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A self-contained microfluidic in-gel loop-mediated isothermal amplification for multiplexed pathogen detection

Chen Chen [a], Pian Liu [b,**], Xing Zhao [a], Wei Du [a], Xiaojun Feng [a,*], Bi-Feng Liu [a]

[a] Britton Chance Center for Biomedical Photonics at Wuhan National Laboratory for Optoelectronics—Hubei Bioinformatics & Molecular Imaging Key Laboratory, Systems Biology Theme, Department of Biomedical Engineering, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China

[b] Cancer Center, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Avenue, Wuhan 430022, China

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ABSTRACT

In this work, we propose a microfluidic in-gel loop-mediated isothermal amplification (gLAMP) containing preloaded reagents as a self-contained microdevice for simultaneous detection of multiple pathogenic bacterial DNA in near point of care settings. The microchip consists of multiple channels with reaction chambers accommodating low-melting-point agarose and all LAMP reagents except the DNA samples, which can be kept at 4 °C for long-term storage. The current microchip enables simultaneous tests of four different bacterial DNA targets. After DNA samples were introduced into the microchip, gLAMP reactions were carried out on a hot plate at 65 °C. The gLAMP reaction conditions including the concentrations of the fluorescence indicator, *Bst* DNA polymerase, primers, and Mg^{2+} ions were further optimized. Under the optimized conditions, detection of foodborne bacteria in serum samples, including *Escherichia coli, Proteus hauseri, Vibrio parahaemolyticus,* and *Salmonella* subsp. *Enterica* were demonstrated with high selectivity and sensitivity (as low as 3 copies/μL). The gLAMP microchip provides a simple and easy-to-operate platform for LAMP-based pathogen detection, which holds high potential for future applications in point-of-care settings.

1. Introduction

Annual worldwide statistics indicates 1.8 million deaths from enteric diseases [1]. It is of unquestionable necessity to rapidly screen foodborne pathogens for clinical diagnosis [2]. Conventional methods for microbe detection involve cultivation of the organism in selective media followed by microbial identification based on morphological, biochemical or immunological characteristics [3]. However, these methods usually suffer from prolonged analysis time, requiring several days to confirm the presence of a pathogenic microbe [4], which cannot meet the requirement for clinical diagnosis, especially in cases of acute infections.

Recently, detection methods based on nucleic acid amplification have been introduced to identify pathogens with high sensitivity and selectivity [5], such as polymerase chain reaction (PCR) [6,7], rolling circle amplification (RCA) [8,9], helicase-dependent amplification (HDA) [10], nucleic acid sequence-based amplification (NASBA) [11,12], recombinase polymerase amplification (RPA) [13,14], and loop-mediated isothermal amplification (LAMP) [15,16]. In particular, LAMP is a promising technique for pathogen detection which relies on a set of oligonucleotide primers to recognize six regions on the target DNA template for DNA amplification by *Bst* DNA polymerase with high strand displacement activity [17,18]. Typically, a few copies of DNA can be amplified to a tremendous amount within an hour. One advantage of LAMP is that it can amplify nucleic acid sequence under isothermal conditions (60–65 °C) with high sensitivity and selectivity [19]. Another advantage is its simplicity for reading the detection results optically by means of adding calcein, a fluorescence metal indicator, and manganous ion to the reaction solution. Initially, calcein binds to manganese ion and its fluorescence remains quenched. As the LAMP reaction proceeds, pyrophosphates are produced as by-products of the LAMP reaction. Consequently, manganese ions are deprived from calcein by pyrophosphates, resulting in the emission of fluorescence [20,21]. In addition, LAMP is tolerant of common inhibitory compounds in clinical sample that typically inhibit PCR

* Corresponding author at: Department of Biomedical Engineering, College of Life Science and Technology, Huazhong University of Science and Technology, Wuhan 430074, China.
** Corresponding author at: Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China.
E-mail addresses: xfeng@mail.hust.edu.cn (X. Feng), liupianamazing@126.com (P. Liu).

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[22–24]. By exploring these advantages, a variety of LAMP-based methods have been developed to perform assays in pathogen detection. For example, Lucchi et al. adopted LAMP for malaria diagnosis by using the ESE-Quant Tube Scanner equipped with temperature settings [25]. Kim and co-workers optimized LAMP for vancomycin-resistant enterococci detection in PCR tubes that were incubated in a PCR thermocycle instrument [26]. Niessen's group introduced real-time LAMP assays for aflatoxin producing fungi quantification and the turbidity was monitored by a Loopamp real-time turbidimeter [27].

LAMP has been demonstrated to be an effective method for detecting a variety of pathogens, such as *Staphylococous aureus* [28], *Salmonella enterica* serovar Typhimurium [29] and *Mycobacterium tuberculosis* [30]. However, conventional LAMP reactions involve multiple wet-bench operations and the use of expensive equipments, preventing its widespread applications in point-of-care testing (POCT). Microfluidic technology can overcome the limitation of conventional LAMP schemes, most remarkably by providing improvements including less consumption of reagents, increased portability and reduced reaction time [30–32]. It provides an opportunity to simply devices for LAMP-based POCT.

To improve the simplicity for POCT, it is necessary to integrate LAMP reaction components into microfluidic devices. One strategy is to preload primers into the microfluidic settings. For example, Zhu et al. and Hoffman et al. grafted modified primers onto the activated surface of microchip [33,34]. Fang's group evaporated primers completely and leaving them in the microchambers [35]. In this type of strategy, samples were often mixed with reaction reagents outside the microchip before loading into the device, adding complexity to the operation procedure. Another strategy is to preload all reaction components except the samples into microfluidic devices. For example, the research group of Jiang reported a capillary-based LAMP containing preloaded reagents for detecting pathogen in a given sample [36]. In comparison, the second type of strategy simplified the operation procedure, which is more suitable for developing LAMP-based POCT devices. However, preloading amplification reagents in liquid solution is unfriendly to long-term storage or transportation.

Microgel has shown potential to stabilize reagent activity for reagent storage and transport in microdevices. Manage et al. previously used acrylamide gels for the storage of PCR reagents in short glass capillaries [37]. Agarose can also provide an effective reaction matrix because of its low fluorescence background and high biocompatibility [38]. In this paper, we demonstrated a novel microfluidic in-gel loop–mediated isothermal amplification (gLAMP) containing preloaded reagents as a self-contained microdevice for simultaneous detection of multiple pathogenic bacterial DNA. A sensitivity of 3 copies/μL was achieved and no cross-contamination was observed during the experiments. The developed gLAMP microchip can be implemented to detect bacteria spiked in serum samples, providing a promising platform for POCT diagnostics.

2. Materials and methods

2.1. Chemicals and reagents

Bst DNA polymerase, ThermoPol Buffer and $MgSO_4$ were purchased from New England Biolabs (U.S.A.). Betaine, mineral oil and low-melting-point agarose were purchased from Sigma-Aldrich (U.S.A.). Calcein and $dNTP_S$ were purchased from Shanghai Sangon Biological Engeneering Co., Ltd. (China). $MnCl_2$ was purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Four sets of LAMP primers targeting the *Proteus hauseri* (*P. hauseri*) *atpD* gene, the *Vibrio parahaemolyticus* (*V. parahemolyticus*) *Tlh* gene [39], the

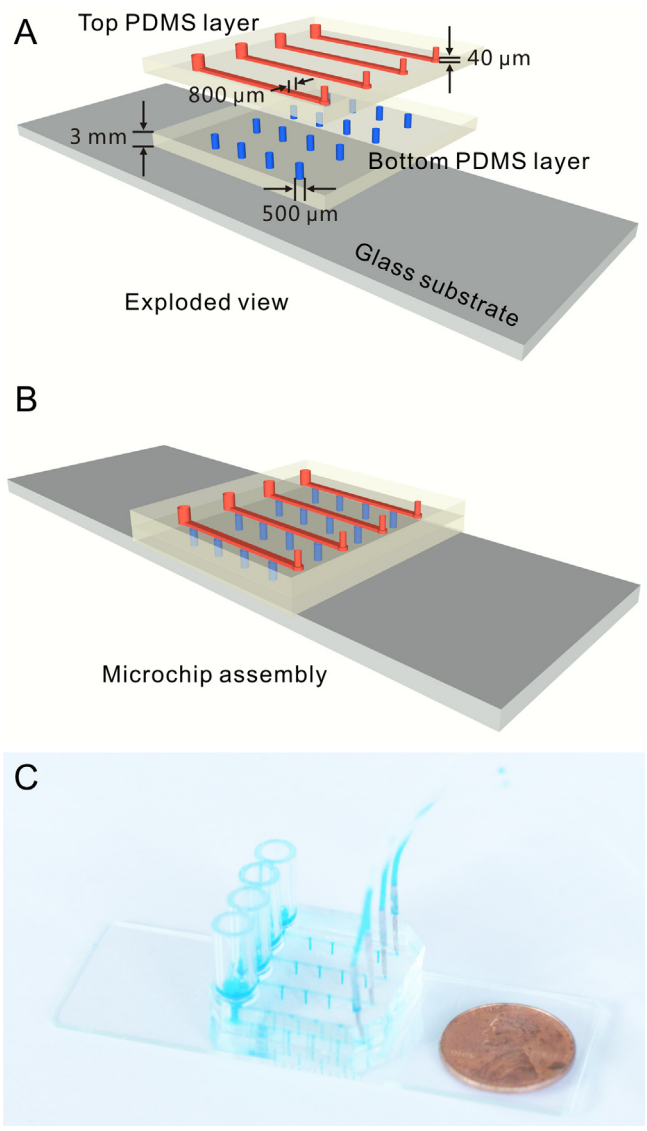

Fig. 1. A PDMS-glass hybrid microfluidic chip for pathogen detection by gLAMP. (A) Exploded view. (B) Microchip assembly. (C) Size comparison between a fabricated microchip and a US one cent coin.

Salmonella subsp. *Enterica* (*Salmonella*) *invA* gene [40], and the *Escherichia coli* (*E. coli*) *malB* gene [41] were synthesized by Shanghai Sangon Biological Engeneering Co., Ltd. (China). All reagents were of analytical grade unless specified otherwise. All solutions were prepared with ultrapure water (Millipore, Bedford, MA, USA). Polydimethylsiloxane (PDMS, Sylgard 184) was purchased from Dow Corning (Midland MI U.S.A.). SU-8^{TM} (GM 1070) was purchased from Gersteltec Sarl (Pully, Switzerland).

2.2. Maintenance of bacterial strains

Bacteria strains including *E. coli*, *P. hauseri*, *V. parahemolyticus*, *Salmonella* were purchased from China General Microbiological Culture Collection Center (CGMCC). *E.coli* were grown on Luria-Bertani (LB) agar plates, *P. hauseri* were grown on Nutrient Broth (NB) agar plates, *V. parahaemolyticus* were grown on 3% NaCl NB agar plates, and *Salmonella* were grown on Beef Peptone Yeast (BPY) agar plates. All bacteria strains were cultured at 37 °C for 16 h. A single colony of each strain was then picked up and grown in the corresponding media at 37 °C overnight. Bacteria number was