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Proof-of-Concept for Speedy Development of Rapid and Simple At-Home Method for Potential Diagnosis of Early COVID-19 Mutant Infection Using Nanogold and Aptamer

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Abstract:
The positive single-stranded nature of COVID-19 mRNA led to the low proof-reading efficacy for its genome authentication. Thus mutant covid-19 strains have been rapidly evolving. Besides Alpha, Beta, Gamma, Delta, and Omicron variants, currently, subvariants of omicron are circulating, including BA.4, BA.5, and BA.2.12.1. Therefore, the speedy development of a rapid, simple, and easier diagnosis method to deal with new mutant covid viral infection is critically important. Many diagnosis methods have been developed for COVID-19 detection such as RT-PCR and antibodies detection. However, the former is time-consuming, laborious, and expensive and the latter relies on the production of antibodies and is not suitable for the early diagnosis of viral infection. Here we proved the concept for the speedy development of a simple, rapid, and cost-effective early at-home diagnosis method by the combination of a new aptamer and existing antibody using the Lateral Flow Nitrocellulose filter. The DNA aptamer specific to spike proteins (S-proteins) is conjugated to gold nanoparticles and serves as a detection probe. And the antibody which is specific to spike proteins that overexpress on COVID viral particles used as a second probe that is immobilized to the nitrocellulose membrane. The aptamer conjugated nanoparticles are incubated with spike proteins for half an hour and tested for their ability to bind to antibodies anchored on the nitrocellulose membrane. The gold nanoparticles are visualized on the nitrocellulose membrane due to interaction between the antigen (spike protein) and antibody. The detection limit for this method was found to be 2.0 ug. Thus, the detection of viral antigen can be obtained within 2 hours, with a cost of less than $5 for the diagnosis reagent. As long as the mutant of the newly emerged viral surface protein is reported, a peptide or protein corresponding to the mutation can be produced by peptide synthesis or gene cloning within several days. An RNA or DNA aptamer can be generated quickly via SELEX. A gold-labeled DNA aptamer specific to spike proteins (S-proteins) serves as a detection probe. Any antibody that has been available on the market to bind the wild-type COVID virus can be used as a second probe that is immobilized to the nitrocellulose. The diagnosis method can be carried out by patients at home if a clinical trial verifies the feasibility of this method.

Keywords Oligonucleotide aptamer, Spike protein, Lateral flow assay, early-stage COVID-19 diagnosis.

Background
COVID-19 emerged in 2019 and rapidly progressed to a pandemic stage within the span of 4-6 months and caused severe repercussions on human health. COVID-19 is named Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) based on its phylogeny and taxonomy. COVID-19 is highly infectious with high transmissibility, and pathogenicity.2-5 Therefore, many diagnosis methods have been developed for COVID-19 detection.6-8 Viral culture is the gold standard for viral infection diagnosis. However, it is time-consuming and requires high biosafety laboratory. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) is a widely used diagnostic method for detecting specific viral nucleic acid sequences. 9-12 Though the RT-PCR method is useful for early detection of the Covid-19, it has its own limitations such as difficulty in sample collection, transportation, and RNA extraction.13, 14 On the other hand, the ELISA test is used for the COVID-19 diagnosis, but its non-specificity limits it due to conserved antigens among different Corona viral species used in the ELISA test. Besides, the method needs at least two weeks post-infection to detect antibodies.15-17 Therefore, developing a simple, rapid, reliable, and point of care COVID-19 diagnosis kit would be helpful to diagnose and isolate the infected people, thus reducing the spread of the viral infection. Serological assays based on recombinant antigens derived viral surface proteins such as Spike (S) and Nucleocapsid (N) proteins are also used in laboratory diagnostics.18, 19 Unlike other methods, the serological test does not need special technical requirements, on top of that, it requires less time, lower sample, and have high detection sensitivity and specificity. Therefore, serological testing can be used as an adjuvant to rRT-PCR for COVID-19 detection. Aptamers are DNA/RNA oligos selected in vitro procedure called SELEX (systematic evolution of ligands by exponential enrichment).20 The selected aptamers can adapt unique tertiary structures and are able to recognize target molecules with high affinity and specificity.21 Yang et al. have reported a DNA aptamer that is specific to SARS-CoV-2 S-protein with high affinity (Kd ~ 5.8 nM).22 Compared with protein antigens, aptamers are easy to obtain in large quantities at a low cost and are considered as useful diagnostic agents.23 In this study, we propose an alternative lateral flow diagnosis method for the early diagnosis of COVID-19. The approach is based on the use of aptamers that can bind to viral proteins such as spike protein or nucleocapsid protein present on the virus's surface.24-26 The DNA aptamers are conjugated to gold nanoparticles via direct covalent conjugation using thiol chemistry or streptavidin and biotin interaction. Gold conjugation to oligonucleotides allowed for various applications in nanotechnology and diagnostics due to their unique physicochemical properties.27, 28 The aptamer-gold complex acts as a detection probe and would specifically bind to viral particles and exhibit color when the samples run against the respective antibodies pre-immobilized on the nitrocellulose membrane, as shown in Figure 1. The gold-aptamer conjugate bind to S-protein and is concentrated into a band upon binding to the pre-immobilized antibody and exhibited red color. Patients can carry out this diagnosis method at home if a clinical trial verifies the feasibility of this method. The developed method can also be extended to detect mutated SARS-CoV2 particles by cloning the mutated antibody and selecting an appropriate oligonucleotide aptamer for spike or nucleocapsid proteins using SELEX. The antibody (Ab) can be from the wild-type virus or It can be any one of the high-title Ab that is currently available in the market. Any available antibody that can bind the wild-type COVID virus can be used as a second probe that is immobilized to the nitrocellulose. The only component that is needed is the aptamer. As long as the mutant sequence of the newly emerged viral surface protein is reported, a peptide or protein corresponding to the mutation can be produced by peptide synthesis or gene cloning. And an RNA or DNA aptamer for the protein can be generated quickly via SELEX. Suppose a clinical trial verifies the feasibility of this method, the diagnosis method can be carried out by patients at home, similar to nitrocellulose for diagnosis of pregnancy.

Materials and methods

Materials for lateral flow study
The DNA aptamer for S-protein sequence was derived from Yang et al. 5′- CAGCACCGACCTTGCTTGGAGTGCTGTGCTCCAGGGCGTTAATGGACA-3′
The DNA strands were purchased from IDT. The bare gold nanoparticles and streptavidin-coated gold nanoparticles were purchased from Sigma-Aldrich, and the commercially available antibody, Rabbit anti-SARS-CoV-2-S2 purchased from Sino Biotech. The nitrocellulose membrane FF120HP Plus was used for the lateral flow study obtained from Cytiva.

DNA-aptamer conjugation to gold nanoparticles
DNA-aptamer conjugation to gold nanoparticles was achieved in two methods. i) DNA-aptamer with 5′-end thiol group conjugation to gold nanoparticles. ii) DNA-aptamer with 5′-biotin conjugations to streptavidin-coated gold nanoparticles.

i) DNA-aptamer with 5′-end thiol group conjugation to gold nanoparticles: The DNA aptamer with a 5′-end thiol group (50 to 100 uM of 100 ul) is treated with TCEP solution at a final concentration of 5.0 mM for 1hr. To the reaction mixture, gold nanoparticles (10X) and dATP (0.1 mM at final concentration) were added and incubated for 45.0 mins. The addition of dATP helps to prevent nonspecific interaction of DNA aptamer to the gold surface. Then, 20.0 mM sodium chloride solution is added to the above reaction mixture and incubated at room temperature overnight. The addition of sodium chloride is to reduces the repulsions between DNA strands and facilitates higher conjugation. The sodium chloride concentration gradually increased to 100.0 mM for 24 hrs. The aptamer conjugated gold nanoparticles are purified by centrifuging at 10,000 rpm for 10.0 mins under cold conditions (4 °C). The gold pallet is washed by repeating it twice by adding 100 to 200 ul of deionized water. The gold nanoparticles are stored in deionized water having 1%BSA, 0.01% triton, 2% sucrose, and 0.02% NaN3.

ii) DNA-aptamer with 5′-biotin conjugations to streptavidin-coated gold nanoparticles: Biotin conjugated DNA aptamers incubated with the S-protein for 30 mins at room temperature, then the mixture incubated with Streptavidin-coated gold nanoparticles for one hour at room temperature. Then, the gold-nanoparticles with the S-proteins are purified from the excess DNA aptamer removed by centrifugation at 1000 rpm. The gold pallet is redissolved in deionized water.

Assembly of lateral flow assay strips
The components needed for the lateral flow assay are assembled on the PVC backing in the order of sample pad, conjugate pads, nitrocellulose membrane, and absorbent pad. Anti-SARS-CoV-2 antibodies are striped onto the nitrocellulose cards using a lateral flow reagent dispenser (LFRD, Claremont BioSolutions, Upland, CA, USA) set to a head speed of 4.5V voltage and Hamilton 100uL syringe using a syringe pump set to a flow rate of 0.2 mL/min. After the four antibodies were striped on the card, the card was dried at room temperature overnight. The strips were cut to 3.3 mm wide and stored under room temperature drying conditions.

Lateral flow assay procedure
The standard S protein samples of 0.25 mg/mL were diluted to different concentrations (0.0, 2.0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, and 250 ug/mL) with 0.1M PBS buffer solution (pH 7.4). Before each test, 5uL of S protein samples with different dilutions were added to a 0.6 mL test tube, then 5 uL 20uM Biotinylated DNA aptamer was aliquoted to the tube. The complex was incubated at room temperature for 30 min followed by adding 15uL Streptavidin-Gold nanoparticles. The complex was further incubated at room temperature for 1 hr and dropped on the conjugation pad. 100uL running buffer (0.1M PBS, 1% BSA, 0.1% Triton x 100) was dropped on the sample pad, and the detection results were observed after 15 min. Furthermore, to evaluate the feasibility of nitrocellulose membrane strip in detecting real patient samples, S-
protein pre-mixed with physiological buffer was used for analysis. In a typical experiment, we collected urine and blood sample from mice. The same analysis was applied.

Results

1. **Two methods for the conjugation of the aptamer to gold nanoparticles**

Aptamers are DNA/RNA sequences with a secondary structure with a high affinity towards their respective analytes with antibody-like specificity. The aptamers are widely used to develop biosensors and diagnostic tools due to their ease of synthesis and cost-effective production. The DNA aptamer selective for S-protein is conjugated to gold nanoparticles using two approaches: i) Conjugation of DNA aptamer for S-protein(spike-aptamer) to gold nanoparticles by thiol-chemistry. ii) Synthesis of the biotin-labeled aptamer and conjugation of the DNA aptamer for S-protein the commercially available streptavidin-coated gold nanoparticles using biotin-streptavidin interaction.

1.1 **Conjugation of DNA aptamer for S-protein(spike-aptamer) to gold nanoparticles by thiol-chemistry**

The DNA aptamer with a 5′-end thiol group is conjugated to gold nanoparticles using reduction chemistry. The DNA aptamer was incubated with TCEP to break disulfide bonds. The DNA aptamer was incubated with gold nanoparticles in the presence of dATP and sodium chloride to facilitate the aptamer conjugation to gold with high fidelity. The aptamer conjugated gold nanoparticles are purified by centrifugation to remove unbound DNA. To confirm the DNA aptamer conjugation to gold nanoparticles, a Cy3-conjugated small complementary DNA fragment is used. The aptamer labeled gold nanoparticles were characterized using 1% agarose gel by hybridizing with its complementary strand harboring Cy3 fluorophore, as shown in figure 2.

1.2 **Synthesis of the biotin-labeled aptamer and conjugation of the DNA aptamer for S-protein to the commercially available streptavidin-coated gold nanoparticles using biotin-streptavidin interaction.**

Using phosphoramidite chemistry, the biotin can be conveniently conjugated to DNA aptamer during solid-phase DNA synthesis. Conjugation of the aptamer to gold-nanoparticles is achieved through biotin-streptavidin interaction. The DNA aptamer with 5′-biotin is incubated with streptavidin-coated gold nanoparticles at room temperature to facilitate the DNA conjugation to gold nanoparticles. The DNA conjugated gold nanoparticles are purified from free DNA using centrifugation. The gold nanoparticles bearing the DNA aptamer were redissolved in deionized water for further use.

2. **Immobilization of the commercially available anti-covid 19 antibodies to the nitrocellulose and design of Lateral flow method for COVID-19 diagnosis**

The proposed method requires two probes, as shown in figure 1. One is a detection probe conjugated to gold nanoparticles using either thiol chemistry or biotin-streptavidin interaction. A DNA aptamer is specific to the SARS-CoV-2 virus S-protein used as a detection probe. The second is a capture probe that is the antibody to the S-protein pre-anchored on the nitrocellulose membrane by placing the antibody on the nitrocellulose membrane at 250.0 ng/ul concentration. Then, the viral samples such as saliva, blood serum, or nasal swabs can be incubated with the detection probe and run over the Spike-antibody pre-immobilized on the nitrocellulose membrane. The complex of viral particles and the detection probe binds to the...
capture probe, concentrates the viral particles into a thick band, and exhibits red color. The visible color can serve as a positive signal for virus detection. As the SARS-CoV-2 viruses are highly infectious and transmissible, we are restricted to using S-proteins instead of specimen samples.

**Figure 1.** Illustration of the design of a fast diagnosis device for COVID-19 infection using a combination of DNA/RNA aptamer and antibodies on a nitrocellulose membrane.

3. **Demonstration of lateral flow using aptamer conjugated gold nanoparticles for spike proteins.**

The importance for Point of care diagnosis (POCD) of human diseases increases as it is simple to perform, specific, low cost, robust, and equipment free. Nanosensing platforms revolutionized molecular diagnostics due to their unique physical and chemical properties, particularly gold nanoparticles suitable for POCD. The gold nanoparticles are widely used for diagnosis due to their unique optical properties arising from their surface plasmon resonance. Besides, the gold nanoparticles are easy to functionalize which allows to conjugate various molecular recognition elements needed for diagnosis. Therefore, we chose gold nanoparticles to develop a detection probe for COVID-19 diagnosis. The selected DNA aptamer is conjugated to gold nanoparticles using thiol chemistry and biotin-streptavidin interaction and serves as a detection probe. The detection probe was incubated with varying concentrations of SARS-CoV-2 spike proteins. And, the capture probe (viz., antibody to the spike protein) is anchored on the nitrocellulose membrane. Then, a later flow test was performed by running the spike-protein detection samples, pre-incubated with the detection probe against the spike-antibodies, which are pre-immobilized on the nitrocellulose membrane. The complex of spike-protein and the gold-nanoparticles binds to the capture probe and concentrates the S-protein into a thick band, exhibiting red color.

**Figure 2.** S-DNA-aptamer conjugation to gold nanoparticles: A) Snap of the gold-nanoparticle gel; B) Ethidium bromide channel; C) Cy3-channel. 1. 1kb ladder; 2. Bare gold nanoparticles; 3. Gold + Cy3-DNA; 4. Gold-DNA-aptamer for spike protein; 5. Gold-DNA-aptamer + Cy3-DNA; 6. Gold-DNA-aptamer for nucleocapsid-protein; Gold-DNA-aptamer for nucleocapsid protein + Cy3-DNA.

3.1 **Lateral flow test for S-protein using aptamer conjugated gold nanoparticles through thiol chemistry.**

DNA aptamers conjugated gold nanoparticles incubated with the respective S-proteins at different concentrations starting from 250.0 ng/ul to 0.0 ng/ul in a serial dilution to detect the minimum detection limit by visualizing the changes of intensity in optical signals at the Test line on nitrocellulose membrane as shown in Fig. 3. The results indicated that the minimum concentration of S-protein that can be detected on nitrocellulose membrane was 7.8 ng/ul. Upon increasing the S-protein concentration, the brightness at the test line is gradually increased.
Figure 3. Concertation limit determination: S-antibody anchored on the nitrocellulose membrane is titrated against S-aptamer conjugated gold nanoparticles through thiol chemistry, after their incubation with s-protein solution from 250 ng/ul to 0 ng/uL.

3.2 Lateral flow test for S-protein using aptamer conjugated gold nanoparticles through biotin-streptavidin interaction.
Aptamer-conjugated gold nanoparticles through biotin-streptavidin interaction were incubated with the respective S-proteins at different concentrations starting from 250.0 ng/ul to as low as 0.0 ng/ul and then applied to the LFA test (Fig. 4, lanes 1-9, & 11). The detection limit was determined by visualizing the optical signal change on the Test line, and it was found to be 2.0 ng/uL. To further demonstrate that the S-protein concentrates at the test line by binding to its antibody with specificity, the S-proteins are covalently linked to the gold nanoparticles using a commercially available protein labeling kit and used as a positive control (Fig. 4, lane 10). Thus, the results demonstrate that the LFA test using DNA aptamer indeed binds to S-proteins, which in turn binds to spike-antibody, showing the aptamers’ potential for the development of an early diagnosis kit. Besides sensitivity, the specificity evaluation of the detection probe binding to spike protein is also an important parameter. There was no coloration at the test line when BSA (250.0 ng/ul) was used instead of spike-proteins, suggesting that the spike-protein binding to its antibodies is specific.

Figure 4. Concertation limit determination: S-antibody anchored on the nitrocellulose membrane is titrated against S-aptamer conjugated gold nanoparticles through biotin and streptavidin interaction, after their incubation with s-protein solution from 250 ng/ul to 0 ng/uL.

3.3 Lateral flow test in different physiological buffers
The gold nanoparticle usually undergoes aggregation in the presence of physiological buffers such as phosphate buffer saline, Fetal bovine serum, saliva, etc. Therefore, the aptamer conjugated gold nanoparticles were tested for their feasibility and stability in different physiological buffers. The gold-nanoparticles and S-protein complex were prepared in phosphate buffer, fetal bovine serum (FBS), and cell culture serum at 125.0 ng/ul concentration. The spike-protein bound gold nanoparticles run against S-antibody pre-anchored on the nitrocellulose membrane. The results showed that the gold nanoparticles are stable and could successfully bind the S-antibody on the nitrocellulose membrane as evident from the red color exhibited by gold nanoparticles as seen in Fig. 5. Therefore, the developed method would be simple, fast, cost-effective, and reliable and might be beneficial for the early detection of the SARS-Cov-2 virus from patient samples.

Figure 5. Photographs of LFA strips used to test S-protein samples using DNA-aptamer conjugated gold nanoparticles in physiological buffers. A) LFA result of S-protein samples in various physiological buffers (1. 1X PBS, 2. 20% FBS, 3. Cell culture media, respectively) using gold nanoparticle conjugated to DNA aptamer through thiol chemistry. B) LFA result of S-protein samples in various physiological buffers (4. 1X PBS, 5. 20% FBS, 6. Cell culture media, respectively) using gold nanoparticle conjugated to DNA aptamer through biotin-streptavidin interaction.

4. Method to produce the surface proteins of the mutant virus
Wildtype S-proteins are used in this study instead of viral particles as a proof of concept for developing an at-home COVID-19 diagnosis kit. As long as the mutant of the newly emerged
viral surface protein is reported, the method can be further extended for mutant strains by producing corresponding proteins to the mutation via gene cloning within a few days.

5. Production of the aptamer against the S-proteins of the mutant virus
A DNA aptamer is used in the current study for the development of a COVID-19 diagnosis kit as a proof of concept. And the method would be extended to mutant S-proteins and viruses by developing corresponding DNA/RNA aptamers against respective mutant S-proteins using SELEX. The SELEX is a well-known method for the development of aptamers which is a relatively easier and cost-effective method.

Discussion
COVID-19 is highly infectious with high transmissibility, pathogenicity, and virulence. The SARS-CoV-2 is believed to be originated from bats and transmitted to humans. Many diagnosis methods have been developed for COVID-19 detection. Among them, rRT-PCR is widely used. However, the technique is challenging to use due to the need for sophisticated instrumentation and highly skilled personnel. The ELISA test is another method for Covid detection, but it is not suitable for early diagnosis as the antibody production takes several days after infection. Thus, these methods are not suitable for at-home early diagnosis. On the other hand, the low proof-reading efficacy of COVID-19 mRNA leads to more mutations. Therefore recently, many mutant covid-19 strains have been reported. The developed aptamer-based lateral flow method would be advantageous since the aptamers can recognize target S-proteins with high affinity. Moreover, the aptamers can differentiate structurally similar CoV-related proteins due to their unique tertiary structures. The developed method for detecting S-proteins using the combination of aptamer and antibody on the nitrocellulose membrane can serve as a proof of concept. And the method would be adopted to diagnose COVID and its mutant strains by cloning respective antibodies and selecting aptamers for mutated spike proteins. A gold-labeled aptamer specific to the mutant S-proteins can serve as a detection probe. Antibodies that can bind to the mutant COVID virus can be used as a probe that is immobilized to the nitrocellulose. As the aptamer conjugated gold nanoparticles are bright in color and exhibit visible red color upon binding to the antibody on nitrocellulose membrane thus, they can be visualized by naked eye without the need for any sophisticated instrumentation. The developed diagnostic method might be applicable for patient samples and reduce the burden, time, and cost of the adjusting diagnosis methods.

Conclusion
The concept of the rapid development of new diagnostic systems to meet the rapid evolution of covid 19 mutations was reported. The approach is to quickly produce a new RNA or DNA aptamer when the sequence of the new mutant has been reported and to combine it with the existing common antibody. The designed and developed COVID-19 detection method using the lateral flow method would be a simple, low-cost, and reliable point of care diagnosis method. The nucleic acid aptamers could specifically find and bind to mutant S-proteins strongly. The aptamer-gold conjugate could bind to SARS-CoV-2 S-protein even at low concentrations (~ 2.0 ng/ul) and exhibit red color upon binding to the spike-antibody present on the nitrocellulose membrane. Thus, the developed method might be applicable to detect SARS-CoV-2 viruses and would be visualized on the LFA strip without the need for any sophisticated instrumentation. The diagnostic method could be extended to the detection of the new mutant COVID strains.

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**Competing Interest Statement**

P.G. is the licensor, grantee, and consultant of Oxford Nanopore Technologies; and the co-founder of ExonanoRNA.

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Illustration of the design of a fast diagnosis device for COVID-19 infection using a combination of DNA/RNA aptamer and antibodies on a nitrocellulose membrane.

Graphical Abstract
Illustration of the design of a fast diagnosis device for COVID-19 infection using a combination of DNA/RNA aptamer and antibodies on a nitrocellulose membrane.
Figure 1
Figure 2
|                        | 1  | 2  | 3   | 4   | 5   | 6   | 7   | 8   | 9   |
|------------------------|----|----|-----|-----|-----|-----|-----|-----|-----|
| S-protein (ng/ul)      | 250| 125| 62.5| 31.3|15.6 | 7.8 | 3.9 | 2.0 | -   |
| Gold-S-aptamer (10 µl) |    |    |     |     |     |     |     | 10.0| µl  |
| 1 %BSA                 |    |    |     |     |     |     |     | 1.0 | µl  |
| 1% Triton              |    |    |     |     |     |     |     | 1.0 | µl  |
|                 | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 |
|-----------------|----|----|----|----|----|----|----|----|----|----|----|
| **S-protein (µg)** | 250| 125| 62.5| 31.3| 15.6| 7.8| 3.9| 2.0| -  | -  | -  |
| **Biotin-oligo (20 µM)** |     |    |    |    |    |    |    |    |    | 5µl|    |
| **STV-Gold**     |    |    |    |    |    |    |    |    |    |    | 15µl|
| **BSA (µg)**     | -  | -  | -  | -  | -  | -  | -  | -  | 250| -  | -  |
| **S-protein/ Gold** | -  | -  | -  | -  | -  | -  | -  | -  | -  | 250| -  |

**Figure 4**
