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Immobilizing and Patterning DNA on Simplified Protein-Free DNA-Based Lateral Flow Assays

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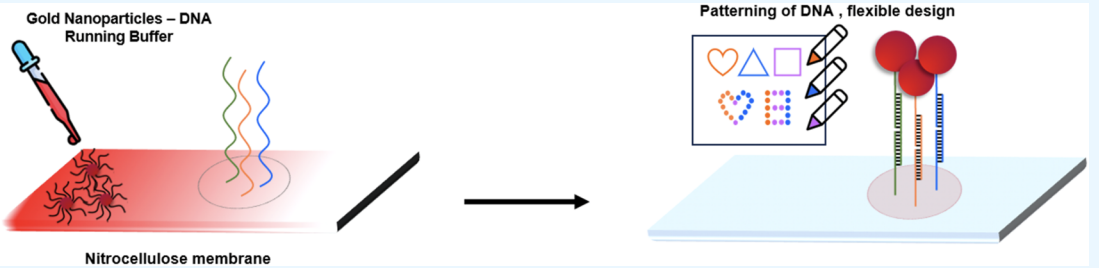

ABSTRACT: This work presents a comprehensive study on DNA-based LFAs that completely avoids the use of proteins or pretreatment buffer. We address key fundamental challenges including optimizing buffer conditions for effective DNA immobilization on paper, understanding sequence-dependent detection, and ultimately developing patterning techniques that allow DNA patterns to appear on paper that exhibit distinct shapes depending on the introduced input sequences.

INTRODUCTION

During the COVID-19 pandemic, there was an urgent need for effective molecular diagnostic techniques to manage the rapidly spreading virus.[1−3] Despite significant efforts, including the widespread use of RT-PCR tests, the initial months of 2020 saw challenges in curbing the virus's spread.[4−7] This situation spurred extensive research into various diagnostic methods, including the development of self-diagnostic kits.[8−10] While PCR technology remained central to these efforts, paper-based lateral flow assays (LFAs) emerged as a widely used alternative due to their simplicity and ease of use.[11−13] However, most LFAs heavily rely on protein-based systems, such as antibody−antigen interactions,[14,15] which can be time-consuming to develop due to the complexity of determining protein structures of unknown viruses. In contrast, genetic sequences can be rapidly identified using sequencing methods, which simplifies the development of diagnostic tools based on genetic information. Hence, there is a critical need to develop LFAs that utilize nucleic acid strands rather than proteins, leveraging faster and more straightforward process of viral sequence identification.[16,17] Previous research has indeed explored the use of DNA on lateral flow assays,[17−20] but often these approaches still rely on proteins for immobilization,[21] incorporate chemical or physical modifications, require pretreatment of lateral flow strips before immobilizing DNA strands,[17] or do not fully leverage the potential for multiplexing or patterning. In this work, we developed a simplified protein-free DNA-based LFA that entirely avoids the use of proteins, chemical modifications, or pretreatment of nitrocellulose paper. It is also the first to demonstrate multistrand patterning of DNA on lateral flow paper without protein-based immobilization, ultimately providing a cost-effective and straightforward assembly method for DNA-based LFAs.

Typically, DNA-based LFAs are built in three steps: (i) DNA strands are initially immobilized on paper, (ii) paper strips are then allowed to dry, and (iii) a running buffer solution containing complementary DNA gold nanoparticles is flowed through to enable visualized detection.

RESULTS AND DISCUSSION

First, we designed immobilizing DNA strands on paper with just water. Previous studies hypothesized that cations reduce the electrostatic repulsion between DNA strands, facilitating the adsorption of DNA onto paper.[17] They emphasized the use of a salt-containing buffer to immobilize DNA, utilizing KCl, $MgCl_2$, and $CaCl_2$ to dissolve DNA before dispensing the salt-treated buffer solution on paper.[17] However, in our approach, we chose to dissolve the DNA in water instead of using a salt-containing buffer to (i) prevent excess salt from accumulating on the paper and (ii) avoid salt-induced nanoparticle aggregation. Furthermore, we hypothesized that omitting the use of salt-containing buffer to dissolve DNA would simplify the workflow of LFA preparation and enhance its versatility for

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various applications, such as depositing multiple DNA strands on the same spot on paper (i.e., multipatterning). Therefore, one set of DNA strands dissolved in water was dispensed in 1, 3, or 5 μL and dried on each Cellulose Nitrate 95 (CN95) paper strip, and then either water or buffer solution (PBS, 0.1 M borate buffer, or NE buffer) was introduced onto each strip to mimic the use of running buffer. In terms of choosing the most optimal buffer condition, different types of buffers, three of the most commonly used ones in biosensors, were tested in comparison: PBS buffer, borate buffer, and NE buffer. The DNA strands were labeled with a Cyanine 3 fluorophore, which allowed fluorescent tracking of the DNA molecules as the capillary action in the nitrocellulose paper carried the DNA solution from one side of the paper to the opposite end. The fluorescence intensity as well as position was measured via a gel reader after each flow of water or buffer solution to check for the remaining amount of DNA strands after having liquid flowed through the paper strip multiple times (Figure 1A).

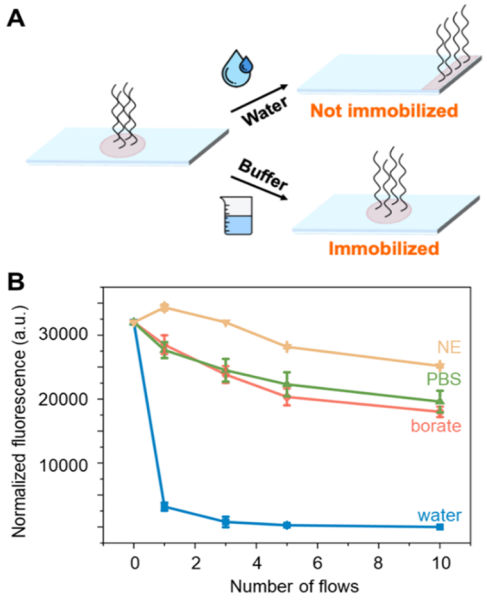

Figure 1. DNA immobilization on paper was tested under various buffer conditions. (A) When just using water, DNA strands were quickly washed away from their initially dispensed position toward the end of the paper strip. However, when buffer was used, without any pretreatment of paper or UV curing, majority of the DNA strands remained in position. (B) PBS, borate buffer, and NE buffer were tested against DEPC-treated water. NE buffer immobilized DNA strands most effectively and no significant difference was found between PBS buffer and borate buffer. The graph represents data for a 1 μL dispense, but the same experiment was also repeated for 3 and 5 μL (see Figure S1).

When just using water, DNA strands were quickly washed away from their initially dispensed position toward the end of the paper strip, and almost all DNA strands were removed before the third set of flow (Figure 1B). However, when buffer was used, the majority of the DNA strands remained in position even until the tenth flow, at which more than 50% of the DNA remained in place (Figure 1B). No significant difference was found between PBS buffer and borate buffer, while NE buffer immobilized DNA strands most effectively. The drastic difference between using water versus buffer showed similar results in all cases of 1, 3, and 5 μL dispenses (Figure S1), suggesting that the running buffer needs to contain salt to prevent DNA strands from becoming mobile. Indeed, confirming our initial hypothesis, even without using a salt-containing solution during the immobilization step, the inclusion of salt in the running buffer alone is sufficient to effectively immobilize DNA.

As previously stated, it is important to note that one must carefully select buffer conditions for DNA-based LFAs since high concentrations of salts may induce aggregation of DNA gold nanoparticles.[22] In this work, borate buffer was used in all proceeding experiments because NE buffer caused nanoparticle aggregation (Figure S2) and borate buffer is generally considered more stable than PBS across various pH ranges.[23]

Once the immobilization ability was confirmed, the hybridization ability between DNA strands anchored on paper and DNA strands functionalized on nanoparticles was tested. To account for different hybridization schemes, two sets of experiments were designed: (i) a 2-strand system and (ii) a 3-strand system (Figure 2A). For the 2-strand system, we designed a complementary sequence overlap of 20 bases. In the 3-strand system, we used a 40-mer "bridge DNA" to connect the DNA strands anchored on nanoparticles with those "capture DNA" strands immobilized on paper. Each connection involved a 20-base complementary hybridization, with 20 bases overlapping on both the nanoparticle and the paper sides. In general, a 2-strand system would necessitate redesigning and synthesizing the full sequences of both "capture DNA" and "particle DNA" whenever the target changes. In contrast, a 3-strand system would be able to reuse parts of the sequences and adjust the "bridge DNA" sequence based on the target. Both 2-strand and 3-strand systems formed distinct patterns only when complementary sequences were available, indicating successful DNA hybridization on protein-free DNA-based LFAs (Figure 2B). For example, when noncomplementary strands were introduced to the system (labeled "NC" in Figure 2B), no hybridization between DNA strands occurred, and therefore no pattern appeared. In the 2-strand system, complementary "particle" and "capture" DNA strands successfully hybridized, appearing in a circular, red pattern on paper. In the 3-strand system, detection was only possible when the "bridge" strand was present. In addition, the position of the thiol modification (e.g., anchoring of DNA on nanoparticles) at the 5′ or 3′ end of the "particle DNA" was tested. As expected, in both 2- and 3-strand systems, the location of the thiol group did not significantly affect the reactions.

Further investigations on the 3-strand system showed that a 2:1 ratio of "bridge DNA" to "capture DNA" exhibited higher sensitivity than 1:1 or 1:2 ratios (Figures 2C and S4A). Such results indicate that having a higher concentration of "capture DNA" strands does not significantly improve the sensitivity performance of the DNA-based LFA and that the number of "bridge DNA" strands is more relevant to the overall detection performance than that of "capture DNA" strands. The role of "bridge DNA" is crucial in the development of DNA-based LFAs with a 3-strand system, as it is the key component responsible for detecting the target. As such, the detection limit of the 3-strand system was tested by progressively diluting the concentration of the "bridge DNA" from 100 μM to 50 pM while maintaining constant concentrations of "capture DNA" and DNA nanoparticles. The results demonstrated that the detection patterns gradually faded, with visual detection possible down to 100 pM but not at 50 pM (Figures 2D and S4B). These results highlight that the DNA-based LFA can

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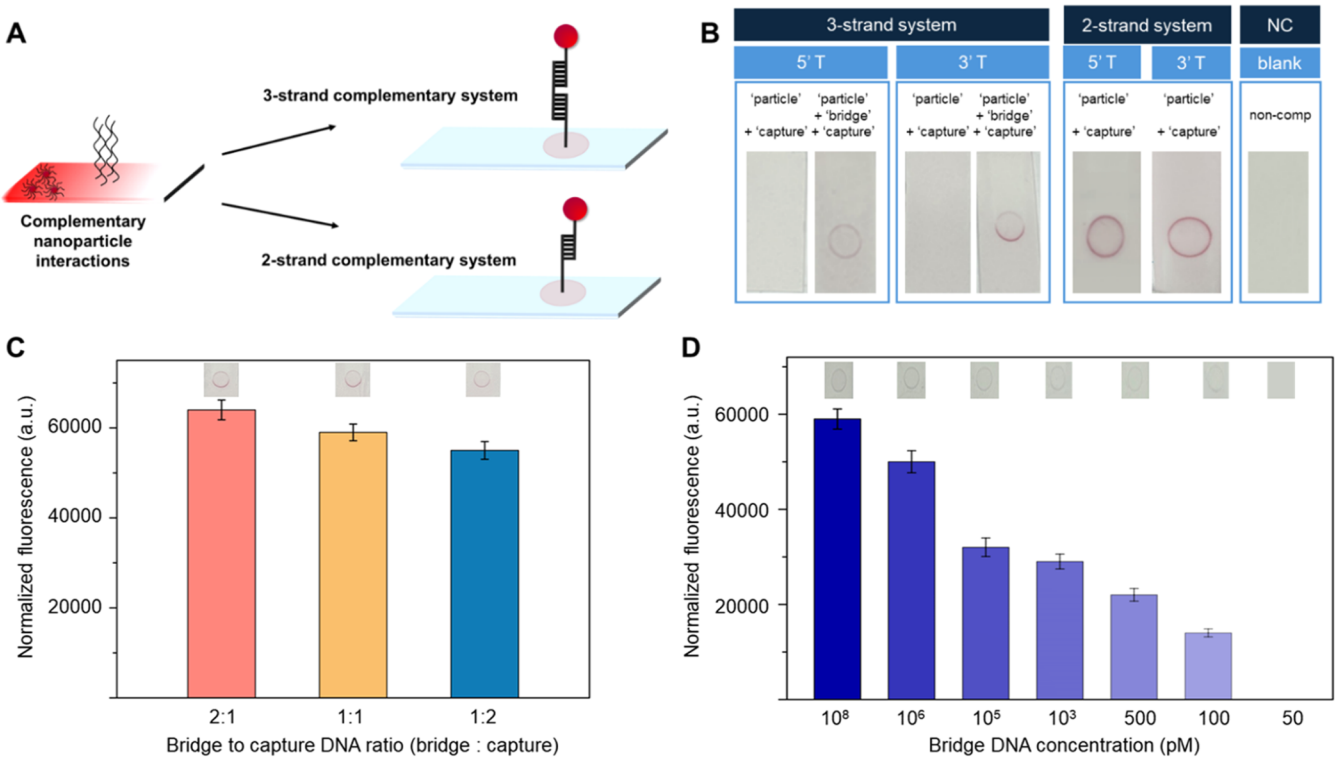

Figure 2. Hybridization ability between DNA strands anchored on paper ("capture DNA") and complementary (or noncomplementary) DNA strands functionalized on gold nanoparticles were tested in a (A) 2-strand versus 3-strand complementary system. (B) In both 2- and 3-strand systems, complementary interactions successfully showed nanoparticle signal (red circle), whereas noncomplementary interactions remained blank (no circle appears on paper). For the 3-strand system, (C) different ratios of "bridge DNA" to "capture DNA" were tested, and (D) various concentrations of "bridge DNA" were tested by progressively diluting the concentration of the "bridge DNA" from 100 μM to 50 pM.

effectively detect targets down to a concentration of approximately 100 pM without requiring complex immobilization methods.

Checking the stability of protein-free DNA-based LFAs is crucial to ensuring consistent performance and reliability over time. Stability testing was performed to confirm the durability of the DNA-based LFAs. Due to the greater complexity of the 3-strand system compared to the 2-strand system, stability testing was primarily focused on the 3-strand DNA-based LFAs. To assess their long-term storage capability, accelerated life testing was performed.[24−26] Specifically, 'capture DNA' strands were deposited on lateral flow paper strips and stored at 50 °C for 1, 3, 5, and 7 days, after which their response was confirmed by reacting them with complementary DNA gold nanoparticles. In each accelerated life testing experiment, it was assumed that every 10 °C increase in the temperature would double the equivalent storage time at room temperature. Based on this assumption, the experimentally tested storage times at room temperature were calculated to be 8, 24, 40, and 56 days, respectively. Each DNA-LFA strip was then reacted with nanoparticles, and photographs of all samples were taken for quantitative signal intensity analysis; visible detection signals were successfully confirmed across all samples (Figure 3).

Additionally, a straightforward test was conducted to observe how long the strips with already-patterned positive signals could maintain their signal intensity (Figure S5). Accelerated life testing was performed by storing the DNA-based LFA strips at 50 °C after reacting with nanoparticles for 1, 4, 8, and 24 hours. The results confirmed that the positive signal remained intact even after prolonged storage time, demonstrating the excellent long-term stability and signal

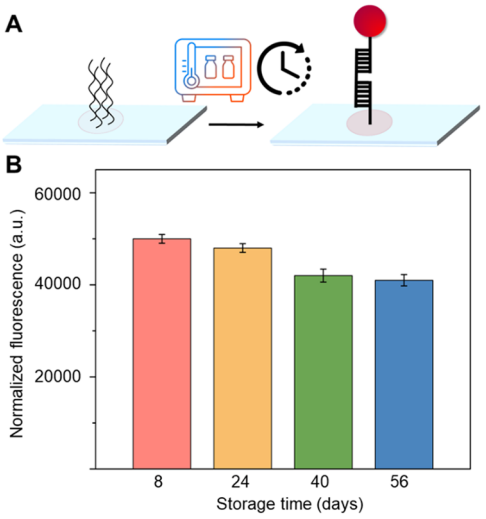

Figure 3. Stability of protein-free DNA-based LFAs was tested via accelerated life testing. (A) DNA-based LFAs were stored for different numbers of days at an elevated temperature and (B) fluorescence results demonstrated that no significant degradation in the signal was found even after long-term storage.

retention of nanoparticle-reacted, protein-free DNA-based LFAs.

Lastly, patterning DNA strands on lateral flow paper strips was successfully achieved in this work, enabling precise control over the placement and formation of specific shapes. A nanoliter inkjet printer was utilized to dispense solutions at specific locations and precisely control the droplet size. This

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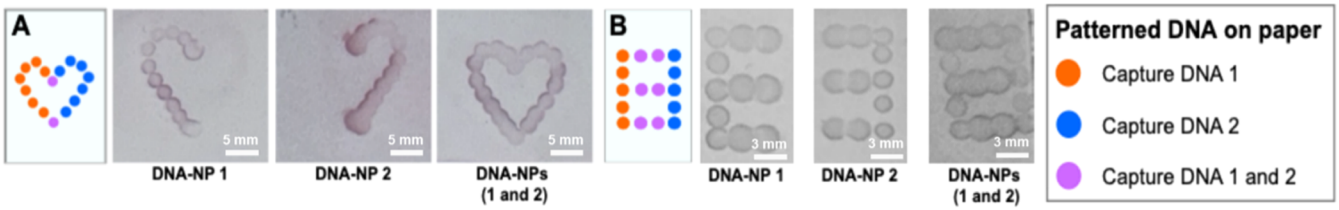

Figure 4. Patterning DNA strands on lateral flow paper strips without the use of proteins was demonstrated by using a nanoliter ink printer. (A) As shown in the schematic, two different strands were immobilized in a shaped pattern on lateral flow paper. Depending on the complementary strand-functionalized particles that were introduced (either strand 1 only, strand 2 only, or both), different patterns appeared on the same piece of DNA-based LFA paper strip. (B) Digital numbers or letters could also be printed on the same piece of paper. Each pattern is about 15 × 15 mm^2 in size.

allowed us to pattern "capture DNA" strands and generate different shapes via complementary interactions with DNA nanoparticles (Figure S6).

Additionally, two differently sequenced "capture DNA" strands were dispensed on either the same or separate spots such that they coexist. For example, we patterned the shape of half a heart with one sequence of "capture DNA" (capture 1) and the other half with another sequence (capture 2). Then, three separate scenarios were tested: (i) DNA nanoparticles with complementary sequences to "capture 1" were introduced, (ii) DNA nanoparticles with complementary sequences to "capture 2" were introduced, and (iii) a mixture of both sets of DNA nanoparticles complementary to "capture 1" and "capture 2" was inserted. As expected, either half of the heart or the complete heart became visualized only when the appropriate complementary DNA nanoparticles were introduced into the system (Figure 4A). In another example, we patterned the shape of a digital number "8" with one sequence (capture 1) and another sequence (capture 2) on the right, while the two sequences overlapped at the center. When DNA gold nanoparticles that are complementary to "capture 1" were introduced, the letter "E" shape appeared, and those that are complementary to "capture 2" produced the number "3". When both DNA nanoparticles were present, the full number "8" formed (Figure 4B). Hence, this paves the way for future advancements in multiplex detection and the ability to selectively design complex patterns on protein-free DNA-based LFAs. Furthermore, our study suggests potential connections to DNA single-molecule research, particularly in areas involving DNA fixation on substrates, including techniques such as single-molecule FRET (smFRET) and DNA stretching assays.[27−30] For example, our protein-free approach may provide additional insights into stable DNA immobilization. Understanding the mechanics and dynamics of DNA−substrate interactions in these contexts could provide further insights into DNA behavior and contribute to advancements in related research fields.[31−33]

CONCLUSIONS

We successfully developed a simple, protein-free, equipment-free, pretreatment-free, and chemical modification-free DNA-based LFA. Our findings indicate that using only water in the immobilizing solution is sufficient, while salt or cation must be added exclusively to the running buffer. In the 3-strand system, increasing the amount of "capture DNA" relative to "bridge DNA" does not necessarily improve sensing performance or efficiency. Therefore, when developing DNA-based LFAs, this finding can be applied to optimize material use efficiently and economically. This sensor can multiplex and selectively detect target DNA at concentrations as low as 100 pM, maintaining the assay stability over time. While 100 pM is below the typical detection limit of protein-based LFAs (ca. 2−200 pM), the sequence specificity and adaptability of DNA-only LFAs offer distinct advantages. Lastly, we demonstrated the ability to pattern complex and diverse shapes, moving beyond basic circles or lines, which highlights the flexibility and innovative potential of our approach in advancing LFA technology. It is important to note that the effectiveness of any LFA method is closely linked to nucleic acid extraction protocols, which can vary depending on sample type and biological or clinical conditions. However, well-established purification techniques, such as silica column or magnetic-bead-based methods, may effectively eliminate potential inhibitors or contaminants, ensuring compatibility with our approach. Future studies will focus on validating this method across diverse conditions and actual patient samples to further enhance its applicability.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c11255.

Experimental methods including buffer preparation, DNA gold nanoparticle conjugation, Biospot nanoink printer operation, and sequence information on oligonucleotides (PDF)

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Notes

The authors declare no competing financial interest.

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