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Equipment-free, salt-mediated immobilization of nucleic acids for nucleic acid lateral flow assays

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ABSTRACT

As the world has been facing several deadly virus crises, including Zika virus disease, Ebola virus disease, severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), and Coronavirus disease 2019 (COVID-19), lateral flow assays (LFAs), which require minimal equipment for point-of-care of viral infectious diseases, are garnering much attention. Accordingly, there is an increasing demand to reduce the time and cost required for manufacturing LFAs. The current study introduces an equipment-free method of salt-mediated immobilization of nucleic acids (SAIoNs) for LFAs. Compared to general DNA immobilization methods such as streptavidin-biotin, UV-irradiation, and heat treatment, our method does not require special equipment (e.g., centrifuge, UV-crosslinker, heating device); therefore, it can be applied in a resource-limited environment with reduced production costs. The immobilization process was streamlined and completed within 30 min. Our method improved the color intensity signal approximately 14 times compared to the method without using SAIoNs and exhibited reproducibility with the long-term storage stability. The proposed method can be used to detect practical targets (e.g., SARS-CoV-2) and facilitates highly sensitive and selective detection of target nucleic acids with multiplexing capability and without any cross-reactivity. This novel immobilization strategy provides a basis for easily and inexpensively developing nucleic acid LFAs combined with various types of nucleic acid amplification.

1. Introduction

Since the beginning of the 21st century, the world has faced several deadly virus crises, including Zika virus disease, Ebola virus disease, severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), and Coronavirus disease 2019 (COVID-19) [1]. The prevalence of viral diseases has been caused by either the evolution of existing viruses or the emergence of new viral species, incurring enormous damage to society [2]. In order to control these threats, one key requirement is new diagnostic tests capable of prompt, accurate identification of viruses at the point-of-care (POC) [3]. The current clinical standard is quantitative reverse transcription-polymerase chain reaction (RT-qPCR) that is only used in centralized hospital laboratories, not in the field, due to its complexity and high cost [4]. In this respect, lateral flow assays (LFAs) have received special attention because they are well suited to facilitate nucleic acid testing at the patient level with minimal instrumentation [5]. Due to their portability, low cost, and simple operation, LFAs have been widely used in various fields, ranging from medical to environmental and safety monitoring [6].

There are two main types of LFA currently in use: a lateral flow immuno-assay (LFA) using an antigen-antibody interaction, and a nucleic acid lateral flow assay (NALFA) using sequence specific DNA hybridization [7]. LFIA is commonly used for the detection of target proteins such as bacteria or viruses [8]. Several serological kits are already on the market to cope with the emergency detection of SARS-CoV-2 antibodies [9]. LFIA based assay for SARS-CoV-2 provide rapid diagnostic results in 15–30 min, which is a relatively short period of time [10,11]. However, they either have unsatisfactory detection

Abbreviations: AuNPs, gold nanoparticles; BSA, bovine serum albumin; LF, lateral flow; LFA, lateral flow assay; LFIA, lateral flow immuno-assay; LOD, limit of detection; LAMP, loop-mediated isothermal amplification; MERS, Middle East respiratory syndrome; NC, nitrocellulose; NTC, no-template control; NALFA, nucleic acid lateral flow assay; POC, point-of-care; RT-qPCR, quantitative reverse transcription-polymerase chain reaction; RPA, recombinase polymerase amplification; SAIoNs, salt-mediated immobilization of nucleic acids; SARS, severe acute respiratory syndrome.

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sensitivity or are unable to detect the early onset of COVID-19, which is evidenced by a poor clinical sensitivity of only 18.4% compared to RT-qPCR [12]. This is possibly because LFIs are difficult to combine with a method of amplifying the target protein. On the contrary, NALFAs can combine the power of exponential enzymatic amplification (e.g., polymerase chain reaction, PCR; recombinase polymerase amplification, RPA; loop-mediated isothermal amplification, LAMP) of the target gene sequence together with the sensitivity and ease of use offered by the LFA technique. Several studies have reported the combined use of nucleic acid amplification with LFA detection [13–15]. The first NALFA was developed by Yu and co-workers for the simultaneous detection of three regions of the SARS-CoV-2 genome (RdRp, ORF3a, and the nucleocapsid [N]-protein gene) combined with LAMP [16]. It facilitates the detection of SARS-CoV-2 with a sufficient limit of detection (LOD) of each gene, showing results comparable to those of RT-qPCR, which is the gold standard [17].

To perform NALFA, capture DNA that specifically recognizes a target must be immobilized on a nitrocellulose (NC) membrane. Biotin-modified capture DNA and streptavidin have been extensively used for this purpose [18]. However, it requires the pre-incubation of the streptavidin and biotin-modified capture DNA and separation of unbound streptavidin molecules to prepare a streptavidin–biotin complex of capture DNA, complicating the preparation of NALFA (Fig. S1A). In addition, since the streptavidin–biotin interaction is used for the immobilization of capture DNA, other molecules (e.g., digoxigenin) and their antibodies (e.g., anti-digoxigenin antibody) are necessary for the generation of colorimetric signals, increasing the overall assay cost. As an alternative, unmodified DNA is directly immobilized on the NC membrane using ultraviolet (UV) irradiation (Fig. S1B) or heat treatment (Fig. S1C) [19,20]. The direct immobilization of capture DNA is highly advantageous but special equipment, such as UV-crosslinkers or heating devices) and importantly, it improves the color intensity signal approximately 14 times compared to methods without SAIoNs (Fig. 1). To demonstrate its utility in biological applications, the new method was applied to analyze various SARS-CoV-2 and human genes (e.g., Orf1b, N, and RPP). The strategy is universally applicable for various capture DNAs and allow for the highly sensitive and selective detection of target nucleic acids with multiplexing capability and without any cross-reactivity.

2. Material and methods

2.1. Reagents and materials

The oligonucleotides used in this study (Table S1) were purchased from Integrated DNA Technologies (Skokie, IL, USA) and Bionics (Seoul, Korea); HAuCl₄, Tween 20, and sodium azide from Sigma-Aldrich (St. Louis, MO, USA); sodium carbonate, sucrose, NaCl, MgCl₂, KCl, and CaCl₂ from Daejung Chemicals & Metals (Gyeonggi-do, Korea); bovine serum albumin (BSA) and PCR premix (RT500S) from Enzymonics (Daejeon, Korea); streptavidin from Biolegend (CA, USA); backing card and absorbent pads from TWOHANDS (Gyeonggi-do, Korea); and NC membrane from Whatman (Maidstone, UK).

2.2. Synthesis of gold nanoparticles (AuNPs)

AuNPs were synthesized by adding 0.04 g of HAuCl₄ to 400 mL of distilled water, followed by addition of 3.2 mL of 1% trisodium citrate. The mixture was boiled for 15 min with stirring, and then cooled to room temperature. Synthesized AuNPs were stored at 4 °C and characterized using the ultraviolet–visible (UV–vis) spectrophotometer (Spectramax iD5 multi-mode microplate reader) from Molecular Devices (CA, San Jose, USA).

2.3. Preparation of streptavidin-coated AuNPs

The AuNPs synthesized in the previous step were centrifuged at 1400 × g for 60 min and prepared to reach an absorbance value at 534 nm (A₅₃₄) of 5 units. To this solution, sodium carbonate and streptavidin were individually added at a final concentration of 1.28 mM and 40 μg/mL, respectively, and were incubated for 1 h at room temperature. Then, BSA was added at the final concentration of 0.1 mg/mL and incubated for 1 h at room temperature. Finally, to remove the unbound streptavidin and BSA, the mixture was centrifuged at 1400 × g for 30 min and resuspended in 10 mM Tris-HCl (pH 8) to reach an A₅₃₄ of 5 units. The prepared streptavidin-coated AuNPs were stored at 4 °C until further use.

2.4. Assembly of LF strips

LF strips consist of three parts: a backing card, an NC membrane, and an absorbent pad. The NC membrane and absorbent pad were connected to each other on the backing card to ensure continuous capillary transfer. First, a 22 × 300 mm absorbent pad was placed on the downstream side of the NC membrane attached to the backing card, overlapping with the NC membrane by 2 mm. Next, the cards were cut into pieces that were 3 mm wide and 43 mm long.
2.5. Confirmation of the pre-treatment effect on DNA immobilization

The pre-treatment buffer was 20 mM sodium borate (pH 8.0) containing 0.8 M KCl, 0.2% (v/v) Tween 20. An amount of 0.3 μL of 300 μM bare capture DNA was dispensed and dried at room temperature for 10 min. Before and after dispensing bare capture DNA, NC membranes were fully immersed in pre-treatment buffer and dried for 10 min. For comparison, 300 μM bare capture DNA was mixed with pre-treatment buffer or each component of the pre-treatment buffer, and an amount of 0.3 μL of above mixtures was dispensed and dried at room temperature for 10 min. Finally, all LF strips were exposed to 254 nm UV light using a UV-crosslinker (Korea Ace Science, Seoul, Korea) at 60 °C for 10 min. After that, the strips were treated by the following conventional methods: for conventional UV-mediated immobilization, prepared strips were exposed to 254 nm UV light using a UV-crosslinker (Korea Ace Science, Seoul, Korea) with a total energy imparted of 90 mJ/cm² at 15 cm distance, for 5 min.

2.6. Various DNA immobilization methods

An amount of 0.3 μL of 300 μM bare capture DNA with or without 0.8 M KCl was spotted on the NC membrane and dried at room temperature for 10 min. After that, the strips were treated by the following conventional methods: for conventional UV-mediated immobilization, prepared strips were exposed to 254 nm UV light using a UV-crosslinker (Korea Ace Science, Seoul, Korea) at 60 °C for 10 min; for conventional heat-mediated DNA immobilization, prepared strips were incubated using a heat block (DAIHAN Scientific, Seoul, Korea) at 60 °C for 10 min; and for no-treatment, prepared strips were incubated at room temperature for 30 min.

2.7. LFA

We mixed 0.3 μL of synthetic DNA product (Table S1) at various concentrations (at 10 nM unless otherwise stated), 3 μL of streptavidin-coated AuNPs, and 26.7 μL of running buffer containing 20 mM sodium borate (pH 8.0), 2% (w/v), sucrose, 0.6 M NaCl, 0.2% (v/v) Tween 20%, and 0.1% (w/v) sodium azide. The LF strips were then dipped into the mixtures and incubated for 10 min. Then, the LF strips were dipped into PBS and incubated for 5 min. The color intensity was then analyzed using Image J software, which was normalized by subtracting the background signal of each strip.

2.8. Detection of synthetic target gene of SARS-CoV-2

For the selectivity test, 1x PCR premix was mixed with 0.12 μM forward primer and 1.2 μM reverse primer for the RPP gene, or 0.03 μM forward primer and 0.3 μM reverse primer for the Orf1b gene, or 0.15 μM forward primer and 1.5 μM reverse primer for the N gene. Then, 10 pM synthetic cDNA of each gene was mixed for asymmetric PCR. For the sensitivity test, 1x PCR premix, 0.1 μM forward primer and 1 μM reverse primer for each gene, and various concentrations of each synthetic cDNA were mixed. Asymmetric PCR was performed in a CFX Connect (Bio-Rad Laboratories, Inc., USA) by using the following pre-determined conditions: initial denaturation at 94 °C for 15 min, followed by 50 cycles of denaturation at 95 °C for 10 s, annealing at 57 °C for 15 s, and extension at 72 °C for 30 s. We added 0.6 μL of each asymmetric PCR amplicon, 3 μL of streptavidin-coated AuNPs, and running buffer up to 30 μL. The strips were then dipped into the mixtures and incubated for 10 min. The color intensity was then analyzed using Image J software, which was normalized by subtracting the background signal of each strip.

3. Results and discussion

3.1. Comparison between various immobilization conditions

Motivated by a recent study reporting that the pre-treatment of the entire LF strip with the solution can improve the signal intensity in LFA [21], we first investigated the effect of pre-treatment on the colorimetric signal in LFA. We pre-treated NC membrane in various conditions with pre-treatment buffer containing 20 mM sodium borate (pH 8.0), 0.8 M KCl and 0.2% (v/v) Tween 20. As shown in LF strip 3 (Fig. 2A), the LFA signal increased when the entire NC membrane was pre-treated before the capture DNA was immobilized. As the pre-treatment step of the entire NC membrane increases usage of the pre-treatment buffer and preparation time, it is more desirable to skip this step, and thus we attempted to achieve this goal by preparing capture DNA that contained components of the pre-treatment buffer (LF strips 4, 5, 6, and 7; Fig. 2B). Interestingly, when capture DNA mixed with pre-treatment buffer was immobilized (LF strip 4 in Fig. 2A), the color intensity was comparable to that of the LF strip pre-treated on the entire NC membrane (LF strip 3 in Fig. 2A). Next, we investigated which components of the pre-treatment buffer were the major contributors to the improved colorimetric signal in the LFA. The colorimetric signal increased substantially when 0.8 M KCl was mixed with the bare capture DNA (LF...
Based on these results, we assumed that the KCl neutralized the negatively charged phosphate backbone of the capture DNA and reduced the intermolecular electrostatic repulsion of the DNA, which consequently mediated the effective immobilization of capture DNA onto the NC membrane, resulting in a more intense colorimetric signal.

We checked the effect of KCl on the various DNA immobilization methods (Fig. 2B). In all three cases (with UV-irradiation, heat treatment, or without any treatment), the colorimetric signal was increased by more than three times by the presence of KCl in comparison to the absence of KCl (Fig. 2B). Importantly, the colorimetric signal of the non-treated LFA in the presence of KCl was superior to the conventional strategies that rely on UV-irradiation or heat treatment without KCl, paving the way for an equipment-free DNA immobilization method.

3.2. Optimization of SAIoNs

Based on these promising results, we optimized the conditions for SAIoNs (Fig. 3). We made efforts to achieve a normalized colorimetric intensity of 0.1 in the non-treated conditions because a normalized colorimetric intensity of 0.1 (as evidenced in Fig. 2B) was high enough to clearly detect the results with the naked eye. Because we found that 0.8 M KCl in the pretreatment buffer increases the immobilization efficiency in the previous experiment (Fig. 2), we started the optimization experiment under this condition. First, we investigated the effect of amine (NH₂) modification and the concentration of the capture DNA (Fig. 3A and Fig. S2A). When 5'-C6 NH₂ modified capture DNA was used, the colorimetric signal was higher than that of bare capture DNA at all concentrations. Thus, NH₂-modified capture DNA at a concentration of 80 µM was selected for the subsequent optimization experiments to minimize the amount of capture DNA. Next, we varied the types of NH₂.
modification in the capture DNA. As shown in Fig. 3B and Fig. S2B, the NH$_2$ modification improved the colorimetric signal in all cases, but the greatest enhancement was observed when using 5'-C12 NH$_2$ capture DNA. Furthermore, we compared the effects of other metal salts (NaCl, MgCl$_2$, and CaCl$_2$) instead of KCl based on our assumption that the neutralization of the phosphate backbone charge of the capture DNA by metal salts would increase the immobilization efficiency (Fig. 3C and Fig. S2C). All tested salts substantially improved the colorimetric signal, with CaCl$_2$ showing the best results and a concentration of 0.8 M CaCl$_2$ showed the clearest signal (Fig. 3D and Fig. S2D). In addition, the mechanism for our SAIoNs technology was proposed in Fig. 3E. It is known that the NC forms dipoles which consist of nitrogen of partial positive charge and oxygen of partial negative charge [22]. Without salt, the negatively charged phosphate group of the DNA backbone is attracted to the nitrogen of the partial positive charge in NC and at the same time is repelled by the oxygen of the partial negative charge in NC. On the other hand, in the presence of salt at optimal concentration, the phosphate group of the DNA backbone and metal ions form a complex by charge-charge interaction [23], which consequently minimizes the repulsive force between DNA and NC. More specifically, the metal ion and phosphate group of DNA-metal complex are arranged to have an attraction with the oxygen of NC and nitrogen of NC, respectively, thereby placing DNA and NC in proximity. However, when a high concentration of salt is present, an excess of metal ions surrounds DNA...
and oxygen of NC, respectively, which makes it difficult for DNA to approach NC due to the repulsive force, leading to the reduction in the immobilization efficiency. Therefore, we assumed that metal salt at optimal concentration increases the chance of non-covalent and irreversible physical adsorption by placing NCs and DNA in proximity [25], consequently improving the immobilization efficiency. Finally, we confirmed the influence of drying time (Fig. S3A). It was observed that the signal increased over time; 30 min drying time was sufficient since a normalized colorimetric intensity above 0.1 was achieved. Notably, the signal at 0 min was also high enough to detect the color intensity signal with naked eye. We confirmed the influence of drying temperature (Fig. S3B). It was observed that the signal increased with increasing temperature. This result suggests that SAIoNs work well even in resource-limited environments, implying that the developed method would be suitable for POC. In addition, the prepared LF strips were stable for long-term storage, as evidenced by no reduction in signal up to 56 days after storage at room temperature or 4 °C (Fig. S4).

### 3.3. Comparison of different DNA immobilization methods

Under the optimized conditions, we compared the normalized colorimetric intensity of our method with other DNA immobilization methods. SAIoNs substantially improved the colorimetric signal over non-SAIoNs methods, whether UV or heat was applied or not (Fig. 4A). The results in Fig. 4B and C show that the colorimetric signal in all cases increased as the concentration of the synthetic DNA product increased. Importantly, the LF strips prepared by our method detected the target DNA at a lower concentration than the method using only UV-irradiation (Fig. 4C). Furthermore, when SAIoNs and UV were used together, the synthetic DNA product was determined at the lowest concentration. The visual limit of detection (LODs) in the three cases, SAIoNs with UV, SAIoNs, and Conventional UV, were approximately...
0.5, 1, and 2 nM respectively. Because UV irradiation or heating is known to increase the immobilization efficiency of capture DNA, this enhancement effect was also applied to SAIoNs. These results confirm that the newly developed SAIoNs have good detection performance and can be used for the effective immobilization of capture DNA onto the NC membrane.

### 3.4. Detection of SARS-CoV-2

The proposed SAIoNs was applied to the detection of SARS-CoV-2 (Fig. 5). For the specific detection, we designed three different capture DNAs, respectively targeting the Orf1b and N gene of SARS-CoV-2, and the human RPP gene, which is conventionally used as an internal control. These capture DNAs were immobilized onto the separate test zones of one LF strip using SAIoNs. After an asymmetric PCR with specifically designed primers (Table S1), the three kinds of single-stranded amplicons (Orf1b, N, and human RPP genes) were mixed with running buffer. The LF strips were dipped into the mixture. Because the reverse primer was modified with biotin, the single-stranded amplicons were labeled with biotin, which was bound to streptavidin-coated AuNPs, generating the colorimetric signal in each test zone. A high sequence specificity between capture DNA and single-stranded amplicons with no cross-reactivities clearly demonstrate the multiplexing capability (Fig. 6A). In addition, the detection sensitivities of the Orf1b and N genes of SARS-CoV-2 were evaluated. When confirmed by visual LOD, they were detectable even at concentrations of 300 aM and 500 aM (Fig. 6B and C). These results support the contention that SAIoNs are sufficiently applicable in the real, practical detection of different pathogenic bacteria or viruses [26].

### 4. Conclusions

In this paper, we introduce a novel technique for the salt-mediated immobilization of nucleic acids (SAIoNs) onto NC membrane. Compared to conventional DNA immobilization methods such as streptavidin–biotin, UV-irradiation, and heat treatment, our method does not require special equipment (e.g., centrifuge, UV-crosslinker, heating device) and can therefore be applied in a resource-limited environment, with the concomitant advantages of reducing production costs and time (Table 1). Through the application testing, our newly developed method shows excellent immobilization performance and reproducibility with the long-term storage stability. However, we confirmed only limited kinds of chloride compounds (NaCl, MgCl2, KCl, and CaCl2) and the effect on NH2 modification; therefore, other types of ionic compounds and DNA modification should be studied in order to ensure more versatile applications. Nonetheless, the new method with the improved performance was successfully validated by the detection of practical targets (e.g., SARS-CoV-2) with multiplexing capability, suggesting that it can be a promising molecular diagnostic platform for the simple and rapid detection of various pathogens.

### CRediT authorship contribution statement

**Jung Soo Park:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **Seokjoon Kim:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **Jinjoo Han:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **Jung Ho Kim:** Methodology, Formal analysis, Investigation. **Ki Soo Park:** Writing – review & editing, Funding acquisition, Resources, Supervision.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

### Supplementary information

The supporting information is available free of charge as the following files: Schematic illustration of conventional DNA immobilization methods (Fig. S1); Optimization of salt-mediated immobilization of nucleic acids (Fig. S2); Signal intensity at various drying conditions (Fig. S3); Storage durability test (Fig. S4); Oligonucleotide sequences used in this study (Table S1).

### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi.org/)

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