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Fabrication of MERS-nanovesicle biosensor composed of multi-functional DNA aptamer/graphene-MoS$_2$ nanocomposite based on electrochemical and surface-enhanced Raman spectroscopy

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ABSTRACT

Middle East respiratory syndrome coronavirus (MERS-CoV) is one of the most harmful viruses for humans in nowadays. To prevent the spread of MERS-CoV, a valid detection method is highly needed. For the first time, a MERS-nanovesicle (NV) biosensor composed of multi-functional DNA aptamer and graphene oxide encapsulated molybdenum disulfide (GO-MoS$_2$) hybrid nanocomposite was fabricated based on electrochemical (EC) and surface-enhanced Raman spectroscopy (SERS) techniques. The MERS-NV aptamer was designed for specifically binding to the spike protein on MERS-NVs and it is prepared using the systematic evolution of ligands by exponential enrichment (SELEX) technique. For constructing a multi-functional MERS aptamer (MF-aptamer), the prepared aptamer was connected to the DNA 3-way junction (3WJ) structure. DNA 3WJ has the three arms that can connect the three individual functional groups including MERS aptamer (bioprobe), methylene blue (signal reporter) and thiol group (linker) Then, GO-MoS$_2$ hybrid nanocomposite was prepared for the substrate of EC/SERS-based MERS-NV biosensor construction. Then, the assembled multifunctional (MF) DNA aptamer was immobilized on GO-MoS$_2$. The proposed biosensor can detect MERS-NVs not only in a phosphate-buffered saline (PBS) solution (SERS LOD: 0.176 pg/ml, EIS LOD: 0.405 pg/ml) but also in diluted 10% saliva (SERS LOD: 0.525 pg/ml, EIS LOD: 0.645 pg/ml).

1. Introduction

Middle East respiratory syndrome (MERS) is a viral acute infectious respiratory disease caused by a new (novel) coronavirus called Middle East respiratory syndrome coronavirus (MERS-CoV) [1,2]. Since it was first reported, MERS has a mortality rate of approximately 35% in 27 countries and thus far MERS-CoV is considered a dangerous virus because there is no vaccine or specific treatment. In addition, large-scale MERS-CoV infections occurred in Korea in May 2015 [3]. So far, CoVid-19 outbreaks in the world. Several methods have been developed to detect MERS-CoV, for example, reverse transcriptase polymerase chain reaction (RT-PCR) [4] and enzyme-linked immunosorbent assay (ELISA) [5]. RT-PCR can be regarded as the gold standard for determining the MERS-CoV and other coronaviruses. However, it can't detect direct virus detection. ELISA generally requires multiple steps and are time-consuming methods. As a good alternative, an electrochemical-based detection method was introduced to direct the detection of virus with several advantages including portability, easy-to-handle, fast detection speed [6]. In addition, Surface-enhanced Raman spectroscopy (SERS) is suggested as a detection technique. SERS is a phenomenon in which the Raman signal of the chemical target material is amplified by resonance between the wavelength of the incident light and the surface free electrons when the chemical target material is adsorbed on the surface of certain metal nanoparticles such as Ag, Au or Cu [7–9]. SERS has the advantage of being able to detect individual components of a multi-component substance due to spectral results, such as fingerprints of biochemical molecules, by detecting unlabeled, non-destructive analytes. Also,

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MERS-NV didn’t contain the genome that can be free from MERS-CoV infection [10]. Based on this interesting technique, for the first time, we devised the MERS-NV-based biosensor composed of multifunctional (MF) DNA aptamer and GO-MoS₂ heterolayer through EC/SERS techniques. MERS-NV can provide a similar structure to the viral envelope but contain no genetic material, therefore having high stability and not being biologically hazardous [11]. MERS nanovesicle (MERS-NV) can be prepared by the extrusion of insect cells expressing spike (S) protein, envelope (E) protein and membrane (M) protein of MERS [10]. In addition to, we synthesized the MERS DNA aptamer that specifically binds to the subunit of the MERS S protein (S1 protein) by SELEX technique. SELEX is a convenient method of producing DNA aptamer through base analysis as only the bound base is extracted by reacting a random base with a specific target molecule [12,13].

To minimize the detection step, we developed an EC/SERS-based biosensor consisting of a multifunctional DNA aptamer and graphene oxide molybdenum disulfide GO-MoS₂ [14]. GO can facilitate the fast electron transfer for EC biosensor preparation as the substrate [15]. In addition to, MoS₂ has been attracted to use of biosensing material due to the SERS enhancement and rapid electron transfer [16,17]. Each end of the DNA 3WJ was modified to perform an individual function including redox property, immobilization and target binding [18]. To detect the MERS-NV, the GO-MoS₂ nanocomposite was synthesized for the EC/SERS biosensor substrate. The Au micro-gap-based biosensor electrode was developed that required small amount of sample in our previous study [19]. The biosensor performances were validated by EIS and SERS. Fig. 1 showed a schematic diagram of the proposed MERS-NV biosensor.

2. Experiment

2.1. Materials

Spike 1 (S1) protein, hemagglutinin (HA) protein, and zika virus envelope (E) protein were purchased from Sino Biology (Beijing, China). Ammonium molybdate tetrahydrate, polyvinylpyrrolidone k90, hemoglobin from horse heart, albumin from bovine serum, amylase from human saliva, human immunoglobulin (IgG) and L-Homocysteine thiolactone hydrochloride were purchased from Sigma-Aldrich (MO, USA), and thiourea was obtained from Tokyo Chemical Industry Co., Ltd., (Tokyo, Japan). Graphene oxide (GO) was purchased from Graphene Supermarket (NY, USA). Streptavidin Mag-Beads was purchased from GenScript (NJ, USA), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS) and EZ-Link Sulfo-NHS-LC-Biotin from Thermo Fisher Scientific (MA, USA). To produce MERS aptamer in the SELEX experiment, the XELEX DNA Core Kit was purchased from Roboklon (Berlin, Germany). For the SELEX experiment, the random DNA library, forward primer and reverse primer were supported by Roboklon, DNA Library Bank 40, 5’-TGA CAC CGT ACC TGC TCT- N40 - AAG CAC GCC AGG TAC TAT - 3’. The sequence information of the forward primer is 5’-ATA GTC CCT GGC GTG CTT-3’. Pfu polymerase extracted from Pyrococcus furiosus was used. For the SELEX experiment, all reagents were supported by Roboklon. The sequence information on the MERS-NV aptamer-tagged MF DNA sequence is: 5’-TGA CAC CGT ACC TGC TCT GCA CTT CCT TCA CCA GAA ACC TGC ACA TCT TCG CCG CGT GAA GCA GGC CAA GGG ACT ATT TGC CAT GTG TAT GTG GG-3’ for Apt/3WJa, 5’- TTT GGG TAG GGC GGG TTG GG CCCA CAT ACT TTG ATC C-3’ for MB/3WJb, and NH2-5’-GGA TCA ATC ATG GCA A-3’ for NH2/3WJc. The oligonucleotides were supported by Bioneer (Daejon, South Korea) and were diluted with water without nucleases. Artificial saliva was purchased from Pickering Laboratories (CA, USA). All chemicals were used as received. Table S1 showed the all sequence information of DNA used in this study.

2.2. MERS aptamer production using in vitro selection

The MERS aptamer, which showed high specificity to MERS S1 protein, was produced using the SELEX technique [20,21]. To modify the conventional method, we introduced the streptavidin-coated magnetic nanoparticle-based SELEX technique (Fig. S1). To prepare the aptamer, 10 μL of EZ-Link Sulfo-NHS-LC-Biotin (2 mM) was reacted with 50 μL of MERS S1 protein (0.25 mg/ml) for 1 h at RT. Biotin-tagged S1 protein enabled binding of the streptavidin-coated magnetic nanoparticles. A 1 ml solution of PBS was added to keep the pH of the solution constant, and then put into a vivaspin column (5000 molecular weight cut off (MWCO), Sigma-Aldrich, MO, USA) at 4000 rpm for 20 min to separate unreacted MERS S1 protein. The supernatant was extracted from the separated reagent as a solution to the vivasin column. 100 μL of Streptavidin MagBeads were added to immobilize the target molecules, MERS S1 protein. Then, the mixture was incubated at 25 °C for 1 h in the shaker. To separate unreacted MERS S1 protein,
500 μL of SELEX Buffer (140 mM NaCl, 5 mM MgCl₂, 2 mM CaCl₂, 20 mM Tris, 0.05% [v/v] Tween 20) was used for washing, and the washing process was repeated 3 times.

To react with the prepared Streptavidin MagBeads-coated S1 protein and a library of random oligonucleotides (DNA Library Bank 40) 20 μL, 10 x SELEX Buffer 20 μL and DI water was added to the reaction for 1 h. The oligonucleotide had more than 150 bases and random bases of 40 mer were used. In addition, 80 mer bases have high target affinity but have non-specific or unwanted binding properties and the universal library is too large to screen the whole. To separate unreacted DNA and MERS S1 protein, a washing process was conducted and MERS S1 protein with random oligonucleotides was eluted and heated at 90 °C for 10 min to release the tangled DNA before amplification. The amplification step is necessary because the total number of nucleic acid molecules in the DNA library is still small during the SELEX step. To solve this problem, DNA was amplification by polymerase chain reaction (PCR).

The PCR process was as follows. First, DNA denaturation was performed to treat two stranded DNA at 95 °C for 150 s and separate it into single stranded DNA. Then, by lowering the temperature of the denatured DNA, two primers were annealed with complementary stranded DNA and kept at 55 °C for 60 s. Finally, in the state where four kinds of substrates (dNTP) coexisted, DNA polymerase was applied to extend the primer. After 72 °C for 180 s and at 70 °C for 300 s, the PCR cycle was repeated 20 times. Subsequently, the amplified DNA was placed in place of DNA Library 40, and the SELEX technique was repeated 15 rounds. The PCR product was purified from an agarose gel to prepare an aptamer having high affinity and specificity to the target. Finally, to confirm the binding of the prepared MERS aptamer and S1 protein, TBE-PAGE was performed at 80 V for 55 min using 8% TBE gel prepared in TBE buffer (889 mM Tris, 2 mM EDTA pH 8.0) [22,23].

After 15 rounds of selection cycle, the PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany). The PCR products were cloned using T blunt cloning and transformed into DH5α competent cells. These steps were supported by Solgent (Daejon, South Korea). 50 colonies were randomly selected for amplification. LB agar plates with ampicillin and kanamycin were used for the transformation. Plasmid DNA sequencing of the selected products was analyzed by Solgent (South Korea).

2.3. Measurement of aptamer binding affinity

In order to confirm the binding affinity of the fabricated aptamer to the S1 protein, an affinity experiment was performed using the bead-based fluorescence binding assay [24-26]. The initial procedure is the same as aptamer production using the SELEX technique, but unlike the DNA Library Bank in the SELEX method tested. The fluorescein phosphoramidite (FAM) group labeled MERS aptamer was added 5 times for each concentration of 1.5 μM, 1 μM, 300 nM, 30 nM, 3 nM, and 100 nL, respectively. The reaction was carried out for 2 h at RT. To remove the unbound DNA, the washing process with SELEX Buffer was performed 3 times. Then, the elution of S1 protein and DNA was performed for 10 min at 90 °C to release the tangled DNA. Subsequently, the fluorescence of the aptamer was measured and the dissociation constant (Kₐ) was calculated. The calculation is as follows:

$$\Theta = \frac{y - y_b}{y_b} = \frac{1}{2}\frac{[DNA \text{Aptamer}]}{K_d + x}\left(\frac{K_d + x}{[DNA \text{Aptamer}] + 4}\right)$$

where Θ is the fraction of bound; x is the protein concentration; y is the observed fluorescence intensity at the n titration; y_b is the fluorescence intensity at the absence protein condition and y_b is the fluorescence intensity at saturating protein concentration.

2.4. Assembly of MF MERS aptamer

The prepared MERS aptamer and MF DNA fragments were connected to prepare a multi-functional aptamer. MB/3WJb were prepared for the Raman signal reporter and amine group-modified DNA 3WJc immobilized to GO-MoS₂ nanocomposite, respectively. It was immobilized using the peptide bond formed by the carboxyl group on the GO surface and the amino group of 3WJc. MF MERS aptamer was heated at 80 °C for 5 min to anneal DNA strands in the same molar ratio as in TMS buffer (40 mM Tris-HCl, 10 mM MgCl₂, 100 mM NaCl) and then it was slowly cooled to 4 °C at a rate of 2 °C/min in the T100™ Thermal Cycler (Bio-rad). The assembly of the multi-functional MERS aptamer was confirmed by 8% TBE-PAGE.

2.5. Production of MERS-NV

Bm5 cells, which were maintained in SF-900 II (Thermo Fisher Scientific K. K., Japan) and supplemented with 10% fetal bovine serum (Thermo Fisher Scientific K. K., Japan), were infected with recombinant BmNPV/S/T/M at the multiplicity of infection (M.O.I.) of 1.0 and cultivated for 4 days. 5 x 10⁶ cells were suspended in PBS (pH 7.4) and extruded 10 times through a 5 μm polycarbonate track-etched membrane disk (GVS Japan K. K., Tokyo Japan) using a mini-extruder (Avanti Polar Lipids, Alabaster, AL, USA). The filtrate was centrifuged by sucrose density gradient centrifugation (20–60%), and the S protein-rich fractions were recovered and dialyzed with PBS. The detailed procedure was described in the previous study [11].

2.6. Fabrication of MERS-NV/MF MERS aptamer/GO-MoS₂ nanocomposite on Au substrates

For synthesizing GO-MoS₂ nanocomposite, we followed the previous study, the functional group was deposited on the surface of MoS₂ and encapsulated by graphene oxide (GO) [27,28]. The detailed explanation and data analysis were described in the Supplementary material (Fig. S2). In order to immobilize the MF MERS aptamer/GO-MoS₂ on Au substrate for the EC/SERS experiment, the Au substrate was cleaned using an acetone solution with sonication for 5 min. Then, it was washed with ethanol and DIW, and then dried with N₂ gas to remove surface residues. Subsequently, cysteamine was dropped on the Au surface to form a self-assembled thin-film to bind between carbonyl group of GO-MoS₂ and amine group of cysteamine with EDC/sulfo-NHS coupling reagent. For activating the reaction, the 5 μL of 200 mM EDC solution and sulfo-NHS (10 μL, 0.2 M) solution were added simultaneously [29]. After that, 30 μL of 1 μM MF MERS aptamer was dropped on the surface of GO-MoS₂ for 3 h with EDC/sulfo-NHS coupling reagent. In this immobilization step, excess biomolecules were removed by deionized water and N₂ gas stream. Finally, the 30 μL of MERS-NV solutions (concentration range: 1 pg/ml to 100 ng/ml) were added to prepared electrode for 1 h. In this reaction step, excess MERS-NV were removed by deionized water and N₂ gas stream.

For confirming the fabrication process, the atomic force microscopy (AFM) (Digital Instruments, USA) was carried out with tapping-mode. For investigating the surface morphology of the sample, the setpoint current, gain values were optimized between the tip and the substrate [30,31].

2.7. Detection of MERS-NV by SERS

The SERS effect was measured using a confocal Raman spectrometer (NTEGRA Spectra, NT-MDT) to detect MERS-NV on the MF MERS aptamer/GO-MoS₂-modified Au substrate [15]. All samples were measured using a 785 nm near-infrared laser with 3 mW laser power in the plane. Raman spectra were averaged from 10 different points in 10 independent samples. Binding the S1 protein of the MERS-NV to MF MERS aptamer gives the Raman signal due to the resonance between the

G. Kim et al. Sensors and Actuators: B. Chemical 352 (2022) 131060

Sensors and Actuators: B. Chemical 352 (2022) 131060

Sensors and Actuators: B. Chemical 352 (2022) 131060

Sensors and Actuators: B. Chemical 352 (2022) 131060

Sensors and Actuators: B. Chemical 352 (2022) 131060
wavelength of the incident light and the surface free electrons when the target is close to certain metal nanosurfaces such as the ones made of GO-MoS\textsubscript{2} molecules. This was shown by the change in the SERS spectrum due to the amplifying effect. In addition, changes were observed because each molecule has its own Raman signal. The measurement required approximately 17 min.

2.8. Detection of MERS-NV by EIS

All electrochemical experiments were conducted using potentiostat (AMETEK’s Versastat3 (USA)) \[14,16\]. A cylinder was fabricated using PDMS on the horizontal gold electrode to construct a working chamber. A Three-electrode system was constructed by inserting the Ag/AgCl reference electrode into the working chamber. All samples were measured for impedance in 5 mM [Fe (CN)\textsubscript{6}]\textsuperscript{4-/3-}, 1 M KCl in PBS Buffer. The electrical properties for impedance measurement are as follows; AC properties of 1 Hz–100 kHz with amplitude of 10 mv RMS; DC properties of 0.25 V (vs. Ref.). Charge transfer resistance ($R_{ct}$) was obtained by setting the equivalent circuit as the ideal Randles circuit, and MERS-NV was quantified using the change in $R_{ct}$ value when the S1 protein of MERS-NV was bound to MF MERS aptamer.

3. Results and discussions

3.1. Construction of multi-functional MERS aptamer

To confirm the affinity between the prepared SELEX PCR product and the target S1 protein, TBE-PAGE was performed (Fig. 2a). The gel result showed the 15th SELEX product (lane 2), 15th SELEX product/MERS S1 protein (lane 4), and 15th SELEX product/myoglobin (lane 6). Compared with the control group of myoglobin with the SELEX product, the SELEX product with S1 protein confirmed that the shape and position of the band of MERS S1 protein changed. These results indicated that the three-dimensional structure was changed when the 15th SELEX product was combined with MERS S1 protein, which confirmed that the 15th SELEX product specifically binds with MERS S1 protein.

After finishing the sequence identification step of the SELEX product, the identified SELEX product was purified through agarose gel electrophoresis and two aptamers were selected. Thirty clones of aptamer PCR products were sequenced, of which, two representative sequences were selected as MERS aptamer candidates. The detailed sequence information is described as follows: (1) S-19 aptamer: 5′-TGA CAC CGT ACC TGC TCT GCA CTT CCT TCA CCA GAA ACC TGC ACA TCT TCG CCG GTG GAA GCA CGC CAA GGG ACT AT-3′, (2) S-12 aptamer: 5′-TGA CAC CGT ACC TGC TCT CGG ACC CAT CTA GCA GTC ACC CAT CAC ACG CGG ATC GGA TTA GTA AGC ACG CCA AGG GAC TAT. The expected 2D structures of S-19 and S-12 aptamers were predicted by the UNAfold program (Fig. 2b, Fig. S3a, S3b), respectively. Subsequently, the target affinity test between the S-19 aptamer and the surface protein of MERS S1 protein was carried out by TBE-PAGE (Fig. 2c). The gel result showed the MERS S-19 aptamer (lane 2), MERS S-19 aptamer/MERS S1 protein (lane 4), and MERS S-19 aptamer/myoglobin (lane 6). Compared with the control group (lane 6), the MERS S-19 aptamer with S1 protein (lane 4) was confirmed to change the shape and position of the MERS S1 protein band. Presumably, these results indicated that the MERS S-19 aptamer was combined with the MERS S1 protein, which confirmed that MERS S-19 aptamer specifically binds with MERS S1 protein.

To determine the $K_d$ value of the fabricated aptamer, fluorescence titration of the S-19 aptamer was performed against the S1 protein. The FAM-labeled S-19 aptamer was added to various concentrations of S1 protein (1.5 μM, 1 μM, 300 nM, 30 nM, 3 nM) (Fig. 2d). In addition, the titration of the S-12 aptamer was carried out as the control experiment (Fig. S3c and d). The fluorescence of the S-19 aptamer was measured and the dissociation constant ($K_d$) was calculated to $K_d = 112.18 \text{ nM} \pm 3.89 \text{ nM}$. In comparison, MERS aptamer-b showed lower affinity ($K_d = 286.98 \text{ nM} \pm 17.7 \text{ nM}$) (Fig. S3d). Based on the test, the three-dimensional structure was changed when the 15th SELEX product was combined with MERS S1 protein, which confirmed that the 15th SELEX product specifically binds with MERS S1 protein.
the S-19 aptamer showed a high affinity to S1 protein.

Fig. 2 predicted the 2D structure of MF MERS aptamer for multifunctional aptamer preparation by UNAFold. To confirm the assembly of MF MERS aptamer, TBM-PAGE was performed (Fig. 2f). The gel clearly shows MERSapt-3WJa (lane 2), MB-3WJb (lane 3), NH2–3WJc (lane 4), and MF MERS aptamer (lane 5). The result is that the designed multifunctional bioprobe can be prepared well. Moreover, a selectivity test between the fabricated MF MERS aptamer and MERS-NV was carried out (Fig. 2g). The MERS-NV was reacted with assembled MF MERS aptamer (lane 2). Lane 4 showed different migration performance, clearly showing the MF MERS aptamer binding MERS-NV and occurred in the upper band. Presumably, the three-dimensional structure was changed by combining MF MERS aptamer and MERS-NV. As a negative control, myoglobin (lane 6) and HA protein (lane 8) were reacted with assembled MF MERS aptamer, respectively. In lane 6 and lane 8, no change in movement was observed compared to lane 4. The result can be interpreted that only MF MERS aptamer showed the selectivity for the MERS-NV. Thus, the designed multi-functional aptamer can specifically detect MERS-NV.

3.2. Investigation of fabricated MERS-NV/MF MERS aptamer on GO-MoS2

To confirm the immobilization of fabricated MERS aptasensor, several experiments were performed. Fig. 3a depicted the schematic diagram of the fabricated MERS-NV biosensor. For confirming the biosensor construction, the immobilization process of MERS-NV/MF MERS aptamer on GO-MoS2 nanocomposite on the Au electrode was investigated by AFM. Fig. 3b showed the AFM results of GO-MoS2 nanocomposite-immobilized Au substrate. Compared with FE-SEM
result of GO-MoS$_2$ nanocomposite (Fig. S2a), the GO-MoS$_2$ nanocomposite immobilized on Au substrate well. The nanocomposite size is around 1.5–2.5 µm. When the MF MERS aptamer was immobilized on GO-MoS$_2$ nanocomposite, the surface topography was entirely changed. Fig. 3c depicted the MF MERS aptamer on GO-MoS$_2$ nanocomposite on the Au electrode. It formed small lumps with sizes around 10–20 nm, presumably, MF MERS aptamer was immobilized on the fabricated substrate well. When the MERS-NV was added, the surface morphology was totally drastically changed (700 nm to 1 µm size) compared to other samples [11]. The MERS-NV showed the well bound with prepared aptasensor for further study (Fig. 3d). Also, the surface roughness and vertical distance analysis were carried out Fig. 3e. The distinct change of surface topography, roughness and vertical distance of each sample can be explained the MERS-NV immobilized well on MF MERS aptamer/GO-MoS$_2$ substrate.

For characterizing the fabricated MERS-NV biosensor, the SERS and EIS experiments were conducted. Fig. 3f exhibited the SERS spectra of the fabricated biosensor. The SERS was used to detect the binding event between probe and target [32–34]. The MB group of MF MERS aptamer on GO-MoS$_2$ acted as the reporter to the Raman signal. By measuring the SERS signal of the MB was confirmed the immobilization of MF MERS aptamer. Fig. 3f and Table 1 showed the most intensive Raman band assignments appearing in the SERS spectra of MF MERS aptamer (MB), GO-MoS$_2$, and MERS-NV obtained from different studies [35–38]. First, the black line in Fig. 3f depicted the SERS spectra of GO-MoS$_2$–modified substrate (Black line), Raman peaks at 1328 and 1602 cm$^{-1}$ were assigned to the D-band and G-band, respectively [35]. And then, the SERS spectra of red line showed the MF MERS aptamer on GO-MoS$_2$–modified substrate. The Raman peaks of MB could be observed at 449, 590, 1394, and 1623 cm$^{-1}$ (Red line) which are assigned to the deformation modes of skeletal deformation of C–N–C mode, skeletal deformation of C–S–C mode, symmetrical stretching of C–N mode, Ring stretching of C–C mode [36,37]. Afterwards, MERS-NV was added onto prepared substrate and measured by SERS (Blue line). Raman peaks were observed in bands 937, 1350, and 2307 cm$^{-1}$ that are significantly different from GO-MoS$_2$ and MF MERS aptamer. S protein–rich fractions were recovered from MERS-NV. BecaS protein band Therefore, the 1350 cm$^{-1}$ band of the S protein can be assigned to the Raman vibrational mode of Amide III [38].

Charge transfer between the MF MERS aptamer and MERS-NV chemically bound to GO-MoS$_2$ changes the chemistry, and as they bind, the three-dimensional structure changes. This phenomenon can be explained by the amplification of the Raman signal, the formation of additional peaks by the chemical enhancement mechanism. It also showed that the MF MERS aptamer binds well with the target MERS-NV.

In addition, the electrochemical experiments including cyclic voltammetry (CV) and EIS were carried out for confirming the electrochemical biosensor application. Fig. S4 showed the cyclic voltammogram of MERS-NV/MF MERS aptamer on GO-MoS$_2$–modified electrode. On the surface of Au working electrodes, ferri–ferrocyanide in the electrolyte exchanges electrons as shown in the equation given below, which leads to the generation of redox current.

\[
[\text{Fe(CN)}_6]^{3-} + e^- \rightarrow [\text{Fe(CN)}_6]^{4-}
\]

Electron exchange is proportional to the area of the electrode surface. Therefore, if the electrode surface is modified by substances such as MF MERS aptamer or MERS-NV, the redox current will be blocked and the Rct will increase. So, MF MERS aptamer will provide a means of detecting MERS-NV on the immobilized electrode surface.

Charge transfer resistance and double layer capacitance can be obtained via EIS, various reactions occurring on the electrode surface can be detected through these signals [39]. Fig. 3g showed the complex impedance of GO-MoS$_2$/Au substrate (Red line), MF MERS aptamer/GO-MoS$_2$/Au substrate (Blue line), MERS-NV/MF MERS aptamer/GO-MoS$_2$/Au substrate (Green line) samples on the complex plane in the range of 1 Hz to 100 kHz. The horizontal axis of the Nyquist plot represents the actual resistance, the intersection of the horizontal axis and the semicircle can be expressed as the resistance in the electrolyte (R$_e$), and the opposite intersection can be expressed as the sum of the charge transfer resistance and the resistance in the electrolyte. As described above, since the charge transfer resistance and the deformation on the electrode surface are closely related, the deformation of the electrode surface can be known by obtaining the diameter of the semicircle. Fig. 3g depicted the R$_e$ of each sample, and the Rct of the MERS-NV/MF MERS aptamer/GO-MoS$_2$/Au substrate samples significantly increased by ~ 6 kΩ compared to the MF MERS aptamer/GO-MoS$_2$/Au substrate samples.

### 3.3. Validation of biosensor performance and clinical test by SERS

To analyze SERS-based biosensor performance, MERS-NV diluted in PBS was added to MF MERS aptamer/GO-MoS$_2$ nanocomposite–modified substrate. To determine the detection limit (LOD) and dynamic range, SERS experiments were performed with 10 samples and recorded and averaged before any analysis. SERS spectra were obtained corresponding to MERS-NV concentrations (0–100 ng/ml). To obtain LOD, serially diluted MERS-NV samples in PBS buffer were used. As shown in Fig. 4a, the SERS peak intensities increased with increasing MERS-NV concentration from 1 pg/ml to 100 ng/ml. The SERS signal of the target molecule can be obtained when the target was near the SERS active nanostructure. The Raman bands of the MERS-NV were observed in the other bands 937, 1350, 2307 cm$^{-1}$ that are significantly different from GO-MoS$_2$ and MF MERS aptamer. S protein–rich fractions were recovered from MERS-NV. BecaS protein band Therefore, the 1350 cm$^{-1}$ band of the S protein can be assigned to the Raman vibrational mode of Amide III [38].

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\[
\text{Fe(CN)}_6^{3-} + e^- \rightarrow \text{Fe(CN)}_6^{4-}
\]

So, LOD was calculated by substituting $y_{blank}$ into the calibration curve.

Besides, the selectivity of the prepared SERS biosensor was tested with other proteins including bovine serum albumin (BSA), myoglobin.
3.4. Validation of biosensor performance and clinical test by EIS

To evaluate the electrochemical performance of the fabricated sensor, EIS was performed with MERS-NV in a concentration range of 1 pg/ml to 100 ng/ml (Fig. 5a and b). EIS was performed by preparing a sample diluted with MERS-NV of the same concentration in 10% saliva. While the Rs-iRct value of the fabricated MF MERS aptamer/GO-MoS$_2$ nanocomposite modified substrate was ~ 4 kΩ, it increased to ~ 8 kΩ when reacted with MERS-NV 1 pg/μL sample, and increased to ~ 16 kΩ when the concentration of MERS-NV was increased to 0.1 μg/μL. The result of performing EIS in 10% saliva sample showed an overall increase in Rct value than the result of performing in PBS buffer (Fig. 5c). MERS-NV was ~ 13 kΩ in 1 pg/μL sample, and as the concentration of MERS-NV increased, the Rct value also increased, increasing to ~ 25 kΩ in 0.1 μg/μL MERS-NV. \( \Delta R_{ct} \) was calculated as follows.

\[
\Delta R_{ct} = \frac{R_2 - R_0}{R_0}
\]

R$_2$ is the Rct value of the MERS-NV sample at different concentrations, and R$_0$ is the Rct value of the sensor that did not react with MERS-NV. Linear regression analysis between the concentrations of \( \Delta R_{ct} \) and MERS-NV was performed to obtain a linear regression line with slope 0.32039 ± 0.014 and intercept 2.4676 ± 0.059 (Fig. 5b). The LOD of this sensor determined through this was 0.4049 pg/ml. The same method was applied to the EIS results performed in 10% saliva to obtain a linear regression line with slope 0.4988 ± 0.01 and intercept 0.32039 ± 0.014 and intercept 2.4676 ± 0.059 (Fig. 5b). The LOD of this sensor determined through this was 0.4049 pg/ml. The same method was applied to the EIS results performed in 10% saliva to obtain a linear regression line with slope 0.4988 ± 0.01 and intercept 0.32039 ± 0.014 and intercept 2.4676 ± 0.059 (Fig. 5b). The LOD of this sensor determined through this was 0.4049 pg/ml.
4.3316 ± 0.1 (Fig. 5d). Through this, the LOD of this sensor at 10% saliva was determined as 0.645 pg/ml. The following relational expression is established between signal at limit of detection ($y_{dl}$) and the signal at blank sample ($y_{blank}$) [39].

$$y_{dl} = y_{blank} + 3S$$

So, LOD was calculated by substituting $y_{dl}$ into the calibration curve. A selectivity test was conducted to check whether the manufactured sensor reacts selectively with MERS-NV (Fig. 5e). The comparative protein used was the protein present in saliva and the surface protein of other respiratory viruses. In the case of reacting another control protein to the manufactured sensor, the $R_{ct}$ value slightly increased, but compared to the MERS-NV of the same concentration, the $R_{ct}$ value was very small and had high selectivity (Fig. 5f). Table 2 showed the comparison table of MERS biosensor from other studies.

4. Conclusion

So far, MERS-CoV showed a high infection, an acute infectious disease, and has no cure method yet. The present study developed the MERS aptamer using SELEX technique and developed the world’s first EC/SERS-based MERS-NV biosensor consisting of an MF MERS aptamer/GO-MoS$_2$ nanocomposite on Au substrate. The MF-MERS aptamer serves as a suitable MERS bioprobe for integrating three functions into a single
DNA structure. The MERS aptamer and MF DNA were identified by TBM.

### Table 2

| No. | Probe | Detection Method | Detection Limit | Sample | References |
|-----|-------|------------------|-----------------|--------|------------|
| 1   | Pyrrolidinyl peptide nucleic acid (apcpPNA) | Paper-Based Colorimetric Assay | 1.53 nM | Synthetic DNA oligonucleotides | [41] |
| 2   | Antibody | SWV | 1.0 pg.ml⁻¹ | Spiked nasal Samples | [42] |
| 3   | ssDNA immobilized on self-assembly shielded AuNPs | Colorimetric Assay(LSPR) | 1 pmol/µl | ORF1a, upE | [43] |
| 4   | MF MERS aptamer/AuNPs | EC/SERS | 0.525 pg/ml (SEROS) 645 pg/ml | Artificial Saliva | Present work |

### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.snb.2021.131060.

### References

[1] R.J. Groot, S.C. Baker, R.S. Baric, C.S. Brown, C. Drosten, L. Enjuanes, S. Perlman, Commentary: middle east respiratory syndrome coronavirus (mers-cov): announcement of the coronavirus study group, J. Virol. 87 (2013) 7790–7792.

[2] R. Bha, J. Budd, D. Ferris, J. Watson, A.T. Curns, D.L. Swardlow, S.L. Gerber, Update on the epidemiology of Middle East respiratory syndrome coronavirus (MERS-CoV) infection, and guidance for the public, clinicians, and public health authorities.—January 2015, MMWR Morb. Mortal. Wkly. Rep. 64 (2015) 61–62.

[3] M.D. Poh, The Korean Middle East respiratory syndrome coronavirus outbreak and our responsibility to the global scientific community, Infect. Chemother. 48 (2016) 145–146.

[4] C. Sun, C. Whan, S.K. Sahr, T. Kamli, L. Rose, L. Lowe, E. Mohare, E. M. Elmass, T. Al-sanaouri, A. Haddadin, D.D. Erdman, Real-time reverse transcription-PCR assay panel for Middle East respiratory syndrome coronavirus, J. Clin. Microbiol. 54 (2016) 67–75.

[5] S. Fukusha, A. Fukuma, T. Kurita, S. Watanabe, M. Shimonmura, K. Shirato, S. K. Melaku, Characterization of novel monoclonal antibodies against the MERS-cov, T. Kurosu, Characterization of novel monoclonal antibodies against the MERS-cov, and their application in species-independent antibody detection by competitive ELISA, J. Virol. Methods 251 (2018) 22–29.

[6] M. Moskovits, Surface-enhanced raman spectroscopy: a brief retrospective, J. Raman Spectrosc. 36 (2005) 485–496.

[7] C.L. Haynes, A.D. McFarland, R.P. Van Duyne, Surface-enhanced raman spectroscopy, Anal. Chem. 346A (2005) 334A–346 A.

[8] Y. Tepedi, U. Anik, Electrochemical biosensors for influenza virus a detection: the potential of adaptation of these devices to POC systems, Sens. Actuators B Chem. 254 (2018) 377–384.

[9] P.L. Stiles, J.A. Dieringer, N.C. Shah, R.P. Van Duyne, Surface-enhanced raman spectroscopy, Annu. Rev. Anal. Chem. 1 (2008) 601–626.

[10] T. Kato, T. Takami, V. Kumar Deo, E.Y. Park, Preparation of virus-like particle mimetic nanovesicles displaying the S protein of Middle East respiratory syndrome coronavirus using insect cells, J. Biotechnol. 306 (2019) 177–184.

[11] A. Zeiri, O. Tum, Characterization and visualization of virus-like particles: a review, Molecular 53 (2013) 92–107.

[12] R. Stoltenburg, C. Reimanem, B. Strehlitz, SELEX—a (r) evolutionary method to generate high-affinity nucleic acid ligands, Biomol. Eng. 24 (2007) 381–403.

[13] Y.X. Wu, V.J. Kwon, Aptamers: the evolution of SELEX, Methods 106 (2016) 21–28.

[14] T. Lee, M. Mohammadiangh, H. Zhang, J. Yoon, H.K. Choi, S. Guo, E. Guo, J. W. Chai, MicroRNA detection: single functionalized pRNA/Gold nanoparticle for ultrasensitive MicroRNA detection using electrochemical surface-enhanced Raman spectroscopy, Adv. Sci. 7 (2020) 1902477.

[15] J. Yoon, T. Lee, J. Jo, B.K. Ooi, J.W. Choi, Electrochemical H2O2 biosensor composed of myoglobin on MoS2 nanoparticle-graphene oxide hybrid structure, Biosens. Bioelectron. 93 (2017) 14–20.

[16] Y. Bai, T. Xu, X. Zhang, Graphene-based biosensors for detection of biomarkers, Micromachines 11 (2020) 60.

[17] J. Yoon, J. Lim, M. Shin, S.N. Lee, J.-W. Choi, Graphene/MoS2 nanohybrid for biosensor, Materials 14 (2021) 518.

[18] Y. Xia, S. Gan, Q. Xu, C. Qiu, P. Gao, S. Huang, A three-way junction aptasensor for lysozyme detection, Biosens. Bioelectron. 39 (2013) 250–254.

[19] S.Y. Park, J. Kim, G. Yim, H. Jung, Y. Lee, S.M. Kim, C. Park, M.-H. Lee, T. Lee, Fabrication of electrochemical biosensor composed of multi-functional DNA/rhodium nanoplate heterolayer for thyroxine detection in clinical sample, Colloids Surf. B 195 (2020), 111240.

[20] C. Tuerk, L. Gold, Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase, Science 294 (1996) 505–510.

[21] R. Wang, J. Zhao, T. Jiang, Y.M. Kwon, H. Lu, P. Jiao, M. Liao, Y. Li, Selection and characterization of DNA aptamers for use in detection of avian influenza virus H5N1, J. Virol. Methods 199 (2013) 362–369.

[22] T. Lee, S.Y. Park, H. Jang, G.H. Kim, Y. Lee, C. Park, J. Min, Fabrication of electrochemical biosensor consisted of multi-functional DNA structure/porous anode nanoparticle for avian influenza virus (H5N1) in chicken serum, Mater. Sci. Eng. C 99 (2019) 511–519.

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**Jeong-Woo Choi:** Contributed to Conceptualization and Supervision. **Taek Lee:** Contributed to Conceptualization and Supervision. **Gahyeon Kim:** Performed the Methodology and Investigation. **Jimmyeong Kim:** Performed the Methodology and Investigation. **So Min Kim:** Contributed to Data curation and Resources. **Tatsuya Kato:** Contributed to Data curation and Resources. **Enoch Y. Park:** Contributed to Data curation and Resources. **Seungwoo Noh:** Contributed validation of work. **Jinho Yoon:** Contributed validation of work. **Chulhwan Park:** Contributed validation of work.

### Authors contribution

The manuscript was written through contributions of all authors. T. L., and J.-W.C. designed the project. G. K. and J. K. performed all experiments, S. K. analyzed the SERs data. T. K. and E.P. synthesized and characterized the MERS-NV. S.N. and C.P. performed the EC and AFM works.

### Declaration of Competing Interest

The authors declare no competing financial interest.

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[23] T. Lee, G.H. Kim, S.M. Kim, K. Hong, Y. Kim, C. Park, J. Min, Label-free localized surface plasmon resonance biosensor composed of multi-functional DNA 3 way junction on hollow Au spike-like nanoparticles (HAuSN) for avian influenza virus detection, Colloids Surf. B 182 (2019), 110341.

[24] J.A. Cruz-Aguado, G. Penner, Determination of ochratoxin A with a DNA aptamer, J. Agric. Food Chem. 56 (2008) 10456–10461.

[25] M. McKeague, R. Velu, K. Hill, V. Bardocz, T. Metézaro, M. DeRosa, Selection and characterization of a novel DNA aptamer for label-free fluorescence biosensing of ochratoxin A, Toxins 6 (2014) 2435–2452.

[26] E.C. Hulme, M.A. Br Trevethick, Ligand binding assays at equilibrium: validation and interpretation, J. Pharm. Pharmacol. 161 (2010) 1219–1237.

[27] J. Yoon, J.W. Shin, J. Lim, M. Mohammadniaei, G.B. Paparao, T. Lee, J.W. Choi, Electrochemical nitric oxide biosensor based on amine-modified MoS2/graphene oxide/myoglobin hybrid, Colloids Surf. B 159 (2017) 729–736.

[28] S. Zeng, S. Hu, J. Xia, T. Anderson, X.Q. Dinh, X.M. Meng, K.T. Yong, Graphene–MoS2 hybrid nanostructures enhanced surface plasmon resonance biosensors, Sensors Actuators B 207 (2015) 801–810.

[29] D. Bartczak, A.G. Kanaras, Preparation of peptide-functionalized gold nanoparticles using one pot EDC/sulfos–NHS coupling, Langmuir 27 (2011) 10119–10123.

[30] T. Lee, S.–U. Kim, J. Min, J.W. Choi, Multilevel biomemory device consisting of recombinant azurin/cytochrome c, Adv. Mater. 22 (2010) 510–514.

[31] T. Lee, A.Y. Kumar, F. Pi, A. Sharma, J.W. Choi, P. Guo, Construction of RNA–quantum dot chimera for Nanoscale resistive biomemory application, ACS Nano 9 (2015) 6675–6682.

[32] A. Rygula, R. Signorini, C. Durante, L. Orian, M. Bhamidipati, L. Fabris, A review on surface-enhanced Raman spectroscopy (SERS) substrates for lipid and protein characterization: sensing and beyond, Analyst 143 (2018) 3990–4008.

[33] R. Pilot, R. Signorini, C. Durante, L. Orian, M. Bhamidipati, L. Fabris, A review on surface-enhanced Raman scattering, Biosens 9 (2019) 57.

[34] G.L. Long, J.D. Winefordner, Limit of detection. A closer look at the IUPAC definition, Anal. Chem. 55 (1983) 712A–724A.

[35] P. Teengam, W. Sangsreb, A. Tuatranont, T. Vitalyan, O. Chailapakul, C. S. Henry, Multiplex paper-based colorimetric DNA sensor using pyrrolidinyl peptide nucleic acid-induced AgNPs aggregation for detecting MERS-CoV, MTB, and HPV oligonucleotides, Anal. Chem. 89 (2017) 5428–5435.

[36] L.A. Layyah, S. Eissa, An electrochemical immunosensor for the corona virus associated with the Middle East respiratory syndrome using an array of gold nanoparticle-modified carbon electrodes, Microchim. Acta 186 (2019) 224.

[37] H. Kim, M. Park, J. Hwang, J.H. Kim, D.R. Chung, K.S. Lee, M. Kang, Development of label-free colorimetric array for MERS-CoV using gold nanoparticles, ACS Sens. 4 (2019) 1306–1312.

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