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Optimization Procedures for the Development of a SERS-Based Lateral Flow Assay for Highly Sensitive Detection of Troponin I †

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Abstract: Continuous and rapid monitoring of cardiovascular diseases (CVD) is critical for the management of emergency situations in healthcare systems. Herein, we performed a quantitative assay using a surface-enhanced Raman scattering (SERS)-based lateral flow immunoassay (LFA) for the early and rapid detection of cardiac Troponin I (cTnI) to optimize the procedures for developing the SERS-based LFA. First, nanoparticles were optimized as SERS tags to obtain a reliable quantitative assay for SERS intensity. Second, for SERS performance, the LFA components and running time were optimized because the LFA component affected the sensitivity of the quantitative assay. Third, the SERS intensity was tested at different laser wavelengths and powers, and optimization of the SERS data collection was performed. The optimized conditions led to strong sensitivity with a 0.02 ng/mL detection limit (LOD) with SERS-based LFA. The development and optimization of SERS-based cTnI LFA sensors shows significant potential for early and rapid detection.

Keywords: lateral flow assay; biosensors; cardiovascular disease (CVD); surface-enhanced raman spectroscopy; POC devices; medical device; healthcare system; gold nanoparticles; biomarker; immunoassay

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1. Introduction

The cardiac troponin I (cTnI) assay has been used to diagnose acute coronary syndrome (ACS) because of its increased sensitivity [1]. Early diagnosis and timely monitoring of changes are important for obtaining early treatment and save time and costs. A lateral flow immunoassay (LFA) is a rapid diagnostic test used in point-of-care testing (POCT). It is part of a multi-billion dollar industry because of its mobility, ease of use, low cost, nonprofessional operators, and rapid diagnostics [2]. LFAs allow small amounts of samples to be analyzed on-the-spot by nonprofessional operators and does not require time-consuming laboratory tests. The results of an LFA are "yes" or "no," and a visualized red line from colloidal gold (Au) nanoparticles is used as a signal label because of the nanoparticles' localized surface plasmon resonance. The LFA procedure initiates at the stationary phase, and then the sample fluid moves forward from the absorbance pad by capillary action. When the target protein that exists in the fluid is captured on the test line region through a sandwich immunoassay immobilized on the zone, the test line appears as a red line of accumulated gold nanoparticles, which is a positive signal, whereas in the absence of the target protein, there is no red line on the test line. However, only yes/no answers can be obtained, and the accumulated signal labels at a certain concentration result in a visual detection result. Therefore, studies have been developed for the quantification in LFA to enhance the sensitivity and reliability of the quantification.

Surface-enhanced Raman scattering (SERS)-based immunoassay have received attention owing to their high sensitive detection of targets in biosensor [3,4]. Au nanoparticles were used as SERS tags for the SERS immunoassay, and the Raman reporter on the Au surface exhibited an enhanced electromagnetic field under laser excitation. Excitation of the localized surface plasmons of Au results in the detection of target biomarkers with high sensitivity using Raman spectroscopy. Although the LFA is a modern technique, the development of a SERS-based LFA is not straightforward.

In this paper, we discuss three optimization procedures used to develop a SERS-based LFA for highly sensitive detection of Troponin I: (a) optimizing gold nanoparticle sizes (30, 50, 80, and 100 nm) for the SERS-based LFA; (b) varying the LFA components and fluid flow time used to recognize Troponin I; and (c) changing the laser wavelengths and laser power for the SERS-based LFA for the analysis of Troponin I. In an SERS-based LFA, these parameters are fundamental for augmenting the sensitivity and detection limits. The SERS-based LFA became more sensitive than visual detection with the naked eye for the quantitative quantification of Troponin I after optimization. In addition, the discussed procedures may inform the development of SERS-based LFA for the highly sensitive detection of various biomarkers.

2. Methods

2.1. Chemicals and Instruments

AuNPs of different sizes were obtained from NanoXact Gold Nanospheres (NanoComposix, San Diego, CA, USA). Malachite green isothiocyanate (MGITC) was purchased from Invitrogen (Carlsbad, CA, USA). Goat-produced anti-rabbit IgG (control line capture antibody) was purchased from Sigma-Aldrich. The rabbit monoclonal antibody against cTnI (detection antibody) was purchased from HyTest (Turku, Finland), and the mouse monoclonal antibody against cTnI was purchased from Gentex (USA). A nitrocellulose (NC) membrane was used (CN110, Satorius, Göttingen, Germany), and PVC backing cards were obtained from TH (TWO HANDS, Seoul, Korea). The absorbance pad was a CFSP223000 (Millipore, Burlington, MA, USA). An LFA dispenser and card cutter were obtained from BTM (Seoul, Korea). Transmission electron microscopy (TEM) images were acquired using a JEM-2010 instrument (JEOL, Tokyo, Japan). Raman spectra and SERS mapping images were obtained using a Raman microscope (Raman spectrometer, Horiba, Tokyo, Japan). Raman images were obtained using a point-mapping system with a 20× objective lens. Mapping images were collected with 50 μm (y-axis) × 170 μm (x-axis) over an area of 350 × 2550 μm for a total of 105 pixelsand SERS intensity of each pixels was collected with 0.25 s acquisition time.

2.2. Preparation of the SERS Tags

Au nanoparticles of 1 mL (OD 1) with 20 nM MGITC were incubated for 30 min at room temperature (RT) and washed by centrifugation at 2000 RCF for 15 min. Conjugated Au-antibody incubated for 1 h at room temperature (RT), and the surface of the conjugates was blocked with 1% skim milk in distilled water (DW) for 30 min at RT. After centrifugation at 2000× *g* for 15 min, the conjugate was concentrated 10 times and stored at 4 °C for the next experiment.

2.3. cTnI Lateral Flow Assay (LFA) and Assay

The NC membrane was used to prepare test and control lines using a line dispenser (BMT, Yangsan, Korea). A dispenser system was set as 0.4 uL/cm of antibody on each line. The test line was treated with 2 mg/mL, whereas the control line was treated with 1 mg/mL anti-rabbit IgG antibody. After dispensing the antibody on the membrane, it was dried for 2 h at 37 °C, and when completely dried, the membrane was cut into strips with widths of 4 mm with a digital programmed cutter (BMT, South Korea). The strips were sealed in a pouch with silica gel to prevent exposure to high humidity. The LFA strips were placed

in an ELISA well containing the solution. Fluid diffusion through capillary action occurred, and the results were collected from the test line under certain conditions.

3. Results and Discussion

3.1. Working Principle

Figure 1 shows a schematic of the SERS-based cTnI LFA platform for the quantitative assay of SERS intensity and the yes/no answers from a commercial cTnI LFA. When the sample contained the target biomarker, it bound to the Au SERS tag and accumulated on the test line by forming a sandwich immunoassay structure, whereas the absence of the target biomarker in the sample resulted in an unbound Au SERS tag on the control line. The conjugated Raman reporter (MGITC) on the Au nanoparticles produces a SERS signal under laser excitation, and the SERS intensity corresponds to the accumulated Au SERS tag, which is proportional to the concentration of the target. The SERS-LFA achieves high sensitivity and suitable quantification.

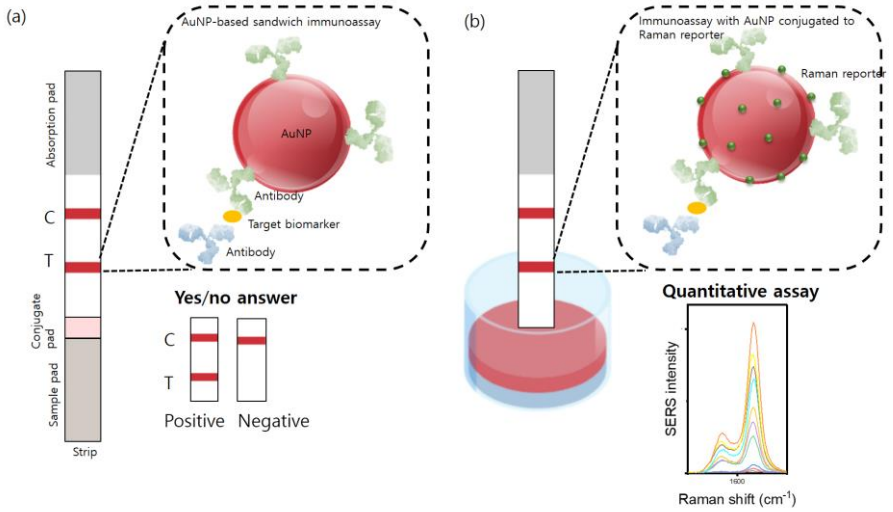

Figure 1. Schematic of (**a**) commercial cTnI LFA strip and (**b**) the SERS-based cTnI LFA.

3.2. Optimization of Gold Nanoparticle Size on SERS-Based LFA for Detection of cTnI

Au nanoparticles have been applied as SERS tags in LFA [5–7], and various sizes have been employed. Here, we investigated Au nanoparticle sizes ranging from 30 to 100 nm as SERS tags for the detection of targets on LFA (Figure 2). The LFA strip results revealed that large SERS tags were detectable at low target concentrations with the naked eye, which was similar to previous research showing increased sensitivity with an increase in Au size [8,9]. However, the SERS intensity showed different results: the 50 nm Au SERS tag showed a strong SERS intensity on the LFA, whereas a large SERS tag decreased the SERS intensity for the detection of high concentrations of cTnI. Previous research revealed that a 45 nm Au SERS tag had higher sensitivity with SERS analysis compared with a 14 nm Au SERS tag [10], and another study reported that a SERS tag size of approximately 50 nm had the highest sensitivity among different SERS tag sizes, which varied in diameter from 26 to 110 nm [4]. An Au size of 50 nm for the SERS tag was applied in this study to increase the sensitivity of the SERS-based LFA for the detection of cTnI.

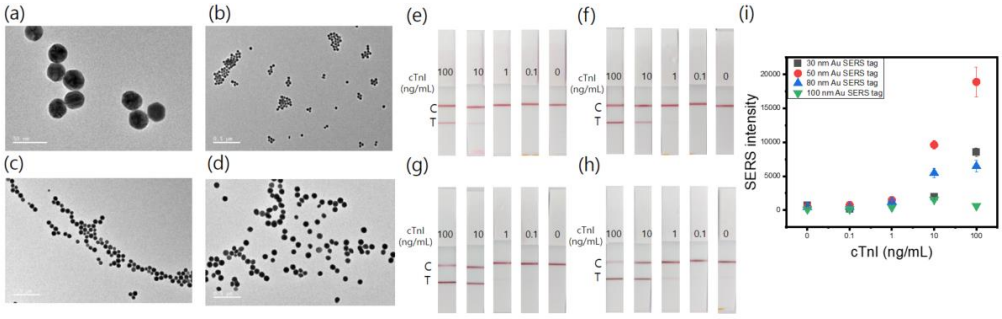

Figure 2. TEM images of Au nanoparticles with sizes of 30 (**a**), 50 (**b**), 80 (**c**), and 100 (**d**) nm. LFA test results achieved with different Au sizes of 30 (**e**), 50 (**f**), 80 (**g**), and 100 (**h**) nm as SERS tags. (**i**) Quantification of the cTnI concentration in SERS-based LFA.

3.3. Optimization of the Running Buffer Formulation and Sample Loading Time for SERS-Based LFA

To optimize the LFA components, the running buffer formulation and sample loading time were evaluated (Figure 3). The running buffer formulation allowed for the formation of homogeneous Au SERS tags with a steady flow on the LFA for reproducible and reliable SERS performance. For optimization of the running buffer, we minimized nonspecific binding (NSB) to avoid false positives that decrease the sensitivity and reproducibility of a reliable quantitative assay. BSA and Tween 20, the selected proteins and surfactants for the simple standard procedure, were tested. Negative samples were tested with 0.1 % to 1% BSA and Tween 20 in the running buffer. It was observed that, the higher the concentration of protein and Tween, the greater the increase in signal intensity on the control line. Although the test-line signal increased, the signal increase on the control line made the test-line signal negligible. In this case, we added 1% BSA and 1% Tween 20 to the running buffer formulation for further experiments.

NSB had an impact on the buffer attributes. A buffer test was designed to determine the minimum NSB required for suitable SERS performance. Low concentrations of buffers (PBS pH 7.4, PB pH 7.4, Tris-HCL pH 8.0, and borate pH 9.0) showed a false-positive reaction on the test line, except for borate buffer pH 9. Although borate buffer had low influence on the buffer concentration at low or high concentrations, it had a low SERS intensity on the control line compared with PBS 150 or PB 100 mM buffer. PBS buffer (150 mM) was selected as the buffer solution to produce a strong control line signal and minimal test line signal.

The SERS intensity can influence the reaction time of the strip dipped in the buffer and sample. As the amount of loaded sample on the NC membrane increases, prolonging the reaction time enhances the SERS intensity by accumulating more Au nanoparticles on the line. A reaction time of 5 to 20 min results in a continuous increase in the SERS intensity on the control line, whereas one of 30 min decreases the signal because of the separation of conjugates on the capture line. To minimize time, a 15 min reaction time was set because of the minimal NSB.

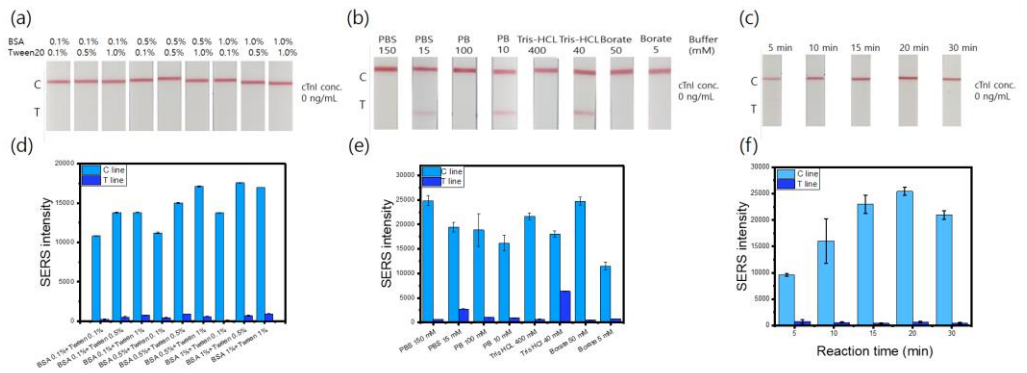

Figure 3. LFA result images for (**a**) protein and surfactant test in running buffer, (**b**) buffer type and concentration test, and running time test. SERS performance on test region and control region–(**d**) protein and surfactant impacts in running buffer, (**e**) buffer type and concentration test, and (**f**) running time test.

3.4. Optimization of Laser Wavelengths and Laser Power for SERS-Based LFA for the Analysis of Troponin I

To optimize the laser wavelengths and power, SERS intensity was collected in Figure 4. It is well known that Raman signals from SERS can be detected near the Au nanoparticle surface through intrinsic localized surface plasmon resonanc (LSPR) [11]. The SERS spectra of the SERS tag on the cTnI LFA test line region were determined under 638 nm and 785 nm laser excitations (Figure 4a,b). A 638 nm laser showed the prominent intensity at 1176, 1368, and 1614 cm^{-1} under 638 nm laser than 785 nm laser. A 638 nm laser was applied for further experiment.

The major Raman spectra of MGITC at 1176, 1368, and 1614 cm^{-1} were due to phenyl-N plus C-C stretching, phenyl-N stretching, and the in-plane benzene v9 mode based on the structure of MGITC [12–14]. Intensity data from three prominent peaks at 1176, 1368, and 1614 cm^{-1} were collected and tested with different concentrations of the target on cTnI LFA. The peak intensity at 1614 cm^{-1} had strong intensity. Data at the 1614 cm^{-1} peak were used to evaluate the SERS-based LFA.

To evaluate the influence of laser power on SERS-based LFA, laser powers from 0.02 mW to 30 mW were applied under 638 nm laser excitation with a 0.25 s acquisition time. As shown in Figure 4d, the normalized SERS intensities were compared for different concentrations to detect cTnI with 1614 cm^{-1} peak intensity of 1614 cm^{-1}. Above 3 mW, 8, 10, and 30 mW laser excitations showed low intensity in the normalized SERS spectra at the highest concentration of cTnI because of damage to the NC membrane by the full laser power. To obtain high sensitivity in the low-concentration detection of LFA, strong SERS intensity data were collected at a 3 mW laser power with 0.25 s as the acquisition time.

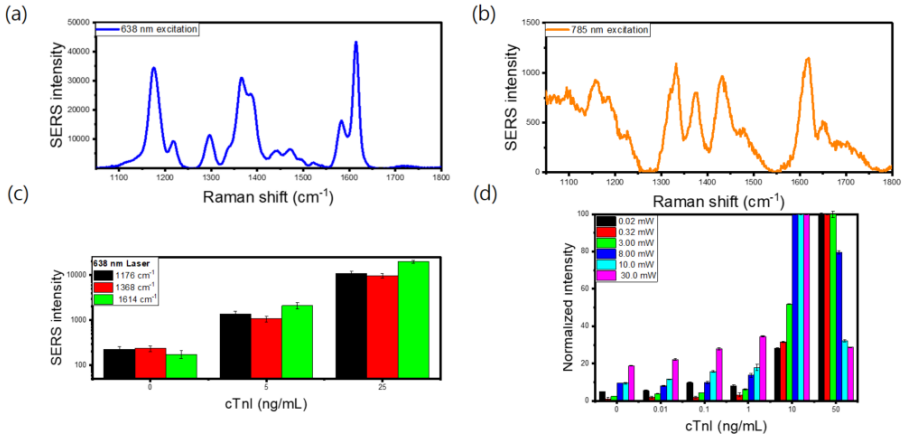

Figure 4. Laser wavelength effects: (**a**) 638 nm and (**b**) 785 nm excitation with 3 mW laser set on the cTnI LFA test region; (**c**) comparison of SERS intensities at 1175 cm^{-1}, 1368 cm^{-1}, and 1614 cm^{-1}; and (**d**) laser power varied from 0.02 mW to 30 mW for SERS-based LFA.

3.5. Performance Evaluation of SERS-Based LFA for the Detection of cTnI

The detection sensitivity of the method was evaluated. Sample solutions were prepared with different concentrations of the target, ranging from 0.001 to 100 ng/mL cTnI (Figure 5). Although the LFA visual images showed a visual line readable with the naked eye at a concentration of 5 ng/mL, images for the quantification of the optical intensity were analyzed using ImageJ software, and they showed a linear range from 1 ng/mL to 50 ng/mL, with an R^2 value of 0.975. The SERS intensity at the 1614 cm^{-1} peak under 633 nm laser exciation was obtained from the SERS spectra. The SERS performance for the detection of cTnI had a detection linear range (DLR) from 0.01 ng/mL 100 ng/mL (R^2 = 0.966). The limit of detection (LOD) was calculated using LOD = 3σ/slope (σ is the standard deviation of the blank solution, and the slope is the slope of the calibaration curve). The LOD for SERS was 0.02 ng/m while that for optical intensity was 1.02 ng/mL. The SERS-based LFA has a lower LOD than the fluorescence method (LOD = 0.049 ng/mL) [6] for the detection of cTnI. In addition, we found that other studies that applied the chemiluminescence method in quantitative assays used to detect cTnI LFA with a Au size of 40 nm acquired a 0.03 ng/mL LOD [1]. SERS data for the quantitative assay can be collected within 1 min without additional processing with the LFA, whereas this chemiluminescent method requires an additional washing step, chemical reaction step, and analysis time that is longer than 15 min. This indicates that the SERS-based LFA can be applied to the detection of cTnI in healthcare systems by matching POCT goals of high sensitivity and rapid detection.

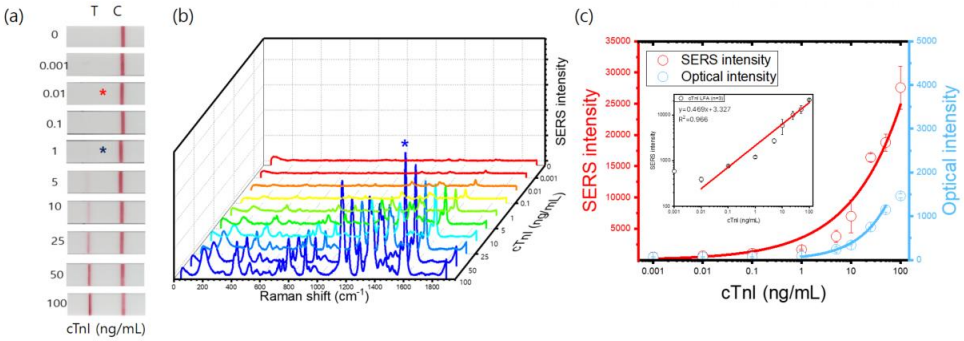

Figure 5. (**a**) cTnI LFA test result after all parameters were optimized. The red and dark blue asterisks indicate the lowest concentration of samples distinguishable by SERS intensity and optical intensity, respectively. (**b**) Average SERS spectra. The blue asterisk indicates the peak at 1614 cm^{-1} for

SERS intensity. (**c**) Calibration curves of optical intensity and SERS intensity for the test line with 50 nm Au SERS tag loaded on cTnI LFA.

3.6. Selectivity Test

To investigate the selectivity of the SERS-based LFA for the detection of cTnI, different biomarkers (10 ng/mL) were tested, including C-reactive protein (CRP), myoglobin (Myo), Tau, and bovine serum albumin (BSA), with a negative control (NC). Figure 6 shows that none of the test proteins showed a positive result in the test region of the cTnI LFA with SERS intensity and optical intensity. This shows that the SERS-based LFA for cTnI has a high sensitivity.

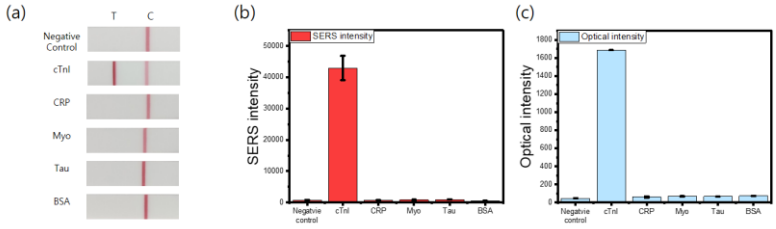

Figure 6. Selectivity test results. (**a**) LFA test results for 10 ng/mL solutions of different target proteins. (**b**) SERS intensity and (**c**) optical intensity for the selectivity test.

3.7. Evaluation of SERS-Based LFA for the Detection of cTnI in Human Serum

We analyzed the clinical applicability of SERS-based LFA for the detection of cTnI in human serum. Different concentrations of cTnI were added to 10% human serum solutions, and their optical intensities were compared with the optical intensity in Figure 7. A 78-fold higher sensitivity was achieved with our method than with the optical sensing method with an LOD 1.56 ng/mL. These results indicate that SERS-based LFA has great potential for the detection of cTnI in clinical assays.

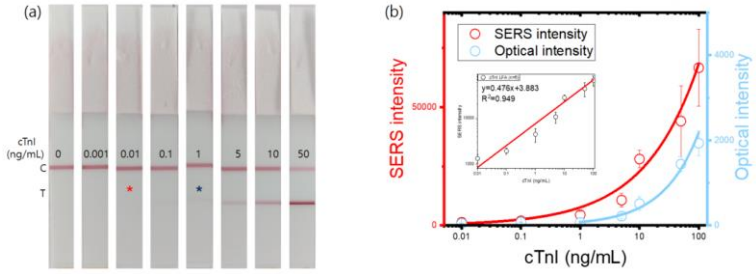

Figure 7. (**a**) Visual images obtained after loading the sample onto the LFA. The red asterisk indicates the lowest concentrations of the sample that are distinguishable by SERS-based LFA, and the dark blue asterisk indicates the lowest concentration of the sample that is distinguishable by optical intensity, while 5 ng/mL cTnI was distinguishable with the naked eye. (**b**) SERS intensity and optical intensity plot obtained from the cTnI LFA test (Inset: Calibration curve for SERS based cTnI LFA).

4. Conclusions

We developed an optimization procedure for the SERS-based LFA by improving the sensitivity of the 50 nm Au SERS tag for high-performance POCT. The optimized system can detect 0.01 ng/mL cTnI (LOD 0.02 ng/mL), which is 78 orders of magnitude lower than the optical intensity achieved with visual Au color on LFA, and showed selectivity with no cross reactivity for CRP, Myo, Tau, and BSA. Here, we demonstrate the potential of SERS-based LFA for highly sensitive and accurate diagnosis in emergency situations at healthcare centers.

Author Contributions: E.S. conceptualized this study, performed the experiments, and wrote the first draft. I.K., C.J. and S.H.P. contributed to the experiment and review of the results to prepare the original draft. All authors have read and agreed to the published version of the manuscript.

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