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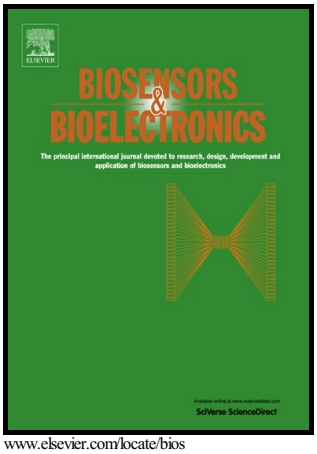

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A new lateral flow strip assay (LFSA) using a pair of aptamers for the detection of Vaspin

Nurul Hanun Ahmad Raston, Van-Thuan Nguyen, Man Bock Gu*

[1]Department of Biotechnology, College of Life Sciences and Biotechnology, Korea University, Anam-dong, Seongbuk-Gu, Seoul 136-713, Rep. of Korea

*E-mail address: mbgu@korea.ac.kr

Abstract

A new lateral flow strip assay (LFSA) using a pair of aptamers has been designed and successfully developed using a pair of aptamers-functionalized with gold nanoparticles (AuNPs). This new LFSA biosensor system utilizes a cognate aptamer duo binding to vaspin, a target protein, at the two different binding sites, and exhibited a sensitive and highly selective response to vaspin. A sandwich-type format in LFSA was developed based on biotin-labeled primary V1 aptamer immobilized on streptavidin coated membrane as a capturing probe and secondary V49 aptamer conjugated with AuNPs as a signaling probe. Using this LFSA, vaspin could be visibly observed within the detectable concentrations of vaspin up to 5 nM in both buffer and serum conditions. The sensitivity of this LFSA developed in this study was ranged from 0.137 to 25 nM in buffer and from 0.105 to 25 nM in spiked human serum, respectively. The limit of detection (LOD) of this LFSA was found to be 0.137 nM and 0.105 nM in buffer and spiked human serum condition, respectively. Therefore, this study has shown successful development of a simple yet effective LFSA for vaspin detection, and this development is not only limited to this target, but also other targets with a pair of aptamers available, without any special and laborious instrumentations. This system will be particularly useful as a screening tool for rapid on-site detection of any targets with a pair of aptamers generated.

Keywords: lateral flow strip assay (LFSA), cognate aptamer duo, Vaspin,sandwich-type

1. Introduction

Lateral flow strip assay, simply called as strip test was firstly developed in 1956, adopted from a logical extension of technology used in latex agglutination test (Plotz et al., 1956). Since then, the biggest successful achievement from strip test lateral technology in biosensor field is the development of pregnancy test strip which has been used all over the world. The lateral technology brings many benefits and become popular platform in biosensors because of its user-friendly format, low production cost, very short time to get test result and easy to use and long term stability over wide range of climates.

However, to date, the practical applications of aptamer-based lateral flow strip platform are still lacking with no successive product introduced to market. Therefore, intensive works are required to overcome the huddle of lateral flow strip aptasensor application in bioanalytical industry. There are two types of lateral flow strip aptasensors designs could be generated which are lateral flow strip aptasensor based-on target induced displacement (Liu et al., 2006; Wang et al., 2011) and sandwich-type assay (Xu et al., 2009; Bruno, 2014; Liu et al., 2009). The lateral flow strip aptasensor based-on target induced displacement is well functioned for a simple on-site small molecules sensing. However, this platform is not preferable to use and also time consuming compared to sandwich-based lateral flow strip assay.

Hence, sandwich-type platform is more convenient to use if the dual aptamers for the target of interest are available. Sandwich-type biosensors are widely applied in field for the detection of

target molecules because the platform offers many probe modification features for a specific signal development and also for enhancing signal generation.

In order to realize the sandwich-type aptasensors, two aptamer probes are needed which basically function as capturing probe and signaling probe. In this case, having dual aptamers (primary aptamer and secondary aptamer) are far better than using antibody coupled, concerning on the advantages of aptamers towards antibodies, such as the use of antibodies are expensive, laborious, temperature sensitive and the antibody affinity to target might decrease by modification.

Recently, Ahmad Raston and Gu, have successfully developed cognate aptamer duo for diabetes diseases biomarker, called Vaspin (Ahmad Raston et al., 2015). This finding has given big impact in aptasensing field, especially for the chances to develop more sensitive and efficient diagnosis point-of-care-test (POCT) devices for on-site Vaspin detection. Vaspin belongs to adipokines group, acts as insulin resistance biomarker protein involved in diabetes-related diseases (Bluher, 2012; Jian et al., 2014; Yan et al., 2014). Therefore, in this report, Vaspin cognate aptamer duo produced were used to further develop lateral flow strip assay-based Vaspin aptasensor.

On the other hand, gold nanoparticles (AuNPs) are known to be the most favorite nanomaterials for the aptasensor development due to their physical and chemical properties. Moreover, the aptamer conjugation process on AuNPs by chemisorption or physical adsorption has been previously well demonstrated (Huang et al., 2005; Li et al., 2004a; Li et al., 2004b; Mirkin et al., 1996; Pavlov et al., 2004). These AuNPs properties offer a simple yet sensitive aptasensors

platform generation. Thus, AuNPs were chosen as the signal producing material, to link with secondary aptamer which later acted as signaling probe in this study.

2. Materials and Methods.

2.1 Materials and reagents

Vaspin and adiponectin were provided by AdipoGen, Inc. (Inchoen, Korea). Bovine serum albumin (BSA), human serum albumin (HSA), and chemical reagents used for binding buffer (BB) were purchased from Sigma Aldrich (USA). The aptamer sequences used in this study are as follow:

Primary aptamer V1: 5'-Biotin- CGTACGGAATTCGCTAGCTGATGGTGTGGCG GGGGCGGCCTGGGGCGGGCCGCCGATGGGATCCGAGCTCCACGTG-3'

Secondary aptamer V49: 5'- Thiolated –CGTACGGAATTCGCTAGCGGTGGC TCTAGGGCCTATCGTTGCGCCGACGGATCCGAGCTCCACGTG-3'

The complementary sequence of V49 (cV49): 5'- Biotin - CAC GTG GAG CTC GGA TCC GTC GGC GCA ACG ATA GGC CCT AGA GCC ACC GCT AGC GAA TTC CGT ACG -3'

All of these aptamers, V1, V49, and cV49 were synthesized and purchased from Genotech, Korea.

2.2 Synthesis of gold-nanoparticles (AuNPs)

The AuNPs were synthesized by using citrate reduction of HAuCl4 protocol as reported previously (Chen et al., 2008). The size and monodispersed production of the AuNPs were confirmed by transmission electron microscopy (Tecnai 20) and UV/Vis spectrophotometry (Ultraspec 6300 pro, Amersham Biosciences, Piscataway, NJ, USA). The synthesized AuNPs were about 13 nm in diameter, spherically shaped, with a deep red color. The concentration of these unmodified AuNPs was estimated by the Beer- Lambert law using an extinction coefficient of $2.43\times108\ M^{-1}\ cm^{-1}$ at 520 nm.

2.3 Preparation of AuNPs-reporter aptamer (V49) conjugates as a signaling probe

The secondary aptamer V49 conjugated with AuNPs was prepared by following our previous protocols (Ahmad Raston et al., 2015; Nguyen et al., 2016). Briefly, ten µl of 5'-thiolated secondary aptamer V49 (50 µM) was stabilized with 10 mM of Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) (2 µl), and incubated 1 hour at room temperature. One ml of prepared AuNPs and 10 µl of thiolated secondary aptamer V49. The mixture was incubated for 16 hours, and subsequently it was aged by NaCl in Tris acetate buffer. The buffer containing 500 mM Tris acetate at pH 8.2 and 1 M of NaCl were added in a drop-wise with gentle hand shaking, and the solution was further incubated for 24 hours. Unconjugated thiolated aptamers were removed by centrifugation at 16,000 rcf for 25 min, and the pellet was washed with 5 mM Tris-HCl for three times. After blocking with 6-mecarpto-1-hexanol, the conjugates were washed with 5 mM Tris-HCl (2 times) and stored in 4ºC for further study.

2.4. Lateral-flow strip design, assembly and preparation for Vaspin detection

The LFSA was designed as shown in scheme Fig.1A. Briefly, the strip contained three overlapping pads placed on a backing card: sample pad (CFSP203000, 20 × 300 mm), HiFlow

Plus NC membrane (HF090MC100, 60 × 300mm), and absorption pad (CFSP203000, 20 × 300 mm). The length of overlapping are 2 mm and 0.5μM biotin-V1 aptamer and biotin-cV49 aptamer (20 μl) were incubated with 100ug/ml streptavidin (30 μl) for 1 hour at 4^{o}C. Then after V1 aptamer-conjugated streptavidins were lined with dispenser (BioDot, ZX101, Irvine, CA) on Nitrocellulose (NC) membrane to form the Test line (V1 aptamer) and control line (cV49), respectively. After that, the DNA aptamers lined on nitrocellulose was dried at 60^{o}C for 30 min for immobilization. Then, PBS buffer containing 50 μg/ml of BSA was flow through the membrane for blocking. Then, the membrane was dried again and the strips were assembled. Finally, the LFSA was cut into 0.4 cm wide strips, and stored in 4^{o}C overnight until use.

2.5. Lateral-flow strip assay of Vaspin

Specificity Test

It is well-known that the flow speed, non-specific binding, and the disturbance in the particle flow were affected by the humidity and temperature. Therefore, to avoid the temperature and moisture effects, this LFSA strips fabricated and stored at 4 ^{o}C were incubated at 30^{o}C as a preconditioning step for 30-min in this study. Then, 0.1 μM Vaspin was mixed with 15 nM of V49-AuNPs for 15 min on slow rotator, as pre-incubation step to improve the binding of target with secondary aptamer (V49) conjugated with AuNPs, and then the mixture was introduced to the strip (total volume 50 μl). After that, 100 μl of washing buffer was flown until clear membrane was observed at non-lined area. Above steps was repeated for other counter targets (Adiponectin and human serum albumin) for specificity test. Buffer was used as control. All the results were further analyzed using imageJ to measure the intensities of Test line and Control line.

Dose dependent test

Similar to specific assay, the prepared strip was incubated at 30^{o}C as precondition for 30 mins. Different Vaspin concentration (1, 5, 10, 25, 50 and 100 nM) was diluted in both buffer and serum. Human serum was diluted up to 10-fold to eliminate the AuNPs aggregation affected by the protein complex in human serum. Then, each sample was mixed with 15 nM of V49-AuNPs for 15 min on slow rotator, and introduced to the strip (total volume 50 µl). Then, 100 µl of washing buffer was flown until clear membrane was observed at non-lined area. The binding buffer was used as negative control. All the results were further analyzed using imageJ to measure the intensities of Test line and Control line.

3. Results and discussion

Test strips were prepared as depicted in **Figure 1A**. Biotin labeled V1 aptamer (as capturing aptamer) was bound to streptavidin which initially aligned on membrane. This line was known to be the test zone. Meanwhile, biotin labeled complementary sequence of V49 aptamer was deposited on the other line of streptavidin on the strip membrane, which was known as control zone.

Upon addition of AuNPs labeled V49 aptamer (as signaling probe) bound to target Vaspin, the primary aptamer on the test zone will capture the other site of Vaspin. Thus, AuNPs color could be observed on the test zone. For the control experiment, complementary V49 aptamer on the control zone will capture the remaining AuNPs labeled V49 aptamer, thus the signal could always be observed as the control.

In order to develop point-of-care test (POCT) lateral flow strip assay for Vaspin detection, the constructed test strip was tested to verify the successive Vaspin targeting, specifically. In this case, 0.1μM of Vaspin and other counter targets proteins (adiponectin and human serum albumin) were incubated with the signaling probe, and then 50 μl of sample solutions were introduced to the sample pad. The results were read out and recorded after 5 min sample loading.

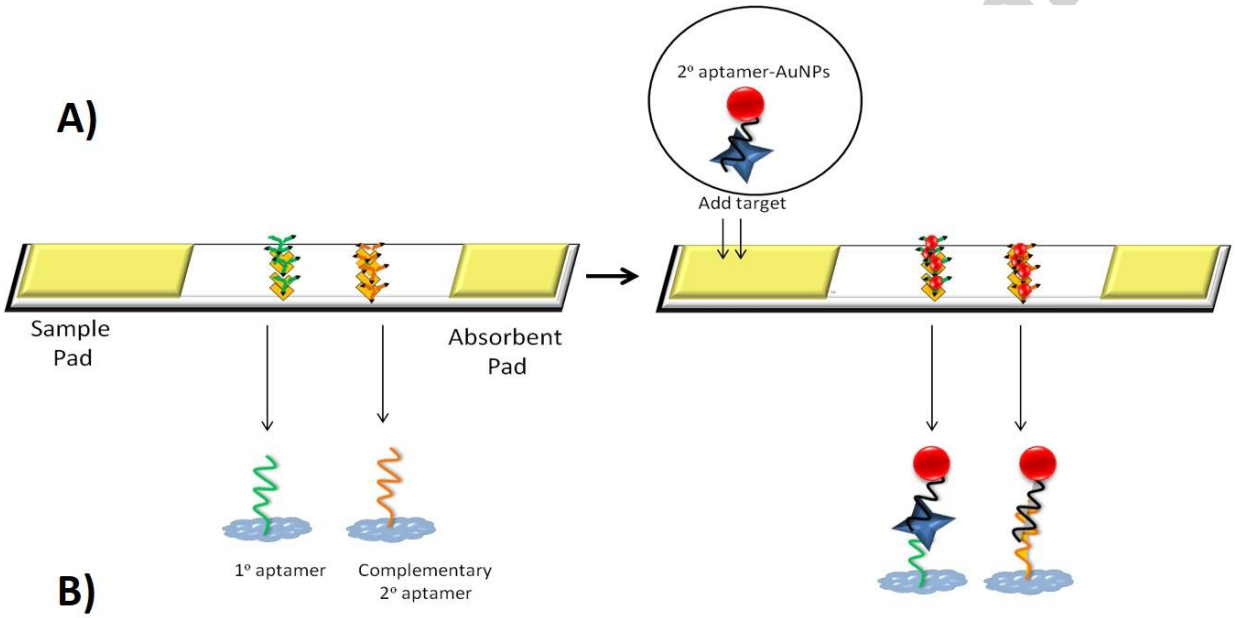
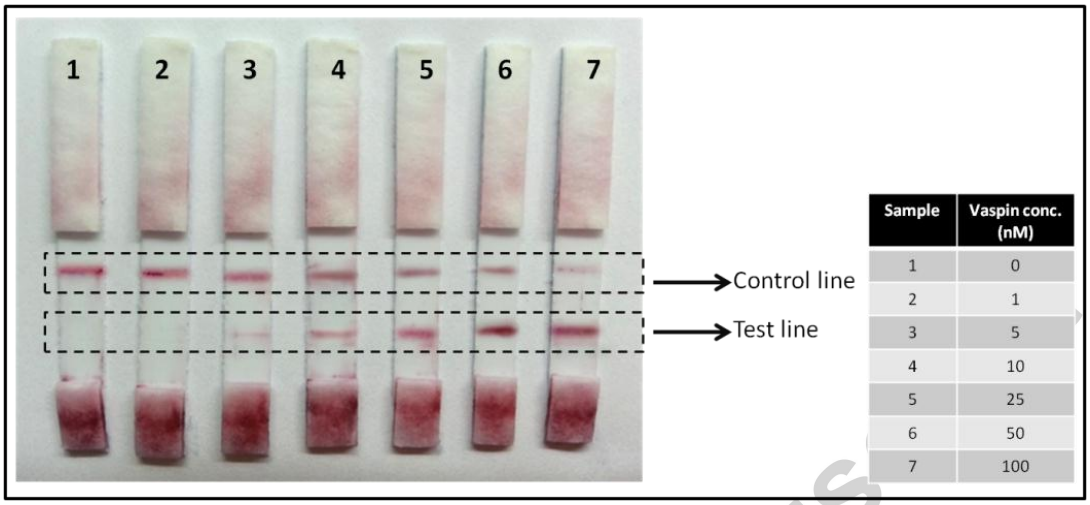


Figure 1. LFSA based on DNA-functionalized AuNPs for on-site detection of Vaspin. A) Schematic diagram of Vaspin detection using lateral-flow strip assay. B) Specific detection of Vaspin using cognate aptamer duo-based lateral flow strip assay. The signal observed was very specific to target Vaspin, while no signal could be seen at test zone of counter targets' strips.

The samples flew through the membrane, laterally, towards absorbent pad. As shown in the Figure 1B, signal could only be observed with the presence of Vaspin, while no signal could be observed on the test zone of adiponectin, HSA and buffer. This result showed that the lateral flow strip assay developed for Vaspin detection was successfully recognized only Vaspin using Vaspin cognate aptamer duo. The amount of V49 aptamers conjugated with AuNPs was calculated to be higher than the highest concentration of Vaspin (100nM) in this study, which is much higher than the normal concentration in human blood samples. Therefore, the detection signal of the Test Line is always guaranteed to be accurately measured.

Interestingly, in this pioneer trial of Vaspin detection using lateral flow strip assay, the control zone was not fully maintained. In one situation, when higher detection of Vaspin presents in test zone, the control zone signal becomes faint. This phenomenon can be attributed by the lack of signal probe. The complementary sequence of the aptamer V49 conjugated with AuNPs was immobilized as a capture probe on Control Line. Therefore, the amount of V49 aptamer-AuNPs should be shared with both Control and Test lines. As shown, the intensities of the control line were decreased with the increasing concentrations of Vaspin, indicating that the amount of aptamer-AuNPs is insufficient for both Test and Control lines at high concentration of Vaspin. To avoid this problem in the future, the amount of aptamer-AuNPs must be higher enough for both the maximum amount aptamer-AuNPs which is needed from Test line (along with the highest concentration of Vaspin) and also control line.

The feasibility of this Vaspin aptasensor-based LFSA was further tested dose dependently. Various concentrations of Vaspin were diluted in binding buffer, and mixed with



Sample	Vaspin conc. (nM)
1	0
2	1
3	5
4	10
5	25
6	50
7	100

Figure 2. Detection of Vaspin by using cognate aptamer duo-based LFSA in buffer condition. Various concentrations of Vaspin were diluted in binding buffer, and mixed with signaling probe to final concentrations of 1, 5, 10, 25, 50 and 100 nM.

signaling probe to final concentrations of 1, 5, 10, 25, 50 and 100 nM. The 50µl of each of the samples was introduced to the prepared test strip, and the results were recorded. As shown in Figure 2, Vaspin was detectable as low as 5 nM, easily seen by naked eye, using the cognate aptamer duo-based LFSA in buffer condition. The result was further analyzed using imageJ software (**Figure S2**) to measure the sensitivity of this LFSA. The results showed that the linear range of this LFSA developed in this study were found to be from 0.137 to 25 nM in buffer condition.

Further experiment was done to investigate the Vaspin aptasensor-based LFSA performance in serum condition. In this case, the same range of Vaspin concentrations was used for the detection of Vaspin spiked in human serum samples as in binding buffer condition.

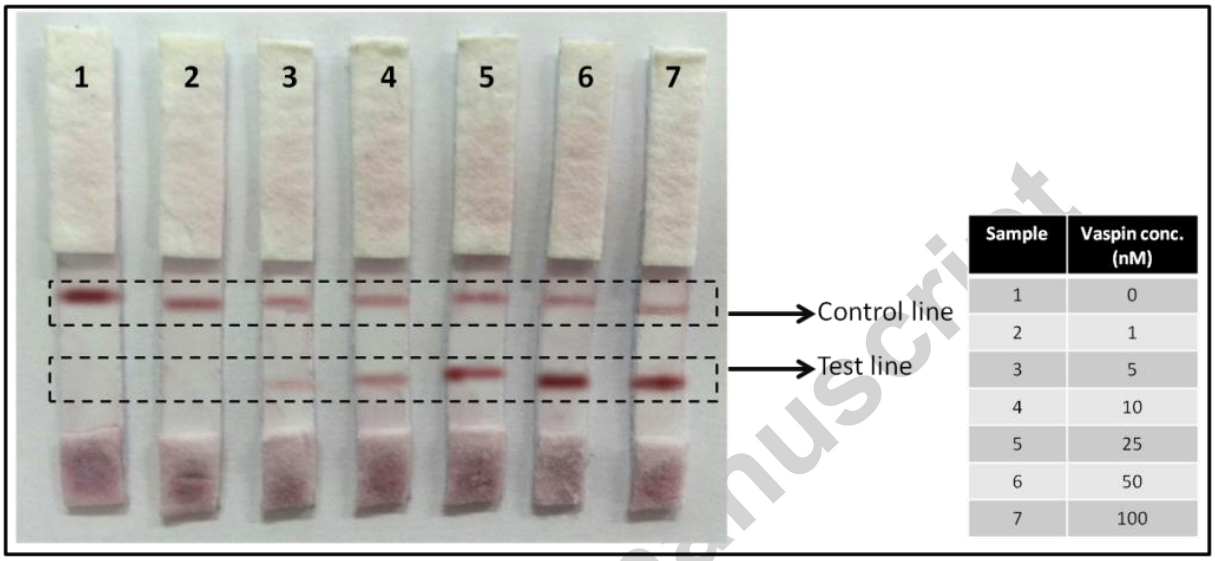


Figure 3. Detection of Vaspin spiked in human serum samples by using cognate aptamer duo based LFSA**.** Various concentrations of Vaspin were diluted in serum samples, and mixed with signaling probe to final concentrations of 1, 5, 10, 25, 50 and 100 nM.

As shown in **Figure 3**, the result shows that the detectable concentration of Vaspin spiked in human serum was found to be 5 nM of Vaspin, which is similar with the buffer condition. Additionally, the dynamic detection range of Vaspin spiked in human serum was found to be from 0.105 to 25 nM (Figure S2, B&D), which is in the range of concentrations of vaspin in real normal human blood samples [0.156 nM; 0.1-7ng/ml]. So, it can be detected with this assay method if the vaspin concentration is increased due to an occurrence or symptoms of a disease in the blood.

The sensitivity of LFSA in binding buffer and spiked human serum was compared to evaluate the effect of non-specific binding of proteins in human serum. As shown in Figure S2, the sensitivities of LFSA developed in this study for the detection of Vaspin were similar both from 0.137 to 25 nM in buffer and from 0.105 to 25 nM in spiked human serum, respectively. Moreover, the limit of detections (LOD) of this LFSA were estimated to be 0.137 nM and 0.105 nM in buffer and spiked human serum condition, respectively, by using a formula, negative sample (buffer) + 3 standard deviations (SDs). This result supports that the performance of this LFSA using vaspin cognate aptamer duo was not disturbed by the presence of complex matrices in human serum. Therefore, the cognate aptamer duo-based LFSA developed in this study should be very useful in rapid analysis of vaspin in real blood samples, and could be used in clinical field.

4. Conclusion

A simple and easy to perform lateral flow strip assay (LFSA) was successfully developed by using a sandwich-type cognate aptamer duo conjugated with gold nanoparticle for the sensitive Vaspin detection. The detectable concentration of Vaspin using this LFSA was found to be 5 nM, in both buffer and serum condition, easily observed by naked eye. Further, the dynamic range of this LFSA were found to be from 0.137 or 0.105 to 25 nM in both buffer and spiked serum, respectively, indicating that the the sensitivity of this LFSA is not much affected. Moreover, the limit of detections (LODs) of this LFSA were found to be 0.137 nM and 0.105 nM in buffer and spiked human serum condition, respectively. Therefore, this cognate aptamer duo-based lateral flow strip assay for vaspin detection developed in this study has a great potential

not only to be a reliable on-site vaspin detection system but also to be an universal platform using a pair of aptamers available.

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Highlights

- A highly sensitive later flow stripe assay (LFSA) using a cognate aptamer-duo, V1 and V49, binding to vaspin at two different sites was successfully developed.
- This dual aptamer-based LFSA system was successfully implemented in serum samples.
- The limit of detections was determined to be 0.137 nM in buffer and 0.105 nM in serum samples, respectively.