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Nucleic acid diagnostics on the total integrated lab-on-a-disc for point-of-care testing

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ABSTRACT

Since the emergence of the lab-on-a-chip technology in 1979, a variety of microfluidic devices have been developed and utilized for chemical and biological applications. Among the microfluidic devices, the centrifugal microfluidic device or lab-on-a-disc (LOAD) has advanced remarkably due to simple operation by the rotation, total integration, and high-throughput capability. Moreover, the centrifugal microdevices do not need complex tubing and pumping systems, which render them ideal for point-of-care testing (POCT) system. Owing to these characteristics, the centrifugal microdevices have been extensively used for bio-diagnostics. In particular, molecular diagnostics, which are regarded as an essential method for definite determination of the targets related with diseases, have been widely applied on the LOAD. In this review paper, we focus on the molecular diagnostics on the LOAD. The steps for the molecular diagnostics such as cell lysis, genome purification, gene amplification, amplicon detection, and data analysis can be performed individually or totally on the LOAD. Future directions of the LOAD in the fields of bio-diagnostics is to realize POCT for U-healthcare monitoring. In this context, the latest LOAD strategies for molecular diagnostics are summarized in this review paper, which would provide an insight for future POCT platform.

1. Introduction

Molecular diagnostic testing focuses on the detection of specific gene sequences of a target pathogen in a tested sample. Traditional molecular diagnostic platforms such as polymerase chain reaction (PCR), hybridization, DNA sequencing, and microarray analysis suffer from high operational cost, complicated and prolonged analytical procedures, requirements for bulky instruments and well-trained staff. These drawbacks limit the applications of traditional diagnostic methods for POCT. The purpose of POCT is to provide rapid diagnosis near the sampling place with low cost, user-friendliness, and little technical training. Thus, the POCT platform plays a role in preventing the spread of disease and improving early medical treatment decisions (Nasseri et al., 2018; Zarei, 2017).

In recent years, molecular diagnostic techniques have been greatly improved toward POCT applications. Several advanced techniques for POCT include paper-based devices (Choi et al., 2015; Ye et al., 2018), lateral flow devices (Choi et al., 2017; Deng et al., 2018; Takalkar et al., 2017), lab-on-a-chip devices (DuVall et al., 2017; Tak For Yu et al., 2015; Zhang et al., 2017), miniaturized PCR platforms (Guarnaccia et al., 2017; Liu et al., 2017), smartphone-based devices (Priye et al., 2016; Wang et al., 2017), and centrifugal LOAD devices (Loo et al., 2019; Miyazaki et al., 2018; Torres Delgado et al., 2018). Among these techniques, LOAD devices stand out as promising candidates for POCT diagnosis, because the LOAD platform offers a huge capacity for total integration, where all the steps for conventional molecular diagnostics can be installed in a single LOAD device. Also, only a simple spindle motor with a sophisticated microfluidic design is enough to perform the operational unit of the molecular diagnosis on a LOAD. When spinning a LOAD platform, the centrifugal force actuates the solution in the centripetal direction, the Coriolis force changes the flowing direction of the liquid, and the Euler force produces turbulent mixing (Strohmeier et al., 2015). Thus, the delicate control of the rotational speed and direction allows us to execute the whole processes of the molecular diagnostics.

Several unit operations for fluidic transportation on the LOAD have been presented, including reagent storage, serial dilution, metering, aliquoting, mixing, incubation, and product detection. On-chip reagent

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storage and serial dilution units have been installed to reduce the required number of manual pipetting steps. Metering and aliquoting into several reaction chambers allow the LOAD device to become a high-throughput platform capable of multiplex detection of various targets in one sample in a single run. The mixing efficiency is considered to be an essential factor for achieving high homogeneity and reproducibility. In the last three years, considerable effort has been invested in the design of molecular diagnostic LOAD devices to achieve fully integrated LOAD platforms. All the analytical processes of a molecular diagnostic test were integrated on a single LOAD device including sample lysis, genome purification, gene amplification, product detection, and data analysis and reporting. This sample-to-answer LOAD prototype aims to provide a new trend of a genetic analyzer, in which a sample was applied into the LOAD platform and the results were obtained within 1 h without any further manual operation steps.

Previous review papers have described many aspects of the centrifugal microfluidic devices such as fundamental units, manufacturing process and diverse applications (Bruijns et al., 2016; Gilmore et al., 2016). Recent progress of the molecular diagnostics on the LOAD is remarkable, so it is timely to review the latest advancement of the fully integrated LOAD for the POCT genetic analysis at this moment. Therefore, we introduce the development of the design of LOAD devices toward total integration to be applied for molecular diagnostics in the last three years (2015–2018) in this review paper.

2. Fluidic handling in a LOAD device

Unit operations are basic building blocks for molecular diagnostics in the centrifugal microfluidic devices. The combination of these unit operations enables us to implement a variety of chemical and biological applications on the LOAD. In this section, we introduce fundamental unit operation of the LOAD to provide an insight for the operation mechanism and for further understanding of the elaborate molecular diagnostic applications.

2.1. Valving

A valving system plays a key role for a step-by-step manipulation of the solution transportation on a disc. Several kinds of valves used in the centrifugal microfluidic devices have been extensively discussed before. This section will review the recent advancement of the passive and active valving techniques, which are adopted to achieve a solution flushing in an orderly manner in the fully integrated LOAD devices. A passive valve is a rotation-speed-dependent valve for which centrifugal pressure or capillary forces control the on/off operation. This kind of a valve has the advantage of simple operation without need of external hardware for controlling the valve. However, their stringent manufacturing requirements are necessary and the valve working conditions can be differ depending on the liquid properties (Zainal et al., 2017; Zhu et al., 2018). Among the passive valves, a capillary valve is the most commonly employed. Capillary valves involve in the operation based on the sudden expansion or contraction of a channel width. The widening of the channel dimension causes a high surface tension force. Thus, the fluidic flow will stop when the centrifugal force is not greater than the surface tension force. Normally, the capillary valve is used in the LOAD to construct the aliquoting structure (Fig. 1A). The capillary valve provides the surface tension force to keep the solution in the aliquot chamber at low spinning speed and releases into the reaction chamber at the high spinning speed.

Another type of the passive valve on the LOAD is the siphon valve. The operation principle is based on the priming of an S-shaped capillary of siphon channels that connect an upstream chamber to a downstream chamber. The control of the centrifugal force versus the capillary force can be tuned by a rotational speed and the channel dimension, and the movement of the liquids can be driven to pass from the inlet through the siphon crest to the outlet (Zhu et al., 2018). For example, at high

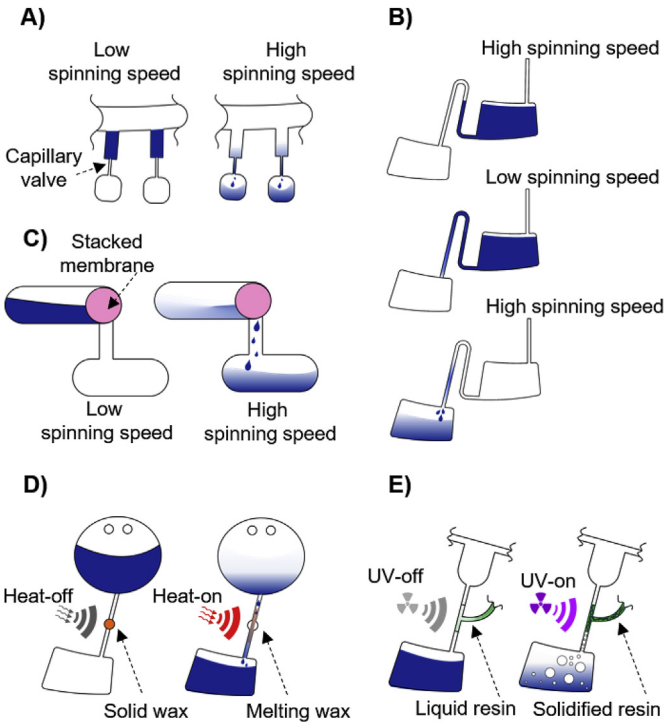

Fig. 1. Valves used in the LOAD such as (A) a capillary valve, (B) a singplex step siphon valve, (C) a membrane assisted valve, (D) a wax valve, and (E) a UV curable adhesive valve.

spinning speed, the S-shape siphon valve locks the flow of the fluid from the upstream chamber to the downstream chamber. At the low spinning speed, the capillary force is dominant over the centrifugal force, unlocking the S-shape siphon valve to allow the solution to flow to the outlet chamber (Fig. 1B). Owing to the hydrophobicity of the polymers used to fabricate the discs, surface treatments such as coating with Vistex agent are necessary to render the siphon valve hydrophilic (Focke et al., 2010).

Membrane resistance valves have been recently utilized for detecting avian influenza viruses on the LOAD. To fabricate the membrane resistance valve, multiple layers of polycarbonate membranes were stacked for assembling the valve layer (Fig. 1C). Due to various pore sizes, the burst pressure of the membrane resistance valves differs (Liu et al., 2018). Thus, at a certain RPM, the solution can be released to perform the downstream processes.

In contrast to the passive valves actuated solely by centrifugal forces, the active valves are functioned by an external trigger, so they require extra instruments on the processing device (Tang et al., 2016; Zainal et al., 2017). Compared with the passive valves, the active valve provides more robust and accurate in controlling the open/close operation of the valve. Therefore, the active valve plays a key role in sealing the solution storage chamber and the reactor chamber. Sayad et al. reported a LOAD installed with a wax valve for sealing the liquid resin chamber, and a UV curable adhesive valve for sealing the reaction chamber of gene amplification (Sayad et al. 2016, 2018). The open/close operation of the wax valve is controlled by a hot air gun, and the liquid UV curable resin was cured to converted to a solid valve by an external UV light (Fig. 1D and E). In despite of high performance of sealing action, these valves need a hot air gun, a UV light, and high stability of the wax and the UV curable resin on the LOAD. A simpler active valve on the LOAD was proposed by a GenePOC company. The wax is initially dried on the surface of the RT-PCR chamber, and during the RT-PCR reaction at high temperature, the wax is melted, floated and plugs the reaction chamber. Moreover, they installed an additional wax valve at the outlet of the reagent prestorage chamber, so the reagents

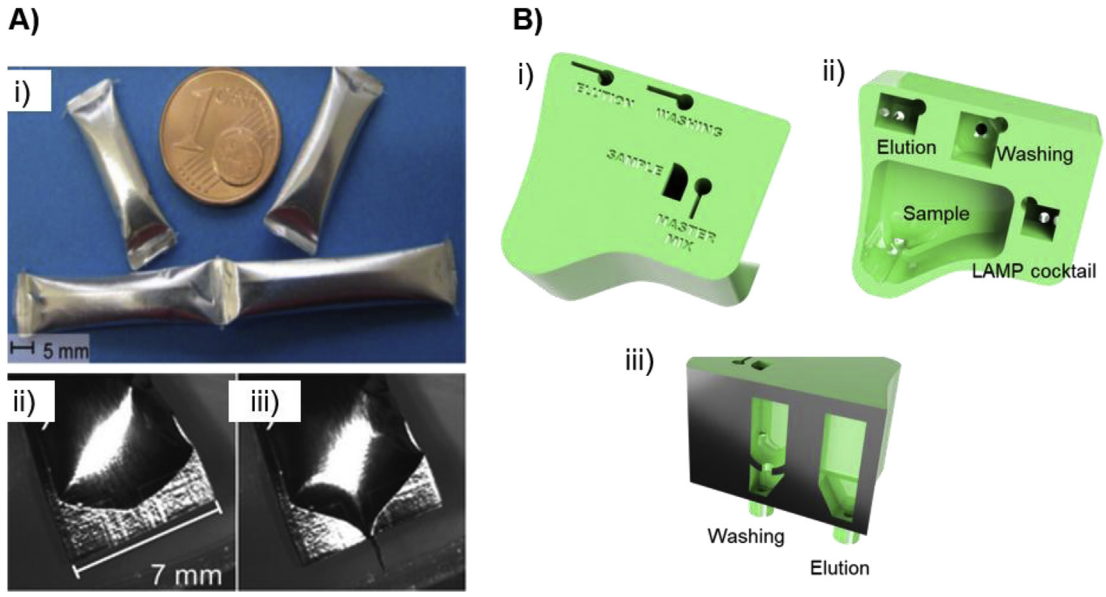

Fig. 2. (A) Miniaturized stick-packs applied on the LOAD (i) Stick-packs filled by the reagents and sealed by ultrasonic welding, (ii) the seal with close state at low spinning speed, and (iii) the seal with opening state at high spinning speed. Adapted from (van Oordt et al., 2013) with permission from The Royal Society of Chemistry. (B) (i) A top view and (ii) a bottom view of the solution loading cartridge, which contains four essential reagents that are released to the LOAD in a step-by-step manner. (iii) A cross-sectional view for the washing and elution solution reservoir. The double rooms for the washing solution for the programmale release to the LOAD. Adapted from (Nguyen et al., 2019) with permission from Elsevier.

could be released at high temperature (GenePOC Inc., 2018).

2.2. Reagent prestorage

Onboard reagent prestorage facilitates the designated use, the reduction of cross-contamination and the enhanced quality control by avoiding the mixing of reagents from different production batches. The prestorage of liquid reagents reduces the need for manual addition of reagents during processing. In general, prestorage can be subdivided into storage of liquid reagents and dried reagents. Long-term prestorage is a considerable challenge due to the nature of the reagents such as buffers and solvents. In the LOAD, the primer is normally prestored in the dried form by coating the primer on the surface of the reaction chamber at room temperature. Liquid reagents can be prestored in a separate container or directly injected in the chambers of a microfluidic device. Stick packs fabricated from a vapor-tight aluminum composite foil were employed for the prestorage of liquid reagents, which can be released through a peelable seal (Fig. 2A). The seal can be broken at a high rotational speed. Stumpf et al. introduced a LOAD with reagent prestorage. The liquid chemicals for RNA extraction were stored in separate stick packs, and the RT-PCR master lyophilizate solid pellet was stored in the reaction chamber (Stumpf et al., 2016). Recently, Nguyen et al. published a LOAD combined with a solution loading cartridge to detect pathogenic foodborne bacteria. In the solution loading cartridge, there are four rooms for storing a sample solution, a washing solution, an elution solution, and a loop-mediated isothermal amplification (LAMP) cocktail (Fig. 2B). The solutions can be orderly released into the centrifugal device by a spinning program (Nguyen et al., 2019).

2.3. Metering

Precise input volumes are required to obtain quantitatively reproducible results with high accuracy. Therefore, metering is an important unit operation in the applications of molecular diagnostics. In the centrifugal microfluidics, metering can be simply achieved by designing an aliquoting structure with an additional waste chamber. The input volumes are splitted to the multiple aliquoting chambers with defined volume, and such an aliquoting enables the execution of multiplex testing in the LOAD devices with high fidelity. Thus, a fully integrated LOAD frequently adopted the simple metering design for multiplex and automatic pathogen detection.

2.4. Mixing

Mixing is a critical step to produce high homogeneity of the mixture solution. Since centrifugal forces are more favorable for separation of liquid rather than for mixing of a solution, mixing is a kind of tricky task on a LOAD. In order to improve mixing efficiency, the use of Coriolis force in combination with modified channel shapes such as square-wave and zigzag patterns has been investigated during the mixing process of LAMP reagents with nucleic acid samples (Strohmeier et al., 2015; Tang et al., 2016). A simple mixing manner in the LOAD is obtained by swirling the disc in a clockwise and counterclockwise direction to generate the Coriolis force, which mimics the mechanical shaking. A more complex mixing method using bubbles was proposed to achieve higher mixing efficiency. Brassard et al. showed that bubbles were pumped into the lysis chamber from the bottom to enhance mixing. However, they needed to set up an external compressed gas for bubble supply (Brassard et al., 2019). Overall, in the past three years, the operational units such as valving, reagent storage, mixing, and metering on the LOAD have not improved significantly. Instead, endeavors to integrate several units on a single LOAD device have been made to realize a fully automatic molecular diagnostic system as described below.

3. Fundamental functions of molecular diagnostics on a LOAD device

3.1. Sample pretreatment

Cell lysis and nucleic acid purification is an initial step and one of the most vital steps for molecular diagnostics. Typical cell lysis methods applied in the diagnostic chips are based on thermal, chemical, electrochemical, and mechanical ones. The most popular type of nucleic acid purification on the LOAD is solid-phase extraction. However, the

on-disc sample pretreatment step still has a room for improvements to achieve a fully integrated LOAD, because these steps generally determine the success rate and the detection sensitivity. The cell lysis step and a DNA purification step require many liquid chemicals, which should be released in a consecutive order and at accurate time on the LOAD. Due to such complex operational and structural requirements, a variety of strategies are proposed including an on-disc direct bioassay without the sample pretreatment (Choi et al., 2016; DiaSorin Molecular LLC. 2016; GenePOC Inc., 2018), an off-disc cell purification combined with an on-disc direct bioassay without DNA purification (Chen et al., 2018), an off-disc cell lysis and DNA purification combined with an on-disc bioassay (Cao et al., 2018; Phaneuf et al., 2018; Sayad et al. 2016, 2018; Seo et al., 2017; Yuan et al., 2018), and an on-disc cell lysis, DNA purification, and bioassay (Liu et al., 2018; Oh et al., 2016a; Park et al., 2017; Stumpf et al., 2016; Nguyen et al., 2019). Among these strategies, the on-disc cell lysis and DNA purification along with the on-disc amplification and detection is close to the total integration and the POCT platform.

Regarding the on-disc cell lysis, chemical lysis is still the most common method for cell membrane disruption despites the requirements of several washing steps. The commercial lysis buffers contain high salts that may inhibit the nucleic acid amplification, so many washing steps are needed, causing high demand of reservoir chambers on the LOAD. Another lysis method used in the LOAD is a mechanical lysis. By adopting micro- or nanoscale beads of glass or magnetic materials, dynamic force and centrifugal force can be combined to rupture cell membranes effectively. This technique shows a 97% lysis efficiency in 3 min (Chen et al., 2018). A thermal-chemical lysis method was also reported for bacterial cell lysis from blood samples. An external heater was equipped in the lysis chamber to assist thermal cell lysis process (Loo et al., 2017). For on-disc DNA purification, the solid-phase extraction is widely accepted. In the past three years, less effort is made on the development of adsorption materials, and more effort is made on how to mimic the same procedure as a conventional off-chip method on a disc. The commonly used adsorption materials are magnetic beads or silica beads. In both cases, three main steps are required including a sample introduction and DNA binding on the beads, a bead washing, and an elution of purified DNAs. For the nucleic acid extraction on the magnetic beads, the magnetic beads are mobilie phase, meaning that the magnetic beads are transferred from the chamber for sample introduction and DNA binding to the chamber for washing to the chamber for elution. In order to transfer magnetic beads between the chambers, an external magnet system with a programmable operation should be equipped (Fig. 3A) (Stumpf et al., 2016). On the other hands, in case of the nucleic acid extraction on the silica beads, the silica beads are stationary phase, meaning that the silica beads are packed in the microchannel with a weir structure (Jung et al., 2015). Such a bead bed is fixed regardless of the rotational speed and direction, meanwhile the sample solution, the washing solution, and the elution solution are serially passing through the silica bead bed channel to obtain the purified DNAs in the end (Fig. 3B). Thus, the sophisticate design of the microchannel and the chambers is required to perform nucleic acid purification on the LOAD with only a spindle motor. Recently, for treatment of a large volume of a sample (up to 1 mL), Nguyen et al. modified the structure of the sample loading cartridge with a large volume and the addition of a delay chamber for releasing solutions in an order, and incorporated the super absorbent polymer in the waste chamber to absorb the sample and washing solution (Nguyen et al., 2019).

3.2. Temperature control

3.2.1. PCR thermocycling

On-chip PCR has demonstrated faster ramping rate and reaction time than tube-PCR due to its low thermal mass with high surface to volume ratio. Typical PCR thermal cycling requires three temperature zones for denaturation (95 °C), annealing (50–65 °C), and extension (72 °C). Normally, a peltier heater has been widely used for thermal cycling in a PCR assay. However, the integration of the peltier heater with a LOAD is rarely reported during the past three years. Czilwik et al. proposed a prototype thermal cycler using circulating air for RT-PCR on the LOAD system. They presented a valve-regulated influx of ambient air for heating and cooling on a PCR LOAD (Czilwik et al., 2015), which enables a closed flow of air with a gradient of temperature on the LOAD (Fig. 4A). The ramping rate in the reaction chamber was recorded as $0.7\,K\,s^{-1}$ for heating and $0.1\,K\,s^{-1}$ for cooling. They performed the detection of H3N2 virus with two gradient temperatures of 95 °C and 60 °C with 45 thermocycles. However, the reaction time was 3.5 h, which should be reduced to be applied for POCT molecular diagnostics (Stumpf et al., 2016).

3.3. Isothermal control

The isothermal amplification technique uses a constant temperature for gene amplification. Thus, the design of the heater and the operation process are much simpler than that of the typical on-chip PCR. During the last three years, numerous LOADs have adapted the isothermal amplification, especially the LAMP (Cao et al., 2018; Jung et al., 2015; Liu et al., 2018; Loo et al., 2017; Oh et al. 2016a, 2016b; Park et al., 2017; Phaneuf et al., 2018; Sayad et al., 2018; Seo et al., 2017; Yan et al., 2017; Yuan et al., 2018), recombinase polymerase amplification (RPA) (Chen et al., 2018; Choi et al., 2016; Law et al., 2018), and nucleic acid sequence–based amplification (NASBA) (Brennan et al., 2017). Since the NASBA reaction requires two different temperatures (65 °C for the denaturation of the RNA secondary structure and 41 °C for the reaction incubation), the RPA reaction (37–42 °C) and the LAMP reaction (60–65 °C) are more frequently applied on the LOAD. Heating sources for the isothermal LOAD platform was resistive heating elements (Law et al., 2018; Loo et al., 2017; Nguyen et al., 2019), a hot air gun, (Sayad et al. 2016, 2018), an infrared emitter (Brennan et al., 2017; Phaneuf et al., 2018), and a heat conductive baseplate (Liu et al., 2018). The heating sources were embedded in the baseplate for heating/cooling the chip (Fig. 4B) or placed above the chip for temperature control. For example, Liu et al. designed a closed heating cavity with one heat-conductive membrane integrated in the baseplate or in the lid (Liu et al., 2018). Sayad et al. utilized a hot air gun as a non-contact heating system. An infrared thermometer was used for surface temperature detection, and a calibrated temp-plate sensor detected the temperature of the inside reaction chamber (Sayad et al., 2016). The application of two thermal sensors improved the precision of the constant temperature in the reaction chamber, but the necessity of a hot air gun with a separate power source made the LOAD system less convenient for total power management. When an aluminum heating plate, an emitter, or a heat conductor is used as a heating source, it directly contacts the LOAD, and one thermal sensor (an IR thermal sensor or a platinum resistance thermometer) is required for thermal detection. The thermal sensor can be embedded in the heating plate or near an emitter to precisely monitor temperature in real-time. Recently, there has been a shift in a heater control from a wired computer connection to a wireless communication, reducing the complexity of the heater system and making it more portable (Liu et al., 2018; Loo et al., 2017).

3.4. Detection

3.4.1. Fluorescence detection

Fluorescence detection is one of the most popular methods for both the off- and on-chip nucleic acid detection owing to its high sensitivity, bioconjugation ability of fluorescent dye to nucleic acids, and no inhibition of dyes for gene amplification. In particular, fluorescence is the preferred choice for real-time quantitative nucleic acid amplification. Similar to the off-chip real-time PCR kits, the LOAD also employs a fluorescence (FAM)–linked-probe such as a Taqman probe, a molecular

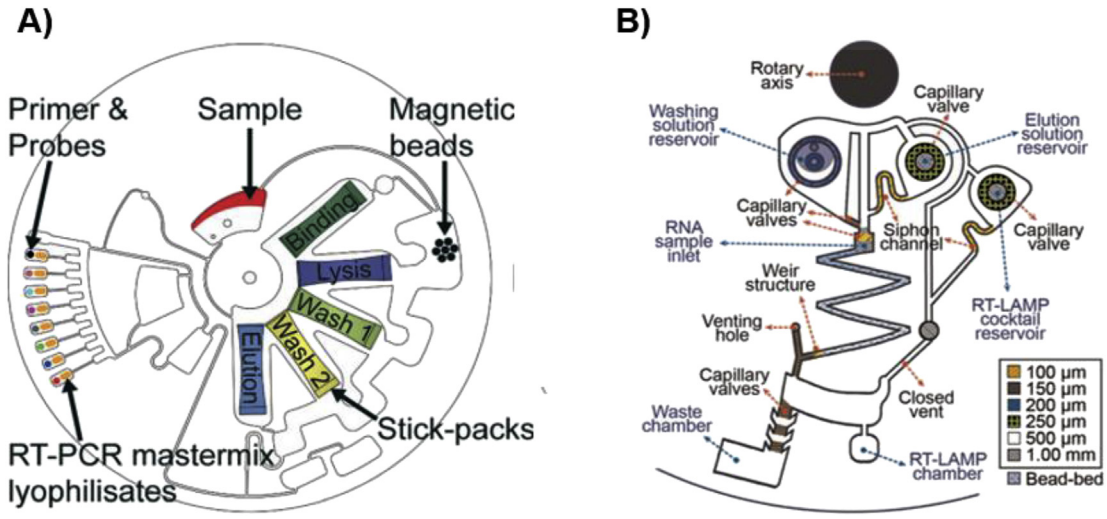

Fig. 3. Two strategies for the solid phase nucleic acid extraction on the LOAD. (A) Magnetic beads move from chamber-to-chamber to perform the nucleic acid binding, washing, and eluting. An external magnet manipulator is necessary to transfer the magnetic beads. Reproduced from (Stumpf et al., 2016) with permission from The Royal Society of Chemistry. (B) Silica beads are packed in the zigzag microchannel and function as the DNA/RNA extraction matrix. The silica beads are stationary during the rotation, and only a spindle motor is required to execute the sample pretreatment. The sophisticated design of the microchannel allows the step-by-step release of the sample, the washing, and the elution solution through the bead bed. Reproduced from (Jung et al., 2015) with permission from Elsevier.

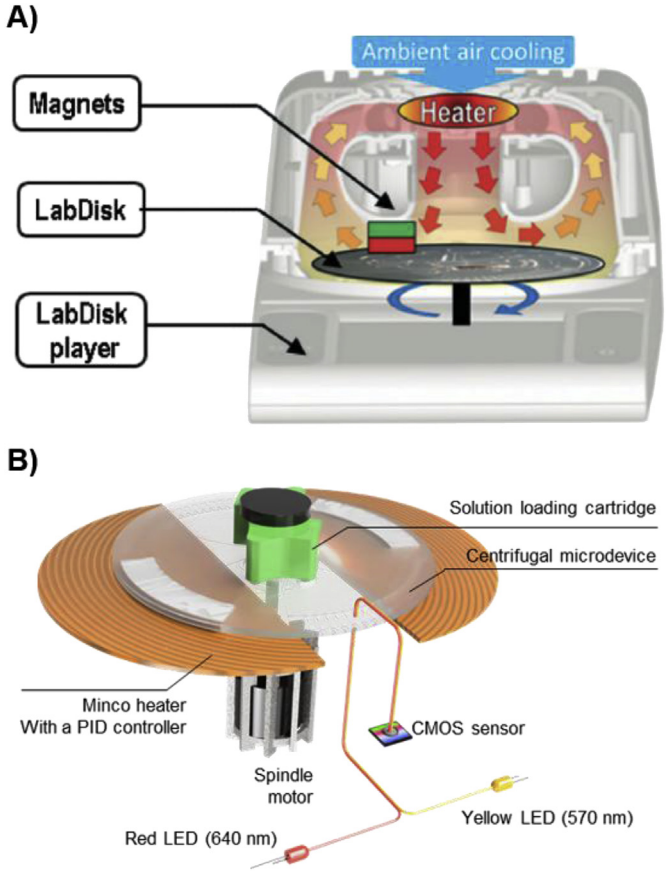

Fig. 4. A heater system for the LOAD. (A) A thermal cycler for RT-PCR based on an air heating system. Reproduced from (Czilwik et al., 2015) with permission from The Royal Society of Chemistry. (B) A Minco heater based isothermal system. Reproduced from (Nguyen et al., 2019) with permission from Elsevier.

beacon and SYBR Green I for real-time detection of the amplicons. Under the excitation of a laser beam or an LED light source, unique fluorescence signal depending on the kind of the fluorophores is emitted and its intensity is recorded by an optical detector. In the most LOADs, the excitation light normally passes straight through the reaction chamber from the top and the emitted fluorescence is detected by the optical sensors (Brennan et al., 2017; Liu et al., 2018; Loo et al., 2017; Phaneuf et al., 2018; Sayad et al., 2018). On the other hand, Choi et al. designed a LED light source located at the sidewall of a microdevice, and the optical detector was on the upper lid perpendicular to the reaction chamber (Choi et al., 2018). However, this design may raise the question of an optical detector maintenance, as the lid is opened frequently.

3.5. Colorimetric detection with naked eyes or UV–vis spectrometry

Because fluorescence-based detection often involves the use of expensive fluorescent labels and optical components such as a laser and PMT detector, alternative methods using colorimetric assay or UV–vis spectrometry are proposed, which are more adequate for POCT with cost-effectiveness. Although several colorimetric indicator dyes were used for detecting amplicons based on the off-chip experiments, the LOAD platform has utilized two approaches for quantifying LAMP amplicons: One is to use Eriochrome Black T (EBT) that is a Mg^{2+} ion indicator (Oh et al. 2016a, 2016b; Seo et al., 2017; Nguyen et al., 2019) and the other is to use a neutral red pH sensitive dye to detect the released H^+ ions during the LAMP reaction (Cao et al., 2018; Yuan et al., 2018). In case of the EBT based colorimetric detection, the LAMP cocktail requires Mg^{2+} ions for the reaction initiation, and then the concentration of free Mg^{2+} ions becomes lower when the LAMP reaction proceeds. Thus, under high initial Mg^{2+} concentrations, EBT produces the complexes of Mg^{2+}-EBT which shows violet color (λ_{max} = 570 nm) in the cocktail. When the target gene is amplified, the released pyrophosphates substracts Mg^{2+} from the complexes, generating free EBT that makes the cocktail solution blue (λ_{max} = 640 nm). Thus, the color change from violet to blue allows us to judge visually whether the target gene is amplified or not. This bioassay was successfully used in the fully integrated LOAD for foodborne pathogen detection (Oh et al., 2016b). Since the colorimetric analysis by naked eyes is sometimes ambiguous, Seo et al. proposed an RGB (Red/Green/Blue) based image analysis, and used the value of the Green/Red and Blue/Red ratio as a numerical indicator to identify negative and positive results (Seo et al., 2017). Recently, Nguyen et al. introduced a miniaturized UV–vis absorption spectrometry on a portable LOAD platform. The integrated UV–vis spectrometry provides a value of

Abs_{640nm}/Abs_{570nm} as a numerical indicator for the EBT mediated LAMP reaction, and the ratio value of 1.0 was set as a threshold to judge the positive or negative results (Nguyen et al., 2019). In case of the pH-sensitive dye assay, the neutral red pH sensitive dye is included in the reaction mixture with the initial value of pH 8.8. When the LAMP reaction occurs, H^+ ions are released, reducing the pH value of the LAMP mixture. So the color of the solution is changed from light brown to pink. This assay was applied on the LOAD for visual detection with naked eyes, not with any numerical indicator (Cao et al., 2018; Yuan et al., 2018).

3.6. Lateral flow strip

Since the lateral flow strip is simple, convenient, and cost-effective, this method has been extensively used in the diverse commercial biosensing kits. Owing to such advantages, some LOADs adopted a lateral flow strip as a detection method in the fully integrated genetic analysis system. In this case, the oligonucleotide primers should be labeled with haptens such as Texas Red or digoxigenin, and the hapten-labeled amplicons are captured by anti–Texas Red or anti–digoxigenin, which are immobilized on the lateral flow strip. Park et al. introduced a fully integrated LOAD, consisting of the sample pretreatment, the LAMP reaction, and the strip detection (Park et al., 2017). They used a metering structure to load 1 μL of the LAMP product to the strip, and a siphone value structure to add a large volume of a ruuning buffer in a sequential order.

4. A fully integrated LOAD platform for molecular diagnostics

The term “fully integrated LOAD platform” refers to a system that integrates all the required steps for nucleic acid testing in a single device. In the past three years, some remarkable works have presented a LOAD platform capable of sample-to-answer molecular diagnostics. The fully integrated LOAD platforms perform the entire molecular diagnostic processes including sample pretreatment, gene amplification, amplicon detection and quantification, and data analysis. Czilwik et al. presented a fully automated LOAD with prestorage reagents for total analysis of bacteria in a serum sample. The proposed LOAD system integrates a magnetic bead–mediated DNA extraction, multiplex PCR preamplification, and multiplex species-specific real-time PCR (Czilwik et al., 2015). The automation features are accomplished by a “LabDisk Player” device that comprises a rotary module, a thermocycler, and a fluorescence monitoring system. Stick packs were employed for on-disc reagent storage, from which the solution could be released with a high centrifugal speed. A gas transfer-magnetophoresis (GTM) method was used for several washing steps. An air thermocycling module was applied to control the PCR temperature. They completed the identification of four multiple pathogens (*Staphylococcus warneri, Streptococcus agalactiae, Escherichia coli,* and *Haemophilus influenzae*) on a single LOAD with a low limit-of-detection (LOD) of 3, 200, 5, and 2 CFU, respectively (Czilwik et al., 2015). However, there are rooms for improvements to be applied for POCT molecular diagnostics. The GTM based DNA extraction processes are complicated, lacking of reproducibility. The air thermocycling module showed slow heating (0.7–0.8 °C s^{-1}) and cooling (0.9–1.0 °C s^{-1}) rates, leading to relatively prolonged reaction time of 2 h 30 min for 35 cycles of RT-PCR (Stumpf et al., 2016). Oh et al. presented a total integrated genetic analysis microsystem, in which the LOAD performs sample pretreatment, DNA isothermal amplification, and colorimetric detection using an EBT-mediated LAMP reaction (Oh et al., 2016a). They used a silica bead–packed channel as a DNA extraction matrix, and a sample solution, a washing solution, and an elution solution were flushed into the bead bed channel in an orderly manner by means of a sophisticated disc design and rotation program (Fig. 5A). The LAMP reaction proceeded on a simple heat block at 63 °C, and the EBT-mediated colorimetric detection was conducted to identify four kinds of bacteria with the LOD of 2.7×10^3 cells/mL in 65 min. This LOAD system also needs further improvements for on-disc cell lysis, on-disc reagent storage, and a digital recording for quantification. Similarly, Park et al. proposed an integrated LOAD platform (Fig. 5B), in which the detection module was replaced by the lateral flow strip (Park et al., 2017).

Loo et al. reported an integrated LOAD to perform DNA extraction, isothermal LAMP reaction and real-time fluorescence detection (Loo et al., 2017). One stand-out feature is to use the microball-grease passive valve for controlling fluidic storage and release. Various microball-grease valves with different opening pressure were applied for each solution chamber to achieve automatic and step-by-step solid phase DNA extraction including washing, eluting, and mixing with the LAMP reagents. They detected *Mycobacterium tuberculosis* and *Acinetobacter baumanii* with the LOD of 10^3 and 10^2 in sputum and in blood, respectively, in 2 h. However, their platform has limitations in terms of throughput, meaning the detection of only one target bacterium for one sample in one run. Moreover, the improvement for increasing the stability of the microball and grease passive valve is required to minimize the environmental effect on the POCT molecular diagnostics.

Choi et al. reported a fully integrated LOAD that was able to perform real-time malaria diagnosis with the LOD of 0.5 parasites/μL using whole blood in 50 min (Choi et al., 2018). The proposed platform consists of a disposable centrifugal disc and a compact analyzer. Each disc contains quadplex testing units for analyzing four samples in one run. The compact analyzer is composed of thermal, optical, electromechanical, and data subsystems. They developed a valve chamber filled with FC-40 oil or air to separate the binding solution, the washing solution, and the reaction chambers. The advancement lays in its scalable and automated sample preparation capability. They also introduced an interface between the LOAD platform and the smartphone allowing the user to record the performance of the system on the smartphone, which is ideal for POCT molecular diagnostics. However, the proposed system has drawbacks in the throughput sampling and the on-disc reagent storage. In addition, an external magnetic manipulator is required for executing the on-chip solid-phase DNA extraction on magnetic beads.

Liu et al. reported an integrated LOAD for performing cell lysis, DNA purification, LAMP reaction, and data report to detect avian influenza virus in 70 min (Liu et al., 2018). They presented a design of an on-disc membrane resistance valve for controlling fluidic flow. Several polycarbonate membranes were stacked to form an outlet, and this stacked membrane valve enabled sequential release of the five essential solutions into a fiber–packed extraction channel. The design of the extraction channel was originally derived from Jung et al., (2015). The fabrication process of the LOAD was complicated, involving the bonding of five layers, and the bonding step requires high-precision alignment with caution.

Most recently, Nguyen et al. developed a fully integrated LOAD and an POCT genetic analyzer. The characteristics of their LOAD is the multiplexing capability of analyzing 20 bacteria for one sample in one run and the treatment of a large volume of sample up to 1 mL. The bacteria cell lysis, DNA purification, EBT mediated LAMP reaction, detection by a UV–vis absorption spectrometry, and data report are fully integrated. They developed a sample loading cartridge to store four-necessary reagents for the LAMP reaction, and combined it with the LOAD for full automatic operation. The POCT analyzer consists of a motor, a heater, a UV–vis detector, and a touch panel (Fig. 4B). The size of the POCT system is 20 cm [length] × 22 cm [width] × 20 cm [height], which is adequate for POCT molecular diagnostics. The multiplex bacterial detection was completed in 1 h with the LOD of 10^2 cells/mL (Nguyen et al., 2019).

A few types of the LOAD are commercially available. DiaSorin Molecular LLC Inc. introduces a LOAD for direct RT-PCR with a portable machine. A variety of reagent kits are available for testing influenza virus A/B, respiratory syncytial virus, *Clostridium difficile*, group A Strep, and HSV 1 & 2 (DiaSorin Molecular LLC. 2016). GenePOC Inc.

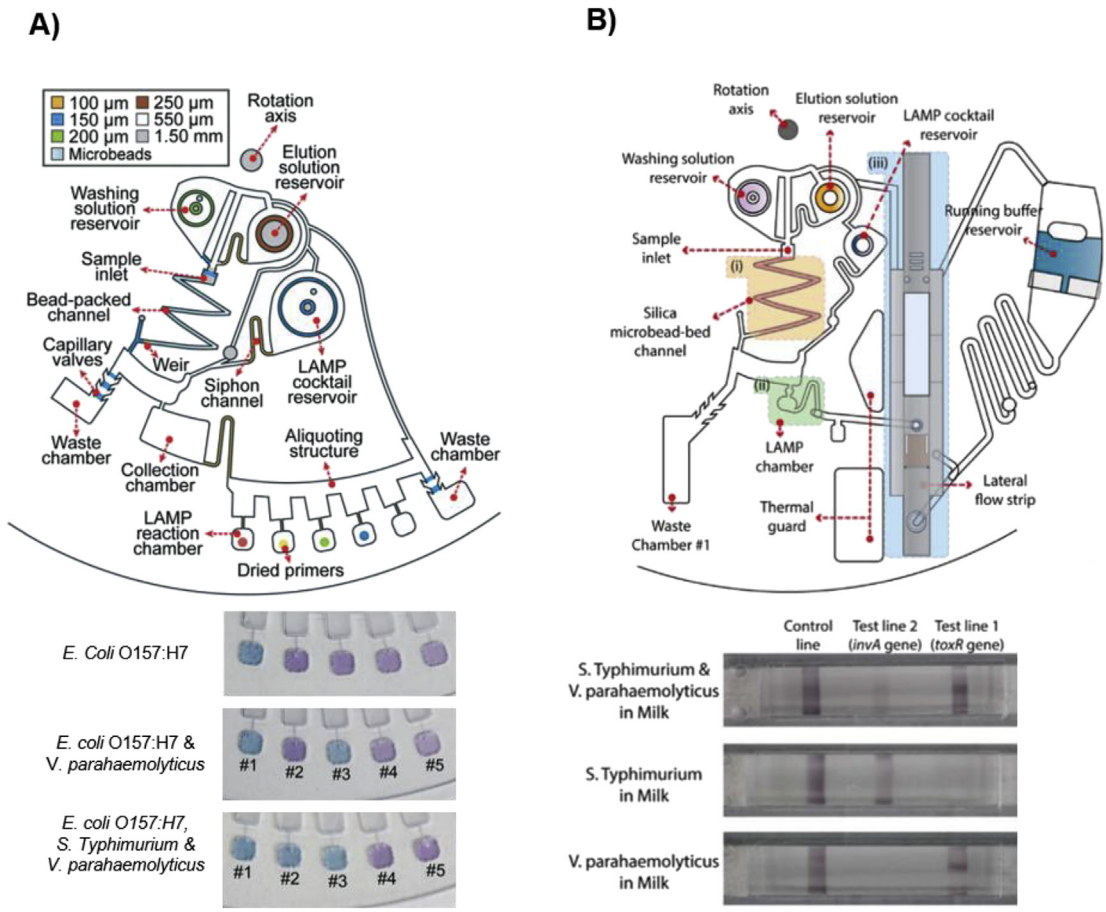

Fig. 5. A fully integrated LOAD platform. (A) The DNA extraction-LAMP reaction-colorimetric detection is integrated on a LOAD for multiplex bacteria analysis. Adapted from (Oh et al., 2016a) with permission from Royal Society of Chemistry. (B) The DNA extraction-LAMP reaction-a strip assay is integrated on a LOAD for multiplex bacteria analysis. Adapted from (Park et al., 2017) with permission from Elsevier.

commercializes a direct RT-PCR LOAD, and the LOAD is installed with an on-disc reagent storage to minimize the manual pipetting steps (GenePOC Inc., 2018). However, the application of this platform is limited for *Clostridium difficile* and Group B *Streptococcus*.

5. Conclusion and prospects

In the past three years, significant progress has been made in the integration of several operation units on a single LOAD device. In terms of the POCT of the molecular diagnostics, all the necessary steps such as sample lysis, solid-phase nucleic acid extraction, target gene amplification, amplicon quantification, and data processing are fully integrated, and the entire processes are automatically operated on a LOAD device for nucleic acid analysis. The cell lysis is mainly based on a chemical method using a lysis buffer. Solid-phase DNA/RNA extraction is normally performed on silica materials. On-disc gene amplification is carried out by means of PCR or isothermal techniques (LAMP, RPA, and NASBA). The real-time amplicon quantification is conducted by a fluorescence detector or a UV–vis absorption spectrometry. Such a total integration allows a sample-to-answer nucleic acid diagnostics, and current endeavors are focused on completing the whole process in 1 h.

To realize the LOAD system for POCT in the future, some improvements are still needed. First, a large volume of the real samples would be needed to be treated on the LOAD or a diluted sample would be obtained to be diagnosed on the LOAD (Antillon et al., 2018). Therefore, either high-volume sample processing capability or preconcentration unit should be further developed on the LOAD. On-chip sample pretreatment for the cell lysis and nucleic acid extraction should increase the efficiency, which affects the limit of detection of the system eventually. Second, the high-throughput and multiplexing capabilities should be improved, considering that many samples need to be analyzed on the spot. Current design is limited to a few sample analysis on a single LOAD at one time. Third, the disc manufacturing technique will be needed for making commercial ones with cost-effectiveness. In general, the LOAD is complex in design and undergoes several fabrication steps including disc etching or molding, hydrophilic coating, bead packing, primer coating, valving unit, and reagent storage. Overall, these processes require high resolution and accurate performance for ensuring the reproducibility. Thus, an affordable LOAD design should be reconsidered to be manufactured in an industrial scale with reliable quality. Last, simple operation with a user-friendly interface should be set up to be applied for POCT. In the sample-to-answer diagnostics, the device should be operable with minimal manual steps. Once the entire reactions are completed, data analysis, reporting, saving, and exporting should be offered with ease. For example, a smartphone-based operation and data report would be ideal in the future. Overall, the LOAD system has recently reached a high maturity level, so we believe that the commercial LOAD system will be utilized for a variety of biomedical diagnostic fields.

CRediT authorship contribution statement

Hau Van Nguyen: Conceptualization, Formal analysis, Writing - original draft. **Van Dan Nguyen:** Conceptualization, Formal analysis, Writing - original draft. **Huynh Quoc Nguyen:** Conceptualization, Formal analysis, Writing - original draft. **Tin Hoang Trung Chau:** Conceptualization, Formal analysis, Writing - original draft. **Eun Yeol**

Lee: Conceptualization, Formal analysis, Writing - original draft. **Tae Seok Seo:** Conceptualization, Formal analysis, Writing - original draft.

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