SUPPLEMENTAL INFORMATION

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

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Table of Contents

S.1 Experimental Section

S.1.1 Chemicals and Materials

LAMP DNA amplification kits were purchased from Eiken Co. Ltd., Japan. QIAamp DNA mini kit for DNA isolation and QIAquick PCR Purification Kit for LAMP products purification were purchased from Qiagen (Valencia, CA). The LAMP reaction mixture contained 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 0.8 M Betaine, 0.5 mM $MnCl_2$, 1.4 mM dNTPs, 8U Bst Polymerase, 1.6 μM each of the inner primer (FIP/BIP), 0.2 μM each of the outer primer (F3/B3), 0.4 μM each of the loop primer (LF/LB). Table S1 lists the sequence information of the LAMP primers and ssDNA probes for *N. meningitidis*[1, 2] and *S. pneumoniae*[3, 4], as well as the negative control probe (NC1)[5] used in this paper. All of them were purchased from Integrated DNA Technologies (Coralville, IA). All of the ssDNA probes were labeled with Cy3 at the 5' end.

Bacteria lysis buffer contained 50 mM Tris buffer (pH 7.5), 4 M urea and 0.1% triton.

Graphene oxide (GO) and Whatman#1 chromatography paper were obtained from Graphene Laboratories (Calverton, NY) and Sigma (St. Louis, MO), respectively. PMMA was purchased from McMaster-Carr (Los Angeles, CA). All other mentioned chemicals were purchased from Sigma (St. Louis, MO) and used without further purification unless otherwise noted. Unless stated otherwise, all solutions were prepared with ultrapure Milli-Q water (18.2 MΩ cm) from a Millipore Milli-Q system (Bedford, MA).

Table S1. Sequence information of LAMP primers and ssDNA probes for *N. meningitidis* and *S. pneumoniae*, and the negative control ssDNA probe (NC1) [1-5]

N. meningitidis ctrA LAMP primer sequences and the probe sequences

Primer	Sequences (5'-3')	No. of bases
FIP	CAAACACACCACGCGCATCAGATCTGAAGCCATTGGCCGTA	41
BIP	TGTTCCGCTATACGCCATTGGTACTGCCATAACCTTGAGCAA	42
F3	AGC(C/T)AGAGGCTTATCGCTT	19
B3	ATACCGTTGGAATCTCTGCC	20
FL	CGATCTTGCAAACCGCCC	18
BL	GCAGAACGTCAGGATAAATGGA	22
Probe	AACCTTGAGCAATCCATTTATCCTGACGTTCT	32

S. pneumoniae lytA LAMP primer sequences and the probe sequences

Primer	Sequences (5'-3')	No. of bases
FIP	CCGCCAGTGATAATCCGCTTCACACTCAACTGGGAATCCGC	41
BIP	TCTCGCACATTGTTGGGAACGGCCAGGCACCATTATCAACAGG	43
F3	GCGTGCAACCATATAGGCAA	20
B3	AGCATTCCAACCGCC	15
BL	TGCATCATGCAGGTAGGA	18
Probe	GCGGATTCCCAGTTGAGTGTGCGTGTAC	28

NC1 (Influenza A) probe sequences

Probe	TGCAGTCCTCGCTCACTGGGCACG	24

S.1.2 Microorganism Culture and DNA Preparation

The *N. meningitidis* (ATCC 13098) and *S. pneumoniae* (ATCC 49619) were obtained from American Type Culture Collection (ATCC, Rockville, MD). *N. meningitidis* was grown on chocolate II agar plates (Becton Dickinson, Sparks, MD). *S. pneumoniae* was grown in TSA II agar plates supplemented with 5% sheep blood (Becton Dickinson, Sparks, MD). All the microorganisms were incubated at 37 °C for 48 h in an aerobic environment with 5% CO_2. DNA isolation and LAMP products purification procedures were followed according to the protocol provided by the manufacturer (Qiagen, Valencia CA) to validate the microfluidic method.

S.1.3 Microfluidic SpinChip Design and Fabrication

As shown in Figure 1, the microfluidic SpinChip has two plates tightened with a screw in a center. The bottom plate (Figure 1a right) contains a LAMP microzone (diameter 4.0 mm, depth 1.5 mm), while the top plate (Figure 1a left) contains 12 detection microzones (diameter 2.0 mm, depth 0.5 mm). A chromatography paper disk (diameter 2.0 mm) was tightly placed inside each detection microzone serving as a three-dimensional (3D) storage substrate for the Cy3-labeled ssDNA probe-functionalized GO nanosensors. The detection microzones are divided into 4 areas, which are designated for *N. meningitidis* and *S. pneumoniae* detection (N.M. and S.P.) with their corresponding ssDNA probes as well as two negative controls (NC1 and NC2). A non-target ssDNA probe, Influenza A probe, is pre-loaded in NC1 detection microzones, whereas no probes are pre-loaded in NC2 detection microzones. In addition, there is one inlet (diameter 1.5 mm) in the top plate, through which samples and reaction reagents are introduced into the LAMP microzone.

All the LAMP microzone, detection microzones, and the paper disks were directly cut by a laser cutter (Epilog Zing 16, Golden, CO) within minutes. The bottom plate was exposed in an oxidizing air Plasma cleaner (Ithaca, NY) for 30 seconds, making the LAMP microzone hydrophilic. The chromatography paper inside the detection microzones absorbed GO and ssDNA probe solutions through the capillary effect. First, 0.8 μL of 0.04 mg/mL GO prepared in water was added into each detection microzone. After it became dry in 5 min at room temperature, 0.8 μL of a ssDNA probe solution (1.0 μM) was then added into each detection microzone and was left to dry at room temperature for 5 min. The two plates were then tightened together with a screw in the center of both plates (Figure 1c). As a result, the hybrid microfluidic SpinChip became ready-to-use.

S.1.4 Assay Procedures

An 18 μL LAMP reaction mixture was prepared in a biosafety cabinet and introduced to the LAMP microzone from the inlet of the microfluidic SpinChip, followed by rotating the top plate to cover the LAMP microzone, and screwing the two plates tightly. Once the SpinChip was closed, by using a heating film that controlled by an inexpensive proportional−integral−derivative (PID)-based temperature controller devised by our laboratory,[6, 7] the SpinChip was heated at 63 ℃ for 45 min for mLAMP reactions, followed by heating at 95 °C for 2 min to terminate mLAMP reactions and to denature the LAMP products (a mixture of stem-loop DNA). After the mLAMP reaction, the SpinChip was turned over and the bottom plate was rotated slowly. LAMP products were distributed to different nanosensor detection microzones separately to hybridize with their corresponding Cy3-labeled ssDNA probes, when the mLAMP microzone in the bottom plate passed by different detection microzones in the top plate. After incubation for 20 min at room temperature, the SpinChip was scanned by a Nikon Fluorescence Microscope (Melville, NY) to measure the fluorescence intensity, using appropriate Cy3 optical filters.

S.1.5 Direct Detection of Pathogenic Microorganisms

Artificial cerebrospinal fluid (ACSF) was prepared according to a published protocol,[8] which contained 119.0 mM NaCl, 26.2 mM $NaHCO_3$, 2.5 mM KCl, 1.0 mM NaH_2PO_4, 1.3 mM $MgCl_2$, 10.0 mM glucose. The ACSF solution was bubbled with 5% CO_2/95% O_2 for 10-15 min, before adding $CaCl_2$ to reach a final concentration of 2.5 mM $CaCl_2$. Then the prepared ACSF solution was filtered with a 0.20 μm filter apparatus and stored at 4 °C for use.

Microorganisms were spiked in ACSF solutions to make artificial 'clinical' ACSF samples. A tiny amount of the bacterial colony of *N. meningitidis* and *S. pneumoniae* was swabbed and suspended in a 1 mL ACSF buffer by adjusting the turbidity to McFarland Standard 0.5 (Key Scientific Products, Stamford, TX), forming an ACSF sample with an approximate bacterial density of 1.5×10^8 CFU/mL. To directly detect the pathogenic microorganisms, 3 μL of the ACSF sample was mixed with 3 μL bacterial lysis buffer to lyse the cells of the pathogenic microorganism and release their DNA. After incubation at room temperature for about 10 min, 1.5 μL of the lysate was directly used to prepare the reaction mixture for the subsequent on-chip mLAMP reaction and qLAMP detection.

S.1.6 Gel Electrophoresis and Fragment Analysis

Gel electrophoresis (Sub-Cell GT, Bio-Rad, CA) was used to investigate and compare the gel electrophoresis-based approach with our approach for mLAMP detection. During gel electrophoresis, LAMP products were resolved by applying 90

V for 1 hour in 1.5% agarose gel. In addition, Applied Biosystems 3130*xl* Genetic Analyzer (Waltham, Mass.) was used to perform fragment analysis of LAMP products to verify the success of the mLAMP reaction and detection.

S.2 Results and Discussion

S.2.1 Gel Electrophoresis-based mLAMP Detection

Before testing on-chip mLAMP, we performed conventional tube-based LAMP reactions by adding extracted DNA samples of *N. meningitidis* and *S. pneumoniae* individually (singleplex LAMP) and both (mLAMP), and investigated the feasibility of the conventional gel electrophoresis-based mLAMP detection method. As shown in Figure S1, although ladder-pattern bands from mLAMP products of both *N. meningitidis* and *S. pneumoniae* could be observed, there were no noticeable differences when compared with the ladder-pattern bands from singleplex LAMP products of *N. meningitidis* and *S. pneumoniae,* respectively. Based on the ladder-pattern bands, we could not conclude if the mLAMP products contained the LAMP products of *N. meningitidis* only, *S. pneumoniae* only, or both. This problem mainly came from the complex mixture of stem-loop different-sized LAMP products from each DNA template DNA, making it almost impossible to distinguish one target from mLAMP! In contrast, each PCR template only generates one single-sized product, which makes PCR advantageous in multiplexed detection.[9]

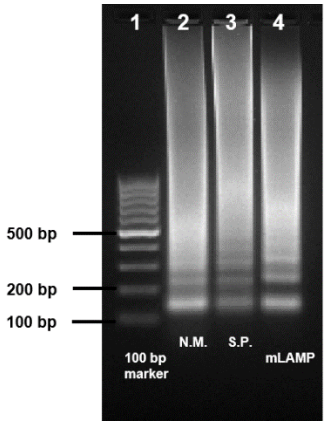

Figure S1. Gel electrophoresis analysis of the products from singleplex LAMP and mLAMP. Lane 1, 100 bp marker; Lanes 2 and 3, singleplex LAMP products of *N. meningitidis* and *S. pneumoniae* respectively; Lane 4, mLAMP products of both *N. meningitidis* and *S. pneumoniae*.

S.2.2 Optimization of ssDNA Capture Probe Concentrations

The concentrations of the ssDNA probes can affect both the fluorescence quenching and fluorescence recovery results, resulting in impacts on the detection sensitivity. Therefore, we first optimized concentrations of the ssDNA probes by testing different concentrations (0.25 μM, 0.50 μM, 0.75 μM and 1.0 μM) of the ssDNA probe targeting the *N. meningitidis* LAMP products (800 ng/μL). Figure S2 shows the fluorescence images of the SpinChip before fluorescence quenching (Figure S2a, left), after quenching (Figure S2a, middle) and after recovery (Figure S2a, right), and their corresponding fluorescence intensities (Figure S2b) at different ssDNA probe concentrations. Three important factors, the fluorescence intensity after recovery, the recovery rate (i.e. the percentages of the fluorescence intensities after recovery to the fluorescence intensities before quenching) and the net fluorescence recovery (i.e. the fluorescence difference between after recovery and after quenching) need to be considered because all of them can directly affect the detection sensitivity. It can be seen that the fluorescence of the ssDNA probe was significantly quenched; low fluorescence intensities below 800 arbitrary unit (a.u.) were observed for all concentrations of the ssDNA probe. The fluorescence intensity after recovery, the recovery rate, and the net fluorescence recovery increased with the increase of the ssDNA concentration. Compared with the lower concentration of 0.25 μM, the fluorescence intensity after recovery, the recovery rate, and the net fluorescence recovery of 1.0 μM of the capture probe increased from 2620 a.u. to 8450 a.u., from 81% to 94%, and from 3 folds to 10 folds, respectively. Therefore, given the higher fluorescence intensity after recovery (8450 a.u.), greater recovery rate (94%) and higher net fluorescence recovery (10 folds increase) observed from the ssDNA probe concentration of 1.0 μM, 1.0 μM ssDNA probe was chosen for the subsequent assays.

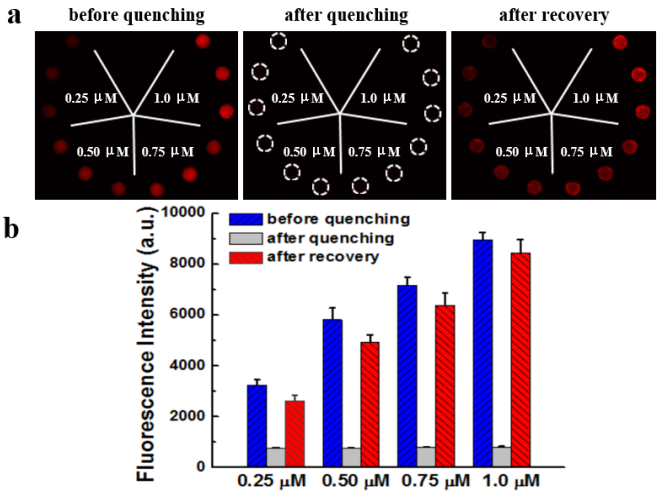

Figure S2. Optimization of the ssDNA probe concentrations. Fluorescence images (a) and the corresponding fluorescence intensities (b) of different concentrations of the ssDNA probe for *N. meningitidis* LAMP products (800 ng/μL) before quenching, after quenching, and after recovery.

S.2.3 Confirmatory Test of μ-mLAMP Detection

The LAMP primers (BIPs) for *N. meningitidis* and *S. pneumoniae* were modified with FAM and Cy3, respectively. Singleplex LAMP and mLAMP reactions were conducted on the SpinChip by using ACSF samples of individual *N. meningitidis*, *S. pneumoniae*, and both. Then, the products from the on-chip singleplex LAMP and mLAMP reactions were collected, purified, and analyzed by fragment analysis. Results were shown in Figure S3. Blue peaks (150, 173, 238 and 253 bp) and green peaks (178, 258 and 267 bp) were major LAMP products from singleplex LAMP amplification of *N. meningitidis* and *S. pneumoniae*, respectively. Some peaks (e.g. 173 and 178 bp) in the mLAMP products from the mixture of *N. meningitidis* and *S. pneumoniae* were too close to be resolved by the conventional gel electrophoresis method, as shown in Figure S1. But thanks to the multiple-color high-resolution fragment analysis, all these major specific peaks from individual *N. meningitidis* and *S. pneumoniae* were well resolved and 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.

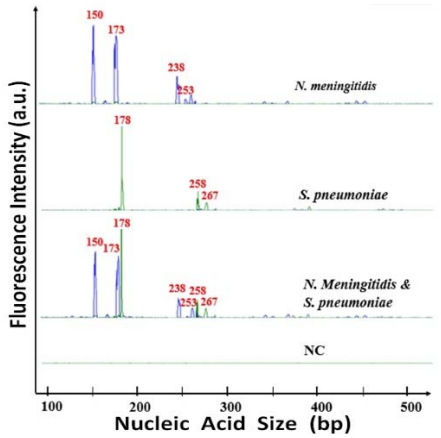

Figure S3. Fragment analysis of the products from singleplex LAMP and mLAMP.

S.2.4 Specificity Test

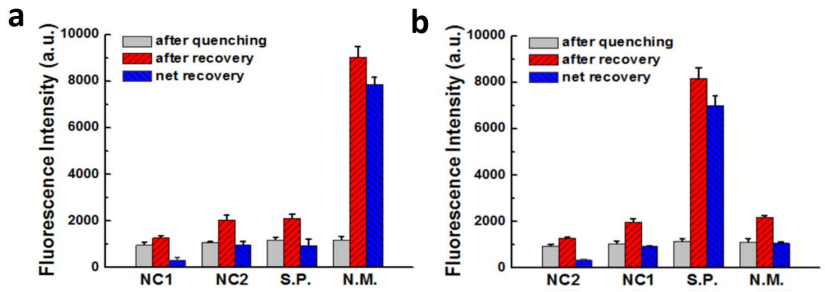

Figure S4. Fluorescence intensities of nanosensor microzones after quenching and after recovery for specificity investigation by testing *N. meningitidis* (a) and *S. pneumoniae* (b) samples with their corresponding and non-corresponding ssDNA probes.

S.2.5 Quantitative Analysis of mLAMP products

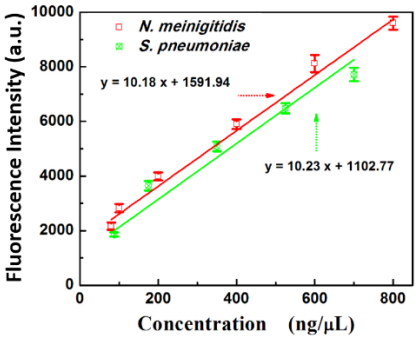

Figure S5. Calibration curves of the fluorescence intensities after recovery versus the target LAMP products concentration of *N. meningitidis* and *S. pneumoniae*, with R^2 of 0.99 and 0.97, and linear detectable range from 80.0-800.0 ng/μL and 87.5-700.0 ng/μL, respectively.

S.2.6 Performance Comparison between Hybrid and Non-hybrid SpinChips

To highlight the benefit from the PMMA/paper hybrid SpinChip, the analytical performance of the hybrid SpinChip was compared with a PMMA non-hybrid SpinChip by investigating the ratios of the signal (recovered fluorescence intensities of samples) to the blank (recovered fluorescence intensities of the NC background) during a time period of 12 days under the same conditions. A series of hybrid and non-hybrid SpinChips were prepared and stored in a dark environment at room temperature. The fluorescence intensities of those different SpinChips were measured after different time periods. As shown in Figure S7a, the ratio of the signal to blank of the hybrid SpinChip (with paper inside) was 3.9 on Day 0 and maintained at 3.4 after 12 days. On the contrary, the ratio of the signal to blank of the non-hybrid SpinChip (PMMA substrate only) was 1.7 on Day 0 and decreased to 1.3 after 12 days. Additionally, it was observed that the non-hybrid SpinChip exhibited hardly distinguishable recovered signals between samples and the blank after Day 2, implying poor detection performance and stability. Therefore, compared to the non-hybrid SpinChip, the hybrid SpinChip with preloaded nanosensors exhibited superior detection performance in terms of the stability and detection sensitivity, which was mainly attributed to the large surface-to-volume ratio of the porous paper substrate in the hybrid system, which improves the reaction kinetics for rapid and sensitive assays.[10]

Furthermore, the shelf life of the ready-to-use hybrid microfluidic SpinChips over a longer time period up to 73 days was studied. As can be seen from Figure S7b, the performance of the SpinChip decreased by ~20% during the first two weeks. After that, it maintained a stable performance by the end of the experimental period. The performance remained higher than 70% during the whole experimental period. It is expected that those SpinChips could maintain close to 100% performance if they were stored in refrigerators during that time period. The stable performance of the SpinChip over a long period of time could be attributed to two reasons. First, GO can protect ssDNA probes against enzymatic cleavage.[11] Second, highly

interwoven paper fibers provide a 3D matrix for preservation of ssDNA probes from harsh environmental elements and prevent ssDNA loss in the form of aerosols in air.[12]

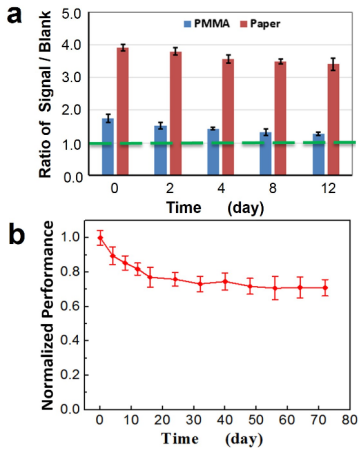

Figure S6. Performance comparison between the hybrid and non-hybrid SpinChips. (a) Ratios of the signal to the blank of the hybrid (with paper inside) and non-hybrid (PMMA only) systems. (b) Study of the shelf life of the ready-to-use hybrid microfluidic SpinChips over a time period of 73 days. The highest fluorescence intensity on Day 0 was used to normalize the rest fluorescence intensities to reflect their performances at different time points.

S.3 Supplementary References

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