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The optimization of a lateral flow immunoassay for detection of aflatoxin B_1 in potable water samples

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***Abstract*— Aflatoxin B_1 (AFB_1) is a highly toxic and carcinogenic metabolite produced by fungi of genus Aspergillus. While AFB_1 contamination in food and crops is well known, little attention has been paid to potable water which is another potential source of AFB_1 contamination that can be ingested by humans. In order to develop a robust, rapidly manufactured sensing device that can monitor the AFB_1 contamination in water, a lateral flow immunostrip based on gold nanoparticles (AuNPs) has been optimized for determination of aflatoxin B_1 in potable water samples. A visual detection limit (LOD) of 0.5ppb was achieved for determination of AFB_1 for the majority of water samples tested. The strip remained functional after being stored at 4°C for 3 months.**

***Index Terms*—Aflatoxin B_1, biosensor, lateral flow immunoassay, potable water**

I. Introduction

Aflatoxins, which are produced by many species of Aspergillus, are the most predominant mycotoxins in nature [1]. Aflatoxins are extremely toxic, mutagenic, teratogenic and carcinogenic compounds that have been implicated as 'causative agents in human hepatic and extrahepatic carcinogenesis' [2, 3]. Aflatoxin B_1 is the most commonly occurring toxin in this group [2]. Food contamination caused by AFB_1 usually occurs in cereals, nuts, beans, spices, dairy products, dry fruits as well as edible oil [4]. The major reason of AFB_1 contamination is due to poor storage conditions such as long exposure time to high relative humidity [5]. As aflatoxin B_1 is considered to be one of the most dangerous mycotoxins, it is listed as a group I carcinogen by the International Agency for Research in Cancer [6]. The EU has set maximum permitted levels for aflatoxin B_1 in nuts, dried fruits, cereals and spices to range from 2-12 μg/kg, while the maximum permitted level for aflatoxin B_1 in infant foods is set at 0.1 μg/kg [7]. Methods developed have mainly focused on detection in the context of food [8, 9].

Aspergillus flavus and *Aspergillus parasiticus* (two major producers of AFB_1) are common forms of 'weedy' molds widespread in nature. Besides soil, they can invade almost all types of organic substrates whenever the conditions are favourable for its growth [10]. This means AFB_1 can be very easily produced in nature and disperses into the water system through aqueous carriers such as rainfall. AFB_1 contamination can also happen during water storage and transportation. This indicates that drinking water has a real potential risk of AFB_1 contamination. Even with very low concentration at ppb levels, the long-term consumption of AFB_1 contaminated water can still become a significant threat to human health [11, 12]. Hence it is necessary to develop a quick and reliable methodology for determination of the AFB_1 concentration in potable water that is easy to commercialize. However, at present there is no relevant research on this field.

Many methodologies have been developed for detection of AFB_1 during the last three decades. High performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) are two reference methods for AFB_1 determination. They show high sensitivity and good ability for quantification [13, 14]. However, they also suffer from many drawbacks such as long assay time, complex sample preparation procedure, high operation and maintenance cost as well as the need for experienced people to carry out the measurement, so they are not suitable for field applications. In addition, some electrochemical sensors with good sensitivity and accuracy to AFB_1 determination have been developed during recent years [15-17]. However, compared to HPLC and ELISA, there is still insufficient evidence to verify these sensors' reliability. Mass production of such sensors reproducibly can be another difficult problem for their commercial application.

Lateral flow immunoassay (LFIA), also called immunochromatographic test strip (ICTS) is one of the most

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popular point-of-care formats and has wide application in food analysis, environmental monitoring as well as clinical diagnosis [18]. Compared to HPLC and ELISA, the lateral flow test offers an inexpensive, relatively rapid assay format. It can be used by people without training, which makes it popular in areas where no sophisticated laboratory equipment is available [19].

Lateral flow immunoassays have been applied in the determination of aflatoxin B_1 in various foods and crops. Some published papers have achieved very low detection limits [3, 18, 20]. There are also some commercial products for mycotoxin detection such as Afla-V AQUA® strips from VICAMTM, which are able to detect 2ppb for total aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) in crops.

However, these devices may have some limitations when translated to measurement of AFB_1 in aqueous environment and later commercialization. First of all, water can have various matrix effects. Microorganisms, metal ions and pH values can all significantly affect sensing performance. This means water samples must go through pretreatment before being measured by LFIA. Secondly, some papers quote some anti-AFB_1 antibodies with high affinity developed in the laboratory, but these antibodies were not subsequently available for general use. To achieve reliable and robust sensors, using commercially available antibodies manufactured to high standards will be very helpful for later commercialization of a sensor. In order to obtain a high sensitivity, conventional AuNP-antibody conjugation methodology usually takes several hours and needs strict control of various parameters during the preparation of gold nanoparticles and AuNP-antibody conjugate such as buffer composition, the amount of antibody and AuNP used during conjugation and purification process. For an industrial manufacturing process, these steps can increase the risk of losing the activity of immunoreagents as well as contamination. As a result, it will be very useful to simplify current procedures.

Based on the specific advantage of LFIA, here we report the development and optimization of a lateral flow immunostrip for determination of AFB_1 in potable water. Compared with the strips reported in the literature, the preparation process is much easier and the total strip can be prepared without automation within one hour. The size of nitrocellulose (NC) membrane applied (25×5mm) is smaller than many published LFA in this area, which can save over 30% of relevant fabrication materials [3, 20]. All the raw materials used are commercially available, which makes it very suitable for later reproduction and improvement. In addition, it shows significant improvement in sensitivity compared to current commercial products on the market. Based on a competitive mechanism, the strip shows visual detection limit of 0.5ppb for the majority of water samples tested and the storage lifetime is good as well.

II. Materials and Methods

A. Reagents and equipment

Aflatoxin B_1, aflatoxin B_1 standard solution (20μg/ml in methanol), bovine serum albumin (BSA), aflatoxin B_1-BSA conjugate, Tween 20, mouse secondary antibodies (anti-IgG), sodium azide, methanol, glycerol, Ethylenediaminetetraacetic acid (EDTA) and sucrose were bought from Sigma-Aldrich. Anti-aflatoxin B_1 antibodies (ab1017, 0.5mg/mL) were from Abcam, Cambridge. InnovaCoat® GOLD-20OD 40nm conjugation kits (230-0010) were from Innova Biosciences. G041 glass fibre conjugate pads (20cm×30cm) and AP1002500 Cellulosic Absorbent Pads (filter Dia. 25 mm) were from Merck Millipore. FF170HP nitrocellulose membranes were obtained from GE Healthcare. CSB-E14087 Aflatoxin B_1 ELISA kit was purchased from CUSABIO® Technology, UK. Phosphate-buffer saline (pH 7.4) was used in the experiments. All the water used in the experiments was deionized.

Equipment used for sample preparation and analysis were: Hettich Zentrifugen® Universal 320R centrifuge, PerkinElmer® Lambda 35 UV/VIS spectrometer, IKA-VIBRAX-VXR S17 Electronic Shaker, PlasmaQuant® PQ 9000 (Elite) high-Resolution Array ICP-OES system and Labsystems® Multiskan Ascent 354 ELISA reader.

B. Preparation of AuNP-antibody conjugate

AuNP-antibody conjugate serves as the biorecognition element in the experiment. The red colour of gold indicates the position of the aggregated antibody on the strip. The preparation of AuNP-antibody conjugate was done according to the kit instructions. Briefly, a certain amount of primary antibody stock solution (the amount of antibody added will be discussed later) was diluted by the antibody diluent to 12μl in a small Eppendorf tube. After dilution, 42μl of reaction buffer was added into the tube and the solution was mixed gently. Then 45μl of the liquid was transferred to the small vial containing 40nm gold nanoparticles. After mixing and 15-minute incubation, 5μl blocking buffer was added and the solution was incubated for another 5 minutes. The conjugate was stored at 4°C for use.

C. Preparation of strip components

The lateral flow strip consists of five parts: nitrocellulose membrane, sample pad, conjugate pad, absorbent pad and PVC backing plate. The coating buffers for the sample pad and conjugate pad were based on the literature with some modification [18, 21, 22]. The sample pad was treated with 0.01M PBS containing 2% BSA, 0.01% sodium azide, 2.5% glycerol and 0.1% Tween 20. The coating solution for conjugate pad was prepared by mixing a certain volume of antibody working solution (20μg/mL) with 0.01M PBS containing 1% BSA, 2.5% sucrose, and 0.02% sodium azide. The volume of antibody working solution dispensed on each conjugate pad was an experimental variable discussed later.

The sample pad (with original size 50mm×20mm) was immersed in coating buffer for around 20 seconds. Then the pad was dried at 37°C for 20 minutes and stored under room temperature for use. In order to ensure the volume of antibody applied on each conjugate pad is the same, the conjugate pad was first cut into small pieces (10mm×5mm) and each pad was

loaded with 25µl coating solution and dried at 37°C for 20 minutes. Then the pads were stored at 4°C. The sample pads were cut into smaller pieces (20mm×5mm) before use.

Nitrocellulose membranes were used without any pretreatment. The test line was dispensed with AFB_1-BSA conjugate and the control line was coated with secondary antibody (0.5mg/mL). Membranes of two different sizes were used in the experiment. One is 40mm×5mm and the other is 25mm×5mm. The positions of test and control lines of these two membranes are shown in Table 1. 1µl coating buffer was applied for each test zone on the membrane. All the membranes were then dried at 37°C for 15 minutes and stored in 4 °C before use.

TABLE 1

POSITION OF THE TEST LINE AND CONTROL LINE OF MEMBRANES WITH DIFFERENT LENGTHS

Membrane length	Test line	Control line
40mm	30mm from the beginning of the membrane	35mm from the beginning of the membrane
25mm	15mm from the beginning of the membrane	20mm from the beginning of the membrane

D. Assembly of the strip

For the 40mm strip, first of all, the NC membrane card was immobilized on the PVC plate with double sided tape. Then the conjugate pad and untreated absorbent pad were pasted on the plate according to the position of the test zones with 2mm overlap with the membrane. Finally, the sample pad was pasted with 2mm overlap of conjugate pad.

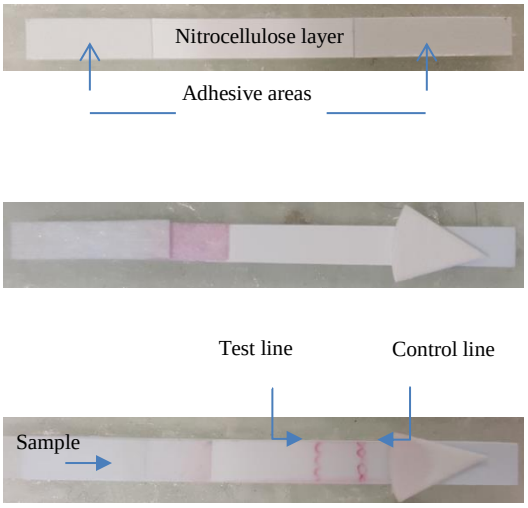


Fig. 1. Blank nitrocellulose membrane (top), assembled lateral flow strip (middle) and strip after the assay (bottom). The positions of the test line and the control remain the same for all the results exhibited later in the paper

The original FF170HP nitrocellulose membrane sheet (300×25mm) was cut into strips (25×5mm). Each strip contains two adhesive areas with nitrocellulose layers in the middle (shown in Fig.1). After removing the protective sheet, the adhesive areas can be used for attaching other sensor components. The laminated sample pad and conjugate pad were pasted together with 2mm overlap with each other. Then the absorbent pad was immobilized with 2mm overlap with NC membrane. The strips were stored in covered petri dishes at 4°C.

E. Determination of metal ion concentrations of water samples with ICP-OES system

To determine whether metal ions could be potential interferents for the assay, Inductive Conductive Plasma Optical Emission Spectroscopy (ICP-OES) was often used to determine trace concentrations of potential interfering metal ions present in water samples [23].

Standard solution (containing Na, Mg, Ca, K, Fe, 500mg/L each in 5% nitric acid) was diluted to different concentrations: 200ppm, 150ppm, 100ppm, 50ppm, 10ppm in 2% nitric acid. Calibration curves were constructed for each of the five metal ions in the standard. Several emission wavelengths were tested for each element and the best one was chosen in the end. Calibration curves were obtained by analysing six concentrations (including the blank control) by ICP-OES systems and then the samples were tested.

Five water samples collected from raw water sources as well as commercial sources were tested including British tap water (Table 2).

TABLE 2

WATER SAMPLE INFORMATION

Sample	Descriptions
1	British tap water
2	Borehole water
3	Lucozade® Sport Fitwater
4	Water from Shepley Spring, Yorkshire
5	Water from Severn River, Trimpley

F. Pretreatment and measurement of water samples by LFIA

40mL of water sample was centrifuged at 4000rpm for 10 minutes and then it was filtered with 1µm filter paper. After filtration, two different strategies were applied. The first was based on chemical precipitation: 0.5mL 1M sodium carbonate was added into the water. After 20-minute incubation, the water was filtered again. The second method took advantage of chelation reaction: 980µl of water samples were mixed with 20µl EDTA (5%) the sample was incubated for 20min.

10µl Aflatoxin B_1 standard solution was first diluted to 0.1µg/mL with 0.01M PBS buffer (pH 7.4). The diluted solution was used as a general stock for later experiments.

Water samples with different AFB_1 concentration levels (0, 0.5, 1.0, 1.5, 2.0ppb) were prepared by spiking a corresponding volume of AFB_1 stock solution into the water. The volume of sample solution applied on each strip is 100µl. After the sample was loaded onto the sample pad, it flows along the membrane driven by capillary force. The results can be read after 15 minutes. There will be one or two red lines forming on nitrocellulose membrane depending on the concentration of AFB_1 in the sample. The detection limit is the lowest AFB_1 concentration that made the colour intensity of the test line significantly weaker than the blank control.

G. Measurement of AFB_1 concentration in water samples with commercial ELISA kit

The extraction methodology used in water sample pretreatment was developed by Song et al. with a small modification, by use of acetic acid instead of formic acid [24].

The oil sample pretreatment and ELISA test were done according to the instructions provided by the ELISA kit manufacturer (CUSABIO® Aflatoxin B_1 ELISA kit, Catalog number CSB-E14087):

For the ELISA experiment, firstly, 50µl of standard or sample solution was added into each well then 50µl of HRP conjugate and 50µl of antibody were added. After gentle mixing, the plate was covered with foil and incubated for 15min at 25°C. After incubation, the plate was washed four times by washing buffer. Then 100µl of TMB substrate was added to each well and the plate was incubated for another 5 minutes. Finally, 50µl of stop solution was added and mixed thoroughly. The optical density of each well was read within 5 min with the microtiter reader set to 450nm. Each sample was measured in duplicate.

H. Evaluation of the strip stability

After the first day of measurement, the unused strips were stored for stability test. Sample pad, conjugate pad and NC membrane were stored separately. Here three storage conditions are tested: (1) 37°C in an incubator (2) 17°C in a lab with air conditioning (3) 4°C in a common refrigerator. All the storage containers were covered with foil to prevent light exposure. For stability measurements, four sets of strip components were taken out and incubated for 20 minutes under room temperature. Then they were assembled into strips. During each experiment, tap water samples with 0 and 0.5ppb AFB_1 were tested.

III. Result and Discussion

A. Principles of competitive lateral flow immunoassay

The competitive assay format is suitable for AFB_1 because of its low molecular weight (312.28 g/mol) [20]. In the competitive mechanism, the signal intensity on the test line is inversely correlated to AFB_1 concentration in sample solution. The explanation is as follows: after loading the sample, AFB_1 in sample solution will react with antibody released from conjugate pad during liquid migration. After the antibodies bind to AFB_1 from sample solution, it cannot be captured by AFB_1-BSA conjugate on the test line any more. As a result, the increasing concentration of AFB_1 in the sample solution leads to reduction of the amount of antibody captured in the test line as well as the resulting signal intensity.

B. Determination of the target detection limit

Although there are several standards set by authorized organizations for the limit of aflatoxin B_1 concentration, they are mainly for food and crops. Up to now, no standard has been set for AFB_1 concentration in water. According to current standards, 2ng/g (set by EU) is the lowest concentration limit of AFB_1 at present. Similarly, this can be applied to water. As the mass of 1ml water is quite close to 1g, the target set in the experiment can be 2ng/ml of water. However, as a semi-quantitative assay, this LOD may be insufficient to ensure the safety of the water because there could be some fluctuation of sensing performance during the actual measurement. As a result, the final target LOD is set to be 1ng/mL.

C. Characterization of nanogold probes

The Visible-spectrum of AuNP, unblocked AuNP-antibody conjugates and blocked AuNP-antibody conjugates was shown in Fig. 2. The surface resonance of AuNP exhibited a peak at 529nm. After the conjugation with antibody, the peak shifted to 532nm. When the conjugates were blocked with quencher buffer, the peak shifted again to 530nm. A significant reduction of absorbency was observed after the addition of antibody and blocking reagents, indicating efficient conjugation and blocking processes during the probes preparation.

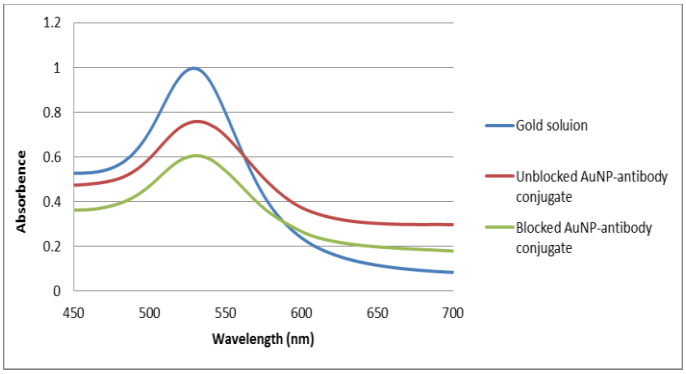


Fig. 2. The Visible-spectrum of AuNP, unblocked AuNP-antibody conjugates and blocked AuNP-antibody conjugates

D. Preliminary verification of using tap water as the standard matrix

British tap water was selected as the standard matrix because of two reasons: Firstly, compared to water from other natural sources, it does not contain high amount of bacteria or other microorganisms [25], which will not cause much interference with the immunoreaction. Secondly, tap water is one of the most important sources of drinking water. As there is no reference method which is suitable for the analysis of AFB_1 in the water, some general verification was done through comparison. Firstly, 0.01M PBS prepared from pure water was

spiked with 1ppb AFB_1. Then the blank control and 1ppb AFB_1 sample were tested with the strip. The signal was compared to those obtained from tap water (Table 3).

TABLE 3

COMPARISON OF SIGNAL BETWEEN BRITISH TAP WATER AND PBS BUFFER

Sample matrix	0.01M PBS (pH 7.4)	British tap water
Blank control		
1ppb AFB_1		

In Table 3, two replicate lateral flow strips are shown, the control strips shown two red strips while one red stripe is found with samples with AFB_1. These two samples show similar signals with 1ppb AFB_1 concentration. As a result, it is reasonable to use British tap water as a standard matrix.

E. Optimization of immunoreagents

One major factor that influences the performance of lateral flow immunoassay is the efficiency of immunoreaction. Effective immunoreaction will help to produce enough immunocomplexes even with very low AFB_1 concentrations and hence improve the sensitivity. For immunoreagents, three major parameters affect the efficiency: the amount of antibody during the conjugation process, the volume of antibody working solution applied on conjugate pad and the concentration of AFB_1-BSA conjugate on the test line. Based on the 40mm×5mm strip, orthogonal tests were carried out to obtain the optimum combination of these three parameters. Four different amounts of antibody 0.7, 0.8, 0.9 and 1.0 μg were tested for conjugation. Table 4 shows some results of the optimization obtained during this step. For each group, the strip was tested with three concentrations of AFB_1 (Blank control, 0.5ppb and 1ppb) in duplicate.

TABLE 4

EXAMPLE RESULTS OF IMMUNOREAGENTS OPTIMIZATION

0.9μg primary antibody during conjugation[a]			
Group number	antibody solution volume/μl	Concentration of AFB_1-BSA conjugate (mg/mL)	Final visual LOD
1	1.0	0.5	0.5ppb
2	1.0	0.4	0.5ppb
3	1.0	0.3	0.5ppb
4	0.875[b]	0.5	0.5ppb
5	0.875	0.4	0.5ppb
6	0.875	0.3	~[c]
7	0.75	0.5	1.0ppb
8	0.75	0.4	~
9	0.75	0.3	~
1.0μg primary antibody during conjugation			
10	1.0	0.5	1.0ppb
0.8μg primary antibody during conjugation			
11	1.0	0.5	1.0ppb

[a] According to the kit instruction, PBS used for antibody storage is not suitable for conjugation process. However, after the PBS underwent a dilution (30times) during the preparation of the reaction mix, no significant negative effect was observed in sensing performance during optimization and sample measurement

[b] Each time 400μl of coating buffer for 16 conjugate pads were prepared. The value 0.875 was achieved by applying 14μl antibody working solution during buffer preparation.

[c] The signal of blank control is too weak so the assay is considered to be invalid.

In a conventional lateral flow assay, though the overall sensing performance is usually considered as the result of integrated interaction among these three factors discussed above, during the experiment, the amount of antibody during conjugation was found to have the most patent influence on both signal intensity and sensitivity while other two parameters mainly affect the signal intensity but their impact on sensitivity is not very obvious. Therefore, it is reasonable to focus on the amount of antibody conjugation for future optimization procedures. According to the overall assay sensitivity and signal intensity, the optimum parameters were selected as: 0.9μg primary antibody during conjugation, 1.0μl antibody working solution on each conjugate pad and 0.5mg/mL of AFB_1-BSA conjugate on the test line.

F. Optimization of other strip parameters

In order to improve the efficiency of device preparation and save materials, NC membrane with 25mm length was tested.

It is reasonable to apply the previous optimization results to the new strip. However, the major challenge is the maintenance of sensitivity. When the new NC membrane was applied direct to AFB_1 spiked samples, the result was not satisfactory because of insufficient reaction time.

To deal with the problem, the flow rate should be slowed down. Glycerol can increase the viscosity of the solution and make liquid move slower. So in this case, we applied glycerol on the sample pad. One advantages of glycerol is it will not

form any crystals on the surface of sample pad. In addition, no interference with the immunoreaction was observed during the experiment. Different concentrations of glycerol were tried and finally 2.5% (v/v) glycerol was applied in the coating buffer.

G. Evaluation of the effect of major metal ions on LFIA performance

As two major metal ions in potable water, the effect of calcium and magnesium on liquid flow and immunoreaction was studied (Tables 5 and 6):

TABLE 5

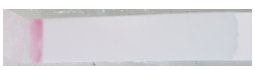
RESULTS OF SOLUTIONS CONTAINING MAGNESIUM APPLIED ON THE LATERAL FLOW STRIP

Solution tested	Signal (blank control)	
	15min	20min
0.01M PBS (pH 7.4)		-
0.0225g/mL $MgSO_4$		
1M $MgCl_2$		

From Table 5, the signal on test zones could still be observed even if with a very high concentration level of magnesium (1M). This indicates Mg^{2+} does not have patent inhibition effect on immunoreactions. However, high concentration of magnesium can slow down the flow rate, which weakens the signal when the sample measurement time keeps constant. Since the concentration of magnesium in potable water could be high, relevant pretreatment is needed.

TABLE 6

RESULTS OF SOLUTION CONTAINING CALCIUM APPLIED ON THE LATERAL FLOW STRIP

Assay	Sample pad	Blocking step (casein)	Calcium concentration	Signal
1	Normal	Not applied	27.5mg/ml	
2	Normal	Applied	27.5mg/ml	
3	Blank	Applied	27.5mg/ml	
4	Blank	Applied	0g/ml (PBS)	
5	Normal	Applied	0.275mg/ml	

For Ca^{2+} shown in Table 6, even though an effective blocking step was applied, the aggregation of AuNP could not be avoided. One possible explanation is that there was some chemical reaction between AuNP and Ca^{2+} when the concentration of calcium ions is high in the water. The reaction caused aggregation and sedimentation of AuNP in the beginning of NC membrane which blocked the liquid flow as well as the resulting signal. Such sedimentation is different from nonspecific binding that was caused by electrostatic interaction between single AuNP and NC membrane so it could not be prevented by a regular blocking step. The amount of gold sedimentation reduced when the concentration of calcium became lower. However, the strip still showed a very poor sensing performance. This indicates calcium has a negative effect on the lateral flow immunoassay, especially when its concentration is high.

H. Measurement of ICP-OES system of water samples

When the concentration of calcium is over 120ppm, the water is considered to be hard [26]. The results in section G indicate some general parameters important for the proper functioning of the strip: it works well in soft water but the performance is not satisfactory in hard water. To determine pretreatment strategies, more data was needed. Therefore, ICP-OES measurement was carried out to determine the concentrations of important ions in the water matrix. The optimum wavelengths of each element and corresponding concentration in sample solution are shown in Tables 7 and 8:

TABLE 7

OPTIMIUM WAVELENGTHS FOR TARGET ELEMENTS IN ICP-OES SYSTEM

Element	Optimum Wavelengths/nm
Sodium	285.301
Magnesium	279.078
Potassium	769.897
Calcium	315.887
Iron	239.563

From Table 8, the borehole and sports water were categorized as hard water while tap water had relatively low ion intensity among all the five samples, especially calcium.

TABLE 8

CONCENTRATIONS OF TARGET ELEMENTS IN WATER SAMPLES

Sample	Na^+/ppm	Mg^{2+}/ ppm	K^+/ ppm	Ca^{2+}/ppm	Fe^{3+}/ppm
1	3.323	6.32	5.488	4.04	~0
2	8.996	9.568	8.856	127.88	~0
3	180.72	151.52	~0	257.12	~0
4	~0	7.935	~0	19.11	~0
5	~0	2.127	~0	14.76	~0

Note: Samples defined in Table 2. ~0 means the concentration of the target ion is very low in sample solution and it was not detected by the system. Since the result depends on the quality of calibration curve, the actual concentration is not necessarily zero.

As shown previously, the performance of the strip is poor when the calcium concentration is above 275ppb. In order to find out more details about if the water with lower hardness can be tested directly on lateral flow strip, borehole water was tested without pretreatment (Fig. 3).

Fig. 3. Test with raw borehole water (without spiking AFB_1)

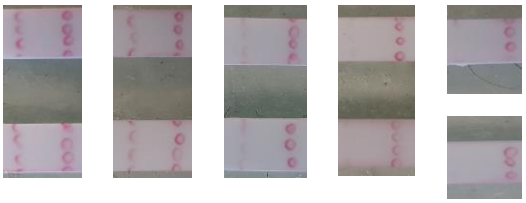
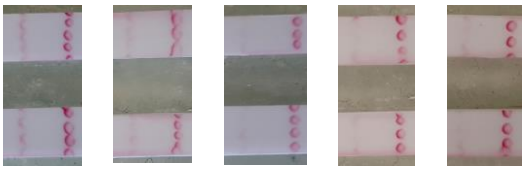
Although the gold aggregation is not as strong as those in previous section, there is still very faint signal on the test line. As the calcium concentration in borehole water (127.88ppm) is very close to the limit of hard water (120ppm), it can be concluded that the strip cannot be used for untreated hard water. Comparing the signals in Fig.3 with those of Table 3, reducing the negative effect caused by calcium is vital to achieve a good sensing performance.

I. Pretreatment and LFIA measurement of water samples

The principles of two pretreatment strategies can be seen as follows: in the presence of CO_3^{2-} in solution, Ca^{2+} and Mg^{2+} will sediment and can be removed through filtration. On the other hand, EDTA can chelate with these two ions and offset their effect with other reactions in water [27]. Borehole water was used as an example during the comparison of the two pretreatment methodologies (Table 9). From Table 9, the signal intensity of the test line is not as strong as the sample treated by sodium carbonate and more gold was bound to the control line.

TABLE 9

RESULTS OF BOREHOLE WATER AFTER PRETREATMENT

Pretreatment method	Blank control	0.5ppb	1.0ppb	1.5ppb	2.0ppb
Chemical precipitation					
Chelation reaction					

However, the signal on the test line became quite weak after the concentration of AFB_1 went above 0.5ppb, showing an increased sensitivity compared to the precipitation method. On the other hand, chemical sedimentation showed some negative effect on the signals on the control line because of a high pH value after the treatment. It also requires a further filtration step, which makes this method more complicated. As a result, EDTA was applied during subsequent sample pretreatment process. The measurements of other water samples are shown in Tables 10 and 11.

TABLE 10

MEASUREMENT OF BRITISH TAP WATER

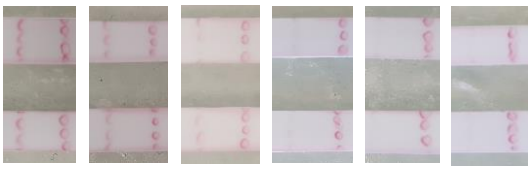
AFB_1 Concentration/ ppb	Blank	0.25	0.5	1.0	1.5	2.0
Signal exhibition						

TABLE 11

SUMMARY OF MEASUREMENT RESULTS OF WATER SAMPLES

Sample	pH	Na^+/ ppm	Mg^{2+}/ ppm	K^+/ ppm	Ca^{2+}/ ppm	Fe^{3+}/ ppm	Visual LOD
1	7.2	3.323	6.32	5.488	4.04	~0	0.5ppb
2	7.6	8.996	9.568	8.856	127.88	~0	0.5ppb
3	6.6	180.72	151.52	~0	257.12	~0	1ppb

4	7.73	~0	7.935	~0	19.11	~0	0.5ppb
5	7.6	~0	2.127	~0	14.76	~0	0.5ppb

Note: The sample number still represents the type of the sample mentioned in Table 2. Sample 6 here used is pure water which is free of AFB_1

From Table 11, the visual LOD of the lateral flow device for most of the samples reaches 0.5ppb. Although the high ion concentration may weaken the sensitivity (sample 3), the LOD is still around 1ppb, which can ensure the concentration of aflatoxin B_1 is within the safety range.

J. Measurement of water samples with ELISA

During lateral flow measurement, most samples are spiked based on the assumption that the original sample is free of AFB_1. However, the actual concentrations of AFB_1 in the original water samples were unknown. As a result, ELISA was applied as a supplement experiment to confirm the original concentration of AFB_1 in the water. Based on the detection range of the kit, samples spiked with 1ppb and 2ppb AFB_1 were also tested as a verification of the extraction strategy. The results were shown in Table 12.

TABLE 12

SUMMARY OF ELISA TEST RESULTS OF SPIKED WATER SAMPLES

Sample	Concentration of spiked AFB_1/ppb	Result of ELISA measurement	Recovery Rate
1	0	0	-
1	1	1.25	107%
1	2	2.664	109.9%
2	0	0	-
2	1	1.45	125%
2	2	2.331	96.2%
3	0	0	-
3	1	0.86	74.1%
3	2	1.703	70.2%
4	0	0	-
4	1	1.43	123%
4	2	2.213	91.3%
5	0	0	-
5	1	1.44	124%
5	2	2.255	93%
6	0	0	-
6	1	0.934	80.5%
6	2	2.49	102.7%

Although the ELISA kit is not as robust as those established procedures developed in the lab, from Table 12, most of the samples still gave a reasonable recovery rate, which indicated an effective extraction operation and accuracy of spiked AFB_1 concentrations. Another significant aspect is that no AFB_1 contamination was detected among the blank controls used in previous experiment. Since the results measured by ELISA is close to the supposed value, it indicated that with a calibrated pipette and proper spiking procedure, the actual concentration of spiked AFB_1 samples, namely, the sensitivity of lateral flow assay can be verified.

K. Test of strip stability

The stability test was carried out after 21 days of storage at 37°C, two months at room temperature and three months in the refrigerator at 4°C. The results show that the strips in the incubator and room temperature have patent denaturation in immunoreagents activity, which resulted in a weak signal in test zones. The strip stored at 4 °C still maintains a good performance. As a result the optimum storage condition of the device will be in the refrigerator (Table 13).

TABLE 13

MEASUREMENT OF STABILITY (REFRIGERATOR)

Date	Blank control	0.5ppb AFB_1	LOD
Day 0			0.5ppb
Day 92			0.5ppb

IV. CONCLUSION

In this study, the determination of AFB_1 in potable water samples was realized through lateral flow immunoassay for the first time. The most effective way to improve the assay sensitivity was to optimize the amount of antibody during conjugation. By pre-treating the water with EDTA, the strip gave robust sensing performance in different aqueous environments that are usually more complicated than those conventional food/feed extracts. The application of glycerol on the sample pad helps to maintain the assay sensitivity when a smaller strip size was used, which is very favourable for future commercialization. The strip shows visual LOD of 0.5ppb of AFB_1 in the majority of water samples tested and a reasonable stability after three months storage in the refrigerator.

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