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A Paper/Polymer Hybrid CD-Like Microfluidic SpinChip Integrated with DNA-Functionalized Graphene Oxide Nanosensors for multiplex qLAMP Detection

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Abstract

A paper/ poly(methyl methacrylate) (PMMA) hybrid CD-like microfluidic SpinChip integrated with DNA probe-functionalized graphene oxide (GO) nanosensors was developed for multiplex quantitative LAMP detection (mqLAMP). This approach can simply and effectively address a major challenging problem of multiplexing in current LAMP methods.

Graphical abstract

A polymer/paper hybrid microfluidic SpinChip integrated with DNA probe-functionalized GO nanosensors was developed for simple and effective multiplex quantitative LAMP (μ -mqLAMP) detection.

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Loop-mediated isothermal amplification (LAMP) is a relatively recent nucleic acid amplification technique, which is simple, rapid, specific, sensitive and cost-effective,¹ demonstrating great potential for various biomarker detection and point-of-care (POC) analysis in low-resource settings.^{1, 2} However, LAMP is often restricted by the lack of simple and robust methods of multiplex LAMP (mLAMP) for its wide applications as multiplex polymerase chain reaction (PCR) does.³ Multiplex LAMP can amplify several DNA targets in one reaction and be employed for simultaneous detection of multiple pathogens, which can provide richer information and more conveniences to end users, consume fewer samples and reagents, and provide faster diagnosis to the patient.⁴ The mLAMP detection is challenging mainly due to complexities from multiple primer sets and complicated ladder-patterned LAMP products from loop-mediated reactions. As a result, the traditional tube-based mLAMP can only identify the occurrence of amplification but cannot effectively identify the specific pathogen.^{3–6} The subsequent gel electrophoresis-assisted detection method is not effective because of the complicated ladder-pattern bands of LAMP products from different targets (see Figure S1 for an example), which may also cause concerns of carry-over contamination.¹ Microfluidic lab-on-a-chip has recently provided a platform for a variety of biomedical applications including human health diagnostics.^{7–17} Our group reported polydimethylsiloxane (PDMS)/paper hybrid microfluidic devices integrated with LAMP for rapid and sensitive infectious disease diagnosis.^{18, 19} With the benefits of multiple compartments in microfluidic devices, multiplexed pathogen detection was reported by performing parallel singleplex LAMP reactions in different independent compartments of a microfluidic chip (note this is not mLAMP).^{20–22} However, it usually can only qualitatively identify the occurrence of amplifications based on the byproducts from the reactions, and the reagent consumption and cost will be doubled, tripled or even more compared with mLAMP detection.^{20, 23} Therefore, a simple, effective and integrated method for multiplex quantitative LAMP detection (mqLAMP) is in great need.

Herein, we developed a poly(methyl methacrylate) (PMMA)/paper hybrid CD-like microfluidic SpinChip integrated with both LAMP amplification and ssDNA probe-

functionalized GO nanosensors on a single device for multiplex quantitative LAMP detection (μ -mqLAMP). Multiple DNA targets can be isothermally amplified in a single microzone (i.e. mLAMP) by a battery-powered heater. Individual targets can be further identified and quantified by different integrated single-stranded DNA (ssDNA) probe-functionalized nanosensors afterward. Dual checkpoints through specific LAMP primers and specific DNA capture probes ensure extremely high specificity of the method. More significantly, quantitation of multiple DNA targets was achieved on the SpinChip. To facilitate seamless integration of the LAMP unit and the nanosensing unit, we designed the microfluidic device in a novel CD-like SpinChip format where LAMP amplicons from a single microzone can be readily transferred to multiple different nanosensing microzones through the spinning of the bottom plate manually, without the use of any complicated pneumatic valves. Therefore, the integrated LAMP and probe nanosensors provided a new simple solution for mqLAMP detection. In this work, we demonstrated the proof-of-concept of μ -mqLAMP by simultaneous detection of *Neisseria meningitidis* (*N. meningitidis*) and *Streptococcus pneumoniae* (*S. pneumoniae*), two leading pathogens causing bacterial meningitis. Bacterial meningitis is a global infectious disease with high fatality (30–60% in developing nations) and morbidity, and becomes fatal within 24 h if not treated promptly.²⁴ Thus, rapid and accurate detection of the exact type of meningitis is vital to the timely prevention and treatment of the disease. The direct mqLAMP detection for meningitis-causing pathogenic microorganisms was achieved without time-consuming sample preparation procedures or the requirement of centrifuges for DNA extraction and purification. The limits of detection (LODs) for *N. meningitidis* and *S. pneumoniae* of as low as 6 copies and 12 copies per assay were achieved in about 1 h. No washing or amplicon purification steps are needed during the whole assay. To the best of our knowledge, for the first time, our study provides a simple and effective microfluidic approach for multiplex qLAMP detection.

The PMMA/paper hybrid SpinChip (see Figure 1) takes advantages from both the substrates and avoids their limitations. For instance, along with ease of liquid handling from PMMA, the introduction of porous paper inside the detection microzones provides a simple 3D substrate for the integration of ssDNA probe-functionalized GO nanosensors on the chip without using any complicated surface modifications. In addition, thanks to the large surface-to-volume ratio of the porous paper, reaction kinetics can be improved for rapid assays.²⁵

The microfluidic SpinChip was designed in an innovative CD-like rotary format to facilitate the integration of both mLAMP reactions and nanosensor qLAMP detection, as shown in Figure 2. The two PMMA plates were not permanently bonded. Instead, they were tightened by a screw in the center, which allowed the rotation of one plate over the other by loosening the screw slightly during the spinning stage. As illustrated in Figure 2-1, during the reagent introduction stage, the top PMMA plate is rotated to align its inlet with the mLAMP microzone in the bottom plate (SpinChip open), thus allowing us to add reagents from the inlet in the top plate to the mLAMP microzone. After the reagent introduction, the top plate is spun manually (see Figure 2-2) and then tightened such that the bottom mLAMP microzone is sealed to avoid reagent evaporation during the mLAMP reaction stage at 63 °C for 45 min. When mLAMP reactions are completed, the SpinChip is heated at 95 °C for 2

min for the reaction termination and thermal denaturation of the LAMP products. Then, the SpinChip is turned over and the original bottom plate is rotated manually over the original top plate, as shown in Figure 2-3. When the mLAMP microzone passes by different qLAMP detection microzones underneath, the mLAMP products will be in contact with the paper disks in different detection microzones. The wicking effect of paper and the gravity of the mLAMP solution assist easy, automatic and even distribution of mLAMP products to all detection microzones, without using any pneumatic valves or pumps. Although only 12 detection microzones are designed in the current SpinChip, these microzones can be scaled up, and the SpinChip provides a simple strategy to distribute reagents to numerous microzones evenly. This feature is especially very useful for applications such as multiplexed detection and high-throughput analysis in low-resource settings.

When mLAMP products are distributed to the detection microzones, amplified target DNA will hybridize with specific target probes immobilized in the paper disk in the detection microzones (i.e. qLAMP detection, see Figure 2-3). The quenched fluorescence of the Cy3-labeled ssDNA capture probes by GO will be recovered (see Figure 1b). In the absence of the target mLAMP products, no obvious fluorescence restoration is detected. Therefore, different targets can be readily identified and quantified from different detection microzones. Previously, we used similar detection principles for the multiplexed detection of food-borne pathogens using aptasensors in a microfluidic device,²⁶ and for the high-sensitivity detection of environmental pollutants in a droplet microfluidic system.¹² Except for one-time reagent addition at the beginning of the assay on the SpinChip, no any further reagent addition and washing steps are required during the whole assay. This significant feature transforms the complicated mLAMP into a very simple one-step assay with good throughput (e.g. multiplexed detection and multiple-sample detection) as well as the capacity of quantitation.

After optimization of the concentrations of specific ssDNA probes (see Figure S2), we conducted mLAMP reactions on the SpinChip, and investigated the performance of our approach for mLAMP detection. ACSF samples with spiked pathogenic microorganisms of both *N. meningitidis* and *S. pneumoniae* were used to mimic real clinic samples. The bacteria were lysed by using a simple centrifuge-free lysis procedure that we developed,¹⁸ which avoided complicated sample preparation procedures. Results shown in Figure 3a and 3b demonstrated the successful mLAMP detection of multiple pathogenic microorganisms in ACSF samples. The recovered fluorescence intensities from *N. meningitidis* and *S. pneumoniae* were about 4 folds higher than those from the negative controls. Compared with the conventional gel electrophoresis, our μ -mLAMP provided a much simpler and more effective method for mLAMP detection. We further confirmed the success of our SpinChip for mLAMP detection by using fragment analysis, as shown in Fig. S3. All major specific peaks from *N. meningitidis* and *S. pneumoniae* were clearly identified in multiplex LAMP of both pathogens (such as peaks of 150 and 173 bp from *N. meningitidis* and 178 bp from *S. pneumoniae*), which further confirmed the success of our microfluidic SpinChip method for mLAMP reactions and detection (See SI for details).

The specificity of our μ -mqLAMP was explored by testing *N. meningitidis* and *S. pneumoniae* DNA samples with their corresponding and non-corresponding ssDNA probes. As shown in Figure 4a and 4b, only the target DNA sample with its corresponding ssDNA

probes generated bright fluorescence. The net recovered fluorescence intensities of *N. meningitidis* and *S. pneumoniae* samples with their corresponding probes were about 8.1 folds and 6.6 folds higher than those microzones with their non-corresponding probes, respectively (see Figure S4a and S4b). The results indicated the high specificity of our approach. Our μ -mqLAMP is capable of not only identification of targets (i.e. qualitative analysis), but also quantitative detection due to the integrated nanosensors, a significant feature of our μ -mLAMP, because most reported on-chip LAMP and mLAMP methods did not achieve quantitative analysis.^{5, 6, 20, 23, 27} mLAMP reactions described in the earlier section amplify DNA of multiple targets by about 10^8 fold,²⁸ which lays a low-cost platform for high-sensitivity detection; while qLAMP can quantify the pathogen concentrations to estimate the infection seriousness. By testing a serial of 10-fold diluted initial template DNA samples, we investigated the detection sensitivity and LODs of our approach for simultaneous detection of *N. meningitidis* and *S. pneumoniae*. The recovered fluorescence intensities corresponding to various copy numbers of initial template DNA were recorded from different nanosensors detection microzones to generate calibration curves, as shown in Figure 4c. It can be seen that there was a linear relationship between the recovered fluorescence intensities and the logarithm of initial copy numbers of the template DNA in the range of $6-6 \times 10^5$ copies and $12-1.2 \times 10^6$ copies per assay for *N. meningitidis* and *S. pneumoniae*, respectively. The greater slope from the curve of *N. meningitidis* than that of *S. pneumoniae* indicated higher LAMP reaction efficiency of *N. meningitidis* than that of *S. pneumoniae*. This may be caused by the intrinsic characteristics of different DNA targets and related primers. Different from *S. pneumoniae*, the primers designed for the *N. meningitidis* LAMP reaction have one more forward loop primer (FL), which can accelerate the amplification. On the basis of the 3-fold standard deviations of the mean fluorescent intensities of the negative control, the LODs for *N. meningitidis* and *S. pneumoniae* were found to be as low as 6 copies and 12 copies per assay, respectively, which were comparable to real-time PCR.^{29, 30} In this work, other than quantitative analysis of the initial target concentrations (i.e. initial copy numbers of DNA templates), we also demonstrated the capability of the nanosensors for quantification of mLAMP amplicons by using a series of diluted *N. meningitidis* and *S. pneumoniae* LAMP products (see Figure S5). The GO nanosensors were able to detect LAMP products of *N. meningitidis* and *S. pneumoniae* at concentrations of as low as 80 ng/ μ L and 87.5 ng/ μ L, respectively. The hybrid SpinChip also demonstrated superior performance and long shelf life time to non-hybrid devices (Fig. S6), as discussed in SI.

In conclusion, we developed a PMMA/paper hybrid CD-like microfluidic SpinChip integrated with DNA probe-functionalized nanosensors for multiplex quantitative LAMP detection (μ -mqLAMP). The integrated mLAMP reactions and nanosensors on the chip provided a simple but superior solution to address difficulties of conventional mLAMP in the identification and quantitation of multiple targets. The innovative CD-like rotary design of the SpinChip allowed a simple and seamless connection between the mLAMP unit and the nanosensor detection unit, without relying on any peripheral pumps and pneumatic values. During the whole assay, after the one-step addition of reagents, no other reagent addition and washing steps were needed, which enabled a “one-step sample-to-answer” strategy for mLAMP reactions and detection. Using the μ -mqLAMP, two types of bacterial

pathogens, *N. meningitidis* and *S. pneumoniae*, were successfully identified and quantified on-chip with high sensitivity and specificity, demonstrating the proof of concept. The LODs for *N. meningitidis* and *S. pneumoniae* of 6 copies and 12 copies per assay were achieved, respectively, indicating high detection sensitivity. Given these significant features, the μ -mqLAMP provides mLAMP an unprecedented opportunity for broad applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Schematic of the PMMA/paper hybrid microfluidic SpinChip for mqLAMP detection. (a) 3D schematic of the exploded view of the SpinChip. (b) Detection principle based on the interaction among the GO, ssDNA probes, and target LAMP products. (c) A photograph of the assembled PMMA/paper hybrid microfluidic SpinChip.

Figure 2. Working principle of the microfluidic SpinChip for mLAMP. One of the plates is manually spun to facilitate three different stages during the whole mqlAMP detection: (1) reagent delivery, (2) mLAMP reaction and denaturation, and (3) mqlAMP detection.

Figure 3.
 μ -mLAMP detection of meningitis pathogenic microorganisms, *N. meningitidis* and *S. pneumoniae*. Fluorescence images (a) and the corresponding fluorescence intensities (b) of nanosensor detection microzones after quenching and after recovery.

Figure 4.

Fluorescence images of nanosensor microzones after recovery for specificity investigation by testing *N. meningitidis* (a) and *S. pneumoniae* (b) samples with their corresponding and non-corresponding ssDNA probes. (c) Calibration curves of the fluorescence intensity after recovery versus the initial copy number of DNA template of *N. meningitidis* and *S. pneumoniae*, with R^2 of 0.98 and 0.97, respectively.