of droplets of submicroliter size. The magnetic bead served as the ssDNA carrier, which could be manipulated and washed with a magnetic force produced from the multifunction controller. Finally, the pyrosequencing protocol was brought to their DMF platform. Figure 4b_1 shows the relation between an area under the curve (AUC) with pyrophosphate at varied concentration in a sequential detection; the numbers on the bar chart indicate the homopolymer length that is approximately equivalent to the amount of pyrophosphate in the reaction. In Fig. 4b_2, a targeted resequencing protocol was used for addition of 52 nucleotide cycles. After assigning a threshold level for each number of nucleotide incorporations, the sequence was read as $TTTGA^{5}$ $CTATT^{10}$ $AAATA^{15}$ $ATCGG^{20}$ $TTGAC^{25}$ $ATTAA^{30}$ $ATAAA^{35}$ $ATTTG^{40}$ $GTTGA^{45}$ $GTTTA^{50}$ $ATCTC^{55}$ $TGGCA^{60}$ GG. Figure 4b_3 shows the result with a pyrosequencing protocol de novo for the same template sequencing. Nucleotides of four kinds were repeatedly added in the same order for 44 cycles. The same sequence is read as Fig. 4b_2). The pyrogram in Fig. 4b_4 shows another testing group with nucleotide addition for 34 cycles for the same template using a resequencing protocol, which proved the repeatability of the pyrosequencing based on the DMF system. So far, the DNA pyrosequencing has been demonstrated with a DMF system. Sample size and reaction time have been decreased by DMF actuation, which are important reasons that a DMF system is used for DNA sequencing. Welch

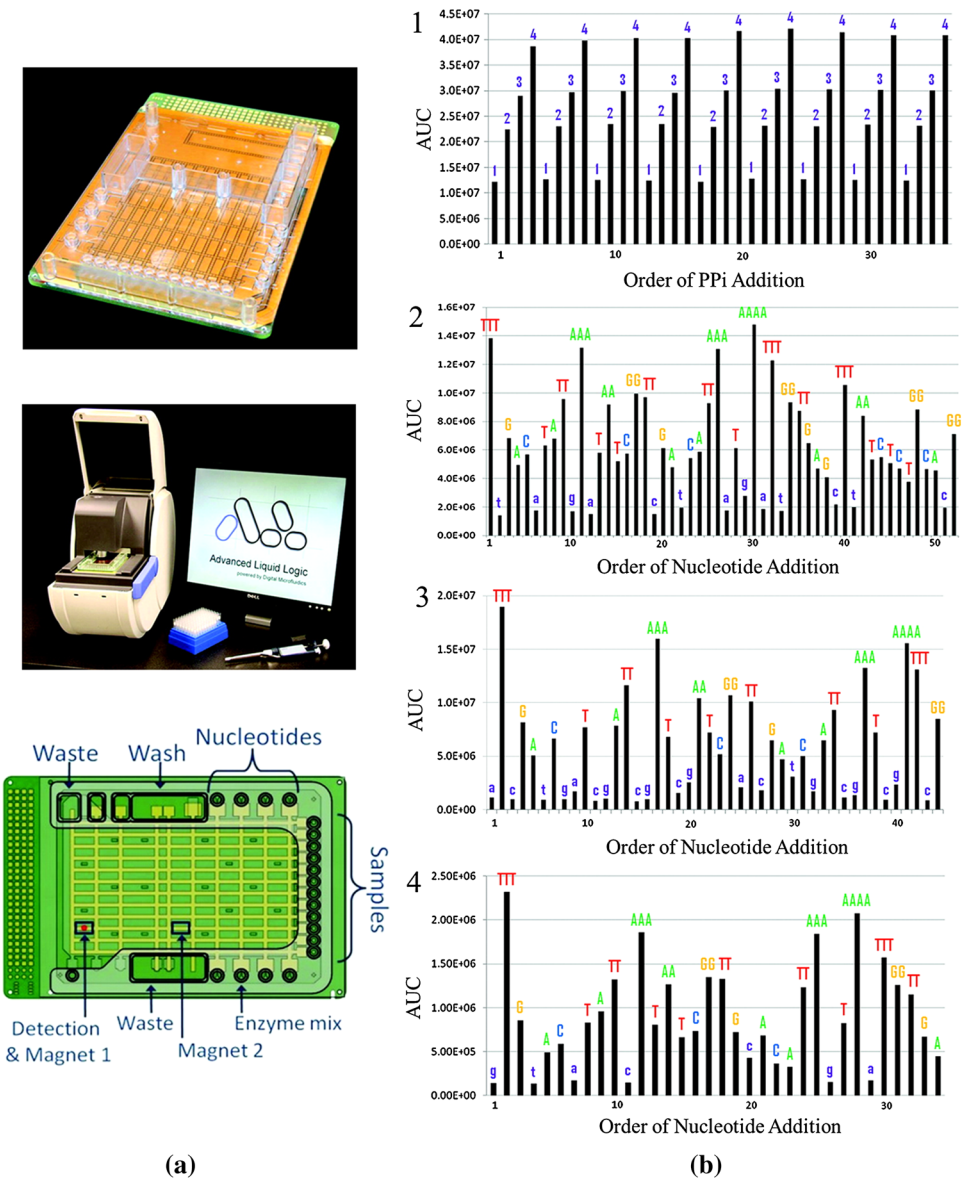

Fig. 4 DMF chip for pyrosequencing. **a** Images of PCB EWOD chip (*left*), system controller (*middle*), and electrodes layout (*right*). $\mathbf{b_1}$ The AUC detection of different concentrations of pyrophosphate. $\mathbf{b_2}$ The pyrogram shows the 52 cycles of nucleotide addition. The nucleotide that was added is indicated by the letter above each *bar* with *uppercase letters* indicating expected incorporations and *lowercase letters* indicating expected mismatches based on the known sequence. $\mathbf{b_3}$ The pyrogram of 44 cycles of nucleotide addition, the same template was used, and the four different bases were repeatedly cycled in the same order. $\mathbf{b_4}$ The pyrogram of 34 cycles of nucleotide addition with the same template, but the synthesis and detection reactions were performed in different droplets and locations on the chip

et al. (2011) tried to discuss the relation between the intensity of the light signal and the luciferase concentration. In this way, a picoliter-scale DMF platform was used for DNA pyrosequencing. This work significantly decreased the necessary amount of reagents and overall cost of DNA sequencing. In conclusion, the DMF system has become a tool for DNA sequencing, which has the potential for a large throughput, multifunction integrated, accurate, and cheap biomedical application because DMF provides features of accurate sample manipulation and requires only a small volume.

4 Protein-based applications

Proteomics researchers study the structures and functions of proteins; these biomolecules are composed of amino acid chains that serve as enzymes for metabolism in the cells and are the components of organs in an organism. Proteins are studied in proteomics research for the compositions, structures, activity patterns, and their roles played in metabolic reactions. The masses and functional groups of proteins can be studied with mass spectrometry (MS). The signatures of the protein functional groups can

be determined by the spectrum generated from MS to elucidate the property of polypeptide chains. The sample preparation processes of the protein MS analysis include protein extraction, crystallization, MS analysis, and the functional conjecture. The DMF system seems to be a suitable tool for protein research because of the features of DMF that include the convenient and accurate manipulation of samples. Protein extraction (Jebrail and Wheeler 2009; Yang et al. 2011; Wijethunga et al. 2011), crystallization (Aijian et al. 2012; Tao et al. 2010; Nelson et al. 2010), and mass spectra (Lapierre et al. 2011; Kirby and Wheeler 2013) have been demonstrated on DMF to investigate the chemical properties of proteins.

4.1 Protein extraction

Wheeler et al. proposed the first DMF method for protein extraction (Jebrail and Wheeler 2009); they used a protein precipitation method to purify proteins in solution. The mechanism of protein precipitation is to decrease the solubility of the protein using a salt, such as ammonium sulfate, as the precipitant reagent. Bovine serum albumin (BSA), fibrinogen (Fb), and myoglobin (Mb) in protein solutions of nL volume were precipitated, rinsed, and resolved in a buffer solution with automated DMF sample manipulation. A parallel-plate EWOD chip of Parylene-C and Teflon-AF as dielectric and hydrophobic layers, respectively, was used in this work. The precipitation and resolubilization steps were as follows: The buffer droplet containing BSA was first mixed with a precipitant droplet. After the BSA precipitate was seen on the extraction electrode after incubation for 5 min at room temperature, the rinsing solution and resolubilizing buffer droplets were used to wash the BSA precipitate and for resolubilization. Proteins of three kinds including BSA (50 mg/mL), Mb (30 mg/mL), and Fb (20 mg/mL) were precipitated and labeled with fluorescence by DMF methods; the fluorescent intensity was compared with the result of a control group. The result indicated that the DMF method is comparable with conventional methods of protein extraction. The authors also used the system for protein extraction from fetal bovine serum (FBS) and cell lysate (CL); these are viscous and sticky solutions for DMF manipulation. The authors concluded that the DMF platform can be applied for protein extraction, which requires no centrifugal step and has a decreased duration of reaction. After 3 years, Wheeler's team proposed another DMF device combining the porous polymer monolith (PPM) for solid-phase extraction (SPE) of proteins (Yang et al. 2011). Different from protein precipitation, solid-phase extraction is another protein separation based on the interactions between a liquid sample and a solid stationary material. Molecules as proteins in the solution are adsorbed and retained by a stationary phase, and subsequently eluted in a solution as a purified form. Here, the polymer PPM served as the solid stationary phase; a column-shaped PPM of radius 1 mm and height 270 μm was made on the DMF chip surface on UV exposure to capture the target molecules. The DMF platform was responsible for protein extraction in four steps—equilibration, sample loading, washing, and elusion. The DMF solid-phase extraction method was proved to have extraction efficiencies similar to the traditional commercial kits for protein extraction.

4.2 Protein crystallization and mass spectrometry

4.2.1 Matrix-assisted laser desorption and ionization (MALDI)-MS

Mass spectral (MS) analysis, the last step in proteomic research, is a tool of analytical chemistry to determine the masses, elemental or isotopic signatures, and relative concentrations of atoms and molecules. During MS analysis, the molecule of a sample is ionized, accelerated, deflected, separated, and detected in its MS according to the ratio of mass to charge. A spectrum, a plot of intensity versus ratio of mass to charge, is produced based on the masses of the molecular ions. Matrix-assisted laser desorption and ionization (MALDI) (Walch et al. 2008) and electrospray ionization (ESI) are two techniques of sample ionization applied in MS for the analysis of biomolecules especially in proteomic research. A major advantage of MALDI-MS is a highly efficient method of sample analysis, which required no tedious extraction, purification, or separation steps. MALDI-MS has hence become a commonly used tool for proteomic investigation. Before the MALDI-MS analysis, macromolecules such as proteins or peptides are extracted and purified. As these sample preparation steps are complicated and tedious, the DMF system has been used for sample processing before MS analysis since 2004 based on its outstanding potential for manipulation of droplets (Wheeler et al. 2004, 2005; Moon et al. 2006). In 2010, Kim's research team designed multifunctional EWOD electrodes on a parallel-plate DMF system (Nelson et al. 2010), which were capable of both actuation of droplets and localized heating. The incubation accelerated the rate of protein reduction, digestion, and crystallization for the matrix-assisted laser desorption and ionization mass spectral (MALDI-MS) analysis. Insulin served as an analyte in the research, which was first reduced by dithiothreitol (DTT) on a heater at 130 °C for 10 s to break the disulfide bonds; via automatic creation, merging, and transport of droplets, the sample preparation could be finished on the DMF platform. The insulin samples were incubated on the heating electrode for protein crystallization. When sample processing steps were

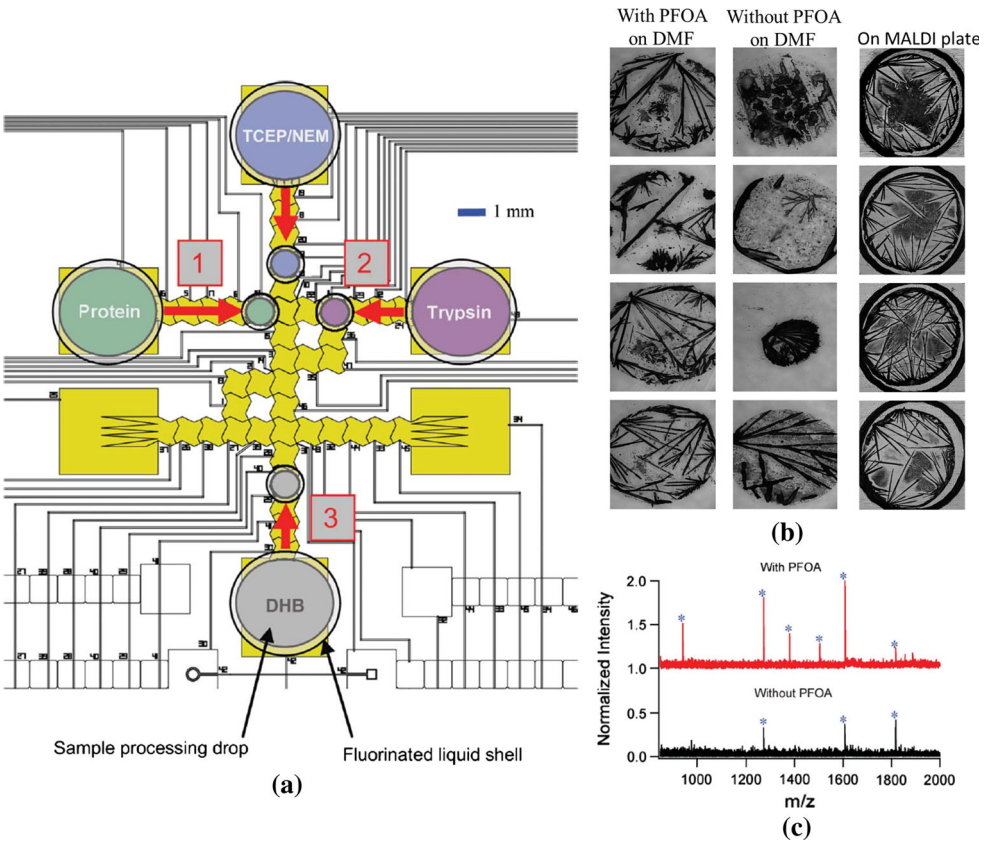

Fig. 5 DMF for proteins extraction at room temperature. **a** The device layout and sample processing steps. *1* A protein droplet was merged with TCEP/NEM for disulfide bond reduction and alkylation. *2* A trypsin droplet was merged with the protein droplet for digestion. *3* The protein digest mixture was merged with two DHB droplets for sample ionization. **b** Comparing the crystal morphology of digested FITC-BSA samples crystallized on the hydrophobic surface of DMF chip and stainless steel MALDI plate. The matrix solution without PFOA exhibited inconsistent sample to sample morphology. **c** MALDI-MS spectra of digested myoglobin crystallized on DMF with or without PFOA

finished, the insulin samples were analyzed with MALDI-MS. In 2012, Wheeler et al. also applied the parallel-plate EWOD chip in protein processing and crystallization for MALDI-MS analysis (Aijian et al. 2012); proteins of various kinds such as lysozyme, cytochrome *c*, myoglobin, and fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA) were analyzed in this research. In contrast with Kim's previous work, all sample processing reactions and crystallization were performed at room temperature. To minimize protein adsorption and to improve crystallization, the authors used a fluorinated liquid to embed the sample droplets; the function of the fluorinated liquid was similar to that of silicone oil, but it was quickly and passively removed on evaporation at room temperature. The procedure of protein crystallization is shown in Fig. 5a. A protein droplet was initially created from the reservoir and merged with TCEP/NEM to reduce the disulfide bond and for alkylation with DMF actuation. The trypsin droplet was created and merged with the protein droplet for digestion. The droplet with the protein digest mixture was merged with two droplets of 2,5-dihydroxybenzoic acid (DHB) for sample ionization. Once the DHB was added, the top plate of the EWOD chip was removed for solvent evaporation and protein crystallization. The authors discussed the function of pentadecafluorooctanoic acid (PFOA) used in the matrix solution crystallization. The result in Fig. 5b revealed that PFOA is able to facilitate the crystallization and to stabilize the crystal morphology. The PFOA also improved the MALDI mass spectra quality as shown in Fig. 5c.

4.2.2 *Surface-assisted laser desorption/ionization (SALDI)-MS*

In work on proteomics, Boukherroub et al. in 2011 used surface-assisted laser desorption and ionization (SALDI) for analysis of peptides (Lapierre et al. 2011). In contrast with MALDI-MS, SALDI-MS is a mass spectral method of analysis for biomolecules free of a matrix. The disadvantages of using an organic matrix for sample ionization are that the sensitivity is small for analysis of compounds of molecular mass less than 1,000 Da, such as peptides, carbohydrates, or lipids, such that the background generated from organic matrix desorption and ionization limited the MS for analysis of small molecules. The organic matrix treatment is tedious and complicates the DMF sample processing. These authors hence used inorganic silicon nanowires for the sample ionization, to replace an organic matrix such as DHB used in MALDI. The DMF platform for sample manipulation was a parallel-plate EWOD chip. Figure 6a and b shows the cross-sectional view of the EWOD chip and the illustration of LDI-MS analysis,

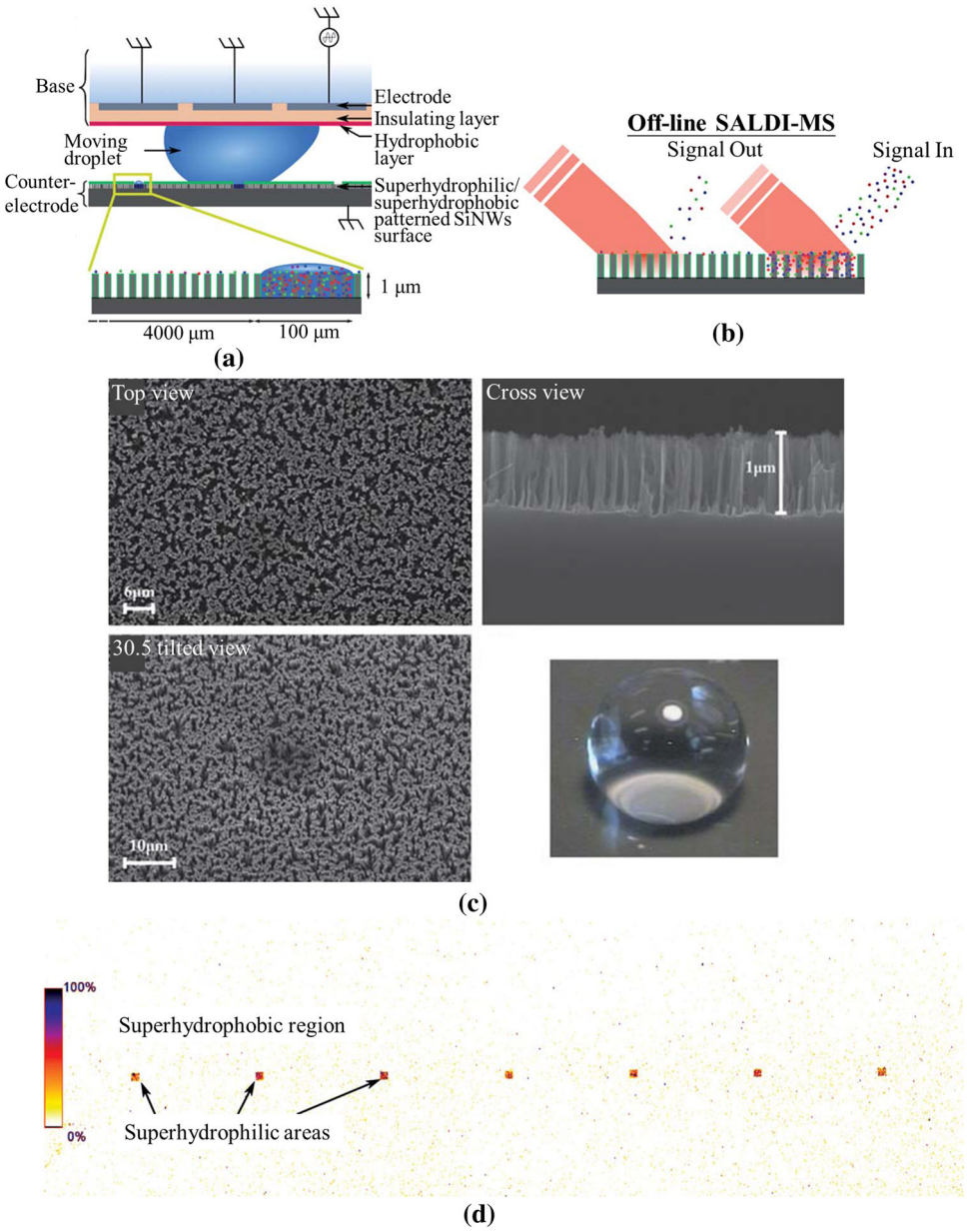

Fig. 6 DMF system applied in SALDI-MS protein analysis. **a** A cross-sectional view of the DMF device and the magnified view of liquid impregnation inside the superhydrophilic area. The *top* plate was designed with droplets actuation electrodes, and the bottom plate was the ground electrode and coated with a layer of superhydrophobic nanowire. **b** Schematic diagram of the LDI-MS analysis on the superhydrophobic silicon nanowire surface (Signal Out) and on the superhydrophilic silicon nanowire region (Signal In). **c** SEM images of the superhydrophobic nanowire on the *bottom* plate and an image of a spherical water droplet deposited on the superhydrophobic nanostructured surface. **d** Small superhydrophilic spots (less than 250 μm) were designed on the superhydrophobic nanowire for sample adsorption

respectively. The sample actuation electrodes were designed on the top plate; the bottom plate for MS analysis was coated with a layer of a superhydrophobic nanowire as shown in the SEM images in Fig. 6c. Some small superhydrophilic spots pf size about 250 μm were made on the superhydrophobic nanowire for sample adsorption as shown in Fig. 6d; a small sample would remain on the small superhydrophilic spots when the droplets passed through the region. Once the samples were trapped on the superhydrophilic areas, the bottom plate was transferred to a MS for peptide analysis. The results of that analysis proved that the DMF system is applicable to both SALDI and MALDI mass spectral analysis.

Wheeler et al. categorized some research into three classes—indirect off-line, direct off-line, and in-line MS analysis (Kirby and Wheeler 2013). Initially in the DMF proteomic research, the microfluidic chips served only as a platform for sample pretreatment. After the protein in the droplets was reduced, digested, and treated with DHB on DMF actuation, the samples were manually transferred to a MS for analysis. The indirect off-line MS analysis required no DMF chip modification, which is a simple and easy way for ranges of DMF applications (Jebrail and Wheeler 2009; Yang et al. 2011; Nelson et al. 2010). The DMF system was improved for further integration with a MS system by geometric design in a direct off-line MS analysis. The DMF chips might be designed as a cartridge, which can be inserted in the MS for protein analysis after DMF sample processing. This sample transfer is more convenient than the indirect off-

line analysis (Aijian et al. 2012; Nelson et al. 2010; Lapierre et al. 2011).

4.2.3 *Eelectrospray ionization (ESI)-MS*

A manual sample transfer in both indirect and direct off-line MS analysis is unfavorable during a DMF analysis. In-line analysis is proposed using sample electrospray ionization (ESI) for totally automatic MS analysis of proteomics. ESI is another nonvolatile method of biomacromolecular ionization used for MS analysis (Karas et al. 2000); an electrolyte-containing peptide or protein solution is pumped into the needle biased to a large potential. The electric field generated at the tip of needle makes the sample solution become dispersed into an aerosol of charged droplets, which is then injected into the vacuum of the MS for analysis. In the in-line ESI–MS system, the sample transfer from the DMF chip to the MS is an automatic and continuous process. There is generally a channel designed to form an interface between a DMF and a MS, such as a tube, capillary, or needle. In this way, sample transfer by hand is no longer necessary after DMF sample processing. In 2012, Roper et al. used an eductor to connect between a DMF device and a MS machine (Baker and Roper 2012). The eductor comprised a transfer capillary of size 150 μm o.d. × 100 μm i.d. and a tapered nozzle. One end of the capillary was placed between the top and bottom plates of a DMF chip, forming a sandwich structure, and the other end was connected with the tapered nozzle. N_2 was pushed into the eductor, which created a pressure gradient at the tip of nozzle and made the sample droplets in DMF platform become pulled into the MS system for analysis through the eductor. Droplets containing caffeine at varied concentrations were mixed on EWOD actuation and merged with droplets of theophylline, and then delivered to the mass spectrometer for analysis.

Another research team led by Wheeler used a DMF device for preparation of dried blood spot (DBS) samples. With the design of a glass capillary emitter, samples were automatically transferred into an ESI–MS for analysis (Shih et al. 2012). Figure 7a shows the sandwiched capillary interface of a DMF and an ESI–MS. The DBS is a blood sample blotted and dried on filter paper, which can be easily stored, delivered, and analyzed. The DBS from a newly born baby was analyzed

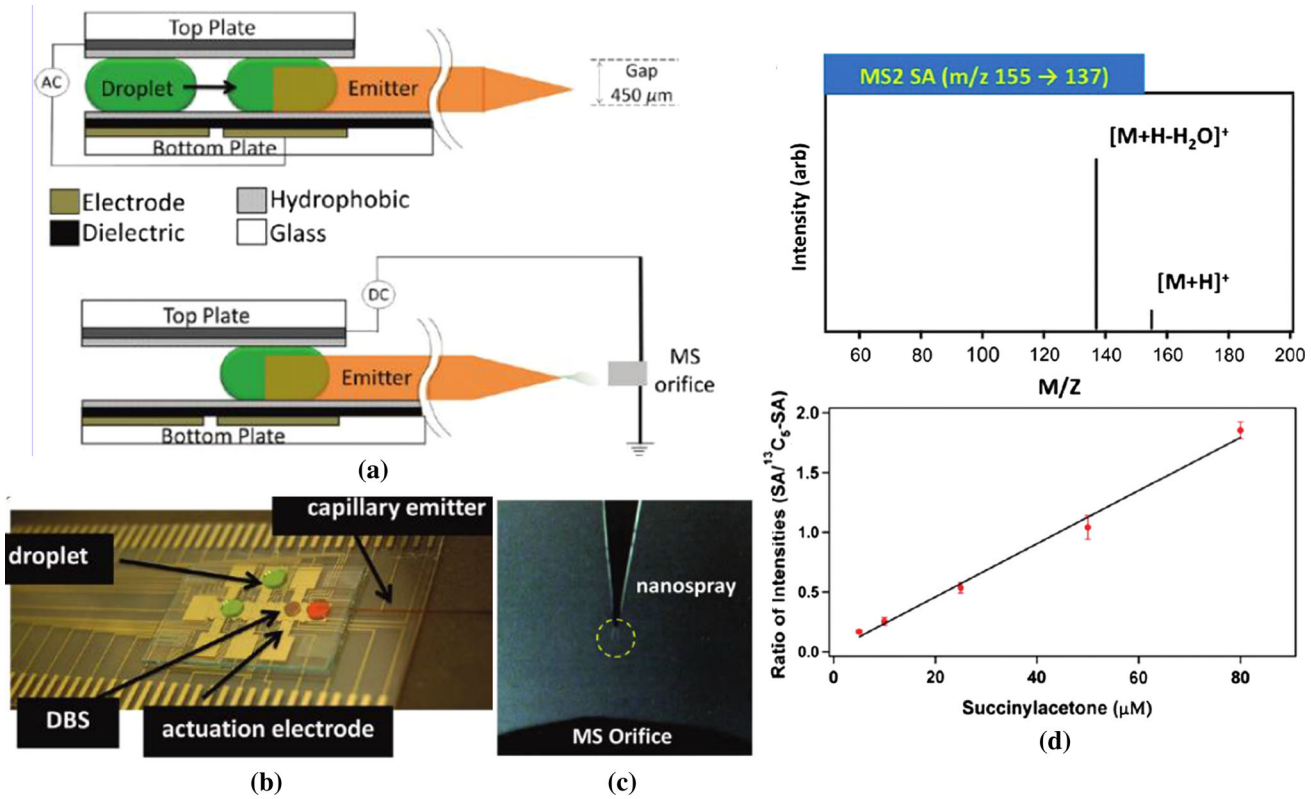

Fig. 7 DMF device for DBS samples preparation. **a** The cross-section view of the parallel-plate EWOD chip with sandwiched capillary for in-line MS sample transferring. **b** An image of the in-line DMF analysis chip with a 3.2-mm-diameter DBS deposited on the bottom plate. **c** Image of spray generated at the tip of the capillary emitter. **d** SA analysis data by MS. The *top* one is the spectrum of derivatized SA after collision-induced dissociation, and the *bottom* one is the calibration curve of spiked SA in dried blood spot punches

on their DMF platform for the evaluation of succinylacetone (SA) as a biochemical hallmark for hepatorenal tyrosinemia, the most severe form of tyrosinemia. At the beginning of the DBS processing, DBS (diameter 3.2 mm) was positioned on the EWOD bottom plate as shown in Fig. 7b. The analytes in DBS were extracted into the solvent droplets followed by EWOD droplets actuation in nine steps. The analyte contained solvent was transported to the entrance of the capillary emitter so that it was spontaneously filled with capillary action. An electric potential was applied between the DMF electrode and the MS to spray the sample into the MS; the image of spray is shown in Fig. 7c. The spectrum in Fig. 7d shows the SA analysis data in the MS. To quantify the SA, their DMF device was useful for the identification of amino acids in DBS.

In Kirby and Wheeler (2013), Wheeler reported another in-line analysis system based on the ESI–MS, proposing a novel and interesting flexible DMF device for the Morita–Baylis–Hillman (MBH) reaction. The flexible chip contained two parts of EWOD electrodes; one region was designed with parallel-plate electrodes for the chemical reaction, and another region with a single-plate electrode design could be folded into the conical ESI emitter at the end of electrode, as shown in Fig. 8a. First, the MBH reagents and the catalyst were loaded into the parallel-plate reservoirs, followed by a DMF-actuated method in six steps. After the chemical reaction, the sample mixture was delivered to the single-plate region and eventually reached the tip of the conical ESI emitter by automatic DMF actuation. The droplet in the emitter was sprayed into the MS under a large DC potential; the ESI–MS emitter and the electrospray plume are seen in Fig. 8b. The result of MS detection is shown in Fig. 8c, d; from the mass spectrum, the signals of two intermediates and the reaction product were analyzed under three time intervals. The flexible DMF platform proposed in this research can be highly integrated with a MS; the folded conical ESI emitter design replaced the capillary and nozzle for sample transfer from the DMF to the MS; we believe that this achievement has represented an exciting milestone for the *lab-on-a-chip*.

4.3 Immunoassays

In biomedical research, an immunoassay is a method to detect the biomolecule concentration in an aqueous solution using antibodies or immunoglobulin (Ig). The concept of a two-site immunoassay is based on the specific binding of a target biomolecule, which is also called an analyte, with an antibody. The analyte would be trapped with the antibody anchored on the surface of the reaction wells. Another detectable label would serve for specific binding with the analyte for signal labeling. The signal of a label is detectable because it emits light or fluorescence. After reaction, the concentration of the analyte can be known when the signal of the label is analyzed.

Without a continuous microfluidic channel, actuation based on a digital droplet makes DMF a powerful tool when applied in the immunoassay. By convenient manipulation of a droplet, complex immunoassay reactions such as washing and incubation steps can be done on the DMF system. In 2008, Pamula's research team proposed a DMF-based immunoassay (Sista et al. 2008) in which magnetic beads were manipulated with actuation by digital droplets as the carrier of the heterogeneous sandwich immunoassay. Beads could be attracted, washed, and resuspended in the reaction solution. Magnetic beads played an important role for immunoassay on the DMF platform. An antibody was modified on the surface of the beads for the recognition of an analyte. After the binding process, the washing protocol was demonstrated by DMF actuation to discard the unbound impurities. The magnetic beads were immobilized with a permanent magnet; the excess supernatant was split with electrowetting electrodes. Heterogeneous immunoassays on human insulin and interleukin-6 (IL-6) were brought to the DMF system. Later in 2010, Kim's research team used a similar immunoassay concept for the specific binding of cells on magnetic beads (Shah et al. 2010). $CD8^+$ T-lymphocytes were recognized and bound with anti-CD8 antibiotics anchored on the surface of the magnetic beads. A magnet was also used in a DMF chip for immobilization of beads, separation, and concentration. These authors claimed that, in the future, this cell-based immunoassay technique might be applied in other tumor cell selection or cell-screening technology. The same year, Wheeler's research team brought the DMF device into a heterogeneous sandwich immunoassay (Miller et al. 2011); they used human immunoglobulin G (IgG), an antibody isotype of one kind, as an analyte. To detect IgG, an anti-IgG antibody was anchored on the surface of the EWOD hydrophobic layer. The label used to measure the signal was FITC-labeled anti-IgG, which emitted fluorescence after binding with the analyte. Actuation of DMF droplets was used for all washing and incubation steps, which replaced traditional tube pipetting. The immunoassay was done on the DMF system; IgG was also detected in serum samples containing interfering proteins. These authors concluded that manipulation of the DMF droplets could decrease the analysis duration and reagent volume of an immunoassay reaction and has potential as a method with large throughput, little waste, and cheap detection of biomolecules. Wheeler published other DMF immunoassay research using magnetic beads that served as carriers for the particle-based immunoassay (Ng et al. 2012). The antibody for an analyte detection was anchored on the

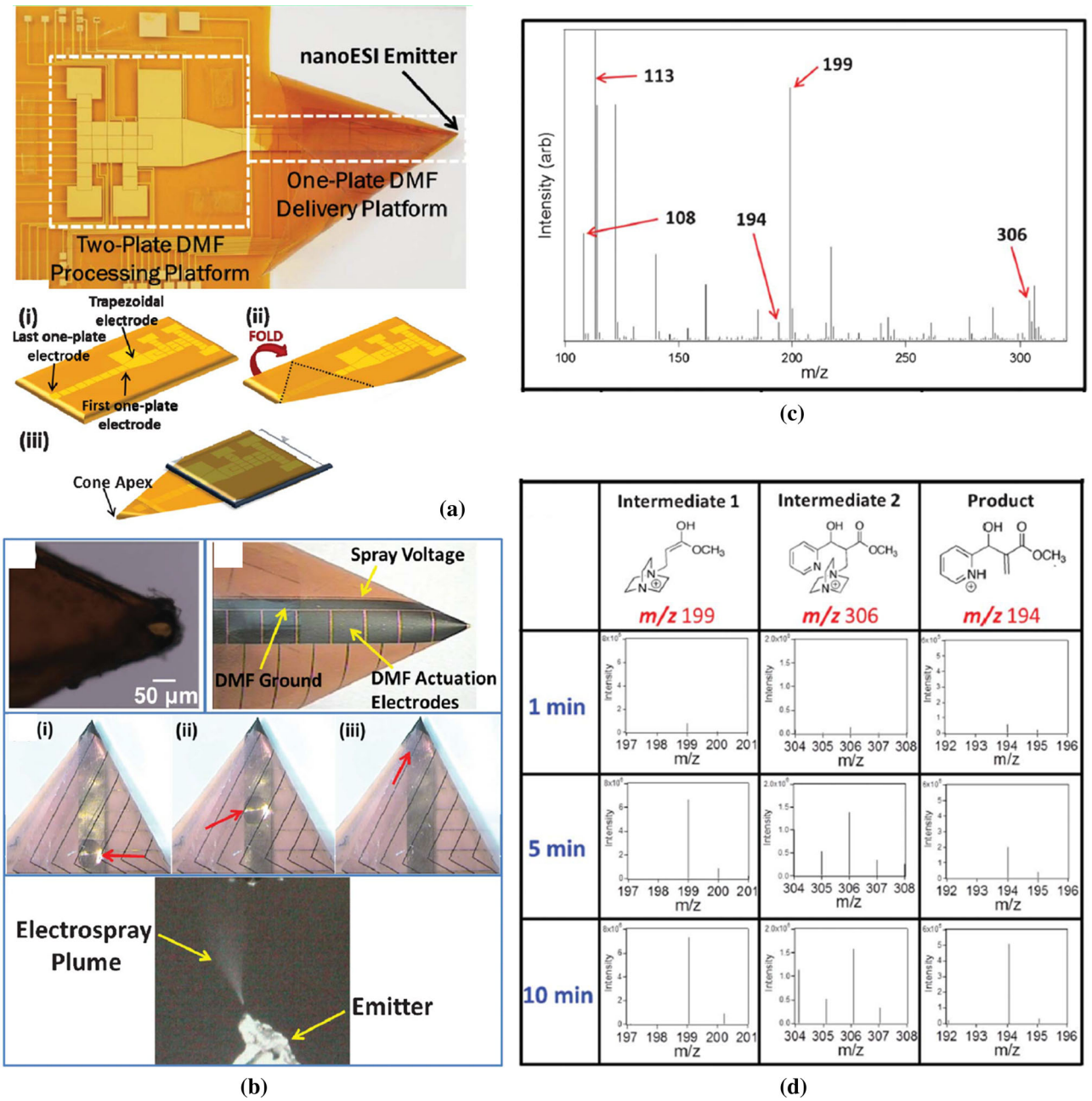

Fig. 8 Flexible DMF chip with for MBH reaction. **a** The flexible EWOD chip design for in-line MS integration, there were two parts of EWOD electrodes for chemical reaction and sample transfer. **b** One region of the chip was folded into the conical ESI emitter, the droplets could be manipulated by the coplanar electrodes design in this region. **c** *Top* one is the mass spectrum of a MBH reaction implemented and analyzed by this in-line MS analysis system, the *five peaks* in the spectrum shows the signals of 2-pyridine carboxaldehyde (m/z 108), DABCO catalyst (m/z 113), intermediate 1 (m/z 199), intermediate 2 (m/z 306), and the product (m/z 194). **d** The intensity of two intermediates and the reaction product were analyzed under three different time intervals

surface of the magnetic beads instead of the surface of an EWOD hydrophobic layer for both noncompetitive and competitive immunoassays as shown in Fig. 9a; the parallel-plate DMF schematic is shown in Fig. 9b. The DMF chip contained ten reservoir electrodes to accommodate the reagents and arrays of droplet actuation electrodes for the sample preparation in the immunoassay. The authors proposed a protocol for supernatant separation washing to increase the washing efficiency. The traditional dilution washing and supernatant separation washing protocols are shown in Fig. 9c and d, respectively. With the excellent separation efficiency (90 %) of magnetic beads proposed in this paper, the supernatant separation is much more efficient than serial dilution, as in Fig. 9e. The

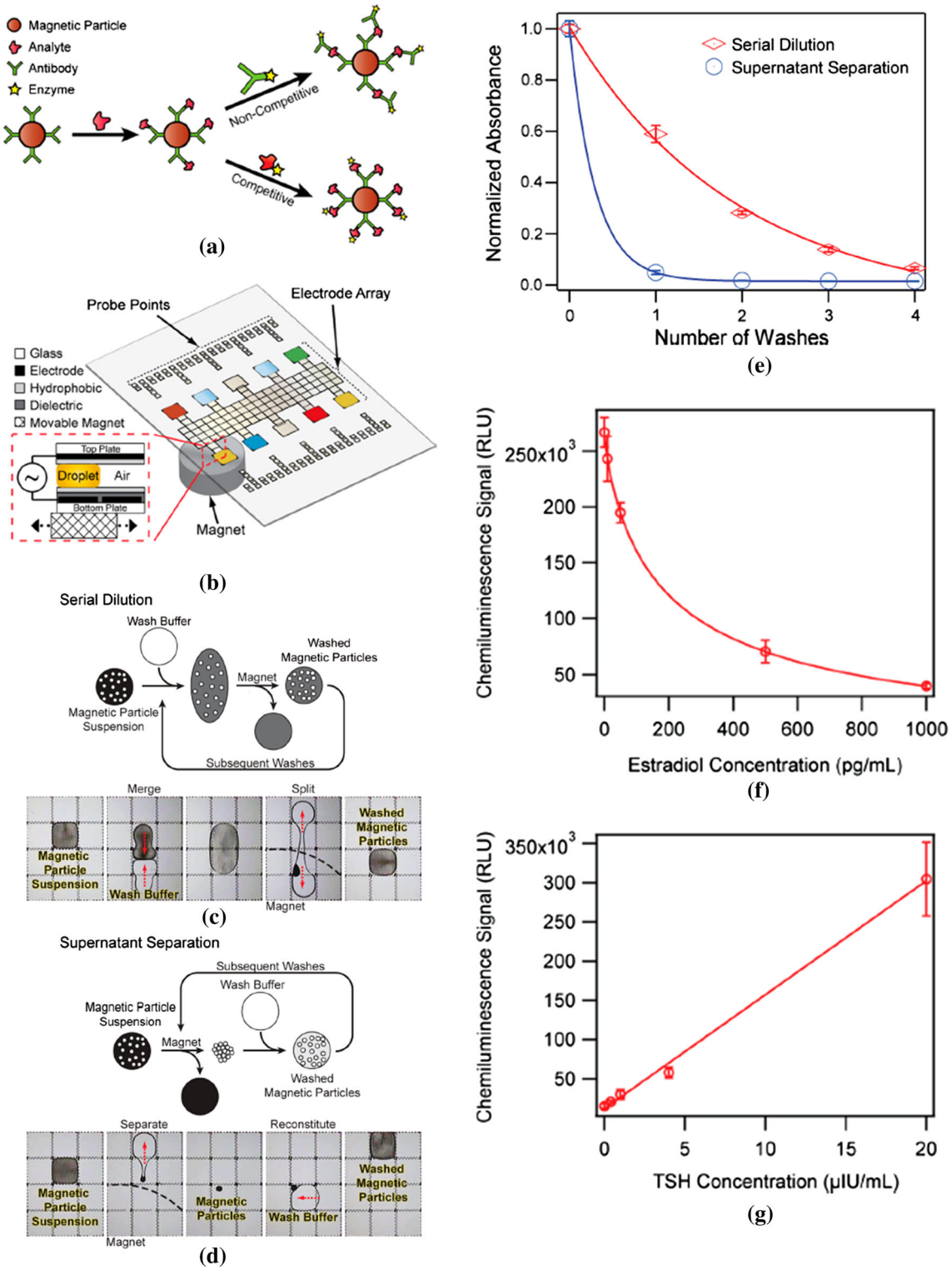

Fig. 9 DMF system for immunoassay by using magnetic beads. **a** Concepts of noncompetitive and competitive immunoassays. **b** Schematic of the parallels-plates EWOD chip. **c** Scheme and video sequence of serial dilution washing protocol. **d** Scheme (*top*) and video sequence (*bottom*) of serial dilution washing protocol. **e** The efficiency of the two dilution methods were compared by measuring enzyme activity in the supernatant after each washing cycle

immunoassays in this work used a thyroid-stimulating hormone (TSH) as a noncompetitive immunoassay analyte and 17β-estradiol (E2) for a competitive immunoassay analyte. The magnetic beads were coated with anti-E2 or anti-TSH, which would bind specifically with E2 or TSH, respectively. The magnetic beads were resuspended in one droplet of a conjugate, which contained HRP-labeled antibody or analyte. After HRP labeling, one droplet of the luminol or enhancer solution was merged with H_2O_2 to activate enzymatically driven chemiluminescence. Between each step, the beads were separated from the solution and washed with DMF actuation to discard unbound impurities. Finally, the authors concluded that their DMF system was successfully applied in the particle-based immunoassay with reduced reagent volumes and analysis time by 100-fold and 10-fold, respectively.

5 Cell-based applications

Many researchers tried to use a microfluidic device for cell-based applications because the designed microfluidic system has the advantages of little consumption of reagents, rapid reactions, and modest cost. When cells were manipulated or cultured in a continuously flowing channel, a contamination problem existed, however, and it was difficult to manipulate a single cell or few cells in the continuous microfluidic devices. Digital microfluidic systems tend to solve the contamination issue and to provide the feasibility for manipulation of single-cell droplets. Much EWOD research was devoted to issues related to cells, such as their separation, their culture, tissue engineering, cell-based biosensors, and drug screening. Contamination of the EWOD chip surface remains a serious problem when the biomedical reagents such as a DNA solution, protein solution, culture medium, buffer solution, or reaction enzymes are used in EWOD researches. Biomolecules would stick on the chip surface and decrease the hydrophobicity of hydrophobic layer, which increases the operating voltage of manipulation of sample droplets. If a hydrophobic surface was seriously contaminated, droplets could never be manipulated again. Chemical additives are sometimes used to prevent protein adsorption (Au et al. 2011). Pluronic in a small dose is a proven nontoxic and safe surfactant, which can be applied in cell culturing or tissue engineering. Luk, Wheeler, and their research team used Pluronic F127 to solve the sticky problems in digital microfluidics (Luk et al. 2008). They observed the protein-sticking problem on the Teflon-AF hydrophobic surface of EWOD devices using a confocal microscope. Pluronic F127 additive (0.08 % mass/volume) was added to the biomedical solutions to decrease biofouling and to enhance protein stability. The authors suggested that the surfactant layers would form at the solid/liquid interface in droplets, which prevents biomolecule such as bovine serum albumin (BSA), fibrinogen (Fb), and casein adsorbing on the hydrophobic surface. Secondary ion mass spectrometry was used to prove their hypothesis.

As DMF was already used for sample droplets containing biomolecules or cells manipulation, some workers tried to use a DMF platform for cell culture, but they found that DMF chips could not be directly used for cell culture; a specific region of a hydrophobic layer must be modified with molecules for cell adhesion, such as selectins, integrins, or cadherins. In 2011, Lammertyn's research team used lift-off fabrication to define the regions of cell culture (Witters et al. 2011). Islands of the poly-L-lysine (PLL) peptide promoting cell adhesion were deposited on the Teflon-AF hydrophobic layer. Cells could attach on these patches and remained viable for up to 3 days.

DMF has become an active topic of research for biomedical application; many investigations of EWOD involved efforts to decrease the voltage for droplet actuation to decrease the adverse effects of actuation on the biomedical sample. In general, the actuation voltage of biomedical sample droplets ranges from 50 to 250 V when applied in biomedical research, which depends on the material and thickness of the hydrophobic and dielectric layers. Some authors have already indicated that charges in the dielectric layer induced with a large voltage would affect cell viability. Most workers on DMF cell culture tried to avoid this serious cell viability problem; a hydrophilic region on the surface of DMF device without an electrode underneath was reserved as the cell culture region. Witters in 2011 proposed a new fabrication to create hydrophilic regions on the hydrophobic surface of an EWOD chip. Parylene-C served as a mask for a dry lift-off to remove the hydrophobic Teflon-AF on the EWOD chip as the cell culture region (Witters et al. 2011). Electrodes of two kinds were designed with a PLL-micropatch (600×600 mm^2) or PLL-micropatch arrays (15×15 μm^2) as the cell culture regions. The cells could be successfully seeded on the cell culture regions by transporting a medium (50 nL) containing HeLa cells onto the cultural electrodes. Wheeler's research group exerted much effort in DMF cell culture research; they proposed an EWOD platform for complete cell culture of a mammalian cell culture in 2010 (Barbulovic-Nad et al. 2010). The device comprised seven reservoirs and an array of actuation electrodes; the latter were used to manipulate droplets (1 μL). They designed adhesion pads near the actuation electrodes for adhesion and culturing of mammalian cells. With this adhesion pad design, they seeded the CHO-K1 cells on the chip surface with DMF droplet transport; the attached cell was dissociated from the surface of the pad

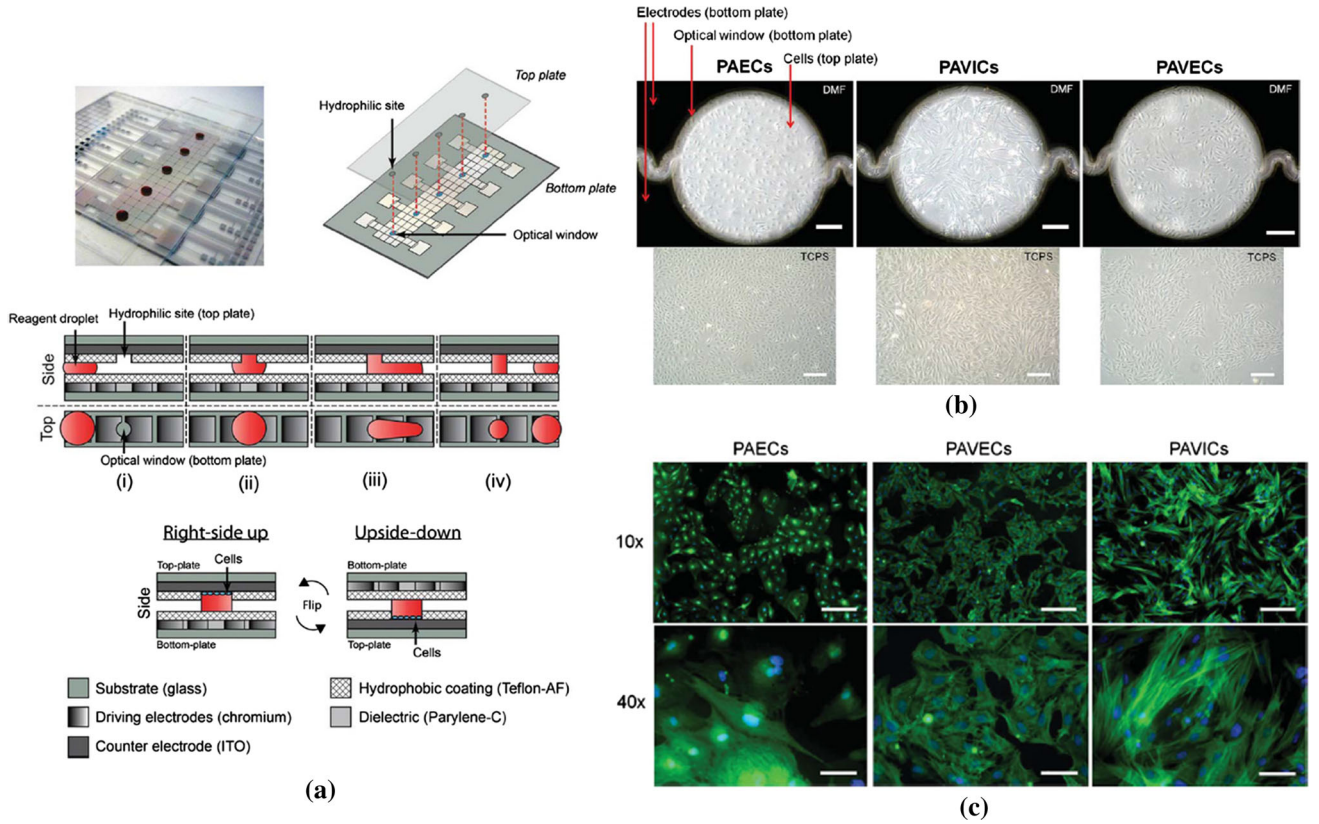

Fig. 10 DMF upside-down platform for cell culture. **a** An image and a schematic diagram of the parallel-plate EWOD chip. **b** *Top* and cross-sectional schematics of passive dispensing on hydrophilic sites. When a droplet was manipulated across the hydrophilic site, part of the droplet remained on the hydrophilic region and formed a virtual microwell. **c** Cross-sectional view of device orientation during experimentation. The chip was maintained right-side up during droplet actuation and was positioned upside-down for cell culturing. **d** Phase-contrast images of PAECs (*left*), PAVICs (*middle*), and PAVECs (*right*) cultured on the DMF. **e** Fluorescent images of PAECs (*left*), PAVECs (*middle*), and PAVICs (*right*) after fixing, permeabilizing, and staining on a DMF device

after delivery of trypsin. In 2012, Wheeler's research team provided an *upside-down* cell culture method by creating regions of cell culture on the top cap of a parallel-plate EWOD chip (Srigunapalan et al. 2012). The fabrication with fluorocarbon lift-off creates circles (diameter 1.5 mm) of a hydrophilic region on the hydrophobic cap for cell attachment; an image and a schematic diagram of the EWOD chip is shown in Fig. 10a. In the beginning of the experiment, a medium droplet containing cells was manipulated across the hydrophilic site, and part of the droplet with cells would remain on the hydrophilic region and formed a virtual microwell as illustrated in Fig. 10b. After the formation of microwell, the DMF chip was positioned upside-down for cell culturing as shown in Fig. 10c. Figure 10d shows the phase-contrast images of cells of three kinds including porcine aortic endothelial cells (PAEC), porcine aortic valve endothelial cells (PAVEC), and porcine aortic valvular interstitial cells (PAVIC) attached to the hydrophilic regions of the top plate and cultured upside-down on the chip for a week. To record fluorescent cell images as shown in Fig. 10e, cell fixation, permeabilization, and staining were done with EWOD droplet manipulation. This stage of cell culture was used in experiments on endothelial cell/monocyte adhesion to evaluate the potential of DMF systems for co-culture and multistep assay.

6 Clinical applications

Portable instruments for immediate recording of human health or analysis of patient blood samples are required today. For this purpose, LOC biochips of various kinds are proposed for clinical diagnosis on chip to bring the profit for human health and to reach the target market. The DMF system has a high potential as a tool for clinical diagnosis based on its excellent sample manipulation. Diagnostic procedures typically include the biomolecule measurement, biomarker detection, biochemical reaction, and cell culture. These related DNA, protein, and cell-related topics have already been presented on the DMF system and discussed in chapters 2–4. By integrating the biomedical analysis and

biochemical reaction on the chip, the DMF system is expected to be a powerful tool for clinical diagnosis. Some researchers already used the DMF system for analysis of whole blood in simple clinical diagnostic processes. Pollack and Mitchell et al. used the DMF as a RT-PCR platform for DNA detection in methicillin-resistant *Staphylococcus aureus* (MRSA) (Hua et al. 2010), *Mycoplasma pneumoniae* (Wulff-Burchfield et al. 2010), and *Candida albicans* (*C. albicans*) (Schell et al. 2012) from infected patient blood specimens. *Mycoplasma pneumoniae* is a bacterium and a human pathogen causing community-acquired pneumonia. *Candida albicans* is a genus of yeasts causing fungal infections. Casper et al. applied the DMF in estrogen extraction and quantification from breast tissue homogenate, whole blood, and serum (Mousa et al. 2009). Estrogen is a female sex hormone in human reproductive physiology, which is also an important biomarker in the pathogenesis of breast cancer.

In 2013, Pamula et al. reported research based on a DMF system with high throughput and rapid analysis for newborn screening (NBS). NBS is a health test to screen the condition of infants at birth for inapparent harmful and fatal disorders. DBS samples were analyzed with a disposable DMF device for performing the five-plex fluorometric enzymatic assays for Pompe, Fabry, Hunter, Gaucher, and Hurler diseases. The analysis steps included sample preparation, loading on a DMF chip, on-chip sample analysis, and data analysis with software. The DMF was responsible for automatic sample manipulation, incubation, and reaction. As a diagnostic method with large throughput, 44 DBS samples were processed within 3 h on a single analyzer; on grouping four analyzers, 176 samples were processed within 3 h. An important point of this research was that the DMF chip was disposable, which means that the cost of their DMF platform has been cut and the reliability was improved; both factors are necessary for a platform for general clinical diagnostics.

7 Conclusions and future outlook

Researchers using biomedical microelectromechanical systems (bio-MEMS) are interested in lab-on-a-chip (LOC) devices and micrototal analysis systems (μTAS). The electrowetting-on-dielectric (EWOD) technique has been used in a digital microfluidic system (DMF) for bio-applications in past decade. Bio-reagents can be prepared, including generation, transport, separation, mix, and reaction on actuation with EWOD electrodes, either parallel plates type or coplanar type of EWOD system. Due to its lots of advantages and portability, an EWOD-based DMF can hence been widely applied in biomedical or chemical fields as a powerful platform. This technology would hence bring considerable advantage for human health and commercial possibilities in the market, such as involving DNA assays, proteomics, cell assays, and immunoassays, as reviewed in the paper. High throughput and small volume analysis of DNA assays or protein array can be accomplished precisely using EWOD DMF, which would be ready for commercial product if contamination and evaporation problems can be well solved. Since cancer has become one of the serious diseases for human, cell-based EWOD DMF has been developing that would become one of the potential products in the future.

References

Abdelgawad M, Wheeler AR (2007) Rapid prototyping in copper substrates for digital microfluidics. Adv Mater 19:133

Abdelgawad M, Wheeler AR (2008) Low-cost, rapid-prototyping of digital microfluidics devices. Microfluid Nanofluid 4:349–355

Abdelgawad M, Freire SLS, Yang H, Wheeler AR (2008) All-terrain droplet actuation. Lab Chip 8:672–677

Abdelgawad M, Watson MWL, Wheeler AR (2009) Hybrid microfluidics: a digital-to-channel interface for in-line sample processing and chemical separations. Lab Chip 9:1046–1051

Aijian AP, Chatterjee D, Garrell RL (2012) Fluorinated liquid-enabled protein handling and surfactant-aided crystallization for fully in situ digital microfluidic MALDI-MS analysis. Lab Chip 12:2552–2559

Au SH, Kumar P, Wheeler AR (2011) A new angle on Pluronic additives: advancing droplets and understanding in digital microfluidics. Langmuir 27:8586–8594

Baker CA, Roper MG (2012) Online coupling of digital microfluidic devices with mass spectrometry detection using an eductor with electrospray ionization. Anal Chem 84:2955–2960

Barbulovic-Nad I, Yang H, Park PS, Wheeler AR (2008) Digital microfluidics for cell-based assays. Lab Chip 8:519–526

Barbulovic-Nad I, Au SH, Wheeler AR (2010) A microfluidic platform for complete mammalian cell culture. Lab Chip 10:1536–1542

Bartlett JM, Stirling D (2003) A short history of the polymerase chain reaction. Methods Mol Biol 226:3–6

Beni G, Hackwood S (1981) Electro-wetting displays. Appl Phys Lett 38:207–209

Berge B (1993) Electrocapillarity and wetting of insulator films by water. Comptes Rendus de l'Academie des Sciences Serie II 317:157–163

Boles DJ, Benton JL, Siew GJ, Levy MH, Thwar PK, Sandahl MA et al (2011) Droplet-based pyrosequencing using digital microfluidics. Anal Chem 83:8439–8447

Chang YH, Lee GB, Huang FC, Chen YY, Lin JL (2006) Integrated polymerase chain reaction chips utilizing digital microfluidics. Biomed Microdevices 8:215–225

Chen C-H, Yao D-J, Tseng S-H, Lu S-W, Chiao C-C, Yeh S-R (2009) Micro-multi-probe electrode array to measure neural signals. Biosens Bioelectron 24:1911–1917

Chen C-H, Su H-C, Chuang S-C, Yen S-J, Chen Y-C, Lee Y-T, Chen H, Yew T-R, Chang Y-C, Yeh S-R, Yao D-J (2010) Hydrophilic modification of neural microelectrode arrays based on multi-walled carbon nanotubes. Nanotechnology 21:485501

Chen C-H, Chuang S-C, Su H-C, Hsu W-L, Yew T-R, Chang Y-C et al (2011) A three-dimensional flexible microprobe array for neural recording assembled through electrostatic actuation. Lab Chip 11:1647–1655

Chen YT, Chang WC, Fang WF, Ting SC, Yao DJ, Yang JT (2012) Fission and fusion of droplets in a 3-D crossing microstructure. Microfluid Nanofluid 13:239–247

Chen C-H, Lin C-T, Hsu W-L, Chang Y-C, Yeh S-R, Li L-J et al (2013) A flexible hydrophilic-modified graphene microprobe for neural and cardiac recording. Nanomed Nanotechnol Biol Med 9:600–604

Cho SK, Moon HJ, Kim CJ (2003) Creating, transporting, cutting, and merging liquid droplets by electrowetting-based actuation for digital microfluidic circuits. J Microelectromech Syst 12:70–80

Cho SK, Zhao YJ, Kim CJ (2007) Concentration and binary separation of micro particles for droplet-based digital microfluidics. Lab Chip 7:490–498

Chokkalingam V, Tel J, Wimmers F, Liu X, Semenov S, Thiele J et al (2013) Probing cellular heterogeneity in cytokine-secreting immune cells using droplet-based microfluidics. Lab Chip 13:4740–4744

Clausell-Tormos J, Lieber D, Baret JC, El-Harrak A, Miller OJ, Frenz L et al (2008) Droplet-based microfluidic platforms for the encapsulation and screening of Mammalian cells and multicellular organisms. Chem Biol 15:427–437

Davoust L, Fouillet Y, Malk R, Theisen J (2013) Coplanar electrowetting-induced stirring as a tool to manipulate biological samples in lubricated digital microfluidics. Impact of ambient phase on drop internal flow patterna. Biomicrofluidics 7:044104

Ding H, Sadeghi S, Shah GJ, Chen S, Keng PY, Kim C-J et al (2012) Accurate dispensing of volatile reagents on demand for chemical reactions in EWOD chips. Lab Chip 12:3331–3340

Dryden MDM, Rackus DDG, Shamsi MH, Wheeler AR (2013) Integrated digital microfluidic platform for voltammetric analysis. Anal Chem 85:8809–8816

Elahi E, Ronaghi M (2004) Pyrosequencing: a tool for DNA sequencing analysis. Methods Mol Biol 255:211–219

Estes MD, Do J, Ahn CH (2009) On chip cell separator using magnetic bead-based enrichment and depletion of various surface markers. Biomed Microdevices 11:509–515

Eydelnant IA, Uddayasankar U, Li BY, Liao MW, Wheeler AR (2012) Virtual microwells for digital microfluidic reagent dispensing and cell culture. Lab Chip 12:750–757

Fan SK, Huang PW, Wang TT, Peng YH (2008) Cross-scale electric manipulations of cells and droplets by frequency-modulated dielectrophoresis and electrowetting. Lab Chip 8:1325–1331

Fan SK, Chiu CP, Lin JW (2009) Electrowetting on polymer dispersed liquid crystal. Appl Phys Lett 94:164109

Fan SK, Yang HP, Hsu WY (2011a) Droplet-on-a-wristband: chip-to-chip digital microfluidic interfaces between replaceable and flexible electrowetting modules. Lab Chip 11:343–347

Fan SK, Hsu YW, Chen CH (2011b) Encapsulated droplets with metered and removable oil shells by electrowetting and dielectrophoresis. Lab Chip 11:2500–2508

Folch i Folch A (2013) Introduction to bioMEMS. CRC Press, Boca Raton

Gong J, Kim CJ (2008) All-electronic droplet generation on-chip with real-time feedback control for EWOD digital microfluidics. Lab Chip 8:898–906

Hao H-C, Chang H-Y, Wang T-P, Yao D-J (2013) Detection of cells captured with antigens on shear horizontal surface-acoustic-wave sensors. J Lab Autom 18:69–76

Hsien-Hua Shen H-YT, Yao D-J (2013) Single mouse oocyte encapsulated in medium-in-oil microdroplets by using a PDMS microfluidic device. Sens Mater 26:85–94

Hua ZS, Rouse JL, Eckhardt AE, Srinivasan V, Pamula VK, Schell WA et al (2010) Multiplexed real-time polymerase chain reaction on a digital microfluidic platform. Anal Chem 82:2310–2316

Huang HY, Wu TL, Huang HR, Li CJ, Fu HT, Soong YK, Lee MY, Yao DJ (2014) Isolation of motile spermatozoa with a microfluidic chip having a surface-modified microchannel. J Lab Autom 19:91–99

Jebrail MJ, Wheeler AR (2009) Digital microfluidic method for protein extraction by precipitation. Anal Chem 81:330–335

Jiang X, Jing W, Zheng L, Liu S, Wu W, Sui G (2014) A continuous-flow high-throughput microfluidic device for airborne bacteria PCR detection. Lab on a Chip 14:671–676

Jiangang W, Ruifeng Y, Xuefeng Z, Ming K, Zheyao W, Litian L (2006) An open-configuration electrowetting-based biofluidics actuation for preventing biomolecular adsorption. In: 1st IEEE international conference on nano/micro engineered and molecular systems, 2006. NEMS '06, pp 1152–1155

Jones TB, Wang KL, Yao DJ (2004) Frequency-dependent electromechanics of aqueous liquids: electrowetting and dielectrophoresis. Langmuir 20:2813–2818

Karas M, Bahr U, Dülcks T (2000) Nano-electrospray ionization mass spectrometry: addressing analytical problems beyond routine. Fresenius J Anal Chem 366:669–676

Kim H, Bartsch MS, Renzi RF, He J, Van de Vreugde JL, Claudnic MR et al (2011) Automated digital microfluidic sample preparation for next-generation DNA sequencing. JALA 16:405–414

Kirby AE, Wheeler AR (2013a) Digital microfluidics: an emerging sample preparation platform for mass spectrometry. Anal Chem 85:6178–6184

Kirby AE, Wheeler AR (2013b) Microfluidic origami: a new device format for in-line reaction monitoring by nanoelectrospray ionization mass spectrometry. Lab Chip 13:2533–2540

Kise DP, Magana D, Reddish MJ, Dyer RB (2014) Submillisecond mixing in a continuous-flow, microfluidic mixer utilizing mid-infrared hyperspectral imaging detection. Lab on a Chip 14:584–591

Koo B, Kim C-J (2013) Evaluation of repeated electrowetting on three different fluoropolymer top coatings. J Micromech Microeng 23:067002

Lapierre F, Piret G, Drobecq H, Melnyk O, Coffinier Y, Thomy V et al (2011) High sensitive matrix-free mass spectrometry analysis of peptides using silicon nanowires-based digital microfluidic device. Lab Chip 11:1620–1628

Lee J, Moon H, Fowler J, Chang-Jin K, Schoellhammer T (2001) Addressable micro liquid handling by electric control of surface tension. In: The 14th IEEE international conference on micro electro mechanical systems, 2001. MEMS 2001, pp 499–502

Lee J, Moon H, Fowler J, Schoellhammer T, Kim C-J (2002) Electrowetting and electrowetting-on-dielectric for microscale liquid handling. Sens Actuators, A 95:259–268

Lin TH, Yao DJ (2012) Applications of EWOD Systems for DNA Reaction and Analysis. J Adhes Sci Technol 26:1789–1804

Lin HC, Liu YJ, Yao DJ (2010) Core-shell droplets for parallel DNA ligation of an ultra-micro volume using an EWOD microfluidic system. JALA 15:210–215

Liu YJ, Yao DJ, Lin HC, Chang WY, Chang HY (2008) DNA ligation of ultramicro volume using an EWOD microfluidic system with coplanar electrodes. J Micromech Microeng 18:045017

Lo S-J, Yang S-C, Yao D-J, Chen J-H, Tu W-C, Cheng C-M (2013) Molecular-level dengue fever diagnostic devices made out of paper. Lab Chip 13:2686–2692

Luk VN, Mo GCH, Wheeler AR (2008) Pluronic additives: a solution to sticky problems in digital microfluidics. Langmuir 24:6382–6389

Malic L, Veres T, Tabrizian M (2009) Biochip functionalization using electrowetting-on-dielectric digital microfluidics for surface plasmon resonance imaging detection of DNA hybridization. Biosens Bioelectron 24:2218–2224

Miller E, Rotea M, Rothstein JP (2010) Microfluidic device incorporating closed loop feedback control for uniform and tunable production of micro-droplets. Lab Chip 10:1293–1301

Miller EM, Ng AHC, Uddayasankar U, Wheeler AR (2011) A digital microfluidic approach to heterogeneous immunoassays. Anal Bioanal Chem 399:337–345

Moon H, Wheeler AR, Garrell RL, Loo JA, Kim CJ (2006) An integrated digital microfluidic chip for multiplexed proteomic sample preparation and analysis by MALDI-MS. Lab Chip 6:1213–1219

Mousa NA, Jebrail MJ, Yang H, Abdelgawad M, Metalnikov P, Chen J, Wheeler AR, Casper RF (2009) Droplet-scale estrogen assays in breast tissue, blood, and serum. Sci Transl Med 1:1ra2

Nelson WC, Kim C-JC (2012) Droplet Actuation by Electrowetting-on-Dielectric (EWOD): a Review. J Adhes Sci Technol 26:1747–1771

Nelson WC, Peng I, Lee GA, Loo JA, Garrell RL, Kim CJ (2010) Incubated protein reduction and digestion on an electrowetting-on-dielectric digital microfluidic chip for MALDI-MS. Anal Chem 82:9932–9937

Ng AHC, Choi K, Luoma RP, Robinson JM, Wheeler AR (2012) Digital microfluidic magnetic separation for particle-based immunoassays. Anal Chem 84:8805–8812

Pollack MG, Fair RB, Shenderov AD (2000) Electrowetting-based actuation of liquid droplets for microfluidic applications. Appl Phys Lett 77:1725–1726

Ronaghi M (2001) Pyrosequencing sheds light on DNA sequencing. Genome Res 11:3–11

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA et al (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350–1354

Sanger F, Coulson AR (1975) A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J Mol Biol 94:441–448

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Schell WA, Benton JL, Smith PB, Poore M, Rouse JL, Boles DJ et al (2012) Evaluation of a digital microfluidic real-time PCR platform to detect DNA of Candida albicans in blood. Eur J Clin Microbiol Infect Dis 31:2237–2245

Seemann R, Brinkmann M, Pfohl T, Herminghaus S (2012) Droplet based microfluidics. Rep Prog Phys 75:016601

Shah GJ, Ohta AT, Chiou EPY, Wu MC, Kim CJ (2009) EWOD-driven droplet microfluidic device integrated with optoelectronic tweezers as an automated platform for cellular isolation and analysis. Lab Chip 9:1732–1739

Shah GJ, Veale JL, Korin Y, Reed EF, Gritsch HA, Kim CJ (2010) Specific binding and magnetic concentration of CD8+ T-lymphocytes on electrowetting-on-dielectric platform. Biomicrofluidics 4:44106

Shah GJ, Ding H, Sadeghi S, Chen S, Kim C-J CJ, van Dam RM (2013) On-demand droplet loading for automated organic chemistry on digital microfluidics. Lab Chip 13:2785–2795

ShaoNing P, Yi-Lun W, Chih-Ting L, Wu MC (2013) Isothermal real-time polymerase chain reaction detection of herpes simplex virus type 1 on a light-actuated digital microfluidics platform. In: The 17th international conference on solid-state sensors, actuators and microsystems (TRANSDUCERS & EUROSENSORS XXVII), 2013 Transducers & Eurosensors XXVII, pp 2791–2794

Shen H-H, Su T-Y, Liu Y-J, Chang H-Y, Yao D-J (2013) Single-nucleotide polymorphism detection based on a temperature-controllable electrowetting on dielectrics digital microfluidic system. Sens Mater 25:643–651

Shen HH, Su TY, Liu YJ, Yao DJ (2013b) SNP detection based on a temperature–controllable EWOD digital microfluidic system. Sens Mater

Shih SC, Yang H, Jebrail MJ, Fobel R, McIntosh N, Al-Dirbashi OY et al (2012) Dried blood spot analysis by digital microfluidics coupled to nanoelectrospray ionization mass spectrometry. Anal Chem 84:3731–3738

Sista R, Hua ZS, Thwar P, Sudarsan A, Srinivasan V, Eckhardt A et al (2008a) Development of a digital microfluidic platform for point of care testing. Lab Chip 8:2091–2104

Sista RS, Eckhardt AE, Srinivasan V, Pollack MG, Palanki S, Pamula VK (2008b) Heterogeneous immunoassays using magnetic beads on a digital microfluidic platform. Lab Chip 8:2188–2196

Srigunapalan S, Eydelnant IA, Simmons CA, Wheeler AR (2012) A digital microfluidic platform for primary cell culture and analysis. Lab Chip 12:369–375

Tang KT, Yao DJ, Yang CM, Hao HC, Chao JS, Li CH, Gu PS (2009) A portable electronic nose based on bio-chemical surface acoustic wave (SAW) array with multiplexed oscillator and readout electronics. AIP Conference proceedings, vol 1137, pp 86–89

Tao X, Chakrabarty K, Pamula VK (2010) Defect-tolerant design and optimization of a digital microfluidic biochip for protein crystallization. IEEE Trans Comput Aided Des Integr Circuits Syst 29:552–565

Tran TM, Lan F, Thompson CS, Abate AR (2013) From tubes to drops: droplet-based microfluidics for ultrahigh-throughput biology. J Phys D-Appl Phys 46:114004

VanGuilder HD, Vrana KE, Freeman WM (2008) Twenty-five years of quantitative PCR for gene expression analysis. Biotechniques 44:619–626

Walch A, Rauser S, Deininger S-O, Hoefler H (2008) MALDI imaging mass spectrometry for direct tissue analysis: a new frontier for molecular histology. Histochem Cell Biol 130:421–434

Watson MW, Abdelgawad M, Ye G, Yonson N, Trottier J, Wheeler AR (2006) Microcontact printing-based fabrication of digital microfluidic devices. Anal Chem 78:7877–7885

Welch ERF, Lin YY, Madison A, Fair RB (2011) Picoliter DNA sequencing chemistry on an electrowetting-based digital microfluidic platform. Biotechnol J 6:165–176

Wheeler AR, Moon H, Kim CJ, Loo JA, Garrell RL (2004) Electrowetting-based microfluidics for analysis of peptides and proteins by matrix-assisted laser desorption/ionization mass spectrometry. Anal Chem 76:4833–4838

Wheeler AR, Moon H, Bird CA, Loo RRO, Kim CJ, Loo JA et al (2005) Digital microfluidics with in-line sample purification for proteomics analyses with MALDI-MS. Anal Chem 77:534–540

Wijethunga PAL, Nanayakkara YS, Kunchala P, Armstrong DW, Moon H (2011) On-chip drop-to-drop liquid microextraction coupled with real-time concentration monitoring technique. Anal Chem 83:1658–1664

Witters D, Vergauwe N, Vermeir S, Ceyssens F, Liekens S, Puers R et al (2011) Biofunctionalization of electrowetting-on-dielectric digital microfluidic chips for miniaturized cell-based applications. Lab Chip 11:2790–2794

Witters D, Vergauwe N, Ameloot R, Vermeir S, De Vos D, Puers R et al (2012) Digital microfluidic high-throughput printing of single metal-organic framework crystals. Adv Mater 24:1316–1320

Witters D, Knez K, Ceyssens F, Puers R, Lammertyn J (2013) Digital microfluidics-enabled single-molecule detection by printing and sealing single magnetic beads in femtoliter droplets. Lab Chip 13:2047–2054

Wulff-Burchfield E, Schell WA, Eckhardt AE, Pollack MG, Hua Z, Rouse JL et al (2010) Microfluidic platform versus conventional

real-time polymerase chain reaction for the detection of Mycoplasma pneumoniae in respiratory specimens. Diagn Microbiol Infect Dis 67:22–29

Yang H, Luk VN, Abelgawad M, Barbulovic-Nad I, Wheeler AR (2009) A world-to-chip interface for digital microfluidics. Anal Chem 81:1061–1067

Yang H, Mudrik JM, Jebrail MJ, Wheeler AR (2011) A digital microfluidic method for in situ formation of porous polymer monoliths with application to solid-phase extraction. Anal Chem 83:3824–3830

Yi UC, Kim CJ (2006) Characterization of electrowetting actuation on addressable single-side coplanar electrodes. J Micromech Microeng 16:2053–2059

Yu YH, Chen JF, Li J, Yang S, Fan SK, Zhou J (2013) Microfabrication of a digital microfluidic platform integrated with an on-chip electrochemical cell. J Micromech Microeng 23:095025

Zhou G, Kamahori M, Okano K, Harada K, Kambara H (2001) Miniaturized pyrosequencer for DNA analysis with capillaries to deliver deoxynucleotides. Electrophoresis 22:3497–3504