Temperature-Responsive Liposome-Linked Immunosorbent Assay for the Rapid Detection of SARS-CoV-2 Using Immunoliposomes

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ABSTRACT: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is the etiological agent of coronavirus disease 2019 (COVID-19), has infected more than 340 million people since the outbreak of the pandemic in 2019, resulting in approximately 55 million deaths. The rapid and effective diagnosis of COVID-19 patients is vital to prevent the spread of the disease. In a previous study, we reported a novel temperature-responsive liposome-linked immunosorbent assay (TLip-LISA) using biotinylated-TLip that exhibited high detection sensitivity for the prostate-specific antigen. Herein, we used immunoglobulin-TLip (IgG-TLip), in which the antibodies were directly conjugated to the liposomal surface to simplify pretreatment procedures and reduce the detection time for SARS-CoV-2. The results indicated that TLip-LISA could detect the recombinant nucleocapsid protein and the nucleocapsid protein in inactivated virus with 20 min incubation time in total, and the limit of detection was calculated to be 2.2 and 1.0 pg/mL, respectively. Therefore, TLip-LISA has high potential to be used in clinic for rapid diagnosis and disease control.

INTRODUCTION
At the end of 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged first in Wuhan, Hubei province, China,1 then rapidly spread to the rest of China and the world. SARS-CoV-2 was recognized as the etiological agent of coronavirus disease 2019 (COVID-19). SARS-CoV-2 is an enveloped, single-stranded RNA virus, and its genomic sequence shares 79.6% homology with SARS-CoV.1,2 The coronavirus has a large pool of genomic RNA, and RNA polymerase is error-prone, leading to the occurrence and amplification of mutation events during infection outbreaks. The evolution of new viral traits may alter the transmissibility, virulence, and pathogenesis of the virus,3 which may challenge the current detection methods and disease control policies.

Coronavirus comprises four basic, elemental structural proteins: the envelope (E), membrane (M), nucleocapsid (N), and spike (S) proteins.4 The S protein usually targets angiotensin-converting enzyme 2 (ACE2) as the entry receptor for the virus5 and is also responsible for cell tropism.6 N proteins package the viral RNA into a helical nucleocapsid structure.4,7 Both S protein and N protein are major viral antigens8,9 and are commonly used as biomarkers in antigen tests, such as ELISA, to diagnose COVID-19 patients.10,11 The N protein is abundantly expressed during infection and has high immunogenic activity.12 It has been shown that the concentration of N protein can be higher than that of S protein in SARS-CoV-2 samples.13 Therefore, the N protein is a promising target for antigen tests of SARS-CoV-2 based on an immunoassay using antibodies including ELISA and immunochromatography. The sensitivity of ELISA for detecting the SARS-CoV-2 N protein may be in the tens to hundreds of picograms per mL, offering a wider concentration range for quantification analysis.14,15 However, conventional ELISA requires several incubation steps and long incubation times to ensure the high sensitivity and specificity of the test results. The detection time is approximately 4.75 h for a commercial
ELISA product. Rapid screening methods with good sensitivity and specificity in situ are in high demand for controlling the spread of disease by ensuring the rapid diagnosis and quarantine of patients. Boccellino and colleagues designed a rapid ELISA that can be performed in the field, reducing the total incubation time to less than 1.5 h. However, the sensitivity of this rapid ELISA was reduced to 50 ng/mL, indicating the importance of incubation time in conventional ELISA methods. A lateral flow immunoassay (LIFA) is another antigen test that allows for rapid detection, taking only 15–30 min to visualize the results, which is faster than laboratory-based testing. The detectable concentration range of recombinant SARS-CoV-2 N protein on testing using several commercial LIFA products has been reported to be more than 5 ng/mL. Despite a short detection time, LIFA exhibits good sensitivity compared with traditional ELISA. However, antigen test sensitivity of below 5 ng/mL may cause false negative results in the early and later phases of COVID-19, resulting in undetected cases and unexpected outbreaks in the community. In addition to the detection of viral proteins, antivirus antibodies and viral RNA are target molecules for diagnosis. The current standard detection method for SARS-CoV-2 is quantitative real-time reverse transcription–polymerase chain reaction (RT-qPCR) because antigen tests usually exhibit lower sensitivity than RT-qPCR. However, RT-qPCR requires RNA extraction, which demands technical proficiency and biosafety level 2 laboratory facilities.

Here, we employed a temperature-responsive fluorescent liposome-linked immunosorbent assay (TLip-LISA) to detect potential target molecules, including viral proteins of SARS-CoV-2, with high sensitivity and specificity. In a previous study, TLip-LISA exhibited high sensitivity for detecting prostate-specific antigen with a good limit of detection (LOD) of 0.97 aM. In this study, instead of using biotinylated-TLip, we performed immunoglobulin conjugation to TLip to reduce the total number of incubation steps and the assay time (Figure 1), thereby enabling rapid detection and subsequent diagnosis, while also developing a point-of-care test that can be used in the field (i.e., in homes, workplaces, and airports) instead of requiring a specialized clinical laboratory. The reduced number of incubation steps also permitted rapid detection of SARS-CoV-2, which may be useful in the event of community outbreaks. Our results indicated that TLip-LISA has high potential to detect target molecules with high sensitivity in a short time and therefore has promising implications for disease control.

## METHODS

**Materials and Reagents.** Hybridomas of SKOT-7 and SKOT-9 were originally established with SARS-CoV and provided by the National Institute of Infectious Diseases (NIID), Japan. Briefly, BALB/c mice were immunized with UV-inactivated SARS-CoV (HKU-39849), and the splenocytes obtained from immunized BALB/c were fused with SP-2/O myeloma to establish the hybridomas. These two monoclonal antibodies, SKOT-7 and SKOT-9, secreted from the hybridomas both targeted the N protein of SARS-CoV-2 via different epitopes. In this study, SKOT-7 was used as a capture antibody immobilized on a plate and SKOT-9 was conjugated to the surface of liposomes as a detection antibody. The SKOT-7-coated plate and the SKOT-9 antibody were obtained from Tokiwa Chemical Industries (Tokyo, Japan). 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was purchased from Nippon Fine Chemical (Osaka, Japan). UV-inactivated SARS-CoV-2 cultured in VeroE6/TMPRSS2 cells (titer: 5.6 × 10^8 TCID_50/mL, RNA copy number: 5.0 × 10^7 copies/μL) was obtained from NIID. The recombinant SARS-CoV-2 N protein was purchased from RayBiotech (Norcross, GA, USA). The following reagents and products were purchased from Wako Pure Chemical Co. (Osaka, Japan): sodium chloride, sodium dihydrogen phosphate, 35% hydrogen chloride, fluorescamine, ethylenediaminetetraacetic acid (EDTA), polyethylene glycol (PEG, molecular weight 10,000), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and a phospholipid assay kit. 2-Iminothiolane hydrochloride was purchased from Kanto Chemical (Tokyo, Japan), and N-acetylcysteine was purchased from Sigma-Aldrich (St. Louis, MO, USA). tert-butyl alcohol (t-BuOH) was purchased from Sigma-Aldrich. Dulbecco’s phosphate-buffered saline (DPBS), used as a solvent and washing buffer, was purchased from Sigma-Aldrich. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was purchased from Nippon Fine Chemical (Osaka, Japan). 1,2-Dipalmitoyl-sn-glycero-3-phosphatidic acid sodium salt (DPPA) and N-[3-maleimidyl-1-oxopropyl]aminopropyl-polylethylene glycol-carbamyl]-distearoyl phosphatidyl-ethanolamine SUNBRIGHT DSPE-020MA (MAL-PEG-DSPE) was purchased from NOF Co. (Tokyo, Japan). The synthesis and characterization of the fluorescent
dye (2-[4-(dibutylamino)phenyl]-4-[(3,3-dimethyl-3H-indol-2-yl)methylene]-3-hydroxycyclobut-2-en-1-one, SQR22) were reported previously. SQR22 was obtained by custom synthesis from the NARD Institute (Kobe, Japan). Octyl glucoside (OG), which was used to destroy the liposomal structure for further lipid concentration analysis, was obtained from Carbosynth (Berks., UK). The Sephacryl s-300HR used for size exclusion chromatography was purchased from GE Healthcare Corporation (Tokyo, Japan). PD-10 was purchased from Cytiva (Tokyo, Japan). The Amicon Ultra centrifugal filter with a 50 kDa cutoff (0.5 mL sample volume) was purchased from Merck Millipore (Burlington, USA).

Preparation of Maleimide-Modified Liposomes (Mal-TLip). The components of the maleimide liposomes were DPPC, DPPA, MAL-PEG-DPPE, and SQR22 at a molar ratio of 86.6:9.6:0.5:3.3. DPPA (sodium salt) was converted to protonated DPPA before use by the following procedures. DPPA (sodium salt) (60 mg) was suspended in ethanol (1.2 mL) containing hydrochloric acid (35%, 15 μL) with a vortex mixer. After precipitating the insoluble sodium chloride by centrifugation (15,000g, 5 min) at room temperature, the supernatant was collected, and the solvent was removed using an evaporator. The residue was dissolved in t-butyl alcohol and freeze-dried overnight. The protonated DPPA powder obtained was used for preparation. The mixed lipids along with SQR22 were dissolved in cyclohexane and freeze-dried overnight. Then, the lyophilized powder was hydrated with 2 mL DPBS to obtain a liposomal dispersion with a lipid concentration of 4 mg/mL. After vortex mixing for 15 min, the liposomal dispersion was extruded twice using an extruder (LIPEX, Norther Lipids Inc., Vancouver, Canada) (preheated to 58 °C) through a 25 mm, 0.2 μm pore-sized, track-etched polycarbonate membrane (Nucleopore, Whatman, Maidstone, UK).

Preparation of the 2-iminothiolane-Modified Antibody (IgG-SH). Traut’s solvent (25 mM HEPES, 140 mM sodium chloride, 3 mM EDTA), HBS (140 mM sodium chloride, 1.5 mM sodium dihydrogen phosphate, 50 mM HEPES), 0.8 mM N-acetylcysteine, and 0.123 mg/mL 2-iminothiolane hydrochloride solution (dissolved in Traut’s solvent) were first prepared. To prepare IgG-SH, 300 μL of SKOT-9 solution (4.21 mg/mL) was mixed with 189 μL of 2-iminothiolane hydrochloride solution at a molar ratio of 1:20 (SKOT-9:2-iminothiolane hydrochloride). The mixture was incubated for 1 h with gentle shaking. Then, 1 mL HBS was added to the mixture, which was loaded onto two 50 kDa Amicon Ultra centrifugal filter devices (0.7 mL each) for centrifugation (15,000g, 10 min) to remove unconjugated IgG-SH and other unreacted molecules to facilitate purification. The obtained products were then subjected to size exclusion chromatography using a Sephacryl s-300HR column to remove unconjugated IgG-SH and other unreacted molecules to obtain pure IgG-TLip.

Characterization of IgG-TLip. To measure the size distribution and mean diameter of IgG-TLip after extrusion, a 10 μL sample of the final product was diluted to 990 μL of DPBS. Then, this 1 mL sample was loaded into a plastic cuvette. The size and poly dispersity index (PdI) of the IgG-TLip were measured by a Zetasizer Nano S90 (Malvern Instruments Ltd., Malvern, UK) using the dynamic light scattering technique. After acquiring data on size-related parameters, the same sample was transferred to a capillary cell mounted onto the apparatus for zeta potential measurement. The measurement of zeta potential followed the manufacturer’s manual. All measurements, including size, PdI, and zeta potential, were conducted in triplicate. The lipid concentration of IgG-TLip was estimated using a phospholipid C kit based on the manufacturer’s instructions (Wako Pure Chemical Corporation). To obtain the SQR22 concentration in IgG-TLip, a standard curve of SQR22 was acquired from the absorbance of different concentrations of SQR22 ethanol solution at 631 nm. An aliquot of the IgG-TLip dispersion (20 μL) was mixed with ethanol (1 mL), and the absorbance of the solution was measured at 631 nm. The concentration of SQR22 in IgG-TLip was then determined based on the standard curve.

To calculate the IgG concentration of the IgG-TLip dispersion, unused IgG-SH solution samples with concentrations of 4, 8, 16, and 32 μg/mL were prepared to obtain a standard curve. Mal-TLip was diluted to an SQR22 concentration of 10 μg/mL. Then, IgG-SH samples (20 μL) were mixed with Mal-TLip samples (20 μL) to prepare standard samples of a Mal-TLip and IgG mixture with a SQR22 concentration of 5 μg/mL and IgG concentrations of 2, 4, 8, and 16 μg/mL. The IgG-TLip samples were diluted with DPBS to a SQR22 concentration of 5 μg/mL. In each group, 40 μL of the TLip and IgG—SH mixture was mixed with 40 μL of OG and incubated in a water bath for 30 min at 50 °C. After incubation, 300 μL of borate buffer (pH 9.0) was added to the sample, followed by vortex mixing. Then, 300 μL of 1 mg/mL fluorescamine was added to the solution. After further vortex mixing, the mixture was incubated in a dark chamber at room temperature for 10 min. Then, 180 μL samples from each group were applied to a 96-well black plate, and the fluorescence values were measured at an excitation wavelength of 381 nm and an emission wavelength of 476 nm, with a gain at 80. The obtained data were used for plotting a standard curve to measure the IgG concentration in the original IgG-TLip sample.

The IgG-TLip sample was first diluted to obtain an SQR22 concentration of 10 μg/mL. Then, 40 μL of the sample was mixed with 1 M of OG solution (40 μL) and incubated for 30 min in a water bath at 50 °C. The sample was mixed with 300 μL of borate buffer, followed by the addition of 300 μL of fluorescamine at a concentration of 1 mg/mL. The mixture was incubated for 10 min at room temperature in a dark environment. The fluorescence of the resultant solution was measured using a microplate reader with the same settings as for the standard curve. The IgG concentration was calculated by extrapolating the fluorescence intensity in the standard curve.

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Table 1. Characterization of IgG-TLip

| size (nm) | PdI  | Z potential (mV) | SQR concentration (μg/mL) | IgG concentration (μg/mL) | lipid concentration (mg/mL) |
|----------|------|-----------------|---------------------------|--------------------------|-----------------------------|
| 198.4 ± 67.3 | 0.099 | −8.09 ± 0.27 | 19.4 | 4.4 | 0.72 |

**N Protein Detection Using TLip-LISA.** Figure 1 shows a conceptual schematic of TLip-LISA measurement using IgG-TLip to detect potential target molecules. N protein samples (100 μL) of different concentrations were added to the wells of a SKOT-7-coated plate and incubated at room temperature for 2 h with gentle shaking. After three washes with 200 μL of DPBS to remove free antigens, 60 μL of IgG-TLip dispersion with a SQR22 concentration of 0.5 μg/mL was added to each well and incubated for 10 min with gentle shaking. The plate was placed on a hot plate (80 °C), and changes in the fluorescence intensity were monitored using a fluorescence detector (FLE1100) with a micro-optic (Probe40100) connected to a computer. In the negative control group, 100 μL of DPBS solution was added to the wells instead of the N protein sample.

**Rapid Detection of N Protein and Inactivated Virus.**

To enable rapid detection and minimize the incubation time, 50 μL of N protein samples, with concentrations ranging from 0.1 to 1000 pg/mL, were added to the wells of the SKOT-7-coated plate and incubated for 10 min. After three washes with 100 μL of DPBS to remove free antigens, 60 μL of IgG-TLip dispersion with a SQR22 concentration of 0.5 μg/mL was added to the wells and incubated at room temperature for 10 min with gentle shaking. The total incubation time was 20 min.

Procedures were slightly altered for inactivated virus detection. The original virus sample (N protein concentration: 31 μg/mL) was first diluted to an appropriate concentration. Then, 10 μL of inactivated solution containing 5% Triton X-100 (Nippon Genetics, Tokyo, Japan) was added to the solution (490 μL). After vortex mixing for 10 s to break down the virus structure, the inactivated virus was diluted to different concentrations for further detection. Then, 50 μL of inactivated virus dispersion of different concentrations was added to the wells of the SKOT-7-coated plate and incubated for 10 min at room temperature. Then, the wells were washed once with 390 μL of DPBS, and finally 60 μL of IgG-TLip with 0.5 μg/mL of SQR22 was added to the wells and incubated for 10 min.

In both cases, the plate was placed on a hot plate (80 °C), and the change in fluorescence intensity was monitored using a fluorescence detector connected to a computer. In each group (four measurements in total), 120 mg of PEG was used to fill the clearance of the air layer between the bottom of the plate and the hot plate to enhance and stabilize the heat transfer efficiency.

**LOD Determination.** The method used to determine the LOD for the TLip-LISA was based on previous studies. A linear fitting line for the N protein concentration and inflection time points was obtained between 0.5 and 5 pg/mL in the regular incubation time experiment. Then, the LOD was determined as the N protein concentration that corresponded to an inflection time equal to the average inflection time of the negative control minus three times the negative control standard deviation. In the experiments involving rapid detection, a linear regression was obtained between 1000 and 0.1 pg/mL for both the N protein and inactivated virus.

**RESULTS**

**Characterization of IgG-TLip.** As shown in Table 1, the obtained IgG-TLip sample showed a narrow size distribution with a low PdI of 0.099. The transmission electron microscopy (TEM) image of IgG-TLip supports the formation of spherical liposomes with a size of around 200 nm (Figure S1). The negative charge of the zeta potential suggested the presence of anionic lipid DPPA, which was used to prevent aggregation by electrostatic repulsion and improve liposome stability. The SQR22 concentration was 19.4 μg/mL after applying the prepared IgG-TLip dispersion to the size exclusion column, and the IgG concentration was determined to be 4.4 μg/mL. The final lipid concentration was 0.72 mg/mL.

**N Protein Detection Using Regular Incubation Time.** Figure 2 shows the representative profiles obtained from the TLip-LISA measurement. The fluorescence intensity values were normalized by setting the intensity to 0 at 0 s. The fluorescence intensity showed an initial decrease, possibly due to the detachment of non-specific binding of IgG-TLip, and then increased to the saturated value because the temperature reached the phase transition temperature for TLip. The graph also exhibits the delayed response of the negative control compared with the positive samples. The inflection time point,
which is defined as the maximum rate reached, was used as the parameter to identify positive and negative samples. Figure 3a shows the results of quantitative detection of N protein using IgG-TLip in the TLip-LISA. The inflection time point was stable and similar for samples, where the concentration of N protein was equal to 5 pg/mL or higher, whereas there was a 4 s difference between the positive samples and the negative control when the N protein concentration was equal to 0.5 pg/mL or lower. Quantification was possible between 0.5 and 5 pg/mL (Figure 3b). The LOD was calculated to be 0.47 pg/mL.

Next, the incubation time for the recombinant N protein solution was shortened from 2 h to 10 min for rapid detection using IgG-TLip. As shown in Figure 4, the inflection time increased as the concentration decreased, and a linear regression line was obtained across a wide range of concentrations (i.e., between 1000 and 0.1 pg/mL). The standard deviations were small, and the coefficient of variation in each group was less than 10%, suggesting consistency in the acquisition of results. The LOD of the recombinant N protein in the rapid detection protocol was calculated to be 2.2 pg/mL.

A similar linear regression line between 1000 and 0.1 pg/mL of N protein was also observed in the inactivated virus sample, as shown in Figure 5. The LOD of N protein for the inactivated virus was calculated to be 1.0 pg/mL. Because of the high transmissibility of SARS-CoV-2, a rapid on-site detection method is vital for controlling the spread of the disease. The LOD of the TLip-LISA was 0.47 pg/mL using the regular incubation time (2 h incubation of N protein and 10 min with IgG-TLip, total 2.17 h), and TLip-LISA sustained high sensitivity when the total incubation time was reduced to 20 min (10 min incubation of N protein and 10 min with IgG-TLip), with a LOD of 2.2 pg/mL for N protein and 1.0 pg/mL for the inactivated virus. The sensitivity of TLip-LISA was much higher than conventional ELISA methods that used the

**DISCUSSION**

Our results revealed that IgG-TLip could successfully detect antigens in the TLip-LISA system. The employment of IgG-TLip greatly reduced the number of incubation steps and the total incubation time, thereby accelerating detection. The average number of SQR22 was calculated to be $2.14 \times 10^4$ due to the large size of IgG-TLip that assisted fluorescence intensity jumped to the detectable level during measurements. Compared with our previous study using biotinylated-TLip to detect prostate-specific antigen, the total incubation time was shortened from 4.75 to 2.17 h, and rapid detection experiments merely required a 20 min incubation time for all reagents. Because of the high transmissibility of SARS-CoV-2, a rapid on-site detection method is vital for controlling the spread of the disease. The LOD of the TLip-LISA was 0.47 pg/mL using the regular incubation time (2 h incubation of N protein and 10 min with IgG-TLip, total 2.17 h), and TLip-LISA sustained high sensitivity when the total incubation time was reduced to 20 min (10 min incubation of N protein and 10 min with IgG-TLip), with a LOD of 2.2 pg/mL for N protein and 1.0 pg/mL for the inactivated virus. The sensitivity of TLip-LISA was much higher than conventional ELISA methods that used the
same pair of antibodies (SKOT-7 and SKOT-9, LOD at 272 pg/mL, Figure S2). These results suggested that the TLip-LISA had potential for rapid biomolecular detection that could be applied to control disease outbreaks, such as outbreaks of COVID-19.

The heating temperature is one of the key factors affecting the sensitivity of TLip-LISA. To study and determine a heating temperature suitable to enlarge the time difference between the positive and negative results for accurate analysis, multiple heating temperatures were tested. It was found that a hot plate at 80 °C was the best to distinguish positive and negative results in terms of time difference (see Supporting Information, Table S2). To apply this heating condition at 80 °C in this TLip-LISA system, two experiments to verify the thermal stability of SKOT-7 and SKOT-9 were conducted. The results reveal that 1 min heating at 80 °C did not significantly damage the binding affinity of these antibodies to N protein (see Supporting Information, Figure S3 and Table S3).

The LOD for SARS-CoV-2 N protein at the picogram level in this study is higher than the LOD for prostate-specific antigen at the attogram level in our previous study using biothin-TLip. Because the sensitivity of immunoassay using antibodies reflects the binding affinity of the antibody for the antigen, the difference in LOD should reflect the binding affinity of the antibodies for the antigen. Another possibility is that the exchange cycle of bound TLip with free TLip due to the reversible bond break events by heating enhances the sensitivity of TLip-LISA as we discussed in the previous paper. The rate of reversible bond break events between the antibody and antigen possibly was much less than that of biotin and streptavidin, which may be the reason why the LOD of the previous study using biothin-TLip was remarkably lower than that of this study. Further investigation is needed to clarify the factors affecting the detection sensitivity in TLip-LISA.

Our TLip-LISA method used specific biomolecular recognition to ensure the selectivity of target molecules. Multiple antibodies anchored to the surface of liposomes enhance the efficiency of binding between antigens and detection antibodies. By employing IgG-TLip, incubation procedures were simplified, and incubation times were greatly reduced, enabling more rapid detection. Conventional ELISA requires incubation processes involving the antigen, biotinylated detection antibodies, streptavidin-HRP (or detection antibody with HRP), and the substrate. By contrast, TLip-LISA only requires two incubation procedures involving the antigen and IgG-TLip, thereby simplifying the process and providing results more rapidly (Figure 1). Moreover, each IgG-TLip conjugate presents multiple IgG on the surface, thereby increasing the binding efficiency between TLip and its target molecule, which also contributes to rapid detection. The devices used were all portable, thereby ensuring that the assay is suitable for use in the field in response to outbreaks of disease.

The cut-off value of the antigen concentration to determine a positive COVID-19 case depends on the reagents (e.g., the binding constant between the antigen and antibody) and methods used. A common cut-off value is around 1.3 pg/mL, which was derived from receiver operating characteristic (ROC) analysis that showed high accuracy for the positive detection of COVID-19. The results of our study showed that TLip-LISA could reach a LOD of 0.47 pg/mL, which meets the criteria for accurate diagnostics. When the incubation time was shortened to 20 min, the sensitivity of TLip-LISA was sustained at a level of 1–2 pg/mL, which remained an acceptable level of sensitivity to identify the presence of viral proteins. Compared with other assays in the literature or market (Table 2), our system exhibited decent sensitivity for N protein detection within a short time. TLipLISA is user-friendly as it shares a similar protocol to the conventional ELISA method. Nevertheless, TLip-LISA can currently only monitor one well per measurement, which is a limiting factor in terms of rapid detection and may hinder the large-scale application of this methodology. The fluorescence detector could be upgraded to connect to multiple micro-optics for multiple sample measurements to increase the detection efficiency and accelerate screening. Fluorescence imaging of multiple wells on a heater would provide another detection format for TLip-LISA. This issue of fluorescence detection while heating for multiple sample measurements is currently under investigation.

Because SKOT-7 and SKOT-9 were originally developed as monoclonal antibodies that react with SARS-CoV N protein, TLip-LISA using SKOT-7 and SKOT-9 could cross-react with SARS-CoV N protein. It has been confirmed that neither SKOT-7 nor SKOT-9 cross-reacts with other human or animal coronaviruses (229E, TGEV, and MHV) by ELISA. We speculate that current TLip-LISA using SKOT-7 and SKOT-9 exhibits no reactivity to these other coronaviruses because the cross-reactivity of the immunosorbent assay basically depends on the specificity of antibodies. Besides, the data from this study were based on cultured inactivated virus and recombinant N protein, no clinical samples and other coronavirus have yet been analyzed using this methodology. Several SARS-CoV-2 variants have been confirmed in clinical samples so far. The sensitivity of the antigen test using an antibody might change when the mutations occur in the epitope of the target antigen recognized by the antibody. The N gene is highly conserved among coronavirus and is more stable with lower mutation rates than the S-protein. In this respect, N protein is preferable to S protein in the development of a variant-independent test method for clinical samples. The sensitivity, specificity, and accuracy of our method will be tested using clinical samples in future studies.

When comparing the results between the N protein and the inactivated virus in the rapid detection experiment, the N protein showed a higher LOD than the inactivated virus. This may be because the SKOT-7 and SKOT-9 antibodies bound more efficiently to the N protein derived from the inactivated virus compared with the commercial recombinant N protein. The conformation reportedly differs between the pure N protein and the RNA-packaged N protein. One study found that the C-terminal dimerization domain of the N protein undergoes a conformational change in response to RNA binding. This structural change might alter the binding certainty, which was confirmed in our study.
affinity between antibodies and the N protein and therefore influence the sensitivity to different targets. We found that a 2 h incubation period did not result in significantly higher sensitivity (LOD: 0.8 pg/mL) over shorter incubation periods in the case of the inactivated virus sample (Figure S4). This may be because the antibody pair used in this study was particularly effective at binding to the N protein and therefore quickly reached a state of equilibrium, whereby antibodies bound to capture and detection antibodies to form a sandwich complex. Thus, longer incubation times did not correlate with significantly lower LOD values than shorter incubation times. This phenomenon was also reflected by the quantitative analysis of TLip-LISA in two experiments. When the incubation time for N protein was 2 h, linear regression was observed between 5 and 0.5 pg/mL, whereas when the incubation time for the antigen was decreased to 10 min, quantification was expanded to between 0.1 and 1000 pg/mL, confirming the quantification ability of TLip-LISA over a short time. This might be due to the shorter incubation time, leading to less opportunity for N protein binding to the capture antibodies. The reliability of the results following the rapid detection of N protein and inactivated virus indicated that TLip-LISA was consistently effective even when the incubation time for the target molecule was reduced by one-sixth that of the regular incubation time.

The current TLip-LISA method had several limitations. The standard deviation obtained with the assay was large, thereby affecting the precision of quantification, as this value in the negative control group was used to calculate the LOD. Therefore, more precise instruments for heating and signal acquisition would enhance the accuracy of quantification analysis. Additionally, no clinical samples have been tested to date. Clinical samples such as nasal swab and saliva are applied for the SARS-CoV-2 antigen test after pretreatment with surfactants such as Triton X-100 to inactive virus and expose N protein by destroying the envelope lipid membrane of the virus. In this study, the inactivated virus sample was pretreated with Triton X-100 at a final concentration of 0.1% because the virus. In this study, the inactivated virus sample was pretreated by destroying the envelope lipid membrane of the N protein by keeping it at 56°C for 30 min, followed by the addition of sodium azide to 0.01% as a preservative. The incubation for antigen capture (PDF)

### CONCLUSIONS

TLip-LISA using IgG-TLip can detect N protein at a LOD of 0.47 pg/mL, with an incubation time of 2.17 h. The use of IgG-TLip both simplified and accelerated the incubation procedure, reducing the total incubation time to 20 min. The results showed that TLip-LISA could achieve rapid detection for both the N protein and inactivated antigen, with a LOD of 2.2 and 1.0 pg/mL, respectively. Our findings indicate that the use of IgG-TLip in TLip-LISA has the potential to considerably accelerate the detection and diagnosis time for important diseases.

### ASSOCIATED CONTENT

- **Supporting Information**
  The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c03597.

Additional data on the TEM image of IgG-TLip; detection of recombinant N protein by ELISA; calculation of the number of IgG and SQR22 molecules on one liposome; influence of heating temperature on time to inflection point in TLip-LISA; verification of the thermal stability of the antibodies; and detection of N proteins in the inactivated virus by TLip-LISA with 2 h incubation for antigen capture (PDF)

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#### Supporting Information

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#### Notes

The authors declare the following competing financial interest(s): R.H., K.S., and S.T. are inventors of the method for detecting antigens using the temperature-responsive liposomes applied in this study and named as inventors on the PCT patent application (PCT/JP2020/021358). The other authors have no conflict of interest to declare in this study.

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