This is an open access article published under a Creative Commons Attribution (CC-BY) License, which permits unrestricted use, distribution and reproduction in any medium, provided the author and source are cited.

ACS AuthorChoice

analytical chemistry

Article

pubs.acs.org/ac

Aptamer Lateral Flow Assays for Ultrasensitive Detection of β-Conglutin Combining Recombinase Polymerase Amplification and Tailed Primers

Miriam Jauset-Rubio,[†] Markéta Svobodová,[†] Teresa Mairal,[†] Calum McNeil,[‡] Neil Keegan,[*,‡] Mohammad S. El-Shahawi,[§] Abdulaziz S. Bashammakh,[§] Abdulrahman O. Alyoubi,[§] and Ciara K. O'Sullivan[*,†,∥]

[†]Nanobiotechnology and Bioanalysis group, Department of Chemical Engineering, Universitat Rovira I Virgili, 43007 Tarragona, Spain

[‡]Institute of Cellular Medicine, Diagnostic and Therapeutic Technologies Group, Newcastle University, Newcastle upon Tyne, NE2 4HH, U.K.

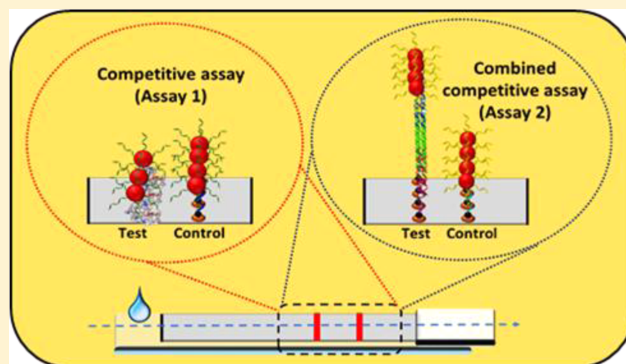
[§]Department of Chemistry, Faculty of Science, King Abdulaziz University, P.O. Box 80203, Jeddah 21589, Kingdom of Saudi Arabia

[∥]Institució Catalana de Recerca I Estudis Avancats, Passeig Lluís Companys 23, 08010 Barcelona, Spain

Downloaded by UNIV ROVIRA I VIRGILI at 06:10:46:756 on June 28, 2019 from https://pubs.acs.org/doi/10.1021/acs.analchem.6b03256.

Supporting Information

ABSTRACT: In this work, different methodologies were evaluated in search of robust, simple, rapid, ultrasensitive, and user-friendly lateral flow aptamer assays. In one approach, we developed a competitive based lateral flow aptamer assay, in which β-conglutin immobilized on the test line of a nitrocellulose membrane and β-conglutin in the test sample compete for binding to AuNP labeled aptamer. The control line exploits an immobilized DNA probe complementary to the labeled aptamer, forcing displacement of the aptamer from the β-conglutin-aptamer complex. In a second approach, the competition for aptamer binding takes place off-strip, and following competition, aptamer bound to the immobilized β-conglutin is eluted and used as a template for isothermal recombinase polymerase amplification, exploiting tailed primers, resulting in an amplicon of a duplex flanked by single stranded DNA tails. The amplicon is rapidly and quantitatively detected using a nucleic acid lateral flow with an immobilized capture probe and a gold nanoparticle labeled reporter probe. The competitive lateral flow is completed in just 5 min, achieving a detection limit of 55 pM (1.1 fmol), and the combined competitive-amplification lateral flow requires just 30 min, with a detection limit of 9 fM (0.17 amol).



Lupin is a leguminous plant consumed extensively in the Mediterranean region due to its' nutritional benefits, being an attractive alternative for gluten-free foodstuffs. It is widely used in a variety of foods, including traditional fermented foods, baked foods, and sauces. In addition, lupin can be grown from temperate to cool climates and is becoming increasingly recognized as an attractive alternative to soy.[1,2] β-conglutin is the predominant conglutin subunit, termed as the anaphylactic Lup an 1, and has been demonstrated to be a potent allergen since 2008, when it was added to the list of allergens requiring mandatory labeling.[3] The allergic reaction to lupin ranges from mild to severe anaphylactic reactions.[4] To allow the specific detection of the Lup an 1 anaphylactic allergen, aptamers have been developed specifically against the β-conglutin subunit.[5−7] Aptamers are short single-stranded DNA or RNA ligands that often adopt a three-dimensional shape for interaction with a wide range of target analytes, having multiple advantages they offer over antibodies (Table S-1).[8]

A variety of methods have been described for the detection of food allergens including the polymerase chain reaction (PCR),[9] enzyme-linked immunosorbent assay (ELISA),[10] as well as mass spectrometry (MS),[11] but these methods are time-consuming and require expensive equipment, significant infrastructure, and trained personnel. Lateral flow assays are an attractive alternative for the detection of food allergens, as they are inexpensive, rapid, portable, and extremely simple to operate and have been developed for a plethora of food allergens, including casein, β-lactoglobulin,[12] glycinin,[13] crustacean proteins,[14] fish proteins,[15] nuts,[16] and fungal alpha-amylase,[17] as has been reviewed extensively elsewhere.[18] In recent years, aptamers have found increasing application in

Received: August 19, 2016
Accepted: October 10, 2016
Published: October 18, 2016

ACS Publications © 2016 American Chemical Society

DOI: 10.1021/acs.analchem.6b03256
Anal. Chem. 2016, 88, 10701−10709

Table 1. Sequences Used in This Work

name	sequence
capture probe test line	5′- gtcgtgactgggaaaacttttttttttttttt-Biotin-TEG-3′
capture probe control line	5′-tgtaaaacgacggccagttttttttttttttt-Biotin-TEG-3′
reporter probe lateral flow	5′-actggccgtcgttttacattttttttttttttt-C6-thiol-3′
aptamer (β-CBA II)	5′-agctccagaagataaattacaggggccggggtggctcaggcaaggggttgacctgtcgtagggattgttttaacaactaggatactatgacccc-3′
forward primer	5′-gttttcccagtcacgac-C3-agctccagaagataaattacagg-3′
reverse primer	5′-tgtaaaacgacggccagt-C3-ggggtcatagtatcctagttg-3′
complementary aptamer	5′-Biotin-ggggtcatagtatcctagttgttaaaacaatccctacgacaggtcaaccccttgcctgagccaccccggcccctgtaatttatcttctggagct-3′
reporter aptamer (β-CBA II)	5′- thiol-C6-agctccagaagataaattacaggggccggggtggctcaggcaaggggttgacctgtcgtagggattgttttaacaactaggatactatgacccc-3′

lateral flow assays, taking advantage of sandwich, competitive, or strand displacement formats (Table S-2). There have been several reports of lateral flow aptamer assays, with targets including thrombin,[19] *Escherichia coli*,[20] *E. coli O157:H7*,[21] Ramos cells,[22] IgE, *Staphylococcus aureus*,[23] *Salmonella enteritidis*,[24] arboviruses,[25] adenosine, cocaine,[26] and ochratoxin A.[27] The sandwich formats take advantage of platforms developed for nucleic acid lateral flow assays (NALFs), in which a biotinylated capture aptamer is immobilized on a streptavidin coated test line, and the target sandwiched between this aptamer and a gold nanoparticle (AuNP) labeled reporter aptamer. The control line is coated with streptavidin linked to a biotinylated DNA probe complementary to the AuNP labeled aptamer. This approach has been used for the LF detection of thrombin[19] and Ramos cells.[22] An alternative approach has been developed for the detection of *E. coli*, in which the capture aptamer is aminated and linked via UV cross-linking to the nitrocellulose membrane, with the reporter aptamer having dual labels of biotin and digoxigenin (dig), the biotin for linking to streptavidin coated AuNPs or quantum dots, and the dig label for capture on the control line by an antidig antibody.[20]

Competitive type lateral flow assays have also been reported for ochratoxin[27] and the HCV core antigen,[28] where competition occurs between the AuNP labeled aptamer with an addended polyA tail, and a DNA probe complementary to the aptamer sequence immobilized on streptavidin coated test lines on the membrane. With increasing concentrations of target in the sample, less aptamer is available for binding to the complementary DNA sequence. The control line consisted of a biotinylated poly T DNA probe which is bound to the poly A tail of the aptamer. In the case of the aflatoxin, a slightly modified approach was pursued with biotinylated aptamer and a DNA probe partially complementary to the aptamer forming a complex in the absence of aflatoxin, which was then detected on the streptavidin coated test line. In the presence of target, the complex did not form and the Cy5 labeled DNA probe was captured by anti-Cy5 antibody on the control line.[29]

In the work reported here, we have evaluated generic methodologies for the aptameric detection of β-conglutin, in search of a robust, simple, rapid, ultrasensitive, and user-friendly lateral flow assay that can be applied to all aptamers. In one approach, we developed a competitive based lateral flow aptamer assay, where β-conglutin immobilized on a nitrocellulose membrane and β-conglutin in the test sample compete for binding to AuNP labeled aptamer. In a second approach, the competition for aptamer binding takes place off-strip, between the target in the test sample and β-conglutin immobilized on magnetic beads. Following competition, aptamer bound to the immobilized β-conglutin is eluted and used as a template for isothermal recombinase polymerase amplification (RPA). RPA was specifically selected due to its simplicity and easy compatibility with lateral flow assays, as neither an initial melting step of the use of multiple primers is required. The RPA exploits the use of tailed primers, resulting in an amplicon of a duplex flanked by single stranded DNA tails, and the amplicon is rapidly and quantitatively detected using a nucleic acid lateral flow with immobilized capture probe and gold nanoparticle labeled reporter probe. The two approaches are compared in terms of detection limits and assay time required.

■ MATERIALS AND METHODS

Materials. Phosphate buffered saline (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4), 1-ethyl-3-(dimethylaminopropyl) carboiimide (EDC), *N*-hydroxysuccinimide (NHS), and all other reagents were purchased from Sigma (Barcelona, Spain). Magnesium chloride, sodium chloride, sodium hydroxide, and hydrochloric acid were purchased from Scharlau Chemie S.A. (Barcelona, Spain). SiGMAG-Carboxyl beads were obtained from Chemicell (Zaragoza, Spain). All solutions were prepared in high-purity water obtained from a Mili-Q RG system (Barcelona, Spain). All DNA oligonucleotides were purchased from BIOMERS (Germany), and β-conglutin was extracted and purified as previously detailed.[30] All primer and probe sequences can be found in Table 1.

Competitive Lateral Flow Assay. *Aptamer Functionalized Gold Nanoparticles.* Gold nanoparticles (AuNPs) with an approximate average diameter of 13 nm were prepared by citrate reduction of $HAuCl_4$ as previously reported.[31] A total of 100 μL of thiolated β-CBA II aptamer modified at the 5′ end was treated with 1 μL of 10 mM TCEP and 2 μL of 500 mM acetate buffer pH 5.2 for 1 h under tilt rotation at room temperature (RT), followed by the addition of 1 mL of AuNPs and incubated for 16 h in dark conditions under tilt rotation. Thereafter, 10 μL of 500 mM Tris acetate buffer pH 8.2 and 100 μL of 1 M NaCl were then slowly added to the solution (10 μL every 20 min) and again incubated for 24 h in dark conditions under tilt rotation. The solution was then centrifuged at 15 000 rpm (Eppendorf Centrifuge 5417R, Spain) for 25 min, washed three times with deionized water, and resuspended in 30 μL of deionized water.

Experiments to Demonstrate Displacement for the Control Line. Control Experiment 1. Maleimide microtiter plates were prepared by pipetting 100 μL of 200 nM thiolated sequence complementary to the β-CBA II aptamer prepared in 10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4 and left to incubate at 4 °C overnight. The plates were subsequently washed with PBS containing 0.05% (v/v) Tween 20 (PBS-Tween) and blocked with 100 μM 6-mercapto-1-hexanol (MCH) via addition of 200 μL per well for 1 h at RT, prior to washing the plate with PBS-tween. An additional blocking step was carried out using 1% w/v skimmed milk with 0.1% v/v

DOI: 10.1021/acs.analchem.6b03256
Anal. Chem. 2016, 88, 10701−10709

Empigen detergent for 30 min at RT. During the last blocking step, a range of concentrations of β-conglutin (10 μM, 100 nM, 1 nM, 10 pM, and 0 pM) were preincubated with a constant amount of biotinylated aptamer (150 nM) and streptavidin-horseradish peroxidase (10 nM SA-HRP) in binding buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM $MgCl_2$, pH 7.4). This preincubated complex was then added to the plate and incubated for 15 min, effectively adding the β-conglutin-aptamer complex to a surface functionalized with the sequence complementary to the aptamer. TMB substrate was subsequently added, and following 5 min color development, the reaction was stopped by the addition of 1 M H_2SO_4, followed by reading the absorbance at 450 nm. Duplicate analyses were carried out to confirm the results.

Control Experiment 2. Maleimide microtiter plates were functionalized with thiolated sequence complementary to the β-CBA II aptamer as described above. During the final blocking step, a range of concentrations of β-conglutin immobilized on carboxylated magnetic beads (0 nM, 8.75 nM, 17.5 nM, 35 nM, and 70 nM) was preincubated with a mixture containing different amounts of biotinylated aptamer (10 nM, 50 nM, and 100 nM) and a constant amount of streptavidin-horseradish peroxidase (10 nM SA-HRP) in binding buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 1.5 mM $MgCl_2$, pH 7.4). Three different strategies were evaluated: (1) All the solution containing beads and supernatant was added to wells of the microtiter plate; (2) the beads magnetically isolated and only the supernatant was added to the wells of the microtiter plate; (3) the beads were magnetically isolated, and only the beads resuspended in binding buffer were added to wells of the microtiter plate. In all cases the incubation time on the plate was 20 min, prior to addition of TMB substrate and subsequent addition of 1 M H_2SO_4, followed by reading the absorbance at 450 nm. Duplicate analyses were performed in order to confirm the results.

Preparation of Lateral Flow Strips. The test strip was prepared by manually cutting strips of 4 mm. The membrane used was FF170HP nitrocellulose (Whatman, Germany), and the absorbent pad was glass cellulose (Whatman, England). The test and the control line were created by drawing a line with an Eppendorf tip containing the biomolecule to be deposited. A biotinylated sequence complementary to the aptamer (60 pmol) was mixed with 20 pmol of streptavidin in PBS buffer for 1 h and then spotted on the membrane to create the control line. For the test line, 2 mg/mL of β-conglutin in PBS buffer was dispensed onto the membrane. Following drying of the membrane, blocking was achieved with 1% w/v skimmed milk powder and 0.1% v/v Empigen detergent for 15 min, under shaking conditions. The membrane was left to dry at RT for approximately 2 h and then stored in the fridge until use.

Lateral Flow Assay. A volume of 9 μL of the aptamer-AuNP conjugate were mixed with 2 μL of sample and 9 μL of binding buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 2.5 mM $MgCl_2$, pH 7.4) for 2 min at RT and applied to the sample pad and allowed to flow through the lateral flow strip.

A range of concentrations of β-conglutin (0−20 μM) was analyzed to explore the sensitivity of the assay. The specificity was evaluated using 2 μM bovine serum albumin (BSA) and streptavidin (SA) as nonspecific controls. The data was normalized by using the highest value corresponding to the intensity of the blank (0 nM β-conglutin). The limit of detection (LOD) was calculated using GraphPad Prism Software and is defined as the top value − 3 × standard deviation (SD) of the top value. Triplicate measurements were performed for each concentration.

The control line was based on the use of an oligomer complementary to the AuNP labeled aptamer sequence. In the case of low concentrations of target β-conglutin, the majority of the reporter aptamer would bind to the immobilized β-conglutin, and a small excess migrates to the control line. In the case of medium to high concentrations of the β-conglutin, small amounts of the reporter aptamer bind to the immobilized β-conglutin, with the rest being complexed with the target β-conglutin, which migrates beyond the test line.

The use of an oligomer complementary to the labeled aptamer as the control line exploited the expected higher affinity of the AuNP labeled aptamer for the immobilized oligomer as compared to its cognate target, β-conglutin, effectively provoking displacement of the labeled aptamer.

Combined Competitive Assay, Recombinase Polymerase Amplification, and Lateral Flow. *Competition Assay.* In the first step, a competitive type-assay was carried out. β-conglutin was immobilized on the surface of carboxyl magnetic beads, as previously described.[6] A range of concentrations of β-conglutin (0−20 μM) were preincubated in individual Eppendorf tubes with 1 nM of the β-CBA II aptamer for 5 min at RT. Subsequently, 10 μL of functionalized beads were added to each Eppendorf tube and left for a further 5 min incubation. Magnetic actuation was applied, and the Eppendorf tubes rigorously washed three times with 500 μL of binding buffer (10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, 2.5 mM $MgCl_2$, pH 7.4) followed by the addition of deionized water and 2 min sonication to elute the aptamer for use as a template in recombinase polymerase amplification (RPA).

Recombinase Polymerase Amplification Assay. RPA was performed in a tube following the indications provided in the TwistAmp Basic kit (TwistDX, Cambridge, England). Briefly, master mix was prepared in a tube containing 480 nM of each tailed primer, template DNA of the desired concentration, 14 mM magnesium acetate, and 1× rehydration buffer. The reaction took place at RT for 15 min.

Reporter Probe Functionalized Gold Nanoparticles. Gold nanoparticles were functionalized with reporter DNA as previously described,[32] with minor modifications. Briefly, 100 μL of reporter DNA probe was mixed with 1 mL of AuNPs. The solution was allowed to incubate for 24 h at 1000 rpm in a thermomixer. Thereafter, 1 M NaCl was gradually introduced every 20 min until a concentration of 0.7 M was reached. The mixture was again incubated for 24 h under the same conditions. Finally, the conjugate was centrifuged at 15 000 rpm (Eppendorf Centrifuge 5417R, Spain) for 30 min and the pellet was washed three times in deionized water in order to clean the conjugate and remove any free DNA. The conjugate was then resuspended in deionized water in the desired volume.

Preparation of Lateral Flow Strip. The lateral flow strip was prepared as described above. The test and the control line were created by dispensing solutions of 20 pmol of streptavidin preincubated (1 h at RT) with 60 pmol of each respective biotinylated probe in PBS buffer. A volume of 10 μL of the reporter probe-AuNP conjugate was mixed with 1 μL of sample (RPA product) and 8 μL of 10× SSC, 3.5% Triton X-100, 0.25% SDS, 12.5% formamide to obtain a final concentration of 4× SSC buffer, 1.4% Triton X-100, 0.1% SDS, 5% formamide,

DOI: 10.1021/acs.analchem.6b03256
Anal. Chem. 2016, 88, 10701−10709

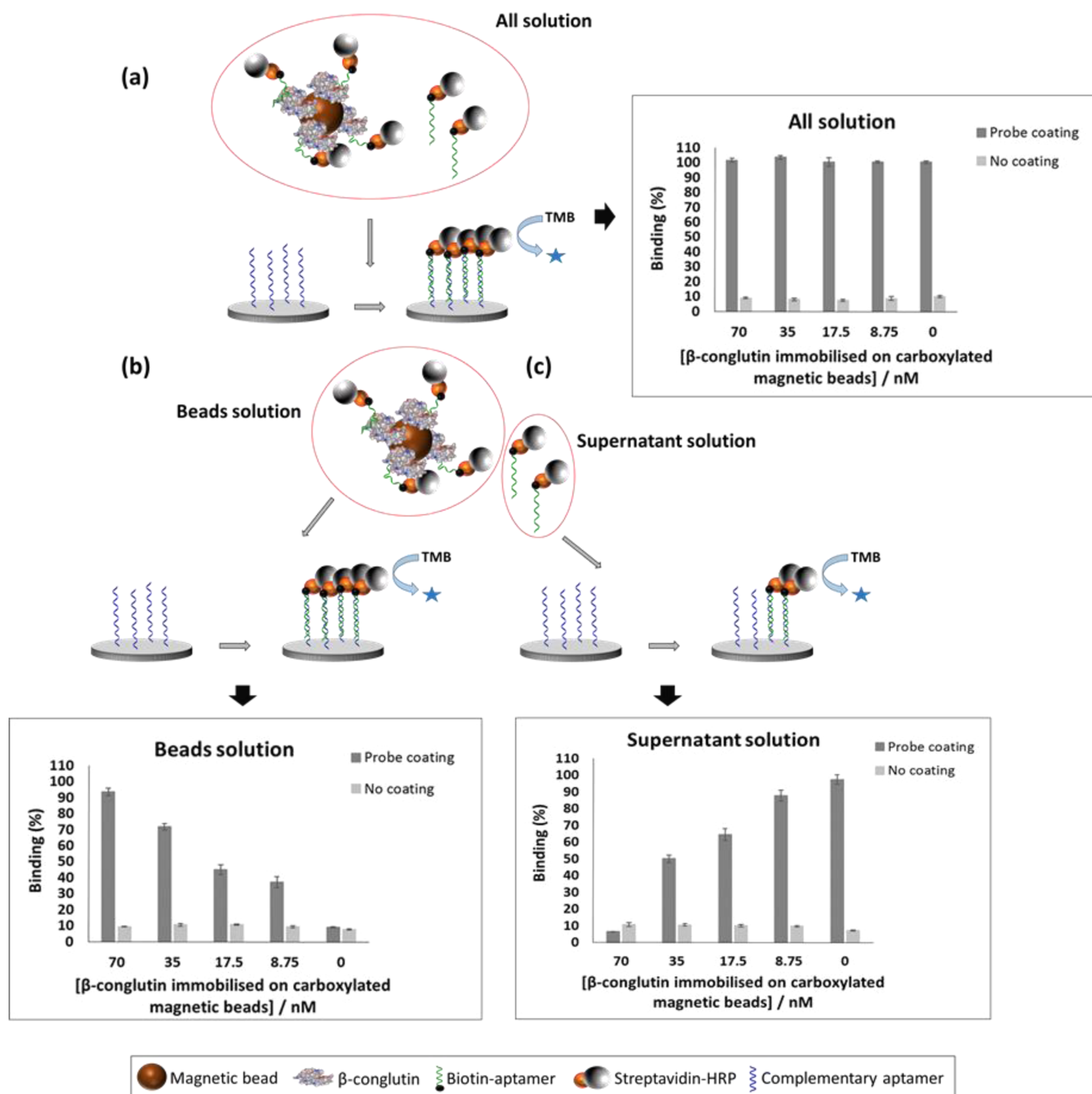


Figure 1. Schematic representation and results of Control Displacement Experiment 2: (a) All solution, containing beads and supernatant is added to the wells of microtiter plate, thus containing all the labeled aptamer; (b) only isolated beads, thus only containing labeled aptamer bound magnetic bead conjugated β-conglutin, increasing concentrations of magnetic bead conjugated β-conglutin resulting in increasing concentrations of bound labeled aptamer; (c) only supernatant, thus only containing free labeled aptamer in solution, increasing concentrations of magnetic bead conjugated β-conglutin resulting in decreasing concentrations of free labeled aptamer in solution.

and incubated for 2 min at RT. The sample was then applied to the sample pad and allowed to migrate through the test strip.

Lateral Flow Assay. In order to test the sensitivity and the specificity, the assay, it was carried out with a range of concentrations of β-conglutin (0–20 μM) and also with a nonspecific DNA sequence (1 μM). The data was normalized by using the highest value corresponding to the intensity of the blank (0 nM β-conglutin). The limit of detection (LOD) was calculated using GraphPad Prism Software and is defined as the top value − 3 × standard deviation (SD) of the top value. Triplicate measurements were performed for each concentration.

RESULTS AND DISCUSSION

Elucidation of Control Line Based on Aptamer Displacement. The control line was designed based on the hypothesis that the aptamer-AuNP has more affinity for its complementary DNA sequence than for its cognate aptameric target, and this could be exploited to provoke displacement of the aptamer-AuNP from the β-conglutin complex to bind to the immobilized complementary sequence. Thus, at high or low concentrations of target β-conglutin, a red line would form at the control line, an approach significantly simpler and inherently less expensive than the control lines developed in previous reports of aptamer based competitive lateral flow assays.[27−29]

Control Experiments on Microtiter Plates to Demonstrate Displacement of AuNP Labeled Aptamer to Form Control Line. In order to corroborate the hypothesis that displacement of AuNP labeled aptamer from the β-conglutin-aptamer complex to form the control line, control experiments were performed. In the first control, the aptamer-AuNP was

DOI: 10.1021/acs.analchem.6b03256
Anal. Chem. 2016, 88, 10701−10709

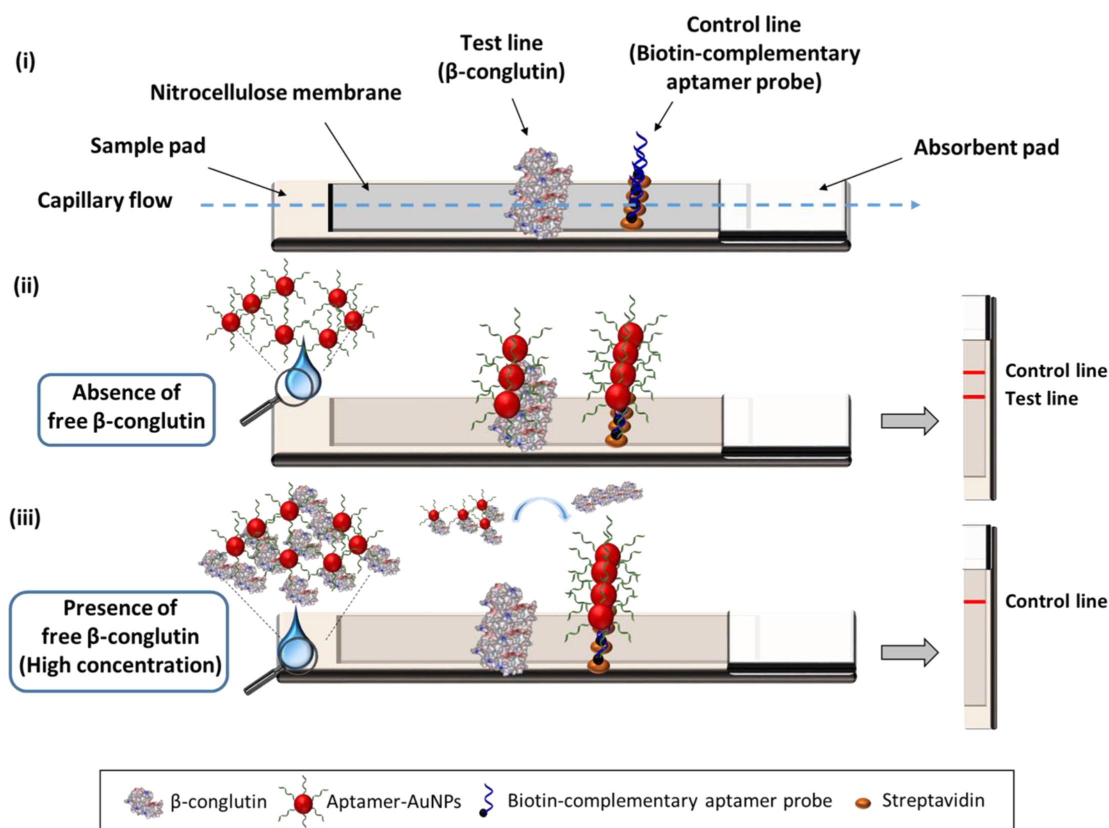


Figure 2. Competitive β-conglutin assay on the strip: (a) schematic representation (i) configuration of lateral flow strip; (ii) absence of sample (negative test): In the absence of free β-conglutin on solution, the conjugate (aptamer-AuNPs) will bind in the test line (immobilized β-conglutin) and in the control line (full complementary probe to the aptamer); (iii) high concentration of the sample (positive test): In the presence of high amount of free β-conglutin on solution, the conjugate (aptamer-AuNPs) will not bind in the test line (immobilized β-conglutin) due to the fact that all the conjugate will bind to free β-conglutin but in the control line (full complementary probe to the aptamer) the conjugate will bind as the aptamer has higher affinity with its full complementary probe than the β-conglutin in solution provoking its displacement.

replaced by the aptamer linked to HRP. A thiolated DNA sequence complementary to β-CBA II aptamer was immobilized in the wells of maleimide coated microtiter plates. A range of concentrations of β-conglutin was mixed with a preincubated mixture of a constant concentration of biotinylated aptamer and SA-HRP. Independent of the concentration of β-conglutin, all biotinylated-SA-HRP aptamer (i.e., the labeled aptamer not bound to the β-conglutin and the labeled aptamer bound to β-onglutin) was observed to bind to the immobilized complementary sequence indicating that any bound labeled aptamer was effectively displaced from β-conglutin (Figure S-1a).

To confirm the observation, two additional controls were carried out. In the first, only SA-HRP was added to each blocked well to demonstrate that SA-HRP was not binding nonspecifically. In the second control, the complementary DNA sequence was not immobilized (no coating), demonstrating that neither β-conglutin nor the aptamer binds nonspecifically. In both cases, no significant signal was observed.

Furthermore, to ensure that the assay was correctly mimicking the lateral flow assay, a competitive assay was carried out by immobilizing the β-conglutin and performing a preincubation of a range of concentrations of β-conglutin with a constant concentration of premixed biotinylated aptamer and SA-HRP, prior to addition of the complex to the plate (Figure S-1b). A trend indicative of a competitive type assay was clearly observed, demonstrating that the microtiter based assay is truly performing as the lateral flow assay does.

In a second experiment to demonstrate the displacement hypothesis, a thiolated DNA sequence complementary to the β-CBA II aptamer was immobilized on maleimide plates. β-conglutin coupled to carboxylated magnetic beads was preincubated with a constant concentration of premixed biotinylated aptamer and SA-HRP. Three different strategies were evaluated to probe the displacement phenomena between the solution-phase complex aptamer-β-conglutin and the immobilized complementary sequence to the aptamer.

In the first case, following preincubation, all the solution containing free β-conglutin, immobilized β-conglutin on the surface of magnetic beads, biotinylated aptamer-SA-HRP (bound to both free (target) and immobilized β-conglutin), was added directly to the wells of the microtiter plate (Figure 1a). In the second case, the beads (functionalized with β-conglutin and complexed with varying concentrations of biotinylated aptamer-SA-HRP following competition) were magnetically isolated and only the supernatant was added to the wells of the microtiter plates (Figure 1c). Finally, the magnetically isolated beads were resuspended in binding buffer and added to the well plate (Figure 1b). In all the cases, controls with no immobilized complementary DNA sequence (no coating) were carried out for all the concentrations in order to check for any nonspecific binding.

DOI: 10.1021/acs.analchem.6b03256
Anal. Chem. 2016, 88, 10701−10709

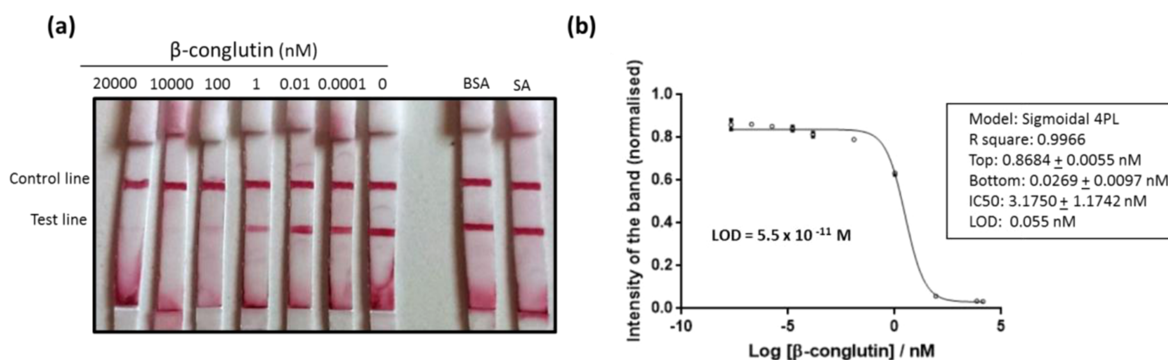


Figure 3. Results of competitive β-conglutin on the lateral flow strip: (a) Strips, control proteins, BSA and streptavidin (SA); (b) calibration curve and statistics values. Data points represent the mean of three repetitions with error bars. Note that only concentrations down to 0.0001 nM are not represented in the pictures.

The results indicated that when all the solution was added directly to the plate, there was always the same absorbance reading, independent of the concentration of β-conglutin, suggesting that all labeled aptamer was bound to its complementary sequence, i.e., labeled aptamer not bound to β-conglutin, as well as labeled aptamer bound to β-conglutin, that is displaced by the interaction between the aptamer and its complementary sequence. This phenomenon was confirmed with the results achieved with the supernatant or the beads, with increasing amounts of β-conglutin functionalized magnetic beads resulting in increasing amounts of labeled aptamer bound to β-conglutin (beads) and decreasing amounts of free labeled aptamer in the solution (supernatant), thus confirming the hypothesis of displacement of aptamer-AuNP for the control line to indicate successful migration through the lateral flow strip.

Competitive Lateral Flow Assay. The competitive lateral flow assay was based on the detection of β-conglutin via competition between the target immobilized on the membrane and the target in the solution. To elucidate the optimal parameters of the aptamer-AuNP conjugate, different dilutions were tested in the absence of target and in the presence of a high target concentration (10 μM), and a dilution of 1 in 2 of the stock AuNP conjugate was observed to be optimum (Figure S-2).

With increasing concentrations of β-conglutin in solution, less aptamer is free to bind to the immobilized β-conglutin on the membrane (Figure 2). As a result, there is a decrease in the intensity of the test line, at concentrations of greater than 10 μM no test line was visible. With all concentrations tested, the red color of the gold nanoparticles was observed at the control line, according to the displacement hypothesis demonstrated using the control experiments on microtiter plates. In the absence, or at very low concentrations of β-conglutin, the AuNP-aptamer mainly binds to immobilized β-conglutin and there is a small excess that migrates to the control line. In the presence of β-conglutin, the AuNP labeled aptamer binds both to the target β-conglutin and the immobilized β-conglutin, and at concentrations of 10 μM and greater, all the aptamer-AuNP conjugate binds to target β-conglutin, and thus in all cases a red stripe is observed at the control line.

To test the specificity of the assay, two nonspecific proteins known to cross-react with a large number of reported aptamers, bovine serum albumin (BSA), and streptavidin (SA) were prepared at 2 μM and incubated for 2 min with the aptamer-AuNPs conjugate prior to addition to the strip.[33] Nonspecific binding was not observed, demonstrating that the aptamer was highly specific for β-conglutin and that the membrane was correctly blocked (Figure 3a).

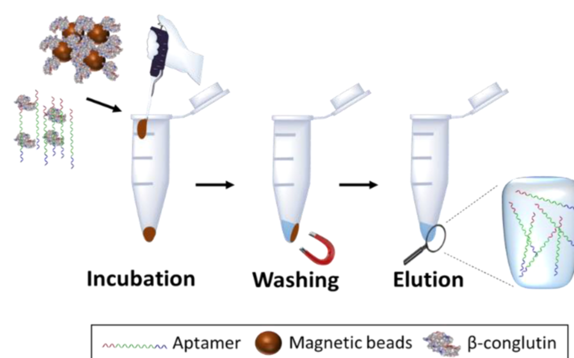
A Smartphone inbuilt camera was used to take an image of the strip followed by analysis using ImageJ software to calculate the intensity of the bands, and these values were then plotted using GraphPad Prism software in order to obtain the LOD. The values were normalized using the highest value corresponding the control of the blank, 0 nM β-conglutin. The LOD achieved was 5.5×10^{-11} M or 1.1 fmol of β-conglutin (Figure 3b). These results were compared with the use of a free mobile application termed IJ_mobile based on ImageJ software and the same results were obtained (data not shown). The entire assay was complete in just 5 min, 2 min preincubation of sample with AuNP labeled aptamer, and 1 min migration on the lateral flow, followed by 2 min incubation.

Combined Competitive Assay with Recombinase Polymerase Amplification and Lateral Flow Detection. A combination of aptamer detection and subsequent aptamer amplification has been reported for the ultrasensitive detection of targets.[34,35] We exploited a combination of the competition assay described above with isothermal amplification with the objective of achieving ultrasensitivity. Following competition, bound aptamer (94 nucleotides in length) was eluted via sonication and used as a template for amplification by recombinase polymerase amplification (RPA). Recombinase polymerase amplification was used as this method of isothermal amplification is remarkable for its simplicity, not requiring any initial melting or multiple primers. Furthermore, we exploited a methodology we previously reported for PCR amplification, where modified primers, referred to as tailed primers, are used to result in amplicons of double stranded DNA with two single stranded DNA tails on each end.[36] This is facilitated by a "stopper", which can be C3, C9, C18, which is located between the primer binding site and the single stranded tail and effectively acts to prevent the polymerase from further elongation. We designed forward and reverse primers with sequences specific to bind to extremes of the eluted aptamer, and flanked by single stranded tails, designed to be complementary to the immobilized and reporter probes. We did not study different length primers, as in our experience, RPA functions highly efficiently with normal length primers. RPA parameters were optimized as recently described.[37]

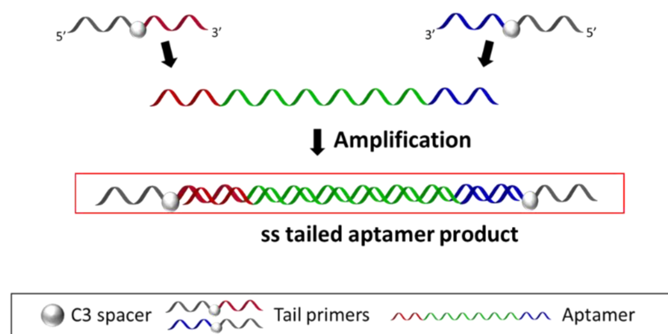
The RPA product was mixed with the reporter probe conjugated with gold nanoparticles (AuNPs) and added to the lateral flow strip. In this case, the lateral flow strip was based on

DOI: 10.1021/acs.analchem.6b03256
Anal. Chem. 2016, 88, 10701−10709

1) Competitive assay



2) RPA amplification of aptamer



3) Lateral flow output

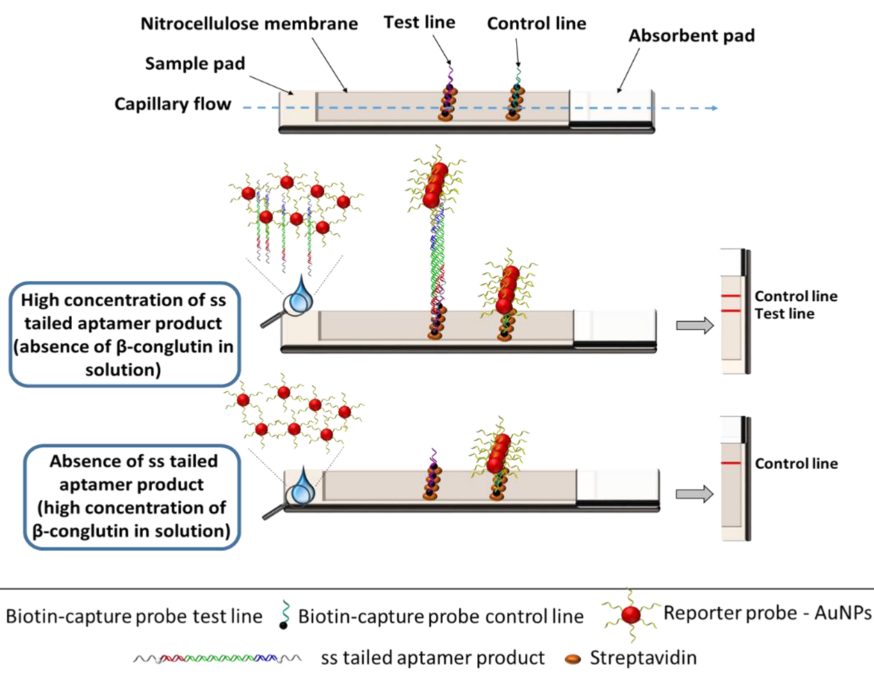


Figure 4. Schematic representation of β-conglutin detection via combined competitive assay (1), recombinase polymerase amplification with tailed primers (2), and lateral flow detection (3).

the immobilization of two biotinylated capture probes (premixed with streptavidin), composing the test line and the control line. In the test line the probe was complementary to the tail in the 5′ region of the amplified aptamer, meanwhile in the control line, the probe was complementary to the reporter probe conjugated with AuNPs. The reporter probe conjugated with AuNPs was also complementary to the other tail at the 3′ end of the amplified aptamer and thus will bind to the amplified aptamer forming a sandwich on the test line and with the

DOI: 10.1021/acs.analchem.6b03256
Anal. Chem. 2016, 88, 10701−10709

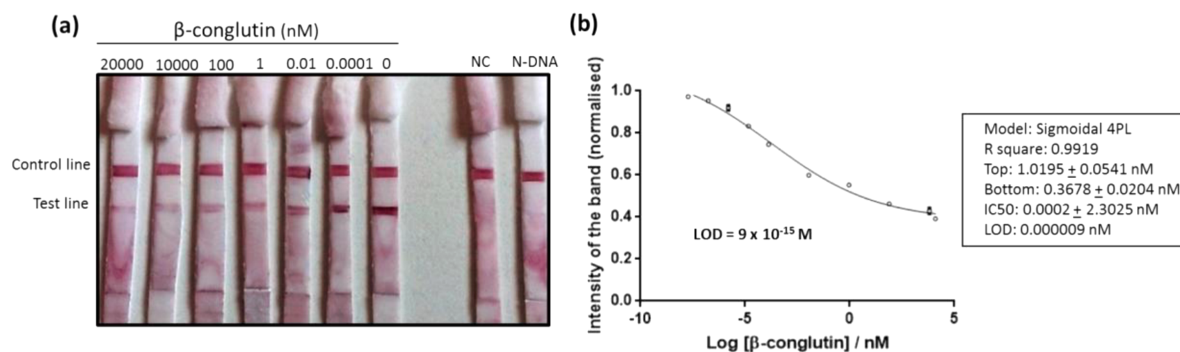


Figure 5. β-Conglutin detection using combination of competitive assay, recombinase polymerase amplification with tailed primers and lateral flow detection: (a) Results of strips, NC (no template negative control), N-DNA (nonspecific DNA); (b) calibration curve and LOD. Data points represent the mean of three repetitions with error bars. Note that only concentrations down to 0.0001 nM are represented in the pictures.

capture probe in the control line, generating a red stripe in both cases (Figure 4).

A range of concentrations of amplified aptamer were tested to evaluate the sensitivity of the assay, and a LOD of 9×10^{-15} M was achieved (Figure 5b), demonstrating the ultrasensitivity achievable upon the combination of aptamer detection with RPA. The specificity of the assay was demonstrated by incubating a nonspecific DNA sequence prior to addition to the lateral strip, and only a band in the control line was observed (Figure 5a).

CONCLUSIONS

We describe two different generic methodologies for lateral flow aptamer assays, which can be applied to any aptamer-target, using the detection of the anaphylactic β-conglutin allergen as a model (Table 2). Direct competition on the lateral flow strip facilitated an extremely rapid test, with the entire assay being completed in less than 5 min, with a 2 min preincubation of aptamer-AuNP and sample, 1 min migration, and 2 min incubation. The control line was achieved via binding of unbound AuNP-aptamer to its complementary sequence via displacement from the β-conglutin, demonstrating a generic methodology for creation of the control line. Previous reports of competitive lateral flow aptamer assays have exploited the use of aptamers modified with a poly A tail, with the control line being functionalized with poly T, which will bind to both complexed and noncomplexed aptamer[38] or, alternatively, an additional AuNP labeled with another DNA sequence was used, with its complementary sequence immobilized on the control line, or the use of an antibody to detect a dual labeled aptamer.[20] However, while these approaches are functional, the use of additional DNA-AuNP or antibodies adds both to the complexity and cost of the assay, while the use of a poly A tail can interfere with aptamer functionality in some cases. While the detection limit achieved using our competitive lateral flow assay, of just 55 pM (1.1 fmol), is impressive, there are cases when better sensitivity would be required. To address these requirements, again using the β-conglutin as a model target, the competitive assay was modified to incorporate an amplification step, in which competition was executed off-strip using functionalized magnetic beads, followed by aptamer elution and its use as a template in isothermal recombinase amplification using tailed primers. Following RPA, the amplicons of duplex DNA flanked by single stranded DNA tails were directly wicked onto the lateral flow strip, achieving a LOD of 9 fM (0.17 amol), with the entire assay being achieved in just 30 min, comprising 12 min competition (including preincubation and postcompetition sonication), 15 min amplification, 1 min lateral flow migration, and 2 min incubation. These generic platforms, combining aptamers with lateral flow, can be applied to a huge range of targets, including other food allergens, pathogens, cancer cells, or fetal cells, for which ultrasensitive detection is critical. Ongoing work is expanding the demonstrated platforms toward multiplex detection.

Table 2. Comparison of Both Methods

	competitive lateral flow assay	combined competitive assay, recombinase polymerase amplification and lateral flow
test line	β-conglutin	biotin-capture probe complementary to amplicon tail
control line	biotin-complementary aptamer probe	biotin-capture probe complementary to reporter probe
conjugate	aptamer-AuNPs	reporter probe-AuNPs
LOD	5.5×10^{-11} M (1.1 fmol)	9×10^{-15} M
assay time	5 min	30 min

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b03256.

Control displacement experiment 1 (Figure S-1), optimization of aptamer-AuNP conjugate concentration (Figure S-2), advantages of aptamers over antibodies (Table S-1), reports of aptamer lateral flow (Table S-2), and calibration curves and statistics values (Table S-3) (PDF)

AUTHOR INFORMATION

Corresponding Authors

*Phone: 0034977558740. Fax: 0034977559667. E-mail: ciara.osullivan@urv.cat.

*Phone: 0044 (0) 191 208 3678. Fax: 0044 (0) 191 208 3678. E-mail: neil.keegan@newcastle.ac.uk.

Funding

This work was funded through the U.K. Engineering and Physical Sciences Research Council (i-sense) (Grant EP/K031953/1) and King Abdulaziz University, under the

financing of the collaborative project "Selection and application of aptamers against anabolic steroids".

Notes

The authors declare no competing financial interest.

Data supporting this publication is openly available under an 'Open Data Commons Open Database License'. Additional metadata are available at: http://dx.doi.org/10.17634/122638-1. Please contact Newcastle Research Data Service at rdm@ncl.ac.uk for access instructions.

■ REFERENCES

(1) Ballester, D.; Zacarías, I.; García, E.; Yañez, E. *J. Food Sci.* **1984**, *49* (1), 14−16.
(2) De Penna, E. W.; Carreño, P.; Urrutia, X.; Lopez, L.; Ballester, D. *J. Food Sci.* **1987**, *52* (5), 1434−1435.
(3) Goggin, D. E.; Mir, G.; Smith, W. B.; Stuckey, M.; Smith, P. M. C. *J. Agric. Food Chem.* **2008**, *56* (15), 6370−6377.
(4) Brennecke, S.; Becker, W. M.; Lepp, U.; Jappe, U. *J. Dtsch Dermatol Ges* **2007**, *5* (9), 774−776.
(5) Jauset Rubio, M.; Svobodová, M.; Mairal, T.; Schubert, T.; Künne, S.; Mayer, G.; O'Sullivan, C. K. *Anal. Bioanal. Chem.* **2016**, *408* (3), 875−884.
(6) Nadal, P.; Pinto, A.; Svobodova, M.; Canela, N.; O'Sullivan, C. K. *PLoS One* **2012**, *7* (4), e35253.
(7) Nadal, P.; Svobodova, M.; Mairal, T.; O'Sullivan, C. K. *Anal. Bioanal. Chem.* **2013**, *405* (29), 9343−9349.
(8) Famulok, M.; Mayer, G.; Blind, M. *Acc. Chem. Res.* **2000**, *33* (9), 591−599.
(9) Cucu, T.; Jacxsens, L.; De Meulenaer, B. *J. Agric. Food Chem.* **2013**, *61* (24), 5624−5633.
(10) Brandon, D. L.; Friedman, M. *J. Agric. Food Chem.* **2002**, *50*, 6635−6642.
(11) Rahman, A. M. A.; Lopata, A. L.; Randell, E. W.; Helleur, R. J. *Anal. Chim. Acta* **2010**, *681* (1−2), 49−55.
(12) Masiri, J.; Barrios-Lopez, B.; Benoit, L.; Tamayo, J.; Day, J.; Nadala, C.; Sung, S.-L.; Samadpour, M. *J. Food Prot.* **2016**, *79* (3), 477−483.
(13) Wang, Y.; Deng, R.; Zhang, G.; Li, Q.; Yang, J.; Sun, Y.; Li, Z.; Hu, X. *J. Agric. Food Chem.* **2015**, *63* (8), 2172−2178.
(14) Koizumi, D.; Shirota, K.; Akita, R.; Oda, H.; Akiyama, H. *Food Chem.* **2014**, *150*, 348−352.
(15) Shibahara, Y.; Ii, T.; Wang, J.; Yamada, S.; Shiomi, K. *Shokuhin Eiseigaku Zasshi* **2014**, *55* (2), 88−93.
(16) Roder, M.; Vieths, S.; Holzhauser, T. *Anal. Bioanal. Chem.* **2009**, *395* (1), 103−109.
(17) Koets, M.; Sander, I.; Bogdanovic, J.; Doekes, G.; van Amerongen, A. *J. Environ. Monit.* **2006**, *8* (9), 942−946.
(18) Baumert, J. L.; Tran, D. H. In *Handbook of Food Allergen Detection and Control*; Flanagan, S., Ed.; Woodhead Publishing Series in Food Science, Technology and Nutrition; Woodhead Publishing: Sawston, Cambridge, U.K., 2015; pp 219−228.
(19) Shen, G.; Zhang, S.; Hu, X. *Clin. Biochem.* **2013**, *46* (16−17), 1734−1738.
(20) Bruno, J. G. *Pathogens* **2014**, *3* (2), 341−355.
(21) Wu, W.; Zhao, S.; Mao, Y.; Fang, Z.; Lu, X.; Zeng, L. *Anal. Chim. Acta* **2015**, *861*, 62−68.
(22) Liu, G.; Mao, X.; Phillips, J. A.; Xu, H.; Tan, W.; Zeng, L. *Anal. Chem.* **2009**, *81* (24), 10013−10018.
(23) Adhikari, M.; Strych, U.; Kim, J.; Goux, H.; Dhamane, S.; Poongavanam, M. V.; Hagström, A. E. V; Kourentzi, K.; Conrad, J. C.; Willson, R. C. *Anal. Chem.* **2015**, *87* (23), 11660−11665.
(24) Fang, Z.; Wu, W.; Lu, X.; Zeng, L. *Biosens. Bioelectron.* **2014**, *56*, 192−197.
(25) Bruno, J. G.; Carrillo, M. P.; Richarte, A. M.; Phillips, T.; Andrews, C.; Lee, J. S. *BMC Res. Notes* **2012**, *5* (1), 633.
(26) Liu, J.; Mazumdar, D.; Lu, Y. *Angew. Chem., Int. Ed.* **2006**, *45* (47), 7955−7959.
(27) Wang, L.; Chen, W.; Ma, W.; Liu, L.; Ma, W.; Zhao, Y.; Zhu, Y.; Xu, L.; Kuang, H.; Xu, C. *Chem. Commun.* **2011**, *47* (5), 1574−1576.
(28) Wang, C.; Zhang, L.; Shen, X. *Nucleosides, Nucleotides Nucleic Acids* **2013**, *32* (2), 59−68.
(29) Shim, W. B.; Kim, M. J.; Mun, H.; Kim, M. G. *Biosens. Bioelectron.* **2014**, *62*, 288−294.
(30) Nadal, P.; Canela, N.; Katakis, I.; O'Sullivan, C. K. *J. Agric. Food Chem.* **2011**, *59* (6), 2752−2758.
(31) Liu, L.; Pan, H.; Du, M.; Xie, W.; Wang, J. *Electrochim. Acta* **2010**, *55* (24), 7240−7245.
(32) Beni, V.; Hayes, K.; Lerga, T. M.; O'Sullivan, C. K. *Biosens. Bioelectron.* **2010**, *26* (2), 307−313.
(33) Bruno, J. G.; Carrillo, M. P.; Phillips, T.; Edge, A. *J. Biomol. Technol.* **2011**, *22* (1), 27−36.
(34) Civit, L.; Pinto, A.; Rodrigues-Correia, A.; Heckel, A.; O'Sullivan, C. K.; Mayer, G. *Methods* **2016**, *97*, 104−109.
(35) Pinto, A.; Bermudo Redondo, M. C.; Ozalp, V. C.; O'Sullivan, C. K. *Mol. BioSyst.* **2009**, *5* (5), 548−553.
(36) Joda, H.; Beni, V.; Willems, A.; Frank, R.; Hoth, J.; Lind, K.; Strombom, L.; Katakis, I.; O'Sullivan, C. K. *Biosens. Bioelectron.* **2015**, *73*, 64−70.
(37) Jauset-Rubio, M.; Sabaté del Río, J.; Mairal, T.; Svobodová, M.; El-Shahawi, M. S.; Bashammakh, A. B.; Alyoubi, A. O.; O'Sullivan, C. K. *Anal. Bioanal. Chem.* **2016**, *408*, 875.
(38) Xu, H.; Mao, X.; Zeng, Q.; Wang, S.; Kawde, A. N.; Liu, G. *Anal. Chem.* **2009**, *81* (2), 669−675.

DOI: 10.1021/acs.analchem.6b03256
Anal. Chem. 2016, 88, 10701−10709