Proceedings of the 14th Annual IEEE International
Conference on Nano/Micro Engineered and Molecular Systems
April 11-14, 2019, Bangkok, Thailand

Sensitive Detection of Multiple Fluoresence Probes based on Surface-enhanced Raman Scattering (SERS) for MERS-CoV

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***Abstract*—Up to now, Middle East respiratory syndrome (MERS) is solely confirmed with polymerase chain reactions (PCR) that is limited to detect a single biomarker on a trial and take long resulting time. Meanwhile, plasmonic paper substrates are eligible as multiplex Surface enhanced Raman scattering (SERS) platform for sensitive detection of multiple dual-labeled PCR probes on single analysis. The plasmonic paper, which contains silver nanodot deposited on 3D cellulose fiber, allows for simultaneous identification and the scalable quantification of fluorescence dyes over broad concentration ranges. Besides, developed biotin-modified probes enable to precisely separate the multiple fluorophores from PCR end-product. The platform will reduce both the number of PCR cycle and analysis time, and be applicable for emergency point-of-care (POC) tests of infectious diseases. It could help to block spread of epidemic disease in a country or world. Furthermore, the rapid diagnostic method could make patients earlier recover from acute severe diseases.**

Keywords— MERS-CoV diagnosis, SERS, Plasmonic paper substrates, Biotin-modified probes, PCR

I. INTRODUCTION

Middle East respiratory syndrome coronavirus (MERS-CoV) is an emerging pathogen that causes severe lower respiratory tract infection and symptoms such as fever, cough and shortness of breath in human [1]. The 2,266 patients have confirmed MERS infection and 35.5 % of mortality rate is measured until 2018. MERS cases are generally recommended to diagnose through real-time reverse-transcription (rRT)-PCR which based on the amplification of unique MERS coronavirus RNA sequences such as envelope (upE) gene and region encoding the open reading frame (ORF) 1a. However, the fluorescence-based real-time PCR process requires expensive specialized equipment and particular settings that are unsuitable as point-of-care test in developing countries. Also, it takes substantial resulting time to screen over two biomarkers due to a broad spectrum of emissions which lead the lack of multiplex capability and intrinsic limitations.

The alternative optical technique, *i.e.,* Surface-enhanced Raman Scattering (SERS) provides highly resolved vibrational fingerprint which is provides an opportunity for multiplex analysis and raises the future possibility of both high-throughput and point-of-care clinical application [2]. Besides, SERS enables sensitive detection of subtle fluorescence dyes in the early RT-PCR cycles. The spectra of SERS signal present peaks with about 1,000 times narrower full width at half maximum (FWHM) than fluorescence signal obtained from same dye reporter. It shows even more sensitivity and specificity, from 10^4 to 10^9 times, than the conventional Raman scattering known as great analytic method for trace detection fields [3]. The properties allow to simultaneously detect a number of fine reporters on a single channel.

In this study, we report multiplex SERS platform based on plasmonic paper substrates and significant dual labeled probe, which goals as superiorly sensitive SERS biosensing application based on LSPR for simultaneous detection of multiple PCR fluorescence probes on single analytic time. The SERS substrates are consisted of deposited silver nanoparticles on 3D cellulose paper to generate a number of inter-particle 'Hot-spot's enhancing the Raman signal. Hot spots incorporated SERS substrates are able to increase the average enhancement above 10^6 that provide single-molecule conditions analysis [4]. The single-stranded DNA probe is labelled with fluorophores and biotin as quenchers. It binds to sequence of target nucleic acid and expresses fluorescence signal after the process of PCR since complementary binding of target DNA broke down connection of quencher molecules and fluorophores. This platform targets the upE and ORF1a on MERS-CoV with PCR probe labeled 3' biotin and 5' fluorophores such as rhodamine 6G (R6G) or 5-Carboxytetramethylrhodamine (TAMRA). The platform could be a valuable tool for rapid molecular diagnosis, high risk and suspected patient of MERS-CoV cases as well as severe infectious diseases.

Acknowledgment: Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HG18C0062, HI14C3229) and the Bio & Medical Technology Development Program of the National Research Foundation (NRF) funded by the Ministry of Science (2016M3A9B6919189).

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II. Experimental Section

A. MERS-CoV targeted PCR preparation

The targeting primer and probe sets were synthesized by standard phosphoramidite chemical techniques (Table.1). Probes were referenced conventional Taqman probe that is consisted of quencer molecules and fluorophores on 3', 5' terminal. We labeled the probes with rhodamine 6G (R6G) and carboxy-tetramethylrhodamine (TAMRA) as reporter dyes and biotin as a quencher. The MERS-CoV DNA was synthesized complementary DNA (cDNA) from an RNA template via reverse transcription with a short complementary primer to target locus.

Table 1.Sequences of forward primer, reverse primer and specific probes to each target gene of MERS-CoV (ORF1a, upE)

Fluorophores	Type	Sequence (5'-3')
TAMRA	FWD 1	CCACTACTCCCATTTCGTCAG
	Rev 1	CAATATCAGTATGTGTAGTGCGC
	Prb 1	[TAMRA]-CAAATTGGCTTGCCCCCACTAATGCTTAT-[Biotin]
R6G	FWD 1	CCACTACTCCCATTTCGTCAG
	Rev 1	CAATATCAGTATGTGTAGTGCGC
	Prb 2	[R6G]-CAAATTGGCTTGCCCCCACTAATGCTTAT-[Biotin]

B. Reverse transcription polymerase chain reaction (RT-PCR)

The cDNA templates were amplified by PCR. Gel electrophoresis with 2% agarose gel confirmed the existence of amplicon and the corresponding sequences between template, specific probes and primers.

C. Preparation of SERS substrates

Large-scale SERS paper substrates was fabricated silver deposited on 0.18-mm-thick cellulose paper through solid-state dewetting method. The thin silver film thermally grown on a hot plate under ambient conditions.

D. Fluorophores separation based on noncovalent bonding

Streptavidin coated magnetic bead was used for this experiments. It was added to solution of PCR products and incubated at room temperature for binding biotin and eliminating the unreacted molecules in end-product. The supernatant, i.g. excluded fluorophores, was obtained by using magnet rack.

E. Fluorescence and SERS measurement

The fluorescence signal was measured by Mithras LB940 Multimode Microplate Reader, while SERS signal was analyzed through Confocal Micro Raman/dark fielded spectrograph system with 633 nm laser and an inverted microscope. The data from fluorophores was screened 500 to 800 nm of emission wavelength and counted average intensity at maximal emission wavelength for 0.10 seconds (556 nm for rhodamine 6G and 582 nm for TAMRA). The SERS spectra were obtained 25 mW excite laser power for 2 seconds acquisition time and mapping on 100 × 100 μm with 10 μm interval.

III. Current Results

In this study, we developed remarkable analytes separation method and SERS platform that allow accurately multiplex detect viral nucleic acid for rapid diagnosis of MERS. The separation method is consisted of biotin and fluorophores conjugated PCR probe and streptavidin coated magnetic beads (Fig.1). It helps obtain accurate result of cDNA amplification after RT-PCR and particularly differentiate optical signals between reporter dyes. The synthesized dual labelled probe, which is respectively specific to sequences of MERS-CoV, was confirmed the complementary base-pairs between target, primer or probe by gel electrophoresis (Fig.2). Also, We approved the area percent and necessary PCR cycle to visualize the bands. Orf1b and upE insufficiently amplified for analysis until 20 cycles. The results indicated that PCR process require at least 40 cycles in order to distinguish the target amplicon bands from negative control and analyze the occupied area percent of gel. After confirmation of primer and probe sets, we modified 5' terminal of dual-labelled PCR probe to biotin performed as quencher. Different fluorophores conjugated orf1a-target-probes were used in this study in order to exclude distinct reactions depended on amplicon structures, amplified extent and so on for definite validation of accuracy and sensitivity of developed whole processes.

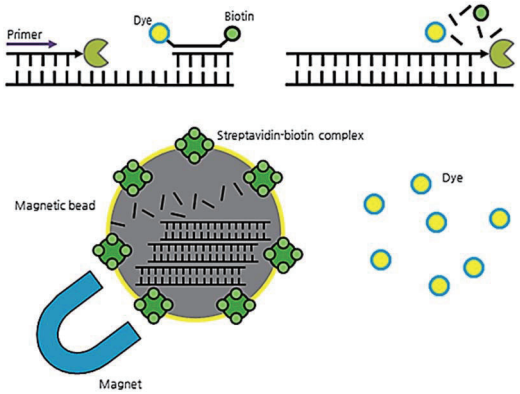


Fig. 1. Experimental scheme of beads based analytes separation method to detect multiple PCR fluorescence probe.

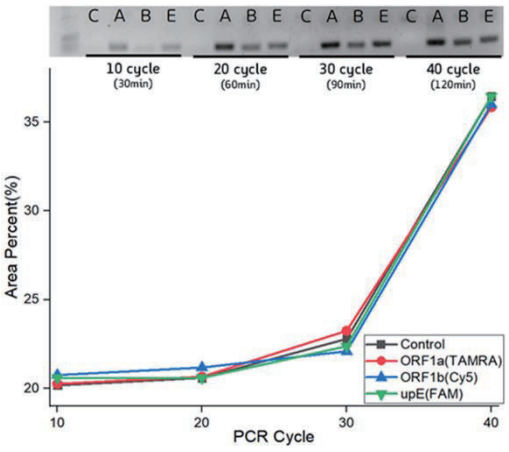


Fig. 2. Band formation and occupied area percent of gel electrophoresis according to number of PCR cycle

In sequence, we employed streptavidin conjugated magnetic beads in PCR end-product. The strongest noncovalent binding complex allow separate fluorophore from the others in PCR end-product, and leads to decrease the background readings [5]. After twice eliminated steps of unnecessary products via formation of streptavidin-biotin complex, the concentration of R6G and TAMRA, which derived from ORF1a targeted probes, declined 1076.5 to 660.32 nM and 113.17 to 29.94 nM, respectively (Fig.3). This results are based on the nonlinear regression analysis of calibration curve that describes relationship between fluorescence intensity and dye concentration ranged from 1 nM to 10^6 nM, whereupon coefficient of determination (R^2) was confirmed as 0.99 about both correlations that implies to get reliable quantified results for analysis of target molecules in sample (Fig.4). In results of DNA concentration measurements, the values declined to 476.77, 397.87, 285.53 ng/µl from R6G-orf1a PCR end-product according to increased separation steps and 459.15, 359.87, 344.17 ng/µl from TAMRA-orf1a amplicon to same steps. These results indicated that bead based separation step eliminated the residues obtained after PCR could affect to analytical results and only extracted reporter dye. In addition, the biotin-modified probes induce precise separation of fluorescence dyes with streptavidin coated magnetic bead and sensitive detection of multiple dyes on post RT-PCR process.

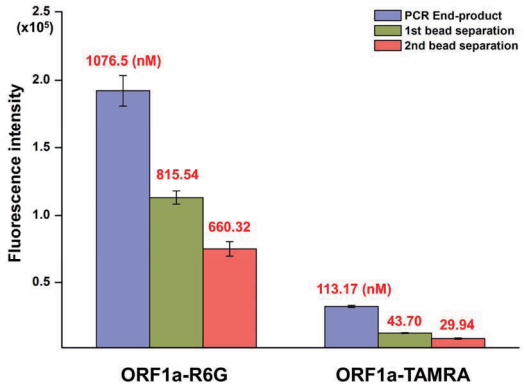


Fig. 3. Changes of fluorophore concentrations according to the increased number of biotin-streptavidin complex based separation.

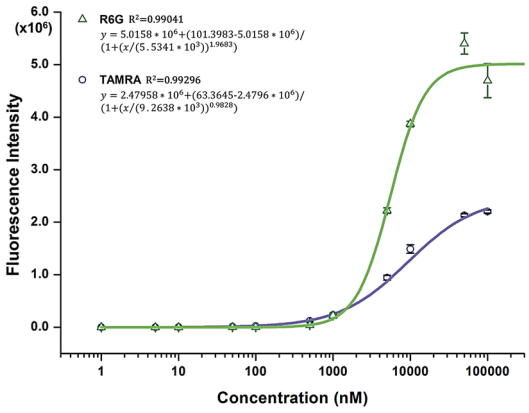


Fig. 4. Linear regression analysis of calibration curve about fluorescence intensity and concentration of rhodamine 6G and TAMRA.

Developed SERS platform enable simultaneously detect MERS-CoV upE and ORF 1a targeted different labeled probe, whereas individual rRT-PCR assays might be utilized to detect both gene. Particular vibrational bands, called SERS fingerprint, allow to distinguish the fluorescence probes and analyze multiple targets at same time. The specific vibrational bands of rhodamine 6G dye labeling ORF1a were observed at 1572, 1361, 1311, 1177 cm-1 due to aromatic C-C stretching and C-H (in-plant) bend (Fig.5). On the other hand, the characteristic TAMRA bands were detected at 1649, 1532, 1352, 1216 cm^{-1} since these are appointed to the aromatic C-C stretching, C-C bridge-band stretching, aromatic C-H bending modes of TAMRA (Fig.5). Also, SERS mapping data indicated that each fluorophores is simultaneously detected on distinct main peak (R6G:1380-1330 cm^{-1}, TAMRA: 1310-1090 cm^{-1}) (Fig.5). The fluorescence dyes, derived from PCR products and beads based separation, were definitely represented on every fingerprint wavelengths and mapping images with signal enhancement by developed SERS platform. The precise multiplex testing lead rapid diagnosis, containment of the epidemic and fast recovery of MERS.

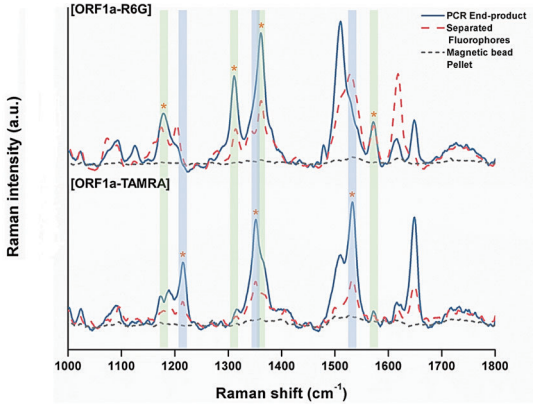


Fig. 5. Raman spectra of rhodamine 6G and TAMRA resulted from ORF1a PCR products, supernatant and pellet after twice separations.

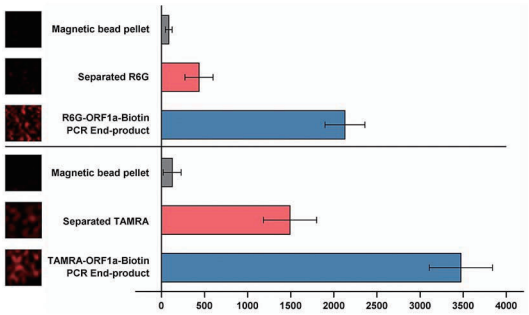


Fig. 6. Average Raman intensity of mapping data on each main peak.

IV. Conclusion

This demonstrated novel assay system is a high efficient tool without any costly experimental apparatus. This combination of bead based separation and paper SERS platform is an advanced method for emergency diagnosis and preventing the spread of infectious diseases in developing countries.

REFERENCES

[1] W. H. Organization. (2015). *Laboratory testing for Middle East respiratory syndrome coronavirus (MERS-CoV)*.Available: http://www.who.int/csr/disease/coronavirus_infections/mers-laboratory-testing/en/

[2] M. Kang, J. J. Kim, Y. J. Oh, S. G. Park, and K. H. Jeong, "A Deformable Nanoplasmonic Membrane Reveals Universal Correlations Between Plasmon Resonance and Surface Enhanced Raman Scattering," (in English), *Advanced Materials,* vol. 26, no. 26, pp. 4510-4514, Jul 9 2014.

[3] A. H. Nguyen, J. U. Lee, and S. J. Sim, "Plasmonic coupling-dependent SERS of gold nanoparticles anchored on methylated DNA and detection of global DNA methylation in SERS-based platforms," (in English), *Journal of Optics,* vol. 17, no. 11, Nov 2015.

[4] S. L. Kleinman, R. R. Frontiera, A. I. Henry, J. A. Dieringer, and R. P. Van Duyne, "Creating, characterizing, and controlling chemistry with SERS hot spots," *Phys Chem Chem Phys,* vol. 15, no. 1, pp. 21-36, Jan 7 2013.

[5] C. E. Chivers, A. L. Koner, E. D. Lowe, and M. Howarth, "How the biotin-streptavidin interaction was made even stronger: investigation via crystallography and a chimaeric tetramer," (in English), *Biochemical Journal,* vol. 435, pp. 55-63, Apr 1 2011.