Biosensors and Bioelectronics 43 (2013) 245–251

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Selection of aptamers against Ara h 1 protein for FO-SPR biosensing of peanut allergens in food matrices

Dinh T. Tran [1,2], Karel Knez [1], Kris P. Janssen, Jeroen Pollet [3], Dragana Spasic, Jeroen Lammertyn *

BIOSYST-MeBioS, KU Leuven–University of Leuven, Willem de Croylaan 42, B-3001 Leuven, Belgium

ARTICLE INFO

Article history:
Received 2 October 2012
Received in revised form
21 November 2012
Accepted 4 December 2012
Available online 20 December 2012

Keywords:
Aptamer
Peanut allergen
Ara h 1
Capillary electrophoresis (CE)
Systematic evolution of ligands by exponential enrichment (SELEX)
Fiber optic surface plasmon resonance (FO-SPR)

ABSTRACT

The rising prevalence to food allergies in the past two decades, together with the fact that the only existing therapy is avoidance of allergen-containing food next to the implementation of anti-allergic drugs, urges the need for improved performance of current assays to detect potential allergens in food products. Therein, the focus has been on aptamer-based biosensors in recent years. In this paper we report for the first time the selection of aptamers against one of the most important peanut allergens, Ara h 1. Several Ara h1 DNA aptamers were selected after eight selection rounds using capillary electrophoresis (CE)-SELEX. The selected aptamers specifically recognized Ara h 1 and did not significantly bind with other proteins, including another peanut allergen Ara h 2. The dissociation constant of a best performing aptamer was in the nanomolar range as determined independently by three different approaches, which are surface plasmon resonance, fluorescence anisotropy, and capillary electrophoresis (353 ± 82 nM, 419 ± 63 nM, and 450 ± 60 nM, respectively). Furthermore, the selected aptamer was used for bioassay development on a home-built fiber optic surface plasmon resonance (FO-SPR) biosensor platform for detecting Ara h 1 protein in both buffer and food matrix samples demonstrating its real potential for the development of novel, more accurate aptamer-based biosensors. In conclusion, the reported aptamer holds a great potential for the detection of Ara h 1 in both the medical field and the food sector due to its high affinity and specificity for the target protein.

1. Introduction

Peanut allergy is one of the most serious, life-threatening food sensitivities since it triggers the highest frequency of severe and fatal reactions. It represents a lifelong disorder with high risk of accidental exposures (Kleber-Janke et al., 1999). Ara h 1 is considered one of the major peanut allergens. It is a glycoprotein with a molecular weight of 63,000 Da and a p*I* of 4.5 (Burks et al., 1991). Ara h 1 exists in food as a homotrimeric complex. It has been shown that this particular tertiary structure contributes to the high allergenicity of the protein even after enzymatic and heat treatments. Thus, domains critical for oligomerization coincide with various protease-resistant fragments that protect molecule from protease digestions and denaturation. Because these domains also contain IgE recognition sites within tertiary structure, numerous of them can survive as large Ara h 1 fragments during gastrointestinal degradation of protein, which is a prerequisite for triggering high titer of IgE and allergenic responses (Maleki et al., 2000). Because the frequency of sensitization by Ara h 1 can be up to 100% of peanut allergic patients (Kleber-Janke et al., 1999), it is used as a marker for monitoring peanut contamination of food products. For these reasons, the development of improved detection methods for Ara h 1 remains priority in ensuring the compliance of food labeling and consumer protection.

To date, several immuno-assay techniques have been described in literature for detection of Ara h 1 including dip stick assay (Mills et al., 1997), ELISA (Pomes et al., 2004, lateral flow assay (Wen et al., 2005), and immunosensors (Huang et al., 2008; Pollet et al. 2011). While these methods are superior in either sensitivity, ease of use, or required assay time, they all rely on antibodies which are sometimes relatively difficult to generate (for instance antibodies against molecules that animals cannot tolerate, such as toxins) (Jayasena, 1999). Moreover, antibodies

* Corresponding author. Tel.: +32 16 321459; fax: +32 16 322955.
E-mail addresses: ttdinh@hua.edu.vn (D.T. Tran), Karel.Knez@biw.kuleuven.be (K. Knez), kris.janssen@biw.kuleuven.be (K.P. Janssen), jeroen.pollet.biosensors@gmail.com (J. Pollet), Dragana.Spasic@biw.kuleuven.be (D. Spasic), jeroen.lammertyn@biw.kuleuven.be (J. Lammertyn).
[1] Both authors equally contributed to this manuscript.
[2] Presnt address: Faculty of Food Science and Technology, Hanoi University of Agriculture, 10000 Hanoi, Vietnam.
[3] Present address: Sabin Vaccine Institute and Texas Children's Hospital Center for Vaccine Development, Department of Pediatrics, Section of Tropical Medicine, Baylor College of Medicine, Houston, TX 77030, USA.

0956-5663/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bios.2012.12.022

also have limited lifetime and may undergo irreversible denaturation due to sensitivity toward temperature variation (O'Sullivan, 2002). On the other hand, single stranded DNA (ssDNA) or RNA oligonucleotides, commonly referred to as aptamers, have the potential to replace or complement antibodies in developing biosensing assays (Min et al., 2008; Savory et al., 2010; Tran et al., 2011) because they are smaller in size, more stable, can be chemically synthesized, and labeled without losing affinity (Jayasena, 1999). Furthermore, aptamers can be selected against various targets (Lee et al., 2004) using powerful molecular selection process known as Systematic Evolution of Ligands by EXponential enrichment (SELEX).

In this paper, we describe the selection and characterization of the first aptamer against the peanut allergen Ara h 1. During eight rounds of the capillary electrophoresis (CE)-SELEX method, we isolated several aptamers, all of which demonstrated high specificity for the target protein and binding affinities within the nanomolar range. One of the aptamers with the lowest dissociation constant was further effectively implemented in bioassays for measuring Ara h 1 protein at various concentrations. Finally this aptamer was applied as a biosensor receptor for detecting and quantifying Ara h 1 which resulted in a sensitive and directly applicable bioassay.

2. Materials and methods

2.1. Materials

Purified natural Ara h 1 was purchased from Indoor Biotechnologies Ltd. (Wiltshire, UK). PCR reagents were acquired from Invitrogen (Merelbeke, Belgium). Trizma base, potassium phosphate dibasic, glycine, and sodium hydroxide were purchased from ACROS (New Jersey, USA). The DNA library, obtained from Integrated DNA Technologies Inc. (Haasrode, Belgium), contained 20 nucleotides in the primer regions and 40 nucleotides in a central randomized region: 5′-TCGCACATTCCGCTTCTACC-N40-TTCGCACACACGGACTTACG-3′. The 5′ end of the library and the forward primer were labeled with 6-carboxyfluorescein.

2.2. Capillary electrophoresis conditions

All CE experiments were performed using a P/ACE MDQ CE system combined with a laser-induced fluorescence (LIF) detector (Beckman Coulter, Inc., Fullerton, CA, USA). Two solutions were tested as electrophoresis buffers: (A) 25 mM trizma base, 192 mM glycine, and 5 mM K_2HPO_4 (TGK pH 8.3) and (B) 8.1 mM Na_2HPO_4, 1.1 mM KH_2PO_4, 1 mM $MgCl_2$, 2.7 mM KCl, 40 mM NaCl (PBS pH 7.4). Buffer solutions were prepared using HPLC gradewater (VWR, Haasrode, Belgium) and filtered through a 0.22 μm filter (Millipore, Bedford, MA, USA). Further details can be found in Supplementary Information.

2.3. Aptamer selection

The DNA library and the ssDNA pool obtained after each round of selection were dissolved in the optimal electrophoresis buffer, followed by heating at 75 °C for 10 min and cooling down on ice to allow proper folding of ssDNA oligonucleotides prior to incubation with the target protein. In the first round, 5 μL of DNA library (100 μM) was incubated with 5 μL of Ara h 1 (12 μM) for 30 min at room temperature. In the following rounds, the enriched DNA pools were incubated with the same amount of Ara h 1 as in the first round. This mixture was subsequently injected into a bare capillary by applying a pressure of 2 psi for 5 s. Separation was carried out at a constant voltage of 15 kV for 6.7 min with normal polarity. During this time DNA–protein complexes migrated to the collection vial containing 30 μL of the buffer. Unbound DNA oligonucleotides remained on the capillary and were later discarded using pressure. Four repeated separations were carried out for each selection round to increase the number of collected sequences.

After separation by CE, the collected DNA oligonucleotides were further PCR amplified and details of conditions used therein are provided as Supplementary Information. The obtained dsDNA was then separated and purified as described previously (Tran et al., 2010). After eight rounds of selection, the enriched pool of candidate DNA sequences was cloned using the same protocol as described in Tran et al. (2010). Twenty-seven colonies were then randomly chosen for sequencing (Genetic Service Facility, University of Antwerp, Belgium). The secondary structures of the selected aptamers were predicted and analyzed using the *mfold* program (Zuker, 2003).

2.4. Binding affinity

The binding affinity of aptamers was determined using three different approaches: affinity capillary electrophoresis and fluorescence anisotropy (the details of which can be found in Supplementary Information), as well as surface plasmon resonance approach.

2.4.1. Surface plasmon resonance

Surface plasmon resonance (SPR) experiments were performed using an in-house developed fiber optic (FO)-SPR method. The SPR device is described in more details in Supplementary Information, while the fabrication of the FO-probe has been extensively described by Pollet et al. (2009). The gold coated FO-probe was, prior to functionalizing with aptamers, incubated with an 11-mercaptoundecanoic acid self-assembling monolayer overnight at 4 °C. Alternatively, in the case of direct immobilization through thiols, probes were immersed in DTT activated (0.1 mM DTT, 1 h) 5′ thiol functional DNA. Afterwards the FO-probe was rinsed thoroughly with ethanol. Next, the carboxyl groups of the 11-MUA monolayer were activated in MES buffer (pH 6.0) and stabilized using a mixture of 0.3 M 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (Pierce Biotech) and 0.4 M N-hydroxy-succinimide (Pierce biotech) during 15 min. The activated fiber was quickly rinsed with MES buffer and dipped in a solution of 100 nM streptavidin in MES buffer. After immobilization of the streptavidin, the remaining activated COOH groups were inactivated with a short amine functional alkane PEG (Polypure, Norway). For the thiol functional DNA a surface 'backfilling strategy' was applied as described by Janssen et al. (2012). The DNA coated fibers were immersed for 3 h in 10 μM of an 8 PEG repeat alkane thiol with an OH end group (Polypure, Norway) dissolved in ethanol. Finally, FO-probes were rinsed and stored in PBS.

Prior to immobilization on the FO-probe, the aptamers were PCR amplified using a 5′ biotin or thiol-labeled forward primer. The PCR product was diluted 1/10 in PBS buffer with 300 mM of NaCl to allow the negatively charged DNA to interact with the negatively charged streptavidin. After 20 min the FO-SPR probe was rinsed again in PBS and heated for 2 min at 95 °C to denature the double stranded DNA. The FO-probe was dipped immediately afterwards in ethanol to precipitate the non-bound complementary strand of the PCR amplicon. The binding reaction between the aptamer and Ara h 1 was monitored by the SPR assay in TGK buffer. The statistical analysis of the kinetic data in the SPR measurements was done based on a 1:1 Langmuir model using

Prism (Graphpad Software, San Diego, CA, US):

$$\frac{d\gamma}{dt} = k_{on}\alpha_o(\beta-\gamma)-k_{off}\gamma \quad (1)$$

where γ is the number of complexes (mol m^{-2}) formed per unit time (s), α_o is the initial concentration of protein (mol L^{-1}), β is the original number of free receptors (mol m^{-2}), k_{on} is the kinetic association rate constant (L mol^{-1} s^{-1}), and k_{off} is the kinetic dissociation rate constant (s^{-1}).

Then the dissociation constant K_d (mol L^{-1}) is calculated as follows:

$$K_d = k_{off}/k_{on} \quad (2)$$

2.5. FO-SPR sandwich bioassay in food samples

For the FO-SPR sandwich bioassay, the same FO probes were used as in the previous experiments. The food samples were prepared according to Pollet et al. (2011). Briefly, a candy bar obtained from a local supermarket was frozen using liquid nitrogen and homogenized using pestle and mortar. The samples were then suspended in preheated (60 °C) Tris–HCl buffer for 10 min in a final volume of 20 mL. Known concentrations of Ara h 1 were added after this step for spiking the samples.Where needed the sample matrix was diluted in TGK buffer.

The robotic arm of the FO-SPR device was programmed to automatically execute a protocol existing of 3 binding steps (Fig. 4). First, a baseline measurement was made in TGK buffer, followed by capturing of Ara h 1 molecules by the aptamer in 100 μL of sample for 10 min. This step was first optimized in TGK buffer and afterwards directly applied on the candy bar matrix. Next, the FO-probe was immersed in TGK buffer containing 10 μg mL^{-1} of a rabbit polyclonal antibody (Indoor Biotechnologies) with affinity for Ara h 1. In a third step, the fiber was dipped for 10 min in a solution containing gold nanoparticles (Au NP) with a mean diameter of 20 nm (BBI International, Cardiff, UK). These Au NPs were first functionalized by adsorbing protein A on their surface. 100 μM protein A was mixed with 5 mL of Au NP (1 OD) after adjusting Au NP pH to 7.0 in 100 μL of carbonate buffer Na_2CO_3(0.2 M). Because protein A recognizes the Fc portion of the antibody, the signal on the FO-SPR sensor will be amplified when the antibody is present. Different concentrations were measured in series by regenerating the aptamer surface on the FO probe with a buffer containing 50 mM NaOH and 1 M of NaCl. The sensor always returned to baseline after regeneration, which indicated full biosensor recovery. Each series of concentrations was measured three times independently using new sensor probes for testing the reproducibility of the sensor tip fabrication as well as reproducibility of the sensor detection performance.

3. Results and discussion

3.1. Testing the buffer effect on aptamer selection

Before starting the aptamer selection, the effect that different buffers have on the selection process was examined. Ideally, it is preferred to identify the buffer which facilitates the binding between ssDNA library and the target protein, stabilizes these complexes, and maintains the fluorescence signal throughout the selection. In view of all these criteria, two buffers were tested: PBS, in which Ara h 1 was originally stored, and TGK, which was proven earlier as a suitable buffer for the selection of aptamers against lysozyme and IgE (Tran et al., 2010; Mendonsa and Bowser, 2004, respectively). Fig. 1 shows the electropherograms of a mixture containing ssDNA and Ara h 1 in the TGK buffer (left panel) and in the PBS buffer (right panel). A small complex was detected, along with the decrease of the free DNA, only in TGK buffer, while there was no complex visible in the PBS buffer under similar conditions (compare red curves in two panels, which correspond to the samples containing 8 μM Ara h 1 protein). Due to very high ionic strength of the PBS buffer, the free ssDNA migrated very slowly in this buffer, with 16.2 min needed for reaching the detector, thus making its peak wider (Fig. 1, right). In addition, the fluorescence signal of ssDNA obtained in the PBS buffer (pH 7.4) was much lower than that in the TGK buffer (pH 8.3). Based on these findings, the TGK buffer was chosen for performing the aptamer selection for Ara h1.

3.2. Selection of aptamers for Ara h 1

In order to select an aptamer against Ara h 1, target protein was incubated with the synthetic ssDNA library, followed by injection of this equilibrium mixture into the capillary. For determination of the aptamer collection window, the protein–ssDNA complex was visualized using the LIF detector. The complex formed between ssDNA and Arah1 protein migrated faster than free ssDNA due to less negative charges, and its corresponding peak was detected at 4 min. The second peak distinguished was that of the free ssDNA, which appeared at 6.5 min. To prevent this fraction from mixing with ssDNA–protein complex, the aptamer collection window was estimated at 5 min (Fig. 1, left panel). The oligonucleotides, collected as part of the complex,

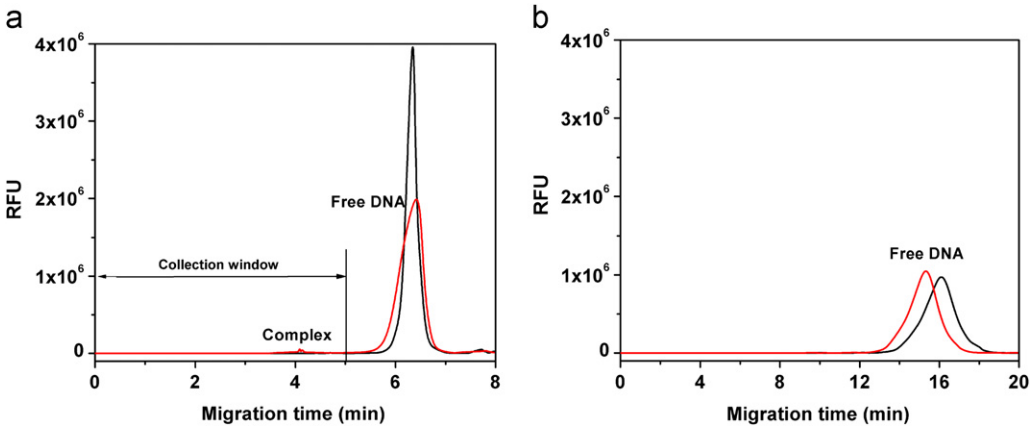

Fig. 1. Testing the buffer effect on aptamer selection. The electropherograms of fluorescently labeled DNA without Ara h 1 (black curves) and with 8 μM Ara h 1 (red curves) in the TGK buffer (left panel) and in the PBS buffer (right panel). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were subsequently PCR amplified and separated into ssDNA to generate a new enriched pool for the next selection rounds. Although negative selections are typically performed to eliminate sequences which have affinity to the stationary support, they were not carried out in this CE-SELEX as selections were done in solution.

The improvement of the selection was estimated using the average K_d value of the pool after each round. Fig. 2 demonstrates that these dissociation constants, calculated using the NECEEM method, were decreasing exponentially. Thus, in round eight, K_d reached the value of 850 nM, which was two orders of magnitude below the bulk value of the DNA library (170 μM). Moreover, as no significant improvement was observed in further rounds of selection, obtained DNA pool was further cloned in pGemt plasmid and sequenced.

Among 27 randomly selected clones, sequence alignment revealed seven groups of identical sequences (A1–A7). These sequences are presented in Table 1 (Supplementary Information) together with frequency of their incidence within these 27 clones. A1 and A6 were the most predominant, representing 48.2% and 33.3% of all sequences, respectively. Comparing sequences of these aptamers revealed that, although A1–A5 were different to a large extent, they had common guanine rich sections (similar sequences are underlined in Table 1, Supplementary Information). A6 and A7, differing in only one nucleotide, shared no consensus sequence with other aptamers, even though short G-triplets were present. Three aptamers, A1, A4, and A6, were chosen based on their abundance for further characterization. It should be noted that the sequences of the selected clones contain a high GC content, making it almost impossible to accurately synthesize them, especially with additional labels on their 5′ end. Therefore, the aptamers used in our experiments were amplified through PCR with the appropriate primers.

3.3. Evaluation of the aptamer binding affinity and specificity

The dissociation constants of three selected aptamers, A1, A4 and A6, were initially determined using the NECEEM method. A1 had the lowest K_d value (450 ± 60 nM), while the other two aptamers had substantially higher dissociation constants, in the low micromolar range (A4, 1770 ± 140 nM and A6, 1210 ± 60 nM). These differences in K_d values pointed to differences in their binding affinity toward the target Ara h 1 protein. Supplementary Fig. 1 depicts the electropherogram of free A1 (left panel) and aptamer in a complex with Ara h 1 protein (right panel) which was used for defining its K_d according to the model given in Supplementary information.

To assess the specificity of the aptamers towards Ara h 1, three selected aptamers A1, A4 and A6 were incubated not only with their target protein, but also with variety of other proteins, including BSA (a non-specific blocking agent in biosensor applications), human anti-Ara h 1 IgE antibody which is secreted when peanut sensitive patents ingest peanut containing foods, and Ara h 2, which is another major peanut allergen. The K_d values were measured using NECEEM and depicted in Fig. 3.The fact that the K_d of all three aptamers were some orders of magnitude higher for the three mentioned proteins than for Ara h 1 points to the absence of cross-reactivity towards non-specific and excellent specificity towards their target protein.

The secondary structure of aptamer A1 (the sequence of its random region being GGGGGGGTCGAGCTGAGTGGATGCGAATCT-GTGGGTGGGC) with the highest affinity for Ara h 1 protein, as determined with the NECEEM method, was further analyzed using the *mfold* program (Zuker, 2003). Because primer regions were part of the ssDNA library — next to the random sequence — during aptamer selection, the entire 80 nucleotide-long aptamer A1 was used for structure prediction. Four secondary structures were retrieved by *mfold* program for submitted A1 sequence, however all with similar values for Gibbs free energy. One of the predicted structures is shown in Supplementary Fig. 2. Although these four models are different to a certain extent, several motifs appeared to be common between them, such as the long stem–loop structure as well as internal loops. These loops, created by constant regions (indicated as underlined sequences in Table 1, Supplementary Information) might be involved in recognizing the target as previously reported (Wilson and Szostak, 1999).

3.4. Characterization of the aptamer A1

Because of favorable properties, only aptamer A1 was studied in more detail. The K_d values of this aptamer were more accurately determined using fluorescence anisotropy (FA) and surface plasmon resonance (SPR).

FA is a simple and effective tool for assaying the interactions of macromolecules. Unlike in CE, in FA the complex formed is always in equilibrium. In this analysis, the dissociation constant of A1 was determined by conducting a titration experiment. Here, more complexes were formed when increasing the protein concentration, leading to an increase in the bound fraction. The binding curve of A1–Ara h 1 measured by FA is represented in Supplementary Fig. 3A. Non-linear least squares analysis was used to estimate the K_d of A1. According to this analysis, the K_d is 419 ± 63 nM, which is close to the value obtained by NECEEM.

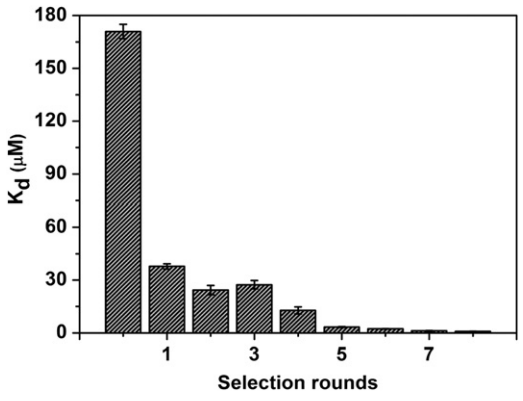

Fig. 2. Aptamer selection. Affinity of the DNA library (first bar) and the enriched DNA pools for binding Ara h 1 protein after each CE-SELEX round. Error bars indicate standard errors.

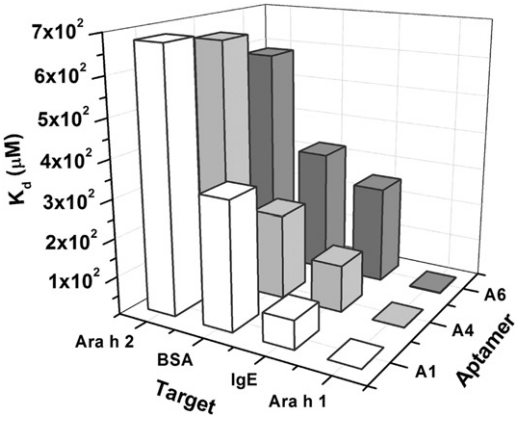

Fig. 3. Evaluation of the aptamer binding specificity. Specificity of the selected aptamers A1, A4 and A6 for Ara h 1, Ara h 2, IgE, and BSA measured with affinity capillary electrophoresis.

Binding affinity of the selected aptamer A1 was further analyzed using SPR, which is a well-known technique for the characterization of affinity events (Yang et al., 2007). An in house developed Fiber Optic SPR (FO-SPR) biosensor(Pollet et al., 2009, 2011; Delport et al., 2012, Knez et al., 2012) was applied to evaluate the binding kinetics between the aptamer and Ara h 1 protein in real-time. However, strong non-specific interaction of Ara h 1 protein was observed already with a bare gold surface, in the absence of any specific receptor. In Supplementary Fig. 3B, this is represented as a shift in the SPR signal of more than 5 nm (bare gold SPR probe). Moreover, this protein could not be removed from the sensor surface even after rinsing the FO probe for 5 min in TGK buffer, further pointing to the genuine surface fouling. Two different strategies were tested for preventing this non-specific interaction. First, the surface was blocked using increasing concentrations of polyethyleneglycol (PEG) back-filling molecules, as previously reported by our group (Janssen et al., 2012). These back-filling molecules are small molecules with a thiol functional group that control the density of DNA molecules on the sensor surface through their displacement while orienting them and fill in the bare gold surfaces in between the DNA strands. PEGs are known for their protein repellant function and are therefore often used to block non-specific binding (Prime and Whitesides, 1991). However, this approach still resulted in interaction of Ara h 1 protein with the surface to a certain extent (Supplementary Fig. 3B, 50 μM thiol–PEG curve). Although this interaction could be blocked through applying very high concentrations of PEG back filling molecules (>100 μM, data not shown), the amount of displaced thiol DNA at these concentrations was too high, thus leaving few receptors available for sensing.

Therefore, second strategy was applied to block this non-specific binding. Using 11-undecanoic self-assembling monolayer (SAM) streptavidin could be covalently immobilized on the gold surface with a standard EDC NHS protocol (Lee et al., 2005). Because streptavidin has four free binding pockets for biotin, the biotinylated aptamer was firmly bound at high density to the FO-SPR probe. Afterwards, the activated carboxyl groups were blocked using a short amine functional alkane chain with 6 PEG repeats. Following this approach, the biosensor surface was maximally blocked for non-specific binding of Ara h 1 protein without displacing the DNA aptamer from the surface. The curve referred to as SAM PEG in Supplementary Fig. 3B shows hardly any deviation from baseline when the sensor was exposed to the Ara h 1 protein at high concentrations.

After immobilization of the aptamer A1 on the FO-SPR, the biosensor was used to monitor binding events in real-time and evaluate the binding kinetics of the sensor. Supplementary Fig. 3C shows the sensorgrams of the SPR assay at various protein concentrations (211 nM, 423 nM, 634 nM, 846 nM, and 1058 nM). There is high correspondence of the model to the measured kinetic data. Based on this model the association rate constant, k_{on}, was estimated to be $19.1\times10^{4}\pm1.7\times10^{4}\,M^{-1}\,s^{-1}$ and the dissociation rate constant, $k_{off}=67.5\times10^{-3}\pm1.0\times10^{-3}\,s^{-1}$. Using these values, a K_d of 353 ± 82 nM was determined. Although this value is somewhat lower compared to those obtained by the NECEEM and FA methods, these small differences in the results are not statistically significant and could be even explained by several facts. Despite CE being a very powerful technique for separation of the DNA–protein complex from the free DNA, it is not the most accurate method for K_d determination. This is due to the fact that under the high voltage applied during CE separation, the equilibrium of the mixture is no longer maintained. As a consequence, the complex constantly dissociates, which causes smearing of the unbound DNA region (Berezovski and Krylov, 2002). The decay of the complex makes it difficult to exactly determine the border between free DNA and dissociated DNA, causing inaccurate peak integration. On the other hand, FA is commonly accepted as an effective technique for studying the affinity of binding between the pure ligand and its target. Because high GC content of selected aptamer influenced the accuracy of its synthesis, FA was performed using aptamer sample obtained through SELEX. However, this sample contained not only the aptamer sequence itself but also the impurities from the dsDNA separation step, as evident by the peak observed with CE-LIF (Supplementary Fig. 1), which could make the K_d determination less accurate in the FA measurement. Based on this, the FO-SPR approach seems like the most appropriate for estimating K_d value in the current study.

3.5. Application of the FO-SPR aptasensor for peanut allergen detection in food matrix

Newly selected aptamers are often only benchmarked in their selection environment. A limited number of aptamers have proven to be truly applicable in the demanding environment such as sample matrix (Müller et al., 2011). Because peanut allergen measurements are important in food applications, the FO-SPR aptasensor was tested on a candy bar matrix spiked with increasing concentrations of Ara h 1 protein.

Candy bars have a very high sugar concentration, which by itself will cause a very large shift in SPR signal due to a change in local density at the sensor surface. This makes detection of the Ara h 1 in real-time in the sample matrix impractical. In order to overcome these limitations, as well as to handle background noise

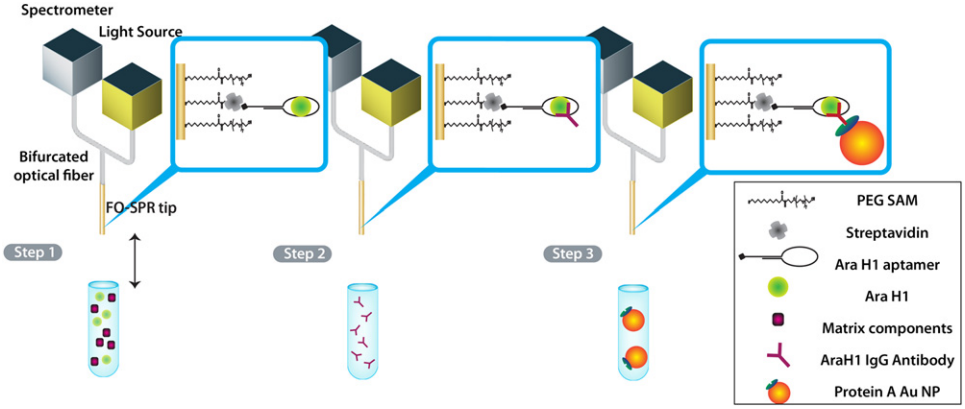

Fig. 4. Schematic representation of multi-step protocol for Ara h 1 protein detection in food matrix samples. Allergen from the food matrix is captured by aptamer on FO-SPR sensor tip followed by sandwich assay formation with polyclonal secondary antibodies which lastly are used for further enhancement of the signal.

deriving from the sample matrix, a multi-step protocol has been developed with a signal amplification strategy (Fig. 4). Similar assay was applied in our previous work where Ara h 1 protein was sandwiched between two antibodies for detection in complex food matrices using the same FO-SPR sensor technology (Pollet et al., 2011). FO-SPR antibody assay proved to be a valuable alternative for ELISA benchmark due to its considerably larger linear dynamic range as well as faster time to result. Therefore, the same bioassay concept was applied in this paper for testing the performance of selected aptamer in the real food matrix on one hand and exploring the advantages of using aptamer as bioreceptor on the other hand. The selected aptamer was first immobilized on biosensor for isolating the allergen from the food matrix (step 1). Next, the FO-SPR sensor was dipped in a buffer with polyclonal secondary antibodies (step 2). After these antibodies were bound to the Ara h 1 protein, the FO-SPR probe was immersed in a solution containing gold nanoparticles (Au NP) with protein A (step 3), which is a bacterial protein with a high affinity for the FC portion of antibodies. Au NPs were applied in this assay to further enhance the signal. Although these hybrid sandwich bioassays between aptamers and antibodies are only a step towards complete substitution of antibodies in analyte detection, they already proved as valuable tools offering some advantages in the sensitivity over traditional bioassays (Kim et al., 2010; Wang et al., 2008).

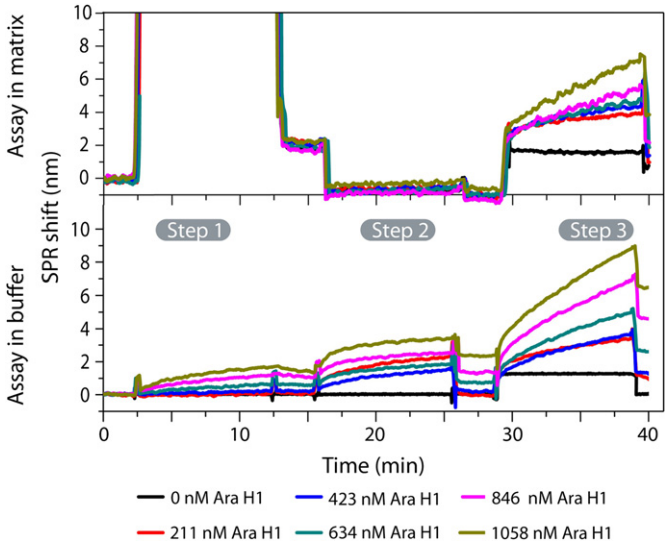

Fig. 5. FO-SPR aptasensor for peanut allergen detection in food matrix. Detection of Ara h 1 protein at various concentrations using the multi-step protocol in TGK buffer (bottom graph) and candy bar matrix (top graph). The discrimination of different Ara h 1 concentrations in candy bar samples is feasible only through Au NPs signal amplification.

This strategy was first tested for detecting Ara h 1 protein in the same buffer previously used for aptamer selection, namely TGK. As shown in Fig. 5 (bottom graph), the signal amplification achieved by the secondary antibody is limited (step 2), whereas much larger shift in SPR signal is triggered by addition of Au NPs (step 3). Furthermore, this signal augmented gradually with increasing concentrations of Ara h 1, verifying the validity of this multistep approach.

Next, the same assay was performed in the candy bar matrix (Fig. 5, top graph), resulting in two clear differences when compared to the assay completed in buffer: first, the appearance of distinctive huge shift in SPR signal caused by sample matrix (step1, Fig. 5), which was completely absent from the buffer assay; second, in the antibody amplification step, no true discrimination could be noticed between different candy bar samples. However, when Au NPs were added, similar signal amplification and dose response could be observed between two assays, pointing to the convincing performance of the assay even in a complex samples, such as food matrix.

To further validate the signal amplification strategy, the shifts in SPR signals observed after each step in the assay were evaluated (Fig. 6). When only using the antibody amplification, the signal did not allow discriminating concentrations below 423 nM of Ara h 1 protein. Contrary to this, the Au NPs sufficiently amplify the signal to detect Ara h 1 below this threshold, even in the sample matrix. The sample matrix was diluted two times and five times prior to spiking. At a twofold dilution, the signal was noticeably lower, presumably because the diffusion of the Ara h 1 protein to the biosensor surface was limited by the viscosity of the matrix. Although these results suggest that the assay time needs further optimization for matrices with higher densities, they already advocate the applicability of the assay even under these conditions. Moreover, as already noticed in our previous

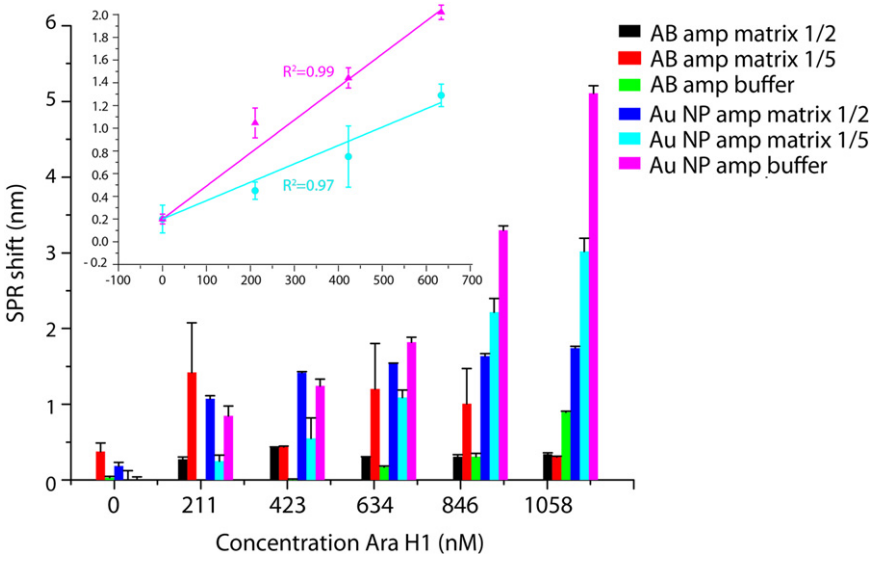

Fig. 6. Quantification of SPR signal shifts and calibration curves obtained with FO-SPR aptasensor. Ara h 1 concentrations below 423 nM could be discriminated in candy bar samples only through Au NPs signal amplification.

work, where Ara h 1 was detected using two antibodies in a similar assay (Pollet et al., 2011), application of Au NPs results in non-linear dose response at higher concentrations. Therefore, the calibration curves (inset of Fig. 6) were based on the linear part of the dose response data (concentrations between 0 nM and 634 nM). When applying the signal to noise ratio of 3, the limit of detection for the Au NPs enhanced assay in the buffer was estimated to be 75 nM. Although Au NPs enhanced assay allows for measurements with aptamer even in the sample matrix, the sensitivity of this aptasensor is lower in comparison with similar reported antibody based bioassays. However, these reported assays rely on several difficult extraction and purification steps, which were not applied in this research, but if implemented would clearly lead to even better performance of the assay (Stephan and Vieths, 2004). Moreover, the strong advantage of our assay is that it does not require any manual handling steps while utilizing universal building blocks.

4. Conclusions

In this paper, we reported for the first time the selection of DNA aptamers against the major peanut allergen protein, Ara h 1. The aptamer has been selected using CE-SELEX, which proved yet again to be a powerful approach for monitoring the aptamer selection process. During the selection, the dissociation constant of the ssDNA pool decreased from the micromolar to the nanomolar range within eight SELEX rounds. The selected aptamers specifically recognized Ara h 1 and did not bind strongly to other proteins, even not to another allergen protein isolated from peanuts, Ara h2. The aptamer had a dissociation constant in the low nanomolar range as measured by three approaches, NECEEM, FA and SPR. Although all three methods have been previously reported in literature as suitable for estimating binding affinities between molecules (Berezovski and Krylov, 2002; Gokulrangan et al., 2005; Pollet et al., 2009), we found that the most appropriate method in this study was SPR due to already mentioned limitations of NECEEM and FA. Therefore, we can report that aptamer with K_d of 353 nM against Ara h 1 has been selected. This aptamer was further implemented into a bioassay for peanut allergen detection both in buffer and food matrix using FO-SPR technology. Successful discrimination of different Ara h 1 protein concentrations using the developed aptasensor further proved high affinity and specificity of this aptamer toward its target. Due to these characteristics, one can conclude that the reported aptamer holds great potential to be used as biorecognition molecule in the development of aptamer-based biosensors for detecting Ara h 1 in both the medical field and the food sector.

Acknowledgments

The authors thank the Flemish Institute for the Promotion of Innovation through Science and Development (Sensors for Food VIS-traject, Flanders' Food), EFRO financing Interreg NanoSensEU, and KU Leuven (IOF-Knowledge platform Nanodiag) for financial support. Hanoi University of Agriculture, Vietnam is acknowledged for great collaboration.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2012.12.022.

References

Berezovski, M., Krylov, S.N., 2002. Journal of the American Chemical Society 124, 13674–13675.
Burks, A.W., Williams, L.W., Helm, R.M., Connaughton, C., Corkrell, G., Obrien, T., 1991. Journal of Allergy and Clinical Immunology 88 (2), 172–179.
Delport, F., Pollet, J., Janssen, K., Verbruggen, B., Knez, K., Spasic, D., Lammertyn, J., 2012. Nanotechnology 23, 1–7.
Gokulrangan, G., Unruh, J.R., Holub, D.F., Ingram, B., Johnson, C.K., Wilson, G.S., 2005. Analytical Chemistry 77, 1963–1970.
Huang, Y., Bell, M.C., Suni, I.I., 2008. Analytical Chemistry 80, 9157–9161.
Janssen, K., Knez, K., Vanysacker, L., Schrooten, J., Spasic, D., Lammertyn, J., 2012. Nanotechnology 23 (23), 1–7.
Jayasena, S.D., 1999. Clinical Chemistry 45, 1628–1650.
Kim, S., Leeb, J., Leec, S.J., Leea, H.J., 2010. Talanta 81, 1755–1759.
Knez, K., Janssen, K., Pollet, J., Spasic, D., Lammertyn, J., 2012. Small 8, 868–872.
Kleber-Janke, T., Crameri, R., Appenzeller, U., Schlaak, M., Becker, W.M., 1999. International Archives of Allergy and Immunology 119, 265–274.
Lee, J.F., Hesselberth, J.R., Meyers, L.A., Ellington, A.D., 2004. Nucleic Acids Research 32, 95–100.
Lee, J.W., Sim, S.J., Cho, S.M., Lee, J., 2005. Biosensors and Bioelectronics 20 (7), 1422–1427.
Maleki, S.J., Kopper, R.A., Shin, D.S., Park, C.W., Compadre, C.M., Sampson, H., Burks, A.W., Bannon, G.A., 2000. Journal of Immunology 164 (11), 5844–5849.
Mendonsa, S.D., Bowser, M.T., 2004. Analytical Chemistry 76, 5387–5392.
Mills, E.N.C., Potts, A., Plumb, G.W., Lambert, N., Morgan, M.R.A., 1997. Food and Agricultural Immunology 9, 37–50.
Min, K., Cho, M., Han, S.Y., Shim, Y.B., Ku, J., Ban, C., 2008. Biosensors and Bioelectronics 23, 1819–1824.
Müller, J., Becher, T., Braunstein, J., Berdel, P., Gravius, S., Rohrbach, F., Oldenburg, J., Mayer, G., Pötzsch, B., 2011. Angewandte Chemie International Edition in English 50 (27), 6075–6078.
O'Sullivan, C.K., 2002. Analytical and Bioanalytical Chemistry 372, 44–48.
Pollet, J., Delport, F., Janssen, K.P.F., Jans, K., Maes, G., Pfeiffer, H., Wevers, M., Lammertyn, J., 2009. Biosensors and Bioelectronics 25, 864–869.
Pollet, J., Delport, F., Janssen, K.P.F., Tran, D.T., Wouters, J., Verbiest, T., Lammertyn, J., 2011. Talanta 83, 1436–1441.
Pomes, A., Vinton, R., Chapman, M.D., 2004. Journal of Food Protection 67 (4), 793–798.
Prime, K.L, Whitesides, G.M., 1991. Science 24, 1164–1167.
Savory, N., Abe, K., Sode, K., Ikebukuro, K., 2010. Biosensors and Bioelectronics 26, 1386–1391.
Stephan, O., Vieths, S., 2004. Journal of Agricultural and Food Chemistry 52 (12), 3754–3760.
Tran, D.T., Janssen, K.P.F., Pollet, J., Lammertyn, E., Anne, J., Van Schepdael, A., Lammertyn, J., 2010. Molecules 15, 1127–1140.
Tran, D.T., Vermeeren, V., Grieten, L., Wenmaekers, S., Wagner, P., Pollet, J., Janssen, K.P.F., Michiels, L., Lammertyn, J., 2011. Biosensors and Bioelectronics 26, 2987–2993.
Wang, J., Renji, L., Xu, J., Xu, D., Chen, H., 2008. Analytical and Bioanalytical Chemistry 390, 1059–1065.
Wen, H.W., Borejsza-Wysocki, W., DeCory, T., Durst, R., 2005. Analytical and Bioanalytical Chemistry 382, 1217–1226.
Wilson, D.S., Szostak, J.W., 1999. Annual Review of Biochemistry 68, 611–647.
Yang, N., Su, X.D., Tjong, V., Knoll, W., 2007. Biosensors and Bioelectronics 22, 2700–2706.
Zuker, M., 2003. Nucleic Acids Research 31, 3406–3415.