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On-chip Nanoplasmonic RT-RPA and CRISPR/Cas12a Assay for Point-of-care Molecular Diagnostics*

Eun-Sil Yu, Hyowon Jang, Jaemyeong Kwon, Hyejeong Jeong, Jaehyeok Park, Taejoon Kang, and Ki-Hun Jeong, *Member, IEEE*

***Abstract*— Rapid and accurate nucleic acid detection at point-of-care (POC) is essential for advancing effective disease diagnosis and management. Here, we report a handheld nanoplasmonic all-in-one setup for on-chip recombinase polymerase amplification (RPA) and real-time fluorescence detection by CRISPR/Cas12a reaction. The all-in-one setup consists of AuNIs-based nanoplasmonic cavity (AuNIs-NC), a disposable plastic-on-polymer (PoP) cartridge, and fluorescence microlens array (FMLA) camera. The AuNIs-NC allows uniform and efficient photothermal heating under white LED illumination due to strong broadband light absorption and internal reflection by randomly distributed AuNIs and thin Al film. This setup allows the RPA and CRISPR/Cas 12a reactions in a single chamber of PoP cartridge, with fluorescence signals monitored by a FMLA camera. The experimental result demonstrates rapid SARS-CoV-2 E gene plasmid DNA detection within 20 min, achieving a detection sensitivity of 10 copies/ul. Testing with 16 clinical samples shows a linear trend with RT-qPCR, indicating the platform's reliable sensitivity and specificity. This compact platform offers affordable and reliable molecular diagnosis, facilitating rapid and scalable POC testing for a range of infectious diseases.**

***Clinical Relevance*— This on-chip real-time RT-RPA and CRISPR/Cas12a assay provides rapid and precise molecular diagnostics at POC using fully integrated plasmonic system.**

I. Introduction

Molecular diagnostics plays a critical role for rapid and sensitive detection of pathogens and genetic marker in the diverse biomedical fields.[1] In particular, real-time quantitative polymerase chain reaction (RT-qPCR) provides high sensitivity and specificity, serving as a gold standard in molecular diagnostics. Nonetheless, this diagnostic method remains constrained for point-of-care (POC) applications due to bulky and costly equipment, long operation time (> 2 hr), and trained personnel.[2] Isothermal nucleic acid amplification (INAA) performs the nucleic acid amplification at constant temperature condition using affordable equipment, allowing rapid and user-friendly diagnostics (<1 hr) in POC. In particular, INAA has widely combined with the CRISPR/Cas reaction to improve the detection limit and accuracy in POC.[3,4] For example, recombinase polymerase amplification (RPA) allows one-pot reaction with CRISPR/Cas enzyme under 50 ℃.[5] However, the one-pot reaction demands a large volume of reagent and sample in a single tube.

Microfluidic-based RPA and CRISPR/Cas reaction allows reduced reagent consumption, low contamination risk, and simultaneous multi-target detection.[6] Nevertheless, the on-chip RPA and CRISPR/Cas assay still consists of two-chamber reaction and struggle with some technical limitations in fluid control, separated bulky detector, and temperature uniformity during the reaction. For example, two-chamber reaction of RPA and CRISPR/Cas requires complex channel design and external fluid controller such as syringe pump or centrifuge.[7] In addition, separated RPA and CRISPR/Cas reaction relies on the end-point detection, which often extends the entire analytical time over 45 min.[8] Recently, a magnetofluidic-driven benchtop system has been equipped with a real-time fluorescence detector for one-pot RPA and CRISPR/Cas assay.[9] This benchtop system still depends on magnetic actuation for sample pre-concentration, high sample volumes, and a high-cost fluorescence detector. Furthermore, diverse isothermal isothermal heater, including hand-warm pouches[10], electrical pads[11], or peltier elements[9], faces practical limitation due to susceptibility to ambient temperature conditions, non-uniform heat distribution, and substantial power consumption.

Here we report on-chip RT-RPA and CRISPR/Cas12a assay using nanoplasmonic all-in-one setup for point-of-care molecular diagnostics (Figure 1). The nanoplasmonic all-in-one setup consists of photothermal heating, on-chip RPA and CRISPR/Cas12a reaction, and real-time fluorescence imaging. The photothermal heating utilizes the AuNIs-based nanoplasmonic cavity (AuNIs-NC). The AuNIs-NC features a sequentially stacked structure composed of randomly distributed AuNIs (SAP-AuNIs), a thin oxide layer, and a thin Al film. This configuration allows strong broadband absorption and internal reflection under white LED (WLED) illumination, leading to uniform photothermal heating. The on-chip RPA and CRISPR/Cas12a reaction simultaneously conduct nucleic acid amplification and target recognition within a single chamber of a small-volume disposable plastic cartridge. The RPA initiates nucleic acid amplification, followed by CRISPR/Cas12a-based recognition of the amplified DNA and collateral cleavage of single-stranded DNA (ssDNA) reporters. The fluorescence microlens array

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E.-S.Yu, J.Kwon, H. Jeong, J. Park, and K.-H. Jeong is with Department of Bio and Brain Engineering, Korea Advanced Institute of Science and Technology (KAIST), Daejeon, Republic of Korea (corresponding author to provide e-mail: kjeong@kaist.ac.kr).

H. Jang and T. Kang is with Bionanotechnology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, Republic of Korea (corresponding author to provide e-mail: kangtaejoon@kribb.re.kr).

(FMLA) camera continuously monitors and captures the fluorescence signals emitted from the ssDNA reporters.

II. Materials and Methods

A. Nanofabrication procedure of AuNIs-NC

Nanofabrication of the AuNIs-NC with Pt-resistance temperature detector (Pt-RTD) involves repeated solid-state dewetting, plasma-enhanced chemical vapor deposition (PECVD), thermal evaporation, and lift-off techniques (Figure 2a). The randomly distributed AuNIs are fabricated on a 4-inch borosilicate glass wafer by using repeated solid-state dewetting of thermally deposited 10 nm Au films. A 100 nm thick silicon dioxide (SiO_2) layer is deposited on the AuNIs by PECVD, followed by 150 nm Al deposition by thermal evaporation. A 500 nm SiO_2 passivation layer is deposited on the Al film to insulate the Pt-RTD, and a 100 nm Pt electrode is patterned on the AuNIs-NC by using metal lift-off.

B. Optical spectrum measurement of AuNIs-NC

All transmittance (T) and reflectance (R) spectra are measured by using a collimated WLED (MCWHL5-C1, Thorlabs, Inc.) coupled spectrometer (SP-2300i, Princeton Instruments, Inc.). The absorbance (A) spectra of AuNIs-NC and 10+10nm AuNIs are calculated based on the measured transmittance and reflectance spectra using the equation: $A = 1 - T - R$.

C. Reagent optimization for on-chip real-time RPA and CRISPR/Cas12a assay

The RPA primer set and crRNA for SARS-COV-2 E gene detection are described as follows. forward primer: 5'-CTGCGCTTCGATTGTGTGCGTACTGCTGCA-3', reverse primer: 5'-GACCAGAAGATCAGGAACTCTAGAAGAATT-3', crRNA: 5'-UAAUUUCUACUAAGUGUAGAUUUACGUUUACUCUCGUGUU-3'. The concentrations of RPA primers, ssDNA reporter, and CRISPR/Cas12a are optimized for RPA and CRISPR/Cas12a assay in a single chamber with small volume by using benchtop qRT-PCR system (StepOnePlus Real-time PCR system, Thermo Fisher Scientific, Inc.). The benchtop PCR reagent volume is set to 2 µl to match the cartridge. The concentrations for RPA primers, ssDNA reporter, and CRISPR/Cas12a are initially selected to ensure maximum fluorescence intensity and minimum threshold time (Tt) in a small volume. The final concentrations are set to 3 µM primer, 1.2 µM ssDNA reporter, and 40 nM CRISPR/Cas12a.

D. Clinical sample acquisition

For the diagnosis of SARS-CoV-2, 16 clinical specimens (nasopharyngeal swabs) from individuals suspected of having SARS-CoV-2 infection were obtained from Gyeongsang National University College of Medicine and stored in universal transport media (UTM, Copan Diagnostics Inc., CA, USA) at -70 ◦C. The Institutional Review Board (IRB) of Gyeongsang National University College of Medicine reviewed and approved this study protocol (IRB approval number: 2022-10-012).

E. RNA extraction and RT-qPCR from SARS-CoV-2 virus

To quantify the cycle threshold (Ct) value, the viral RNA of the SARS-CoV-2 sample was initially extracted using the ExiPrep™ 96 Viral DNA/RNA Kit (Bioneer, Seoul, South Korea) with the Bioneer extractor system, in accordance with the manufacturer's instructions. Subsequently, RT-qPCR was conducted using the Takara One-Step TB Green® PrimeScript™ RT-PCR Kit II (Kusatsu, Shiga, Japan) in a manner consistent with the protocol outlined by the manufacturer. Briefly, the extracted RNA was combined with the components of the quantitative reverse transcription polymerase chain reaction (qRT-PCR), as well as primers specific to the open reading frame (ORF) gene. This mixture was then subjected to a five-minute reverse transcription at 42 °C, a one-second PCR at 95 °C, and 50 cycles of five seconds at 95 °C and 30 seconds at 60 °C. The CFX opus Real-Time System (Bio-Rad) was used to measure the fluorescent signal during each cycle of the PCR step, and the system's built-in software (CFX maestro) was utilized to obtain the Ct values. The following is the sequence of the primers that were used to detect the ORF gene. Forward primer: 5'-TGCCTTGGTGATATTGCTGC -3', Reverse primer: 5'-TACCCGCTAACAGTGCAGAA -3'.

III. Results and Discussions

A. Photothermal efficiency enhancement of AuNIs-NC

The optical image shows the fabricated Pt-RTD on AuNIs-NC (Figure 2b, left). A focused ion beam scanning electron microscopy (FIB-SEM) image shows the cross-sectional structure along A-A', clearly displaying the well-fabricated AuNIs-NC and Pt-RTD layers (Figure 2b, right). The AuNIs-NC allows broadband absorption in the visible range and further increases a 1.5-fold absorption through transmitted light blocking and multiple reflections by the thin Al film (Figure 2c). The surface temperature of the microchamber area is monitored using an infrared (IR) camera during the maintenance of the AuNIs-NC at 50 ℃ under WLED illumination (Figure 2d). The microchamber area shows a uniform heat distribution with minimal temperature variation of less than 1 ℃.

B. Fully integrated all-in-one setup for on-chip real-time RPA and CRISPR/Cas assay

The on-chip real-time RPA and CRISPR/Cas12a assay utilized all-in-one setup with photothermal heating base, fluorescence optical module, and plastic-on-polymer (PoP) cartridge. The photothermal heating base features a WLED on the AuNIs-NC. The fluorescence optical setup includes an FMLA camera, excitation (480/40) and emission (520/20) bandpass filters, and a blue LED. The PoP cartridge is fabricated by bonding polypropylene and polyester film with double-sided adhesive tape and is positioned between the heating base and the optical module. The physical dimension of all-in-one setup is 80 mm × 55 mm × 20.6 mm (Figure 3a). The FMLA camera captured the fluorescence array images during photothermal heating. The all-in-one setup equipped with AuNIs-NC exhibits a clear fluorescence signal from 100 µM FITC in the PoP cartridge, compared to AuNIs without the Al layer. (Figure 3b). The AuNIs-NC maintains an isothermal temperature though WLED modulation with feedback control. WLED initially turns on to reach the target temperature for RPA and CRISPR/Cas12a assay and repeatedly turns on and off to maintain 45 ℃ based on Pt-RTD signals collected by

LabVIEW. The temperature fluctuation is less than 0.7 ℃ (Figure 3c).

C. *On-chip real-time RPA and CRISPR/Cas12a assay*

The on-chip real-time RPA-CRISPR/Cas12a assay is demonstrated using SARS- CoV-2 E gene plasmid DNA samples. The different plasmid DNA concentrations of E gene are utilized to confirm the detection sensitivity of the on-chip real-time RPA and CRISPR/Cas assay (Figure 4a). The maximum fluorescence intensity (Imax) increases with the DNA concentration as shown in the real-time fluorescence profiles. The inset graph shows the linear correlation between maximum intensity and DNA concentration, which also confirms the detection limit as 10 copies/µl in the on-chip real-time RPA-CRISPR/Cas12a assay. A threshold time (Tt) is defined as time to 0.2 Imax for rapid detection. All DNA concentration reach the Tt within 20 min (Figure 4b).

D. *Clinical validation test*

Clinical validation test is performed to evaluate the sensitivity and specificity of on-chip RT-RPA and CRISPR/Cas12a assay compared to conventional RT-qPCR. Reverse transcription (RT) is further incorporated into the on-chip RPA and CRISPR/Cas12a assay for direct detection of the SARS-CoV-2 RNA virus. An extracted RNA is added to a reagent mix with reverse transcriptase, recombinase, polymerase, primers, ssDNA reporter, and CRISPR/Cas12a, then loaded into the PoP cartridge. A total of 16 patient-derived samples are utilized for the clinical validation, consisting of 11 positive (P) and 5 negative (N) cases. The heatmap visualizes the comparison of clinical validation results between the on-chip RT-RPA and CRISPR/Cas12a assay and the RT-qPCR for each sample. The yellow and green gradient indicate the Imax of the on-chip RT-RPA and CRISPR/Cas12a assay and cycle threshold (Ct) from the RT-qPCR, respectively (Figure 5a). All clinical samples show the similar color pattern and moderate linear trend between Imax and Ct (Figure 5b). As a result, the clinical validation in on-chip condition provides 100% of sensitivity and specificity.

This nanoplasmonic all-in-one setup allows on-chip RPA and CRISPR/Cas assay for the detection of diverse infectious pathogens by adjusting primers and crRNAs beyond SARS-CoV-2. Although the current setup provides broad diagnostics potential, the absence of a sample preparation step limits direct clinical application. Integration of microfluidic-based nucleic acid extraction or extraction-free approaches can further improve point-of-care applicability.[12]

IV. Conclusion

A compact nanoplasmonic all-in-one setup combines photothermal heating and real-time fluorescence detection for on-chip RPA and CRISPR/Cas12a assays. This setup allows rapid SARS-CoV-2 detection within 20 min with a sensitivity of 10 copies/ul and shows a linear trend with RT-qPCR results in clinical validation. Adjustment of primers and crRNAs expands the detection capability to a broad range of infectious pathogens. Furthermore, adoption of microfluidic acid extraction or extraction-free strategies could advance the platform toward a fully integrated point-of-care system for decentralized disease monitoring and rapid clinical decision-making.

V. Figures

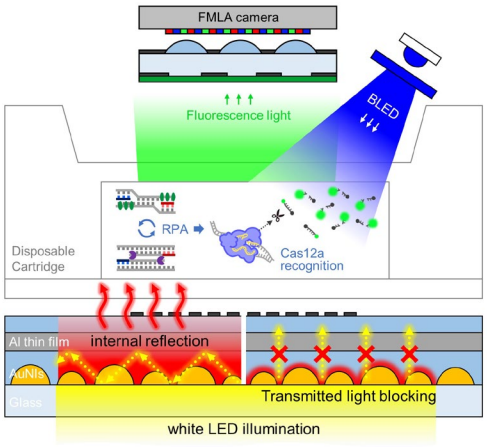

Figure 1. A schematic illustration of on-chip real-time RPA and CRISPR/Cas12a assay.

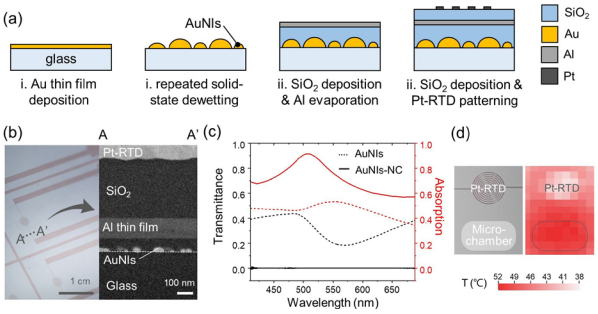

Figure 2. photothermal heater of AuNI-based nanoplasmonic cavity. (a) Nanofabrication steps for AuNIs-NC with platinum-resistance temperature detector (Pt-RTD). (b) Cross-sectional FIB-SEM image of AuNI-NC. The left image shows the optical image of Pt-RTD. (c) Spectral absorbance of AuNIs-NC. (d) uniform surface heat distribution of AuNIs-NC.

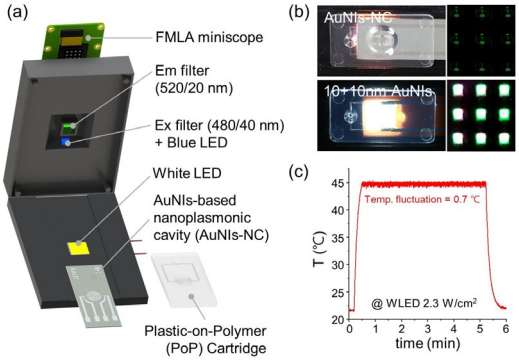

Figure 3. Experimental setup for on-chip RPA and CRISPR/Cas12a assay. (a) All-in-one configuration with photothermal heating base, fluorescence optical module, and PoP cartridge. (b) Real-time fluorescence imaging during white LED illumination. (c) Isothermal temperature maintenance by white LED modulation at 45 ℃.

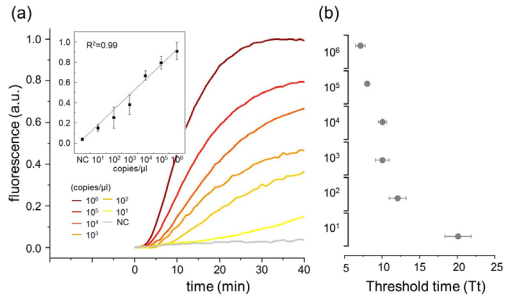

Figure 4. On-chip real-time RPA and CRISPR/Cas12a assay using SARS-CoV-2 plasmid DNA templates. (a) The real-time fluorescence profiles with different plasmid DNA concentrations. (b) Threshold time (Tt = $0.2I_{max}$) depending on the plasmid DNA concentrations.

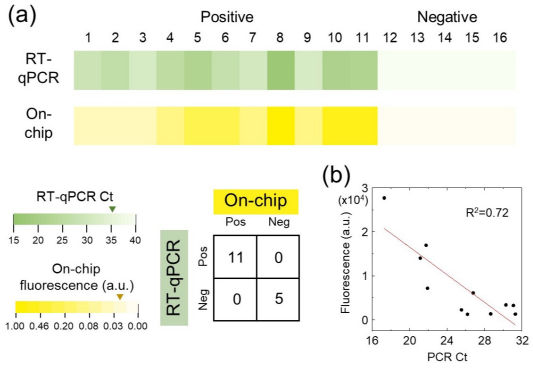

Figure 5. Clinical validation for 16 patient samples (11 positive and 5 negative) using on-chip real-time RT-RPA and CRISPR/Cas12a assay. (a) heatmap displays the fluorescence intensity of on-chip RT-RPA and CRISPR/Cas12a assay (yellow) with the RT-qPCR cycle threshold (green). (b) Correlation between fluorescence intensity and Ct.

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